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MINIREVIEW

From Latent Tuberculosis Infection to Tuberculosis. News in Diagnostics (QuantiFERON-Plus)

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Abstract

It is estimated that one third of the world's population have latent tuberculosis infection and that this is a significant reservoir for future tuberculosis cases. Most cases occur within two years following initial infection. The identification of individuals with latent tuberculosis infection is difficult due to the lack of an ideal diagnostic assay and incomplete understanding of latent infection. Currently, there are three tests: the oldest tuberculin skin test, T-SPOT.TB and the latest QuantiFERON-Plus for the detection of *Mycobacterium tuberculosis* infection. The interpretation of the test results must be used in the conjunction with a patient's epidemiological history, risk assessment, current clinical status, radiography and microbiological methods to ensure accurate diagnosis.

Key words: *Mycobacterium tuberculosis*, interferon-gamma release assays, latent tuberculosis infection, tuberculin skin test, tuberculosis

Introduction

The World Health Organization estimated that one third of the world's population has latent tuberculosis infection (LTBI) and the risk of progressing to TB is very heterogeneous (WHO, 2008). LTBI provides a potential reservoir for the reactivation and future development of active TB (Rangaka *et al*., 2012; Turetz and Ma, 2016). The active disease develops in 5–10% of those with LTBI over the course of their lifetimes (Turetz and Ma, 2016; Salgame *et al*., 2015). The highest risk of the progression from LTBI declines exponentially. Most TB cases occur within the first two years after a person has been infected (Mack *et al*., 2009; Salgame *et al*., 2015). In countries with intermediate (for example Poland) and high incidence of TB it is not practical to provide mass treatment for LTBI (Salgame *et al*., 2015; Korzeniewska-Koseła, 2016). It is well established that only a minority of patients with LTBI will develop TB (Mack *et al*., 2009). However, among children, immunocompromised individuals and patients receiving biological treatment, the risk of the progression to TB is significantly higher (Salgame *et al*., 2015). A major component of TB control is the identification of patients with LTBI in risk groups and the provision of chemoprophylaxis to prevent the development of active TB in those infected (Turetz and Ma, 2016; Uplekar *et al*., 2016). In Poland, prior to biological treatment, children and older patients are treated prophylactically. LTBI is a state of persistent T-cell responses to *Mycobacterium tuberculosis* antigens without clinical symptoms or signs of active TB, such as cough, hemoptysis, fever, night sweats, weight loss and opacity in chest radiographs (Mack *et al*., 2009; Lim, 2016; Getahun *et al*., 2015). There is no diagnostic gold standard for LTBI and direct identification of LTBI is not possible. Diagnostic tests are designed to identify the immune response against *M. tuberculosis*. Currently, there are two accepted methods for LTBI identification: the *in vivo* tuberculin skin test (TST) and *ex-vivo* interferon-gamma release assays (IGRAs). Two IGRAs are commercially available: QuantiFERON-TB Gold Plus (Qiagen, Germany) and T-SPOT.TB (Oxford Immunotec, UK). Both IGRAs are approved by the U.S. Food and Drug Administration and Conformité Européenne (Rangaka *et al*., 2012; Turetz and Ma, 2016; Salgame *et al*., 2015, Pai *et al*., 2014). However, none of the assays mentioned above, can distinguish between LTBI and active TB and none can identify which patients with LTBI will develop active TB (Rangaka *et al*., 2012; Turetz and Ma, 2016; Lim, 2016).

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Tuberculin Skin Test

The TST has been continuously in use for 100 years in clinical medicine and has been used to identify people with latent *M. tuberculosis* infection (Mack *et al.*, 2009). A TST is performed by intradermal injection of a purified protein derivative (PPD) of tuberculin, on the palmar surface of the forearm, at a volume of 2 units. The induration at the injection site is measured after 48–72 hours, by measuring the diameter of the area of induration, transversely to the long axis of the forearm (Borkowska *et al.*, 2011; Kruczak *et al.*, 2009; Kang *et al.*, 2005). In the case of patients who are latently infected with TB, tuberculin will stimulate a delayed type hypersensitivity (DTH) response *via* T lymphocytes. Tuberculin induces DTH where T cells and macrophages produce lymphokines that cause oedema, fibrin deposition, and inflow of other inflammatory cells (Turetz and Ma, 2016; Borkowska *et al.*, 2011; Kruczak *et al.*, 2009; Lalvani, 2007). In Poland, a RT23 type (renset tuberculin, 23 series) of PPD tuberculin has been used since 1966, produced at the Institute of Serum and Vaccine in Copenhagen (Borkowska *et al.*, 2011). TST is interpreted on the basis of the diameter and the clinical characteristics of a patient (Turetz and Ma, 2016). The TST has limitations, however. False positive and negative results can occur. There are 2 causes of false positive results: Bacillus-Calmette-Guerin (BCG) vaccination and nontuberculous mycobacterial infections (Rangaka *et al.*, 2012; Turetz and Ma, 2016; Pai *et al.*, 2014). In populations vaccinated with BCG, the skin reaction may be positive in some individuals even after 15 years following vaccination (Borkowska *et al.*, 2011). The specificity of the test is low because tuberculin contains more than 200 different antigens from microorganisms other than *M. tuberculosis*. In addition, false negative responses can occur if the patient is too young or too old, in immunocompromised patients (HIV infection), as well as in cases of those taking immunosuppressive medications or in those with active TB (Turetz and Ma, 2016; Pai *et al.*, 2014; Borkowska *et al.*, 2011). In Poland, where the whole population is vaccinated with BCG, it is important to establish whether the positive result of the TST is connected with a previous vaccination or with ongoing *M. tuberculosis* infection (Borkowska *et al.*, 2011).

Interferon Gamma Release Assays

The IGRAs are an alternative to the TST for the diagnosis of LTBI. These assays identify cellular immune responses to *M. tuberculosis* by measuring interferon-gamma (IFN-γ) after stimulation of T cells with *M. tuberculosis*-specific antigens (Turetz and Ma, 2016; Diel *et al.*, 2011). Two tests are available: T-SPOT.TB is based on the Elispot-enzyme-linked immunospot and QuantiFeron TB Gold Plus on the enzyme-linked immunosorbent assays (ELISA) technique. In the case of the T-SPOT.TB, whole blood is used and the test is based on measurement of the number of peripheral mononuclear cells that produce IFN-γ after stimulation with two antigens: early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP 10). Both antigens are encoded in the region of difference 1 (RD1) (Trajman *et al.*, 2016; van Ingen *et al.*, 2009). The second test, QFT-Plus, uses specialized whole blood collection tubes. The antigens used in this test are a peptide cocktail simulating the ESAT-6 and CFP 10. QFT-Plus comprises two distinct TB antigen tubes and both tubes contain ESAT-6 and CFP 10. TB1 tube is designed to elicit cell mediated immunity (CMI) responses from CD4+ T-helper lymphocytes and the TB2 tube contains an additional set of peptides targeted to the induction of CMI responses from CD8+cytotoxic T lymphocytes (2015b). The QFT-Plus measures the level of IFN-γ in the peripheral blood by the ELISA technique (Trajman *et al.*, 2016). The antigens used in the IGRAs do not cross-react with the vaccination strain *Mycobacterium bovis* BCG and with most species of nontuberculous mycobacteria (NTM). However, the RD1-coding region of antigens ESAT-6 and CFP 10, similar to that of *M. tuberculosis*, is present in *Mycobacterium kansasii*, *Mycobacterium szulgai*, *Mycobacterium marinum*, and *Mycobacterium riyadhense* (Diel *et al.*, 2011; van Ingen *et al.*, 2009; Hermansen *et al.*, 2016). The presence of similar antigens in NTM theoretically lowers the specificity of the IGRAs in diagnosing LTBI (van Ingen *et al.*, 2009). Compared to TST, IGRAs have better specificity, positive and negative controls, clear interpretation criteria and require only one visit in the clinic (Pai *et al.*, 2014).

Sensitivity and specificity TST, QFT, T-SPOT.TB

There is no method to truly confirm the diagnosis of LTBI, because we do not have a gold standard for diagnosing LTBI. The sensitivity of IGRAs or TST for LTBI diagnosis is typically assessed in patients with active TB, treating this group as a surrogate for LTBI (ECDC, 2011). The specificity of TST and IGRAs reflects the true negative rate of patients tested for LTBI. Populations with a recognizable low risk of *M. tuberculosis* infection introduce a surrogate for a group, free of *M. tuberculosis* infection (ECDC, 2011). Menzies *et al.* (2007) calculated the sensitivity and specificity of 3 tests based on 56 studies. Pooled sensitivity was lowest for the TST (70%), higher for QFT (76%) and the highest for T-SPOT.TB (88%). Pooled specificity was the lowest

for the TST (66%), higher for T-SPOT.TB (92%) and the highest for QFT (97%). The meta-analysis proved that no tests had high sensitivity. IGRAs were more specific than the TST in populations vaccinated with BCG (Menzies *et al*., 2007). The meta-analysis of Diel *et al*. (2010) showed that the pooled sensitivity of TST was 70% compared to 81% for the QFT and 88% for the T-SPOT.TB. The specificity of the QFT was 99% and 86% for the T-SPOT.TB. This meta-analysis included 25 studies (Diel *et al*., 2010). Both meta-analyses had similar results. Hoffman *et al*. (2016) prepared the first evaluation of the new test generation called QuantiFeron TB Gold Plus (QFT-Plus) in comparison with the older version of QuantiFeron TB Gold In Tube (QFT). QFT analyses IFN-γ released only by CD4+ T-helper cells after stimulation with *M. tuberculosis* antigens while QFT-Plus analyses the response of CD8+ cytotoxic T lymphocytes. Hoffman *et al*. (2016) counted the sensitivity of the new method based on data from 163 patients, including 77 health care workers and 86 suspected cases of TB. QFT-Plus produced 87.9% true-positive results, which was interpreted as demonstrating increased sensitivity compared to 80% for QFT in the meta-analysis (Hoffmann *et al*., 2016; Sester *et al*., 2011; Barcellini *et al*., 2016). This is one of the first such studies and therefore has some limitations, so further studies are needed to confirm these findings (Hoffmann *et al*., 2016).

Predictive value of IGRAs

A clear understanding of the predictive value of IGRAs for the development of active TB disease is necessary (Lim, 2016). The positive predictive value (PPV) for the progression of LTBI is the probability that in the case of an individual with a positive test, there is real a risk of developing active TB disease later in their life. The negative predictive value (NPV) is the probability that a patient with a negative test does not have LTBI and therefore will not develop active TB (Hermansen *et al*., 2016). The NPV is high in low-endemic countries, whereas the PPV of both the TST and IGRAs is low in these countries. Therefore, currently, only targeted testing in specific high-risk groups is recommended. Studies assessing the PPV of IGRAs show heterogeneous results (Hermansen *et al*., 2016). In 2015, Tuberculosis Network European Trials Group (TBNET) calculated the PPV and NPV of the QFT test, noting results of 1.9% and 99.9%, respectively (Zellweger *et al*., 2015) while Hermansen *et al*. (2016) in Denmark, a TB low endemic country, showed a high NPV (99.85%) and a low PPV (1.32%) for the same test. Their study included a 5-year retrospective cohort study assessing the risk of TB among patients with positive and negative QFT results (Hermansen *et al*., 2016). Lim (2016) analysed 3 studies in a low-TB- incidence countries and confirmed that IGRA has a very high NPV (99.5%) and a low PPV (about 4%) for future active TB (Lim, 2016; Hermansen *et al*., 2016; Zellweger *et al*., 2015; Sloot *et al*., 2014). Detecting LTBI and the need for treatment in specific cases should focus on patients with the highest risk of reactivation of TB. Current diagnostics of LTBI are deficient with limited PPV for the development of active TB (Turetz and Ma, 2016). There are a limited number of studies of the predictive value of IGRAs in countries with intermediate or high incidence of TB.

Summary

Standard diagnostic methods for an active TB diagnosis are known and have clear guidelines. A number of studies concerning the issue have been published. The diagnosis of LTBI however lacks gold standard. There are indirect tests: TST, QFT and T-SPOT.TB for detection of *M. tuberculosis* infection that must be used in conjunction with the patient's epidemiological history, risk assessment, current medical status, radiography and microbiological methods. The sensitivity, specificity and predictive values of IGRAs for the diagnosis of LTBI in low, intermediate and high-TB incidence settings should be the subject of further studies. New studies are also needed to explore the use of the new generation assay of QFT-Plus for the diagnosis of LTBI and active TB in various populations. QuantiFERON – Plus can be used as an adjunct tool in the diagnosis of active TB, but certainly cannot be used solely and indiscriminately, separate from other clinical epidemiological and radiological factors.

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MINIREVIEW

Application of Metagenomic Analyses in Dentistry as a Novel Strategy Enabling Complex Insight into Microbial Diversity of the Oral Cavity

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Abstract

The composition of the oral microbiome in healthy individuals is complex and dynamic, and depends on many factors, such as anatomical location in the oral cavity, diet, oral hygiene habits or host immune responses. It is estimated at present that worldwide about 2 billion people suffer from diseases of the oral cavity, mainly periodontal disease and dental caries. Importantly, the oral microflora involved in local infections may spread and cause systemic, even life-threatening infections. In search for etiological agents of infections in dentistry, traditional approaches are not sufficient, as about 50% of oral bacteria are not cultivable. Instead, metagenomic analyses are particularly useful for studies of the complex oral microbiome – both in healthy individuals, and in patients with oral and dental diseases. In this paper we review the current and future applications of metagenomic studies in evaluation of both the composition of the oral microbiome as well as its potential pathogenic role in infections in dentistry.

Key words: dental caries, endodontics, metagenomics, periapical abscess, periodontitis

Introduction

The oral microbiome plays a very important role both in health as well as in disease (Duran-Pinedo and Frias-Lopez, 2015; Strużycka, 2014; Xu and Gunsolley, 2014). It is now known – based on 16S rRNA gene sequence analyses – that the bacterial flora of the oral cavity exceeds 1000 taxa (Dewhirst *et al*., 2010; Zehnder *et al*., 2015). It consists of "protective" bacteria, transient invaders and opportunistic microflora of specific niches in the oral cavity (Zehnder *et al*., 2015).

Even in healthy individuals the composition of the oral microbiome is complex and dynamic, depending on many factors, such as anatomical location in the oral cavity (supragingival or subgingival plaque, tongue, mucous membrane lining the oral cavity), diet, oral hygiene habits or host immune responses (Xu and Gunsolley, 2014). Disruption of a symbiotic relationship between the oral microbiome and the host results in dysbiosis, which may cause overgrowth of pathogenic microflora and diseases of the oral cavity (Belibasakis and Mylonakis, 2015). Furthermore, oral health may affect the health status of the host leading to systemic infections.

It is now estimated that about 2 billion people suffer from oral diseases, such as periodontal disease and dental caries. They are therefore some of the most prevalent infectious diseases of humans (Xu and Gunsolley, 2014; He *et al*., 2015). Importantly, the oral microflora may cause not only local infections, but contributes also to the pathogenesis of systemic – even life-threatening infections – such as infective endocarditis, bacterial meningitis or brain abscess (Hsiao *et al*., 2012; Maurer *et al*., 2009; Mang-de la Rosa *et al*., 2014). However, microbial composition in different conditions affecting the oral cavity remains unknown. The use of traditional microbiological methods (culturing and identification of microorganisms) is unsatisfactory, as it is known at present that at least 50% of the oral microflora cannot be cultured, as revealed by genetic studies, including metagenomic strategies. Furthermore, microbial populations may be studied with the use of modern "omics"

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techniques combined with thorough bioinformatics analyses, detecting not only metagenome (DNA-based analysis), but also metatranscriptome (RNA level), metaproteome (protein composition of the oral microbiome) and metabolome (functional activity of a studied microbial population) (Duran-Pinedo and Frias-Lopez, 2015).

Amongst the above mentioned analytical strategies, metagenomic analyses are particularly useful for studies of the oral microbiome. They allow not only evaluation of bacterial composition in different locations and conditions in the oral cavity, but also contribute to the detection of novel, not yet described, but potentially pathogenic species. Furthermore, they allow detection of even non-viable bacteria (Belda-Ferre *et al.*, 2012).

Metagenomics strategies

Metagenomics – while being still a relatively novel science – has already helped to disclose many complicated, and often unexpected, relationships between the human microbiome and diseases it may cause (PadmanabHan and Wang, 2013; Alcaraz *et al.*, 2012; Xu and Gunsolley, 2014). Originally, metagenomics mostly found application in ecological analyses, and it was called environmental genomics or ecogenomics. However, currently it finds a broad application also in medical sciences, as clinical metagenomics revealed to be useful in detection and analysis of non-cultivable microorganisms (both commensal and pathogenic) and their mutual relationships in the community which they form within the host or in the particular anatomical location.

Microbial metagenomics may involve one of two possible strategies (Fig. 1). The first one is a targeted strategy called deep amplicon sequencing (DAS). It employs a pre-sequencing PCR amplification step, during which a particular taxonomic marker (*e.g.* 16S rRNA, *rec*A or *rpo*B gene) is amplified selectively. Afterwards, the thorough bioinformatic analysis and assignments of the assembled, individual reads into the appropriate operational taxonomic units (OTUs, clustering closely related individuals into one group), further taxonomic classification is possible. This leads to answering the question "Who is there?", *i.e.* enables a deep insight into overall microbial diversity of the particular environment. Although, the DAS strategy is slightly biased, mainly due to the preliminary PCR step, the progress in sequencing technologies and *in silico* analytical methodologies currently allows efficient taxonomic community characterization with application of this strategy and makes DAS being commonly used in various environmental and clinical applications (Scholz *et al.*, 2012; Turaev and Rattei, 2016).

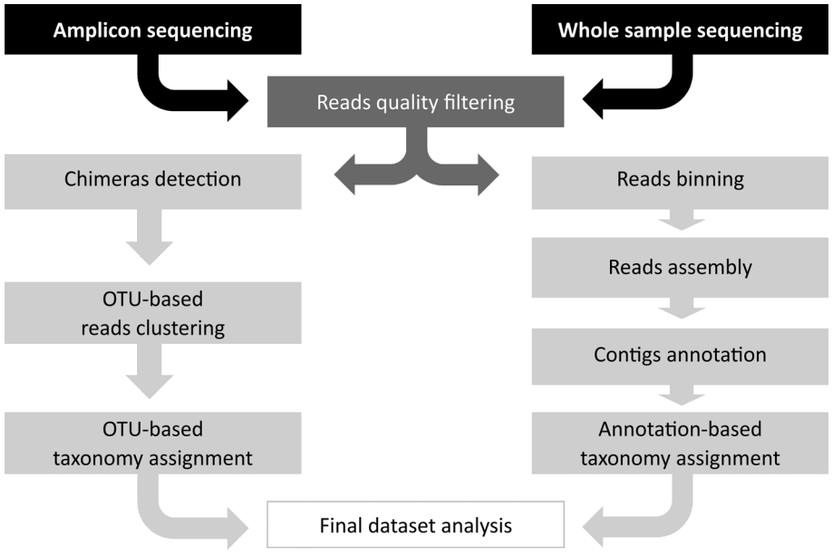

Fig. 1. Metagenome sequencing strategies.

Two types of metagenomic sequencing approaches, amplicon (on the left) and whole sample (on the right) sequencing, require specific library preparation and provide different sequencing data. The first step, common for both strategies, involves adaptor and quality-based reads trimming and length filtering. Amplicon sequencing, the strategy based on pre-PCR amplification of specific *loci* may result in point mutations and chimeras creation during that step. The later one should be filtered from the analyzed dataset as they could result in misleading biodiversity distribution. Then, the reads (or assembled read pairs) are clustered into operation taxonomy units (OTUs) based on their percentile identity between each other, i.e. 97% commonly used for 16S rRNA gene. Afterwards, representative sequences for each OTU are assigned to proper taxonomy group based on sequence similarity. The whole sample sequencing does not require previous pre-library DNA processing as the whole genomic DNA, including plasmid and phage DNAs, is sequenced. This kind of approach results in much bigger dataset. The reads are binned (sorted) into groups that might refer to individual genomes. After that, the reads may be assembled using either *de novo* (preferred for environmental samples) or reference-based assembly. Resulting contigs are then annotated and their taxonomy is assigned and used for biodiversity calculations.

The second metagenomics strategy is much broader, as it leads to answering not only the question "Who is there?", but also "What are they doing?". This strategy is known as shotgun metagenomics. According to this approach after the isolation of the total DNA from a particular sample, the total nucleic acid content of a sample is sequenced either directly or after applying an enrichment step, which might be a capture-based approach or subtraction prior to sequencing. The application of the high-throughput sequencing leads to generation of enormous number of short reads, which in the first step of bioinformatic analysis have to be assembled into contigs. Then, their taxonomic classification and functional assignments may be performed (Scholz *et al*., 2012).

Both above mentioned strategies find application in analyses of the oral cavity microbiomes. However, many researchers underline the need for standardized sampling methods for metagenomic studies of the oral microbiome. This would ensure reliable results, which will make it possible to compare the microbiome in different intraoral locations and in diverse clinical conditions of health and disease. Bacterial flora composition may be influenced by many factors, such as anatomical location (*e.g.* soft palate, hard palate, tongue, tooth surface, supra- or subgingival sample) or other factors (*e.g.* diet, smoking and oral hygiene habits) (Xu and Gunsolley, 2014; Wu *et al*., 2016). Xu and Gunsolley (2014) also indicate that sampling methods differ significantly. In dental caries specimens should be taken precisely from the affected tooth, avoiding contamination from subgingival sites. On the other hand, there is a risk of human DNA contamination of the samples taken from periodontitis lesions (Xu and Gunsolley, 2014). Even different tools for supragingival sample collection (*e.g.* cotton swabs *vs* loop-like devices) may influence the results of metagenomic studies (Xu and Gunsolley, 2014).

Bacterial metagenome in dental caries

Dental caries is one of the most common diseases in many parts of the world despite a decline in its rate in some regions due to prevention programmes (Belibasakis and Mylonakis, 2015; Gross *et al*., 2012; Gooch *et al*., 2009; Edelstein, 2006; Petersen *et al*., 2005). It may affect even very young children shortly after the eruption of their milk teeth. It is estimated by the WHO, that worldwide 60–90% of school children and nearly 100% of adults have dental caries (WHO, 2012). As indicated above, clinically it can have a severe course, which may even require hospitalization, with some fatal cases (Gross *et al*., 2012; Colak *et al*., 2013). Similarly, in adults dental caries may cause severe, even life-threatening complications. Most authors believe that dental caries constitutes an infectious, transmissible and polymicrobial disease, which results from a shift within the biofilm community of the oral cavity, however its etiology and pathogenesis remain unsolved (Gross *et al*., 2012; Simon-Sorro *et al*., 2014; Belibasakis and Mylonakis, 2015).

At present there are three major hypotheses of the etiology of dental caries: the specific, the non-specific, and the ecological plaque hypothesis (Aas *et al*., 2008; Xu and Gunsolley, 2014). Therefore, verification of these hypotheses is urgently needed and possible with the use of modern molecular techniques comprising metagenomic analyses of the oral microbiome. This would contribute to an improvement in diagnosis, treatment and prevention of caries and its complications, such as dental pulp necrosis and periapical abscess (Alcaraz *et al*., 2012; Belda-Ferre *et al*., 2012).

According to the specific plaque hypothesis, only a few bacterial species, such as *Streptococcus mutans* and *Streptococcus sobrinus*, are actively involved in the initiation of dental caries (Alcaraz *et al*., 2012; Xu and Gunsolley, 2014; Karpinski and Szkaradkiewicz, 2013; Kuramitsu and Wang, 2011). Apart from mutans streptococci, also lactobacilli and *Actinomyces* spp. may contribute to the development of dental caries (Beighton, 2005). There is, however, a conflicting opinion for and against this hypothesis as some authors claim that 10% of subjects with rampant caries in permanent teeth do not have detectable levels of *S. mutans* (Aas *et al*., 2008). It is even postulated by some researchers that the association of mutans streptococci and caries is weak and no greater than for other bacteria, and that the mere presence of *S. mutans* and *S. sobrinus* in dental plaque does not account alone for the cariogenic potential of such biofilms, as caries occurs in the absence of these species and their presence does not necessarily indicate caries activity (Gross *et al*., 2012; Beighton, 2005; Belda-Ferre *et al*., 2012; Kuramitsu and Wang, 2011; Simon-Sorro *et al*., 2014).

The non-specific plaque hypothesis maintains that caries is caused by a heterogenous mixture of many bacterial species and results from the overall activity of the total plaque microflora (Aas *et al*., 2008). Other dental plaque bacteria – apart from mutans streptococci, lactobacilli and *Actinomyces* spp. – also possess some characteristics thought to be important in cariogenicity. It seems likely that interaction of different bacteria may cause initiation of caries, and therefore the plaque flora may be non-specific in nature.

The ecological plaque hypothesis suggests that cariogenic flora of the oral microbiome constitutes only a minority of the total community and caries results from an imbalance of the metabolic activity of the resident microflora in the dental biofilm, due to changes in local environmental conditions (Astorga *et al.*, 2015). A diet rich in carbohydrates causes prolonged pH

change, which promotes tooth demineralisation and the growth of acid-tolerant and acid-producing bacteria (*e.g.* mutans streptococci and lactobacilli), while eliminating acid-labile species (Astorga *et al.*, 2015).

In a recent study Zhou *et al.* (2016) applying high-throughput metagenomic sequencing reported that in dental caries a synergistic effect may influence microbial community assembly and the co-prevalence of the pathogenic genera. In contrast to these findings, in caries-free individuals the authors found that the function of clustered genera was more random and competition appears to play a more significant role in the oral microbiome. They also found, that the following genera were more abundant in the caries group in comparison to healthy subjects: *Veillonella*, *Bifidobacterium*, *Selenomonas*, *Olsenella*, *Parascardovia*, *Scardovia*, *Chryseobacterium*, *Terrimonas*, *Burkholderia* and *Sporobacter.*

Metagenomic studies help to elucidate the potential role of oral bacteria in the initiation and establishment of a dental plaque. Many authors report that *S. mutans* is not present in all patients with dental caries (Gross *et al.*, 2012). Instead, in these individuals other streptococci are predominant (*e.g. Streptococcus salivarius*, *S. sobrinus*, and *Streptococcus parasanguinis*) as well as strains of *Veillonella* spp. Detailed metagenomic analysis may therefore contribute to modification of current treatment of this disease and establishment of effective prophylactic measures.

Bacterial diversity in endodontics and purulent complications of severe dental caries

Progression of dental caries may cause pulpitis, infection of the root canal and tooth necrosis (Belibasakis and Mylonakis, 2015; Zehnder *et al.*, 2015). Further expansion of the infection may lead to periapical abscess and apical periodontitis (Narayanan and Vaishnavi, 2010). The course of disease appears to depend on the interaction between the microbial flora and the host's immune system (Zehnder *et al.*, 2015).

It is estimated that periapical abscesses and accompanying pain (which can be excruciating) constitute about 56% of all non-traumatic dental emergencies (Hsiao *et al.*, 2012). Infection may complicate the outcome of endodontic treatment and the survival of the tooth (Hsiao *et al.*, 2012). Furthermore, pathogens involved in purulent complications may spread to the circulation, causing systemic diseases and infections in anatomically distant organs and sites (Pappa and Jones, 2005; Sequeira and Rocas, 2013; Robertson, 2015). However, despite major progress in endodontic techniques and many chemicals being available for root canal treatment, periapical abscesses remain the main cause of tooth loss and severe, even life-threatening complications.

Periapical abscesses constitute an enclosed environment, separated from the oral cavity. Indeed, recent metagenomic analyses revealed that bacterial composition in the root canal and abscess samples differs significantly from the microflora present in the oral cavity (Hsiao *et al.*, 2012; Tavares *et al.*, 2010). Metagenomic studies are therefore needed to characterize bacterial flora present in the endodontic system and in periapical abscesses in order to establish their etiology and proper treatment (Ribeiro *et al.*, 2011). This can be done by 16S rDNA sequence analysis. Using this approach Ribeiro *et al.* (2011) were able to detect in samples from root canals of 12 untreated asymptomatic teeth, on average 10 different bacterial taxa per root canal (range: 3–21), out of which as many as almost 66% represented non-cultivable bacteria. Earlier, Siqueira *et al.*, (2000) identified up to 17 taxa in a single root canal.

Molecular studies made it possible to detect uncultivable bacteria present in root canals of teeth with apical periodontitis, such as *Spirochaetes*, *Synergistetes* and *Dialister* (Munson *et al.*, 2002; Zehnder *et al.*, 2015). Recent approaches using 16S rRNA gene pyrosequencing revealed high diversity of bacteria in the apical portion of infected root canals (Siqueira and Rocas, 2009; Siqueira *et al.*, 2011). It now appears that bacterial flora present in different types of endodontic infections comprises as many as >460 bacterial taxa, classified in 100 genera and 9 phyla (Siqueira and Rocas, 2009). Most of them represent *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. Tavares *et al.* (2011) evaluated the microbiota of 32 samples obtained from the root canal system of deciduous teeth with pulp necrosis. In their study the mean number of species detected was 19 per sample, with a range from 3 to 66.

Santos *et al.* (2011) examined the root canal content of 8 teeth with chronic apical periodontitis and compared it with the aspirate from 9 abscesses of endodontic origin. They found, using a high-throughput multiplexed 16S rRNA gene pyrosequencing analysis that bacteria from the genus *Peptostreptococcus*, but also *Fusobacterium*, *Atopobium*, *Parvimonas*, *Dialister*, *Porphyromonas* and *Prevotella* were much more common in abscesses as compared to chronic root canal infections. It stands in agreement with a study by Sequeira and Rocas (2009), who found that the most prevalent species in apical abscess aspirates from 42 patients were *Fusobacterium nucleatum*, *Parvimonas micra* and *Porphyromonas endodontalis*. Other common taxa were *Olsenella uli*, streptococci and *Eikenella corrodens*. In another study Sequeira and Rocas (2013) confirmed that the most common genera found in acute apical abscesses are *Fusobacterium*, *Parvimonas*, *Prevotella*, *Porphyromonas*, *Dialister*, *Streptococcus*, and *Treponema*. On the other hand, Hsiao *et al.* (2012) found in 8 patients that although strains of *Prevotella* spp. and

Fusobacterium spp. were most prevalent in samples taken from the diseased endodontic sites, *Streptococcus* spp. were not common in these specimens. In this study, using next-generation sequencing of 16S rDNA amplicons, they found that the most common microbial species present in the samples from endodontic lesions were *Granulicatella adiacens*, *Eubacterium yurii*, *Prevotella melaninogenica*, *Prevotella salivae*, *Streptococcus mitis*, and *Atopobium rimae* (Hsiao *et al*., 2012). In a study by Ribeiro *et al*. (2011) in 12 samples from root canals the most prevalent bacterial species identified by metagenomic investigations were *Atopobium rimae*, *Dialister invisus*, *Prevotella oris*, *Pseudoramibacter alactolyticus*, and *Tannerella forsythia*. In a study of 32 root canal samples from deciduous teeth with pulp necrosis, the most prevalent taxa were *Prevotella intermedia* (96.9%), *Neisseria mucosa* (65.6%), *Prevotella nigrescens* (56.2%) and *Tannerella forsythia* (56.2%), while *Aggregatibacter (Haemophilus) aphrophilus* and *Helicobacter pylori* were not detected (Tavares *et al*., 2011).

It remains to be evaluated on a larger group of patients what is the etiological role of particular bacteria – or their specific compositions – in endodontic infections. Multiple species present in the root canals and/or periapical abscesses may result in network of interactions, which may affect their pathogenicity (Siqueira and Rocas, 2013). Understanding of the microflora associated with different forms of endodontic infections is necessary for improvement of the success of endodontic treatment (Narayanan and Vaishnavi, 2010).

Metagenomic studies in periodontal diseases

Periodontal disease may be defined as a cluster of infectious inflammatory conditions (gingivitis and different forms of periodontitis) which in a severe form may affect even 10–15% of the global population and is the major cause of tooth loss in adults (Belibasakis and Mylonakis, 2015). Apart from genetic factors, its development is strongly related to the polymicrobial biofilm formed by oral bacteria on the tooth surface, which stimulates pro-inflammatory responses in the surrounding tissues and their destruction (Liu *et al*., 2012; Belibasakis and Mylonakis, 2015). It can often resolve by good oral hygiene which eliminates the biofilm formed by bacteria (Xu and Gunsolley, 2014). On the contrary, built-up of bacteria may contribute to development of severe periodontitis, which may lead to systemic complications, such as infective endocarditis as well as diabetes, pneumonia, low birth weight in infants, inflammatory bowel disease, systemic autoimmune disease and colon cancer, to name a few (Xu and Gunsolley, 2014; Han and Wang, 2013; Moodley *et al*., 2013; Zarco *et al*., 2012; He *et al*., 2015; Liu *et al*., 2012). Apart from insufficient oral hygiene, other modifiable risk factors have been identified, such as tobacco use, excessive alcohol consumption, poor diet and nutrition, obesity and psychological stress, which contribute to periodontal disease prevalence (Petersen and Baehni, 2012).

Several metagenomic studies have indicated that samples of subgingival plaque from periodontitis patients contained different flora than in healthy subjects (Liu *et al*., 2012; Xu and Gunsolley, 2014; Griffen *et al*., 2012). Furthermore, a shift has been demonstrated in the oral microbiome from Gram-positive bacteria predominant in healthy individuals to Gram-negative microflora in patients suffering from periodontal disease. Indeed, Wang *et al*. (2013) observed that strains of *Streptococcus* spp. (13.7–41.3%), *Haemophilus* spp. (2.0–25.8%), *Rothia* spp. (0.9–16.7%), and *Capnocytophaga* spp. (3.1–13.0%) predominated in samples from individuals without periodontitis, while other genera comprised less than 10.0% of the microflora. In contrast, in specimens from individuals with periodontal disease the most prevalent were strains of *Prevotella* spp., which amounted to 14.4–44.7% of the bacterial communities.

The predominant species isolated from dental plaques from patients with periodontal disease are *Porphyromonas gingivalis* (which has many virulence factors and an ability to evade the host's immune response), *Aggregatibacter actinomycetemcomitans* (causing aggressive periodontitis), *Treponema denticola*, and *Tannerella forsythia* (which may have invasive properties). Similarly, Wang *et al*. (2013) showed a higher proportion of anaerobic Gram-negative bacteria classified in the genera *Prevotella*, *Leptotrichia*, *Veillonella*, *Porphyromonas*, and *Treponema*, in samples from periodontitis patients in comparison to microflora in samples from healthy individuals. Therefore, these species were considered by many authors as specific pathogens of periodontal disease. It should be noted, that further microbiological studies revealed a strong correlation between periodontal disease and the proportions of some bacteria cultured from dental plaques, *e.g. Prevotella intermedia*, *Fusobacterium nucleatum*, *Selenomonas noxia*, *Aggregatibacter actinomycetemcomitans*, and *Eubacterium nodatum* (Slots and Genco, 1984; Tanner, 2015). However, the use of culture-independent molecular techniques allowed to identify other groups of bacteria prevalent in samples from patients with periodontal disease, including the following genera: *Megasphaera*, *Parvimonas*, *Desulfobulbus*, and *Filifactor* (Kumar *et al*., 2005; Colombo *et al*., 2009).

Elucidation of pathogenesis of periodontitis and an association between its progression and specific pathogens – or their composition – require further studies, particularly metagenomic analyses (Wang *et al*., 2013; Jorth *et al*., 2014). Wang *et al*. (2013) used metagenomic sequencing of 16 samples from patients with 4 different

clinical forms of periodontal disease to evaluate functional potential of detected bacterial microflora. They observed a strong correlation between the composition of identified microflora and periodontal disease status. They also claim that they were successful in identifying an essential ("core") disease-associated set of bacterial taxa. Jorth *et al.* (2014) confirmed a high diversity of microbial flora composition in patients with periodontits, however they found that disease-associated communities exhibit conserved changes in metabolic profiles and virulence gene expression.

Surprisingly, recent studies suggest that bacterial species present in low quantities in oral samples cannot be ignored as they may play a significant role in the oral microbiota, including inflammatory processes observed in periodontitis (Kawamura and Kamiya, 2012; Hajishengallis *et al.*, 2011; Wang *et al.*, 2013). Using a metagenomic approach, Wang *et al.* (2013) identified low-abundance genera, which were associated with periodontitis, including *Alistipes*, *Bulleidia*, *Butyrivibrio*, and *Parabacteroides*. They also claim that several functional genes and metabolic pathways (*e.g.* bacterial chemotaxis, flagellar assembly, and toxin biosynthesis) were over-represented in the microbiomes in periodontal disease, in comparison to the oral microflora in healthy individuals (Wang *et al.*, 2013). Furthermore, they found a large number of phages in samples from both healthy individuals and patients with periodontal disease. They postulate that phages may modify the oral microflora and therefore may also play an indirect role in the pathogenesis of oral diseases.

The future of metagenomics in dentistry

Metagenomics has the potential to revolutionize clinical diagnostics (Miller *et al.*, 2013). It enables simultaneous detection of all microorganisms in a clinical sample, including uncultivable, rare and novel pathogens. Furthermore, metagenomic techniques may help explain the role of variability in microbiome composition and function in relation to pathogenesis of infectious diseases.

Metagenomic analyses have been done so far on a relatively small number of dental patients and healthy individuals. Further studies are therefore urgently needed to establish true composition of the oral microbiome in health and disease. Metagenomic and related molecular techniques also offer perspectives for evaluation of pathomechanism of different dental infections and subsequently proper management of them. Function-based metagenomic analyses have already helped to discover new resistance mechanisms and potential targets for antimicrobial therapy, therefore evaluation of the oral resistome is very important (Sukumar *et al.*, 2016; Tansirichaiya *et al.*, 2016). As Zarco *et al.* (2012) stated, metagenomics may contribute not only to more effective diagnostic and therapeutic techniques, but also to personalized dental medicine. This may help to develop effective prophylaxis of oral diseases, such as dental caries and periodontitis, which according to the recent estimates affect as much as a quarter of the world's human population.

Conflict of interest

The authors dec0lare that there are no conflicts of interest.

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ORIGINAL PAPER

Deletion of *ato*R from *Streptococcus pyogenes* Results in Hypervirulence in a Mouse Model of Sepsis and is LuxS Independent

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Abstract

Group A *Streptococcus* (GAS) is a Gram-positive human pathogen that causes a variety of diseases ranging from pharyngitis to life-threatening streptococcal toxic shock syndrome. Recently, several global gene expression analyses have yielded extensive new information regarding the regulation of genes encoding known and putative virulence factors in GAS. A microarray analysis found that transcription of the GAS gene *M5005_Spy_1343* was significantly increased in response to interaction with human polymorphonuclear leukocytes. *M5005_Spy_1343* is predicted to encode a member of the LysR family of transcriptional regulators and is located upstream of a putative operon containing six genes. Five of these genes have sequence similarity to genes involved in short-chain fatty acid metabolism, whereas the sixth gene (*lux*S) is found in many bacterial species and is involved in quorum sensing. Unexpectedly, inactivation of the *M5005_Spy_1343* gene resulted in hypervirulence in an intraperitoneal mouse model of infection. Increased virulence was not due to changes in *lux*S gene expression. We postulate that short-chain fatty acid metabolism is involved in GAS pathogenesis.

Key words: *Streptococcus pyogenes*, *ato*, host-pathogen interactions, short chain fatty acid synthesis, virulence factors

Introduction

Group A *Streptococcus* (GAS) is a Gram-positive human bacterial pathogen that causes a variety of diseases ranging from pharyngitis to life-threatening streptococcal toxic shock syndrome (Sitkiewicz and Hryniewicz, 2010). The molecular mechanisms responsible for these different diseases are an intense area of GAS research. Several studies have examined the global gene expression changes in GAS occurring as it encounters distinct niches within the host (Graham *et al.*, 2002; 2005; Klenk *et al.*, 2005; Shelburne *et al.*, 2005; Sitkiewicz and Musser, 2006; Virtaneva *et al.*, 2005; Voyich *et al.*, 2004; Voyich *et al.*, 2003; Musser and DeLeo, 2005). For example, an expression microarray analysis examined the transcriptional response of GAS to interaction with human polymorphonuclear leukocytes (PMNs) (Voyich *et al.*, 2003). An important discovery was that transcription of the *M5005_Spy_1343* gene (*spy1343*) was upregulated two-fold 60 min post-PMN contact. *Spy1343* encodes an inferred 298-amino acid protein that belongs to the LysR family of transcriptional regulators.

The *spy1343* open reading frame (ORF) is located downstream and is divergently transcribed from a putative operon containing six genes (Lyon *et al.*, 2001) (Fig. 1). The first three genes *(ato*BDA*)* share sequence similarity with the *ato*BDA operon in *Esherichia coli* (Jenkins and Nunn, 1987a) that encodes enzymes involved in short-chain fatty acid (SCFA) degradation. In *E. coli*, AtoB is an acetoacetyl-CoA acetyltransferase (thiolase II), and AtoA and AtoD form the two subunits of acetate CoA-transferase. These enzymes also can participate in metabolic pathways other than SCFA degradation, such as butanoate metabolism, synthesis and degradation of ketone bodies, and fatty acid biosynthesis *via* pathway 2 (www.brenda.uni-koeln.de). Two other genes present in the putative GAS operon (*M5005_Spy_1347* and *M5005_Spy_1348*) encode proteins with sequence similarity to 3-hydroxybutyrate dehydrogenase and 3-hydroxybutyrate permease, respectively. These enzymes are involved in butanoate metabolism and synthesis and degradation of ketone bodies. Interestingly, upon exposure of GAS to human blood, the transcript level of all 5 genes (*M5005_Spy_1344-1348*)

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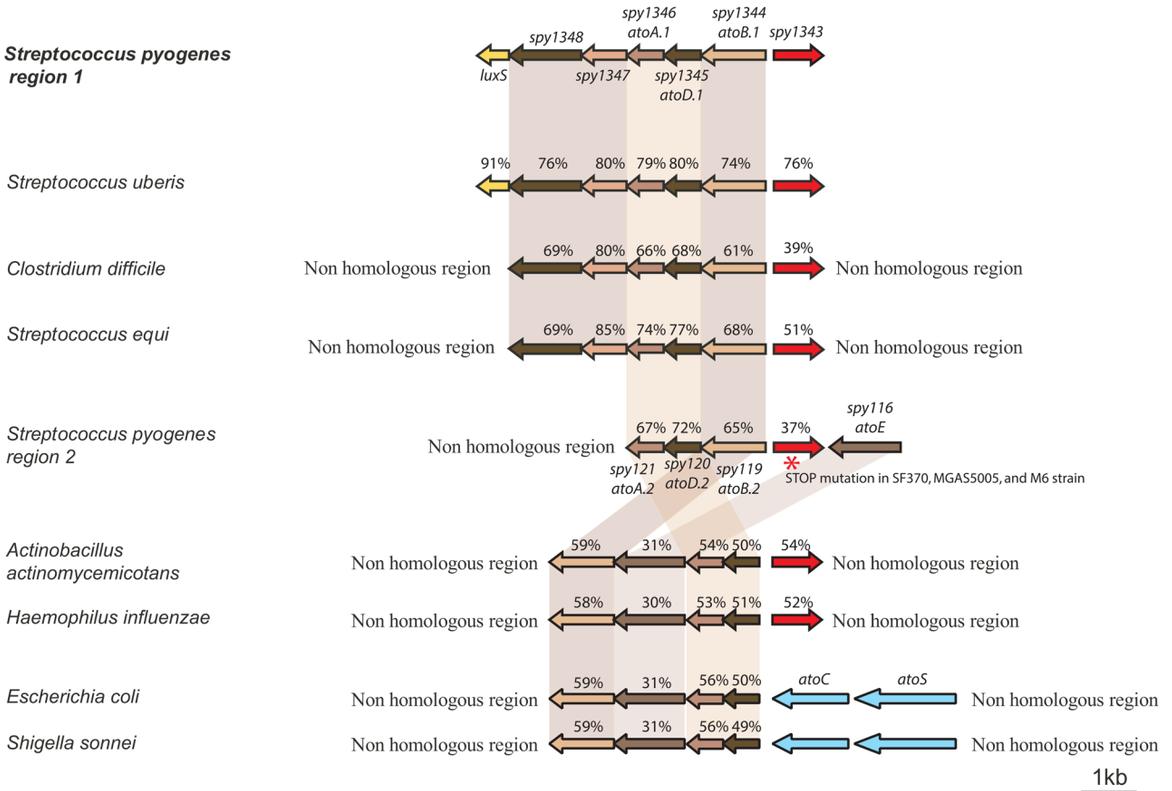

Fig. 1. Homologous regions encoding *lux*S and *ato* genes in GAS and other bacteria.
Red arrows represent *M5005_spy1343* and its homologs. Beige shading indicates regions of high sequence similarity. Percentages denote amino acid identity compared to GAS.

decreased concomitantly with the increase in *spy1343* transcript, suggesting that *spy1343* negatively regulates expression of these five genes (Graham *et al.*, 2005).

The last gene in the putative operon has sequence similarity to *lux*S. LuxS is found in a diverse array of bacterial species and is involved in quorum sensing *via* processing of auto-inducer II (Waters and Bassler, 2005; Jimenez and Federle, 2014). In GAS, LuxS affects the production of at least 3 known virulence factors including streptococcal pyrogenic exotoxin B (SpeB), streptolysin S (SLS) and M protein (Lyon *et al.*, 2001; Marouni and Sela, 2003).

The organization of the *ato* gene region is nearly identical in sequenced GAS genomes, and the inferred homologous proteins have 98% to 100% identity. Additionally, we determined by sequence similarity searches (NCBI BLAST) and operon organization (FGENESB at www.softberry.com) that similar *ato* operons are present in bacteria that are distantly related to GAS, such as *Clostridium difficile*, *Actinobacillus actinomycemicotans*, *Haemophilus influenzae*, and *Shigella sonnei* (Fig. 1). The *ato* operon in *E. coli* and *S. sonnei* is regulated by the *atoSC* two-component gene regulatory system (Jenkins and Nunn, 1987b). However, *A. actinomycemicotans* and *H. influenzae* genomes contain an upstream regulatory gene that encodes a protein with greater than 50% identity to *spy1343*. Additionally, all sequenced GAS genomes contain a second *ato*BDA operon. However in several strains, including MGAS5005, the putative regulator contains a truncation mutation resulting in a stop codon at amino acid 139, suggesting that this protein is not fully functional (Fig. 1).

The studies reported herein were motivated by the observation that the *spy1343* transcript was up-regulated in response to interaction of GAS with human PMNs, the presence in the region of a gene (*lux*S) encoding a known regulator of GAS virulence genes, and the conserved inter-species and cross-genus operon organization. We show that *spy1343* regulates *ato* genes, and that a *spy1343* mutant is hypervirulent in a mouse model of sepsis. We demonstrate that the hypervirulent phenotype is not caused by changes in *lux*S transcription.

Experimental

Materials and Methods

Bacterial strains and culture conditions. Serotype M1 strain MGAS5005 was used in this study. The genome sequence has been published (GenBank acc.

No. CP000017), and MGAS5005 is representative of contemporary clinical M1 isolates (Sumby *et al.*, 2005). GAS were grown in Todd-Hewitt broth (Difco Laboratories) supplemented with 0.2% yeast extract (THY medium) at 37°C in an atmosphere of 5% CO_2, 20% O_2. THY medium or tryptose agar with 5% sheep blood (Becton Dickinson) was used as solid media. THY agar supplemented with spectinomycin (150 µg/ml) was used for selection of the antibiotic-resistant mutant derivative strain. Cloning experiments were performed with *E. coli* DH10B (Invitrogen). Ampicillin (100 µg/ml) or spectinomycin (150 µg/ml) was used for selection of *E. coli* clones when required.

DNA techniques. Restriction and modification enzymes were purchased from New England BioLabs or ThermoFisher. Plasmid DNA from *E. coli* was isolated with a QIAprep Spin Miniprep Kit (Qiagen). Chromosomal DNA was isolated from GAS using the DNeasy Tissue Kit (Qiagen) as described by the manufacturer, with slight modification (Sitkiewicz and Musser, 2006). Southern hybridization was performed with the ECL system according to the manufacturer's instructions (Amersham).

Transformation of GAS. GAS was transformed as described previously (Sitkiewicz and Musser, 2006). Briefly, cells were grown to an OD_{600} of 0.2–0.25 in medium containing L-threonine and sucrose, washed, and frozen in aliquots. The GAS-DNA mixture was subjected to a pulse of 1.8 kV, 25 µF, 400 Ohms, and transformed cells were incubated 2–3 hours at 37°C without antibiotics and plated onto selective media.

Construction of the Δ*spy1343* mutant strain. The coding sequence of *spy1343* was replaced with a spectinomycin-resistance cassette (spc) by a double crossover strategy. Upstream and downstream sequences of *spy1343* were amplified in PCR reactions with primer pairs 1343 5'F/1343 5'R and 1343 3'F/1343 3'R (Table I). The PCR products were cloned into pUC19 (Fermentas) and pSTblue-1 (Novagen) vectors to generate plasmids p1343-5' and p1343-3', respectively. The spc containing the *add9* gene was excised from plasmid pSL60-2 (Lukomski *et al.*, 2000) with *Sma*I and cloned into the

Table I
Primers and probes used in this study

Primer name	Sequence (5'→3')
1343 5'F	CCCAAACATATGCGGTGCTGAGTTGATACATAG
1343 5'R	CCCAAACCCGGGGGATTCTCCTTGTCTTATCAATTGC
1343 3'F	CCCAAACCTAGGCATGGACGACTTGCTACAGTC
1343 3'R	CCCAAAACCGGTGGAGCGCTCCGCTAAGCGTG
1343L	TCTGTTAAGCCATCATGAACAAG
1343R	ATCGGTCAGACTTTGTTCTTTAC
1348F	GGGTCTTGGTAGGTGTTATTG
1348R	TTTGTGGTTAAGTCCTGTCAATGCTAAG
1347F	ATCATGTTGACAGCACCATTTATTG
1347R	GAACAAGTGGGTATAGCACTTC
spcF	CCCGGGAATACATGTTATAATAACTATAAC
atoA F	CCAACTATATTCCTGAAGGTGTAAC
atoA R	CAGTCAGAGGAAGGGTGCATTTG
1343-taq-F	AGTGGGAAGATTTAACAGATGAACAA
1343-taq-R	GACGCTCGCAAGCTTCAAG
atoB-taq-5'	CAAGACAAGTTGCAGTCCAC
atoB-taq-3'	GCGCAGCTAATTGGATTG
luxS-taq-3'	AAAGGAGAGCAATCAATCATC
luxS-taq-5'	CCGGTTTGCATACCATTG
proS5'	TGAGTTTATTATGAAAGACGGCTATAGTTTC
proS3'	AAT AGC TTC GTA AGC TTG ACG ATA ATC
Probe name	Sequence (5'→3')
luxS	CTTACTTGCCAAGCTCATCCGCCAAC
atoB	TCCAGAACCGCAAACCATACTAATGTGAAAGC
1343	TGCCCTATTTGATCCTAGTTTCATGGTTCACC
proS	TCGTAGGTCACATCTAAATCTTCATAGTTG

*Pml*I site of p1343-3' to generate plasmid p1343-3'spc. The orientation of the spc was determined by PCR with primers spcF/1343 3'R. The *spy1343* fragment and spc from p1343-3'spc were cloned into p1343-5' to generate a plasmid with a DNA cassette containing the upstream and downstream fragments of *spy1343* flanking the *add9* gene. The entire fragment was amplified using PCR primers 1343 5'F and1343 3'R and purified product was transformed into strain MGAS5005. Transformants were selected on THY medium containing spectinomycin. Chromosomal DNA isolated from spectinomycin-resistant colonies was screened by PCR using primers 1343L/1343R, which annealed outside of the targeted integration site, thereby amplifying different-size products in mutant *versus* wild-type clones. Inactivation of the target gene was further confirmed by sequencing of the appropriate chromosomal region, and single integration was confirmed by Southern hybridization using a probe that annealed to the spc.

Mouse infection experiments. GAS strains used for mouse infection studies were grown in THY medium to exponential phase ($OD_{600} \sim 0.5$), harvested, washed twice with cold PBS, and frozen at –70°C in aliquots. The number of CFUs used to inoculate mice was determined by plating GAS on sheep blood agar. Thawed aliquots of wild-type strain MGAS5005 and the isogenic mutant strain were adjusted to the same CFU/ml by diluting with PBS prior to injection. Outbred CD-1 Swiss male mice (4–6 weeks old) (Harlan) were used for intraperitoneal inoculation with a dose of 1×10^7 CFUs. Mortality and morbidity were monitored every 2 h for the first 48 h after infection, and every 6 h for the next 5 days.

TaqMan analysis of gene transcription. MGAS5005 and the Δ*spy1343* mutant strain were grown in THY medium to an OD_{600} of 0.2 (early-exponential), 0.5 (mid-exponential), 1.2 (late-exponential), or 1.8 (stationary phase). Two independent cultures for each strain represented two biological replicates. RNA was isolated as described previously (Sitkiewicz and Musser, 2006). cDNA was generated using SuperScript III reverse transcriptase (RT) and random hexamers (Invitrogen), and TaqMan reactions were performed in quadruplicate using Platinum Quantitative PCR SuperMix (Invitrogen). Primer/probe sets (Table I) used for Taqman reactions were: atoB-taq-5'/atoB-taq-3'/atoB probe for *ato*B (*M5005_Spy_1344*); luxS-taq-5'/luxS-taq-3'/luxS probe for *luxS*; 1343-taq-F/1343-taq-R/1343 probe for *spy1343* and proS5'/proS3'/proS probe for internal *pro*S standard. The transcript level of the genes of interest was normalized to *proS* transcript and compared between the wild-type and Δ*spy1343* mutant strains using $\Delta\Delta C_T$ method (ABI, 2005).

RT-PCR. cDNA was generated using SuperScript III RT (Invitrogen) from RNA collected for TaqMan analysis with primers luxS-taq 3', 1348R and *ato*A-R according to the manufacturer's instructions. First-strand cDNA was purified using S.N.A.P. columns (Invitrogen) and amplified in PCR reactions with primer pairs luxS-taq-3'/luxS-taq-5', luxS-taq-3'/1348F and luxS-taq-3'/1347F (*lux*S transcript); 1348R/1638F, 1348R/1347F and 1347R/1347R (*M5005_Spy_1348* transcript); *ato*AR/*ato*B-taq-5' (*ato*A transcript). MGAS5005 genomic DNA served as a positive control for PCR, while the negative control used template generated in the absence of RT.

Results

Construction and characterization of the Δ*spy1343* isogenic mutant strain. To determine if *spy1343* is involved in regulation of the putative *ato* operon, we inactivated the gene by allelic replacement using standard methods (Sitkiewicz and Musser, 2006). The mutant strain was confirmed to have the correct construction by PCR and DNA sequence analysis (data not shown) and gene transcript studies (see below). The Δ*spy1343* isogenic mutant strain had no observed phenotypic difference from the parent strain during growth in laboratory media. For example, the growth of the wild-type and mutant strains was identical in THY medium (Fig. 2), and colony morphology was identical on blood agar plates (data not shown).

***spy1343* regulates transcription of the putative *ato* operon.** To test the hypothesis that *spy1343* regulates transcription of the genes in the putative *ato* operon, we compared transcript levels of *spy1343* and the *atoB* gene in the wild-type and Δ*spy1343* mutant strains during early-, mid-, late-exponential, and stationary growth phases. TaqMan analysis indicated that the level of *spy1343* transcript was constant throughout growth in the wild-type strain, and as expected, transcript was not detected in the Δ*spy1343* mutant strain (Fig. 3A). Interestingly, the *atoB* transcript level was decreased two-fold in the Δ*spy1343* mutant compared to wild-type in early-exponential phase, and then gradually attained wild-type transcript levels by stationary phase (Fig. 3B, red bars). This result indicates that *spy1343* is not a direct negative regulator of the *ato* genes.

Deletion of *spy1343* results in hypervirulence in mice. Because the level of *spy1343* transcript increased when GAS interacted with human PMN *ex vivo* (Graham *et al.*, 2005), we hypothesized that this gene played a role in GAS-host interactions. To test this hypothesis, we compared the virulence for mice of the wild-type and Δ*spy1343* mutant strains after intraperitoneal inoculation, as described previously (Sitkiewicz and Musser, 2006). Unexpectedly, mice infected with the Δ*spy1343* mutant strain died significantly more rap-

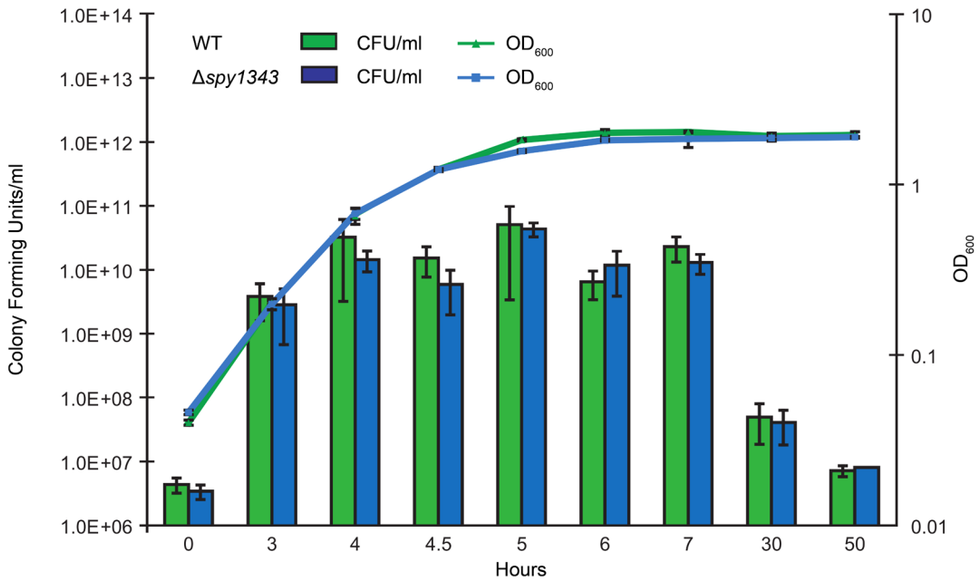

Fig. 2. Growth curve of MGAS5005 (WT) and Δ*spy1343* mutant strains in THY medium. Growth was determined both by OD_{600} measurement (right) and plating aliquots taken at the indicated times on THY agar plates (left).

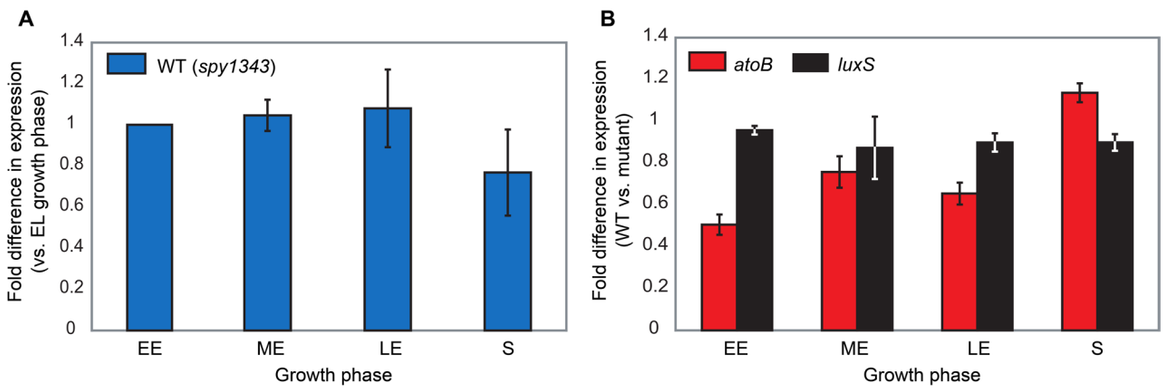

Fig. 3. TaqMan analysis of *spy1343*, *ato*B, and *lux*S expression.

(A) Transcript levels of *spy1343* in the wild-type (WT) strain during early-exponential (EE), mid-exponential (ME), late-exponential (LE) and stationary (S) growth phases. Results were standardized to an internal control gene (*pro*S) and compared between growth points using the $\Delta\Delta C_T$ method. Transcription in early exponential phase (EE) was used as basal level and differences in transcription during other phases were compared to it. (B) Comparison of transcript levels of *ato*B and *lux*S in wild-type and Δ*spy1343* mutant strains. Transcript levels for each gene at EE, ML, LL and S phases were determined separately for each strain, standardized to *pro*S and compared between strains using the $\Delta\Delta C_T$ method.

idly and in greater numbers than mice infected with the wild-type strain ($P = 0.006$; Fig. 4).

Hypervirulence for mice is not caused by altered *lux*S expression. LuxS in GAS regulates expression of several virulence factors, including SLS, SpeB and M protein (Lyon *et al.*, 2001; Marouni and Sela, 2003). To determine if the mouse hypervirulence phenotype was associated with changes in *lux*S transcript level, we compared the transcript level of *lux*S in the wild-type and Δ*spy1343* mutant strains during growth in THY medium. In contrast to *ato*B, the *lux*S transcript level was constant in all four stages of GAS growth tested and was independent of *spy1343* (Fig. 3B, black bars). This result suggested that *lux*S was not under the same transcriptional regulation as *ato*B. To seek additional support for this hypothesis, we used reverse-transcription polymerase chain reaction (RT-PCR) to characterize the length of transcripts generated from the *ato* promoter. cDNAs were synthesized using purified RNA by RT reactions with primers specific for *luxS*, *M5005_Spy_1348* (*spy1348*), or *atoA* (*M5005_Spy_1346*) (See Fig. 5, legend). When cDNA generated with the *lux*S primer was used as template, only primers that amplified internal fragments of the *lux*S gene yielded a PCR product. That is, use of the *lux*S primer with primers designed to amplify larger transcripts from the *ato* region failed to generate a detectable amplicon (Fig. 5, panels A-C). When cDNA generated with *ato*A or

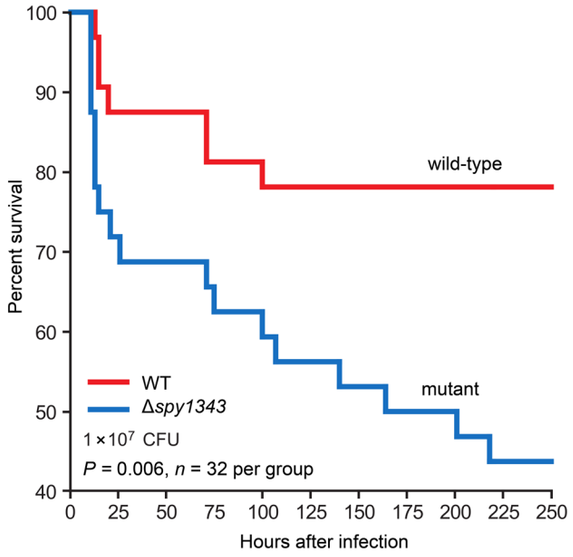

Fig. 4. Survival of mice after intraperitoneal inoculation with wild-type MGAS5005 (WT) or Δ*spy1343* mutant strains.
Kaplan-Meier curves were generated and datasets were compared (logrank test) using GraphPad Prism v. 4.02.

spy1348 primers was used as template, amplification products were obtained with primers that annealed throughout the region (Fig. 5, panels D-G). Taken together, these RT-PCR results demonstrated that *M5005_Spy_1344-1348* yield a multi-gene transcript consistent with an operon organization. These results strongly support the hypothesis that the *luxS* transcript is separate and distinct from this multi-gene transcript.

Discussion

The predicted product of *spy1343* is a member of the LysR family of DNA-binding proteins. These gene regulators have a conserved N-terminal helix-turn-helix domain followed by a less-conserved C-terminal domain which is thought to participate in binding of a co-inducer (see below) (Schell, 1993). In addition to their structural similarities, LysR family members are often transcribed from divergent promoters that are located close to, or overlap, the promoter of their target gene(s). The putative promoter regions of *spy1343*

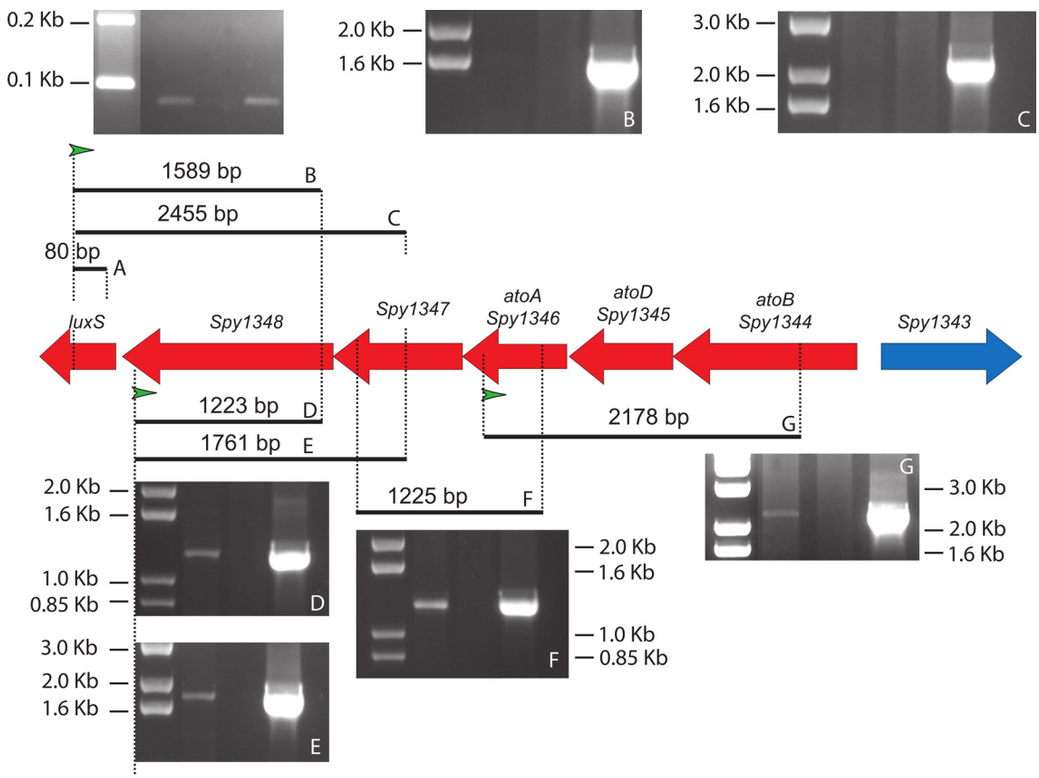

Fig. 5. Analysis of transcripts derived from the *M5005_Spy_1344-luxS* region.
Red and blue arrows represent ORFs; green arrowheads denote location of primer used for cDNA synthesis; thick horizontal black lines represent expected length of products obtained by PCR amplification of cDNAs. cDNA template generated with primer *luxS-taq-3'* was used in PCR reactions with primer pairs *luxS-taq-3'*/*luxS-taq-5'* (A), *luxS-taq-3'*/1348F (B), and *luxS-taq-3'*/*ato*AF (C). cDNA template generated with primer 1348R was used in PCR reaction with primers 1348R/1348F (D), 1348R/1347F (E), and 1348R/atoAF (F). cDNA generated with primer *ato*AR was used in PCR reaction with primers *ato*AR/*ato*B-*taq*-5' (G). Each gel shows four lanes which contain, from left to right: lane 1, DNA ladder (A, 100 bp DNA ladder (Invitrogen); B-G, 1 kb DNA ladder (Invitrogen)), lane 2, PCR with cDNA template, lane 3, negative control (RT was omitted from cDNA synthesis), lane 4, positive control (genomic DNA).

and the *ato* operon overlap, which is consistent with the genes sharing a common regulator (Schell, 1993; Zaim and Kierzek, 2003). Many LysR proteins also bind a co-inducer, such as ions (NhaR), flavonoids (NocR), octopine (OccR), N/O-acetylserine (CysB), or indoglycerol phosphate (TrpI) for optimal activity (Schell, 1993).

Our TaqMan analysis showed that *spy1343* regulates transcription of the *ato* genes. *ato*B transcript levels were decreased in the *spy1343* mutant strain (Fig. 3B), suggesting that *spy1343* acts as an activator rather than a repressor. This result is consistent with the regulation of the *ato* operon in *E. coli*, which is activated by the upstream *atoCS* two-component system(Jenkins and Nunn, 1987b). However, in a previous report by Graham *et al.* (2005), *spy1343* (designated SPy1634) and the *ato* genes (designated SPy1637-41) had an inverse transcriptional profile. That is, the transcript level of *spy1343* increased over time, whereas the level of *ato* gene transcripts decreased (supplemental Table I in Graham *et al.*, 2005). This result suggested that *spy1343* functions as a repressor. The difference in our data could be attributed to several factors. First, the signals encountered by GAS upon contact with human blood, which contains multiple cell types and other factors, are likely different from those present during culture in laboratory media. Second, the microarray data noted changes in transcript level at increasing times (30, 60, and 90 min) after contact with human blood, whereas in our TaqMan analysis, the greatest difference in transcript levels occurred during early-exponential phase, approximately 2 h after sub-culture in THY broth. These qualitative and temporal differences in our methods could have affected the results. Finally, many LysR family members are known to require a co-inducer for full activity. Absence of this co-inducer during culture in broth could influence the *spy1343*-promoter interaction. Despite these differences, our data clearly indicate that *spy1343* regulates transcription of *ato* genes, and we propose that *spy1343* be named *ato*R, for *ato* regulator.

Inspection of the published GAS genome sequences revealed a second *ato*RBDA locus present in GAS strains (region 2 *M5005_Spy_0116-0121*, Fig. 1). The putative regulatory proteins share only 37% identity, and downstream from $atoR_2$ there is an additional gene not present in region 1. The predicted product of this ORF shares sequence similarity to *ato*E from *E. coli,* which encodes a short-chain fatty acid transporter (NCBI BLAST). Region 2 also lacks M5005_Spy_1347 and M5005_Spy_1348, which are involved in butanoate metabolism and transport. Additionally, $atoR_2$ in both sequenced M1 strains and the M6 genome (Sumby *et al.*, 2005; Banks *et al.*, 2004; Ferretti *et al.*, 2001), has a frameshift mutation that introduces a stop codon at amino acid 139. Therefore, a truncated and possibly non-functional regulator is predicted in these strains. And lastly, the microarray data from Graham *et al.* show that there is no clear pattern of transcriptional regulation of the ato_2 genes upon GAS contact with human blood (supplemental Table I in Graham *et al.*, 2005). Unlike the five genes in region 1, which had an identical trend in transcription levels, and decreased with increased exposure times, the transcript level of $atoB_2$ and $atoD_2$ increased at 60 min after culture in blood, whereas $atoA_2$ transcript level was decreased at 60 min. Taken together, these data suggest that the ato_2 locus has a different specificity, function, and regulation than *ato* region 1.

Inactivation of *ato*R resulted in hypervirulence in our mouse model of GAS sepsis. TaqMan and RT-PCR analyses indicated that transcriptional control of *lux*S is independent of the *ato* genes, and therefore changes in *lux*S transcription likely were not responsible for this phenotype. This result suggests that hypervirulence is due to changes in transcription of the *ato* operon. Although our studies did not directly address the function of the *ato* genes in GAS, based on the conserved operon organization and sequence similarity with *ato* genes of known function, it is likely that proteins encoded by the GAS *ato* genes participate in SCFA metabolism.

Several reports have linked SCFA metabolism to bacterial virulence. For example, exposure of *Salmonella dublin* to SCFAs increased RpoS-dependent transcription of *spv*ABCD, genes involved in pathogenesis (El-Gedaily *et al.*, 1997). Additionally, the presence of SCFAs in the distal ileum has been shown to increase expression of proteins required for *Salmonella typhimurium* epithelial cell invasion (Lawhon *et al.*, 2002), and exogenous SCFAs increased *S. typhimurium* invasion of eukaryotic cells (Durant *et al.*, 1999). SCFAs produced by *Porphyromonas gingivalis* (Kurita-Ochiai *et al.*, 1995) and other anaerobic bacteria can down-regulate host immune responses by inhibiting phagocytosis, cytokine production, and lymphocyte proliferation (Kurita-Ochiai *et al.*, 1995; Eftimiadi *et al.*, 1990). Succinate, a SCFA produced by *Bacteroides* during infection, has been shown to inhibit migration of PMNs and phagocytic killing by inhibiting the respiratory burst (Rotstein *et al.*, 1987). Of note, *Aggregatibacter actinomycetemcomitans*, which also carries a similar *ato* region as GAS, some species of *Bacteroides*, and *Porphyromonas gingivalis*, are oral pathogens that can inhabit the same host niche as GAS. Finally, SCFAs can induce apoptosis in a variety of cell types (Kurita-Ochiai *et al.*, 1995; 2002).

Research on GAS has traditionally focused on virulence factors such as extracellular toxins and adhesins. Our work suggests that manipulation of GAS metabolic properties can influence virulence through the accumulation of certain byproducts during infection. Our data suggest that *ato*R is a positive regulator of the *ato*

operon, and therefore the Δ*ato*R mutant strain could be impaired in degrading SCFA *in vivo*. An increase in the local concentration of SCFA could inhibit the host immune system and account for the observed hypervirulence.

Acknowledgments

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ORIGINAL PAPER

Expression of the Fluoroquinolones Efflux Pump Genes *acr*A and *mdf*A in Urinary *Escherichia coli* Isolates

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Abstract

Escherichia coli is one of the most frequent causes of urinary tract infections. Efflux system overexpression is reported to contribute to *E. coli* resistance to several antibiotics. Our aim in this study was to investigate the relation between antibiotic resistance and the expression of the efflux pump genes *acr*A and *mdf*A in *E. coli* by real-time reverse transcription-PCR. We tested the *in vitro* susceptibilities to 12 antibiotics in 28 clinical isolates of *E. coli* obtained from urine samples. We also determined the minimum inhibitory concentrations of levofloxacin to these samples. We then revealed significant correlations between the overexpression of both *mdf*A and *acr*A and MICs of levofloxacin. In conclusion, we demonstrated that the increased expression of efflux pump genes such as *mdf*A and *acr*A can lead to levofloxacin resistance in *E. coli*. These findings contribute to further understanding of the molecular mechanisms of efflux pump systems and how they contribute to antibiotic resistance.

Key words: *Escherichia coli*, *acr*A and *mdf*A genes, efflux pump, levofloxacin, overexpression

Introduction

Antimicrobial abuse is one of the major factors contributing to the development and maintenance of antimicrobial resistance in bacteria. The issue is of utmost importance in many developing countries, where the sale of antimicrobials is often unrestricted (Okeke *et al*., 2005).

In Egypt, *Escherichia coli* is among the most common pathogens isolated from patients with urinary tract infections and it has a high prevalence of resistance to antibiotics (Shaheen *et al*., 2004; Mohamed Al-Agamy *et al*., 2006).

Bacterial resistance to antimicrobial agents is a threat to public health in Egypt. The consequences of resistance affect not only the ability to treat the infection, but also the cost and duration of treatment (Shaheen *et al*., 2004; Mohamed Al-Agamy *et al*., 2006).

Fluoroquinolone resistance is on the rise. The emergence of this resistance in nearly all species of bacteria was documented soon after the introduction of these compounds for clinical use (Acar and Goldstein, 1997).

Levofloxacin resistance has been shown to increase more than 5-fold in 6 years. Rapid emergence of resistance may have been fuelled by the increase in the rate of levofloxacin prescribing (Johnson *et al*., 2008). A study showed that the annual rates of levofloxacin resistance of *E. coli* were 29.49% in 2005, 26.51% in 2006, 40.21% in 2007, 43.20% in 2008, and 31.75% in 2009 (Jang *et al*., 2011). The persistent increase in fluoroquinolone resistance rates influences patient management and demands a change in some existing guidelines for the treatment (Paterson, 2004; Wagenlehner *et al*., 2011; Han *et al*., 2012).

The overexpression of efflux pumps contributes to multidrug resistance (MDR) in *E. coli.* Almost all Gram-negative bacteria have genes for efflux pumps. Two transporters, AcrAB and MdfA, which belong to two distinct families of efflux pumps, have the ability to efflux quinolones in *E. coli*. Most of these efflux genes are either underexpressed or not expressed at all. When they are overexpressed, they are associated with MDR in *E. coli* (Yang, 2003; Yasufuku *et al*., 2011).

The AcrAB-TolC system of *E. coli* is among the better characterized resistance-nodulation-cell division superfamily (RND) systems. In addition to recognizing many fluoroquinolones, this system exports a diversity of agents including tetracycline, β-lactams, chloramphenicol, erythromycin, rifampicin, disinfectants, dyes, and organic solvents. AcrA is a lipoprotein situated in

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the periplasm; the inner membrane protein, AcrB, is a proton-motive-force transporter of the RND type. Strains lacking in the AcrAB proteins are hypersusceptible to many quinolones; on the other hand, fluoroquinolone-resistant efflux mutants frequently overproduce the periplasmic protein AcrA. Such mutants display decreased susceptibility to ciprofloxacin, tetracycline, ampicillin, and chloramphenicol. Removal or inactivation of proteins that suppress expression of AcrAB (AcrR and AcrS) reduces fluoroquinolone susceptibility (Drlica *et al*., 2012).

MdfA is a paradigm of multidrug-resistance antiporters. It is a member of the major facilitator superfamily (MFS) and is a transmembrane-spanning integral inner membrane protein (Edgar and Bibi, 1997).

In laboratory-generated mutants, overexpression of the efflux pumps AcrAB or MdfA have been shown to cause three to six fold increase in fluoroquinolone resistance (Yang, 2003). The simultaneous overexpression of AcrAB and MdfA results in synergistic increases in resistance to fluoroquinolones (Yang, 2003).

The overexpression of AcrA and MdfA in quinolone resistance in *E. coli* has been thoroughly investigated, yet data about it in Egypt, up until the date this paper has been written, has been really scarce.

Our aim in this study was to investigate the relation between antibiotic resistance and the expression of the efflux pump genes in *E. coli* by real-time quantitative polymerase chain reaction (RT-qPCR).

Experimental

Material and Methods

***E. coli* strains and growth conditions.** A total of 28 non-duplicate *E. coli* isolates were used in this study obtained from patients with urinary tract infection (UTI) treated in Damanhour General Hospital, Damanhour, Egypt between the years 2013 and 2014. Post treatment isolates were excluded from this study. The isolates were identified with conventional biochemical tests. All isolates were deemed to be clinically significant ($>10^5$ CFU/ml). They were selected according to their sensitivity to levofloxacin in order to correlate their sensitivity profile with the overexpression of efflux pump genes. *E. coli* isolates with intermediate susceptibility were not classified as being resistant. The strains were propagated at 37°C in Luria-Bertani (LB) broth.

Susceptibility testing. The following 12 antimicrobial discs were selected according to CLSI guidelines (CLSI, 2015): amoxicillin (AML), amoxicillin-clavulanic acid (AMC), cefuroxim (CMX), cefalexin (CL), cefepime (FEP), ceftriaxone (CRO), chloramphenicol (C), gentamycin (CN) tetracycline (TE), levofloxacin (LVX), meropenem (MEM), azithromycin (AZM) (Oxoid Ltd., Basingstoke, United Kingdom) on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, United Kingdom). Zone sizes were measured to the nearest millimetre. Using the published CLSI guidelines, the susceptibility or resistance of the organism to each drug tested were then determined. *E. coli* ATCC 25922 (LGC Standards GmbH, Wesel, Germany) was used as a control (CLSI, 2015).

Determination of the minimum inhibitory concentration (MIC) to levofloxacin. Determination of the MIC of levofloxacin was done by using the two-fold dilution method according to CLSI guidelines (CLSI, 2015). MICs were performed in 96-well microplates (Greiner, Wemmel, Belgium). Briefly, serial two fold dilutions of 2x strength antibiotic were performed in sterile distilled water. An overnight culture of the tested isolate in Mueller-Hinton Broth (MHB) was diluted in fresh double strength MHB till it reaches the concentration of 10^5 CFU/ml.

Fifty µl suspension of the organism in double strength MHB was added to the well with 0.05 ml of the antibiotic solution, and the microtiter plates were incubated aerobically at 35°C. The MIC was then defined as the lowest concentration of the antibiotic in which there is no visible growth after overnight incubation. Results were recorded in µg/ml after incubation at 35°C for 18 hours (CLSI breakpoints for levofloxacin are ≤ 2 µg/ml for susceptibility and ≥ 8 µg/ml for resistance).

RNA extraction and quantification of RNA expression using RT-qPCR (Taylor *et al*., 2010). All *E. coli* isolates were cultured at 37°C for 14 h on MacConkey agar plates (Oxoid Ltd., Basingstoke, United Kingdom), then cultured in 4 ml of Luria-Bertani medium (Oxoid Ltd., Basingstoke, United Kingdom) for 8 h. Total RNA was obtained from mid-logarithmic growth-phase bacterial cultures with Isolate II RNA Micro Kit (Bioline, Michigan, USA) (Pfaffl *et al*., 2002; Keeney *et al*., 2008). Genomic DNA contamination was removed by an on-column RNase-free DNase I digestion. RNA purity was assessed spectrophotometrically by measuring the $OD_{260/280}$ ratio by NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific – Delaware, USA). RNA integrity was assessed by visualization of one large and one small band of RNA after electrophoresis (Fleige and Pfaffl, 2006).

The quantification protocol was performed immediately following the quality control assessment. The *acr*A and *mdf*A genes were quantified by RT-qPCR using a SensiFAST™ SYBR No-ROX One-Step Kit (2x) (Bioline, United Kingdom) in a StepOne™ Real-Time PCR System (StepOne™ system, Applied biosystems, USA). The reaction mixture was prepared in a total volume of 20 µl dispensed in MicroAmp® optical microplates

Table I
Primers used in this study

Gene	Primer (5'-3')	Amplicon length (bp)	Reference
*mdf*A F *mdf*A R	CGGCAACGATATGATTCAAC CAGTGACAGTTTCTCGCCTA	523	Yasufuku *et al.*, 2011
*acr*A F *acr*A R	CTCTCAGGCAGCTTAGCCCTAA TGCAGAGGTTCAGTTTTGACTGTT	107	Swick *et al.*, 2011
*gap*A F *gap*A R	ACTTCGACAAATATGCTGGC CGGGATGATGTTCTGGGAA	215	Yasufuku *et al.*, 2011

(ThermoFisher Scientific, Michigan, USA). Each reaction mixture contained 0.5 µM of both forward and reverse primers, 10 µl of SensiFAST™ SYBR No-ROX One-Step Kit (2x) mix, 0.2 µl of reverse transcriptase, 0.4 µl of Ribosafe RNA inhibitor, 4 µl of template RNA, and nuclease-free water to complete the volume. The oligonucleotide primers for the amplification were adapted from prior studies and are listed in (Table I) (Swick *et al*., 2011; Yasufuku *et al*., 2011).

PCR-grade water was used as a negative control. Genomic DNA from the *E. coli* ATCC 25922 strain was the positive control. The melting curve analysis ensured that only a single PCR product was amplified. A no-RT control was included by omitting the reverse transcriptase from the reaction.

Each sample was placed in triplicate on a 48-well plate and subjected to one-step reverse transcription at 50°C for 30 min, 40 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s and extension at 72°C for 30 s. The *Cq* means of almost all samples in our study were less than 30 (Yasufuku *et al*., 2011).

Using the Applied biosystems stepone™ system software the relative quantification of the expression of the target genes was calculated relative to *gap*A, a housekeeping gene, and then the expression rate of each efflux pump gene was determined by using *E. coli (*ATCC 25922) as the control (Pfaffl *et al*., 2002; Bohnert *et al*., 2007).

Correlation of overexpression of efflux pump genes with MICs of each antibiotic. The overexpression of efflux pump genes was identified as ≥ 2-fold gene expression compared to that of the standard strain *E. coli* (ATCC 25922) in qRT-PCR (Keeney *et al*., 2008). The correlation of the overexpression of efflux pump genes with the MICs of levofloxacin was then determined. The correlations between the pattern of resistance and the expression of both genes together with the MICs of levofloxacin have also been determined.

Statistical analysis. Statistical analyses were performed by using Statistical Package for Social Sciences (SPSS) software version 17 with Spearman's correlation test; $p < 0.05$ was considered statistically significant.

Results

Susceptibility testing. Susceptibility testing for the 12 antibiotics was performed according to CLSI guidelines (CLSI, 2015). It showed that 79% of the isolates were MDR. MDR is most commonly defined as resistance to ≥ 3 classes of antibiotics (Magiorakos *et al*., 2012). The resistance patterns of the isolates (the number of antibiotics to which each isolate is resistant) to the studied antibiotics are shown in (Table II).

Table II
Susceptibility of antimicrobial agents

Antibiotic	No. (%) of isolates (n = 28)		
	Susceptible	Intermediate	Resistant
AML	0 (0)	1 (3.6)	27 (96.4)
AMC	10 (35.7)	6 (21.4)	12 (42.8)
CMX	6 (21.4)	4 (14.3)	18 (64.3)
CL	6 (21.4)	2 (7.1)	20 (71.4)
FEP	14 (50)	0 (0)	14 (50)
CRO	10 (35.7)	2 (7.1)	16 (57.1)
C	20 (71.4)	0 (0)	8 (28.6)
CN	14 (50)	0 (0)	14 (50)
TE	10 (35.7)	0 (0)	18 (64.3)
LEV	0 (0)	3 (10.7)	25 (89.3)
MEM	28 (100)	0 (0)	0 (0)
AZM	8 (28.6)	0 (0)	20 (71.4)

Minimum Inhibitory Concentration (MIC) and gene expression. Determination of the MIC of levofloxacin was done by using the two-fold dilution method according to CLSI guidelines (Clinical and Laboratory Standards Institute. 3rd ed. 2015). All the tested isolates (100%) were levofloxacin resistant. Results of determination of MIC of levofloxacin are shown in (Table III).

Expression of *acr*A and *mdf*A genes. Among the 28 isolates, 23 isolates (82.1) % showed overexpression of *mdf*A ranging from 2–34.47 folds, and 22 (78.6%) showed overexpression of *acr*A ranging from 2–9.52 folds (Table III).

Table III
Resistance pattern of strains, MIC of levofloxacin and analysis of quantification of RNA expression using qRT-PCR

Sample no.	Resistance pattern	MIC of Levofloxacin (μg/ml)	Expression of	
			*mdf*A*	*acr*A*
1	8	32	2.52	2.66
2	11	128	4.93	7.71
3	11	32	2.49	0.43
4	4	64	4.90	3.93
5	10	128	11.95	9.18
6	2	8	1.15	1.45
7	6	8	1.03	0.89
8	10	64	2.83	6.44
9	9	64	3.47	9.52
10	4	32	1.70	8.18
11	10	256	34.47	2.55
12	9	256	11.45	15.1
13	6	128	4.07	8.65
14	8	32	2.6	3.05
15	11	64	3.96	2.87
16	3	32	1.78	2.43
17	9	128	7.76	7.98
18	11	64	2.94	3.32
19	8	256	5.58	6.93
20	10	32	2.67	3.32
21	2	8	1.06	0.95
22	10	128	7.93	7.39
23	4	32	2.55	1.89
24	9	64	4.32	3.99
25	6	32	2.78	2.09
26	10	32	3.65	1.24
27	8	128	6.44	7.84
28	6	64	3.99	2.33

* Average of three independent replicates

Correlation of overexpression with MIC and patterns of antibiotic resistance. The overexpression of efflux pump genes was defined as ≥ 2-fold gene expression compared to that of the standard strain *E. coli* (ATCC 25922) in qRT-PCR. A strong positive correlation of the overexpression of both of *mdf*A and *acr*A with MICs of levofloxacin was detected (Spearman correlation 0.93 and 0.72 respectively, $P < 0.05$) by bivariate analysis.

Moderate positive correlation was found between the expression of *mdf*A and the pattern of antimicrobial resistance of each isolate (Spearman correlation 0.45, $P < 0.05$). This was not the case with the expression of *acr*A where no significant correlation with the pattern of antimicrobial resistance was found.

Moderate positive correlation was also found between levofloxacin MIC and the pattern of antimicrobial resistance of each isolate (Spearman correlation 0.45, $P < 0.05$)

Discussion

Multidrug resistance is a growing public health concern worldwide (Okeke *et al.*, 2005). The increasing incidence of resistance among pathogenic *E. coli* makes the treatment difficult with an unpredictable outcome (Kariuki *et al.*, 2007). Resistance to quinolones has reached 50% in some parts of the world (Kronvall, 2010). This leads to a challenge in the use of this class of drugs. It is now necessary to investigate the possible molecular mechanisms by which *E. coli* strains acquire antibiotic resistance in an attempt to prevent further spread of these kinds of resistant strains and prevent the occurrence of new resistant ones.

In the present study we examined 28 *E. coli* isolates, all isolates (100%) were levofloxacin resistant and 79% were MDR. Similar to our results, Swick *et al.* (2011) found that no isolate categorized as MDR was flouroquinolone susceptible. They also suggested the possibility of an underlying correlation between fluoroquinolone resistance and MDR, this was confirmed in our study where moderate positive correlation was found between levofloxacin MIC and the pattern of antimicrobial resistance of each isolate (Spearman correlation 0.45, $P < 0.05$)

There is a widespread, escalating use of fluoroquinolones. Even contrary to guidelines that recommend them in only select situations, the frequent use of fluoroquinolone antibiotics has been observed in nearly all settings. Quinolone antibiotics now have exceeded sulfa antibiotics in recent years as the most commonly prescribed treatment for outpatient UTIs in women (Kallen *et al.*, 2006).

We quantified the expression of the efflux pump genes known to affect fluoroquinolone resistance in a single quantitative study and we used *gap*A as a house keeping gene. Housekeeping genes provided internal controls, which were used as a measure of mRNA expression levels irrespective of growth condition (Szabo *et al.*, 2004). Jandu *et al.* (2009) reported that the expression of *gap*A was similar when detected under four different growth conditions so it was chosen as an internal control for our study.

We found that most of the levofloxacin resistant isolates overexpressed both *mdf*A and *acr*A genes (82.1% and 78.6% respectively). This was with agreement of other studies, where overexpression of *acr*A together with *acr*B led to a three- to six-fold increase in fluoroquinolone resistance, while augmenting the level of *acr*AB through the overexpression of the tran-

scriptional regulator, *sdi*A, resulted in about the same (approximately six-fold) increase in drug resistance. Yasufuku *et al*. (2011) revealed a significant correlation of the overexpression of *mdf*A with higher MICs of levofloxacin (Rahmati *et al.*, 2002; Yang, 2003).

In general the more severe the MDR phenotype, the higher the likelihood that the isolate also overexpressed *acr*AB (Nikaido and Pagès, 2012). Han *et al*. (2012) found that out of 89 patients with fluoroquinolone resistant isolates, 49.4% overexpressed *acr*AB.

A synergy in quinolone resistance was shown when *acr*AB was overexpressed simultaneously with *nor*E or *mdf*A. Deletion of *acr*AB alone and all of the three pumps together had the same effect on the susceptibility of fluoroquinolones. The authors also found that the strain with *acr*AB deletion were the most susceptible when compared to mutants strains with deletion in *nor*E and/or *mdf*A. The maximum quinolone resistance mediated by efflux pumps was suggested to be ~10-fold, irrespective of any increase in production of these pumps (Yang, 2003).

Studies conducted with efflux pump deleted (ΔAcrAB) strain delayed the emergence of resistance suggesting that the inhibition of AcrAB efflux pump could be a strong strategy for slowing the development of resistance in clinically important Gram-negative bacteria (Singh *et al*., 2012)

In our study 17.9% and 21.4% of the levofloxacin resistant isolates didn't show over expression of *mdf*A and *acr*A respectively, thus flouroquinolone resistance may be contributed to other mechanisms such as gyrase or topoisomerase IV mutations. Morgan-Linnell *et al*. (2009) reported mutations in gyrase and/topoisomerase IV in 100% of the high-level-resistant *E. coli* clinical isolates, while over production of the efflux pump (AcrA) contributed approximately 33% to the overall quinolone resistance. As to the *mdf*A gene, one of the levofloxacin susceptible isolates showed very slight overexpression.

It should be noted that expression of pump genes can be induced by natural substrates encountered during infection. For example, indole and bile, which would be encountered in the mammalian gut, induce expression of *acr*AB in *E. coli* and *Salmonella* (Sun *et al*., 2014).

Though increased efflux pump expression correlates with increase in levofloxacin MIC, we did not compare expression of efflux pumps in levofloxacin sensitive strains. Thus, this has to be investigated as efflux pump expression might be high in these strains too if they are resistant to other antibiotics since both pumps are broad spectrum. Some studies have found that resistance to antibiotics through the same efflux pump is common (Poole, 2005).

An approach of blocking the efflux pump protein or gene has been studied. This falls under the class of biological inhibition of efflux pumps. The efflux pumps could be switched off with the means of specific antibodies (Yoshihara and Inoko, 2011). The genes encoding these pumps or their regulators could be blocked by means of the antisense strategies. The antisense approach has been revealed to work for AcrAB efflux pump in *E. coli* and has also been patented (Oethinger and Levy, 2001; 2011).

Efflux pump inhibitors can also be used for the evaluation of the effect of efflux pumps on resistance emergence, however there are two major concerns with such inhibitors. First, some of these inhibitors have been found to be substrates of efflux pumps themselves, thus questioning their credibility (Lomovskaya and Bostian, 2006). Second, the concentrations required for efflux pump inhibitory activity are often associated with toxicity and hence, are not clinically relevant (Schmitz *et al*., 1998).

While, only one Gram-negative bacterial species has been evaluated in our study, it is imperative to investigate other bacterial species to increase the clinical relevance of our results. Different inhibitory techniques can be utilized to study the different efflux pumps. These should not only be effective against multiple efflux pumps in one bacterial species but also against efflux pumps in more than one bacterial species.

In conclusion, we demonstrated a significant positive correlation between the overexpression of AcrA and MdfA efflux pumps and the resistance to levofloxacin in *E. coli* clinical isolates. These findings contribute to further understanding of the molecular mechanisms of efflux pump systems and how they contribute to antibiotic resistance and suggest that developing new EPI could lead to treatments for flouroquinolones resistant bacteria.

Resistance emergence presents a debilitating challenge in the management of infectious diseases. Data from this study might add to our understanding of resistance development. Our findings suggest that the inhibition of efflux pumps could be a potential strategy to thwart the problem of antibiotic resistance. Considering the genetic plasticity in the bacteria, these findings just seem to be the tip of the iceberg and much needs to be unravelled in the future.

Literature

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ORIGINAL PAPER

Antimicrobial Effects of Platelet-rich Plasma against Selected Oral and Periodontal Pathogens

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Abstract

Antimicrobial properties of platelet rich plasma (PRP) against various microorganisms have been recently pointed out. PRP could be an alternative to conventional antibiotics in preventing oral and periodontal infections. We examined whether PRP has *in vitro* antimicrobial properties against *Aggregatibacter* actinomycetemcomitans, *Prophyromonas gingivalis*, *Staphylococcus aureus* and *Candida albicans*. PRP and platelet-poor plasma (PPP) were obtained from whole blood of 10 healthy volunteers and 10 periodontitis patients. *In vitro* laboratory susceptibility was carried out using the modified agar diffusion method by measuring the diameters of inhibition zones on agar plates coated with selected microbial strains. Both calcium chloride (CaCl2) activated and non activated samples were tested. Both activated PRP and PPP, of both patients and controls, effectively inhibited the growth of *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans*. However, a statistically significant difference in favor of PRP was found indicating more susceptibility to PRP than PPP ($p < 0.05$). Non activated PRP and PPP exhibited negative zones of inhibition against the studied microorganisms. There was no activity against *S. aureus*. No statistically significant difference was found between the antimicrobial effects of PRP and/or PPP obtained from patients and controls ($p > 0.05$). We conclude that PRP is a potentially useful substance against oral and periodontal pathogens. Activated PRP was found to be more active than activated PPP and the activation of coagulation is a fundamental step. Additionally, the antimicrobial activity of PRP and/or PPP seems not to be affected by periodontitis.

Key words: antimicrobial activity, oral infection, platelet-rich plasma

Introduction

Platelet-rich-plasma (PRP) is an autologous concentration of platelets in plasma developed by gradient density centrifugation. It has been increasingly used in a variety of medical fields. The effectiveness of this procedure lies in the delivery, up on stimulation, of a wide range of platelet growth factors mimicking the physiologic wound healing and reparative tissue processes (Werner and Grose, 2003). Current evidence suggests that platelets have multiple functional attributes indicative of an integral role in antimicrobial host defense (Yeaman *et al*., 1998; Tang *et al*., 2002). These functions include navigation toward the inflammatory chemoattractant N-f-etLeuPhe, expression of Fc and complement C3a/C5a receptors, and the capacity to generate antimicrobial oxygen metabolites. Platelets interact directly with microorganisms, contribute to clearance of pathogens from the bloodstream, and significantly participate in antibody dependent cell cytotoxicity against microbial pathogens (Yeaman, 1997; Yeaman and Bayer, 1999). Moreover, platelet α-granules contain catecholamines, serotonin, osteonectin, von Willebrand factor, proaccelerin and other substances (Anitua *et al*., 2005; Bielecki *et al*., 2006). These are released in high concentrations after platelet aggregation and may also have antibacterial effects. Yeaman *et al*. (1998) and Tang *et al*. (2002) had shown that platelet microbicidal proteins released after platelet activation demonstrate potent activities against many gram-negative, gram-positive and fungal pathogens. Collectively, these findings suggest that human platelets possess, and can be stimulated to release, several antimicrobial polypeptides (Krijgsveld *et al*., 2000). The antimicrobial potential of

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platelets might be increased through their concentration in PRP (Drago *et al*., 2013).

Despite extensive use in clinical procedures, only few studies have investigated PRP's microbicidal activity (Bielecki *et al*., 2007; Moojen *et al*., 2008; Anitua *et al*., 2012; Burnouf *et al*., 2013; Drago *et al*., 2013). Mariani *et al.* (2015) tested the effect of PRP against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*, as species potentially involved in bone, soft tissue and wound infections (Zalavras *et al*., 2004). In the current study, we have selected four microorganisms that are commonly found in oral and periodontal infections and investigated whether PRP could have *in vitro* antimicrobial effects against them.

Aggregatibacter actinomycetemcomitans and *Porphyromonas gingivalis* are major periodontal pathogens. *A. actinomycetemcomitans* is a nonmotile, capnophilic, Gram-negative, coccobacillus, and a facultative anaerobe. *P. gingivalis* is a nonmotile, coccobacillus, and an obligate anaerobe. Both pathogens produce a number of virulence factors and damaging metabolites that are associated with initiation and progression of periodontal destruction. Adjunctive antimicrobial therapy along with appropriate mechanical therapy helped in the elimination of these pathogens. The linking of the inductive and antimicrobial effects of PRP may be beneficial for the treatment of periodontal disease (Teles *et al*., 2006).

S. aureus has been associated with dentoalveolar infections and oral mucosal lesions. Studies suggested that the presence of *S. aureus* in saliva was a significant risk factor for aspiration pneumonia (Terpenning *et al*., 2001; El-Solh *et al*., 2004). Oral *S. aureus* may also serve as a reservoir for cross-infection to other patients, as well as health care staff (Smith *et al*., 2003). Additionally, some studies have demonstrated that *S. aureus* plays a role in exacerbating dental diseases by forming a biofilm with the causative pathogens of periodontal diseases (Smith *et al*., 2001; Cuesta *et al*., 2010; Passariello *et al*., 2012).

Oral candidiasis is the most common fungal infection encountered in general dental practice. It manifests in a variety of clinical presentations and can occasionally be refractory to treatment. It is caused by commensal *Candida* species. The most commonly implicated strain is *C. albicans* which is isolated in over 80% of oral candidal lesions (Farah *et al*., 2010).

The development of new, cost-effective and safe measures to prevent and control oral and periodontal infections is important. The present study was undertaken to test the *in vitro* antimicrobial activity of activated and non activated PRP and PPP against *A. actinomycetemcomitans*, *P. gingivalis*, *S. aureus* and *C. albicans*.

Experimental

Materials and Methods

Study design and donors. The study was conducted in compliance with good clinical practice guidelines and the Declaration of Helsinki. The subjects of this study were patients who visited the outpatient clinic of the Faculty of Dentistry, October 6 University. Ten patients diagnosed with generalized chronic periodontitis (Armitage, 1999) (mean age ± SD: 45.2 ± 3.1 years) and ten periodontally healthy subjects with no clinical attachment loss and a probing pocket depth less than or equal to 3mm (mean age ± SD: 42.7 ± 3.4 years) were included. All subjects received verbal and written information about the study and signed consent forms before enrollment in the study. Criteria for exclusion were the use of antibiotics or other antimicrobial and/or anti-inflammatory medicaments during the previous 3 months, smoking, pregnancy, suffering from systemic disorders and/or presence of infection. Subjects with hemoglobin concentrations < 11 g/dl and platelet numbers $\leq 150 \times 10^3$/µl, were also excluded from the study.

Collection of samples. Sub-gingival samples were taken from the ten chronic periodontitis patients after rinsing the mouth with distilled water. Samples from the sub-gingival pockets were collected with a Gracey 7/8 curette, after relatively isolating the zone with cotton rolls and high-power suction and removing supra gingival biofilm. Each patient contributed a total of four samples collected from the deepest pocket of each patient's dentition quadrant. Samples from all patients (40 samples) were placed in sterile PBS (phosphate buffer solution, pH 7.4) transport medium, and kept at –4°C until processing. The samples were processed within 6 hours.

Microbiological procedures. The isolates were all purified and identified to species level. Tenfold serial dilutions were prepared in sterile phosphate buffered saline. Appropriate dilutions were plated onto non-selective 5% horse blood agar plates (Oxoid no. 2; Oxoid Ltd, Basingstoke, England) supplemented with haemin (5 Fg/l) and menadione (1 Fg/l) (BA), and on trypticase soy-serum bacitracin-vancomycin (TSBV) plates (Slots, 1982) for selective isolation and growth of *A. actinomycetemcomitans.* TSBV plates were incubated at 35°C in 10% CO_2/90% N_2 for 4 days.

Identification of isolates. Microscope studies with Gram coloring and biochemical tests were used to identify the microorganisms. Identification of *A. actinomycetemcomitans* and *P. gingivalis* isolates was carried out using the criteria and techniques of Slots (1986; 1987). *P. gingivalis* was identified based on microscopic morphology, Gramreaction and detection of a trypsin-like activity based on the degradation of benzoyl-DL-

arginine-2-naphthylamide (Sigma, St Louis, MO, USA) (van Winkelhoff *et al.*, 1988). Identification of *A. actinomycetemcomitans* was based on the characteristic colony morphology (star-like inner structure) on the TSBV plate, and a positive catalase reaction upon exposure to 3% H_2O_2. *S. aureus* were grown in plates containing Mannitol Salt Agar (Becton Dickinson, USA) and was identified in this media by the production of golden color zones around the colonies. Additionally, catalase and coagulase tests were performed (Murray *et al.*, 1999).

For the isolation and identification of *C. albicans*, we used the exhaustion technique in plates of chromogenic agar media, CandiSelect (Bio-Rad, France), which is a specific culture media for the growth of yeasts. Blue colonies were identified as *C. albicans*.

Platelet rich plasma preparation. PRP was prepared following a one-step procedure according to Anitua's protocol (Anitua *et al.*, 2008). Peripheral blood (4.5 ml) from each donor was taken by venipuncture into 5 ml blood-collecting tubes containing 0.5 ml of sodium citrate solution (3.8%) as anticoagulan and centrifuged using a laboratory centrifuge (Centurion, Scientific limited, UK) at 460 × g for 8 min, thus obtaining three layers: platelet-poor plasma (PPP) on the top of the tube, PRP in the middle and erythrocytes at the bottom of the tubes. Subsequently, PPP component was removed. PRP, located on the red blood cell pellet, was carefully harvested avoiding leukocyte collection. The platelet and leukocyte counts in the peripheral blood, PRP and PPP were measured automatically using a hematology analyzer. According to Anitua *et al.* (2008) platelet concentration in PRP is at least twice the concentration in whole blood; while leukocyte concentration is consistently lower ($< 10^3$ white blood cells/μl). Platelets should be almost absent in PPP. PRP and PPP were activated shortly before use by the addition of 10% calcium chloride (50 μl per ml of PRP or PPP).

Determination of antimicrobial activity. Antimicrobial activity of PRP, PPP against *A. actinomycetemcomitans*, *P. gingivalis*, *S. aureus* and *C. albicans* were carried out according to the modified agar diffusion method. Both CaCl2 activated and non activated samples were tested. Controls were performed using CaCl2 *per se* at a final concentration of 4.5 mM.

An overnight culture of *S. aureus* and *C. albicans* and three day culture of *A. actinomycetemcomitans* and *P. gingivalis* were adjusted to reach a cell density of approximately 10^7 to 10^8 cfu/ml and was surface inculcated on BHA plates, then fixed volumes of PRP with CaCl2, PPP with CaCl2 and CaCl2 were aseptically added, by using separate micropipettes, to cups made in these plates. Plates were incubated for 24 h for *S. aureus* and *C. albicans* at 37°C and for 3–7 days under microaerophilic condition for *A. actinomycetemcomitans* and *P. gingivalis*. Twenty four hours after incubation, activity was assessed by measuring the zones of inhibition, which were recorded as the diameter in millimetres across the centre of the embedded discs.

Fig. 1. Antimicrobial activity of platelet rich plasma against *A. actinomycetemcomitans* using disc diffusion method.

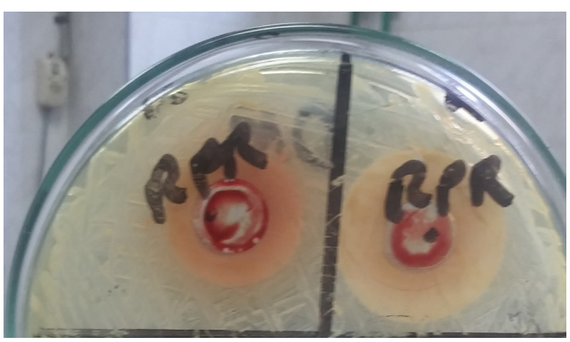

Fig. 2. Antimicrobial activity of platelet rich plasma against *P. gingivalis* using disc diffusion method.

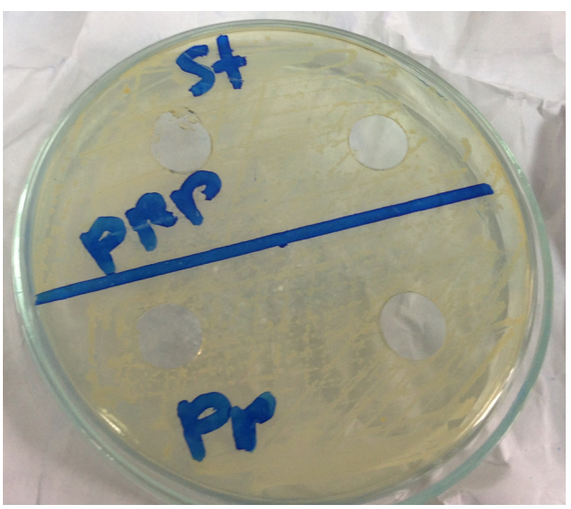

Fig. 3. Antimicrobial activity of platelet rich plasma and platelet poor plasma against *S. aureus* using disc diffusion method.

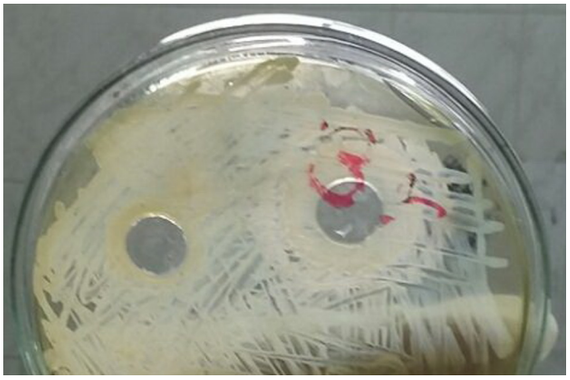

Fig. 4. Antimicrobial activity of platelet poor plasma and calcium chloride against *P. gingivalis* using disc diffusion method.

Statistical analysis. The data are presented as the mean ± SD. Statistical differences were evaluated using the Mann-Whitney U test with a p-value ≤ 0.05 considered to be significant.

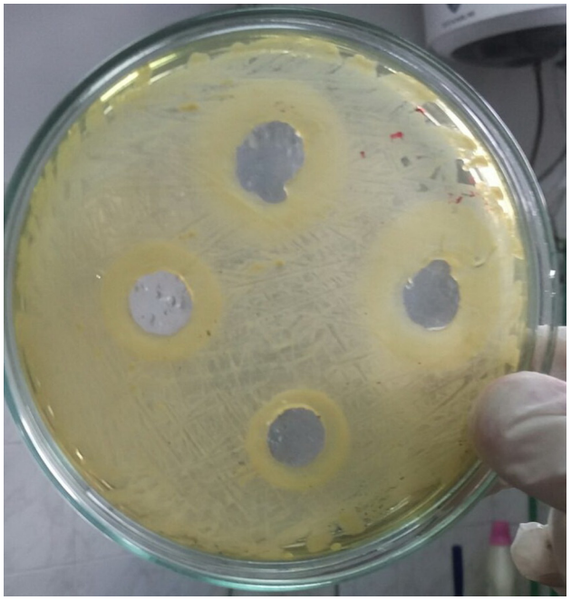

Fig. 5. Antimicrobial activity of platelet rich plasma, platelet poor plasma and calcium chloride against *C. albicans* using disc diffusion method.

Results

No significant difference has been found between platelet concentration in PRP pooled blood samples of periodontitis patients and controls ($p > 0.05$). It has been found to be about 2.5 times higher than in whole blood (Table I). Platelets were almost absent in PPP of both patients and controls ($< 10^2$ platelets/ml) (Table I). All pooled PRP and PPP was efficiently leukocyte-depleted ($< 10^3$ white blood cells/ml).

In vitro antimicrobial activity of PRP and PPP (patients and controls) showed that both of them effectively inhibited the growth of *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* but not *S. aureus*. A statistically significant difference in favor of PRP has been found indicating more susceptibility to PRP than PPP ($p < 0.05$). Only activated samples displayed an antimicrobial activity, whereas non activated samples did not exhibit any effect, suggesting that the activation of coagulation is a fundamental step (Table II, III).

Among the tested microorganisms *A. actinomycetemcomitans* was found to be the most sensitive to both PRP and PPP while *C. albicans* the least. Activated PRP of the 10 periodontitis patients exhibited mean zones of inhibitions of 29 ± 0.1, 25 ± 0.4 and 20 ± 0.1 mm against *A. actinomycetemcomitans, P. gingivalis* and *C. albicans* respectively. While activated PPP of the same patients exhibited mean zones of inhibition of 25 ± 0.4, 18 ± 0.3 and 17 mm against *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* respectively (Table II). Activated PRP of 10 controls exhibited mean zones of inhibitions of 30 ± 0.2, 26 ± 0.6, 19 ± 0.05 mm against *A. actinomycetemcomitans, P. gingivalis* and *C. albicans* respectively and activated PPP exhibited mean zones of inhibitions 23 ± 1, 20 ± 1.1 and 15 ± 0.99 mm against *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* respectively (Table III). Calcium chloride *per se* exhibited zones of inhibitions of 17 ± 03, 12 ± 0.1, 14 ± 0.3 mm against

Table I
Characteristics of the sample presented as mean ±SD

Variable	Periodontitis group (Mean ± SD)	Control group (Mean ± SD)	(p-value)
Age(years)	45.2 ± 3.1	42.7 ± 3.4	> 0.05
Gender Male Female	 **6** **4**	 **5** **5**	> 0.05
Mean platelet concentration in whole blood (platelets /ml)	215×10^3	210×10^3	> 0.05
Mean platelet concentration in PRP (platelets /ml)	480×10^3	462×10^3	> 0.05
Mean platelet concentration in PPP (platelets /ml)	–	–	> 0.05

Table II
Microbiological screening for the antimicrobial activity of the activated PRP, activated PPP, non-activated PRP and non-activated PPP obtained from 10 periodontitis patients and $Cacl_2$ *per se* against *A. actinomycetemcomitans*, *P. gingivalis*, *C. albicans* and *S. aureus* by agar diffusion method

Name of microorganism	Diameter of zone of inhibition ± standard deviation (SD) in mm					
	Activated PRP	Non-activated PRP	Activated PPP	Non-activated PPP	$Cacl_2$	PRP *vs* PPP (*p*-value)
A. actinomycetemcomitans	29 ± 0.1	–	25 ± 0.4	–	17 ± 03	< 0.05
P. gingivalis	25 ± 0.4	–	18 ± 0.3	–	12 ± 0.1	< 0.05
C. albicans	20 ± 0.1	–	17	–	14 ± 0.3	< 0.05
S. aureus	–	–	–	–	–	

– No inhibition zone

Table III
Microbiological screening for the antimicrobial activity of the activated PRP, activated PPP, non-activated PRP and non-activated PPP obtained from 10 controls and $Cacl_2$ *per se* against *A. actinomycetemcomitans*, *P. gingivalis*, *C. albicans* and *S. aureus* by agar diffusion method

Name of microorganism	Diameter of zone of inhibition ± standard deviation (SD) in mm					
	Activated PRP	Non-activated PRP	Activated PPP	Non-activated PPP	$Cacl_2$	PRP *vs* PPP (*p*-value)
A. actinomycetemcomitans	30 ± 0.2	–	23 ± 1	–	17 ± 03	< 0.05
P. gingivalis	26 ± 0.6	–	20 ± 1.1	–	12 ± 0.1	< 0.05
C. albicans	19 ± 0.05	–	15 ± 0.99	–	14 ± 0.3	< 0.05
S. aureus	–	–	–	–	–	

– No inhibition zone

A. actinomycetemcomitans, P. gingivalis and *C. albicans* respectively (Table II, III). Activated PRP and Activated PPP of patients and controls exhibited negative zone of inhibition against *S. aureus.*

The difference in the antimicrobial activity between PRP and PPP of patients and controls against the studied microorganisms was not significant ($p > 0.05$).

Discussion

It is impossible to separate wound healing and infection. The use of PRP may be advantageous compared to conventional antibiotic treatments since PRP is less likely to induce antibiotic resistance and PRP's antimicrobial and healing-promoting properties may have a synergistic effect on infection prevention. Moreover, PRP is inherently biocompatible, and safe. In the current study, we tested the antimicrobial activity of PRP, PPP, with and without activation against four microorganisms, *A. actinomycetemcomitans*, *P. gingivalis*, *S. aureus* and *C. albicans.* We used samples from both periodontally healthy individuals and patients with chronic periodontitis to investigate potential donor variations and whether the antimicrobial effect of PRP could be affected by the presence of chronic periodontitis.

Although leukocytes have been proposed as an additional source for cytokines, microbicidal proteins and myeloperoxidase activity (Hampton *et al*., 1996), the few available published data do not support the increased microbicidal activity of PRP due to the leukocyte component (Bielecki *et al*., 2007; Moojen *et al*., 2008; Anitua *et al*., 2012). Therefore in this study we used the Anitua's protocol (Anitua *et al*., 2008) that allows the production of leukocyte-poor PRP. Originally bovine thrombin was used as an activating agent, but the rare and major risk of coagulopathy from antibody formation has restricted the routine use of bovine thrombin. Calcium chloride, used in the current study, offer an alternative *in vitro* activation means (Anitua, 1999).

In the current study, we have found that PRP, and to a lesser extent, PPP has antimicrobial effects against *A. actinomycetemcomitans*, *P. gingivalis* and *C. albicans* but not *S. aureus*. Only activated samples were able to inhibit microbial growth, suggesting that the activation of coagulation is a fundamental step. No significant difference was found between *in vitro* antimicrobial effect of PRP and PPP isolated from periodontitis patients and healthy controls.

To the best of our knowledge, only Yang *et al.* (2015) have investigated the antimicrobial activity of PRP and other plasma preparations against periodontal

pathogens. The authors found that all plasma preparations can inhibit bacterial growth, with PRP showing the superior activity. In the current study, the activity of PRP against *A. actinomycetemcomitans* and *P. gingivalis* is comparable to that of Yang *et al.* (2015). These results confirm the beneficial role of PRP in the treatment of periodontal disease.

Activated PRP and activated PPP didn't inhibit the growth of *S. aureus*. These results are contradictory to those obtained by Bielecki *et al.* (2007). They performed a microbiological examination of PRP on 20 healthy volunteers; a strong activity comparable to gentamicin and oxacillin for PRP against methicillin-sensitive *S. aureus* was demonstrated. According to the former authors, PRP also inhibited the growth of methicillin – resistant *S. aureus* and *Escherichia coli*. Moreover, a study by Moojen *et al.* (2008) investigated the effect of various platelet concentrate on *S. aureus*, they found a strong effect of PRP activated by thrombin (PLG) but not non activated PRP. They concluded that the decrease in microbial load is mainly attributed to the effect of activation by thrombin. However, they found that the strong antimicrobial effect of PLG seems to be limited to the first hours after application. Additionally, Jia *et al.* (2010) reported that PRP forms into PLG after activation with bovine thrombin can inhibit *S. aureus* reproduction *in vitro* and can effectively prevent bone infection *in vivo*. The reasons for the inconsistency between their results and the results of the current study may be explained by differences in the bacterial isolates and the increased frequency of antimicrobial use in minor surgical procedures which may lead to increased antimicrobial resistance in our population. Additionally, in the current study, CaCl2, which may have a weaker and more prolonged activation time, has been used for activation instead of thrombin (Li *et al.*, 2013). Moreover, the different protocol used for PRP preparation can lead to products with different cellular components and biological characteristics. The difference in the sensibility of the method (modified agar diffusion method) used to evaluate the susceptibility to platelet concentrate should also be considered (Ehrenfest *et al.*, 2013; Assirelli *et al.*, 2014; Cavallo *et al.*, 2014).

Another important obtained result is the observation that PRP and PPP were less active against *C. albicans* than against *A. actinomycetemcomitans* and *P. gingivalis*. This result is consistent with the findings of Tang *et al.* (2002), who tested *in vitro* antimicrobial activity of antimicrobial peptides isolated from human platelets, and noticed that they were more potent against bacteria than fungi.

In contrary to Li *et al.* (2013) study, PPP has demonstrated antimicrobial effect. However, a greater susceptibility for activated PRP was observed. The antimicrobial effect of PPP might be explained by the presence of antimicrobial peptides and other factors in plasma, either released from the platelets during normal functioning or by breakdown of some platelets during the blood processing (Wu *et al.*, 1994).

Future research should focus on the analysis of the contribution of individual PRP and PPP components, its antimicrobial capacity compared to antibiotics and its exact antibacterial spectrum. Moreover, despite the clinical relevance of these results, it is clear that the *in vitro* behavior of bacterial plates may not mimic the *in vivo* environment of the oral cavity and *in vitro* susceptibility to antimicrobials should only be regarded as a guide to potential *in vivo* activities. Future clinical trials should be done to investigate the potential practical implications of the findings of this study as well as the modality of clinical application of platelet concentrates.

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ORIGINAL PAPER

A Comparative Study: Taxonomic Grouping of Alkaline Protease Producing Bacilli

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Abstract

Alkaline proteases have biotechnological importance due to their activity and stability at alkaline pH. 56 bacteria, capable of growing under alkaline conditions were isolated and their alkaline protease activities were carried out at different parameters to determine their optimum alkaline protease production conditions. Seven isolates were showed higher alkaline protease production capacity than the reference strains. The highest alkaline protease producing isolates (103125 U/g), E114 and C265, were identified as *Bacillus licheniformis* with 99.4% and *Bacillus mojavensis* 99.8% based on 16S rRNA gene sequence similarities, respectively. Interestingly, the isolates identified as *Bacillus safensis* were also found to be high alkaline protease producing strains. Genotypic characterizations of the isolates were also determined by using a wide range of molecular techniques (ARDRA, ITS-PCR, $(GTG)_5$-PCR, BOX-PCR). These different techniques allowed us to differentiate the alkaliphilic isolates and the results were in concurrence with phylogenetic analyses of the 16S rRNA genes. While ITS-PCR provided the highest correlation with 16S rRNA groups, $(GTG)_5$-PCR showed the highest differentiation at species and intra-species level. In this study, each of the biotechnologically valuable alkaline protease producing isolates was grouped into their taxonomic positions with multi-genotypic analyses.

Key words: 16S rRNA gene sequence, alkaline protease, alkaliphilic *Bacillus*, ARDRA, ITS-PCR, *rep*-PCR

Introduction

Alkaliphilic bacteria, which are also called as extremophiles, can grow at high pH conditions. They split into two groups as alkaliphiles and alkalitolerants. While alkaliphiles grow optimally at pH 9.0 and also at higher alkaline conditions like pH 10.0, they cannot grow at or below pH 7.0. On the other hand, alkalotolerants can grow both at high (like pH 10.0) and neutral pH values. The enzymes of these alkaliphilic bacteria have a high demand for many industrial branches due to their stability at high pH values (Horikoshi, 1999; Kumar and Takagi, 1999). Of these enzymes, alkaline proteases take the lead and have been used in many areas such as detergent, medicine, food, leather, pharmaceuticals, biological waste elimination and textile industry. Alkaliphilic *Bacillus* strains, which are one of the well-known and well-studied alkaline protease producers, secrete very stable alkaline proteases against pH, temperature, and detergent additives (Ito *et al*., 1998; Horikoshi, 1999). In many countries, obtaining legal permission is necessary in order to produce industrial enzymes and the types of microorganisms that produce enzymes with industrial importance should be identified to use the enzymes commercially (Arellano-Carbajal and Olmos-Soto, 2002). Several PCR based nucleic acid fingerprinting methods have been used to characterize and differentiate *Bacillus* strains when 16S rRNA gene sequencing failed to give the information at subspecies and strain level. 16S-23S intergenic transcribed spacer region PCR (ITS-PCR), BOX and $(GTG)_5$-PCR as repetitive element sequence-based PCR (*rep*-PCR) which are powerful methods to screen the several parts of bacterial genome, have been used to identification, differentiation and comparing the bacterial genome diversity (Freitas *et al*., 2008; Cihan, 2013). In the present study, 56 rod shaped bacteria, capable of growing under highly alkaline conditions were isolated from different regions of Turkey. Carbon sources as a nutrient factor and the pH of the culture medium have a critical importance in the alkaline protease production. As a primary purpose of this study, we aimed to determine the optimal alkaline

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production and enzyme activity conditions by using different growth parameters in order to cover the needs for determining different alkaliphilic and alkalotolerant bacterial strains. Thus, two different culture media having two different pH values were used for cultivation and the enzyme activity experiments were carried with a buffer having two different pH. In addition, we aimed to compare and combine the alkaline protease production capacities of the isolates with their phylogenetic data. In this context, besides 16S rRNA gene sequence similarities, amplified rDNA (Ribosomal DNA) restriction analysis (ARDRA), internal transcribed spacer (ITS)-PCR, $(GTG)_5$ and BOX-PCR as a repetitive extragenic palindromic (*Rep*-PCR) were applied as nucleic acid fingerprinting techniques to obtain detailed information about the taxonomic position of the isolates at the subspecies and strain level.

Experimental

Materials and Methods

Strains. In this study, water and soil samples were collected from different areas of Turkey. The isolates used in this study, their origins and their isolation sources are presented in Table I. In order to isolate new alkaliphilic bacilli having alkaline protease activities, samples were mostly collected from extreme environments having alkaline and saline conditions or containing sulfur and soda. For the bacterial isolation, the soil (0.2–0.4 g) and the water samples (0.5 ml) were inoculated in to Nutrient Broth (pH 9.0) and were cultivated at 37°C by shaking at 200 rpm for 48 h. The turbid cultures were diluted with sterile saline solution and transferred onto Skim Milk Agar plates, which include 0.1% glucose, 2% peptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 7H_2O$, 0.5% skim milk (sterilized separately) (Denizci *et al.*, 2004). After sterilization, the pH of the medium was adjusted to 9.0 by adding 10% Na_2CO_3. The isolates, which gave a clear zone around the colonies due to the hydrolysis of skim milk were selected as an alkaline protease producing strains (Denizci *et al.*, 2004; Tekin *et al.*, 2012).

Following *Bacillus* strains were also used as reference strains; *Bacillus licheniformis* DSM 13, *Bacillus coagulans* DSM 1^T, *Bacillus subtilis* ATCC 6633^T, *Bacillus alcalophilus* DSM 485^T, *Bacillus subtilis* DSM 1971, *Bacillus clausii* DSM 8716^T, *Bacillus cohnii* DSM 6307^T, *Bacillus horikoshii* DSM 8719^T, *Bacillus gibsonii* 8722^T, *Bacillus agaradhaerens* DSM 8721^T, *Bacillus halodurans* DSM 497^T and *Bacillus pseudalcaliphilus* DSM 8725^T. The alkaline protease production capacities of all the isolates and reference strains were qualitatively screened on Skim Milk Agar medium.

Alkaline protease production and quantitative determination of enzyme activity. Two different medium was used for enzyme production. The first medium, which contains casein was prepared according to Gessesse and Gashe (1997) (0.5% Casein, 0.5% Peptone, 0.2% Yeast extract, 0.5% NaCl, 0.02% $MgSO_4 7H_2O$, 0.01% $CaCl_2 2H_2O$, 0.1% K_2HPO_4). The second medium, which contains starch, was prepared according to Denizci *et al.* (2004) (1.0% starch, 0.5% yeast extract, 0.1% K_2HPO_4 and 0.02% $MgSO_4 7H_2O$). The pH of the media was adjusted to pH 7.5 (for alkali-tolerant and facultative alkaliphiles) and 9.5 (for alkaliphiles and obligate alkaliphiles) and the incubation was carried out for 48 h and 72 h. The extracellular alkaline proteases were obtained from the culture supernatant as described by Tekin *et al.* (2012) and the same procedures were applied for the determination of alkaline protease activity. All enzyme activity assays were carried out in triplicate (technical replica) from triplicate cultivations (biological replica) and the results were calculated as mean standard values. Analysis of variance with repeated measures was performed using the Software IBM SPSS Statistics (Version 22, USA). Enzymatic activity means and standard deviation were calculated. Univariate analysis of variance was employed on the data with nutritional supplements (casein or starch)-alkaline protease activity, pH-alkaline protease activity, incubation period-alkaline protease activity, and enzyme reaction buffers having different pH values-alkaline protease activity were tested for significance. Main effects and interaction were also tested for significance.

The parameters for alkaline protease production and activity were summarized at Fig. 1. While determining the alkaline protease production capacities of the isolates, the enzyme activity values per pellet-wet weight (U/g) were determined. The micro molar extinction value of tyrosine, used at enzyme activity formula, was determined by measuring the optic densities of different concentration of tyrosine dilutions at spectrophotometry (660 nm). Tyrosine micro molar extinction value was calculated as 0.0011 µM/ml. One unit of alkaline protease activity was defined as the amount of the enzyme capable of producing 1 µg of tyrosine in 1 min under standard assay conditions.

Morphologic and physiologic characterization of the isolates. Actively growing cells on Nutrient Agar plates (pH 7.0 and 9.0) at 37°C were used for cell and colony morphology analyses. The formation of the spores (spore shape, position in vegetative cell and swelling property) and motility were tested by using 18–24 h Nutrient Broth cultures supplemented with 5 mg/l $MnSO_4 \cdot 4H_2O$ and observed on the phase-contrast microscope (Suzuki *et al.*, 1976). Colony morphology of the isolates determined by using 18 h-old cultures

Table I
Diversity and origin of the bacterial isolates

Bacterial isolates	Sample	Origin
APT1, APT2, APT5, APT8, APT9	Soil	Besevler, Ankara
APT10, APT11, APT12	Soil	Hazar Lake, Diyarbakır coast
APT13a, APT14, APT20b	Mud	Hazar Lake, Diyarbakır coast
APT23, APT24, APT25, APT26	Soil	Sulu Ada, Adrasan, Antalya
APT30	Soil	Acısu Deresi, Baskoy, Kastamonu
APT32	Water	Burdur Lake, Burdur
APT34	Mud	Burdur Lake, Burdur
APT35, APT36	Soil	Burdur Lake, Burdur
APT37, APT38	Water	Güvercinlik Cave, Guneysinir, Konya
APT39, APT40, APT41	Soil	Güvercinlik Cave, Guneysinir, Konya
APT42	Mud	Avlan Lake, Elmalı, Antalya
APT43	Water	Avlan Lake, Elmalı, Antalya
APT44	Mud	Kükürtlü Su, Demre İcmeleri, Antalya
APT47	Water	Kükürtlü Su, Demre İcmeleri, Antalya
APT48	Mud	Burguç Su Kaynagı, Demre, Antalya
TG11, TG20	Soil	Salt Lake, Aksaray
A107, A131, A151, A185	Sediment	Omerbeyli, Germencik, Aydin
A111, A325, A331	Soil	Omerbeyli, Germencik, Aydin
A363	Soil	Yavuzkoy, Salavatli, Aydin
B16	Water	Urganlı, Turgutlu, Manisa
B65	Soil	Urganlı, Turgutlu, Manisa
C83ca, C91, C92	Water	Buharekent, Tekkehamam/Tekkekoy, Denizli
C234, C235, C236, C241, C244, C251, C265	Soil	Buharekent, Tekkehamam/Tekkekoy, Denizli
D311	Water	Doganbey, Seferhisar, İzmir
E114, E287	Sediment	Altinsu, Kozakli, Nevsehir
E215	Soil	Baglica, Kozakli, Nevsehir

on Nutrient Agar plates at 37°C. Gram staining, catalase and amylase activities were carried out according to the methods of Claus and Berkeley (1986). The optimal pH for growth was defined in Nutrient Broth, which was adjusted to different pH values from 7.0 to 13.0 after incubating at 37°C for 24–48 h. The temperature

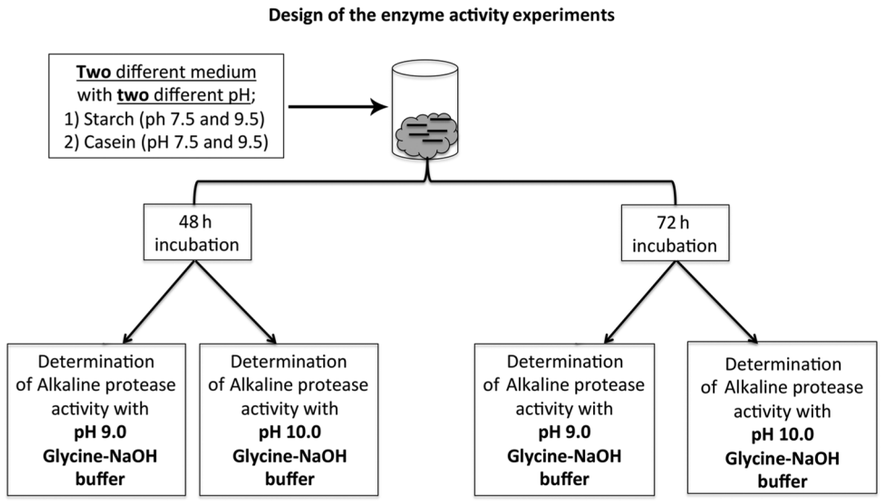

Fig. 1. The design of the enzyme activity experiments.

requirements were tested in Nutrient Broth at pH 10.0 after 24–48 h incubation at different temperatures (5–70°C). The salinity (0–10% NaCl) tolerance for growth was tested in Nutrient Broth and after 24–48 h incubation at 37°C (Nielsen *et al.*, 1995). The growth properties of the isolates were determined according to their optic density at 660 nm. All characterization assays were performed with monocultures in triplicates.

Amplification and sequencing of 16S rRNA gene. Genomic DNA extraction, 16S rRNA gene amplification, purification of the PCR products and sequencing reactions were carried out as previously described (Tekin *et al.*, 2012). In phylogenetic analyses, the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and the evolutionary history was derived using the Neighbor-Joining method (Saitou and Nei, 1987) with the bootstrap values based on 1000 replicates (Felsenstein, 1985). The phylogenetic tree was constructed with the MEGA package version 4 (Tamura *et al.*, 2007).

Amplified ribosomal DNA restriction analysis (ARDRA) of 16S rRNA gene. The same PCR amplicons that used for sequencing reactions were taken into ARDRA analysis of the 16S rRNA gene primed by 27F/1492R (Tekin *et al.*, 2012). The PCR products were digested with the restriction enzymes with Fast digest *Alu*I, *Hae*III and *Taq*I restriction enzymes (MBI Fermentas). ARDRA profiles were detected and statistically analyzed as previously described (Cihan *et al.*, 2011). Finally, the individually examined all the ARDRA analyses were then taken into a cumulative cluster analysis which combined all these tests in a dendrogram by using the GelCompar II software packages (Applied Maths, Belgium). In clustering analyses, according to the presence or absence of DNA bands and also to their densities, the similarity shade limits of 16.66, 33.33, 49.99, 66.66 and 83.3% values were used with GelCompar II software (Applied Maths, Belgium). In this context, the bacteria displaying lower similarities than 97% were denoted as having unique distinctive profiles; the ones having similarities between 97.0% and 100% were determined as sharing similar profiles and then were implied as displaying the same profiles.

PCR based fingerprinting analyses of *Rep* elements and intergenic 16S-23S rRNA gene. Repetitive elements (*Rep*-PCR) genomic fingerprintings (BOXA1- and $(GTG)_5$-PCR) and intergenic transcribed spacers (ITS) between 16S and 23S rRNA genes were performed according to Cihan (2013). Distinctive ITS-PCR, BOXA1 and $(GTG)_5$-PCR fingerprintings were analyzed by the GelCompar II software packages (Applied Maths, Belgium). Similarities of the digitized profiles were calculated using Dice correlation and an average linkage (UPGMA) dendrogram was obtained. The individually examined all the ITS-, $(GTG)_5$- and BOX-PCR fingerprintings were then taken into a cumulative cluster analysis which combined all these tests in a dendrogram by using the GelCompar II software packages (Applied Maths, Belgium). This combined results containing dendrogram was presented in this paper, instead of showing all the three individual cluster analyses of these fingerprinting tests. In clustering analyses according to the presence or absence of DNA bands and also to their densities, the similarity shade limits of 16.66, 33.33, 49.99, 66.66 and 83.3% values were used in GelCompar II software. In this context, the bacteria displaying lower similarities than 83 % were denoted as having unique distinctive profiles, the ones having similarities between 83.3.0% and 99.9% were determined as showing similar profiles and the ones with 100% similarity were implied as displaying the same profiles.

For the all PCR based reactions, the DNA templates were analyzed spectrophotometrically on Nanodrop (NanoDrop 1000 Spectrophotometer V3.7, Thermo Fisher Scientific Inc, Wilmington, DE, USA). Then the concentrations of genomic DNA samples were diluted to 200 ng/µl as stock DNA. For the each PCR based experiment same amount of DNA was used from 200 ng/µl stocks to standardize the band density.

Results

Alkaline protease producing isolates. All of the 56 isolates determined as alkaline protease producers due to growing and producing proteolytic zone on skim milk agar plates at different pH values (7.0, 9.0 and 10.0) as reference *Bacillus* strains. Qualitative proteolytic activity was expressed as a diameter of clear zones in mm (data not shown).

Quantitative alkaline protease production. Enzyme activity values of the isolates and reference strains per pellet-wet weight (U/g) generally were differed between 353-103125 U/g. Isolates E114 and C265 were produced the highest amounts of alkaline protease (103125 U/g) when compared with the other isolates and reference strains. Besides these two isolates APT11, B65, APT43, C251 and C234 were also showed higher alkaline protease production than the reference strains at the different parameters which were summarized in Fig. 1. Alkaline protease production capacities of the seven highest enzyme producing strains and the reference strains were showed in graphs on Fig. 2. Prominently, it's assessed that 70% of the bacteria showed high alkaline protease activity after 48 h incubation. In addition, 76% of the isolates displayed high enzyme activity after incubating at the casein containing medium. Also, 73% of the isolates were carried out the highest enzyme activity measurements at the Glycine-NaOH reaction buffer at pH 9.0. However, it's revealed that

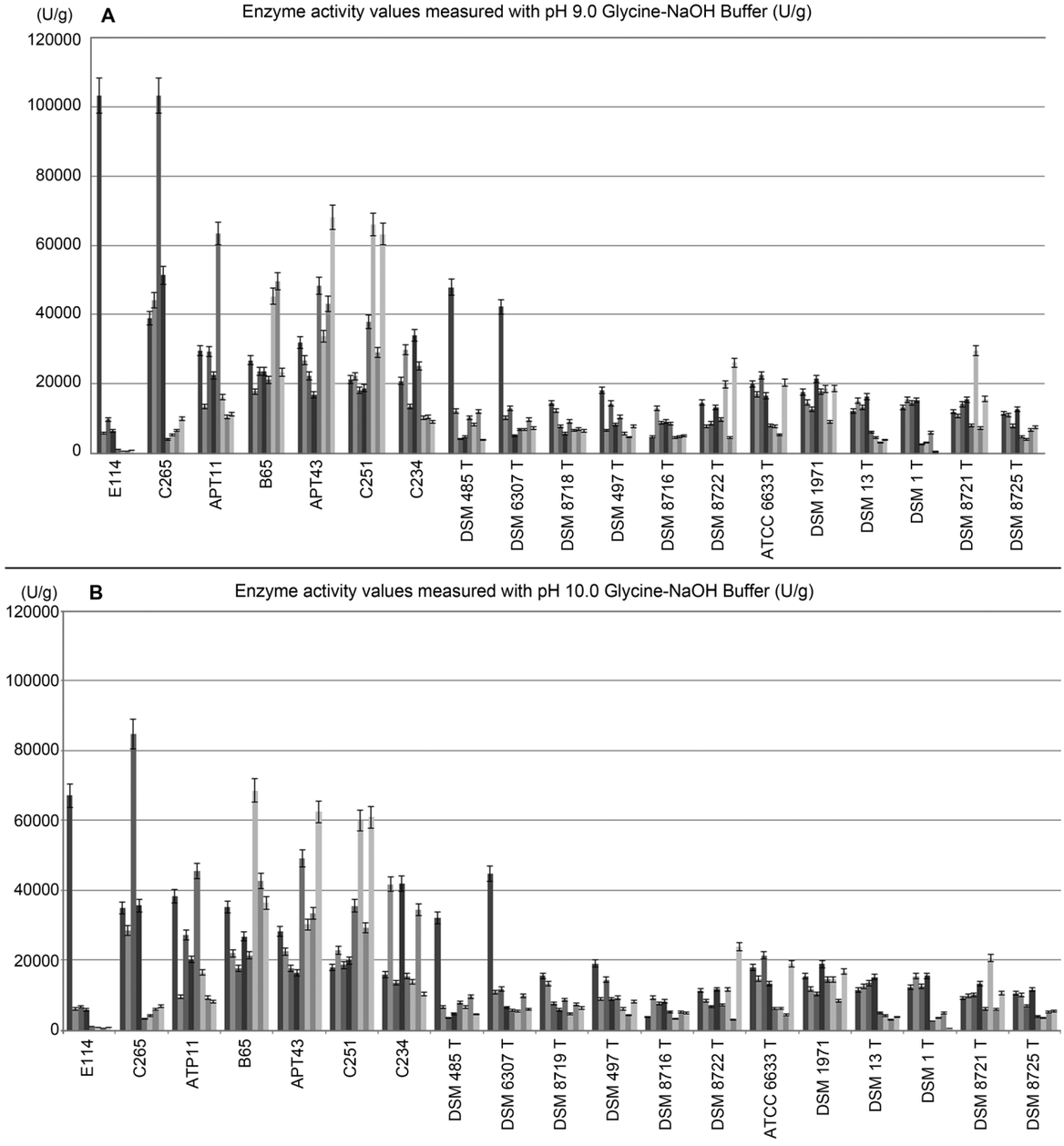

Fig. 2. Alkaline protease activities of the highest enzyme producing bacterial isolates and reference strains with (A) pH 9.0 and (B) 10.0 Glycine-NaOH enzyme reaction buffer.

effect of pH on growth medium wasn't significant. As a conclusion, 56 isolates and 12 reference strains generally showed their highest alkaline protease activity at casein containing medium after 48 h incubation with activity at pH 9.0 Glycine-NaOH buffer. Besides these findings, it can reveal that the pH of the media showed variability according to the physiological requirements of the isolates and reference strains. Additionally, we can conclude that the detection of proteolytic zone at skim milk agar does not reflect the alkaline protease production capacity of the isolates; however, proteolytic zone detection can be assessed to determine the ability of alkaline protease production.

Statistical analysis of quantitative alkaline protease activity. Effect of different carbon (starch) and nitrogen (casein) sources, pH (7.5 and 9.5), incubation period (48–72 h) and Glycine-NaoH buffers adjusted with different pH values (pH: 9.0 and 10.0) on the production of alkaline protease activity is summarized and illustrated in Figure 3A-C. Three sets of experiments were carried out for all the strains and it was observed that there was a drastic statistical difference between

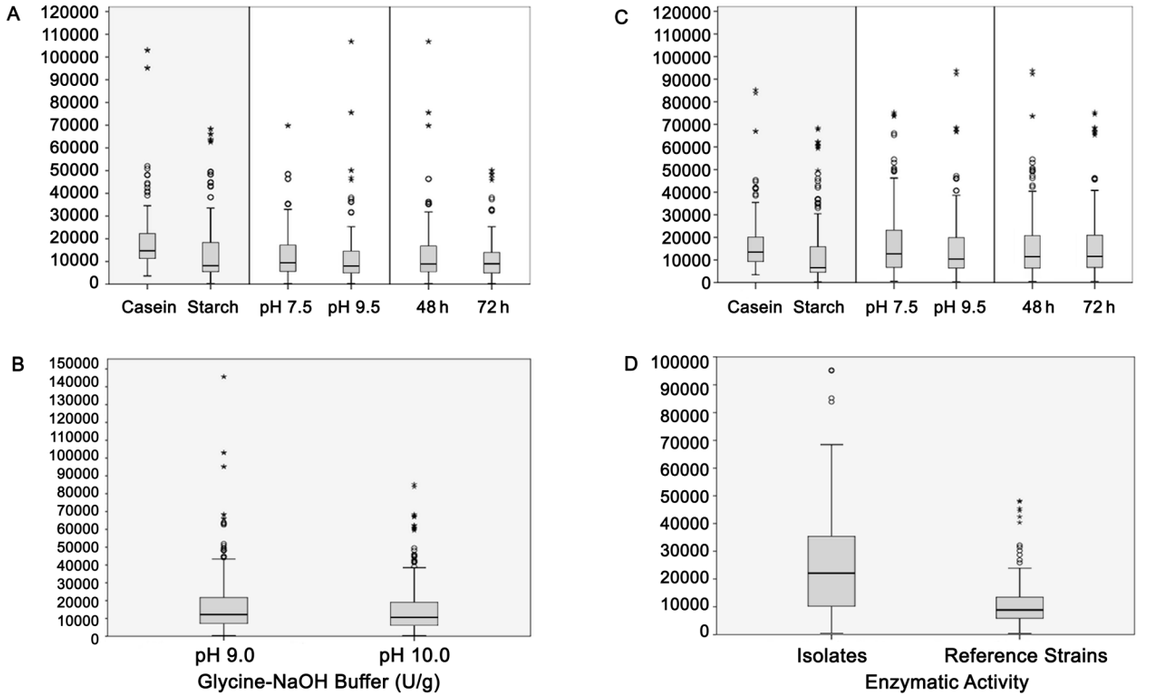

Fig. 3. Statistical analysis of alkaline protease activities.

(A) pH 9.0 Glycine-NaOH buffer; Casein-starch supplement, pH 7.5–9.5 media pH, 48–72 h incubation; (B) pH 10.0 Glycine-NaOH buffer; Casein-starch supplement, pH 7.5–9.5 media pH, 48–72 h incubation; (C) Overall enzyme activity comparisons between Glycine-NaOH buffer having pH 9.0 and 10.0; (D) Overall enzyme activity comparisons between isolates and reference strains.

the casein and starch for alkaline protease production ($p < 0.01$) (Fig. 3A, B). Depending on enzymatic assays performed with both pH 9.0 Glycine-NaOH and pH 10.0 Glycine-NaOH Buffers, it was concluded that supplementation with casein (casein) enhanced production of the enzyme only, and there was no significant differences between pH 9.0 and pH 10.0 Glycine-NaOH buffers (Fig. 3C). Also, the alkaline protease activity between the groups of pH (7.5–9.5) – alkaline protease activity and incubation period (48–72 h) – alkaline protease activity was not found to be statistically significant with both Glycine-NaOH buffers [(pH 9.0 buffer = pH-alkaline protease; $p = 0.776$, incubation period-alkaline protease; $p = 0.076$), (pH 10.0 buffer = pH-alkaline protease; $p = 0.174$, incubation period-alkaline protease; $p = 0.087$)] (Fig. 3A, B). Finally, pH, buffer conditions, and incubation period had no significant effect on alkaline protease activity, but overall alkaline protease activity of the isolates was ~2 fold in comparison with the overall alkaline protease activity of the reference strains ($p < 0.01$) (Fig. 3D).

Morphological and physiological characterization of the isolates. All of the 56 isolates were found to be Gram-positive, endospore-forming and motile bacilli. They were also found positive for catalase activity except strain APT26. Colony morphologies and spore formation differed depending on the species. Forty-seven of the isolates showed amylase activity. Growth was observed at pH 9.0–12.0 (optimum pH 7.0–10.0), at 25–70°C (optimum 30–50°C) and at 0 to 10% concentrations of NaCl (optimum 0–7%) (Data not shown).

Phylogenetic analysis. Sequences of the isolates of 16S rRNA gene were analyzed in order to determine their phylogenetic position. Approximately 1500 bp length 16S rRNA gene sequence data of all the isolates have been deposited in the GenBank databases and all the accession numbers were given in the phylogenetic tree (Fig. 4). The isolates and reference strains were phylogenetically clustered into 28 groups on the basis of their individual 16S rRNA gene sequence homologies to their closest relatives (Fig. 4). Groups of the isolates of 16S rRNA gene and their similarity percents to their closest relatives were also detailed in Table II. The isolates were found to be belonged to the families *Bacillaceae*, *Planococcaceae* and *Paenibacillaceae* from order *Bacillales*. The isolates which clustered into *Bacillaceae* family diverged among 4 different genera (*Bacillus*, *Virgibacillus*, *Lysinibacillus* and *Exiguobacterium*). The isolates belonging to *Planococcaceae* and *Paenibacillaceae* families were also clustered into 2 different genera from *Sporosarcina* and *Paenibacillus*, respectively. In comparison analyses, isolate APT23 displayed more heterogenic 16S rRNA gene sequence similarity to *Bacillus cereus* DSM 31^T (98.3%) and *Bacillus anthracis*

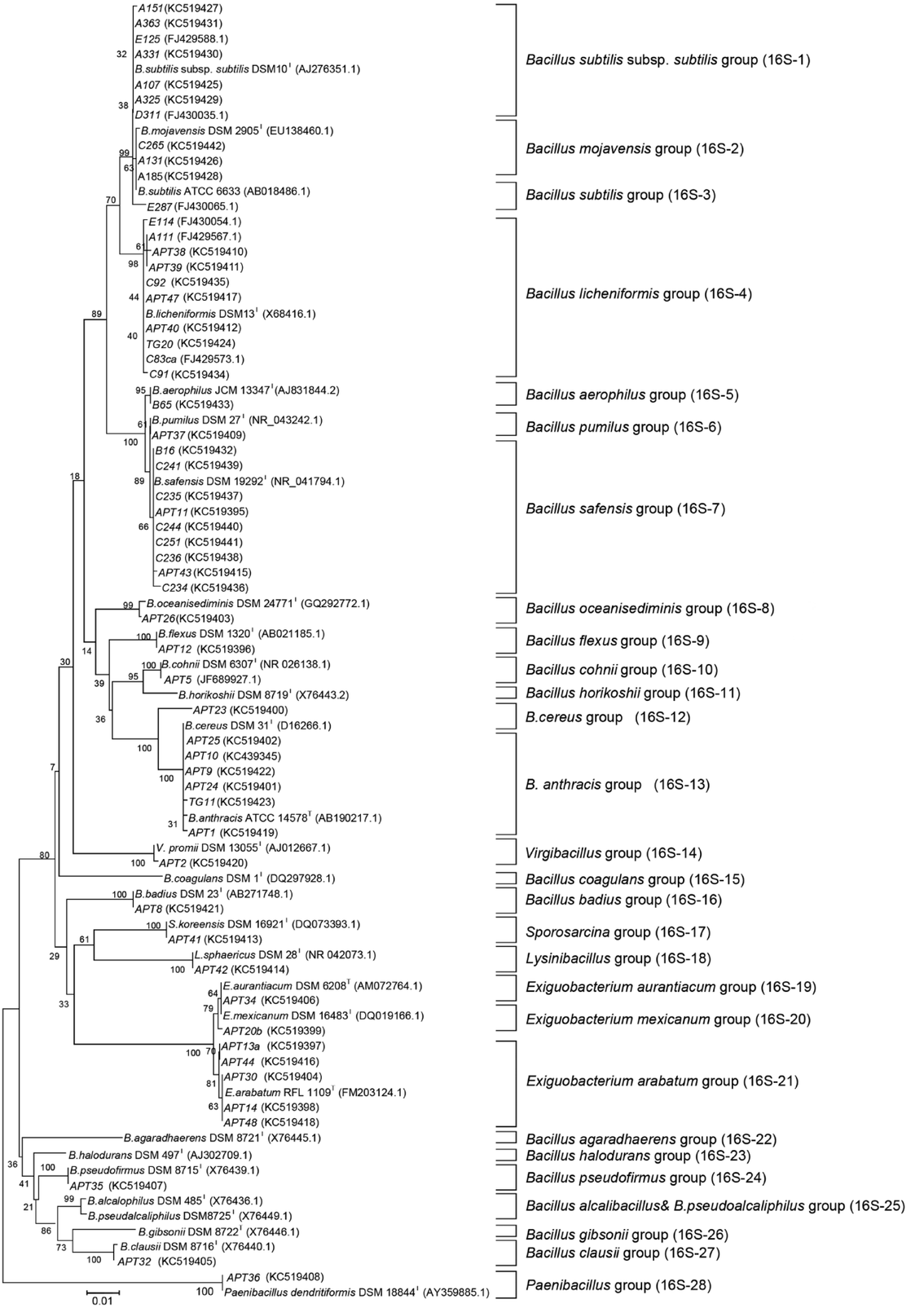

Fig. 4. A phylogenetic tree based on the 16S rRNA gene sequences.

The tree was generated by neighbor-joining method. Bootstrap values (%) are based on 1.000 replicates and shown for branches with more than 30% bootstrap support. Bar indicates 0.01 substitutions per 100 nucleotide positions.

Table II
The species groups of the isolates and the number of the bacteria belonging to these groups derived from 16S rRNA gene nucleotide sequences

16S rRNA Gene Groups	Number of the isolates	Isolates and similarity percentages (%)
Bacillus subtilis	1	E287 (97)
Bacillus subtilis subsp. *subtilis*	7	A363 (99.9), E215 (99.8), A151 (99.9), D311 (99.9), A325 (99.9), A107 (99.8), A331(99.6)
Bacillus mojavensis	3	A185 (99.8), C265 (99.8), A131 (99.8)
Bacillus licheniformis	10	C83ca (99.9), C92 (99.9), C91 (99.8), A111 (99.8), APT38 (99.7), APT39 (99.7), APT47 (99.6), TG20 (99.5), E114 (99.4), APT40 (99.3)
Bacillus aerophilus	1	B65 (100)
Bacillus pumilus	1	APT37 (99.8)
Bacillus safensis	9	B16 (100), APT43 (99.9), C235 (100), C244 (100), C241 (100), C234 (99.9), C236 (99.9), C251 (99.9), APT11 (99.7)
Bacillus oceanisediminis	1	APT26 (99.6)
Bacillus pseudofirmus	1	APT35 (99.9)
Bacillus clausii	1	APT32 (99.7)
Bacillus flexus	1	APT12 (99.9)
Bacillus cohnii	1	APT5 (99.9)
Bacillus cereus	1	APT23 (98.3)
Bacillus anthracis	6	APT10 (100), APT24 (100), APT9 (100), APT25 (100), APT1 (99.9), TG11 (99.9)
Virgibacillus proomii	1	APT2 (99.9)
Sporosarcina koreensis	1	APT41 (99.7)
Lysinibacillus sphaericus	1	APT42 (99.8)
Exiguobacterium arabatum	5	APT14 (99.9), APT13a (99.9), APT44 (99.9), APT48 (99.9), APT30 (99.8)
Exiguobacterium aurantiacum	1	APT34 (99.9)
Exiguobacterium mexicanum	1	APT20b (99.4)
Paenibacillus dendritiformis	1	APT36 (99.1)

(99.2%) reference strains. The rest of the isolates clustered between % 99.1 and 100 similarity values to their type strains as indicated at Table II.

Benchmark of alkaline protease production with phylogenetic clusters. It is obvious that genus *Bacillus* heads a list in the alkaline protease in producing bacteria. Similarly in our study, the highest alkaline protease producing isolates, E114 (103125 U/g) and C265 (103125 U/g) were clustered in *B. licheniformis* and *Bacillus mojavensis*, respectively (Fig. 2 and 4). Other highest alkaline protease producing strain B65 (68506 U/g) was clustered with *Bacillus aerophilus*, and isolates APT11 (63429 U/g), APT43 (68082 U/g), C251 (65939 U/g) and C234 (41834 U/g) were clustered with *Bacillus safensis*. In addition, the isolate APT36 was grouped within *Paenibacillus dendritiformis* (3824 U/g), identified as the lowest alkaline protease producer within all isolates and the reference strains. Notwithstanding, while APT23 was clustered with *B. cereus*, APT1, APT9, APT10, APT24, APT25 and TG11 were clustered with *B. anthracis*, which are class III pathogen strains. Despite of the alkaline protease production capacities of these isolates, as they produced higher amount of protease than most of the strains, they are not sufficient for the industrial enzyme production due to their pathogenic identity (Table IIIA, B). Consequently, 49 isolates except these 7 isolates were clustered within non-pathogenic strains. Within the context of optimal alkaline protease production conditions, it was assessed that while *B. safensis* group isolates produced highest alkaline protease in the starch medium, the isolates belonging to *B. licheniformis*, *B. anthracis* and *Exiguobacterium* groups produced their highest alkaline protease in the casein medium. According to alkaline protease production capacity of the isolates against to reference strains, it is observed that all of the isolates belonging to *B. subtilis* and *B. subtilis* subsp. *subtilis* group displayed higher enzyme production levels than the reference strains of *B. subtilis* DSM 1971 and *B. subtilis* ATCC 6633^T. Similarly, the isolate APT36 grouped as *B. clausii*, and the isolate APT5 belonging to *B. cohnii*, showed higher alkaline protease production capacities than their reference strains *B. clausii* DSM 8716^T and *B. cohnii* DSM 6307^T, respectively (Table IIIA, B).

The *Rep*-PCR and ITS-PCR fingerprintings of the isolates. The isolates and the reference strains were

Table IIIA
16S rRNA groups of the isolates and their individual optimum alkaline protease activity conditions

16S rRNA species groups	Bacteria	Alkaline protease activity (U/g)	The highest alkaline protease activity conditions			
			Medium	pH of the medium	Incubation time (h)	pH of glycine-NaOH buffer
Bacillus subtilis subsp. *subtilis*	A363	44864	Casein	7.5	48	9.0
	E215	43725	Starch	9.5	48	9.0
	A151	29906	Starch	7.5	48	9.0
	D311	53350	Starch	7.5	48	10.0
	A325	27726	Casein	7.5	72	9.0
	A107	22471	Casein	9.5	48	9.0
	A331	22367	Casein	9.5	72	9.0
Bacillus subtilis	E287	41708	Casein	9.5	48	9.0
	DSM1971	21450	Casein	9.5	72	9.0
	ATCC6633	22458	Casein	9.5	48	9.0
Bacillus mojavensis	A185	33950	Starch	7.5	48	9.0
	C265	103125	Casein	9.5	48	9.0
	A131	30425	Casein	7.5	48	9.0
Bacillus licheniformis	C83ca	12844	Casein	7.5	72	10.0
	C92	18116	Casein	9.5	48	9.0
	C91	18700	Casein	7.5	48	10.0
	A111	17233	Casein	9.5	72	9.0
	APT38	7398	Casein	7.5	48	10.0
	APT39	9888	Casein	7.5	48	9.0
	APT47	26308	Casein	7.5	48	9.0
	TG20	10529	Casein	7.5	48	10.0
	E114	103125	Casein	7.5	48	9.0
	APT40	11648	Casein	7.5	48	10.0
	DSM13	16323	Casein	9.5	72	9.0
Bacillus aerophilus	B65	68506	Starch	7.5	72	10.0
Bacillus pumilus	APT37	31656	Casein	9.5	72	9.0
Bacillus safensis	B16	42961	Starch	7.5	48	9.0
	APT43	68082	Starch	9.5	72	9.0
	C235	37492	Starch	9.5	48	9.0
	C244	65939	Starch	7.5	72	9.0
	C241	34490	Starch	7.5	48	10.0
	C234	34375	Starch	9.5	48	10.0
	C236	53763	Starch	9.5	72	9.0
	C251	65939	Starch	7.5	72	9.0
	APT11	63429	Starch	7.5	48	9.0

implemented to the *Rep*-PCR and ITS fingerprinting analyses. These fingerprinting results, the fingerprinting groups and the individual 16S rRNA gene groups were presented in Fig. 5, Table IV, Table V A-B respectively. The isolates, having unique distinctive profiles, were indicated in Table V A-B. In the individual cluster analyses of the *Rep*-PCR containing BOX- and $(GTG)_5$-PCR fingerprintings, totally 33 to 51 clusters were obtained, whereas 28 clusters were obtained from ITS-PCR fingerprintings. According to these results *Rep*-PCR products, especially $(GTG)_5$-PCR fingerprintings were generated a high number of bands giving discriminative information below species and subspecies level between the endospore-forming bacilli isolates when compared with ITS-PCR fingerprintings and 16S rRNA phylogenetic analyses. Additionally to these findings, the clusters obtained with ITS-PCR fingerprints and 16S rRNA phylogenetic analyses showed codependency and sustained the relation between the isolates. In conclusion, the cluster analyses of the *Rep*- and ITS-PCR

Table IIIB
16S rRNA groups of the isolates and their individual optimum alkaline protease activity conditions

16S rRNA species groups	Bacteria	Alkaline protease activity (U/g)	The highest alkaline protease activity conditions			
			Medium	pH of the medium	Incubation time (h)	pH of glycine-NaOH buffer
Bacillus pseudofirmus	APT35	38019	Casein	9.5	48	9.0
Bacillus clausii	APT32	34035	Starch	9.5	48	9.0
	DSM 8716^T	12950	Casein	7.5	72	9.0
Bacillus flexus	APT12	31549	Casein	9.5	48	9.0
Bacillus cohnii	APT5	15400	Casein	7.5	48	10.0
	DSM 6307^T	44688	Casein	7.5	48	10.0
Bacillus cereus	APT23	14491	Casein	7.5	48	9.0
Bacillus anthracis	APT10	20689	Casein	9.5	48	9.0
	APT24	22756	Casein	9.5	72	9.0
	APT9	23623	Casein	7.5	48	9.0
	APT25	16800	Casein	9.5	48	9.0
	APT1	20442	Casein	9.5	48	9.0
	TG11	29081	Casein	7.5	48	9.0
Bacillus badius	APT8	24420	Casein	7.5	48	9.0
Virgibacillus proomii	APT2	22358	Casein	9.5	48	9.0
Sporosarcina koreensis	APT41	13180	Casein	7.5	48	10.0
Lysinibacillus sphaericus	APT42	27762	Starch	9.5	48	10.0
Exiguobacterium aurantiacum	APT34	20006	Casein	9.5	72	9.0
Exiguobacterium mexicanum	APT20b	8054	Casein	9.5	48	10.0
Exiguobacterium arabatum	APT14	9598	Casein	7.5	48	9.0
	APT13a	24844	Casein	7.5	48	9.0
	APT44	36300	Casein	9.5	48	9.0
	APT48	13292	Casein	7.5	48	10.0
	APT30	16454	Casein	7.5	48	10.0
Paenibacillus dendritiformis	APT36	3824	Casein	9.5	48	9.0
B. alcaliphilus	DSM 486^T	47850	Casein	7.5	48	9.0
B. pseudalcaliphilus	DSM 8715^T	12668	Casein	9.5	72	9.0
B. agaradhaerens	DSM 8721^T	29578	Starch	7.5	72	9.0
B. halodurans	DSM 497^T	18993	Casein	7.5	48	10.0
B. horikoshii	DSM 8719^T	15461	Casein	7.5	48	10.0
B. gibsonii	DSM 8722^T	26171	Starch	9.5	72	9.0
B. coagulans	DSM 1^T	15538	Starch	7.5	72	9.0

Table IV
Number of clusters for both isolates and reference strains obtained from individual 16S rRNA genes, ARDRA profiles and ITS-, BOX- and GTG-PCR DNA fingerprintings

Number of	16S rRNA	ITS-PCR	BOX-PCR	GTG-PCR	*Alu*I	*Taq*I	*Hae*III
Standard Clusters	6	7	5	10	6	3	0
Isolate Clusters	22	21	28	41	20	21	27
Total Clusters	28	28	33	51	26	24	27

fingerprintings allowed us to differentiate these isolates and reference strains genetically from each other.

***Alu*I, *Hae*III and *Taq*I-ARDRA analyses of the *Anoxybacillus* isolates.** The amplified PCR products of the isolates were subjected to digestion with *Alu*I, *Hae*III and *Taq*I restriction enzymes in comparison with reference strains. The individual *Alu*I-, *Hae*III- and *Taq*I-ARDRA cluster analyses of the digitized band-

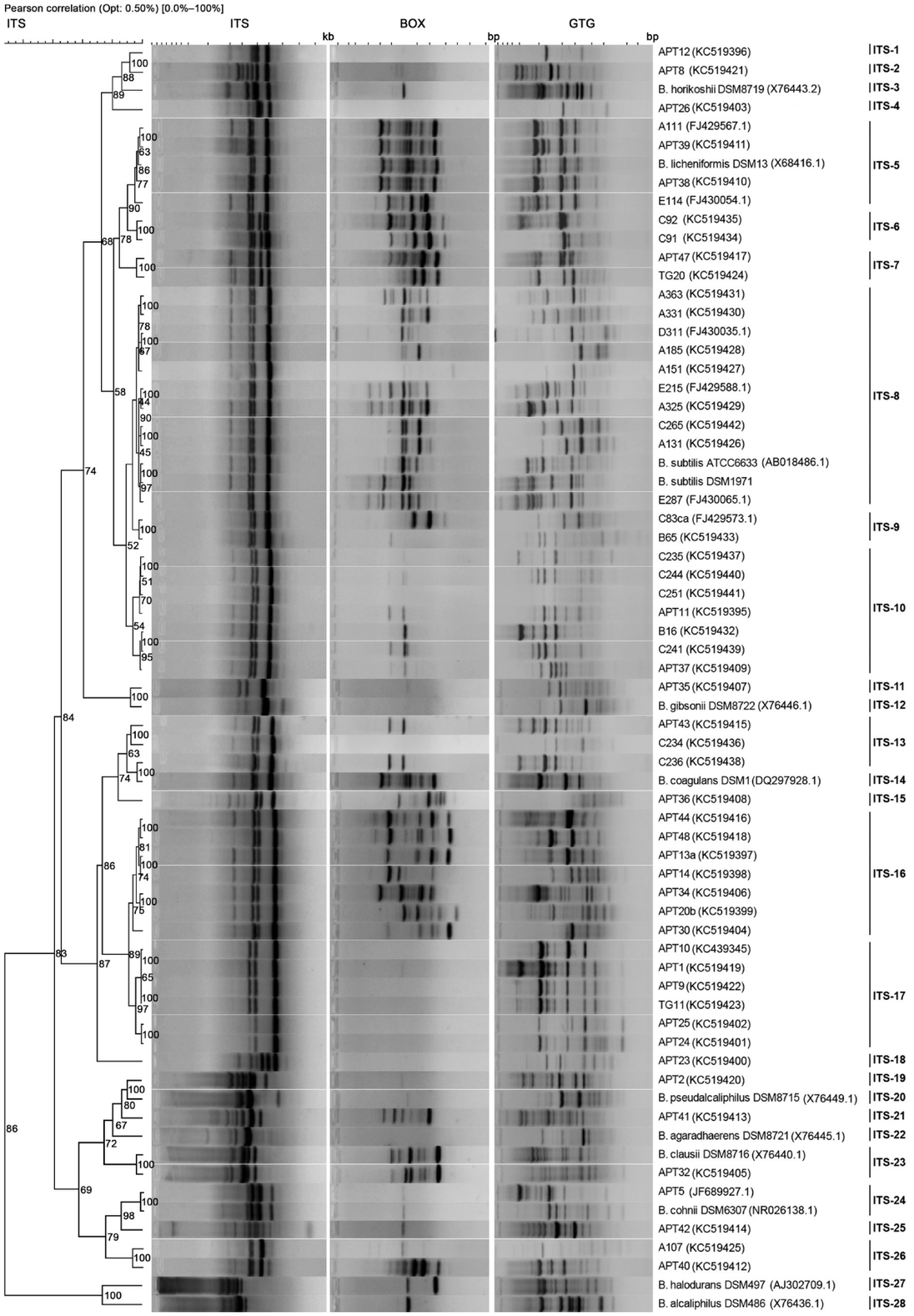

Fig. 5. The cumulative cluster analysis of representative digitized banding patterns, generated by ITS-, BOX- and GTG-PCR profiles from isolates and reference strains.

The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. Due to the correlation between 16S rRNA groups, the numbers of clusters obtained from the ITS-PCR cumulative analysis were indicated in the right side of the figure.

Table VA
Individual *Rep*- and ITS-PCR fingerprinting groups against to 16S rRNA groups

16S rRNA species groups	Isolate/reference strain	16S rRNA group	ITS group	BOX group	GTG group
Bacillus subtilis subsp. *subtilis*	A363	16S-1	ITS-8	BOX-27	GTG-44
	E215	16S-1	ITS-8	BOX-28*	GTG-44
	A151	16S-1	ITS-8	BOX-25*	GTG-16*
	D311	16S-1	ITS-8	BOX-30	GTG-45
	A325	16S-1	ITS-8	BOX-11	GTG-41*
	A107	16S-1	ITS-26	BOX-31*	GTG-46*
	A331	16S-1	ITS-8	BOX-11	GTG-1*
Bacillus subtilis	E287	16S-2	ITS-8	BOX-29*	GTG-43*
	DSM1971	–	ITS-8	BOX-27	GTG-42*
	ATCC6633	16S-2	ITS-8	BOX-30	GTG-45
Bacillus mojavensis	A185	16S-3	ITS-8	BOX-26	GTG-10
	C265	16S-3	ITS-8	BOX-26	GTG-10
	A131	16S-3	ITS-8	BOX-26	GTG-10
Bacillus licheniformis	C83ca	16S-4	ITS-9	BOX-10	GTG-14*
	C92	16S-4	ITS-6	BOX-9	GTG-21
	C91	16S-4	ITS-6	BOX-10	GTG-12*
	A111	16S-4	ITS-5	BOX-4	GTG-22
	APT38	16S-4	ITS-5	BOX-4	GTG-22
	APT39	16S-4	ITS-5	BOX-4	GTG-22
	APT47	16S-4	ITS-7	BOX-8*	GTG-24*
	TG20	16S-4	ITS-7	BOX-7	GTG-20*
	E114	16S-4	ITS-5	BOX-9	GTG-21
	APT40	16S-4	ITS-26	BOX-7	GTG-25*
	DSM13	16S-4	ITS-5	BOX-5*	GTG-22
Bacillus aerophilus	B65	16S-5	ITS-9	BOX-24*	GTG-9*
Bacillus pumilus	APT37	16S-6	ITS-10	BOX-32	GTG-6*
Bacillus safensis	B16	16S-7	ITS-10	BOX-32	GTG-4
	APT43	16S-7	ITS-13	BOX-33	GTG-7
	C235	16S-7	ITS-10	BOX-23	GTG-3
	C244	16S-7	ITS-10	BOX-23	GTG-4
	C241	16S-7	ITS-10	BOX-32	GTG-4
	C234	16S-7	ITS-13	BOX-14	GTG-3
	C236	16S-7	ITS-13	BOX-33	GTG-7
	C251	16S-7	ITS-10	BOX-23	GTG-5
	APT11	16S-7	ITS-10	BOX-33	GTG-5

The isolates, having unique distinctive profiles, were indicated with "*"

ing patterns derived from the isolates and the reference strains can be seen in Fig. 6. The numbers of clusters obtained from these three ARDRA profiles and the isolates, having unique distinctive profiles, were shown in Table VIA and VIB. In individual cluster analysis based on the *Alu*I-, *Hae*III- and *Taq*I-ARDRA profiles of the amplified 16S rRNA genes, totally 26, 27 and 24 clusters were observed when the presence or absence of the restriction fragments and also the density of these DNA bands were considered. The highest group numbers were determined by *Hae*III-ARDRA analyses. However, *Hae*III-ARDRA band patterns did not show any correlation with 16S rRNA clusters. Both *Hae*III- and *Taq*I-ARDRA groups showed a similar restriction band patterns between phylogenetically distinct strains and reference strains, and could not give any discriminative result even in genus level. This can be the result of the evolutionary conserved property of 16S rRNA gene. On the contrary to *Hae*III- and *Taq*I-ARDRA analyses, *Alu*I-ARDRA analysis was found to be superior on the

Table VB
Individual *Rep*- and ITS-PCR fingerprinting groups against to 16S rRNA groups

16S rRNA species groups	Isolate/reference strain	16S rRNA group	ITS group	BOX group	GTG group
Bacillus oceanisediminis	APT26	16S-8	ITS-4*	BOX-14	GTG-15*
Bacillus pseudofirmus	APT35	16S-24	ITS-11*	BOX-14	GTG-11*
Bacillus clausii	APT32	16S-27	ITS-23	BOX-2*	GTG-19*
	DSM8716	16S-27	ITS-23	BOX-1*	GTG-26*
Bacillus flexus	APT12	16S-9	ITS-1*	BOX-14	GTG-13*
Bacillus cohnii	APT5	16S-10	ITS-24	BOX-14	GTG-51*
	DSM6307	16S-10	ITS-24	BOX-14	GTG-38*
Bacillus cereus	APT23	16S-12	ITS-18*	BOX-14	GTG-37*
Bacillus anthracis	APT10	16S-13	ITS-17	BOX-14	GTG-36*
	APT24	16S-13	ITS-17	BOX-14	GTG-35
	APT9	16S-13	ITS-17	BOX-14	GTG-33
	APT25	16S-13	ITS-17	BOX-14	GTG-35
	APT1	16S-13	ITS-17	BOX-14	GTG-34*
	TG11	16S-13	ITS-17	BOX-14	GTG-33
Bacillus badius	APT8	16S-16	ITS-2*	BOX-14	GTG-28*
Virgibacillus proomii	APT2	16S-14	ITS-19*	BOX-14	GTG-50*
Sporosarcina koreensis	APT41	16S-17	ITS-21*	BOX-12*	GTG-47*
Lysinibacillus sphaericus	APT42	16S-18	ITS-25*	BOX-14	GTG-2*
Exiguobacterium aurantiacum	APT34	16S-19	ITS-16	BOX-17*	GTG-39*
Exiguobacterium mexicanum	APT20b	16S-20	ITS-16	BOX-18*	GTG-48*
Exiguobacterium arabatum	APT14	16S-21	ITS-16	BOX-19*	GTG-29
	APT13a	16S-21	ITS-16	BOX-22	GTG-27
	APT44	16S-21	ITS-16	BOX-20*	GTG-27
	APT48	16S-21	ITS-16	BOX-21*	GTG-30*
	APT30	16S-21	ITS-16	BOX-22	GTG-29
Paenibacillus dendritiformis	APT36	16S-28	ITS-15*	BOX-13*	GTG-17*
B. alcaliphilus	DSM486	16S-25	ITS-28*	BOX-16*	GTG-49*
B. pseudalcaliphilus	DSM8715	16S-25	ITS-20*	BOX-14	GTG-18*
B. agaradhaerens	DSM8721	16S-22	ITS-22*	BOX-14	GTG-31*
B. halodurans	DSM497	16S-23	ITS-27*	BOX-3*	GTG-40*
B. horikoshii	DSM8719	16S-11	ITS-3*	BOX-15*	GTG-8*
B. gibsonii	DSM8722	16S-26	ITS-12*	BOX-14	GTG-32*
B. coagulans	DSM1	16S-15	ITS-14*	BOX-6*	GTG-23*

The isolates, having unique distinctive profiles, were indicated with "*"

other restriction enzyme digestions for differentiating the reference strains within 6 unique clusters. Also, the differences at species and genus level were correlated with 16S rRNA phylogenetic groups.

Discussion

Because of the high value of the alkaline proteases, many new data regarding the alkaline protease producing *Bacillus* strains have been publishing during the last years (Niyonzima and More, 2014; Sari *et al.*, 2015). Especially *B. licheniformis*, *B. subtilis*, *B. amyloliquifaciens* and *B. mojavensis* are the most preferred alkaline protease producing *Bacillus* species due to their high enzyme production capacities and their non-toxic properties. Some of the alkaline proteases, produced by *B. licheniformis* were patented and they have been widely using as laundry detergent additives (Kumar and Takagi, 1999). Also, Haddar *et al.* (2009) announced that serine alkaline proteases produced from *B. mojavensis*, are effaceable for the industrial usage. Likewise, in our study the highest alkaline protease producer strains E114 and C265 were identified as *B. licheniformis* and

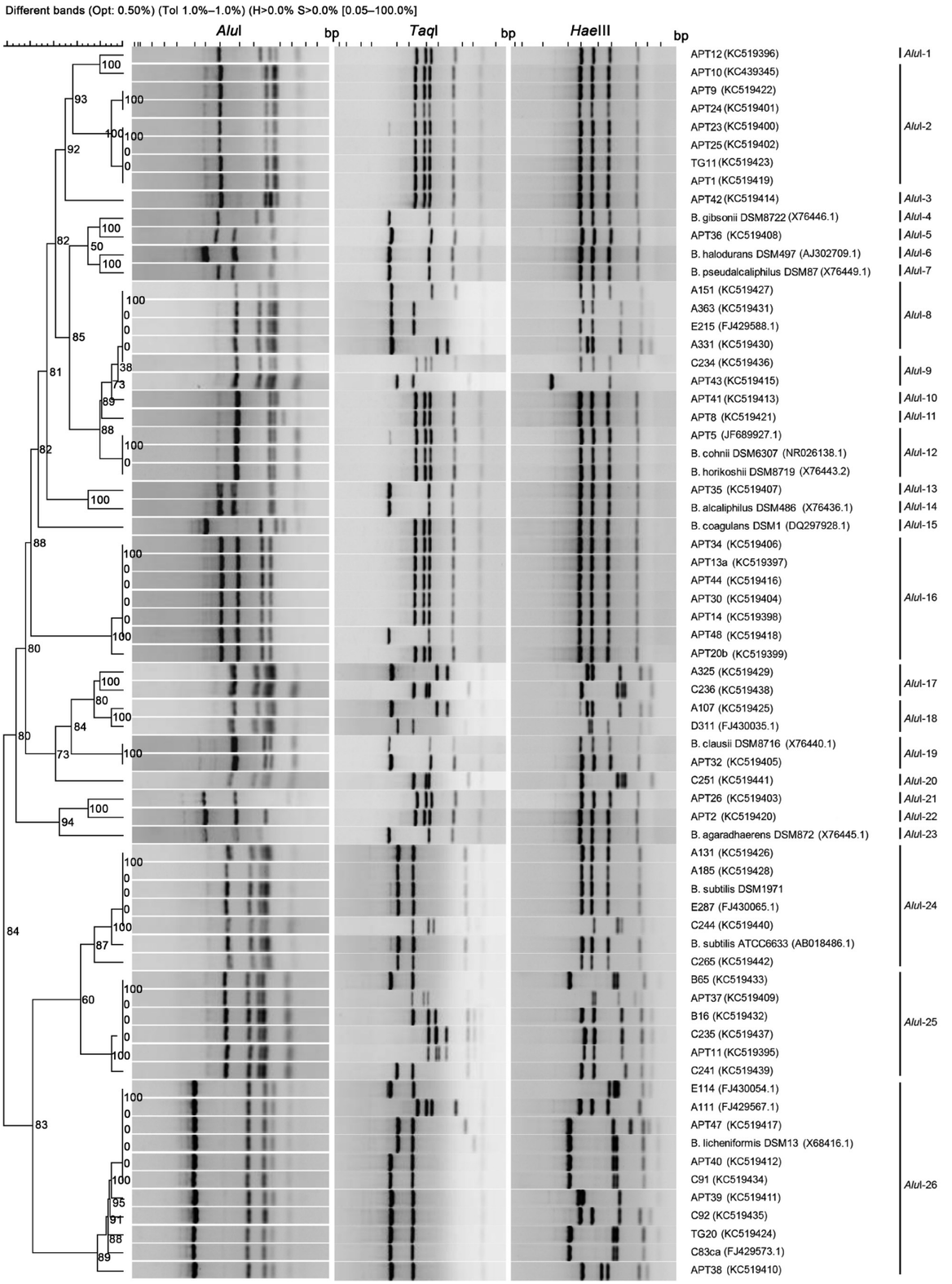

Fig. 6. The cumulative cluster analysis of representative digitized banding patterns, generated by ARDRA profiles from isolates and reference strains.

The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Dice coefficient. Due to the correlation between 16S rRNA groups, the numbers of clusters obtained from the *Alu*I profile cumulative analysis were indicated in the right side of the figure.

Table VIA
Individual ARDRA profile groups against to 16S rRNA groups

16S rRNA species groups	Bacteria	16S rRNA group	*Alu*I group	*Taq*I group	*Hae*III Group
Bacillus subtilis subsp. *subtilis*	A363	16S-1	*Alu*I-8	*Taq*I-5	*Hae*III-1*
	E215	16S-1	*Alu*I-8	*Taq*I-5	*Hae*III-14*
	A151	16S-1	*Alu*I-8	*Taq*I-17*	*Hae*III-10
	D311	16S-1	*Alu*I-18	*Taq*I-2	*Hae*III-5*
	A325	16S-1	*Alu*I-17	*Taq*I-8	*Hae*III-9*
	A107	16S-1	*Alu*I-18	*Taq*I-8	*Hae*III-17*
	A331	16S-1	*Alu*I-8	*Taq*I-8	*Hae*III-15*
Bacillus subtilis	E287	16S-2	*Alu*I-24	*Taq*I-1	*Hae*III-26*
	DSM1971	–	*Alu*I-24	*Taq*I-1	*Hae*III-6
	ATCC6633	16S-2	*Alu*I-24	*Taq*I-1	*Hae*III-6
Bacillus mojavensis	A185	16S-3	*Alu*I-24	*Taq*I-2	*Hae*III-6
	C265	16S-3	*Alu*I-24	*Taq*I-2	*Hae*III-6
	A131	16S-3	*Alu*I-24	*Taq*I-2	*Hae*III-6
Bacillus licheniformis	C83ca	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-22
	C92	16S-4	*Alu*I-26	*Taq*I-5	*Hae*III-25*
	C91	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-22
	A111	16S-4	*Alu*I-26	*Taq*I-15*	*Hae*III-10
	APT38	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-3*
	APT39	16S-4	*Alu*I-26	*Taq*I-5	*Hae*III-12*
	APT47	16S-4	*Alu*I-26	*Taq*I-2	*Hae*III-23*
	TG20	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-22
	E114	16S-4	*Alu*I-26	*Taq*I-7*	*Hae*III-7*
	APT40	16S-4	*Alu*I-26	*Taq*I-6	*Hae*III-24
	DSM13	16S-4	*Alu*I-26	*Taq*I-4*	*Hae*III-24
Bacillus aerophilus	B65	16S-5	*Alu*I-25	*Taq*I-6	*Hae*III-19*
Bacillus pumilus	APT37	16S-6	*Alu*I-25	*Taq*I-22*	*Hae*III-13*
Bacillus safensis	B16	16S-7	*Alu*I-25	*Taq*I-24	*Hae*III-16*
	APT43	16S-7	*Alu*I-9	*Taq*I-3	*Hae*III-27*
	C235	16S-7	*Alu*I-25	*Taq*I-23	*Hae*III-11
	C244	16S-7	*Alu*I-24	*Taq*I-24	*Hae*III-20*
	C241	16S-7	*Alu*I-25	*Taq*I-3	*Hae*III-11
	C234	16S-7	*Alu*I-9	*Taq*I-13*	*Hae*III-8
	C236	16S-7	*Alu*I-17	*Taq*I-21	*Hae*III-2*
	C251	16S-7	*Alu*I-20*	*Taq*I-21	*Hae*III-21*
	APT11	16S-7	*Alu*I-25	*Taq*I-23	*Hae*III-18*

The isolates, having unique distinctive profiles, were indicated with "*"

B. mojavensis, respectively, according to their 16S rRNA gene sequence similarities. Additionally to these species strain B65 identified as *B. aerophilus*, strains APT11, APT43, C251 and C234 were identified as *B. safensis*. Both *B. aerophilus* and *B. safensis* species previously have not been defined as alkaline protease producing *Bacillus* species. Therefore, in the scope of this study, we conclude that these species may be new alkaline protease producing *Bacillus* species and also can be a potential for new alkaline protease sources. Their non-pathogenic property also supports their industrial usage.

Due to its evolutionary protected property, 16S rRNA gene sequences provide distinguishing the microorganisms at the genus level but also its conserved property fails while differentiating the closely related species at subspecies level (Clarridge, 2004). Therefore, when 16S rRNA gene sequence similarities show 97.0% or more similarity within the closest relative species, the Ad Hoc Committee recommends the DNA-DNA hybridizations to determine the novel species (Stackebrandt *et al.*, 2002; Logan *et al.*, 2009). In our study, all of the isolates show higher 16S rRNA gene sequence

Table VIB
Individual ARDRA profile groups against to 16S rRNA groups.

16S rRNA species groups	Bacteria	16S rRNA group	*Alu*I group	*Taq*I group	*Hae*III Group
Bacillus oceanisediminis	APT26	16S-8	*Alu*I-21*	*Taq*I-10	*Hae*III-4
Bacillus pseudofirmus	APT35	16S-24	*Alu*I-13*	*Taq*I-20	*Hae*III-8
Bacillus clausii	APT32	16S-27	*Alu*I-19	*Taq*I-19	*Hae*III-8
	DSM8716	16S-27	*Alu*I-19	*Taq*I-20	*Hae*III-8
Bacillus flexus	APT12	16S-9	*Alu*I-1	*Taq*I-10	*Hae*III-4
Bacillus cohnii	APT5	16S-10	*Alu*I-12	*Taq*I-11	*Hae*III-4
	DSM6307	16S-10	*Alu*I-12	*Taq*I-10	*Hae*III-4
Bacillus cereus	APT23	16S-12	*Alu*I-2	*Taq*I-11	*Hae*III-8
Bacillus anthracis	APT10	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	APT24	16S-13	*Alu*I-2	*Taq*I-14*	*Hae*III-4
	APT9	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	APT25	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	APT1	16S-13	*Alu*I-2	*Taq*I-9	*Hae*III-8
	TG11	16S-13	*Alu*I-2	*Taq*I-10	*Hae*III-8
Bacillus badius	APT8	16S-16	*Alu*I-11*	*Taq*I-12	*Hae*III-4
Virgibacillus proomii	APT2	16S-14	*Alu*I-22*	*Taq*I-12	*Hae*III-4
Sporosarcina koreensis	APT41	16S-17	*Alu*I-10*	*Taq*I-10	*Hae*III-4
Lysinibacillus sphaericus	APT42	16S-18	*Alu*I-3*	*Taq*I-10	*Hae*III-4
Exiguobacterium aurantiacum	APT34	16S-19	*Alu*I-16	*Taq*I-12	*Hae*III-4
Exiguobacterium mexicanum	APT20b	16S-20	*Alu*I-16	*Taq*I-9	*Hae*III-4
Exiguobacterium arabatum	APT14	16S-21	*Alu*I-16	*Taq*I-9	*Hae*III-4
	APT13a	16S-21	*Alu*I-16	*Taq*I-12	*Hae*III-8
	APT44	16S-21	*Alu*I-16	*Taq*I-9	*Hae*III-8
	APT48	16S-21	*Alu*I-16	*Taq*I-20	*Hae*III-8
	APT30	16S-21	*Alu*I-16	*Taq*I-9	*Hae*III-8
Paenibacillus dendritiformis	APT36	16S-28	*Alu*I-5*	*Taq*I-19	*Hae*III-8
B. alcaliphilus	DSM486	16S-25	*Alu*I-14*	*Taq*I-16	*Hae*III-8
B. pseudalcaliphilus	DSM8715	16S-25	*Alu*I-7*	*Taq*I-18	*Hae*III-8
B. agaradhaerens	DSM8721	16S-22	*Alu*I-23*	*Taq*I-16	*Hae*III-8
B. halodurans	DSM497	16S-23	*Alu*I-6*	*Taq*I-16	*Hae*III-8
B. horikoshii	DSM8719	16S-11	*Alu*I-12	*Taq*I-10	*Hae*III-4
B. gibsonii	DSM8722	16S-26	*Alu*I-4*	*Taq*I-18	*Hae*III-8
B. coagulans	DSM1	16S-15	*Alu*I-15*	*Taq*I-9	*Hae*III-4

The isolates, having unique distinctive profiles, were indicated with "*"

similarities than 97% with their closest relative species. Especially in order to use the high alkaline protease producing strains at industrial purposes, their species have to be determined with hybridizing their DNA to their closest relative species. However, before using this expensive method, their suitable closest reference strains have to determine correctly. In this study, the nucleic acid fingerprinting techniques used allow us to determine the most suitable reference strain and avoid the DNA:DNA hybridization between the less similar strains (Cihan *et al.*, 2011).

Intergenic Transcribed Spacers PCR (ITS-PCR) is one of the most suitable nucleic acid fingerprinting technique by distinguishing the species and intraspecies levels. It provides high range variety according to evolutionary highly protected 16S rRNA gene sequences. In parallel with our study, ITS-PCR groups were better correlated with 16S rRNA groups than $(GTG)_5$ and BOX-PCR groups. Logan *et al.* (2009) and Daffonchio *et al.* (1998a; 1998b) reported that 6 species of the *B. cereus* group (*B. anthracis*, *B. cereus*, *B. mycoides*, *B. pseudomycoides*, *B. thuringiensis* and *B. weihenstephanensis*) were showed similar ITS band profile. Therefore, they concluded that this technique is not effective to differentiate *B. cereus* species. Similarly, Huang *et al.* (2012) reported that ITS-PCR is not effective to differentiate

B. cereus and *B. subtilis* specie. However, contrary to these studies *B. cereus*, *B. anthracis* and *B. subtilis* species showed different ITS band profiles and distinguished from each other successfully in this study (Fig. 5). Our results also showed that ITS-PCR was not discriminative for *B. subtilis* and *B. mojavensis* species, which were identified by 16S rRNA gene sequences. However, it obviously illuminated the diversity for *B. licheniformis* and *B. safensis* groups at intraspecies level.

The main difference of *Rep*-PCR from the other fingerprinting techniques is that it provides scanning of the repetitive sequences on complete bacterial genomes (Versalovic *et al.*, 1994). The discriminative efficacy of BOX and $(GTG)_5$ elements on taxonomic classification of endospore forming bacilli were showed at various studies (Freitas *et al.*, 2008; Logan *et al.*, 2009; Cihan *et al.*, 2011). According to our results, BOX-PCR profiles correlated with 16S rRNA groups. Especially the species of *B. subtilis*, *B. subtilis* subsp. *subtilis*, *B. licheniformis*, *B. safensis* and the species of genus *Exiguobacterium* discriminated at intraspecies level. However, BOX elements of *B. anthracis* and *B. cereus* could not amplified with the same PCR conditions of other samples. Similarly Freitas *et al.* (2008) reported that BOX elements of some samples were not amplified while the reactions were successful for the other isolates. We concluded that new PCR conditions have to be determined for *B. anthracis* and *B. cereus* species when BOX-PCR was used as a discriminative fingerprinting method. Contrary to the BOX-PCR, $(GTG)_5$-PCR efficiently distinguished the isolates at species and intraspecies level. Especially *B. cohnii*, *B. anthracis* and *Exiguobacterium* groups, which were not discriminated by ITS and BOX-PCR, were distinguished at intraspecies level with a higher resolution. Similarly, Freitas *et al.* (2008) reported that they were carried genomic fingerprint analysis with $(GTG)_5$, BOXA1R and ERIC (Enterobacterial Repetitive Intergenic Consensus) PCR primers on many different *Bacillus* isolates and of these techniques $(GTG)_5$-PCR provided a wide variety of band profiles. Again similarly, at their study $(GTG)_5$-PCR was not efficient grouping the microorganism according to BOX and ERIC-PCRs. Also, De Clerck and De Vos (2004) highlighted the efficiency of $(GTG)_5$-PCR when distinguishing the intraspecies level. ARDRA analyses are carried on evolutionarily highly conserved 16S rRNA gene. Despite the conserved property of 16S rRNA gene, *Alu*I restriction fragments correlated with 16S rRNA gene sequence analyses and the isolates partly distinguished at species and intraspecies level. rRNA genes are organized as multiple gene families and it is known that they express from 1 to 15, different copy numbers (Klappenbach and Dunbar, 2000). The mixed groups obtained by *Hae*III and *Taq*I ARDRA analysis may occur because of the different copy numbers of 16S rRNA gene. Many studies were carried out on ARDRA analysis of 16S rRNA gene with different restriction enzymes. But, according to our knowledge this is the first study analyzing the ARDRA profiles of alkaline protease producing strains belonging to the *Bacillaceae* family. In conclusion, by this study, we determined the alkaline protease production capacities of the each isolates and reference strains according to; their carbon source requirements (casein or starch containing growth media), the effect of growth time on enzyme activities (48 and 72 h incubation) and also the effect of pH on enzyme activities (Glycine-NaOH buffer at pH 9.0 and 10.0) by applying various parameters. Moreover, we identified the taxonomic positions of these numerous endospore-forming bacilli, alkaline protease producing isolates in a polyphasic approach which leads to determine their appropriate taxonomic levels by investigating their phenotypic and genotypic diversity (White *et al.*, 1993; Mora *et al.*, 1998). According to the literature, this is the first report that compares many DNA fingerprint techniques with on alkaline protease production capacities of the *Bacillus* strains. Moreover, many novel *Bacillus* species were introduced as alkaline protease producers and enumerated according to their enzyme production capacities.

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ORIGINAL PAPER

Isolation and Characterization of Phosphate-Solubilizing Bacteria from Mushroom Residues and their Effect on Tomato Plant Growth Promotion

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Abstract

Phosphorus is a major essential macronutrient for plant growth, and most of the phosphorus in soil remains in insoluble form. Highly efficient phosphate-solubilizing bacteria can be used to increase phosphorus in the plant rhizosphere. In this study, 13 isolates were obtained from waste mushroom residues, which were composed of cotton seed hulls, corn cob, biogas residues, and wood flour. NBRIP solid medium was used for isolation according to the dissolved phosphorus halo. Eight isolates produced indole acetic acid (61.5%), and six isolates produced siderophores (46.2%). Three highest phosphate-dissolving bacterial isolates, namely, M01, M04, and M11, were evaluated for their beneficial effects on the early growth of tomato plants (*Solanum lycopersicum* L. Wanza 15). Strains M01, M04, and M11 significantly increased the shoot dry weight by 30.5%, 32.6%, and 26.2%, and root dry weight by 27.1%, 33.1%, and 25.6%, respectively. Based on 16S rRNA gene sequence comparisons and phylogenetic positions, strains M01 and M04 belonged to the genus *Acinetobacter*, and strain M11 belonged to the genus *Ochrobactrum*. The findings suggest that waste mushroom residues are a potential resource of plant growth-promoting bacteria exhibiting satisfactory phosphate-solubilizing for sustainable agriculture.

Key words: 16S rRNA, mushroom residues, phosphate solubilizing bacteria, tomato plant growth

Introduction

In a terrestrial ecosystem, soil microorganisms are important players in the rhizosphere of plants involved in the recycling of nutrients and crucial for long-term soil sustainability (Grönemeyer *et al.*, 2011). After nitrogen, phosphorus (P) is the second major essential macronutrient for plant development in soil, and the lack of P limits plant growth (Nautiyal, 1999; Yu *et al.*, 2011). However, most agricultural soils contain large reserves of total P, which is typically within the range of 0.2–5 g P/kg and with an average of 0.6 g P/kg; moreover, P accumulation partly depends on regular chemical fertilizer application (Fernández *et al.*, 2007). Nevertheless, many soils throughout the world are P-deficient because most of P in nature exists in various organic and inorganic forms; in addition, the concentration of free phosphorus available to plants in fertile soils is generally not higher than 10 μM, even at pH 6.5 at which P is most soluble (Rodríguez and Fraga, 1999; Gyaneshwar *et al.*, 2002).

Although chemical fertilizers are added to the soils, plants can only utilize few amounts of phosphatic fertilizers that are often continuously applied; the remaining amount, which is almost 75–90% of added P fertilizer, is rapidly converted into insoluble complexes, such as calcium phosphate, aluminum phosphate, and iron phosphate in the soil (Gyaneshwar *et al.*, 2002; Vassilev and Vassileva, 2003; Alam and Ladha, 2004). Consequently, chemical fertilizers are frequently applied during crop planting, but its regular use is costly and produces undesirable environmental impacts, such as soil and water contamination. Therefore, P is often regarded a limiting nutrient in agricultural soils (Guiñazú *et al.*, 2010; Yu *et al.*, 2011).

Given the negative environmental impacts of chemical fertilizers and increasing costs, the utilization of phosphate-solubilizing bacteria (PSB) is advantageous for sustainable agricultural practices (Gyaneshwar *et al.*, 2002). PSB could convert these insoluble phosphates into available forms for plant *via* acidification, chelation, exchange reactions, and production of gluconic

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acid (Chung *et al.*, 2005; Gulati *et al.*, 2010). They may also promote plant growth by secreting plant hormones, such as indole acetic acid and cytokinin (Poonguzhali *et al.*, 2008). Currently, many PSB belonging to *Pseudomonas*, *Bacillus*, *Rhizobium*, *Agrobacterium*, *Burkholderia*, *Achromobacter*, *Micrococcus*, *Aerobacter*, *Enterobacter*, *Flavobacterium*, and *Erwinia* have been isolated from soils (Rodríguez and Fraga, 1999). These bacteria can grow in media containing calcium-phosphate complexes as the sole source of P, solubilize and assimilate a large proportion of P, and release P in high amounts. Phosphate is solubilized *via* organic acid synthesis and released by microorganisms (Puente *et al.*, 2004). This reaction, appearing as a halo or clear zone on the plate, is used to assess the P-solubilizing activity of these bacteria. Undoubtedly, the selection of considerably efficient PSB strains as possible inoculants will be a promising way to release large amount of P from soil to improve the current status of extensive chemical fertilizer usage.

Currently, China has become the largest mushroom consuming country; accordingly, a large amount of mushroom residues, which are a kind of solid organic wastes, have been produced annually (Li *et al.*, 2015). Thus, the ecological environment of the planting area is suffering from large mushroom residues characterized by soil pollution and difficult degradation. Nevertheless, mushroom composting has recently attracted significant attention and been regarded an environmentally friendly and sustainable alternative for the management and recycling of organic wastes (Sæbø and Ferrini, 2006; Liu *et al.*, 2015). For instance, mushroom residues can be fermented for use as growing substrates in vegetable seedling breeding. In addition, mushroom compost is an artificial ecosystem that harbors a complete spectrum of microbial diversity (Johri, 2011); thus, isolating PSB from mushroom compost is significant. To date, rare information is available for the screening of PSB from wasted mushroom residues. Therefore, the present work mainly aimed to isolate and characterize native PSB from waste mushroom residues. Their effect on promoting growth of tomato seedlings were also evaluated under greenhouse condition.

Experimental

Materials and Methods

Collection of mushroom residues. In this study, mushroom residues were collected from the experimental basements in Hefei city (117.27°E, 31.85°N). These residues were composed of cotton seed hulls mixed with corn cob (CC), cotton seed hulls mixed with wood flour (CW), cotton seed hulls mixed with biogas residues (CB), and wood flour (WF). All samples were stored at 4°C until analysis and isolation.

Isolation of phosphate solubilizing bacteria. The serially dilution from 10^{-3} to 10^{-5} was achieved by transferring 1 ml of mushroom residue solution from each preceding attenuation stage to the next. Approximately 0.1 ml volume of the resulting solution was then placed on the National Botanical Research Institute's Phosphate growth medium (NBRIP) contained g/l: glucose, 10.0; $Ca_3(PO_4)_2$, 5.0; $MgCl_2 \cdot 6H_2O$, 5.0; $MgSO_4 \cdot 7H_2O$, 0.25; KCl, 0.2 and $(NH_4)_2SO_4$, 0.1, including 0.5% $Ca_3(PO_4)_2$ as an insoluble P source for selectively screening the bacteria capable of releasing inorganic phosphate from tricalcium phosphate (TCP) (Nautiyal, 1999). Sterile medium served as a control. The PSB were identified by clear halo zones around their colonies after 3 days of incubation at 30°C. Experiments were performed in triplicate. Furthermore, the colonies that had larger solubilization zones were further purified. Thirteen PSB strains thus screened were selected for further analysis. All the isolates were designated as M01-13.

Phenotypic characterization of PSB isolates. Physiological and biochemical characteristics of the bacterial isolates were examined according to the methods described in Bergey's Manual of Determinative Bacteriology Edition 8.0 (Holt *et al.*, 1994). All strains were characterized by Gram staining and light microscopy. The direct observation of isolated colonies was served as the first characterization comprising the color, shape, elevation, margins, diameter, and texture. Such traits as endospore, catalase, and starch hydrolysis were characterized (Yu *et al.*, 2011).

Assay of phosphate solubilizing ability. As the plate assay is not considered a reliable method in determining a strain as phosphate solubilizer (Johri *et al.*, 1999), the pure cultures were further screened in liquid medium containing $Ca_3(PO_4)_2$ at a concentration of $5\,g \cdot l^{-1}$ as insoluble P source. Strains were grown in 30 ml liquid medium shaken ($190 \times g$) at 30 ± 1°C for 20 h. One milliliter culture was then transferred to a 300-ml flask containing 80 ml medium previously. Sterile water-inoculated medium was treated as a control. Three Erlenmeyer flasks for statistical replication were used to incubate in the dark on a gyratory shaker ($190 \times g$) at 30 ± 1°C for 3 days. The supernatant of the medium was used to assess P released into the solution. Phosphorus in the culture was determined by the molybdenium blue method with a spectrophotometer at a wavelength of 700 nm (Watanabe and Olsen, 1965).

Characterization of plant growth promoting traits. Indole acetic acid (IAA) production was measured by the colorimetric method (Gordon and Weber, 1951). The isolates were cultivated in a minimal medium (Park *et al.*, 2011) at 25°C for 7 days in a shaking incubator at $120 \times g$. Bacterial cells were removed from the culture

broth by centrifugation (1.5 ml of bacterial suspension). Supernatants were vigorously mixed at a 1:2 ratio with salkowski's reagent, and incubated in the dark for 30 min at 25°C. The absorbance of the final solution was measured at 530 nm. The concentration of IAA in the culture medium was determined using the standard curve of pure IAA (Sangon Biotech Co., Ltd., Shanghai, China).

Siderophore production was determined using an Fe-deficient mineral salt medium (MSM) (Park *et al*., 2011). The selected strains were inoculated in the MSM and incubated in a shaking incubator at 25°C for 3 days at 174 × g. The cell-free culture supernatants were assayed for siderophore production according to the method former described (Schwyn and Neilands, 1987).

Cellulase production was determined in casein yeast extract agar (contained g/l: casein, 5.0; yeast extract, 2.5; glucose, 1.0; agar, 15.0 dissolved in distilled water) medium amended with 1% carboxymethyl cellulose (Teather and Wood, 1982). After 72 h of incubation at 28°C, the agar was flooded with an aqueous solution of congo red (1 mg/ml) for 15 min. The colonies surrounded by clear halos were considered positive for cellulase production.

Proteolytic activity was determined by plating bacteria onto casein yeast extract agar plates containing with 7% skim milk powder (Kumar *et al.*, 2005). After 72 h incubation at 28°C, a clear zone surrounding the colonies is considered as positive.

Sequencing of 16S rDNA gene. Characterization to the genus level of each selected PSB strain was performed by partial sequencing of the 16S ribosomal DNA gene. Genomic DNA was extracted by the phenol/chloroform method (Sambrook *et al.*, 1989) and amplified using PCR amplification of the 16S rDNA. The primers 27 f (5'-GAGATTTGATTCTGGCTCAG-3') and 1495 r (5'-CTACGGCTACCTTGTTACGA-3') were used for amplification. The 50 µl PCR mixtures contained: 0.5 µM of each primer, 200 µM dNTPs, 3 mM $MgCl_2$, PCR reaction buffer (50 mM KCl, 20 mM Tris-HCl at pH 8.0), 1 U Taq DNA polymerase (Promega, USA), and 2 µl of template DNA. The amplifications were carried out in a thermocycler (AB, USA). The PCR was performed as follows: hot start at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 40 s, and extension at 72°C for 3 min. A final extension step was carried out at 72°C for 7 min. After the reaction, 5 µl of the PCR reaction was analyzed in 1.5% agarose gels containing 1 µg/ml of ethidium bromide and photographed with the gel electrophoresis image system. Sequences of the 16S rDNA nucleotide were determined by Sangon Biotech (Shanghai) Co., Ltd. The sequences obtained in this study were analyzed by BLAST algorithm for comparison of a nucleotide query sequence against public nucleotide sequence database to find the closely related bacteria (Yu *et al*., 2011). The nucleotide sequences of the 16S rDNA were subjected to BLAST analysis with the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). Sequences with high similarity scores were downloaded from the NCBI database. A phylogenetic tree was constructed using molecular evolutionary genetics analysis (MEGA 4.0) (Tamura *et al.*, 2007). All sequences were deposited in the GenBank sequence database, and the accession numbers are listed in Table I.

Plant growth promotion assay. Tomato seeds (*Solanum lycopersicum* L. Wanza 15) were surface sterilized according to a previous method (Yegorenkova *et al.*, 2001). The seeds were placed in sterile petri dishes,

Table I
Identification of bacterial isolates based on 16S rDNA partial sequence analysis.

Isolate	Organisms identified	Accession number	Closest type strain in RDP data base	16S rDNA identity (%)
M01	*Acinetobacter* sp.	KT964802	*Acinetobacter* sp. 66A1; GQ178052	99.0
M02	*Klebsiella* sp.	KT964803	*Klebsiella* sp. P058; KC252799	99.0
M03	*Enterobacter* sp.	KT964804	*Enterobacter* sp. 3242O2; KF598876	99.0
M04	*Acinetobacter* sp.	KT964805	*Acinetobacter baumannii*; RSO15; KM502224	99.8
M05	*Acinetobacter* sp.	KT964806	*Acinetobacter baumannii*; RSO15; KM502224	99.8
M06	*Bacillus megaterium*	KT964807	*Bacillus megaterium* DPBS17; EU249559	99.0
M07	*Bacillus* sp.	KT964808	*Bacillus megaterium;* F1; FJ009385	99.0
M08	*Bacillus megaterium*	KT964809	*Bacillus megaterium,* DPBS17; EU249559	99.0
M09	*Paenibacillus taichungensis*	KT964810	*Paenibacillus* sp. H420; KJ943997	98.8
M10	*Paenibacillus taichungensis*	KT964811	*Paenibacillus* sp. L202; KJ944125	99.6
M11	*Ochrobactrum* sp.	KT964812	*Ochrobactrum* sp. SCU-B91; KJ000782	99.0
M12	*Sphingobacterium* sp.	KT964813	*Sphingobacterium* sp. 21; NR_074508	95.0
M13	*Sphingobacterium* sp.	KT964814	*Sphingobacterium* sp. 21; NR_074508	95.0

Note: Accession number, the accession number of the strains deposited in the Genbank (NCBI).

properly watered, and germinated in darkness for 2 days at 25°C.

Germinated seeds were inoculated by immersion in appropriate bacterial suspensions (10^8 cells/ml) for 30 min at 28°C. Control seeds were soaked in distilled water, transferred to glass tubes (Diameter 5 cm × Length 10 cm) containing 80 ml of semi-solid Hoagland medium amended with 1% TCP (Hoagland and Arnon, 1950), and kept in a greenhouse at 25°C with a photoperiod of 16 h light and 8 h dark. The dry weights of shoots and roots were calculated after 15 days.

Statistical analysis. All data in the present study were subjected to analysis of variance (ANOVA), and means were separated by the Fisher's protected least significant difference (LSD) test using the SPSS package (version 19.0). Differences obtained at the $P \leq 0.05$ level were considered significant.

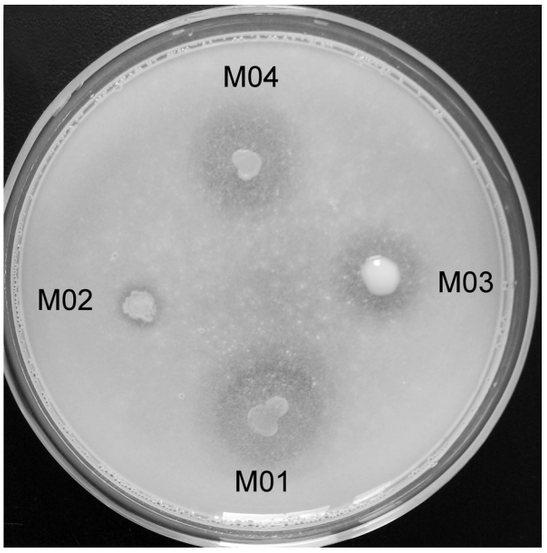

Fig. 1. Dissolved phosphorus halo of isolates on NBRIP solid medium. Strains M01, M02, M03, and M04.

Results

Phenotypic characterization. Thirteen isolates were obtained from mushroom residues by selective NBRIP medium (Fig. 1). Out of 13 isolates, four strains isolated from CC, one strain was separated from each CW and CB, and seven strains were derived from WF (Table II). Most of the strains were Gram-negative, motile rods, and non-endospore forming (Fig. 2). The colonies were milky white and white. The catalase test was positive for twelve isolates. The starch test was positive for ten isolates, and the VP test was positive for four bacterial strains. Nine isolates showed cellulase production and eight ones had proteolytic activity. However, only four strains could utilize of glucose to produce acid (Table III).

Plant growth-promoting trait characterization. Out of 13 tested isolates, eight (61.5%) strains produced IAA within the range of 8.06–62.43 mg/l. Three isolates produced more than 50 mg/l of IAA, and strain M07 showed the highest IAA production (62.43 mg/l). Six (46.2%) isolates produced siderophores, with two strains producing them at high amounts. All the strains can solubilize phosphate ranging within

Table II
Dissolved phosphorus ratio of isolates on NBRIP solid mediumfrom four mushroom residues.

Isolate	Source[a]	Diameter of colonies (cm)	Diameter of hydrolysis circle (cm)	Cultivation time (d)	Dissolved phosphorus ratio[b]
M01	CC	0.6	2.1	4	**0.875**
M02	CC	1.1	2.0	4	0.455
M03	CC	0.7	2.0	4	0.714
M04	CC	0.4	1.8	4	**1.125**
M05	CW	0.6	2.0	4	0.833
M06	CB	0.8	1.3	4	0.406
M07	WF	0.8	1.1	4	0.344
M08	WF	0.4	1.1	4	0.688
M09	WF	0.4	1.0	4	0.625
M10	WF	1.1	1.5	4	0.341
M11	WF	0.5	1.8	4	**0.900**
M12	WF	0.6	1.2	4	0.500
M13	WF	0.4	0.7	4	0.438

[a] CC, Cotton seed hulls + Corn Cob; CW, Cotton seed hulls + Wood flour; CB, Cotton seed hulls + Biogas residue; WF, Wood flour.

[b] Dissolved phosphorus ratio equal to diameter of hydrolysis circle divided by diameter of colonies and cultivation time.

Table III
Selected physiological and biochemical characteristics of phosphate solubilizing strains from waste mushroom residues*.

Isolate	Colony Morphology[a]	Gram stain	Physiological and biochemical characteristics						
			Endos[b]	Cellu[c]	Prote[d]	Catalase	Starch	VP[e]	Fermentation Test (glucose)[f]
M01	RE MW	–	ND	+	+	+	–	–	Acid
M02	RE MW	–	ND	–	–	+	+	–	–
M03	RE MW	–	ND	+	+	+	+	–	Acid
M04	RE MW	–	ND	–	+	+	+	–	–
M05	RE MW	–	ND	+	–	+	–	+	–
M06	RE MW	+	+	+	+	+	+	–	–
M07	IR WH	+	+	+	+	+	+	+	–
M08	RE MW	+	+	+	+	+	+	–	–
M09	RE WH	+	+	+	–	+	+	–	–
M10	RE WH	+	+	–	–	+	+	–	–
M11	RE WH	–	ND	–	–	–	–	–	–
M12	RE WH	–	ND	+	+	+	+	+	Acid
M13	RE WH	–	ND	+	+	+	+	+	Acid

Note: * +, positive; –, negative.
[a] RE, regular; IR, irregular; MW, milky white; WH, white; [b] Endos, endspore; ND, not detected; [c] Cellu, cellulase production; [d] Prote, proteolytic activity; [e] VP (Voges–Proskauer test); [f] Utilization of glucose to produce acid.

17.31–60.87 µg/l. Strains M01, M04, and M11 solubilized phosphate at 54.91, 60.87, and 54.41 µg/ml, respectively (Table IV). In total, five isolates displayed three plant growth-promoting traits.

Phylogenetic analysis. Based on the phylogenetic analysis of the 16S rDNA partial sequence, strains M01, M04, and M05 were identified as *Acinetobacter* sp. Strains M02 and M03 were identified as *Klebsiella* sp. and *Enterobacter* sp., respectively. Moreover, three strains were identified as *Bacillus* sp. Strains M06, M07, and M08 were identified as *B. megaterium*. M09 and M10 were *Paenibacillus taichungensis*, and strain M11 was identified as *Ochrobactrum*. M12 and M13 were both

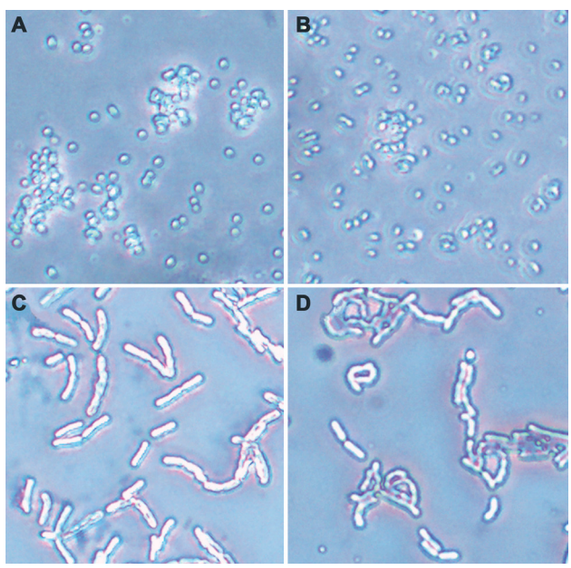

Fig. 2. Single-cell form of isolated strains under optical microscope view. (A) M02, (B) M03, (C) M07, and (D) M10. Bar represents 10 µm.

Table IV
Plant growth-promoting traits of phosphate-solubilizing isolates.

Isolate	Plant growth promoting traits		
	IAA production[a] (mg/l)	Siderophore production[b]	Phosphate solubilization[c] (µg/ml)
M01	–	–	54.91 ± 1.25
M02	11.27 ± 0.85	–	22.94 ± 0.23
M03	13.03 ± 0.79	–	25.66 ± 1.35
M04	10.45 ± 0.65	–	60.87 ± 1.62
M05	–	++++	34.88 ± 1.39
M06	56.32 ± 1.52	+++++	19.45 ± 1.21
M07	62.43 ± 1.52	+++	17.31 ± 1.04
M08	50.87 ±1.10	++++	26.54 ± 1.20
M09	8.06 ± 0.77	++	23.07 ± 0.94
M10	19.82 ± 1.14	++	21.34 ± 2.11
M11	–	–	54.41 ± 1.31
M12	–	–	35.25 ± 0.84
M13	–	–	21.64 ± 1.11

Note: [a] Production of IAA determined in MM liquid medium amended with L-tryptophan after 6 d of growth.
[b] +++++, very high; ++++, high; +++, moderate; ++/+, low; –, not detected.
[c] Amount of phosphorus solubilized into a NBRIP liquid medium.

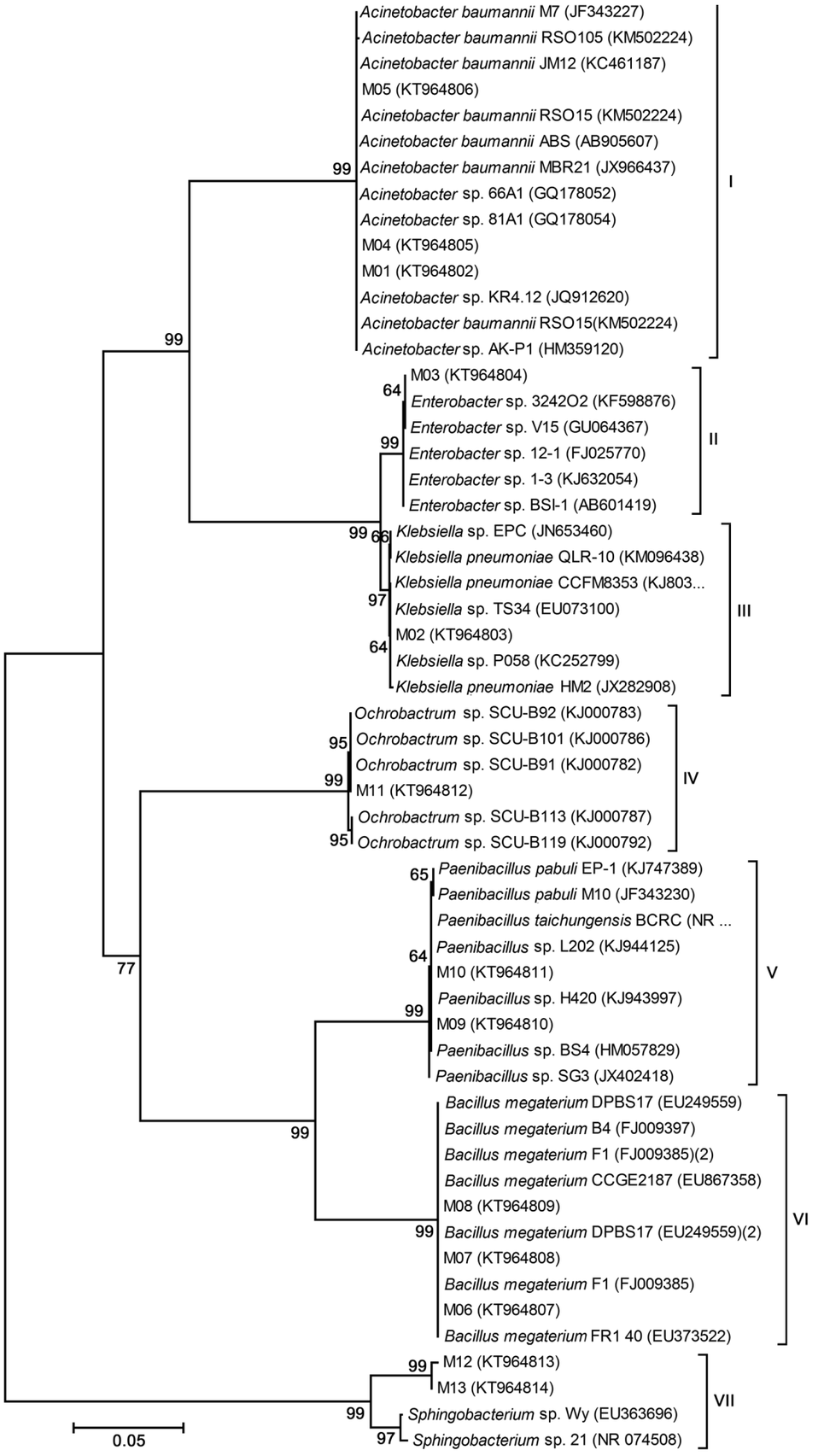
Acinetobacter baumannii M7 (JF343227)
Acinetobacter baumannii RSO105 (KM502224)
Acinetobacter baumannii JM12 (KC461187)
M05 (KT964806)
Acinetobacter baumannii RSO15 (KM502224)
Acinetobacter baumannii ABS (AB905607)
Acinetobacter baumannii MBR21 (JX966437)
Acinetobacter sp. 66A1 (GQ178052)
Acinetobacter sp. 81A1 (GQ178054)
M04 (KT964805)
M01 (KT964802)
Acinetobacter sp. KR4.12 (JQ912620)
Acinetobacter baumannii RSO15(KM502224)
Acinetobacter sp. AK-P1 (HM359120)
I
M03 (KT964804)
Enterobacter sp. 3242O2 (KF598876)
Enterobacter sp. V15 (GU064367)
Enterobacter sp. 12-1 (FJ025770)
Enterobacter sp. 1-3 (KJ632054)
Enterobacter sp. BSI-1 (AB601419)
II
Klebsiella sp. EPC (JN653460)
Klebsiella pneumoniae QLR-10 (KM096438)
Klebsiella pneumoniae CCFM8353 (KJ803...
Klebsiella sp. TS34 (EU073100)
M02 (KT964803)
Klebsiella sp. P058 (KC252799)
Klebsiella pneumoniae HM2 (JX282908)
III
Ochrobactrum sp. SCU-B92 (KJ000783)
Ochrobactrum sp. SCU-B101 (KJ000786)
Ochrobactrum sp. SCU-B91 (KJ000782)
M11 (KT964812)
Ochrobactrum sp. SCU-B113 (KJ000787)
Ochrobactrum sp. SCU-B119 (KJ000792)
IV
Paenibacillus pabuli EP-1 (KJ747389)
Paenibacillus pabuli M10 (JF343230)
Paenibacillus taichungensis BCRC (NR ...
Paenibacillus sp. L202 (KJ944125)
M10 (KT964811)
Paenibacillus sp. H420 (KJ943997)
M09 (KT964810)
Paenibacillus sp. BS4 (HM057829)
Paenibacillus sp. SG3 (JX402418)
V
Bacillus megaterium DPBS17 (EU249559)
Bacillus megaterium B4 (FJ009397)
Bacillus megaterium F1 (FJ009385)(2)
Bacillus megaterium CCGE2187 (EU867358)
M08 (KT964809)
Bacillus megaterium DPBS17 (EU249559)(2)
M07 (KT964808)
Bacillus megaterium F1 (FJ009385)
M06 (KT964807)
Bacillus megaterium FR1 40 (EU373522)
VI
M12 (KT964813)
M13 (KT964814)
Sphingobacterium sp. Wy (EU363696)
Sphingobacterium sp. 21 (NR 074508)
VII
99
99
99
99
66
97
64
99
95
99
95
65
64
77
99
99
99
99
97
0.05

Fig. 3. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence shows the position of isolated strains with the species of each genus downloaded from the NCBI database. Bootstrap percentage values as obtained from 1000 resamplings of the data set are given at the nodes of the tree. Only values higher than 50% are shown. Bar represents 0.05 substitutions per nucleotide position.

Sphingobacterium sp. (Table I). On the basis of the neighbor-joining method, a total of 13 strains were clustered into seven genus groups, and the three major groups were *Acinetobacter*, *Bacillus*, and *Paenibacillus* (Fig. 3).

Plant growth promotion. Three isolates displaying sufficient phosphorus production were chosen to determine their beneficial effects on tomato growth under greenhouse conditions. Tomato shoots grew better than controls when inoculated with the three isolates. All three strain M01, M04 and M11 significantly promoted the shoot dry weight by 30.5%, 32.6% and 26.2%, and root dry weight by 27.1%, 33.1% and 25.6%, respectively, compared to those of the control (Fig. 4).

Discussion

Phosphorus in soil is important for plant development, and the lack of P limits plant growth. Although chemical fertilizers are added to the soils, plants can only utilize low amounts of phosphatic fertilizers. In this case, the selection of highly efficient PSB will practically increase phosphorus in plant rhizosphere. Various PSB have been isolated from different plant roots (Yu *et al.*, 2011; Afshan *et al.*, 2015). Hence, PSB can be regarded as one kind of plant growth-promoting rhizobacteria, which are widely considered as alternatives to common biofertilizers (Vessey, 2003; Hafeez *et al.*, 2006; Adesemoye *et al.*, 2009). In addition, the presence of P-solubilizing microbial population in soils will be considered a positive indicator of utilizing the microbes as biofertilizers for crop production (Afshan *et al.*, 2015). Mushroom waste residues are an artificial ecosystem that harbors a complete spectrum of various bacteria (Johri, 2011). In the present study, a total of 13 isolates were obtained from mushroom residues. Selective NBRIP medium was used for isolation according to their phosphate-solubilizing ability. Our results confirmed that different PSB could be isolated from mushroom resides.

Currently, numerous attempts have been made to isolate the effective plant growth-promoting bacteria according to different criteria, such as auxin and siderophore production or nitrogen-fixing activities (Ding *et al.*, 2005; Chopade *et al.*, 2009; Park *et al.*, 2011; Majeed *et al.*, 2015; Ullah and Bano, 2015). The beneficial effect of PSB in maintaining adequate levels of mineral nutrients, particularly P, in crop production had also been previously reported (Afshan *et al.*, 2015). In the present study, phosphate-dissolving ability was considered a criterion for isolating highly effective PSB strains from waste mushroom residues according to the dissolved phosphorus halo. Out of 13 isolates, seven strains were derived from WF, thereby indicating that wood flour favored the growth of PSB. Considering the isolation of PSB from mushroom resi-

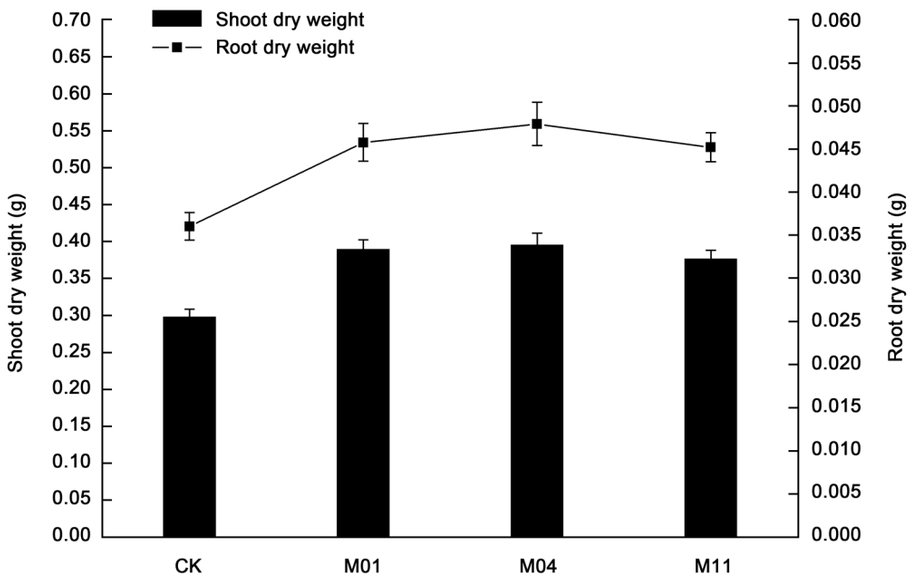

Fig. 4. Growth promotion effects of phosphate-solubilizing isolates on tomato shoot and root dry weights. Significant differences tested according to Fisher's protected LSD at $^{*}p \leq 0.05$. Values are the means ± standard deviations of three experiments.

dues, the amount of isolates in the current study was lower than that reported by Yu *et al.* (2011), but higher than in other reports (Afshan *et al.*, 2015). However, bacteria are considerably diverse because of different crops and soil types. To our knowledge, only few reports have identified the bacterial species in mushroom compost.

IAA production by bacteria isolated from different crops, such as wheat and rice, had already been reported (Park *et al.*, 2005; Afshan *et al.*, 2015). In the current study, about 61.5% of the isolates produced IAA within the range of 8.06–62.43 mg/l, which indicated a substantial variability among isolates for IAA production. Furthermore, approximately 46.2% of strains produced siderophores, and all the strains solubilized tricalcium phosphate. The amount of IAA detected in the present study is close to that reported by Afshan *et al.* (2015), but lower than that reported earlier (Park *et al.*, 2005). These differences may be attributed to the different sources and substantial variability among bacteria. IAA production is also an indicator of plant growth-promoting rhizobacteria (Chopade *et al.*, 2009), thus indicating that the mushroom residues under investigation have bacteria that can enhance plant growth. Previous reports showed that the phosphate solubilization ability of *Pseudomonas* sp. and *Bacillus* sp. is 90 and 60 μg/ml, respectively (Nautiyal, 1999). The same amount of phosphate solubilization abilities were obtained from the mushroom residues in the present study. However, the soluble phosphate released was also lower than that reported by Hafeez *et al.* (2006) and Yu *et al.* (2011). Considering the genus and sources, bacteria may show different phosphate solubilization abilities. In total, about 38.5% of isolates displayed three types of plant growth-promoting traits, hence suggesting the possibility to isolate PSB from mushroom resides.

All the strains displaying phosphate-solubilizing traits were identified based on a 16S rDNA partial sequence. Out of 13 sequenced isolates, 3, 3, and 2 isolates belonged to the cluster of *Acinetobacter* sp., *Bacillus*, and *Paenibacillus* sp., respectively. One isolate belonged to each of the genera *Klebsiella* sp., *Enterobacter* sp., and *Ochrobactrum* sp. Two strains belonged to *Sphingobacterium* sp. The number of isolates belonging to the genera *Acinetobacter* and *Bacillus* was higher than those from other groups, which mean that these two genera are dominant in mushroom waste residues. In addition, *Bacillus* spp. is dominant in root-adhering soil (Laguerre *et al.*, 1994). Based on the neighbor-joining phylogenetic tree constructed from 16S rRNA gene sequences, a total of seven genera were identified in the present study. Therefore, these bacterial strains exhibiting phosphate-solubilizing activity presented large spectrum of microbial diversity, and the present results were consistent with previous findings (Johri, 2011). Notably, strain M11 belonged to genus *Ochrobactrum*, which will help us further understand this genus.

Considering the low solubility of phosphorus in plant rhizosphere soil, three highest phosphorus-dissolving isolates were used to determine their beneficial effects on tomato growth under greenhouse conditions. The inoculation of three isolates M01, M04, and M11 significantly increased the tomato's shoot and root dry weight. This finding indicated that PSB could stimulate early root growth in tomato seedling. Simultaneous growth promotion as the result of PSB inoculations leads to an increase in the yield of maize and other cereals (Ullah and Bano, 2015). The significant increase in plant dry weight caused by the inoculation with PSB strains has also been reported in other plants, such as walnut plant seedlings and wheat (Yu *et al.*, 2011; Afshan *et al.*, 2015). The present results were in agreement with those reported for greenhouse experiments. Hence, inoculation with PSB might have released considerable amount of available P in plant root, which were utilized by plants in the pot experiment. Based on 16S rRNA gene sequence comparisons and phylogenetic positions, isolates M01 and M04 belonged to the genus *Acinetobacter*, and M11 belonged to the genus *Ochrobactrum* sp. To date, few reports have revealed that genus *Ochrobactrum* possesses phosphate-dissolving ability. The use of PSB as inoculants would minimize the negative impact of chemical fertilizers on the environment and promote plant growth. Taken together, the selection of an efficient PSB strain as possible inoculants should be based not only on the laboratory assays and greenhouse trails, but also in field experiments. Further studies should be focused on the practical applications in the field.

Conclusion

A total of 13 strains were isolated in this study. Most of the isolates displayed plant growth-promoting traits, thereby indicating that they have doable function for enhancing plant growth. The present findings suggest that waste mushroom residues are a potential resource of plant growth-promoting bacteria. Furthermore, the obtained isolates exhibited satisfactory phosphate-solubilizing activity for application in sustainable agriculture.

Disclosure statement

The authors declare that they have no competing interests.

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ORIGINAL PAPER

Dipicolinic Acid Release and the Germination of *Alicyclobacillus acidoterrestris* Spores under Nutrient Germinants

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Abstract

The presence of *Alicyclobacillus*, a thermoacidophilic and spore-forming bacterium, in acidic fruit juices poses a serious problem for the processing industry. A typical sign of spoilage in contaminated juices is a characteristic phenolic off-flavour associated with the production of guaiacol. Spores are formed in response to starvation and in a natural environment re-access the nutrients, *e.g.*: L-alanine and AGFK – a mixture of asparagine, glucose, fructose and potassium, triggers germination. The aim of this study was to estimate the impact of L-alanine and AGFK on the germination of the spores of two *Alicyclobacillus acidoterrestris* strains and to evaluate the relationship of the germination rate with dipicolinic acid (DPA) release. The spores were suspended in apple juice or in buffers at pH 4 and pH 7, followed by the addition of L-alanine and AGFK. Suspensions were or were not subjected, to a temperature of 80°C/10 min and incubated for various periods of time at 45°C. Optical density (OD_{660}) was used to estimate the number of germinated spores. The amount of DPA released was determined using HPLC. The results indicate that the degree of germination of *A. acidoterrestris* spores depended on the strain and time of incubation and the nutritious compounds used. The data obtained show that the amount of DPA released correlated to the number of *A. acidoterrestris* spores germinated.

Key words: *Alicyclobacillus acidoterrestris*, AGFK mixture, dipicolinic acid, L-alanine, spore germination

Introduction

Alicyclobacillus acidoterrestris, a gram-positive, thermoacidophilic, spore forming bacterium, is a frequent contaminant of juices and is a common spoilage microorganism in the processing industry. Due to its ability to undergo sporulation in an acidic environment, and to spoil juices by producing undesirable off-flavours (Sokołowska, 2014; Tianli *et al*., 2014), comprehensive knowledge about the germination of *A. acidoterrestris* spores is of general interest. The germination of *A. acidoterrestris* spores, and their subsequent outgrowth or inactivation, can be induced by external factors such as high hydrostatic pressure (Wuytack *et al*., 2000; Vercammen *et al*., 2012; Sokołowska *et al*., 2013; 2015; Porębska *et al*., 2015a) or supercritical carbon dioxide (Bae *et al*., 2009; Porębska *et al*., 2016). The data indicate that within *Bacillus* species, the germination of spores can also be induced by various nutrients: amino acids, purine nucleosides, sugars (Lovdal *et al*., 2012), L-alanine (Parades-Sabja *et al*., 2011; Kuwana and Takamatsu, 2013; Cruz-Mora *et al*., 2015), ions and combinations of these, and a mixture of asparagine, glucose, fructose and potassium ions (AGFK) (Gosh *et al*., 2012; Stewart *et al*., 2012).

The mechanism of spore germination is very complex and has been the subject of many studies (Setlow *et al*., 2008; Parades-Sabja *et al*., 2011; Luu and Setlow, 2014; Bevilacqua *et al*., 2015; Luu *et al*., 20 15; Troiano *et al*., 2015). Nutrients initiate spore germination by binding to the germination receptors (GRs), located in the spore's inner membrane which rapidly degrades the cortex peptidoglycan. Water is taken up, calcium dipicolinate (Ca-DPA) is released and a variety of spore constituents are degraded by hydrolytic enzymes. Although proteins which are likely candidates for DPA channels in the spore membranes have been identified, as yet there is no understanding of how the *Ger* receptors interacti on triggers the earliest events in spore germination, including the release of DPA and cations (Setlow *et al*., 2006).

Each *Ger* receptor can detect a specific germinant, including amino acids, nucleosides, sugars and cations.

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Thus, expressing multiple *Ger* receptor operons allows spores to respond to structurally diverse compounds. Different *Ger* receptors can cooperate to recognize a single germinant or multiple germinants (Moir *et al*., 2002; Moir, 2006; Ross and Abel-Santos, 2010; Mongkolthanaruk *et al*., 2013).

The mechanism whereby individual GRs can cooperate to recognize a single germinant or multiple germinants to allow or accelerate germination is not known. However, it has been suggested that either various individual GRs form complexes in the spore's inner membrane or there is a mechanism which integrates signals from various individual GRs or GR complexes and that this integration determines the ultimate rate of germination (Yi *et al*., 2011). For example L-alanine and AGFK mixture as the nutrient germinants in the spore germination process may be involved in integrating signals from different GRs (Parades-Sabja *et al*., 2011; Wang *et al*., 2015). During spore germination, Ca-DPA release is preceded by the loss of resistance to heat, the release of Na^+, K^+, H^+, and Zn^{2+}, and a significant change in the elastic light-scattering intensity from individual spores (Luu and Setlow, 2014).

In the case of *Bacillus subtilis*, the GRs are encoded by homologous tricistronic *GerA*, *GerB* and *GerK* operons. Each of these GRs contains A, B and C sub-units, all of which are required for the function of the individual GR. The *GerA* receptor responds to either L-alanine or valine, while the *GerB* and *GerK* receptors together are essential for germination in a AGFK mixture (Yi *et al*., 2011; Stewart *et al*., 2012; Chen *et al*., 2014).

In general, the model for bacterial spore germination for *Bacillus* and *Clostridium* is known, and is associated with the expression of *Ger* proteins. The *Ger* family germination protein can also be found in *Alicyclobacillus* [http://www.ncbi.nlm.nih.gov/protein/YP_003184683.1?report=genpept].

L-alanine is a common germinant for both *Bacillus* and *Clostridia* species. L-alanine-mediated germination has been characterized mostly in *B. subtilis* spores (McCann *et al*., 1996; Ramirez-Peralta *et al*., 2012). However, L-alanine can also induce the germination of *B. cereus* (Barlass *et al*., 2002), *B. anthracis* (Fisher and Hanna, 2005), *B. megaterium* (Christie and Lowel, 2007), *B. lichenoformis* (Lovdal *et al*., 2012), *C. botulinum* (Broussolle *et al*., 2002), *C. sporogenes* (Broussolle *et al*., 2002), *C. perfringes* (Parades-Sabja *et al*., 2008) and *C. sordellii* (Ramirez and Abel-Santos, 2010). In fact L-alanine seems to be the most ubiquitous germinant for bacterial spores. There are data indicating that high hydrostatic pressures of 200 MPa – 400 MPa also trigger germination through the *GerA*, *GerB* and *GerK* receptors (Parades-Sabja *et al*., 2011).

To understand the molecular determinants of *Ger* receptor interactions and their effect on germinant recognition, the kinetic method can be used to study bacterial spore germination (Abel-Santos and Dodatko, 2007; Akoachere *et al*., 2007; Ramirez and Abel-Santos, 2010; Zhang J. *et al*., 2011; Zhang P. *et al*., 2010; 2014; Brunt *et al*., 2014). Spore germination can be analysed by optical density decreases (Terano *et al*., 2005; Akoachere *et al*., 2007; Brunt *et al*., 2014; Nagler *et al*., 2015; Porębska *et al*., 2015a).

The aim of this study was to characterize the process of spore germination in two *A. acidoterrestris* strains, initiated by L-alanine and AGFK, and to evaluate the relationship between DPA release and the germination of *A. acidoterrestris* spores induced by these biochemical substances. This study is an attempt to expand the current state of knowledge concerning the mechanism of the *A. acidoterrestris* spore germination process, the variations in spore population and the factors stimulating this process.

Experimental

Materials and Methods

Tested organisms. The *A. acidoterrestris* strains TO-169/06 and TO-117/02 used in this study were isolated from Polish concentrated apple juice, using the International Federation of Fruit Juice Producers' method (2004/2007). These strains were selected from among eight wild strains tested previously (Skąpska *et al*., 2012; Porębska *et al*., 2015a; 2015b; 2016). TO-117/02 was the strain highly resistant to temperature and HHP and TO-169/06 was the sensitive one.

Spore production. Spores were produced based on a method described by Sokołowska *et al.* (2012). Just before the experiments, the spores (> 95% phase bright – ungerminated) were suspended in apple juice (11.2 Bx, pH 3.4) or in a McIlvain buffer solution of pH 4.0 and pH 7.0. L-alanine (50 mM) or AGFK (50 mM) were then added to the samples, which were afterwards subjected, or not subjected, to a temperature of 80°C/10 min and incubated at 45°C (Bevilacqua *et al*., 2014). The number of spores in the suspensions was approximately 6 log cfu/ml for determining spore germination using the drop in optical density, and approximately 9 log cfu/ml for determining the release of dipicolinic acid.

Optical density measurement. The optical density (OD_{660}) of the spore suspensions was measured at a wavelength of 660 nm in a UV/Visible spectrophotometer Ultrospec 2000 (Pharmacia Biotech Ltd., England). To estimate the effect of the process parameters on the dynamics of spore germination, the drop in optical density was expressed as the ratio (OD_{660} during germination/OD_{660} before germination) × 100% (Terano *et al*., 2005; Kato *et al*., 2009; Pandey *et al*., 2013;

Bevilacqua *et al*., 2014; Porębska *et al*., 2015a). The optical density was measured 10 min after the addition of L-alanine or AGFK, after incubation for 30 min, for 4 h, and after overnight incubation of the same samples at 45°C (optimum growth temperature).

Determining the release of dipicolinic acid. Quantification of the DPA concentration in the samples 10 min after the addition of nutrient germinants was performed using the HPLC method (Warth, 1979). A Waters 2695 Separations Module with Waters 2996 Photodiode Array Detector system and SunFire C8 Column, (5 µm, 4.6 mm × 250 mm) with SunFire C8 Guard Pre-column, (5 µm, 4.6 mm × 20 mm) were used.

Samples containing 50 to 1000 µM DPA (0.1 to 2 mg of spores per ml) in 0.2 M potassium phosphate, pH 1.75, were prepared and centrifuged at 17000 × *g* for 10 min at 4°C and filtered through a 0.2-µm membrane filter. All the samples were stored at −70°C prior to the HPLC analysis. Ten µl samples were injected onto the column at approximately 12-min intervals. Elution was carried out with 1.5% tertamylalcohol in 0.2 M potassium phosphate, pH 1.75, at a flow rate of 1.0 ml/min at 25°C. The eluent was filtered and degassed. The stock solution of pH 1.75 buffer contained 3 M H_3PO_4 and 1 M KH_2PO_4. The peak heights at 271 nm were measured at 0.02 or 0.20 absorbance units, full scale.

To determine the total amount of DPA in the spore suspensions, 3 ml of each individual batch was sterilized at 121°C for 20 min and then analysed (Reineke *et al*., 2013a).

Data analysis. Analysis of variance and Duncan's multiple-range test, using StatSoft® Statistica 7.1, was used to test the significance of the differences ($p < 0.05$) between the drop in optical density. The assays were performed using two independent samples. Microsoft Office Excel 2010 was used for linear regression and to calculate the coefficient of determination (R^2) and coefficient of correlation (r).

Results and Discussion

According to the literature, optical density measurement allows a rapid assessment of spore germination (Terano *el al*., 2005; Porębska *et al*., 2015a). When spores begin to germinate, optical density decreases, and begins to rise again when the outgrowth stage begins. Previously, Porębska *et al.* (2015a) showed that 1 log cfu/ml germination of *A. acidoterrestris* spores under high hydrostatic pressure resulted in a 4.5% decrease in optical density ($R^2 = 0.84$). The subsequent rapid release of Ca-DPA under external factors is accompanied by the activation of the spores CLEs which degrade the cortex and cause a loss in the optical density of the spore (Reineke *et al*., 2013b). The germination of *A. acidoterrestris* spores induced by nutrient germinants was assessed by measuring the optical density and monitoring DPA release. Porębska *et al.* (2015b) showed that 2 log cfu/ml germination of *A. acidoterrestris* spores under high hydrostatic pressure resulted in 8.7 µM DPA being released from the spore suspension ($R^2 = 0.89$). Fig. 1 shows the dynamics of the germination of *A. acidoterrestris* 169/06 spores in apple juice with nutrient germinants – L-alanine and AGFK.

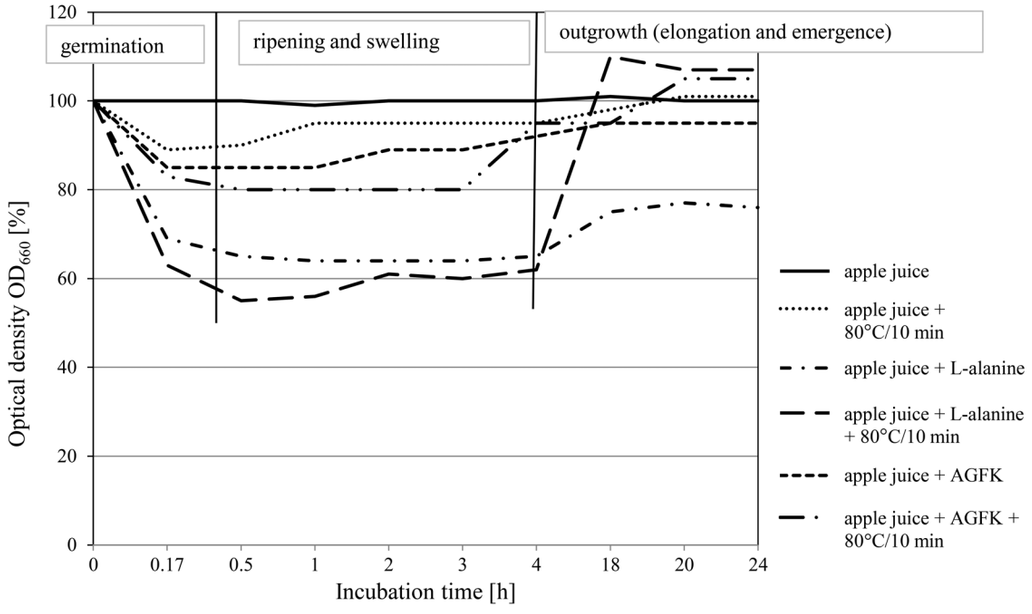

Fig. 1. Dynamics of the germination of *A. acidoterrestris* 169/06 spores in apple juice with nutrient germinants.

Germination occurred and there was also a significant drop (of 15%) in optical density after 10 min incubation at 45°C in the samples treated with AGFK. A higher (17%) drop in optical density was observed when the samples were additionally subjected to a temperature of 80°C/10 min and incubated at 45°C. The samples with L-alanine indicated a 31% drop in optical density. The best results (a 37% decrease in optical density) were observed when the samples of apple juice with L-alanine were additionally subjected to a temperature of 80°C/10 min and incubated at 45°C. It was expected that thermal treatment would determine a reduction in absorbance, as it is known that slight heat shocks can additionally activate spores (Byun *et al*., 2011). Similar results were obtained by Bevilacqua *et al.* (2014), who reported promoting effect of L-alanine on spore germination. During incubation at 45°C for a further 4 hours, no change in optical density was observed. After further incubation overnight, in favourable conditions, a significant increase in the optical density of apple juice with germinants and heat treatment was achieved. The highest increase was noticed in apple juice with L-alanine. This indicates that L-alanine and heat treatment promoted spore germination. The germinated spores transformed into vegetative cells and a subsequent growth of *A. acidoterrestris* in apple juice was observed (Fig. 1).

The specificity of the nutrient germinants is strain and species specific and probably reflects the adaptation of spore formers to their specific environmental niches. Some pathogenic spore formers require specific nutrient germinants. In models for bacterial spore germination the release of DPA through a DPA channel, presumably composed at least partly of SpoVA proteins, which leads to the activation of CwIJ, whereas changes in the cortex strain might activate SleB. These two redundant CLEs (cortex lytic enzymes) degrade the PG cortex, allowing the completion of germination and initiation of spore outgrowth (Parades-Sabja *et al*., 2011). In our study, to complete the germination of the spores and return them to a vegetative form, incubation with nutrient germinants and treatment with heat shock was necessary. Similar results were observed by Terano *et al.* (2005) and Brunt *et al.* (2014). They found that germination was initiated following the recognition of small molecules (germinants) by GRs located in the spore's inner membrane, and that the addition of L-alanine initiated spore germination but had no effect on the rate or overall germination process in the case of *Clostridium* sp.

Germination as a decrease in the optical density of a suspension of *A. acidoterrestris* 169/06 spores in apple juice and buffers with different pH after 10 min incubation at 45°C is presented in Figure 2. The highest germination and highest DPA release was observed in apple juice and buffer pH 4 with L-alanine and after heat treatment. In buffer pH 7, germination was suppressed. This could be associated with the acidophilic nature of these bacteria. The results indicate that the nutrients present in apple juice can promote the germination of *A. acidoterrestris* spores in the presence of L-alanine. These results confirmed that L-alanine induces spore germination (Barlass *et al*., 2002).

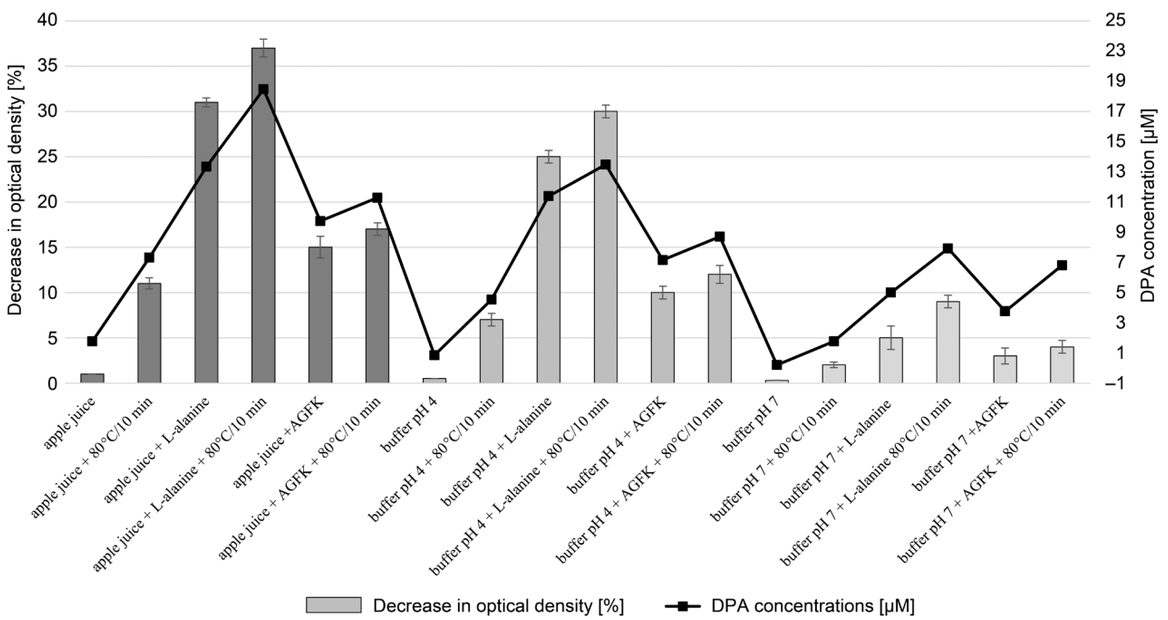

Fig. 2. Germination as a decrease in optical density and DPA released from *A. acidoterrestris* 169/06 spores in buffers at low and neutral pH and in commercial apple juice.

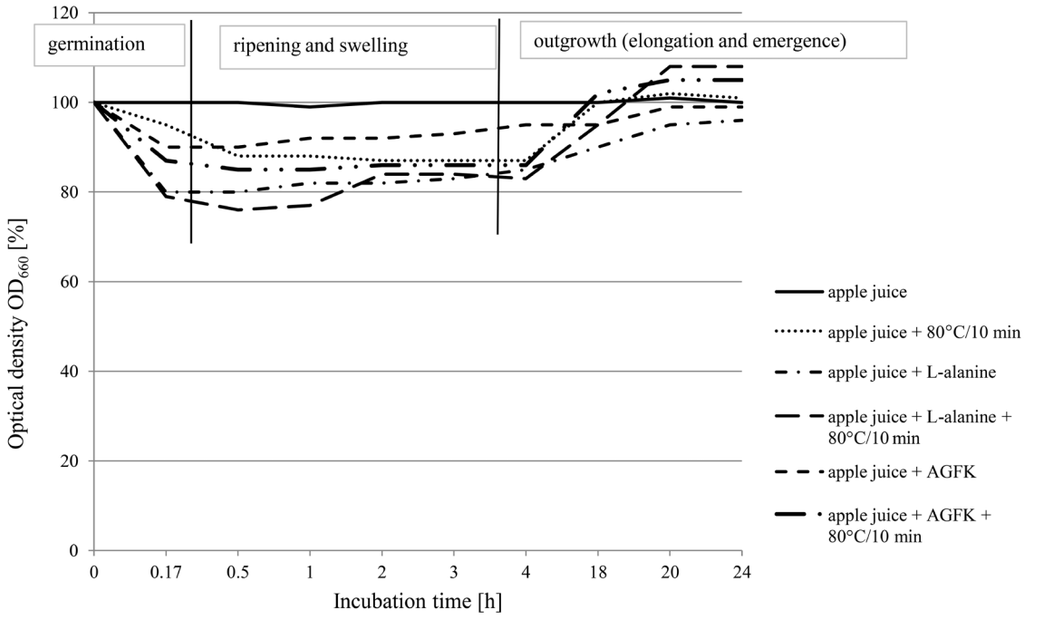

Fig. 3. Dynamics of the germination of *A. acidoterrestris* 117/02 spores in apple juice with nutrient germinants.

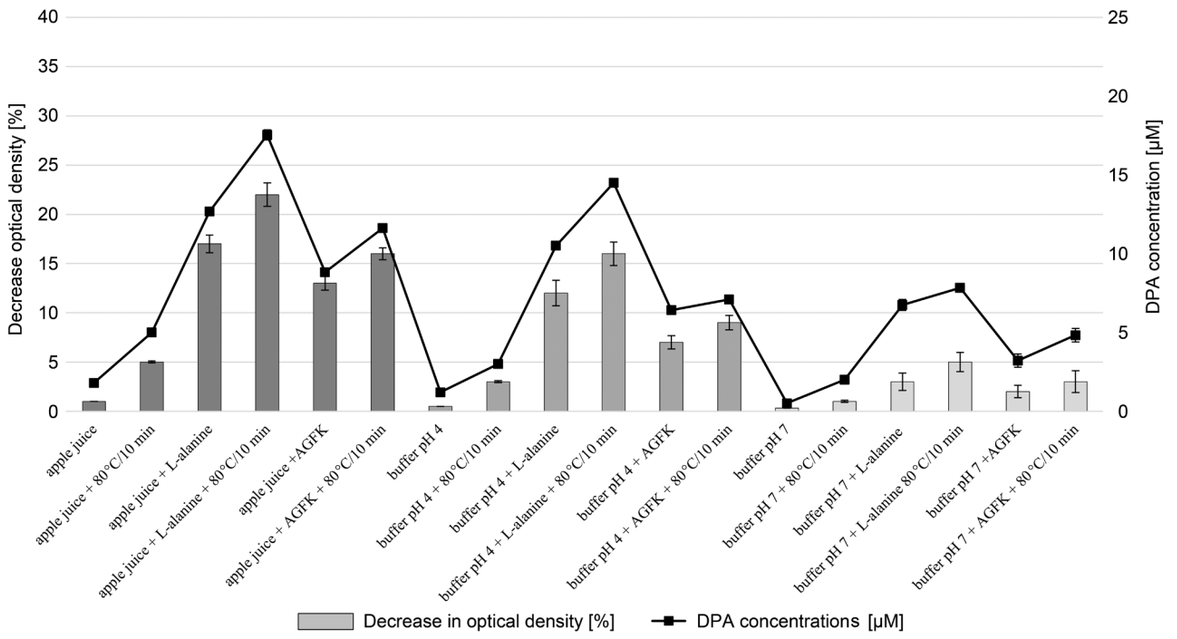

Fig. 4. Germination as a decrease in optical density and DPA released from *A. acidoterrestris* 117/02 spores in buffers at low and neutral pH and in commercial apple juice.

Some aspects of *A. acidoterrestris* spore germination triggered by nutrients and heat treatment were investigated by Terano *et al.* (2005). *A. acidoterrestris* spores showed efficient germination after heat activation in potato dextrose medium (pH 4) and commercial fruit juices. The same treatment was lethal for germinated spores. Germination was weaker in buffer pH 4, but increased to levels comparable to growth in apple juice in the presence of L-alanine. In phosphate buffer pH 7, germination was suppressed.

The results obtained under the same conditions for spores of the second *A. acidoterrestris* TO-117/02 strain, showed the same trend, but at lower values, indicating a weaker germination process (Fig. 3–4).

The germination of *A. acidoterrestris* 117/02 spores in apple juice and buffers of different pH is presented in

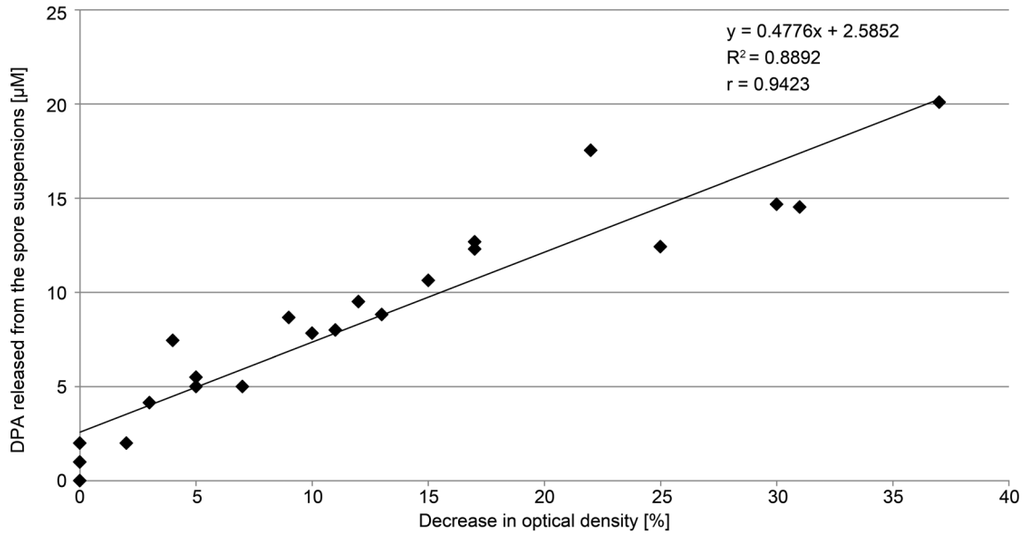

Fig. 5. DPA released from the spore suspensions *vs* decrease in optical density as a measure of the number of germinated *A. acidoterrestris* spores.

Figure 4. The results indicate that the nutrients present in apple juice can promote the germination of *A. acidoterrestris* spores with the addition L-alanine. In many cases, nutrients such as L-alanine are those found in environments favoured by growing bacteria derived from spores. Nutrients trigger spore germination *via* their interaction with germinant receptors, proteins that recognize and respond to specific nutrients such as sugar, amino-acids and cations. Spores of the bacteria germinate better with co-germinants such as, in the case of our study, glucose from apple juice with L-alanine and AGFK or with glycine (Parades-Sabja *et al*., 2011) and lysozyme (Bevilacqua *et al*., 2014).

In the second part of our study, we focused on examining the process of DPA release. An early event in spore germination is DPA release, a process that likely requires proteins in the inner spore membrane and prior to DPA release, there is also a release of monovalent ions (Cabrera-Martinez *et al*., 2003).

The data presented in Fig. 2 and 4, showing the dynamics of DPA release, derived from processes conducted under the same conditions as the experiments in which the germination phenomenon was investigated. DPA concentrations were measured after 10 min incubation with a nutrient germinant.

The total amount of DPA present in *A. acidoterrestris* TO-169/06 spores (released during sterilization) was 50.3 µM, and 42.7 µM for the TO-117/02 strain, respectively (data not showed). The highest amount of released DPA was achieved during the incubation of spores in apple juice with L-alanine and after additional heat treatment and was 20.1 µM for TO-169/06 spores (40% of the total DPA) (Fig. 2) and 17.5 µM for TO-117/02 spores (41% of the total DPA) (Fig. 4).

The relationship between DPA release after incubation with nutrient-induced germination of *A. acidoterrestris* spores is presented in Figure 5. A good correlation ($R^2 = 0.8892$, $r = 0.9423$) between these variables was observed. A similar phenomenon was observed by Porębska *et al*. (2015b), who claimed that the amount of DPA released correlated to the amount of germinated *A. acidoterrestris* spores treated with high hydrostatic pressure. Setlow *et al*. (2003) claimed that dodecylamine may be correlated with DPA concentrations and trigger spore germination by directly or indirectly activating the release of DPA from the spore core, through the opening of channels for DPA in the spore's inner membrane.

Strains of bacteria possess an efficient germination machinery for L-alanine and AGFK germination. In our study we observed that AGFK had a less impact on germination of *A. acidoterrestris* spores than L-alanine. AGFK is often less efficient, because the GerB genes are more diverged, and the two germinant receptor operons of unknown function could have been lost from the genome in these strains (Cabera-Martinez *et al*., 2003).

The *A. acidoterrestris* strains have conserved the *GerA* receptor function, confirming its importance, at least in the natural environments of these strains. It also seems likely that *Ger* proteins have a direct effect on the release of DPA that takes places early in germination and during germination induced by nutrient germinants. This step of germination is crucial with regard to loss of resistance and it is therefore of great interest for a variety of food preservation techniques and further research.

Until now, no studies have been reported on the DPA release of *A. acidoterrestris* spores in L-alanine and AGFK.

Conclusions

The presence of L-alanine and AGFK in the environment stimulated spore germination. The results indicate that the degree of germination of *A. acidoterrestris* spores depended on the strain and medium. Optical density is a fast and effective method for estimating the dynamics of spore germination. The results showed a greater decrease in optical density after incubation with L-alanine than with AGFK, and a subsequent increase of this parameter after overnight incubation at 45°C. It is worth noting that heat treatment additionally stimulated the increase in the rate of germination of spores. This indicates the start of the next phase, outgrowth. These results may show that the strains of *A. acidoterrestris* tested are dominated by the presence of *GerA* receptors. A slight decrease in the germination of spores was observed with an increase in the pH, while a lower pH was conducive to germination. The nutrients in apple juice also can stimulate *A. acidoterrestris* spores to germinate. The process of DPA release from the spores depended on the strain and biochemical substances. The amount of DPA released correlated to the amount of *A. acidoterrestris* spores germinated.

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ORIGINAL PAPER

Microbial Biomass and Enzymatic Activity of the Surface Microlayer and Subsurface Water in Two Dystrophic Lakes

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Abstract

Nutrient and organic matter concentration, microbial biomass and activities were studied at the surface microlayers (SML) and subsurface waters (SSW) in two small forest lakes of different water colour. The SML in polyhumic lake is more enriched with dissolved inorganic nitrogen (0.141 mg l^{-1}) than that of oligohumic lake (0.124 mg l^{-1}), the former also contains higher levels of total nitrogen (2.66 mg l^{-1}). Higher activities of lipase (V_{max} 2290 nmol l^{-1} h^{-1} in oligo- and 6098 in polyhumic) and glucosidase (V_{max} 41 nmol l^{-1} h^{-1} in oligo- and 49 in polyhumic) were in the SMLs in both lakes. Phosphatase activity was higher in the oligohumic SML than in SSW (V_{max} 632 vs. 339 nmol l^{-1} h^{-1}) while in polyhumic lake was higher in SSW (V_{max} 2258 nmol l^{-1} h^{-1} vs. 1908 nmol l^{-1} h^{-1}). Aminopeptidase activity in the SSW in both lakes was higher than in SMLs (V_{max} 2117 in oligo- and 1213 nmol l^{-1} h^{-1} in polyhumic). It seems that solar radiation does inhibit neuston microbial community as a whole because secondary production and the share of active bacteria in total bacteria number were higher in SSW. However, in the oligohumic lake the abundance of bacteria in the SML was always higher than in the SSW (4.07 vs. 2.69×10^6 cells ml^{-1}) while in the polyhumic lake was roughly equal (4.48 vs. 4.33×10^6 cells ml^{-1}) in both layers. Results may also suggest that surface communities are not supplemented by immigration from bulk communities. The SML of humic lakes may act as important sinks for allochthonous nutrient resources and may then generate considerable energy pools for microbial food webs.

Key words: dystrophic lakes, enzymatic activity in lakes, neuston, bacteria in surface microlayer

Introduction

At the air-water interface, the surface microlayer (SML) is physico-chemically distinct compared to the subsurface water (SSW) and is characteristically enriched with nutrients and organic matter (Dietz *et al.*, 1976; Södergren, 1993; Hunter, 1997; Münster *et al.*, 1998; Franklin *et al.*, 2005; Stolle *et al.*, 2009). The presence of the surface film and surface tension properties causes the SML to be a unique ecotone inhabited by microorganisms called neuston to distinguish from the subsurface plankton (term first used by Nauman 1917 and cited in Cunliffe *et al.*, 2011). For bacterioneuston, it is considered to be a stressful habitat on the one hand, but on the other – it might favour bacterial heterotrophic activity through the accumulation of organic matter (OM) (Cunliffe *et al.*, 2011). Most organic matter inputs into water bodies are not directly utilisable by bacteria, which cannot access organic material that is larger than 600 Da (Weiss *et al.*, 1991). Therefore, bacteria induce extracellular enzymes that hydrolyse polymers and oligomers into labile monomers that can pass through cell membranes (Chróst, 1991; Weiss *et al.*, 1991; Hoppe *et al.*, 1991 cited in Williams and Jochem, 2006). Upon exposure to solar radiation, which takes place more intensively in the SML and in the photic zone, active enzymes can be released (Boavida and Wetzel, 1998). The SML is a challenging habitat in which neustonic microbial communities must rapidly respond, with possible effects on the overall bacterial metabolism (Santos *et al.*, 2009).

However, our knowledge of the biology of the SML in humic lakes is still insufficient (Södergren, 1993; Münster *et al.*, 1998; Hillbricht-Ilkowska and Kostrzewska-Szlakowska, 2004; Kostrzewska-Szlakowska, 2005). Much more work has been done in oceans (Dietz *et al.*, 1976; Hermansson and Dahlbäck, 1983; Hunter, 1997; Stolle *et al.*, 2009; Cunliffe *et al.*, 2011). It is believed that these processes that cause enrichment in the SML are very similar in marine and freshwaters (Hardy, 1997).

In this study, we quantitatively characterise the activity of bacteria living in the SML and simultaneously

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those living in the SSW, in two humic lakes of different water colours and assessed the relationship between bacterial activities and various environmental parameters of these lakes. We try to answer the following questions: (1) how do SMLs in lakes of different trophy state behave; (2) does the solar radiation inhibit bacterioneuston activities; (3) are SMLs directly supported from the underlying waters; (4) may SML act as sinks for allochthonous nutrient resources and thus may generate new energy pools for microbial food webs.

Experimental

Materials and Methods

Sampling. Two dystrophic, humic lakes (Kruczy Staw 53°39'42"N, 21°24'21"E and Smolak 53°43'29"N, 21°36'09"E) from north-eastern Poland were sampled monthly from April to October 2008 and some analyses in 2009. SML samples were collected by using Larsson plate. This method was chosen as fast and appropriate for sampling bacteria, microalgae and chemical compounds (Larsson *et al*., 1974; Hillbricht-Ilkowska and Kostrzewska-Szlakowska, 2004). Considering the great dynamics of SML, we sampled the lakes during calm weather (wind speed under 0.3 m s^{-1}) to minimize this variability. This procedure collected volumes of water corresponding to the layer thickness of 0.61 ± 0.13 mm in oligo- and 0.50 ± 0.15 mm in the polyhumic lakes. SSW samples (from 0.5 m depth) were collected using a Limnos apparatus. To compare the concentration of variables in the SML versus SSW, the enrichment factor ($Ef = SML/SSW$) was calculated (Hunter, 1997). Mean enrichment factors (Table I) were estimated as the average from monthly data. Water samples were kept in dark bottles at ambient temperature until laboratory processing within a few hours.

Table I

Mean values for total nitrogen (TN; mg l^{-1}), dissolved inorganic nitrogen (DIN; mg l^{-1}); total phosphorus (TP; µg l^{-1}), orthophosphates (PO_4-P; µg l^{-1}), total and dissolved carbon (TOC, DOC; µg l^{-1}), SUVA, chlorophyll *a* (chl *a*; µg l^{-1}), bacterial numbers (BN; 10^6 cell ml^{-1}). Mean enrichment factors (Ef) were estimated as averages from monthly calculated enrichment factors.

	Oligohumic			Polyhumic		
	SML	SSW	Ef	SML	SSW	Ef
TN	0.97	0.72	1.4	2.66	2.43	1.1
DIN	0.124	0.103	1.2	0.141	0.087	1.5
TP	60.5	86.3	0.8	106.7	110.2	1.0
PO_4-P	3.1	13.5	0.9	17.6	34.6	0.7
TOC	15.1	13.5	1.1	54.1	52.9	1.1
DOC	13.7	12.3	1.1	47.2	48.0	1.0
SUVA	8.6	9.2	1.0	31.7	32.5	1.0
chl *a*	11.1	10.4	1.2	39.3	75.0	0.5
BN	4.07	2.69	1.7	4.48	4.33	1.1

Physico-chemical analyses. During each sampling period, physical conditions (temperature, pH, which were recorded electronically OXI 195 *in situ*), inorganic and organic nutrients, chlorophyll *a* concentrations, bacterial abundance, and ectoenzyme kinetics were determined at each lake and in each water layer. Photosynthetically active radiation (PAR) was measured with a light meter [LI-COR Biosciences (LI-250A), USA] very closely over the surface of the water and a few mm below. Water colour was measured in portable photometer (Hanna Instruments HI93727) in 0-500 Platinum Cobalt Units range.

The chemical analyses were measured in unfiltered (total) and filtered (GF/F – dissolved) samples. The total and dissolved phosphorus (TP and DP), orthophosphates (PO_4-P), ammonia (NH_4-N), nitrate (NO_3-N) and nitrite (NO_2-N) were analysed according to Golterman and Clymo (1978) and total and dissolved nitrogen (TN and DN) according to Dowgiałło (1984). Dissolved inorganic nitrogen (DIN) was calculated as a sum of ammonium, nitrate and nitrite (Williams and Jochem, 2006). Direct determination of calcium (Ca), iron (Fe), magnesium (Mg) and manganese (Mn) in aqueous matrices by atomic absorption spectrometry were analysed according to Flame Atomic Absorption Spectrometry (FAAS, 2007) in Schimadzu AA 660. Total and dissolved organic carbon (TOC and DOC) were determined by high temperature catalytic combustion (Schimadzu TOC 5050A). Specific ultraviolet absorbance at 254 nm (SUVA) was defined as the UV absorbance at 254 nm divided by the DOC concentration (Chow *et al*., 2003).

Chlorophyll *a* concentration (chl *a*) as a proxy for phytoplankton biomass was analysed spectrophotometrically after extraction with 96% hot ethanol (Marker *et al*., 1980). Samples of 0.2–0.75 ml volume of water were concentrated on glass fibre filters GF/F (Whatman, 25 mm).

Bacterial analyses. The number of DAPI (4',6-diamidino-2-phenylindole) stainable bacteria (BN) was determined by direct counting of cells suspended on 0.2 µm, black polycarbonate membrane filters (Millipore) under epifluorescence microscope according to Porter and Feig (1980). For bacteria counting, a computer image analyzing system composed of a Nikon epifluorescence E450 microscope, Nikon Digital Camera DXM 1200F and NIS elements software (Nikon) was used. Bacterial biomass (BB) was calculated from the biovolume and content of C per μm^{-3} 360 fg C according to Arvola *et al*. (1996) and Tulonen (1993).

Secondary production (BP) was measured by the incorporation of ^{3}H-thymidine (^{3}H-TdR according to Chróst *et al*., 1988).

Table II
Substrates used for enzyme activity measurement.

Enzyme	Fluorogenic products of reaction	Substrate	Increasing substrate concentration in the sample (μM)
L-leucine-aminopeptidase (AMP)	AMC (7-amino-4-methylcoumarin hydrochloride (Sigma)	LMCA (L-leucine-4-methyl-7-coumarinylamide) (Fluka)	3.125; 6.25; 10; 12.5; 15; 20; 25
lipase	MUF (4-methylumbelliferone) (Sigma)	MUFB (4-methylumbelliferyl butyrate) (Sigma)	3.125; 6.25; 10; 12.5; 15; 20; 25; 50.0
β-glucosidase (Glu)	MUF (4-methylumbelliferone) (Sigma)	4-Methylumbelliferyl-*β-D* -glucoside (Sigma)	1,25; 2,5; 5,0; 7,5; 10,0; 15,0; 20,0
acid phosphatase (APA)	DiFMU (6,8-difluoro-7-hydfoxy-4-methylcoumarin) (Sigma)	DiFMUP (6,8-difluoro-4-methylumbeliferyl phosphate) (Sigma)	3.125; 6.25; 10; 12.5; 15; 20; 25; 50.0

The share of active bacteria with intact membranes (MEM+) was determined using LIVE/DEAD *Bac*Light Bacterial Viability Kits (Schumann *et al*., 2003). For 1 ml subsamples, a mixture of two *Bac*Light Kits: SYTO 9 and propidium iodide was added (1:1), then incubated for 15 min, filtered through 0.2-μm pore-size black polycarbonate membrane filters Millipore (Millipore Corporation, Billerica, MA, USA, 2013) and enumerated using an epifluorescence microscope (the same as in BN analyses). The percentage contribution of MEM+ bacteria was calculated as the ratio of MEM+ to the sum of MEM+ and MEM- bacterial cells.

Extracellular enzyme activity was measured on the base of the degradation rate of increasing concentrations of four artificial fluorogenic substrates (Table II) as described previously by Chróst (1991) and Hoppe (1993). To create the samples set for kinetics measurement, we used 3.8 ml of sample in case of buffered ones and 3.9 ml for unbuffered samples for each substrate concentration. The increasing concentration of substrates for each examinated enzyme was used (Table II). A 0.1 ml of 40 fold concentrated substrates (separately for each concentration) was used to obtain desired substrate concentration (final sample volume – 4 ml). Samples for measuring lipase and beta-glucosidase (Glu) were buffered after incubation to pH 9.0 (0.1 ml of Tris-HCL, final concentration 25 μM), to intensify the MUF fluorescence. L-leucine-aminopeptidase (AMP) and acid phosphatase (APA) were determined by the *in situ* pH. Calibration curves were created independently for each sample and water layer by preparing an increasing concentration of fluorogenic reaction products in 0.2 filtrated (Millipore polycarbonate filters), autoclaved water derived from examinated lake. Fluorescence of the reaction products (described in Table II) was determined in a Shimadzu RF 1501 spectrofluorometer at zero time and after 0.5–1.0 h of sample incubation (incubation temp.: 20°C). The tested enzyme-substrate systems followed first-order Michaelis-Menten kinetics. The plot of the reaction velocity (v) against substrate concentration [S] displayed a rectangular hyperbola relationship, described by the equation $v = Vmax * [S]/(Km + [S])$. Nonlinear regression analysis was applied to calculate the kinetic parameters of enzymatic reactions by means of PC software Origin 6.1 (OriginLab Corporation, Northampton).

Statistical analyses. To compare the mean values between both layers (surface microlayer and bulk water) or between lakes, the nonparametric Wilcoxon test for matched pairs was performed. A critical p-value of < 0.05 was always applied. Canonical Analyses (CA) was used in an effort to use water biological and chemical parameters to explain the residual variance in the four response parameters, SML and SSW in two lakes. Statistics were performed in Statistica v. 6. Due to specific method for collection of surface waters we used no replications.

Results

Physical and trophic parameters. The water temperature ranged from 3–5°C in November to 12–14°C in April to maximal over 22°C in July and August in both lakes. The pH value fluctuated around 5.0 and generally was slightly higher in SMLs. PAR reaching the surface waters during the study period varied between 120 and 2300 μmol m^{-2} s^{-1} depending on the weather and season. We calculated that the SML absorbs 60% (40–70%) of PAR radiation.

The polyhumic lake was more eutrophic (Table I). Concentrations of TP were almost twice as high in the SML in the polyhumic than in the oligohumic lake (in the SSW more than 1.3 times). Differences between lakes were statistically significant in the case of the SML ($p = 0.043$, $n = 5$, Wilcoxon test). Although, PO_4-P were also higher in the polyhumic lake-close to six times in the SML and in the SSW – 2.6 times, differences were statistically significant between both lakes in SSWs ($p = 0.028$, $n = 7$, Wilcoxon test). TN concentrations

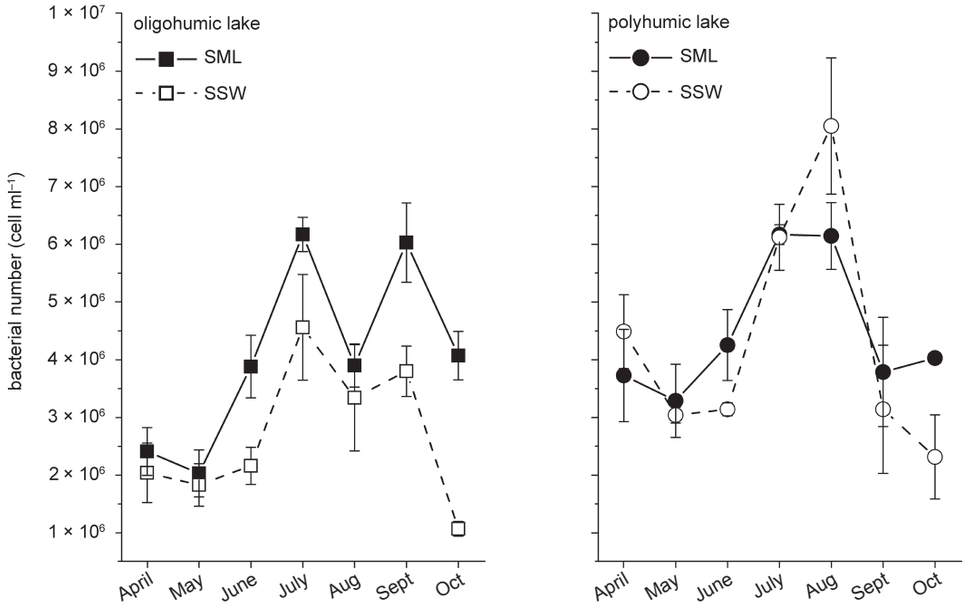

Fig. 1. Bacterial number in the surface microlayer (SML) and subsurface water (SSW) in oligohumic and polyhumic lakes.

were nearly three times higher in the SML and more than 3.0 in the SSW in polyhumic lake, statistically important differences were only in the lower layer ($p = 0.023$, $n = 7$, Wilcoxon test) (Table I). The DIN was almost at the same level in the SML in both lakes, but in the SSW it was higher in the oligohumic lake. The SML was enriched with nitrogen (Ef – ranged from 1.4 for TN in the oligohumic lake and 1.1 in the polyhumic, to 1.5 for DIN in the polyhumic and 1.2 in the oligohumic). The differences between layers were statistically significant in both lakes in the case of DIN ($p = 0.042$ and 0.043, respectively) and in TN only in the oligohumic lake ($p = 0.028$, $n = 7$). The concentrations of TP and PO_4-P were generally higher in the SSW in both lakes, but the differences were not statistically significant.

The concentrations of chl *a* were almost equal for neuston and plankton samples in the oligohumic lake. In the polyhumic lake, the concentration of chl *a* was lower in the SML (Ef = 0.5) and differences were statistically significant ($p = 0.043$, $n = 5$). The concentration of chl *a* in the polyhumic lake was higher than in the oligohumic by 3.5 times in the case of the SML and more than 7 times in the SSW (Table I).

The concentrations of TOC and DOC were almost 4.0 times higher in the polyhumic than in the oligohumic lake (Table I). To distinguish the aromaticity of DOC in the studied lakes, SUVA values were calculated. In the oligohumic lake, SUVA ranged from 8.6 in the SML and 9.2 in the SSW, while in the polyhumic lake they were 31.7 and 32.5, respectively. However, the differences between layers were not statistically significant in both lakes. These data correlated with water colour, almost transparent in the oligohumic lake (39 in the SML and 29 in SSW), and dark brown in the polyhumic (688 and 631, respectively). Through all seasons, the water colour of the SML was higher, however, only in the oligohumic lake were the differences significant ($p = 0.018$, $n = 7$).

Bacterial parameters. In the SML of both lakes and in the SSW of the polyhumic lake the BN varied between 4.0 and 4.5×10^6 cells per ml; however, in the SSW of the oligohumic lake the number was as low as 2.7×10^6 ml^{-1} (Fig. 1). Only in the oligohumic lake the differences of mean BNs between layers were statistically significant ($p = 0.018$, $n = 7$). The abundance of bacteria started from spring at a low level with the highest number during the summer and decreasing during autumn. The Ef reached 1.7 only in the oligohumic lake (1.1 in the polyhumic lake). BB changes followed the similar pattern as the BN in the polyhumic lake. During the end of the season, the SML was still enriched in BB (Fig. 2). A different situation was observed in the oligohumic lake where much higher biomasses in the SML were observed with one exception (June) throughout the season. Mean values for the studied season were 376 μg C l^{-1} in the SML and 208 μg C l^{-1} in the SSW in the oligohumic lake, while in the polyhumic lake they were 228 μg C l^{-1} and 221 μg C l^{-1}, respectively. The SML was enriched in BB in both lakes, but only in the oligohumic lake were the differences significant ($p = 0.042$, $n = 7$). The enrichment factors for biomasses were – 1.9 in the oligohumic lake and 1.2 in the polyhumic lake.

During the spring (April and May) and autumn (November), there were no differences in the contri-

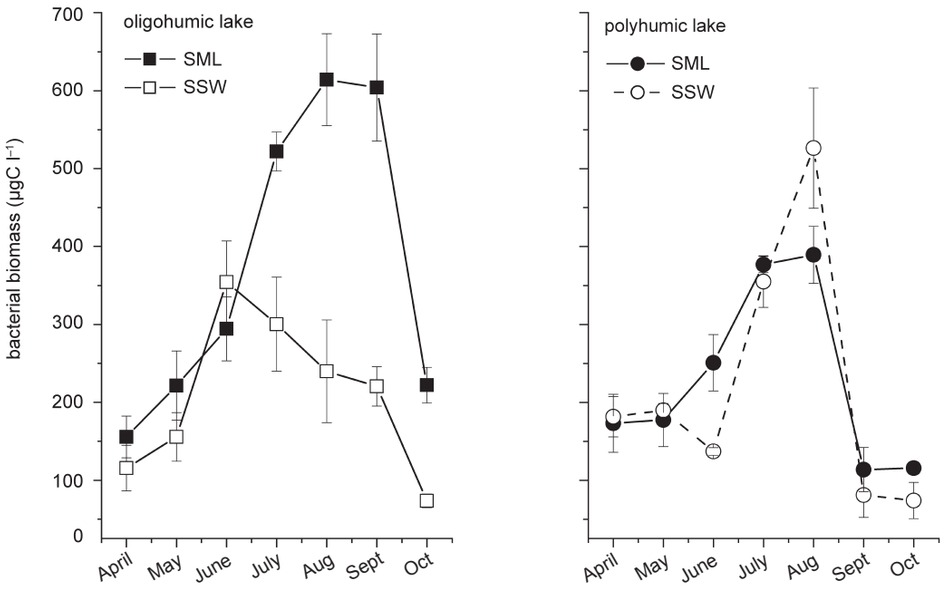

Fig. 2. Changes in bacterial biomass in the surface microlayer (SML) and subsurface water (SSW) in oligohumic and polyhumic lakes.

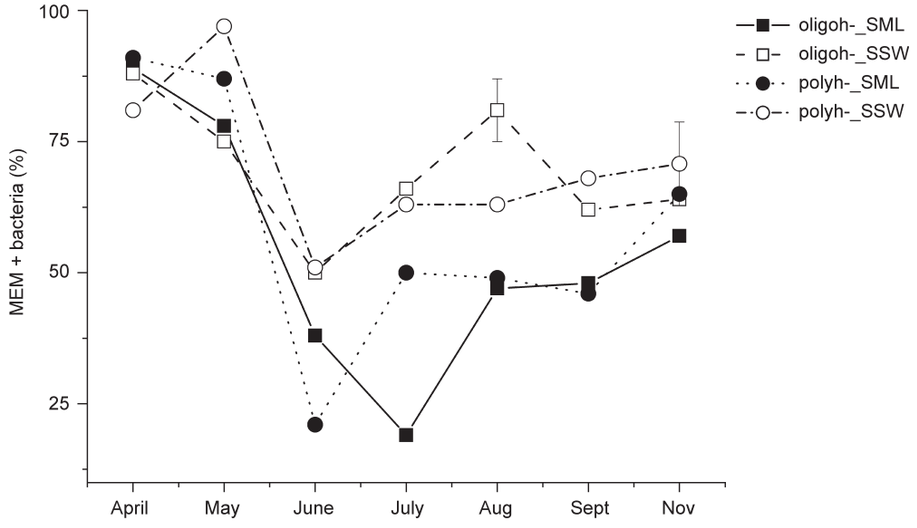

Fig. 3. Percentage contribution of active bacteria with intact membranes (MEM+) to the sum of MEM+ and MEM- bacteria in the surface microlayer (SML) and subsurface water (SSW) in oligohumic (oligoh-) and polyhumic (polyh-) lakes.

bution of active bacteria with an intact membrane to the sum of MEM+ and MEM– bacteria between the SML and SSW in both lakes (Fig. 3). The percent of active bacteria started from over 80% in the spring and fell to about 60% during the autumn. Throughout the summer, active bacteria were found in the SSW. Differences in the mean percentage of MEM+ bacteria were not important between lakes, but between SML vs. SSW they were statistically significant for the polyhumic lake ($p = 0.043$, $n = 7$).

BP was about half as much in the poly- than in the oligohumic lake (11.5 µg C l^{-1} d^{-1} in the SML of the polyhumic lake and 8.2 µg C l^{-1} d^{-1} in the oligohumic lake, while in the SSW they were 24.1 and 15.8 µg C l^{-1} d^{-1}, respectively; Fig. 4). Differences were observed only for the polyhumic lake and were statistically significant ($p = 0.046$, $n = 6$). In both lakes, ^{3}H-TdR incorporation of the bacterioplankton was up to 3 times higher compared to that of bacterioneuston throughout the study season, however, differences were not statistically significant (Efs were 0.7 in the oligo- and 0.5 in the polyhumic lake).

The relative magnitude of extracellular enzyme activities determined in this study ranked; in polyhumic

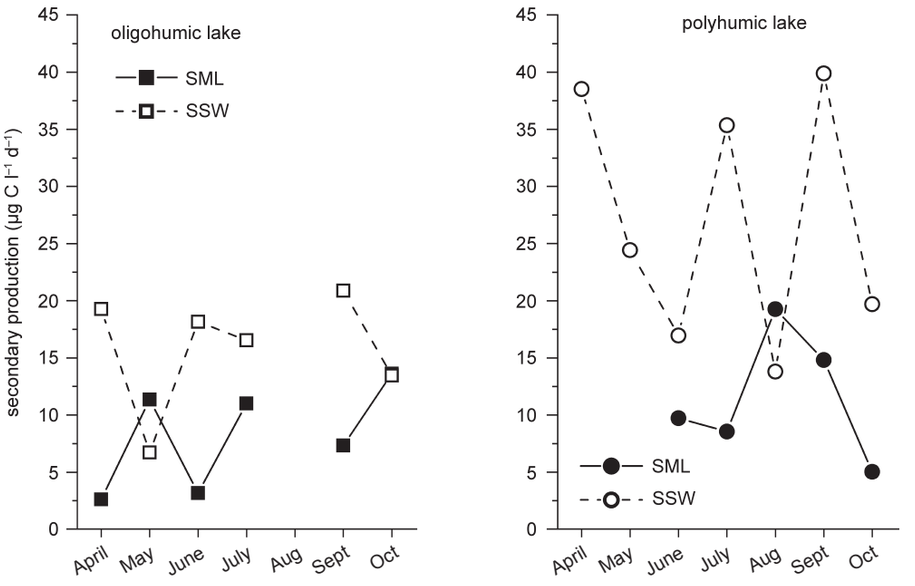

Fig. 4. Secondary production in the surface microlayer (SML) and subsurface water (SSW) in oligohumic and polyhumic lakes.

lake: lipase > APA > AMP > Glu; in oligohumic: lipase > AMP > APA > Glu (Table III). APA activity was higher in the polyhumic lake (V_{max} was 1908 nmol l^{-1} h^{-1} in the SML and 2258 nmol l^{-1} h^{-1} in the SSW, while in the oligohumic lake it was as low as 632 and 339 nmol l^{-1} h^{-1}, respectively (Fig. 5). Differences between lakes and layers were statistically significant only in the SSW ($p = 0.043$, $n = 7$). APA activity was higher in the polyhumic lake than in the oligohumic by 11 times in the SSW and ca. five times in the SML. However, the differences between APA and TP or orthophosphates were not statistically significant. The enrichment factor (Ef) was much higher in the oligohumic lake (1.9) and just below 1.0 in the polyhumic one. Of all the measured enzymes, only the potential maximal activity of AMP was higher in the oligohumic (1471 nmol l^{-1} h^{-1} in the SML and 2117 nmol l^{-1} h^{-1} in the SSW) than in the polyhumic lake (861 and 1213, respectively), but differences were statistically significant only in the SSW ($p = 0.028$, $n = 6$) (Fig. 5). In both lakes higher activity of AMP was observed in the SSW. Lipase activity was more than twice higher in the polyhumic lake (6098 nmol l^{-1} h^{-1} in the SML and 3732 nmol l^{-1} h^{-1} in the SSW) than in the oligohumic one (2290 nmol l^{-1} h^{-1} in the SML and 2238 nmol l^{-1} h^{-1} in the SSW; Fig. 5). Differences between lakes were statistically significant in both layers on the same level ($p = 0.028$, $n = 6$), and bacteria were more active in the SML of the polyhumic lake (Efs 1.6). In both lakes, glucosidase was on the same level (about 30–50 nmol l^{-1} h^{-1}) and differences between lakes were not statistically important. Neustonic bacteria were more active in both lakes (Efs 1.1–1.4).

Table III
Mean values for ectoenzyme affinity (K_m; µM), potential maximal activity (V_{max}; nM h^{-1} l^{-1}) for glucosidase (Glu), acid phosphatase (APA), aminopeptidase (AMP); and lipase (lipase).

	Oligohumic		Polyhumic	
	SML	SSW	SML	SSW
Glu				
K_m	142.0	168.3	59.3	30.7
V_{max}	41	29	49	43
APA				
K_m	0.8	2.9	22.8	36.8
V_{max}	632	339	1908	2258
AMP				
K_m	93.6	92.2	65.2	41.3
V_{max}	1471	2117	861	1213
Lipase				
K_m	44.5	90.3	59.3	30.7
V_{max}	2290	2238	6098	3732

Discussion

Bacteria are a key component in aquatic ecosystems due to their wide biodiversity, their capacity to survive in extreme environments like in the surface microlayer, and their large variety of metabolic activities (Cunliffe *et al.*, 2011). Bacteria also play a major role in OM cycling and, subsequently, in sustaining nutrient turnover (Chróst, 1991). However, conflicting results concerning surface films can be found in the literature (Dietz *et al.*, 1976; Hermansson and Dahlbäck, 1983; del

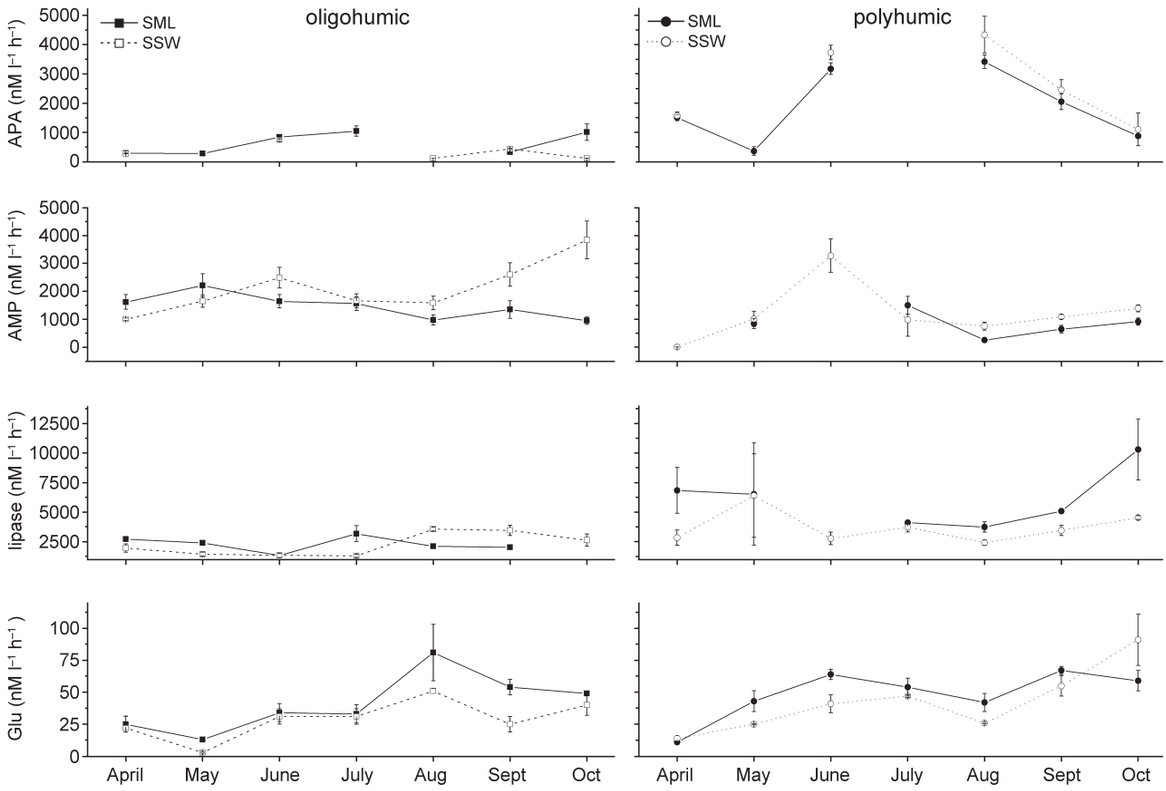

Fig. 5. Enzymatic (APA – alkaline phosphatase, AMP – aminopeptidase, Glu – glucosidase, and lipase) activity (Vmax) of the surface microlayer (SML) and subsurface water (SSW) in oligohumic and polyhumic lakes.

Giorgio and Scaraborough, 1995; Münster *et al.*, 1998; Kuznetsova and Lee, 2001; Mudryk and Skórczewski, 2004; Kostrzewska-Szlakowska, 2005; Santos *et al.*, 2009; Stolle *et al.*, 2009). We need to keep in mind that the SML undergoes highly dynamic spatial and temporal changes (Kuznetsova and Lee, 2001). The key issue may be the surface microlayer sampling methods used. Different devices sample different depths of the surface water of a microlayer, thus, influencing the collected samples (Münster *et al.*, 1998; Stolle *et al.*, 2009; Cunliffe *et al.*, 2011).

Nutrient and organic matter content (Table I) was roughly concordant with data obtained by Münster *et al.*, (1998) for Finnish, humic lakes and Södergren (1993) for Swedish lakes.

Hermansson and Dahlbäck (1983) and Joux *et al.* (2006) showed no significant differences between the fraction of active cells in the SML and in the bulk water. Kuznetsova *et al.* (2004) found that the percentage of bacteria with damaged membranes was frequently lower in the SML than in the SSW. In opposition to these data, in dystrophic lakes the percentage of bacteria with damaged membranes were higher in SMLs (Fig. 3). It seems that the function of bacteria, based on their total numbers, may have to be revised to accommodate large variations in the proportion of metabolically active cells (del Giorgio and Scaraborough 1995). However, they revealed that both based on literature data and their own from 24 studied Canadian lakes, the percent of active bacteria was between 21 and 23%. Schumann *et al.* (2003) discovered that in the eutrophic freshwater lakes bacteria with intact cells accounted for 70%, while in the mesotrophic only 42%. In our lakes, the means for the season percent of active bacteria were slightly higher in the polyhumic lake in the SML (61 versus 54%) but were similar in the SSW (about 70%). It could be concluded that (1) more active bacteria were found in the SSW and (2) variability is large (19–97%) depending on the trophic status of the waters, sampling data and investigated layer.

We cannot conclude that irradiance had detrimental effects on the neustonic algal community because chl *a* was enriched in the SML of the oligohumic lake and depleted in the polyhumic one. In addition, bacteria preferred to stay in the SML (Efs higher than 1 in both lakes; Table I). Cunliffe *et al.* (2011) showed 78% similarity of the bacterial communities of SML and SSW samples, Santos *et al.* (2009) pointed to high structural similarities between bacterial communities in both layers while Franklin *et al.* (2005) suggested that the marine bacterioneuston contains a distinct community of bacteria.

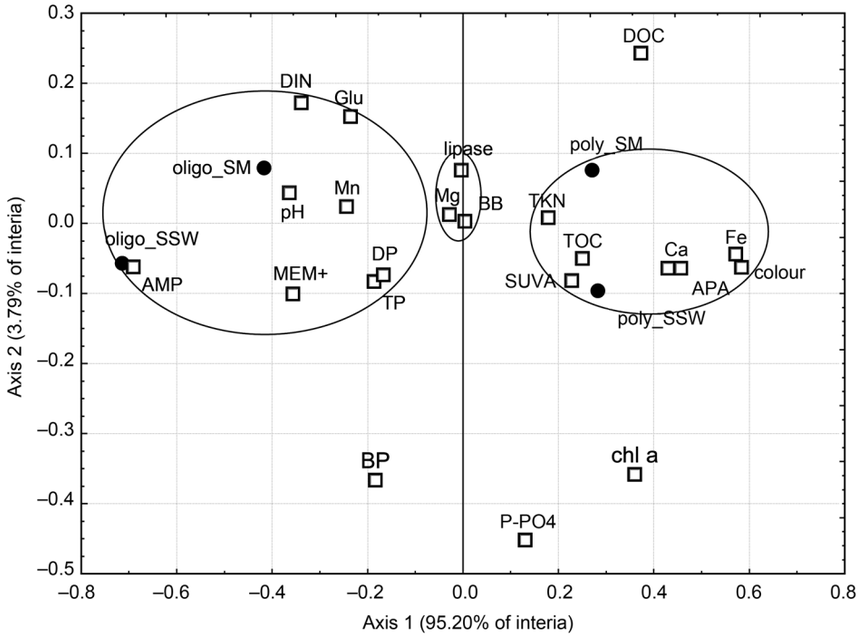

Fig. 6. CA analysis of studied variables in the surface microlayer (SML) and subsurface water (SSW) in oligohumic (oligo-) and polyhumic (poly-) lakes.

DIN – dissolved inorganic nitrogen, TKN – total Kjeldahl nitrogen, TP – total phosphorus, DP – dissolved phosphorus, PO_4-P – ortophosphorans, TOC – total organic carbon, DOC – dissolved organic carbon, BB – bacterial biomass, BP – secondary production, MEM+ – percentage contribution of active bacteria with intact membranes, Fe – iron, Ca – calcium, Mg – magnesium, Mn – manganese, AMP – aminopeptidase, Glu – glucosidase, APA – alkaline phosphatase and lipase.

Münster *et al.* (1998) stated that although the biofilm microbial communities in the SML can be assumed to receive generally higher environmental impacts like UV-irradiance, heavy metals, pollutants and others, there were significantly higher microbial activities and bacterial growth rates in the SML samples compared to those in the SSW. However, Hermansson and Dahlbäck (1983) also did not observe any effects of sunlight on the activity of bacteria in the surface samples. In the lakes studied here, this statement is only true in the case of lipase and glucosidase activity in both lakes and APA in the oligohumic one. Other activities measured such as: secondary production, AMP and APA in the polyhumic lake were higher in the SSW. However, in both lakes we observed a higher concentration of phosphorus, especially orthophosphates, in the SSW and a slightly enriched SML of DOC. In addition, Santos *et al.* (2009) stated that the activity of the studied extracellular enzymes was higher in bacterioneuston than in bacterioplankton, probably due to stimulation of extracellular enzyme synthesis by high concentrations of polymeric OM in the SML. It was also varying in the studied humic lakes, whereas some enzymatic activities were higher in the SML (lipase and glucosidase in both lakes and phosphatase in the oligohumic lake) and some in the SSW (aminopeptidase in both lakes and phosphatase in the polyhumic lake). Münster *et al.* (1992) found that the activity of extracellular enzymes produced by aquatic microheterotrophs depends on lake water pH. Phosphatases and glucosidases have their relative maxima of activity at pH = 5.4, while aminopeptidases had their maxima in the neutral pH region. Generally, we used standard fluorogenic substrates with one exception, we used an unusual substrate for APA, (DiFMUP (6,8-difluoro-4-methylumbeliferyl phosphate), which exhibits strong fluorescence at acidic pH and allowed us to directly measure the activity of acid phosphatases in the acidic water samples without alkaline buffer addition after sample incubation. In the majority of published studies a substrate for alkaline phosphatases were used so comparing the results obtained from these two methodological approaches may be inaccurate.

Stolle *et al.* (2009) have reported that ^{3}H-TdR incorporation of the bacterioneuston in the Baltic Sea was reduced by 50 to 80% compared to that in the underlying water. The same situation was observed in humic lakes and thymidine incorporation of the bacterioneuston was reduced by 50 to 70% compared to bulk water. This indicates that the DOM pool of the SML may not be readily available for bacteria (Santos *et al.* 2009). However, opposing data was reported by Münster *et al.* (1998) measuring α-Glucase, which was 4–5 times higher in bacterioneuston compared to

bacterioplankton. Joux *et al.* (2006) observed that the intensity of bacterial production in the Mediterranean Sea was highly variable.

CA analysis was used to investigate the relation between water biology and chemistry variables in two layers-SML and SSW of two humic lakes with different water colours (Fig. 6). For layers in both types of lakes, almost all of the explained variables were due to the first axis (95.20% of inertia) with a negligible influence from the second axis (3.79% of inertia). Concentrations of iron, calcium, orthophosphates, total nitrogen, total and dissolved carbon and water colour, SUVA, and some biological aspects like APA and chlorophyll *a* were positively related to both analysed layers of the polyhumic lake. Concentrations of total and dissolved phosphorus, DIN, Mn, pH and some biological elements connected with bacteria like secondary production, percent of active bacteria with intact membranes, Glu, AMP correlated with both layers in the oligohumic lake. On the contrary, the model could not explain BB, Mg or potential activity of lipase. In addition, the results suggest that inherent properties of organic matter as indicated by TOC and DOC, SUVA, Fe were related to chl *a* in the polyhumic lake and were negatively related with most attributes of bacteria like production, MEM+ and some enzymatic activity. APA, which was used as an indicator of P deficiency, correlated negatively with concentrations of phosphorus but positively with orthophosphates. Nonetheless, the low percentage of second axis (however both explaining almost 99% of cases) if we put '0' line – two layers of two lakes are placed in each quadripartite. Thus, it seems that SML and SSW showed lake-specific differences in all studied parameters and that was SML is generally supported from the underlying waters.

It could be stated that: (1) Polyhumic lake are characterised as more eutrophic with higher concentrations of organic matter and nutrients compared to oligohumic lake, then the surface microlayer of the polyhumic lake was enriched in forms of phosphorus and ammonium, while enrichments in nitrogen and bacterial abundance were higher in the oligohumic one. (2) The bacteria activity pattern in the SML versus SSW was variable. Activities expressed as lipase and glucosidase V_{max} were higher in the SMLs in both studied lakes and as phosphatase V_{max} just in the oligohumic lake. The aminopeptidase V_{max} were higher in the SSW in both lakes and phosphatase V_{max} only in the polyhumic lake. (3) Solar radiation does inhibit neuston microbial community as a whole; it has a pronounced inhibitory effect on the surface microbial activity characteristics as an bacterial production and share of active bacteria with intact membranes, however, in the oligohumic lake, we noticed stable higher total abundance of bacteria in SML. (4) SML and SSW showed lake-specific differences in all studied parameters and SMLs are not directly supported from the underlying waters (SSWs). (5) The SMLs of small humic lakes is an inhospitable microhabitat in the same way as in other water bodies (controlled by dynamic physical processes like intense solar radiation, wind, changing temperature, waves, etc.) but to a larger extent may act as an important sinks for allochthonous nutrient resources and may then generate significant energy pools for microbial food webs.

Acknowledgments

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ORIGINAL PAPER

Suppressive Effect of *Trichoderma* spp. on toxigenic *Fusarium* species

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Abstract

The aim of the present study was to examine the abilities of twenty-four isolates belonging to ten different *Trichoderma* species (*i.e.*, *Trichoderma atroviride*, *Trichoderma citrinoviride*, *Trichoderma cremeum*, *Trichoderma hamatum*, *Trichoderma harzianum*, *Trichoderma koningiopsis*, *Trichoderma longibrachiatum*, *Trichoderma longipile*, *Trichoderma viride* and *Trichoderma viridescens*) to inhibit the mycelial growth and mycotoxin production by five *Fusarium* strains (*i.e.*, *Fusarium avenaceum*, *Fusarium cerealis*, *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium temperatum*). Dual-culture bioassay on potato dextrose agar (PDA) medium clearly documented that all of the *Trichoderma* strains used in the study were capable of influencing the mycelial growth of at least four of all five *Fusarium* species on the fourth day after co-inoculation, when there was the first apparent physical contact between antagonist and pathogen. The qualitative evaluation of the interaction between the colonies after 14 days of co-culturing on PDA medium showed that ten *Trichoderma* strains completely overgrew and sporulated on the colony at least one of the tested *Fusarium* species. Whereas, the microscopic assay provided evidence that only *T. atroviride* AN240 and *T. viride* AN255 formed dense coils around the hyphae of the pathogen from where penetration took place. Of all screened *Trichoderma* strains, *T. atroviride* AN240 was also found to be the most efficient (69–100% toxin reduction) suppressors of mycotoxins (deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, nivalenol, zearalenone, beauvericin, moniliformin) production by all five *Fusarium* species on solid substrates. This research suggests that *T. atroviride* AN240 can be a promising candidate for the biological control of toxigenic *Fusarium* species.

Key words: antagonism, dual culture bioassays, fungal interactions, microscopic observations, mycotoxins

Introduction

Fusarium species are considered to be some of the most important plant pathogens, causing head blight in small grain cereals and ear rot in maize. These pathogenic species produce a wide range of mycotoxins, such as trichothecenes (TCTs), fumonisins (FBs), zearalenone (ZEN), beauvericin (BEA) and moniliformin (MON), which have been determined to be common contaminants of cereal grains and derived products (Bottalico and Perrone, 2002; Jestoi *et al*., 2008; Goetz *et al*., 2010). Among the toxigenic *Fusarium* species that have been associated with infected grain are *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium cerealis*, and *Fusarium temperatum* (Bottalico and Perrone, 2002; Logrieco *et al*., 2002a; 2002b; Goetz *et al*., 2010; Amarasinghe *et al*., 2014; Czembor *et al*., 2014). *F. graminearum* is capable of producing two major types of mycotoxins: estrogenic ZEN and type B trichothecenes, such as deoxynivalenol (DON) and, depending on the chemotype, its acetylated forms 3-acetyl-deoxynivalenol (3-AcDON) or 15-acetyl-deoxynivalenol (15-AcDON), as well as nivalenol (NIV) (Bily *et al*., 2004; Glenn, 2007). The main mycotoxins biosynthesized by *F. culmorum* include DON, 3-AcDON, NIV, fusarenone X (FUS) and ZEN (Glenn, 2007; Wagacha and Muthomi, 2007). NIV, FUS and ZEN are also formed in cereals by *F. cerealis* strains (Logrieco *et al*., 2003; Amarasinghe *et al*., 2014). However, *F. avenaceum* and *F. temperatum* do not produce TCTs or ZEN, but they do produce other mycotoxins such as enniatins (ENs), BEA and MON (Logrieco *et al*., 2002b; Jestoi *et al*., 2008; Scauflaire *et al*., 2012). *F. temperatum* additionally produces FB_1 (Scauflaire *et al*., 2012).

As has been repeatedly demonstrated, *Fusarium* mycotoxins pose a significant health risk for humans and animals through food and feed prepared from contaminated cereal crops (Bennet and Klich, 2003; Glenn, 2007). Therefore, different strategies have been adopted

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to reduce *Fusarium* mycotoxin contamination in cereals (Palazzini *et al*., 2007). One of the more economically and environmentally attractive options is considered to be the use of biological control agents (BCAs) acting as natural antagonists to *Fusarium*.

Trichoderma spp. are among the most studied and promising microorganisms used in a biocontrol system (Benitez *et al*., 2004; Woo *et al*., 2014). Species of the ascomycete genus *Trichoderma* (teleomorph *Hypocrea*) are found in many ecosystems of all climatic zones, but the most common and natural habitat of these fungi is known to be soil and wood with symptoms of decay (Samuels, 2006; Kubicek *et al*., 2008; Druzhinina *et al*., 2011; Strakowska *et al*., 2014). These species succeed in various heterotrophic interactions, exhibiting saprotrophic and mycoparasitic (necrotrophic hyperparasitic, mycotrophic) lifestyles (Druzhinina *et al*., 2011). Their antagonistic abilities *i.e.* activities towards plant pathogens, such as *Botrytis cinerea*, *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*, *Verticillium dahilae*, and *Sclerotinia* spp. (Harman *et al*., 2004; Verma *et al*., 2007; Druzhinina *et al*., 2011) are a combination of several mechanisms, including nutrient and/or space competitions, antibiosis associated with the secretion of antibiotic metabolites and direct mycoparasitism, which involves the production of cell-wall-degrading enzymes (Vinale *et al*., 2008a; 2008b; Druzhinina *et al*., 2011). In addition, *Trichoderma* strains used as biocontrol agents are able to induce plant defense against pathogens and to promote plant growth (Hermosa, 2012; Nawrocka and Małolepsza, 2014).

Several studies indicate that *Trichoderma* species are effective biocontrol agents for phytopathogenic *Fusarium*. Although, particular attention has been paid to *Trichoderma harzianum* strains. It has been shown that seed treatment of maize with *T. harzianum* T22 and Th-8 strains reduces *Fusarium verticillioides* kernel colonization and FBs contamination under controlled and natural conditions as well as induces systemic resistance in maize against this pathogens (Nayaka *et al*., 2008; Ferriego *et al*., 2014a; 2014b). *T. harzianum* T16 and T23 strains have been found to be effective antagonists towards *F. verticillioides* and FBs production in maize kernels in liquid as well as agar medium (Altinok, 2009). Furthermore, *T. harzianum* isolate THF2/3 has been reported to reduce DON production by *F. graminearum* in agar medium bioassays (Cooney *et al*., 2001). Recently, it has been documented that *Trichoderma gamsii* 6085 and *Trichoderma atroviride* AN35 strains are able to suppress TCTs production by *F. graminearum* and *F. culmorum* species on natural substrates (Buśko *et al*., 2008; Matarese *et al*., 2012). In addition, *T. atroviride* AN35 grown in dual culture bioassays on rice (in a competition assay system) with *F. graminearum* and *F. culmorum* strains has also been found to substantially inhibit ZEN production (Gromadzka *et al*., 2009; Popiel *et al.*, 2008). However, very little is known on the impact of the various *Trichoderma* species on the phytopathogenic *Fusarium* and their capacity to inhibit mycotoxin production on solid substrates.

Therefore, the aim of this study was to examine the antagonistic ability of twenty-four isolates belonging to ten different *Trichoderma* species, including species that are not yet fully recognized as important biological control agents (BCA), against mycotoxigenic *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. graminearum* and *F. temperatum* species.

Experimental

Materials and Methods

Fungal isolates. The twenty-four *Trichoderma* isolates and five *Fusarium* strains investigated in this study were selected from the culture collection of the Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland and are listed in Table I. Four selected *Trichoderma* strains (Table I) are deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS). All the studied *Trichoderma* strains sourced from different ecological niches in Poland had previously been identified to the species level by sequence analysis of the internal transcribed spacer regions ITS-1 and ITS-2 of the nuclear rDNA and a fragment of the translation-elongation factor 1-alpha (tef1) gene by Błaszczyk *et al.* (2011) and Jeleń *et al.* (2014). *Fusarium* strains of five species (*F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2870 and *F. temperatum* KF 506) were identified using species-specific markers validated during earlier studies (Stępień *et al*., 2011; Wiśniewska *et al*., 2014).

Dual culture bioassay. Antagonistic activities of all *Trichoderma* isolates towards *Fusarium* species were assessed in dual cultures on potato dextrose agar (PDA) medium. Pure cultures of *Trichoderma* and *Fusarium* were re-cultured aseptically from stock slants onto 8.5 cm diameter Petri dishes. They were grown on PDA for 7 days at 25 ± 2°C and were subsequently used as inoculum. Mycelial disks of 3 mm diameter from colony margins of each pathogen-antagonist combination were placed in Petri dishes containing 15 ml of PDA medium, 8.0 cm apart and diametrically opposed to each other. Each dual culture was set up in triplicate. The control consisted of individual cultures of the pathogen and antagonist. Plates were incubated at 25 ± 2°C, 12 h/12 h darkness/light. The radial growth of each fungus was measured daily with a ruler for

Table I
Trichoderma strains analysed for antagonistic activity against *Fusarium* species.

Culture code/ CBS[a] no.	Origin		NCBI GenBank Assession no.[b]	
	Locality	Source	ITS	*tef1*
T. atroviride				
AN152/ CBS136453	Central Poland	triticale kernel	HQ292792	HQ292957
AN182/CBS136454	Central Poland	forest wood	HQ292794	HQ292965
AN206/CBS136455	Central Poland	mushroom compost	HQ292804	HQ292960
AN240	Karkonosze Mts, SP	forest wood	JX184119	JX184096
AN497	Gorce Mts, SP	forest wood	JX184119	JX184096
T. citrinoviride				
AN262	Central Poland	forest wood	HQ292847	–
AN393	Gorce Mts, SP	forest wood	JX184109	JX184086
T. cremeum				
AN392	Gorce Mts, SP	forest wood	JX184117	JX184094
T. hamatum				
AN120	Central Poland	forest wood	HQ292855	–
AN277	Central Poland	forest wood	HQ292857	–
T. harzianum				
AN150	Central Poland	forest wood	HQ292878	–
AN278	Central Poland	forest wood	HQ292890	–
AN360	Tatra Mts, SP	forest wood	JX184113	JX184090
T. koningiopsis				
AN143	Central Poland	forest wood	HQ292929	HQ292992
AN251	Karkonosze Mts, SP	forest wood	HQ292939	HQ292993
T. longibrachiatum				
AN197	Eastern Poland	mashroom factory	HQ292780	–
AN213	Central Poland	mashroom compost	HQ292781	–
T. longipile				
AN359	Tatra Mts, SP	forest wood	JX184115	JX184091
T. viride				
AN255	Karkonosze Mts, SP	forest wood	JX184121	JX184098
AN401	Gorce Mts, SP	forest wood	JX184122	JX184099
AN430	Central Poland	forest wood	HQ292926	HQ293014
AN826	Karkonosze Mts, SP	forest wood	JX184122	JX184099
T. viridescens				
AN323	Tatra Mts, SP	forest wood	JX184127	JX184103
AN405/CBS136460	Gorce Mts, SP	forest wood	JX184127	JX184103

a – The culture collection of the CBS-KNAW Fungal Biodiversity Centre,Utrecht, the Netherlands
b – The same NCBI GenBank Accession number assigned to the isolates possessing identical alleles in the locus ITS or *tef1*

4 days (until contact). After 14 days of incubation, a qualitative evaluation of antagonism was carried out based on the modified scale of Mańka (1974): +8, antagonist completely overgrew the pathogen and entire medium surface; +6, antagonist occupies 85% of the medium surface; +4, antagonist occupies 75% of the medium surface, 0, antagonist occupies 50% of the medium surface (Popiel *et al*., 2008).

Microscopic observations. Co-cultures of *Trichoderma* spp. with *Fusarium* spp. were performed on PDA medium in Petri dishes. After solidification of the medium, a sterile strip (20 mm) of cellophane membrane (50-µm thick) was placed in the middle of each plate. Mycelia were collected from pure, actively growing colonies of each of *Trichoderma* and *Fusarium* species. Colonies were inoculated on opposite sides of Petri dishes at a distance of 5 mm from the edge. Incubation of co-cultures was performed at room temperature. After 7, 14 and 21 days of incubation, the cellophane membrane was cut with a razor blade in sterile

conditions and the mycelia were placed onto a drop of distilled water on a microscope slide and examined. Observations were carried out using a light microscope (Olympus CX-41-1 with UC-30 camera, Olympus, Japan). After the first 7 days, the samples were screened mainly for loops of the *Trichoderma* around *Fusarium* hyphae. After the following days of incubation the samples were screened for the anatomical damage of the *Fusarium* and other structures indicating potential mycoparasitism.

Solid substrate bioassay. In order to evaluate the ability of *Trichoderma* to inhibit mycotoxin production by *Fusarium* species, dual cultures were established on solid substrates (rice kernels) following the methods described by Buśko *et al.* (2008). The assay was performed using all of the *Fusarium* strains and five selected *Trichoderma* isolates characterized by the highest antagonistic potential against *Fusarium* species in dual cultures on PDA medium. Rice kernels (50 g) were added to 15 ml distilled water, left overnight and autoclaved at 121°C for 30 min in a 300 ml Erlenmeyer flask. The flasks were inoculated with four disks (4 mm in diameter) cut from the advancing edge of 7-day PDA culture of pathogen and/or antagonist. Three replicates for each experimental set (antagonist, pathogen and antagonist/pathogen variants) were performed. Uninoculated rice was used as negative controls. The incubation was carried out at 25 ± 2°C 12 h/12 h of darkness/light for 21 days. Flasks were shaken daily to prevent clumping and provide aeration.

Mycotoxin extraction and analysis

Chemicals and reagents. Mycotoxin standards (ZEN, DON and its derivatives 15-AcDON and 3-AcDON, NIV, MON and BEA) were supplied by Sigma-Aldrich (Steinheim, Germany). Acetonitrile and methanol (HPLC grade), disodium tetraborate, 2-mercaptoethanol, potassium hydroxide, sodium dihydrophosphate and *o*-phosphoric acid were also purchased from Sigma-Aldrich. Potassium chloride, dipotassium phosphate, *n*-hexane and *t*-butyl-ammonium hydroxide were purchased from POCh (Poland). Water of HPLC grade from Merck Millipore water purification system was used for analyses.

Sample preparation. Rice culture samples were powder-ground in a WŻ-1 laboratory mill (the Research Institute of Baking Industry Ltd., Bydgoszcz, Poland). ZEN, DON, NIV, 3-Ac-DON and 15-Ac-DON were extracted with acetonitrile:water (90:10, v/v) solution. MON and BEA were extracted with acetonitrile:methanol:water (16:3:1, v/v/v). A solvent mixture was used at a ratio of 2.5 ml of solvent per 1 g of ground samples. Extractions were performed by blending the samples with the solvents at high speed for 2 min in an H 500 blender jar (POL-EKO, Poland). The extract was filtered through filter paper (No. 5, Whatman), a glass microfiber filter (GF/B, Whatman) and a syringe filter (CHROMAFIL PET-45/15MS) and then the filtrate was collected. Where the toxin content in the sample was below the detection limit, the sample was purified by immunoaffinity columns (ZearalaTest, WB) according to the method of Visconti and Pascale (1998) for ZEN, and by using carbon columns for the determination of DON, MON, 3-Ac-DON and 15-Ac-DON by the method of Tomczak *et al.* (2002), Kostecki *et al.* (1999) and Yang *et al.* (2013) respectively. The filtrate was analyzed by HPLC chromatography according to the methodology described below.

HPLC analysis. The chromatographic system consisted of a Waters 2695 high-performance liquid chromatograph (Waters, Milford, USA), a Waters 2475 Multi λ Fluorescence Detector and a Waters 2996 Photodiode Array Detector. Millenium software was used for data processing. DON, NIV and MON analyses were performed according to the method described by Kostecki *et al.* (1999) and Tomczak *et al.* (2002) while 3-AcDON, 15-AcDON was detected by the method of Yang *et al.* (2013). Analysis of ZEN and BEA was performed according to Visconti and Pascale (1998) and Jestoi *et al.* (2008), respectively. The detection limit for DON, NIV, BEA and MON was 0.01 g kg $^{-1}$, for 3-AcDON, 15-AcDON was 1.0 g kg^{-1} and for ZEN was 0.3 g kg^{-1}. Recovery rates for NIV, DON, 3-AcDON, 15-AcDON, BEA and MON were 75, 87, 76, 75, 85, 90%, respectively. ZEN recovery was in a range from 97 to 99%. Relative standard deviation (R.S.D.) was below 1% for ZEN, below 5% for DON, 3-AcDON, 15-AcDON, NIV and BEA, and below 7% for MON. The recovery of mycotoxins were measured in triplicates by extracting toxins from solid substrates spiked with 1.0–100 g kg^{-1} of compounds. In order to confirm the presence of ZEN, a Photodiode Array Detector was used. Mycotoxin was identified by comparing retention times and UV spectra of purified extracted samples with pure standards. Quantification of mycotoxins was carried out by comparing peak areas of the analyzed samples to the calibration curve of peak areas obtained with authentic mycotoxin standards.

Statistical analysis. To analyse the inhibition (%) of pathogen growth on the fourth day after co-inoculation, inhibition coefficients and their standard errors were calculated according to the formula: (Rc-R) / Rc × 100, where Rc, R are the estimates of radial growth of a pathogen in control and dual culture, respectively (Edington *et al.* 1971). The same procedure was also applied to calculate relative toxin reduction (%), based on estimated toxin production level. The final assessment of inhibitory effectiveness of the antagonists was based on observations from visual assessment of colony state at day 14 of co-incubation. To identify the most

and the least efficient antagonists the values, expressed on a nominal scale (modified scale of Mańka, 1974), were averaged and compared.

To generally assess the influence of antagonists on the growth of pathogens during the initial days of the experiment the analysis of regression of pathogens' growth curves was performed. Linear regression was calculated for each pathogen in the presence/absence of antagonists (each combination in triplicate), based on measurements from days 0 to 4. Analysis of the variance of pathogen colony growth was performed for all pathogen-antagonist interactions on all days of observations at significance level $\alpha = 0.001$. To identify the effects of particular antagonists on pathogen colony size, Duncan's Multiple Range Test ($\alpha = 0.05$) was performed for (raw) growth observations at day 4 of co-incubation.

Statistical analysis of the results was performed using Genstat statistical software (regression analysis, ANOVA, modeling, correlation analysis) and R (Duncan test).

Results

Evaluation of the antagonistic activity of *Trichoderma* strains in dual cultures on PDA medium. Antagonistic tests showed that all *Trichoderma* strains significantly reduced *F. avenaceum* and *F. culmorum* growth during the initial days of co-incubation on PDA medium. Furthermore, five strains (AN240, AN262, AN359, AN826, AN323) significantly reduced the mycelial growth of *F. cerealis*, *F. graminearum* and *F. temperatum* (Supplemental online material 1 and 2). Among twenty-four *Trichoderma* strains screened for their antagonistic activity against five *Fusarium* species in dual cultures on PDA medium, thirteen strains (AN152, AN240, AN497, AN262, AN277, AN143, AN251, AN213, AN359, AN401, AN430, AN826, AN323) were able to inhibit mycelial growth of all investigated pathogens on the fourth day after co-inoculation (Table II). These strains provided from 1% to 80% inhibition (Supplemental online material 3). The remaining eleven *Trichoderma* strains (AN182, AN206, AN393, AN392, AN120, AN150, AN279, AN360, AN197, AN255, AN405) displayed a suppressive effect on mycelial growth of only *F. culmorum*, *F. avenaceum*, *F. graminearum* and *F. temperatum* (Table II, Supplemental online material 3). As shown in Table II and Supplemental online material 3, the range of action of *Trichoderma* strains against each of the *Fusarium* species was different. After 4 days of incubation, the estimated growth inhibition of *F. avenaceum* by *Trichoderma* ranged from 62% to 80%. The colony diameter of this species was significantly affected, compared to the control, by *T. harzianum* AN150. The growth inhibition of *F. cerealis* by *Trichoderma* ranged from 2% to 64% and the highest inhibitory effect on pathogen growth was achieved by *Trichoderma longibrachiatum* AN213. The *F. culmorum* growth inhibition rate caused by *Trichoderma* ranged from 27% to 80%. The maximum growth suppression of this species was recorded as a result of interaction with *Trichoderma koningiopsis* AN143. The growth inhibition of *F. gramineraum* ranged from 1% to 64% and *F. temperatum* ranged from 1% to 60%. The colony diameter of these pathogens was significantly retarded by the *T. atroviride* AN206 and *Trichoderma cremeum* AN392 strains, respectively.

The antagonistic profiles of all *Trichoderma* strains from qualitative evaluation of the interaction between the colonies after 14 days of co-culturing on PDA medium were present in Table III. Of the 24 *Trichoderma* strains, one strain, namely AN240, was efficiently antagonistic against the entire target *Fusarium* species in that it received the best evaluation (*i.e.*, 8) according to Mańka's classification (1974). Whereas, four *Trichoderma* strains (AN152, AN251, AN255, AN430) completely overgrew and sporulated on the colonies of all except one of the *Fusarium* species tested (Table III).

To investigate the mode of action of the antagonists, microscopic observation was conducted of the mycelium of confronting fungi in the interaction zone.

Supplemental online material 1
Analysis of variance of the growth rate of pathogens in the presence of antagonists/control across days; significance of all factors and their interactions.

Factor	*Df*	Sum Sq	Mean Sq	F value	Pr (> F)
Pathogen	5	14802	2960	2279.20	< 0.001 ***
Antagonist	24	6536	272	209.68	< 0.001 ***
Day	3	115885	38628	29739.56	< 0.001 ***
Pathogen × antagonist	120	15282	127	98.05	< 0.001 ***
Pathogen × day	15	7484	499	384.13	< 0.001 ***
Antagonist × day	72	5954	83	63.66	< 0.001 ***
Pathogen × antagonist × day	360	15481	43	33.11	< 0.001 ***

Supplemental online material 2

Linear regression analysis. Growth rate of *Fusarium* spp. facing *Trichoderma* spp. compared with the growth rate of *Fusarium* spp. in the control conditions (first row). R2 – determination coefficient; Δ slope – difference of growth rate (mm per day) and its significance. Δ int (intercept) – difference of elevation of the regression line. and its significance (determined only if Δ slope insignificant at < 0.001).

Pathogen	*F. avenaceum* KF 2818				*F. cerealis* KF 1157				*F. culmorum* KF 2795				*F. graminearum* KF 2870				*F. temperatum* KF 506			
	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope
T. atroviride																				
control	0.73	13.0		−7.4	0.95	8.0		−3.1	0.87	14.6		−7.5	0.96	11.6		−3.7	0.93	6.6		−2.9
AN152	0.97	−8.5	***	6.3	0.97	−2.8	***	2.0	0.97	−9.2	***	7.1	0.87	−3.5	***	0.6	0.98	−0.4		2.3
AN182	0.97	−7.1	***	6.1	0.92	0.0		1.9	0.88	−7.1	***	4.9	0.93	−4.5	***	1.5	0.91	−2.1	***	1.8
AN206	0.94	−8.0	***	6.8	0.93	1.6	*	−0.3	0.92	−9.0	***	7.9	0.97	−7.4	***	4.4	0.97	−1.4	**	1.2
AN240	0.97	−8.8	***	8.1	0.98	−2.9	***	1.5	0.97	−9.5	***	6.5	0.96	−3.5	***	2.7	0.97	−1.3	**	1.7
AN497	0.97	−8.5	***	6.3	0.92	−2.5	**	3.0	0.94	−5.9	***	4.8	0.94	−3.2	***	0.6	0.95	−1.6	**	0.9
T. citrinoviride																				
AN262	0.96	−8.3	***	7.0	0.96	−3.6	***	1.7	0.89	−4.5	***	4.1	0.90	−2.9	**	1.1	0.74	−1.7	***	1.5
AN393	0.97	−8.4	***	7.4	0.93	0.8		0.9	0.89	−6.0	***	4.7	0.95	−0.7		0.4	0.97	−1.3	*	1.3
T. cremeum																				
AN392	0.91	−8.2	***	5.7	0.90	3.9	***	−2.3	0.98	−4.1	***	5.5	0.96	−0.5		1.3	0.99	−3.8	***	3.1
T. hamatum																				
AN120	0.94	−8.0	***	6.1	0.90	0.5		0.3	0.92	−2.7	**	2.7	0.92	0.0		−1.7	0.94	−1.6	***	1.3
AN277	0.97	−8.8	***	6.3	0.92	−2.1	**	0.1	0.98	−7.5	***	5.9	0.96	−3.3	***	1.5	0.89	−1.0	*	0.5
T. harzianum																				
AN150	0.93	−9.5	***	6.5	0.92	3.9	***	−3.3	0.75	−6.2	***	4.6	0.82	−3.1	***	−2.3	0.97	−1.3	**	1.3
AN279	0.94	−7.0	***	6.0	0.95	0.6		1.0	0.97	−9.3	***	7.1	0.96	−4.2	***	1.8	0.96	−1.4	**	1.3
AN360	0.98	−7.8	***	6.2	0.94	0.5		0.1	0.93	−7.8	***	4.7	0.94	−1.7	*	−1.1	0.98	−0.3		0.5

Supplemental online material 2 – continued

Linear regression analysis. Growth rate of *Fusarium* spp. facing *Trichoderma* spp. compared with the growth rate of *Fusarium* spp. in the control conditions (first row). R2 – determination coefficient; Δ slope – difference of growth rate (mm per day) and its significance. Δ int (intercept) – difference of elevation of the regression line. and its significance (determined only if Δ slope insignificant at < 0.001).

Pathogen	*F. avenaceum* KF 2818				*F. cerealis* KF 1157				*F. culmorum* KF 2795				*F. graminearum* KF 2870				*F. temperatum* KF 506			
	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope
T. koningiopsis																				
AN143	0.83	−9.6	***	5.8	0.92	−2.1	**	0.1	0.98	−11.4	***	6.9	0.96	−4.8	***	2.3	0.97	−1.6	***	1.5
AN251	0.98	−7.8	***	6.2	0.96	−0.8		0.6	0.96	−6.4	***	6.4	0.98	−6.4	***	2.9	0.76	−1.5	**	1.4
T. longibrachiatum																				
AN197	0.95	−7.8	***	5.2	0.95	0.7		1.1	0.97	−10.4	***	6.5	0.93	−6.3	***	4.1	0.98	−1.6	***	1.3
AN213	0.94	−9.5	***	8.4	0.95	−5.1	***	3.2	0.97	−7.6	***	5.3	0.96	−2.4	**	0.4	0.87	−0.4		−0.5
T. longipile																				
AN359	0.98	−7.8	***	6.1	0.92	−3.2	***	1.4	0.97	−4.0	***	4.5	0.94	−2.3	**	−0.5	0.97	−1.6	***	1.3
T. viride																				
AN255	0.97	−7.9	***	5.6	0.91	1.8	*	1.9	0.96	−8.3	***	7.2	0.97	−6.2	***	3.3	0.88	−1.1	*	1.0
AN401	0.97	−7.9	***	6.3	0.99	−2.7	***	2.1	0.91	−4.9	***	4.5	0.93	−1.4		−1.9	0.92	−2.8	***	2.3
AN430	0.98	−7.7	***	6.2	0.96	−0.3		1.0	0.98	−9.5	***	6.9	0.93	−4.8	***	1.9	0.97	−1.0	*	0.9
AN826	0.91	−6.8	***	5.2	0.99	−2.7	***	2.1	0.86	−4.8	***	2.3	0.98	−6.4	***	2.3	0.94	−1.4	**	1.6
T. viridescens																				
AN323	0.98	−7.8	***	5.8	0.98	−2.8	***	2.4	0.97	−10.3	***	8.3	0.94	−5.4	***	3.0	0.89	−1.7	***	1.7
AN405	0.93	−7.2	***	6.2	0.96	2.4	**	−1.1	0.91	−9.8	***	5.8	0.93	−2.0	*	0.3	0.89	−1.4	**	0.5

Significance codes: '***' for < 0.001; '**' for < 0.01 ; '*' for < 0.05

Table II
Duncan's Multiple Range Test (α = 0.05). Multiple comparisons procedure revealed pairwise differences between antagonists' influence on pathogen growth and assigned identity classes in terms of pathogen colony size.

Pathogen / Antagonist	*F. avenaceum* KF 2818			*F. cerealis* KF 1157			*F. culmorum* KF 2795			*F. graminearum* KF 2870			*F. temperatum* KF 506		
	Means[a]	Std[b]	M[c]	Means	Std	M	Means	Std	M	Means	Std	M	Means	Std	M
control[d]	60.67	1.15	a	30.00	0.00	d	61.00	1.73	a	45.67	0.58	a	26.67	5.77	a
T. atroviride															
AN152	17.67	1.15	efg	19.33	1.15	g	20.00	0.00	hi	35.00	0.00	d	22.67	2.31	cd
AN182	22.33	0.58	bc	30.00	0.00	d	26.67	2.89	efg	25.00	0.00	f	17.67	1.15	f
AN206	19.00	1.73	def	35.00	0.00	c	20.00	0.00	hi	16.33	1.15	i	19.67	0.58	ef
AN240	16.33	1.15	gh	19.67	0.58	g	19.33	0.58	hi	29.33	0.58	e	20.00	0.00	ef
AN497	17.67	1.15	efg	20.00	0.00	g	31.67	2.89	de	30.67	1.15	e	20.00	0.00	ef
T. citrinoviride															
AN262	17.33	0.58	fgh	16.67	0.58	h	35.00	0.00	cd	30.67	1.15	e	19.67	0.58	ef
AN393	17.33	0.58	fgh	30.00	0.00	d	30.67	1.15	def	39.67	0.58	b	20.33	0.58	def
T. cremeum															
AN392	20.33	0.58	cde	43.67	1.15	a	40.00	0.00	bc	40.00	0.00	b	10.67	1.15	h
T. hamatum															
AN120	20.67	1.15	cd	35.00	0.00	c	44.33	1.15	b	45.00	0.00	a	20.00	0.00	ef
AN277	16.33	0.58	gh	22.33	2.52	f	28.00	0.00	efg	30.00	0.00	e	23.00	2.65	bc
T. harzianum															
AN150	12.33	0.58	j	45.00	0.00	a	28.67	14.43	efg	35.00	0.00	d	20.00	0.00	ef
AN279	21.00	0.00	bcd	30.33	0.58	d	19.67	0.58	hi	30.00	0.00	e	20.00	0.00	ef
AN360	20.00	0.00	cdef	30.00	0.00	d	25.00	0.00	fgh	38.00	0.00	c	25.33	0.58	ab
T. koningiopsis															
AN143	15.00	0.00	hi	22.33	2.52	f	12.00	0.00	j	25.00	0.00	f	20.00	0.00	ef
AN251	20.00	0.00	cdef	28.33	0.58	e	30.00	0.00	def	20.00	0.00	h	19.67	0.58	ef
T. longibrachiatum															
AN197	19.33	0.58	def	30.67	0.58	d	16.33	0.58	ij	20.00	0.00	h	20.00	0.00	ef
AN213	13.67	1.15	ij	10.67	1.15	i	26.00	0.00	efg	35.33	0.58	d	26.33	0.58	a
T. longipile															
AN359	20.00	0.00	cdef	20.33	0.58	g	40.00	0.00	bc	35.00	0.00	d	20.00	0.00	ef
T. viride															
AN255	19.00	0.00	def	35.00	0.00	c	23.33	0.58	gh	20.00	0.00	h	23.00	0.00	bc
AN401	19.33	1.15	def	20.33	0.58	g	35.00	0.00	cd	39.00	0.00	bc	15.00	0.00	g
AN430	20.00	0.00	cdef	29.33	0.58	de	19.33	0.58	hi	24.33	2.31	f	22.00	0.00	cde
AN826	23.33	5.77	b	20.33	0.58	g	35.00	0.00	cd	20.00	0.00	h	20.00	0.00	ef
T. viridescens															
AN323	20.00	0.00	cdef	19.33	0.58	g	16.33	1.15	ij	22.67	2.31	g	20.00	0.00	ef
AN405	20.00	0.00	cdef	40.00	0.00	b	20.33	0.58	hi	35.00	0.00	d	20.33	0.58	def

a – Average colony size of the pathogen in the presence of the antagonist – a mean of three replicates
b – Standard error for three replicates
c – Values within columns followed by the same letter are not significantly different according to the test for α = 0.05.
d – The control consisted of individual cultures of the pathogen in the absence of the antagonist

After 7/14 days of incubation two of the 24 isolates, namely AN240 and AN255, showed coiling structures and branched hyphae with at least one of all five *Fusarium* species (Fig. 1A-B). For AN240, coiling was observed on all Petri dishes when growing in co-culture with *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum* strain (Fig. 1). However, AN255 produce dense coils around the hyphae of *F. graminearum* in all Petri dishes. The analysis of the area of intermingling contact (pathogen-antagonist)

Supplemental online material 3

Estimated inhibition (%) of mycelial growth of *F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2878 and *F. temperatum* KF 506 by *Trichoderma* species after 4 days of co–incubation on PDA medium.

Pathogen / Antagonist	*F. avenaceum* KF 2818		*F. cerealis* KF 1157		*F. culmorum* KF 2795		*F. graminearum* KF 2870		*F. temperatum* KF 506	
	I[a]	se[b]	I	se	I	se	I	se	I	se
T. atroviride										
AN152	70.88	1.37	35.56	2.05	67.21	3.05	23.36	1.27	15.00	4.05
AN182	63.19	1.41	0.00	2.43	56.28	3.16	45.26	1.15	33.75	3.70
AN206	68.68	1.38	–16.67	2.65	67.21	3.05	64.23	1.07	26.25	3.83
AN240	73.08	1.37	34.44	2.06	68.31	3.04	35.77	1.20	25.00	3.85
AN497	70.88	1.37	33.33	2.07	48.09	3.26	32.85	1.22	25.00	3.85
T. citrinoviride										
AN262	71.43	1.37	44.44	1.97	42.62	3.34	32.85	1.22	26.25	3.83
AN393	71.43	1.37	0.00	2.43	49.73	3.24	13.14	1.34	23.75	3.88
T. cremeum										
AN392	66.48	1.39	–45.56	3.04	34.43	3.46	12.41	1.35	60.00	3.32
T. hamatum										
AN120	65.93	1.39	–16.67	2.65	27.32	3.58	1.46	1.42	25.00	3.85
AN277	73.08	1.37	25.56	2.15	54.10	3.19	34.31	1.21	13.75	4.07
T. harzianum										
AN150	79.67	1.35	–50.00	3.10	53.01	3.20	23.36	1.27	25.00	3.85
AN279	65.38	1.40	–1.11	2.45	67.76	3.04	34.31	1.21	25.00	3.85
AN360	67.03	1.39	0.00	2.43	59.02	3.13	16.79	1.32	5.00	4.25
T. koningiopsis										
AN143	75.27	1.36	25.56	2.15	80.33	2.95	45.26	1.15	25.00	3.85
AN251	67.03	1.39	5.56	2.37	50.82	3.23	56.20	1.10	26.25	3.83
T. longibrachiatum										
AN197	68.13	1.38	–2.22	2.46	73.22	3.00	56.20	1.10	25.00	3.85
AN213	77.47	1.35	64.44	1.83	57.38	3.15	22.63	1.28	1.25	4.33
T. longipile										
AN359	67.03	1.39	32.22	2.08	34.43	3.46	23.36	1.27	25.00	3.85
T. viride										
AN255	68.68	1.38	–16.67	2.65	61.75	3.10	56.20	1.10	13.75	4.07
AN401	68.13	1.38	32.22	2.08	42.62	3.34	14.60	1.33	43.75	3.54
AN430	67.03	1.39	2.22	2.41	68.31	3.04	46.72	1.15	17.50	4.00
AN826	61.54	1.41	32.22	2.08	42.62	3.34	56.20	1.10	25.00	3.85
T. viridescens										
AN323	67.03	1.39	35.56	2.05	73.22	3.00	50.36	1.13	25.00	3.85
AN405	67.03	1.39	–33.33	2.87	66.67	3.05	23.36	1.27	23.75	3.88

a – Inhibition (%)
b – Standard error for three replicates

after 14/21 days of incubation revealed differences in the hyphal morphology of the pathogen as vacuolization of hyphae and plasmolysis of mycelium (Fig. 1C). For the other *Trichoderma*/*Fusarium* combinations, mycoparasitic signs were either only observed occasionally on some of the replicates or were not observed at all (date not shown).

Evaluation of the antagonistic activity of *Trichoderma* strains in dual cultures on solid substrates. Five *Trichoderma* strains (AN152, AN240, AN251, AN255, AN430) selected on the basis of dual culture bioassay on PDA medium, and showing the highest antagonistic ability against at least four of all five *Fusarium* species (Table III), were further tested for

Table III
The final assessment of the interaction between the *Trichoderma*/*Fusarium* colonies after 14 days of co-culturing on PDA medium.

Pathogen Antagonist	*F. avenaceum* KF 2818	*F. cerealis* KF 1157	*F. culmorum* KF 2795	*F. graminearum* KF 2870	*F. temperatum* KF 506	Average[a]
T. atroviride						
AN152[b]	8	6	8	8	8	7.6
AN182	6	6	6	8	8	6.8
AN206	6	6	8	8	8	7.2
AN240	8	8	8	8	8	8
AN497	8	8	0	6	6	5.6
T. citrinoviride						
AN262	4	6	4	4	6	4.8
AN393	6	4	4	4	6	4.8
T. cremeum						
AN392	4	0	0	4	6	2.8
T. hamatum						
AN120	6	4	0	0	4	2.8
AN277	6	6	8	6	6	6.4
AN150	6	0	0	4	6	3.2
AN279	6	4	6	6	6	5.6
AN360	6	4	4	4	6	4.8
T. koningiopsis						
AN143	8	6	8	8	6	7.2
AN251	8	6	8	8	8	7.6
T. longibrachiatum						
AN197	6	4	6	6	6	5.6
AN213	6	6	4	4	6	5.2
T. longipile						
AN359	6	4	4	4	6	4.8
T. viride						
AN255	8	6	8	8	8	7.6
AN401	6	4	4	0	6	4
AN430	8	8	8	8	6	7.6
AN826	4	4	4	6	6	4.8
T. viridescens						
AN323	6	6	8	8	8	7.2
AN405	8	0	4	6	6	4.8

a – The values in modified Mańka (1974) scale (+8, antagonist completely overgrew the pathogen and entire medium surface; +6, antagonist occupies 85% of the medium surface; +4, antagonist occupies 75% of the medium surface, 0, antagonist occupies 50% of the medium surface) after visual assessment of the colony state (3 replicates, all observations equal in all cases) were averaged to identify the most efficient antagonist.
b – *Trichoderma* strains (grey) showing the highest antagonistic ability against at least four of all five *Fusarium* species

their ability to inhibit *Fusarium* mycotoxin production in dual cultures on rice kernels. *Fusarium* species used in the experiments represented five chemotypes (ZEN, DON and its derivatives 15-AcDON and 3-AcDON, NIV, MON, BEA). The estimated amount of mycotoxin production by these species after 21 days of incubation and co-incubation with *Trichoderma* on rice medium is shown in Table IV. The effect of *Trichoderma* strains on *Fusarium* mycotoxin synthesis in dual culture bioassay on rice after 21 days at 25 ± 2°C is presented in Table V. The experiments revealed that all *Trichoderma* strains assayed reduced mycotoxin production by *Fusarium* species from 21% to 100%, except for strain AN430 that showed no suppressive effect on ZEN production by *F. culmorum*. As shown in Table V, the presence of each of the four *Trichoderma*

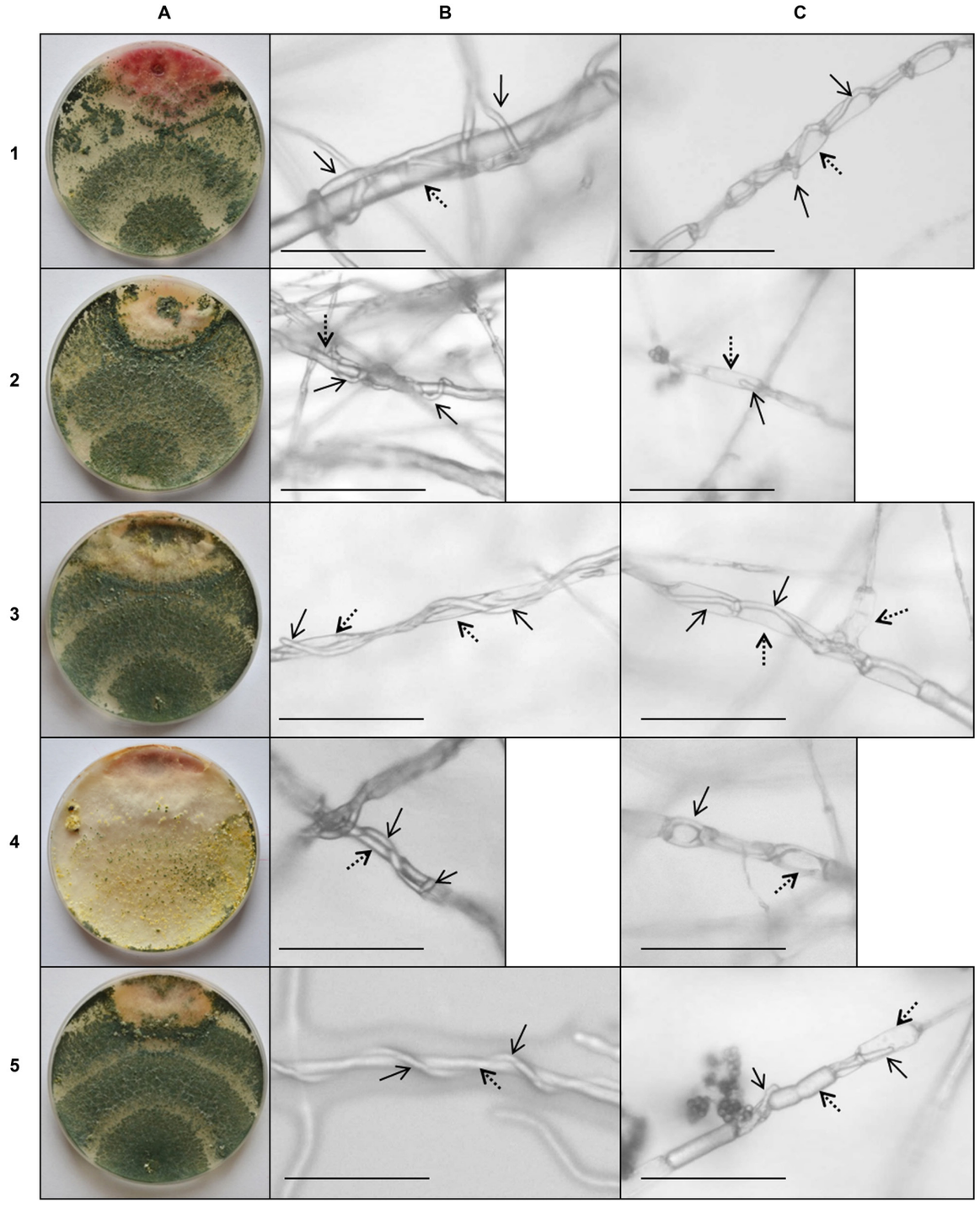

Fig. 1. Interactions between *T. atroviride* AN240 and *F. avenaceum* KF 2818 (1), *F. cerealis* KF 1157 (2), *F. culmorum* KF 2795(3), *F. temperatum* KF 506 (5) and between *T. viride* AN 255 and *F. graminearum* KF 2870 (4) in dual cultures on PDA medium: A – dense sporulation and overgrowth of antagonist on pathogen after 14 days of co-incubation; B – coiling of pathogen hyphae by antagonist after 7/14 days of co-incubation; C – antagonist hypha inside a partial degraded hypha of pathogen after 14/21 days of co-incubation. The black arrow indicate the hyphae of *Trichoderma* strain and the dotted arrow indicate the hyphae of *Fusarium* strains. Bar = 50 µm.

strains completely (100%) inhibited MON production by *F. avenaceum*. *Trichoderma atroviride* AN240 markedly (>99%) reduced the level of both BEA and MON synthesized by *F. temperatum*. In the presence of *Trichoderma viride* AN430, *F. cerealis* produced 92% less NIV than when alone. However, among the four

Table IV
Mycotoxin production (mg kg^{-1}) by *F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2870 and *F. temperatum* KF 506 on solid substrates (rice kernels) in the presence/absence of the *Trichoderma* strains (*T. atroviride* AN152, AN240, *T. koningiopsis* AN251, *T. viride* AN255, AN430).

Combination		Mycotoxin (mg kg^{-1})													
		DON		NIV		ZEN		3-AcDON		15-AcDON		BEA		MON	
		Means	se[a]	Means	se	Means	se	Means	se	Means	se	Means	se	Means	se
F. avenaceum KF 2818	control[b]	ND[c]		ND		ND		ND		ND		ND		1.08	0.18
	AN152													ND	
	AN240														
	AN255														
	AN430														
F. cerealis KF 1157	control	ND		19.24	0.85	ND		ND		ND		ND		ND	
	AN152			3.33	0.48										
	AN240			5.43	0.62										
	AN255			1.94	0.09										
	AN430			1.52	0.09										
F. culmorum KF 2795	control	11.06	4.71	43.81	2.5	45.76	16.96	5.77	1.16	ND		ND		ND	
	AN240	0.37	0.53	1.91	0.07	7.46	3.52	0.01	0.01						
	AN251	2.71	1.76	8.35	0.27	36.2	34.46	0.86	0.99						
	AN255	1.7	1.7	5.15	0.11	0.34	0.48	0.01	0.02						
	AN430	0.55	0.54	7.24	0.1	46.62	4.17	1.36	1.14						
F. graminearum KF 2870	control	39.79	1.19	49.72	0.43	34.27	0.81	ND		2.96	0.23	ND		ND	
	AN240	1.93	0.62	9.01	0.63	0.21	0.3			0.16	0.23				
	AN251	3.94	0.97	7.41	0.21	8.04	1.05			0.93	0.18				
	AN255	3.34	0.22	4.94	0.12	0.58	0.56			0.36	0.51				
	AN430	1.34	0.95	5.48	0.18	0.98	0.68			0.73	0.52				
F. temperatum KF 506	control	ND		ND		ND		ND		ND		19.59	1.66	63.34	2.09
	AN240											0.01	0.01	0.08	0.04
	AN251											7.56	1.28	0	0
	AN255											0.22	0.04	10.24	1.65
	AN430											0.54	0.28	1.47	0.68

a – Standard error for three replicates
b – The control consisted of individual cultures of the pathogen in the absence of the antagonist.
c – Not detected (*i.e.* < LOD)

Trichoderma strains, AN240 and AN255 showed the best capacity for reducing DON, 3-AcDON, ZEN, NIV concentrations during co-incubation with *F. culmorum* and DON, 15-AcDON, ZEN, NIV accumulation in dual cultures with *F. graminearum*.

Discussion

As is well known, antagonism by *Trichoderma* is a combination of several mechanisms, including nutrient and/or space competitions, antibiosis associated with the secretion of antibiotic metabolites and direct parasitism or mycoparasitism, which involves the production of cell-wall-degrading enzymes (Vinale *et al.*, 2008a; 2008b; Druzhinina *et al.*, 2011). The powerful tool for the study of the antagonistic activity of *Trichoderma* against some phytopathogenic fungi is considered to be the dual culture assays (Almeida *et al.*, 2007; Qualhato *et al.*, 2013; Schöneberg *et al.*, 2015). In the present study, this approach was used in the preliminary screening of 24 *Trichoderma* isolates, belonging to 10 species for *in vitro* antagonism towards five toxigenic *Fusarium*. The results of this work revealed a great variability in the level of biological activity between *Trichoderma* species and even among strains of the same species towards the same *Fusarium* strains. Moreover, the results obtained in the present study showed a different degree of biological activity of the same antagonistic agent against distinct *Fusarium* species (*i.e.* that the antagonism of the same *Trichoderma* strains varies when it is confronted with different *Fusarium* species).

Table V
Estimated reduction (%) of toxins production of five *Fusarium* species (*F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2878 and *F. temperatum* KF 506) by *T. atroviride* AN152, AN240, *T. koningiopsis* AN251 and *T. viride* AN255, AN430 strains in dual culture on solid substrates (rice kernels).

Combination		DON		NIV		ZEN		3- AcDON		15-AcDON		BEA		MON	
		R[a]	se[b]	R	se	R	se	R	se	R	se	R	se	R	se
F. avenaceum KF 2818	AN152	–[c]		–		–		–		–		–		100.00	5.29*
	AN240													100.00	5.29*
	AN255													100.00	5.29*
	AN430													100.00	5.29*
F. cerealis KF 1157	AN152	–		82.69	1.94	–		–		–		–		–	
	AN240			71.78	1.99										
	AN255			89.92	1.92										
	AN430			92.10	1.92										
F. culmorum KF 2795	AN240	96.63	15.34	95.63	1.82	83.70	27.17	99.83	10.47	–		–		–	
	AN251	75.53	15.78	80.95	1.85	20.88	34.20	85.16	10.58						
	AN255	84.63	15.51	88.24	1.83	99.26	26.81	99.77	10.47						
	AN430	95.00	15.35	83.47	1.84	–1.88	38.29	76.50	10.75						
F. graminearum KF 2870	AN240	95.16	1.53	81.87	0.53	99.39	1.49	–		94.48	8.73	–		–	
	AN251	90.11	1.53	85.09	0.52	76.55	1.53			68.47	9.14				
	AN255	91.61	1.53	90.07	0.52	98.32	1.49			87.84	8.78				
	AN430	96.63	1.53	88.99	0.52	97.14	1.49			75.23	8.98				
F. temperatum KF 506	AN240	–		–		–		–		–		99.95	3.42	99.87	1.37
	AN251											61.41	3.66	100.00	1.37
	AN255											98.88	3.42	83.83	1.39
	AN430											97.26	3.42	97.68	1.37

a – Reduction (%)
b – Standard error for three replicates
c – Not applicable
* The standard error of the estimate for MON toxin comes only from variation in the control conditions. All antagonists reduced MON completely (toxin was not found in the measurements).

These findings are consistent with several earlier studies on the antagonistic potential of *Trichoderma* species against *F. graminearum* and *F. cerealis*, *Fusarium solani*, *R. solani*, *Sclerotinia rolfsii*, *Sclerotinia sclerotiorum* (Dubey *et al.*, 2007; Inch and Gilbert, 2007; Shaigan *et al.*, 2008; Amin *et al.*, 2010; Anees *et al.*, 2010; Qualhato *et al.*, 2013; Schöneberg *et al.*, 2015) and could suggest that the *in vitro* antagonistic potential of *Trichoderma* is determined by types of antagonist/pathogen interactions unique and specific for each strain. It is worth noting that this type of interaction might also depend on the experimental conditions, as was recently observed by Schöneberg *et al.* (2015).

Dual-culture assay on PDA medium clearly documented that all of the *Trichoderma* strains used in the present study were capable of influencing the mycelial growth of at least four of all five *Fusarium* species on the fourth day after co-inoculation, when there was the first apparent physical contact between antagonist and pathogen. Ten of *Trichoderma* strains – AN152, AN182, AN206, AN240, AN497, AN277, AN143, AN255, AN430, AN323, AN405 – completely overgrew and sporulated on the colony at least one of the tested *Fusarium* species. Whereas, the microscopic assay provided evidence that only *T. atroviride* AN240 and *T. viride* AN255 formed dense coils around the hyphae of the pathogen from where penetration took place. Coiling has been considered to be an indicator for mycoparasitic potential, which could play an important role in making contact with the pathogen (Benítez *et al.*, 2004; Schöneberg *et al.*, 2015). In the present study, not all highly antagonistic *Trichoderma* strains were able to coil around the hyphae of the pathogen. This observation is in accordance with previous reports where no correlation between coiling and other features of antagonistic potential of *Trichoderma* was found (Almeida *et al.*, 2007; Anees *et al.*, 2010; Schöneberg *et al.*, 2015) and supports the statement that coiling could also depend on various biotic and abiotic factors and may be part of a more general response to a filamentous substrate (Inbar and Chet, 1992; Almeida *et al.*, 2007).

As observed here, those *Trichoderma* strains which displayed the highest antagonistic activity against at least four *Fusarium* species in co-culturing on PDA medium, namely AN152, AN240, AN255, AN430 and AN251, were also found to be effective suppressors of mycotoxin production by these pathogens on solid substrates. A similar study performed by Cooney *et al.* (2001) showed that *T. harzianum* isolate THF2/3 grown in a competition assay system with *F. graminearum* displayed an inhibitory effect on mycelial growth and trichothecene mycotoxin (DON) production by *Fusarium*. The capacity of highly antagonistic *T. gamsii* and *T. atroviride* strains to inhibit trichothecene and ZEN production by *F. graminearum* and *F. culmorum* on rice kernels was also described by Buśko *et al.* (2008), Popiel *et al.* (2008) and Matarese *et al.* (2012). Furthermore, Matarese *et al.* (2012) clearly demonstrated that the reduction of DON production by *Fusarium* species is correlated with the reduction of the pathogen biomass by *Trichoderma*. This finding could suggest that the ability all of the *Trichoderma* strains tested in the present study to suppress mycotoxin synthesis by *Fusarium* species on solid substrates was also the result of their high activity related to pathogen growth inhibition. The only exception is *T. viride* AN430. As was shown here, this strain was not able to reduce ZEN production by *F. culmorum* KF 2795, whereas the level of the other mycotoxins synthesized by KF 2795 was markedly reduced by AN430. Thus, the cause could not be as that described by Matarese *et al.* (2012). It is supposed that the presence of AN430 could stimulate the pathogen to selectively overproduce ZEN (Elmholt, 2008). However, the explanation of this aspect requires further studies.

In the present work, the most efficient strain overall was *T. atroviride* AN240 which was able to reduce mycelial growth and mycotoxins production of *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum* species. To the best of our knowledge, this is the first report of the suppressive effect of *T. atroviride* on five toxigenic *Fusarium* species. The only reports of an efficient antagonistic strain of *T. atroviride* towards *F. avenaceum*, *F. culmorum* and *F. graminearum* were available from Buśko *et al.* (2008) and Popiel *et al.* (2008). The results of present study indicate that *T. atroviride* AN240 strain was able both to inhibit the pathogen's growth before the contact in confrontation plates and to overgrow and sporulate on the pathogen's colony. Moreover, the microscopic observation revealed a direct development of AN240 on the mycelium of *Fusarium* species. This research has suggested a possible antibiosis and mycoparasitism of AN240 and the production of antifungal components and extracellular metabolites by this antagonist (Druzhinina *et al.*, 2011; Verma *et al.*, 2007). Many studies of the *in vitro* biological activity of *Trichoderma* against fungal plant pathogens demonstrated the involvement of cell-wall degrading enzymes (CWDEs), which are also capable of acting synergistically with diffusible, volatile and non-volatile secondary metabolites and a complex system for fungal prey detection (Almeida *et al.*, 2007; Qualhato *et al.*, 2013). It is significant to note that our associated studies (unpublished data) on the examination of the 186 *Trichoderma* strains originating from wood with decay symptoms and analyses of their ability to produce hydrolytic enzymes have shown *T. atroviride* AN240 as the best producer of β-1,3-glucanase – an enzyme which have been found to be directly involved in the hydrolysis of the fungal pathogen's cell walls (Druzhinina *et al.*, 2011). Furthermore, in our previous study on the formation of the 6-n-pentyl-2H-pyran-2-one and other volatile compounds by different *Trichoderma* species grown on PDA medium, *T. atroviride* was found to be the most efficient species in this respect (Jeleń *et al.*, 2014). In the light of this and our previous findings, it can be assumed that *T. atroviride* AN240 represents a good candidate for the biological control of toxigenic *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum*. However, it will be necessary to verified the reproducibility of the suppressive effect of AN240 also on *Fusarium* species (*i.e.*, *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum*) originating from other fungal collections and to undertake the field trials in order to determine the ability of these strains to control of mycotoxin production in grain as well as to reduce toxigenic *Fusarium* inoculum levels in cereal debris under natural conditions.

Conclusion

The present survey has led to the emergence of the most efficient antagonist – *T. atroviride* AN240, able to inhibit the mycelial growth, completely overgrown and sporulated on the pathogen's colony, as well as to reduce the level of mycotoxins produced by *F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2870 and *F. temperatum* KF 506. Considering the biological activity of this strain, being the synergistic effect of the several factors, it can be concluded that AN240 is a candidate fungus for the biological control of toxigenic *Fusarium* species by reducing their inoculum, as well as preventing mycotoxin accumulation in plant tissues.

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ORIGINAL PAPER

Screening and Identification of Yeasts Antagonistic to Pathogenic Fungi Show a Narrow Optimal pH Range for Antagonistic Activity

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Abstract

Microbes have evolved ways of interference competition to gain advantage over their ecological competitors. The use of secreted antagonistic compounds by yeast cells is one of the prominent examples. Although this killer behavior has been thoroughly studied in laboratory yeast strains, our knowledge of the antagonistic specificity of killer effects in nature remains limited. In this study, yeast strains were collected from various niches and screened for antagonistic activity against one toxin-sensitive strain of *Saccharomyces cerevisiae* and three pathogenic fungi. We demonstrate that some strains with antagonistic activity against these pathogenic fungi can be found in antagonist culture tests. These yeasts were identified as members of *Trichosporon asahii*, *Candida stellimalicola*, *Wickerhamomyces anomalus*, *Ustilago esculenta*, *Aureobasidium pullulans*, and *Pichia kluyveri*. The results indicated that the antagonistic activity of these killer yeasts has a narrow optimal pH range. Furthermore, we found that the antagonistic activity of some species is strain-dependent.

Key words: antagonistic yeasts, interference competition, pathogenic fungi

Introduction

Two broad types of competition are generally recognized: interference and exploitative. Interference competition is a direct form of competition in which an organism actively interferes with another organism's ability to obtain resources. A common strategy for interference competition is the production of antibiotic compounds or toxins, by which cells inhibit the growth of sensitive competitors (Cornforth and Foster, 2013). The use of secreted antagonistic compounds by microbes is one prominent example (Burgess *et al*., 1999; Jousset *et al*., 2014; Schmitt and Breinig, 2002).

The most common method for selecting probiotic microbes involves testing the inhibition potential of the antagonistic compounds produced by candidate strains against known pathogens. Biological control using microbial agents has been suggested as an effective approach against pathogens, and antibiotics produced by microorganisms have garnered particular attention in research (Kohanski *et al*., 2010; Mathur and Singh, 2005). Bacterial species producing antibiotics have been used as biocontrol agents against pathogenic fungi (Gebreel *et al*., 2008; Yilmaz *et al*., 2006). In addition, because antagonistic yeasts produce toxic secondary metabolites (antibiotics), they have been used in commercial-scale yeast production including fermentation, formulation, storage, and handling (Wisniewski *et al*., 2007; Wisniewski and Wilson, 1992).

Based on the aforementioned literature, this study investigated antagonistic activity against these human pathogenic fungi. We used single killer-toxin-sensitive strain of *Saccharomyces cerevisiae* (Chang *et al*., 2015) and three pathogenic yeasts identified by the large subunit (LSU) of ribosomal DNA (rDNA) sequences isolated from the little black biting midge (*Forcipomyia taiwana*) (Chou *et al*., 2015): *Aureobasidium pullulans* JYC1003, *Pseudozyma aphidis* JYC1050, and *Trichosporon asahii* JYC1040. To confirm these species, the ITS1-5.8S-ITS2 regions of their rDNA were also sequenced. These yeasts are emergent opportunistic pathogens that cause infections in immune-compromised individuals.

A. pullulans is classified as a dematiaceous fungus, commonly known as black yeast because of its melanin production. *A. pullulans* can cause corneal and dermal infections in immunocompromised patients (Girardi *et al*., 1993). Despite the importance of *A. pullulans* in biotechnology (Molina-Leyva *et al*., 2013), and although it rarely causes cutaneous infections in humans, several recent studies have reported its pathological significance (Molina-Leyva *et al*., 2013; Vlchkova-Lashkoska *et al*., 2004). *P. aphidis* is rarely

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reported as pathogenic; it is an opportunistic yeast typically isolated from plants and rarely from clinical specimens. Chen *et al*. (2011) isolated *P. aphidis* from the tissues of a patient with a swollen limb, and it has also been isolated from patients with astrocytoma (Hwang *et al*., 2010). However, knowledge regarding the pathogenicity of *Pseudozyma* genus is limited. *Trichosporon* spp. are opportunistic pathogens that can cause invasive infections and are associated with a high mortality rate in immunocompromised patients (Girmenia *et al*., 2005). *T. asahii* (previously *Trichosporon beigelii*) has wide infectious ability, even in healthy individuals; it can cause white piedra and hypersensitive pneumonia, as well as papular skin lesions, which are often fatal in immunocompromised patients (Bayramoglu *et al*., 2008; Wolf *et al*., 2001). To date, over 100 cases of hematogenously disseminated infections caused by this life-threatening yeast have been reported (Ebright *et al*., 2001; Krcmery Jr *et al*., 1999).

Because high levels of fungicide engender resistance in fungal pathogens, and because of growing public concerns over health and environmental hazards, new methods for controlling fungal diseases must be developed. Therefore, the objectives of this study were to screen and identify yeasts antagonistic to the aforementioned pathogenic fungi, and to investigate the influence of pH values on the antagonistic activity.

Experimental

Materials and Methods

Yeast isolation. The yeast samples were collected from various niches, including wine, flowers, insects and fruits and the like. They were transported to the laboratory in sealed plastic bags placed in an icebox and sent to the laboratory. The samples were maintained at a low temperature (4°C) prior to the yeast isolation procedures. The yeasts were isolated using an enrichment technique involving a malt extract medium (30 g/l of malt extract (Sigma-Aldrich, USA), 5 g/l of peptone (Sigma-Aldrich, USA)) supplemented with approximately 2–3 ml of 100% lactic acid (Sigma-Aldrich, USA). The samples were placed in 15-ml test tubes and incubated on a rotary shaker at 30°C for 3 h. A loopful of the enriched culture was streaked onto malt extract agar supplemented with approximately 2–3 ml of 100% lactic acid. Yeast colonies of different morphologies were selected and purified by cross-streaking on malt extract agar. Purified yeast strains were suspended in a YPD medium (1% yeast extract (Sigma-Aldrich, USA), 2% peptone, 2% dextrose (Sigma-Aldrich, USA)) supplemented with 15% v/v glycerol (Sigma-Aldrich, USA) and maintained at –80°C.

Yeast genomic DNA extraction. One milliliter of young yeast cultures was transferred to a 1.5-ml tube and centrifuged at 13,000–16,000 *g* for 1 min. The supernatant was discarded, and the cell pellet was suspended in 200 µl of a lysis buffer (2% Triton X-100 (Sigma-Aldrich, USA), 1% sodium dodecyl sulfate (Sigma-Aldrich, USA), 100 mM sodium chloride (Sigma-Aldrich, USA), 10 mM Tris (pH 8.0) (ACROS Organics, USA), and 1 mM ethylenediaminetetraacetic acid (EDTA) (Panreac Quimica, Spain)), to which 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1, (Sigma-Aldrich, USA)) and 0.3 g of acid-washed glass beads (0.45–0.52 mm) was added and gently mixed. The samples were vortexed for 5 min to disrupt the cells and then centrifuged at 13,000–16,000 *g* for 5 min. The aqueous layer of each sample was then transferred to a clean tube, followed by the addition of 400 µl of 95% ethanol and 16 µl of 3M sodium acetate (pH 5.2) (Panreac Quimica, Spain). The samples were mixed through inversion and centrifuged at 13,000–16,000 *g* for 5 min. The pellets were then washed with 300 µl of 70% ethanol, and the samples were centrifuged at 13,000–16,000 *g* for 2 min before the supernatant was discarded. Subsequently, the ethanol solution was aspirated for 30 min to dry the pellets. Finally, genomic DNA from each sample was suspended in 100 µl of a Tris-EDTA buffer (pH 8.0).

Yeast identification. Sequences of the LSU rDNA were determined from the polymerase chain reaction (PCR) products of the genomic DNA extracted from the yeast cells. The LSU rDNA, including the D1/D2 domain, 5.8S rDNA, and internal transcribed spacer (ITS) regions, were amplified using a PCR with the universal primers ITS-1 (5'-TCCGTAGGTGAACCTGCG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett, 1997). PCR proceeded as follows: initial denaturation at 95°C for 5 min followed by repeated denaturation at 95°C for 1 min, annealing at 48–55°C for 30 s, and elongation at 72°C for 1 min 40 s for 35 cycles; the final elongation lasted 5 min. The DNA sequencing of these samples was performed at Tri-I Biotech, Inc. A BLAST search of the nucleotide sequences was conducted through the National Center for Biotechnology Information homepage (http://www.ncbi.nlm.nih.gov). According to the guidelines of Kurtzman and Robnett (1998), yeast strains with 0–3 nucleotide differences among them were identified as conspecific or sister species, and those with > 6 nucleotide substitutions as distinct species.

Analysis of the antagonistic activity. For the antagonistic activity assay, first, 100 µl of the lawn cell culture (approximately 1×10^7 cells) was spread on low-pH (pH 4.7, 5, 5.5, and 5.7) methylene blue (MB) plates (containing 1% yeast extract, 2% peptone, 0.1 M citric acid, and 0.003% MB dye). Two microliters of killer

cell culture (approximately 2×10^7 cells) was then spotted on the same plate. The lawn cells were one killer-toxin-sensitive strain of *S. cerevisiae* and three pathogenic fungi: *A. pullulans*, *T. asahii* and *P. aphidis*. MB is a dye that stains dead cells blue; living cells also absorb the dye, but the active enzymes within them process (reduce) the dye, rendering it colorless. This simple assay can be used for detecting yeast colonies containing dying or dead cells (Kucsera *et al.*, 2000). The MB plates in our study were incubated at 22°C for 4 d until a clear killing zone or colored dead colonies appeared.

Results

Yeast antagonists against *S. cerevisiae* and pathogenic fungi. In our study, 128 yeast strains isolated from samples of different natural sources were screened for antagonistic activity. The data showed that three isolates are antagonistic to the toxin-sensitive strain of *S. cerevisiae* (*T. asahii* JYC122 and JYC2122, and *Pichia kluyveri* JYC2095) (Fig. 1); three isolates are antagonistic to *A. pullulans* JYC1003 (*T. asahii* JYC122 and JYC2122, and *Wickerhamomyces anomalus* JYC2063) (Fig. 2); two isolates are antagonistic to *T. asahii* JYC1040 (*T. asahii* JYC122 and JYC2122) (Fig. 3); and five isolates are antagonistic to *P. aphidis* JYC1050 (*T. asahii* JYC122 and JYC2122, *Ustilago esculenta* JYC2036, *A. pullulans* JYC2095, and *Candida stellimalicola* JYC2120) (Figs. 4 and 5).

From the results, we determined that the killer *T. asahii* strains JYC122 and JYC2122 showed antagonistic activity against *T. asahii* JYC1040 at pH 4.7. These three isolates are different strains of the same species; hence, we also investigated whether *T. asahii* 1040 exhibits antagonistic activity against *T. asahii* JYC122 and JYC2122. Notably, *T. asahii* JYC1040 showed no antagonistic activity under any of the pH conditions we tested (Fig. 6).

The influence of pH value on the antagonistic activity. In nature, yeasts live in fermenting organic material, which is generally acidic. Thus, most yeast

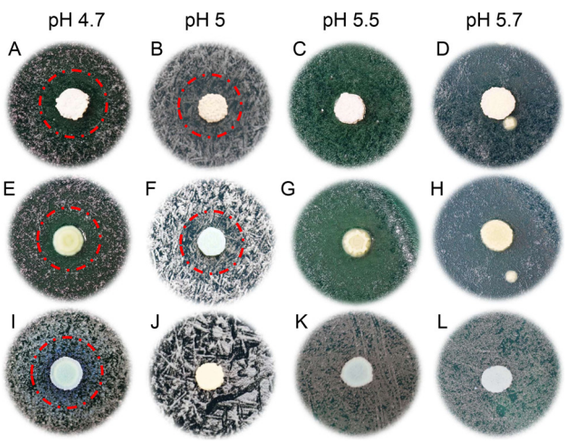

Fig. 1. *T. asahii* JYC122 (A-D) and JYC2122 (E-H), and *P. kluyveri* JYC2095 (I-L) are antagonistic to sensitive strains of *S. cerevisiae*.

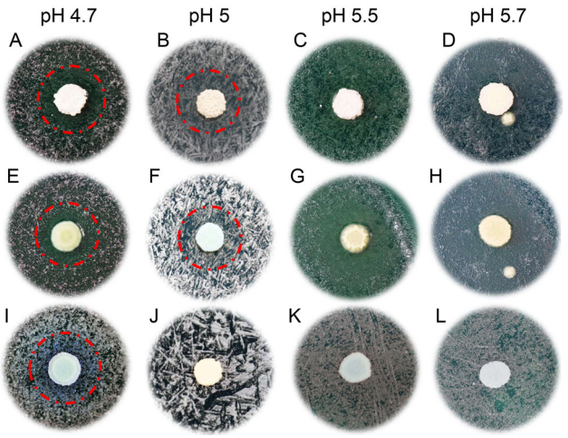

Fig. 2. Three isolates, *T. asahii* JYC122 (A-D) and JYC2122 (E-H), and *W. anomalus* JYC2063 (I-L), are antagonistic to *A. pullulans*.

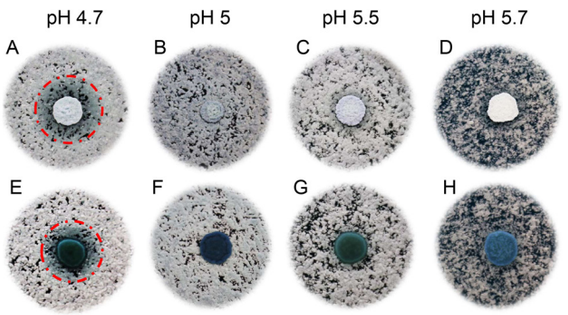

Fig. 3. Two isolates, *T. asahii* JYC122 (A-D) and JYC2122 (E-H), are antagonistic to *T. asahii* JYC1040.

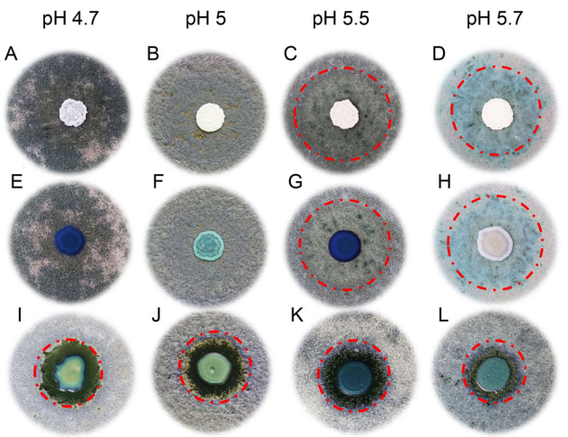

Fig. 4. Three isolates, *T. asahii* JYC122 (A-D) and JYC2122 (E-H), and *U. esculenta* JYC2036 (I-L), are antagonistic to *P. aphidis* JYC1050.

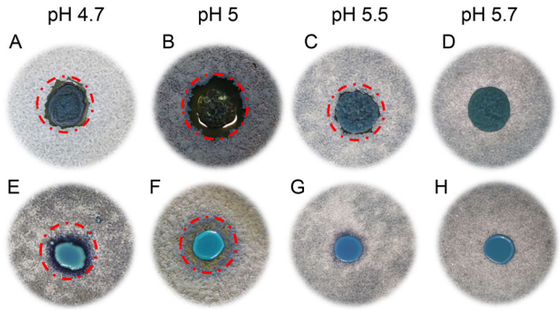

Fig. 5. Two isolates, *A. pullulans* JYC2095 (A-D) and *C. stellimalicola* JYC2120 (E-H), are antagonistic to *P. aphidis* JYC1050

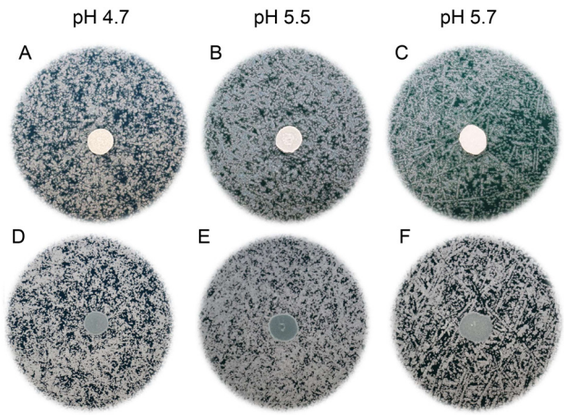

Fig. 6. *T. asahii* JYC1040 shows no antagonistic activity against *T. asahii* JYC122 (A-C) or JYC2122 (D-F) under any of the pH conditions tested.

media are mildly acidic. However, the fermentative activities of yeast alter the pH as the culture grows, which may change the antagonistic activity. Thus, we investigated the effect of pH on antagonism by observing the killing effects on MB plates incubated at 22°C for 4 d.

The inhibition haloes produced by *T. asahii* JYC122 and JYC2122 were clearer at pH 4.7 (Fig. 1A-E) and pH 5 (Fig. 1B-F) than at pH 5.5 (Fig. 1C-G) or pH 5.7 (Fig. 1D-H) when the lawn cells were a sensitive strain of *S. cerevisiae*. Furthermore, when the killer was *P. kluyveri* JYC2095, *S. cerevisiae* was surrounded by a blue precipitated halo – indicative of cellular death at pH 4.7 (Fig. 1I) – but not at pH 5, pH 5.5, or pH 5.7 (Fig. 1J-L). Similar results against *A. pullulans* JYC1003 were obtained from *T. asahii* JYC122 and JYC2122, and *W. anomalus* JYC2063 (Fig. 2). When the killer was *T. asahii* JYC122 or JYC2122, the lawn cells *T. asahii* JYC1040 were surrounded by a halo at pH 4.7 (Fig. 3A-E) but not at pH 5, pH 5.5, or pH 5.7 (Fig. 3B-D, F-H). Finally, when the lawn cells were *P. aphidis* JYC1050, *T. asahii* JYC122 and JYC2122 exerted stronger killer effects at pH 5.5 (Fig. 4C-G) and pH 5.7 (Fig. 4D-H) than at pH 4.7 (Fig. 4A-E) or pH 5 (Fig. 4B-F). *U. esculenta* JYC2036 exerted killer effect under all pH conditions we tested (Fig. 4I-L). *A. pullulans* JYC2095 exerted stronger killer effects at pH 4.7, pH 5, and pH 5.5 than at pH 5.7 (Fig. 5A-D). By contrast, *C. stellimalicola* JYC2120 showed the antagonistic behavior only at pH 4.7 and pH 5 (Fig. 5E-H).

These results indicated that the same lawn cells showed different sensitivities to different killer yeasts at the same pH value. Furthermore, the antagonistic activity of some killer yeasts had a narrow optimal pH range. However, several killer isolates that we found are also opportunistic pathogens (*T. asahii* JYC122 and JYC2122, and *A. pullulans* JYC2065). Despite the considerable biotechnological potential of these isolates, their pathogenicity should not be overlooked when considering them for future applications.

Strain-dependent antagonistic activity. We observed that *A. pullulans* shows antagonistic activity against *P. aphidis*. *A. pullulans* is a yeast-like fungus that can be found in different environments; it is notable for its phenotypic plasticity, and it is adaptable to various stressful conditions (*e.g.* hypersalinity, acidity, alkalinity, cold, and oligotrophy) (Slepecky and Starmer, 2009). Thus, antagonistic activity may be another characteristic of this polyextremotolerant species. Sixty-one *A. pullulans* strains were tested, and three of them (4.9%) were positive for the killer character (JYC1119, JYC1135, JYC1213). These strains were antagonistic to *P. aphidis* JYC1050 (Fig. 7), but not all the *A. pullulans* strains exhibited antagonistic activity against the other species we tested. Notably, *A. pullulans* JYC1135 and JYC1213 exerted stronger killer effects at pH 4.7, pH 5, and pH 5.5 than at pH 5.7 (Fig. 7E-L). However, *A. pullulans* JYC1119 exerted the killer effect only at pH 4.7 and pH 5, not at pH 5.5 or pH 5.7 (Fig. 7A-D). These results indicated the possibility that *A. pullulans* produces a unique antagonistic substance in response to *P. aphidis* but shows variety.

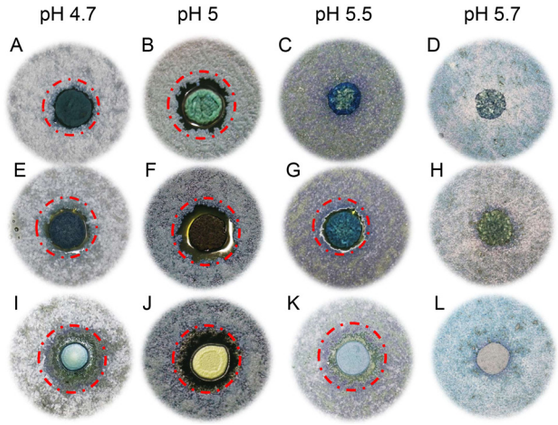

Fig. 7. *A. pullulans* JYC1119 (A-D), JYC1135 (E-H), and JYC1213 (I-L) exhibit antagonistic activity against *P. aphidis* JYC1050.

Discussion

The production of killer toxins is a well-established phenomenon in yeasts. The killer activity is detectable only when a suitable sensitive strain is tested and the killer strain is immune to its own toxin. The effect of a killer toxin depends on both its own potency and the susceptibility of lawn cells under selected conditions. Killer toxins are proteinaceous substances produced by some groups of yeast called "killer yeasts", and these toxins kill sensitive strains of other yeasts. In general, they exhibit maximum activity under conditions of acidic pH (Liu *et al*., 2013).

To evaluate the existence of a killer phenotype in a collection of yeast isolates, we used one toxin-sensitive strain of *S. cerevisiae* and three pathogenic fungi of potential clinical interest. The assays were performed within a narrow pH range likely to determine a killer phenotype, based on a previous study demonstrating that most killer toxins are stable and able to act only at acidic pH values. Kashiwagi *et al*. (1997) found that the SMK toxin secreted by the KK1 strain of the halotolerant yeast *Pichia farinosa* exhibits maximum killer activity under conditions of acidic pH and high salt concentration. The SMK toxin is composed of alpha and beta subunits, which tightly interact with each other under acidic conditions but are easily dissociated under neutral conditions and lose their killer activity. Similarly, the killer toxins of *Kluyveromyces wickerhamii* and *W. anomalus* maintain their killing activities in a pH range compatible with wine-making conditions and peak at pH 4.4 (Comitini *et al*., 2004). However, some killer toxins are stable within a wide pH range. For example, the killer toxins produced by *Hansenula mrakii* are stable at pH 2–11, those by *Hansenula saturnus* at pH 3–11, and those by *Tilletiopsis albenscens* at pH 3.5–8 (Marquina *et al*., 2002).

In this study, *T. asahii* demonstrated broad killing activity against various yeast species. The broad killer phenomenon has been reported for other *Trichosporon* species including *Trichosporon japonicum* (Senter *et al*., 2011) and *Trichosporon porosum* (Kulakovskaya *et al*., 2010) and seems to be a common character in the genus (Golubev, 2006), although the killing phenomenon exhibited by *T. asahii* has yet to be investigated. The members of *Trichosporon* are fungi that commonly inhabit the soil, but several species occur as a natural part of the skin microbiota of humans and other animals (Chagas-Neto *et al*., 2008; Zhang *et al*., 2011). They belong to the basidiomycetous yeasts and have no sexual reproduction phase. Some yeast isolates exerted killer effects against more than one species, suggesting that the produced killer toxins could have a large spectrum of action. Alternatively, these yeasts could produce more than one killer toxin, similar to the killer toxins K1, K2, and K28, produced by *S. cerevisiae* (Schmitt and Breinig, 2002).

In our study, several yeast strains were determined to be antagonistic to pathogenic fungi. Therefore, understanding how the toxins kill these pathogens is crucial, because the molecular mechanisms of this killer action could be helpful in developing strategies for fighting harmful fungi. In killer yeast, toxin secreting strains are frequently infected with double-stranded RNA viruses that are responsible for killer phenotype and toxin secretion in the infected host. Most viral toxins act as ionophores and disrupt cytoplasmic membrane function by forming cation-specific plasma membrane pores (such as K1, K2, and zygocin) that kill non-infected and sensitive yeast cells by disrupting cytoplasmic membrane function. In contrast, the *S. cerevisiae* K28 toxin enters susceptible cells by receptor-mediated endocytosis and act in the nucleus by blocking DNA synthesis and subsequently causing a G1/S cell cycle arrest (Liu *et al*., 2013). Because the ability of yeasts to produce killer toxins is strain-dependent and the pH greatly influences toxin synthesis, it is also crucial for protein engineering must aim for toxin stability in a broader pH range.

In the future, these yeast antagonists can be used in treatments for fungal infections. The isolated antagonists can also be applied in food industries to reduce contamination by undesirable yeasts or other fungi.

Conclusion

The spectrum of action and the activity of yeast killer toxins are affected by temperature, salinity and pH of media. In the present work we determined the antagonistic activity against one toxin-sensitive strain of *S. cerevisiae* and three pathogenic fungi of yeasts isolated from various niches including wine, flowers, insects and fruits *et al*. In this study, we found that some strains with antagonistic activity against these pathogenic fungi can be found in antagonist culture tests. The assays were performed in a narrow range of pH, and this parameter shows a strong influence in the determination of killer phenotype. We further showed that the antagonistic activity of some species is strain-dependent.

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Conflict of Interests

The authors have not declared any conflict of interests.

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SHORT COMMUNICATION

Use of Amplification Fragment Length Polymorphism to Genotype *Pseudomonas stutzeri* Strains Following Exposure to Ultraviolet Light A

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Abstract

Changes in ultraviolet light radiation can act as a selective force on the genetic and physiological traits of a microbial community. Two strains of the common soil bacterium *Pseudomonas stutzeri*, isolated from aquifer cores and from human spinal fluid were exposed to ultraviolet light. Amplification length polymorphism analysis (AFLP) was used to genotype this bacterial species and evaluate the effect of UVA-exposure on genomic DNA extracted from 18 survival colonies of the two strains compared to unexposed controls. AFLP showed a high discriminatory power, confirming the existence of different genotypes within the species and presence of DNA polymorphisms in UVA-exposed colonies.

Key words: *Pseudomonas stutzeri*, AFLP, polymorphisms, UVA exposure

Microorganisms play a pivotal role in regulating fundamental biogeochemical processes in terrestrial and marine systems. Changes in ultraviolet light (UV) radiation, soil warming and desiccation may be strong selective forces acting on the phylogenetic and physiological composition of microorganisms. We chose to focus on *Pseudomonas stutzeri* since it is a non-fluorescent denitrifying bacterium with a wide distributional range and it occurs as an opportunistic pathogen in humans (Lalucat *et al.*, 2006). *P. stutzeri* is a Gram-negative bacterium known for its high propensity nitrogen fixation and opportunistic pathogenicity. In addition, *P. stutzeri* strains have also been described to exert xenobiotic degradation capacity, mainly naphthalene and other similar aromatic compounds (Brunet-Galmés *et al.*, 2012). In fact, several strains of this species are involved in nitrogen fixation, whilst others participate in the degradation of pollutants or interact with toxic metals, processes that could be influenced by environmental factors (Lalucat *et al.*, 2006). This species was also recently isolated from the oral cavity of asymptomatic health care workers, suggesting the possibility that *P. stutzeri* may behave as a commensal strain occasionally tuning pathogenic in immunocompromised patients (Lima *et al.*, 2015).

In this study, two *P. stutzeri* strains with different origins and optimal growth temperatures were used to assess bacterial viability following exposure to UV radiation.

In addition, we evaluated the applicability of amplification length polymorphism analysis (AFLP) as a tool for genotyping *P. stutzeri* strains.

P. stutzeri DSM 7136, isolated from aquifer cores and with an optimum growth temperature of 30°C, and type strain DSM 5190, isolated from human spinal fluid, with an optimum growth temperature of 37°C were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany).

Bacterial cultures were grown in Nutrient Broth (Liofilchem S.r.L., Teramo, Italy) and incubated with shaking at their optimal temperatures until the exponential growth phase was reached ($O.D._{\lambda 600\ nm}$ 0.7). Strains were maintained on Nutrient Agar at 4°C (Liofilchem S.r.L., Teramo, Italy) for the duration of the study.

The effect of ultraviolet light B (UVB) (280–315 nm) and ultraviolet light A (UVA) (315–400 nm) radiation was evaluated by *P. stutzeri* survival following different exposure lengths and periods. The UV-light-generating lamps consisted of two fluorescent narrowband tubes emitting in the 340 nm band (UV-A TLD 15 W,

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Philips, Milano, Italy) or in the 280–315 nm range (UVB TL 20W/01 RS SLV, Philips, Milano, Italy), respectively. We calibrated the UV lamps measuring their potency at a distance of 30 cm from the biological sample (UVA = 11.6 W/m^2; UVB = 5.26 W/m^2) by a radiometric-calibrated detection system consisting of an InstaSpec IV CCD detector head coupled to an MS125 spectrograph (Oriel Instruments, Milano, Italy). Experiments were set up by plating aliquots (100 µl) of bacterial suspensions obtained from cultures of both *P. stutzeri* strains in exponential growth phase, adjusted to a concentration of 2.7×10^8 cell/ml and serially diluted (10^{-1} to 10^{-5}). Plates were immediately exposed for 15, 30, 60 and 90 minutes to UVB light, using an UVB lamp (5.65 W/m^2) positioned at 10 cm distance from the plates. Non-exposed replica plates represented negative control. Following an 18 h incubation at 30°C and 37°C for *P. stutzeri* DSM 7136 and DSM 5190 respectively, CFU counts revealed the expected CFU numbers in the unexposed plates, but no colonies were detectable on the UVB exposed plates, at any of the exposed times examined (data not shown). Previous data published on several Gram-negative bacteria showed a decreasing survival rate following irradiation with UVB and ultraviolet light C (UVC), with the higher survival rates observed post-exposure to UVA (Santos *et al.*, 2013).

Experiments were then repeated using a UVA lamp (11.6 W/m^2), positioned at 10 cm distance from the samples, with the aim to determine the appropriate exposure period resulting in approximately 90–95% killing of bacterial cells (Fig. 1). Plates were incubated overnight at the appropriate temperatures. Colony forming units (CFU) were then counted on each plate.

Surviving bacteria (4–16%) were recovered following a 22-minute exposure, with the environmental strain showing the highest UVA susceptibility ($P < 0.0001$ following unpaired *t*-test, Fig. 1). Further analysis encompassing a wider set of clinical and environmental strains will be necessary to confirm whether environmental isolates are more susceptible to UVA selective pressure.

Nine single colonies and one non-exposed colony (control) per strain were used for genomic DNA extraction and amplification fragment length polymorphism (AFLP) analysis. Genomic DNA was extracted from 5 ml late exponential-phase cultures of *P. stutzeri* as described by Grüntzig *et al.* (2001), with minor modifications. Genomic DNA was extracted from lysed cells following three phenol chloroform-isoamyl alcohol treatments. An equal volume of the chloroform-isoamyl alcohol mixture was added to the recovered aqueous layer (24:1, vol/vol). Nucleic acids were then precipitated with 1 ml isopropanol and 0.3 M ammonium acetate. Following a 30 min incubation at room temperature, precipitated DNA was recovered by centrifugation. The DNA pellet was washed once with 70% ethanol and suspended in 50 µl of TE, pH 8.

The presence of genomic DNA modifications in *P. stutzeri* strains following UVA exposure was evaluated by AFLP analysis. This technique is a high-resolution genotyping method that allows evaluation of strains relatedness as well as strain replacement/maintenance and the extent of micro-evolutionary events (Tavanti *et al.*, 2007; Xiang *et al.*, 2010; Vos *et al.*, 1995). However, to the best of our knowledge, this molecular method has not yet been applied to characterize *P. stutzeri* strains. Preliminary *in silico* analysis of AFLP applied to the genome sequence of *P. stutzeri*

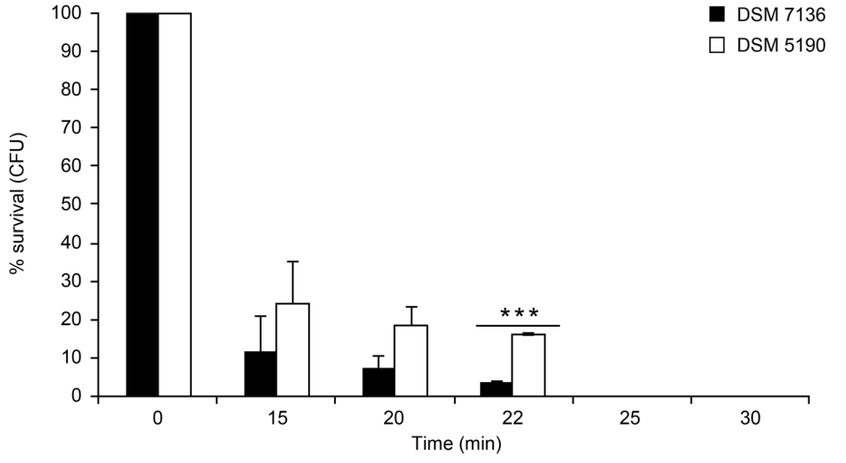

Fig 1. *P. stutzeri* survival following different exposure times to a UVA lamp (11.6 W/m^2), positioned at 10 cm distance from Petri dishes. Two different strains of *P. stutzeri* DSM 5190 (isolated from human spinal fluid) and DSM 7136 (isolated from aquifer cores) were tested. Data are expressed as mean ± standard error of mean of 4 independent experiments. ***$P < 0.0001$.

A1501 assisted us in the selection of the appropriate set of restriction enzymes, adaptors and primers available. Among those, HindIII-0 (5'-GACTGCGTAC CAGCTT-3') and Cy5-labelled EcoRI-0 (5'-GACT GCGTACCAATTC-3') primers were chosen to perform AFLP. Genotyping by AFLP was performed on 60 ng of genomic DNA as previously described (Tavanti *et al.*, 2007). The restriction enzyme combination EcoRI/HindIII was used in the first restriction/ligation step, with a HindIII (5'-AGCTGGTACGCAGTC-3') and EcoRI (5'-CTCGTAGACTGCGTACC-3') adaptor concentration of 0.45 μM. Pre-selective, selective amplifications and gel electrophoresis conditions were performed as previously described, using Eco0/HindIII0 primer combination (Hensgens *et al.*, 2009). AFLP profiles, ranging from 100 to 900 bases, were exported as a TIFF file and analyzed with the TotalLab TL120 software package (Nonlinear Dynamics Ltd, UK). DNA bands obtained for each isolate were size-matched. Each AFLP fragment was analysed and labelled by time and by the surface of the fluorescent peak it formed. Background fluorescence was subtracted from each lane and the surface of each peak was determined to better quantify each AFLP fragment. The sum of all peak surfaces for each AFLP profile was defined as "lane volume" and set as 100% and peak surfaces were expressed as a percentage of the "lane volume". Only those fragments, which were at least represented as 0.5% of the lane volume, in at least one of the isolates, were included in the analysis (Hensgens *et al.*, 2009). Consequently, bands with a relative intensity of less than 0.03% of the lane volume were not included in the analysis.

Computerised analysis of the profiles obtained clearly indicated that the two isolates have completely different AFLP-genotypes, demonstrating that this technique can be successfully applied to molecular typing of this bacterial species. This finding is in agreement with previous data obtained using different methods, demonstrating the existence of several genomovars within the *P. stutzeri* species (Sikorski *et al.*, 1999; Sikorski *et al.*, 2005). In particular, strain *P. stutzeri* DSM 5190 is described as belonging to genomovar 1, while *P. stutzeri* DSM 7136 was assigned to genomovar 9 (Sepúlveda-Torres *et al.*, 2001). Indeed, the two isolates shared only few bands (n = 8) out of the approximately 100 fragments obtained for each strain (Fig. 2). Such a low number of fragments shared by the two *P. stutzeri* patterns could be statistically expected for unrelated species (Krauss,

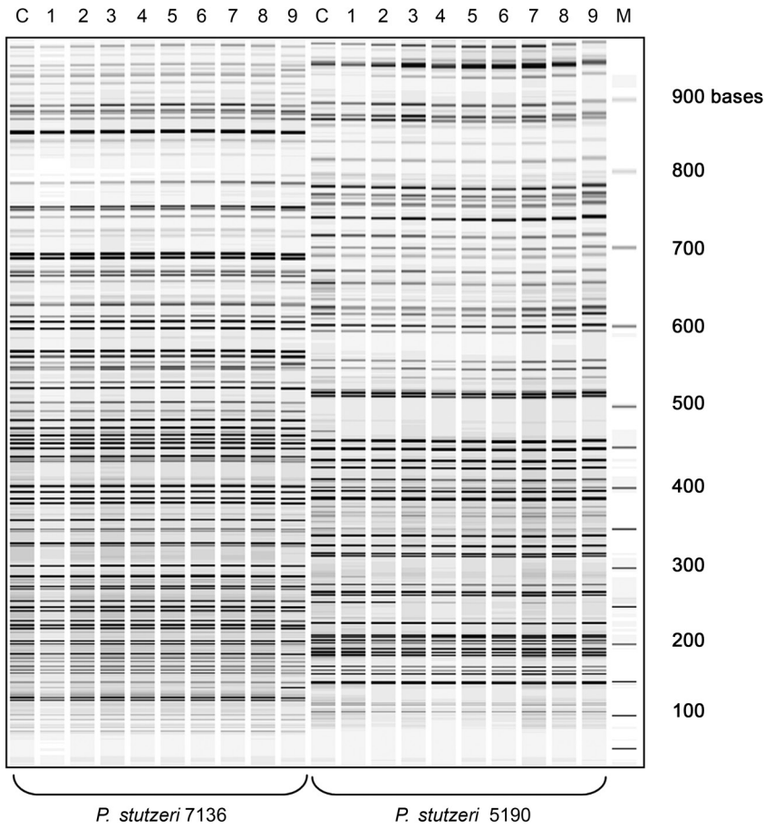

Fig 2. AFLP profiles of *P. stutzeri* DSM 7136 and DSM 5190 strains.
Lanes 1–9, patterns obtained from UVA-exposed independent colonies; Lane C, unexposed control colonies.
M – molecular weight marker.

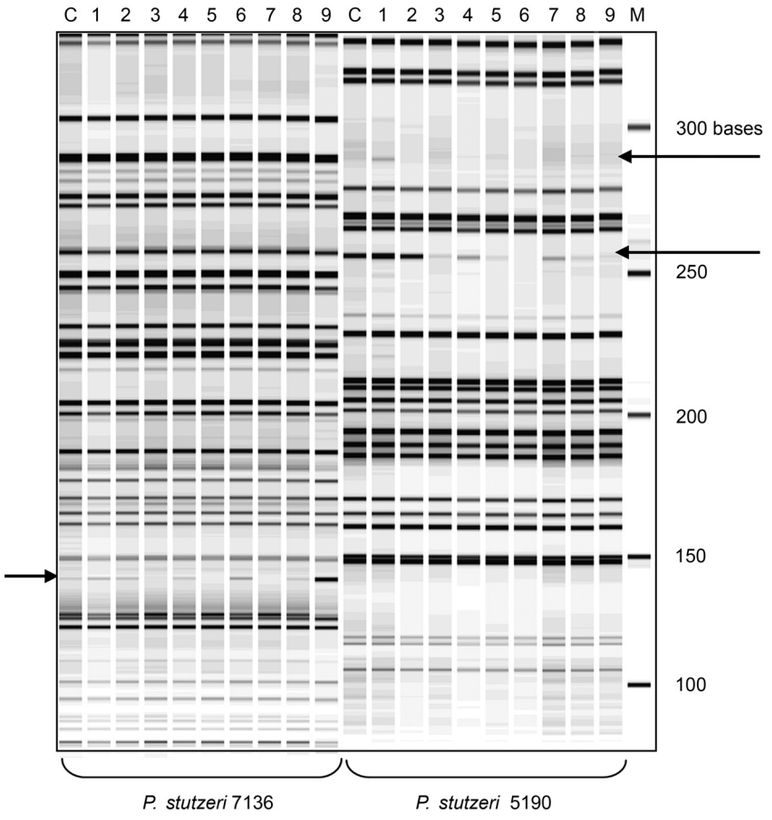

Fig 3. AFLP profiles within the range of 100–300 bases, showing minor changes (indicated by black arrows) in DNA patterns obtained from independent UVA-exposed (lanes 1–9) and unexposed control colonies (lane C) for both *P. stutzeri* isolates. M – molecular weight marker.

2000). Therefore, AFLP profiles indicates a high degree of genetic diversity, which could be in agreement with the definition of "genomovar", a term coined to describe different genomic species lacking of discriminative phenotypes (Lalucat *et al.*, 2006). However, more strains belonging to different genomovars should be included in the analysis to confirm this hypothesis.

As expected, AFLP profiles were highly similar in all selected UVA-exposed colonies and control unexposed colony within each strain. However, for both strains minor differences could be observed in the AFLP profiles following UVA exposure (Fig. 3). In addition, the two different *P. stutzeri* isolates showed different polymorphic sites in their respective AFLP profiles, which were not shared by all colonies analysed per strain. This could confirm the genetic diversity of the two isolates used in this study, although we cannot exclude the possibility that these polymorphisms may have also arisen naturally by culturing the bacteria over time and in the absence of UV-induced stress.

The results presented here confirm that AFLP is a robust typing technique, which can be successfully applied for the molecular characterization of *P. stutzeri* strains. In fact, different genomovars gave distinctive AFLP patterns. In addition, AFLP profiles obtained from UVA-exposed colonies of both *P. stutzeri* strains showed the presence of polymorphisms, which could be related to UVA exposure. However, due to the limited number of colonies screened it is not possible to ascertain the origin of these polymorphisms.

Given the involvement of both of UVA and UVB in bacterial inactivation, it will be very interesting to determine the nature of the observed polymorphisms. Future studies will be performed to investigate further the molecular mechanisms by which polymorphisms in this species arise, in order to better understand the effect of natural light on bacterial populations.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AT and LL designed the study. RS and CR processed the data, MZ and CGE completed data analysis. AT wrote the manuscript. All authors read and approved the final manuscript.

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SHORT COMMUNICATION

Vital Staining of Bacteria by Sunset Yellow Pigment

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Abstract

In this study, we describe a method for discriminating pathogenic bacteria with a dye. First, we determined that among several colours tested, the sunset yellow pigment easily coloured *Escherichia coli* bacteria yellow. Next, we demonstrated that *E. coli* O157:H7, *Shigella flexneri* O301, *Staphylococcus aureus* and *Bacillus subtilis* could all be well marked by sunset yellow pigment. Finally, we performed bacterial viability assays and found there was no effect on bacterial growth when in co-culture with sunset yellow. Our results suggest that sunset yellow is suitable pigment to dye microorganisms.

Key words: bio-safety, pathogenic bacteria detection, sunset yellow, vital staining

In the global effort to cure many human diseases, a large number of pathogenic bacteria is cultured for different types of experiments (Jünger *et al*., 2012). However, numerous accidents have been reported when laboratories mishandled deadly germs (Cohen *et al*., 2004; Tucker, 2003). Moreover, leaks of contaminated waste, spilled bacterial or viral cultures, and defective airtight seals remain common occurrences in many laboratory settings. Accordingly, to improve the level of biological safety, an indicator that can detect spilled pathogenic bacteria is needed. There are numerous methods to stain bacteria in order to characterize and discriminate between them (Salleh *et al*., 2012); however, most dyes have been found to be toxic and inhibit bacterial growth (Salleh *et al*., 2011). To date, only few studies have investigated non-poisonous methods of dyeing cells in culture (Pérez-Díaz and McFeeters, 2009), therefore, a more effective staining method is needed.

Here, we present a convenient and safe method of microbial staining with the sunset yellow food dye. Using this food dye, we were able to trace the source of the leak or pathogenic microorganism without affecting bacterial growth, which we believe is necessary to allow for further characterization.

First, we investigated the ability of several pigments, such as coccinellin, erythrosine and FD&C Yellow No. 6 (sunset yellow, $C_{16}H_{10}N_2Na_2O_7S_2$), to dye bacteria, and found the pigment sunset yellow to be the most potent dye. Furthermore, previous studies have shown that sunset yellow is a synthetic dye and a small molecule that can easily dissolve in water. Accordingly, these characteristics allow sunset yellow to affix to bacteria when the dye is added to the nutrient medium. The ability of sunset yellow to colour bacteria offers a new way to indicate the trail of microorganisms, allowing researchers to physically monitor their spread. Accordingly, based on these characteristics, we believe this dye may become a good candidate for colouring bacteria and monitoring their spread.

Escherichia coli strain DH5α, *E. coli* O157:H7, *Shigella flexneri* O301, *Staphylococcus aureus* and *Bacillus subtilis* were used in this study. *E. coli* was grown in Luria Bertani (LB) medium aerobically at 37°C, while other strains were grown in LB medium in both liquid broth and on agar plates. We utilized coccinellin, erythrosine and FD&C Yellow No. 6 (sunset yellow, $C_{16}H_{10}N_2Na_2O_7S_2$) dyes in this study. A coloured LB culture media with the addition of sunset yellow was used

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to culture *E. coli* strain DH5α, thereby colouring the bacteria (De Mey *et al*., 2008). The pigment doses used in all experiments ranged from 0.05 g/ml to 5 g/ml. All cultures were put into 5 ml eppendorf tubes and centrifuged at 10.000 × *g* for 5 min to allow for bacterial collection (Ngwa *et al*., 2013). Sunset yellow plating medium was used to grow *E. coli* at 37°C for 24–48 h. To test the ability of sunset yellow to dye pathogenic bacteria, *E. coli* O157:H7 and *S. flexneri* O301 were cultured in media containing sunset yellow. Again, different dilutions of sunset yellow pigment were used to culture *S. aureus* and *B. subtilis*.

Colony count assays were performed to determine the effect of sunset yellow on bacterial viability. *E. coli* DH5α cells were serially diluted (10^{-5}, 10^{-6} or 10^{-7}). Subsequently, 50 µl of diluted *E. coli* DH5α cells were added to 5 ml of a 43°C solution of 1% agarose in 100 mM NaCl, 10 mM sodium phosphate and 1% trypticase soy broth. Next, the entire 5-ml sample was poured over a conventional trypticase soy agar underlay. After incubating overnight, the colonies in the overlay were counted (Ferreira *et al*., 2010). Using the results of the plate counts, a growth curve was created to determine the incubation time and amount of *E. coli* required for the coloured culture.

Alternatively, turbidity readings of a 10-hour *E. coli* DH5α culture were taken every hour and PD was measured at a wavelength of 600 nm (Choi *et al*., 2010). Different inoculum sizes were set to 1% and 0.1% of the bulk volume. A growth curve was plotted using the results of the changes in OD values during the 10-hour growth. All the data were statistically evaluated with SPSS/13.0 software (SPSS Inc., Chicago, IL). Values of $P < 0.05$ were considered statistically significant. All the results are expressed as the mean ± standard deviation (SD).

During the *E. coli* culture, we could easily observe the turbidity of the media in each tube after 5 hours. We noted that sunset yellow was easily able to colour the bacteria yellow (Fig. 1A). The collected precipitate showed the coloured culture and the pigment successfully turned the bacteria yellow (Fig. 1B). More importantly, if some pathogenic bacteria had spilled out during the process, we would have been able to find them and promptly decontaminate the area. After culturing with the dye, we were able to observe the shape, size and traits of the bacteria (Fig. 1C).

When the sunset yellow plating medium was used, the *E. coli* bacterial colonies were coloured after growth at 37°C for 24–48 h. These methods were similar to those used by Zimmermann *et al*. (1978). Those colonies were adhered on the surface of the filter paper and were coloured yellow when compared with the control group colonies, which possessed sunset yellow staining that was not well adhered on the surface of the bacterial cells. More importantly, it could colour these cells. Thus, the prediction regarding the sunset yellow colouring mechanism proved to be correct. In Fig. 1D, tubes 1 and 3 are the cultures of O157:H7 and *S. flexneri* O301, respectively, in LB culture medium. It was apparent that O157:H7 in tube 2 and *S. flexneri* O301 in tube 4 were well labelled. The same phenomenon appeared in the groups of *S. aureus* and *B. subtilis* in Fig. 1E and 1F, respectively. In this way, the spillage of pathogenic bacteria could easily be detected. The pathogenic bacterial colonies of *S. aureus* and *S. flexneri* O301 in Fig. 1G show the clearly coloured cells. Compared with the uncoloured bacterial cells in Fig. 1A and B, *S. flexneri* O301 germinated and was marked well, as shown in images (Fig. 1C and D). The same results were found for *E. coli* O157:H7, as shown in images (Fig. 1E and F). Pathogenic bacteria are only a small portion of all bacterial families; however, the vital staining method is universal.

We performed colony counting and determined that sunset yellow dye did not inhibit bacterial growth. *E. coli* was cultured for 12–16 hours in coloured LB before being spread onto solid LB. Sunset yellow was shown to be an ideal dye that had little influence on bacterial growth (Fig. 2A). *E. coli* strain DH5α was cultured overnight and then diluted (10^{-5}, 10^{-6}, 10^{-7} or 10^{-8}). One hundred microliters of the diluted sample was then plated on solid LB with and without sunset yellow. These solid LB cultures were placed into a 37°C incubator and cultured overnight (Adler *et al*., 2011). Bacteria cultured in these coloured media were then sub-cultured in media without dye. Next, we chose the 5^{th} culture generation and applied the same methods of examination. As shown in Fig. 2B, those bacteria grew well and exhibited little difference compared with the control groups. Fig. 2C and D showed that at different bacterial loads (1% or 0.1%), the bacteria could grow in both normal LB and media containing sunset yellow LB. The *E. coli* cultured with sunset yellow added to the LB appeared to grow slightly slower than *E. coli* in normal LB between 1 and 4 hours. The growth was identical at 4 to 10 hours (Conrad *et al*., 2010; Cox *et al*., 2000). These results showed that there was no difference in bacterial growth and we concluded that sunset yellow pigment had no effect on bacterial growth.

Previously, rats were given FD&C Yellow No. 6 by gavage at 0, 60, 100, 200, 400, 600 or 1000 mg/kg body weight/day on days 0–19 of gestation. At the doses given, FD&C Yellow No. 6 was neither toxic nor teratogenic (Poul *et al*., 2009; Sasaki *et al*., 2002). The stability of this dye had been shown by Nevado *et al*. (1998). This method of staining with sunset yellow was a comprehensive, effective, stable, straightforward and safe way to stain some types of microorganisms. Although several studies have provided unequivocal evidence that

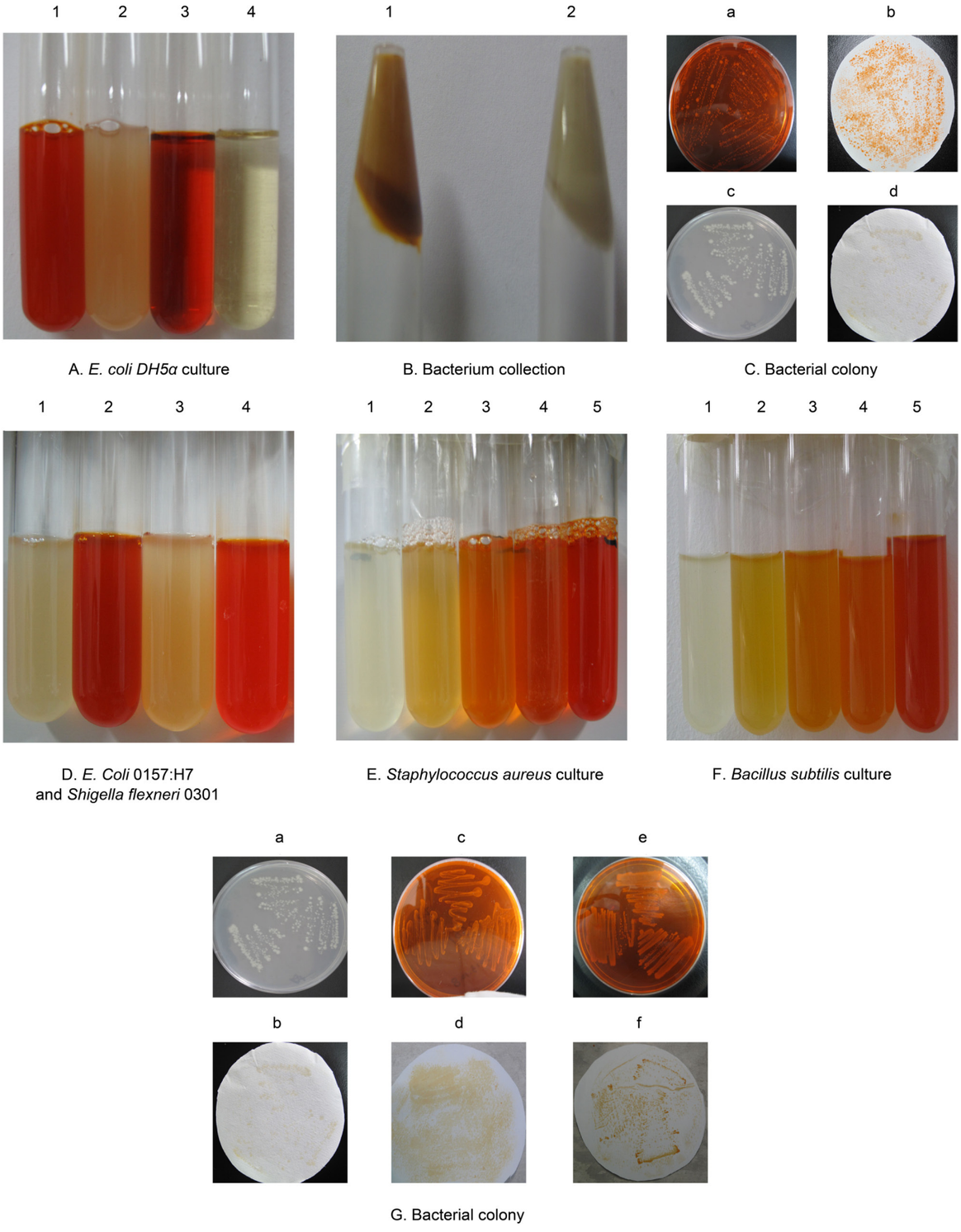

Fig. 1. The direct observation of *E. coli* DH5α, *E. coli* O157:H7, and *S. flexneri* O301 that had been stained with sunset yellow.

(A) The LB that was used to culture DH5α contained the sunset yellow dye (tube 1) or without pigment (tube 2). And tubes 3 and 4 contained sterile test media. (B) The precipitates were collected from 80 ml of culture. Tube 1 contained the precipitate collected from the sunset yellow LB, and tube 2 contained the precipitate collected from the normal LB. (C) The *E. coli* colonies grown in normal LB plating medium are shown in c and d, and those grown in sunset yellow plating medium are shown in a and b. (D) Sunset yellow LB was used to culture *E. coli* O157:H7 and *S. flexneri* O301. Different densities of sunset yellow LB were used to cultivate *S. aureus* (E) and *B. subtilis* (F). (G) The pathogenic bacterial colonies in d and f were *S. aureus* and *S. flexneri* O301, respectively, while the control group in Figure a was *S. flexneri* O301.

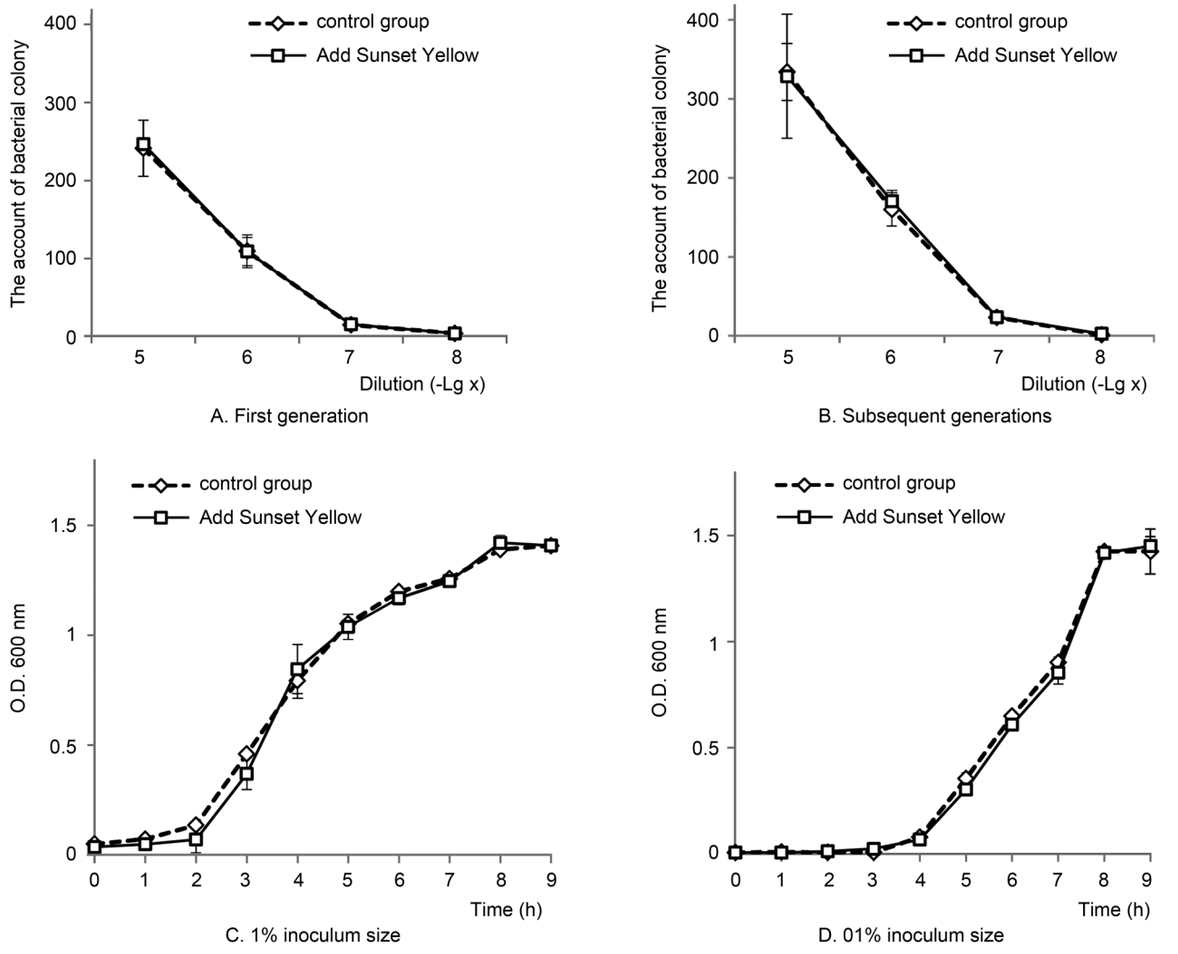

Fig. 2. Effects on the growth of *E. coli*.

(A) The counts of bacterial colonies at different dilutions after their first generation. These 4 dilutions demonstrate that there was no influence on *E. coli* DH5α on the number of colonies formed from generation to generation using new LB from beginning to end. These 4 dilutions show the growth rates of the experimental and control groups were nearly the same. (C) The one-step growth curve of *E. coli* DH5α. (D) The one-step growth curve of re-beginning to *E. coli* DH5α (0.1% inoculum size). From these results, we determined that there was no effect on the growth of *E. coli*.

bacteria isolated from humans, such as *E. coli*, *Shigella* sp., *S. aureus* and *Bacillus* sp., have the ability to cleave the azo linkage of sunset yellow to produce aromatic amines, which induce urinary bladder cancer, the most commonly used Sunset Yellow may not produce such adverse cytotoxic, mutagenic, or carcinogenic effects (Chung *et al.*, 2008).

In summary, in this study, we described a novel method of using a coloured dye to stain several types of pathogenic bacteria in culture. Importantly, the culture media, with added sunset yellow pigment, allowed for bacterial growth while simultaneously colouring all progeny. Therefore, we propose that this new coloured-LB culture system might provide a new method for labelling bacteria, especially pathogenic bacteria. The hope of this research is that after treatment with the sunset yellow pigment, any spillage or leak of pathogenic bacteria from culture will become visible and will be easy to monitor. The use of the sunset yellow dye as an indicator of bacterial distribution is both time- and cost-effective. Moreover, this method can be used for a much broader application in the dying of other types of microbes.

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SHORT COMMUNICATION

Outpatient Antibiotic Consumption Fluctuations in a View of Unreasonable Antibacterial Therapy

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Abstract

Unreasonable antibacterial therapy is suspected to be the main reason of emergence of multi-resistant bacteria. The connection between seasonal variability of antibiotic use and reasonable antibacterial therapy has been described. We examined the issue basing on the data obtained from the primary care system in Szczecin (Poland) in order to verify the situation in this region of Central Europe. Increase in antibiotic consumption in a viral infection season was proved to be statistically significant. Statistically significant differences in various drug forms dispensation were also observed. Increased consumption of antibiotics in seasons of influenza-like illnesses might be connected with a lack of proper diagnostics or numerous cases of bacterial co-infections.

Key words: antibacterial agents, drug resistance, drug usage fluctuations, outpatient infection treatment, unreasonable antibacterial therapy

The attitude to antibacterial therapy needs to be fundamentally changed. After the period of indisputable efficient outcomes of treatment (Aminov, 2010) and intensive search for new substances with a wide spectrum activity and better pharmacological characteristics, a crisis in antibiotic therapy has emerged (Davies, 2014). This situation results from the underestimated plasticity of bacterial genomes and ability to accumulate features, which allow bacteria to endure environment which is hostile and rich in antibacterial substances. A vital example of this kind of environment is an organism of a person treated with antibiotics (Ambur *et al.*, 2009). Increasing frequency of multi-drug resistance hinders the ability to treat patients in life-threatening conditions. Unreasonable antibacterial therapy is suspected to be the main reason of the problem.

The most common causes of the community acquired respiratory infections in children over 3 years old and adults are viruses (respectively up to 85% and 95%): respiratory syncytial virus (RSV), influenza and parainfluenza viruses, adenoviruses and even rhinoviruses (Korppi *et al.*, 2004; Macfarlane *et al.*, 2001). According to Jennings *et al.* (2008), bacterial pathogens isolated from adults were *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Legionella pneumophila* and *Staphylococcus aureus*.

Large national, European and worldwide programs have been started in order to improve the situation (WHO, 2014a; National Institute of Allergy and Infectious Diseases, 2014; National Medicines Institute, 2014; ECDC, 2014). In the presented study the relevance between the number of doses of different antibiotic types prescribed for patients and autumn-winter season with the increased number of viral infections was examined.

Data on the number of systemic-use antibiotics prescribed between July 2009 and June 2014 was obtained from a leading twenty-four-hour pharmacy located in the city center of Szczecin, Poland, and was grouped according to the drug's active substance. Due to its location and local conditions, for most of the analyzed period, the pharmacy, aside from its regular activity, was the only one to serve patients at night and on public holidays. Data obtained from an internal pharmacy system have been converted into the consumed DDDs (defined daily doses) according to WHO guidelines and ATC/DDD Index 2014 (WHO, 2014b) and divided according to a month of prescription. Months from October to March were classified as viral infections "season", which correlates with autumn/winter period in the Polish climate zone. Months from April to September were classified as "out of season", which

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is characterized by a significant decrease in viral infections in Europe (Bollaerts *et al.*, 2013). Data has also been divided according to antibiotic classes and drug form type. Afterwards, data was converted into DDDs per 1000 inhabitants. As a part of those calculations, a number of all prescriptions in the analyzed pharmacy was used, as well as a number of all prescriptions in all pharmacies in Szczecin over the respective periods of time. Data on prescriptions dispensed in the entire city has been obtained from the records of the Polish National Health Fund (NFZ). The population of Szczecin, according to the Central Statistical Office Report (2013) (Central Statistical Office, 2013) was calculated as 408 502 people. In order to determine the age structure of patients for whom antibiotics in 2012/2013 season and 2013 out-of-season periods were prescribed, prescriptions were divided by patients' age. Differences between numbers of DDDs consumed in season and out of season, noticed in the analyzed data, were statistically tested using Statsoft STATISTICA 10 software. Statistical tests were chosen according to Statsoft guidelines based on Cobb (1998) publication, depending on results' distribution in the analyzed groups: *t*-test or Mann-Whitney *U* test.

Conducted research comprised information on prescriptions for 42 997 drug packages with antibiotics for systemic use, which relates to 369 909 DDDs of active substances, dispensed in a four-year period. Averages of DDDs consumption per 1000 inhabitants in different seasons and out of season period are shown in Table I. The number of prescribed penicillins, cephalosporins and macrolides (which are the most frequently prescribed antibiotic classes) in season always surpassed the quantity in "out of season" periods. As far as lincosamides are concerned, the trend was exactly opposite. For tetracyclines, quinolones, and sulphonamides the results are divergent in different years. As far as different drug forms were analyzed, the increase in season was noticed in both tablets and suspensions.

Overall fluctuations in the number of the prescribed antibiotics are shown in Fig. 1.

Fluctuations were significantly different for various classes of antibiotics, as shown in Table I. The largest differences were noticed for cephalosporins (*e.g.* 60.71 DDDs/1000 inhabitants in season 2012/2013 in comparison to 26.96 DDDs/1000 inhabitants in out-of-season 2012), macrolides (*e.g.* 79.69 DDDs/1000 inhabitants in season 2012/2013 in comparison to 36.27 DDDs/1000 inhabitants in out-of-season 2012), penicillins (*e.g.* 145.90 DDDs/1000 inhabitants in season 2009/2010 in comparison to 74.09 DDDs/1000 inhabitants in out-of-season 2009), sulphonamides (*e.g.* 11.17 DDDs/1000 inhabitants in season 2012/2013 in comparison to 5.59 DDDs/1000 inhabitants in out-of-season 2012) and tetracyclines (*e.g.* 40.52 DDDs/1000 inhabitants in season 2012/2013 in comparison to 29.23 DDDs/1000 inhabitants in out-of-season 2012).

The scale of the observed differences depended also on the type of a drug form. The term "Tablets" also refers to capsules. The term "Suspensions" also refers to syrups and solid dry forms intended for dissolution in water and oral administration as a suspension. Taking into account pharmaceutical practice, suspensions are predominantly prescribed in treatment of children. Larger differences were observed for suspensions (*e.g.* 34.74 DDDs/1000 inhabitants in season 2009/2010 in comparison to 11.95 DDDs/1000 inhabitants in out-of-season 2009) than for much more often prescribed tablets (*e.g.* 384.06 DDDs/1000 inhabitants in season 2012/2013 in comparison to 220.63 DDDs/1000 inhabitants in out-of-season 2012). Statistical analysis results are shown in Table II.

For the age groups 0–10, 21–30 and over 70 years old there were prescribed the highest number of anti-

Table I
Averages of systemic-use antibiotic consumption (DDDs per 1000 inhabitants) in viral infections seasons (Oct-Mar) and out of seasons (Apr-Nov).

DDDs per 1000 inhabitants	out of season 2014	season 2013/ 2014	out of season 2013	season 2012/ 2013	out of season 2012	season 2011/ 2012	out of season 2011	season 2010/ 2011	out of season 2010	season 2009/ 2010	out of season 2009
TOTAL	370.08	428.24	329.82	443.72	245.30	263.40	228.54	361.69	282.62	316.67	186.46
Penicillins	164.57	189.71	147.95	197.75	110.14	117.95	98.39	173.37	131.43	145.90	74.09
Cephalosporins	48.60	59.08	39.12	60.71	26.96	30.72	23.13	35.73	26.47	28.27	12.75
Macrolides	45.97	65.00	42.55	79.69	36.27	44.25	33.62	60.90	45.15	53.07	31.15
Lincosamides	23.82	20.89	20.96	19.60	14.18	13.59	15.36	14.12	12.79	11.38	9.11
Tetracyclines	48.15	47.61	39.79	40.52	29.23	31.12	33.07	46.85	39.18	42.73	31.65
Quinolones	23.84	30.38	27.02	29.60	20.29	15.96	14.07	16.08	18.11	21.16	17.53
Sulphonamides	9.66	8.59	7.60	11.17	5.59	6.86	6.02	10.68	6.09	9.70	6.43
Suspensions	37.09	47.15	31.62	59.47	24.48	27.59	19.55	63.72	24.27	34.74	11.95
Tablets	332.46	380.85	297.97	384.06	220.63	235.64	208.61	297.57	258.05	281.56	174.04

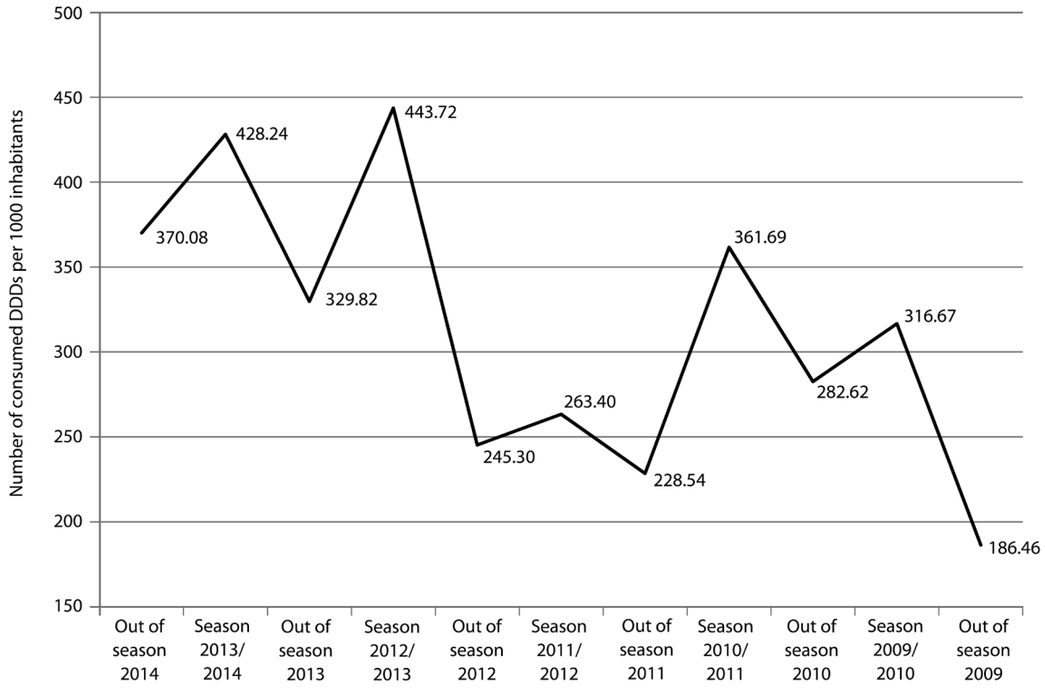

Fig. 1. Overall fluctuations in the number of the prescribed antibiotics (DDD per 1000 inhabitants).

biotics, based on the number of prescriptions in season 2012/2013 and out of season period in 2013. The largest increase in the number of prescriptions in the abovementioned season in comparison with out-of-season period was observed in age groups 41–60 and 71 and older.

According to the data collected by the European Centre for Disease Prevention and Control (ECDC, 2013a) consumption of antibacterial agents for systemic use (primary care sector, no hospital use) as well as antibiotic distribution systems vary significantly in European countries. In Scandinavian countries, *e.g.* in Sweden antibiotic consumption is at the lowest level in Europe (*e.g.* in 2012 – 14.1 DDDs per 1000 inhabitants per day) (ECDC, 2013b), which can be directly linked with prescription-only distribution system, high awareness among specialists, patients and also efficient national efforts to reduce the problem of bacterial resistance (Malmvall *et al.*, 2007). The opposite situation is in France, where the consumption of antibiotics is at

Table II
Averages of antibiotic consumption in division by antibiotic group and drug form with statistical analysis results.

	Overall antibiotic consumption (DDDs per 1000 inhabitants)				
	Season average	Out of season average	Increase in season [%]	Statistical significance	p value
	362.75	270.29	34.21%	significant	0.000066
Classification by antibiotic group					
Cephalosporins	42.90	28.76	49.18%	significant	0.001201
Quinolones	22.64	19.94	13.49%	insignificant	–
Lincosamides	15.92	15.74	1.11%	insignificant	–
Macrolides	60.58	39.02	55.28%	significant	0.000004
Penicillins	164.94	120.16	37.27%	significant	0.000239
Sulphonamides	9.40	6.62	41.93%	significant	0.002510
Tetracyclines	41.77	36.00	16.03%	significant	0.033546
Classification by drug form					
Suspensions	46.53	24.53	89.72%	significant	0.000003
Tablets	315.94	245.44	28.72%	significant	0.001265

the highest level in Europe (in 2012–29.7 DDDs per 1000 inhabitants per day) (ECDC, 2013b), which might be connected with the wide availability of antibiotics and frequent prescribing by primary care practitioners (Humphreys, 2011; Grimaldi-Bensouda *et al.*, 2014). In Polish healthcare system, antibiotics for systemic use are prescription-only drugs, nonetheless the awareness of the problem of unreasonable antibacterial therapy seems to be quite low (Panasiuk *et al.*, 2010; Godycki-Cwirko *et al.*, 2014). Therefore Poland is placed in the middle of the ECDC list (in year 2012 –22.6 DDDs per 1000 inhabitants per day) (ECDC, 2013b).

The connection between seasonal variability of outpatient antibiotic use in European countries and reasonable antibacterial therapy has already been described by Goossens *et al.* (2005) in *Lancet*. In countries where antibiotic consumption has been highest (with France as a leading country), fluctuations have been most significant. On the contrary, in countries with low consumption of antibiotics and restricted policy for antibiotics usage, seasonal fluctuations have been very low. Comparing the data from the aforementioned article and ECDC data, the situation in Poland over 10 years (2002–2012) has not changed substantially. The seasonal variability was significant and antibiotics consumption in year 2002 was estimated at around 21.4 DDDs per 1000 inhabitants per day (ECDC, 2013b), so at even a slightly lower level than in 2012.

According to the treatment recommendations presented by the National Medicines Institute (2010) 90–95% of cases of acute pharyngitis and tonsillitis in adults are caused by viruses (*e.g.* rhinoviruses, coronaviruses, adenoviruses, Epstein-Barr Virus, Coxackie, *Herpes simplex*, influenza and parainfluenza viruses). From five to ten percent of these cases are caused by streptococcal infections (*e.g. Streptococcus pyogenes*). According to the abovementioned document, only about 0.5–2% of cases of rhinitis and sinusitis are caused by bacterial infections (e.g. *Streptococcus pneumoniae*, *Haemophilus influenzae*). The aforementioned bacterial infections and a small percent of viral diseases followed by bacterial infections can justify a slight increase in antibiotics consumption during season in all analyzed countries. Unreasonable prescribing of antibacterial substances for likely viral infections might cause a more significant increase in antibiotic intake in the viral infections season.

Worldwide medical recommendations, in the USA as well as in Poland and other European countries (National Medicines Institute, 2010; L'Agence Nationale de Sécurité du Médicament et des Produits de Santé, 2003; CDC, 2014) require differentiation between viral and bacterial etiology. According to these recommendations, it is based on medical history and analysis of symptoms but also on rapid infection tests like RS virus test (Slinger *et al.*, 2004), influenza A/B tests (Ko *et al.*, 2013) and streptococcal antigen tests (Lean *et al.*, 2014). Despite well-defined recommendations, this kind of rapid diagnostic tests is restricted in the primary care system in Poland. The decision on the course of treatment is usually empirical and not preceded by comprehensive diagnostics. Because of the lack of rational antibiotic prescriptions in the light of microbial diagnostics, the most frequently used antibacterial agents are wide-spectrum penicillins and cephalosporins. The high prevalence of macrolides (especially azithromycin) might result from a simple dosing scheme and shortage of information about potential penicillin hypersensitivity in patients. Particularly high fluctuations in the consumption of oral suspensions might indicate that children are the largest group of patients subjected to unjustified antimicrobial therapy.

Obtained results show that unreasonable antibiotic treatment still poses a serious problem in Szczecin area. Despite the introduction of the Polish National Antimicrobial Surveillance Program in 2004, which propagates rational antibacterial therapy (Hryniewicz, 2011; Mazińska and Hryniewicz, 2010; National Medicines Institute, 2014), further actions need to be taken. Unfortunately, in the Polish primary care system, antimicrobial agents are still too frequently and unreasonably prescribed for the treatment of cold-like infections, especially in children (0–10 years old), young adults (21–30 years old) and elderly people (over 70 years old). Furthermore, rapid diagnostics tests are not common in Polish primary care system. It is essential to enhance the knowledge about different causes of infections and methods of differentiating them among physicians and pharmacists.

Presented study shows that changes in attitude to rational antibacterial therapy proceed too slowly and need permanent monitoring. According to ECDC data, the situation in various European countries hasn't changed significantly over past decade. In the era of globalization and common travelling of people between countries and continents, the problem of multi-resistant bacteria has become international and might be escalating in the following years.

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SHORT COMMUNICATION

Susceptibility of Vascular Implants to Colonization *in vitro* by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*

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Abstract

We compared association of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa and Enterococcus faecalis* with nine vascular implants after co-culture. Vascular implants were composed of various materials such as warp knitted polyester (with or without gelatin and silver ions), expanded polytetrafluoroethylene and biological materials – surface treated porcine pericardial patch and Omniflow II. The lowest overall number of associated bacteria was detected for polytetrafluoroethylene implants and porcine pericardial patch. The highest overall number of associated bacteria was detected for Omniflow II implant. The major source of variation, *i.e.* primary factor influencing colonization, is the implant type (56.22%), bacterial species is responsible for only 1.81%, and interaction of those two factors – 13.09% of variation.

Key words: biofilm, graft infection, Omniflow II, porcine pericardial patch, vascular implant

Application of synthetic vascular implants has considerably improved treatment results in vascular surgery. However, implanting artificial bypasses might bring about complications of which graft infection seems to pose a major threat. The incidence of the infection is 7% (Herrera *et al*., 2009) and it is correlated with a high mortality rate (up to 75% in the aortic segment) and an extremely high risk (up to 70%) of a complete or partial loss of the limb. In most cases, the vascular implant gets contaminated by microorganisms during an operation. A surgeon makes the patient's vessels accessible by incising or puncturing the inguinal region. Being naturally colonized by numerous microorganisms, this region is considered one of the major risk factors for infection of vascular implant (Antonios *et al*., 2006).

Staphylococcus aureus strains and coagulase negative *Staphylococcus* spp. are responsible for 70–90% infections of vascular bypasses (Bozoglan *et al*., 2016). They produce glycocalyx, which on the one hand, facilitates their adhesion to the surface of the prosthesis, and on the other hand, it is a component of biofilm which serves as protection against phagocytes, antibodies and antibiotics (Bandyk *et al*., 1991). Of gram-negative bacteria, *Pseudomonas aeruginosa* is the most common etiological factor contributing to vascular implant infections. It causes about 10% of such infections. Infections caused by *P. aeruginosa* often result in the occurrence of false aneurysms and dissection of the vascular wall in the infection site. Esterase and alkaline protease, produced by *P. aeruginosa*, are responsible for these processes (Chiesa *et al*., 2002).

Gram-positive cocci, including *Enterococcus faecalis*, have recently become highly contributive factors in infections of vascular implants. Several years ago, these bacteria were considered relatively harmless and were characterized as possessing slight virulence traits. Despite being commonly found in human environment, they contributed only to a small percentage of infections. A characteristic feature of *Enterococci* is their natural resistance to cefalosporins, thus introduction of cefalosporins to perioperative prophylaxis on a great scale led to a great dominance of *Enterococci* in intestinal flora. This, in turn, resulted in an increase in the number of dangerous infections induced by these bacteria in hospitalized patients. Pathogenicity of *Enterococci* is associated with production of proteins

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which facilitate adhesion and colonization of the surface of an artificial prosthesis as well as formation of protective biofilm (Bronk and Samet, 2008).

Unsuccessful treatment of infected vascular implants makes scientists implement new strategies in order to lessen the risk of infections. Infection-resistant implantable material might appear to be that innovation. Such material should be characterized with low thrombogenicity and immune indifference.

Implantable materials, currently used in vascular surgery, can be divided into three groups: synthetic, biosynthetic and biological. Professional literature does not, however, contain collective comparative studies on susceptibility of various implantable materials to infections.

Vascular prostheses, made of polyethylene terephthalate (Dacron) and polytetrafluoroethylene (PTFE) have been commonly applied for more than 50 years. PTFE is commonly believed to be less susceptible to colonization than Dacron. This observation was earlier confirmed in *in vitro* studies (Schmitt *et al*., 1986), which revealed that most bacteria are prone to adhere to Dacron, rather than to PTFE. Bacterial adhesion to synthetic material, which vascular implants are made of, is closely associated with its colonization, which increases the risk of postoperative infections/complications in patients. However, clinical studies, comparing complications after implanting Dacron or PTFE, did not confirm that one material outdoes the other. Patency indices for bypasses manufactured from both the synthetic materials, and the number of infection-induced complications in both the groups were similar (Post *et al*., 2001; Jensen *et al*., 2007).

Scientists had high hopes that biosynthetic prostheses would make infection-resistant implantable material. One of such prostheses is a composite bioprosthesis, Omniflow II. Its complex structure consists of polyester mesh (a supportive element and framework for biological component) which is coated with ovine collagen (facilitating permeation of the graft wall through the recipient's vessels and tissues). The ability of Omniflow II prostheses to easily heal in the recipient's body has been known for more than 20 years (Werkmeister *et al*., 1995). This property makes them an alternative for autologous material in the process of creating a vascular bypass (Dünschede *et al*., 2015) or arteriovenous access for dialysis (Palumbo *et al*., 2009). Application of Omniflow II prostheses in treatment of synthetic prosthesis infections is an interesting observation. In 2012 Töpel *et al*. (2012) presented the first benefits of Omniflow II prostheses which replaced infected bypasses, located below inguinal ligaments. In 2015 Krasznai *et al*. (2016) presented reconstruction of infected synthetic bypasses with Omniflow II prostheses in the aortoiliac segment. Most authors claim that Omniflow II prostheses are highly beneficial since they are resistant to re-infections and degenerative changes. In a study conducted on rat models, Bozoglan *et al*. (2016) compared PTFE and Omniflow II biosynthetic prostheses in terms of their resistance to *S. aureus* infections and the authors arrived at completely different conclusions. They confirmed that Omniflow II prosthesis is more susceptible to infections than PTFE prosthesis.

For dozens of years scientists have also been conducting clinical studies on application of biological prostheses. They include patches, implants and valves produced from bovine or porcine pericardium, prepared according to the No-React technology (multistage detoxification process with the use of glutaraldehyde). Due to this technology, biological material is biocompatible with recipient's tissues and the biocomponent is protected against calcification. Biological material which has been prepared with the application of the above technology gets covered with endothelium within 6 weeks. The endothelial coating provides a natural barriers against infections. After conducting studies for longer than a decade, Musci *et al*. (2013) presented positive findings regarding application of biological prostheses in treating infectious endocarditis. Those positive results might encourage for a wider use of such prostheses. In a different study, Avsar *et al*. (2013) presented early results of using bovine internal mammary arteries, also prepared in the No-React technology. A number of patients were implanted 33 femoropopliteal bypasses, 100% of which remained patent one year following the surgery. The authors did not observe infections of the grafts, either.

The aim of our study was to compare susceptibility of selected synthetic and biological implantable materials, applied in vascular surgery, to colonization *in vitro* by *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. faecalis*.

Despite the fact that, *in vitro* experiments using abiotic medical devices and laboratory cultures lack the component of host produced proteins and specific protein-interactions, co-culture allows comparison of colonization using controlled condition by quantification of implant related bacteria.

In the study we tested 9 types of implants (numbered in the text 1 through 9), all types and their manufacturers are listed in Table I. The implants were made from various materials such as warp knitted polyester (with additional surface covering substances such as gelatin and silver ions), expanded polytetrafluoroethylene and two biological implants-surface treated porcine pericardial patch and Omniflow II.

To determine number of bacteria associated with implants, we used strains that represent the most common causes of vascular implant infections *i.e. S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 14990), *P. aeruginosa* (ATCC 27853) and *E. faecalis* (ATCC 29212). Overnight cultures of each of the strains were diluted

in 75 ml of fresh TSB medium in 300 ml flask and fragments of sterile implants were placed in the diluted culture. Implants were incubated with bacteria for 24 h at 37°C with gentle mixing (100 rpm) to assure contact of the whole implant surface with the bacterial culture. After 24 h bacterial culture was decanted and implants were washed five times with 100 ml of sterile buffered saline (PBS). After the wash, each implant fragment was transferred to 7 ml of sterile PBS in a glass tube and bacteria associated with the implant were detached by sonication for 5 minutes on ice (Branson Sonifier 250, micro tip, output control 6, constant duty cycle). PBS with detached bacteria was serially diluted, plated on TSA solid medium and colony forming units (CFU) from 2–3 dilutions for each experimental variant were counted the next day. The experiment was repeated independently for at least 3 fragments for each implant/bacterial species combinations. To calculate the number of bacteria per cm^2 of the implant, we used the formula

$$CFU/cm^2 = \frac{(CFU \text{ per } 1 \text{ ml}) \times 7}{(\text{implant surface in } cm^2) \times 2}$$

Outliers from the datasets were removed using Grubbs' test (Grubbs, 1950) and the datasets were compared using 1 or 2 way ANOVA, to analyze one (species or prosthesis type) or two factor influence (species and prosthesis type).

We detected high colonization rate of all types of devices (Fig. 1) with median ranging from $1{,}94 \times 10^6$ CFU/cm^2 (implant no. 3) to almost 100 times higher value

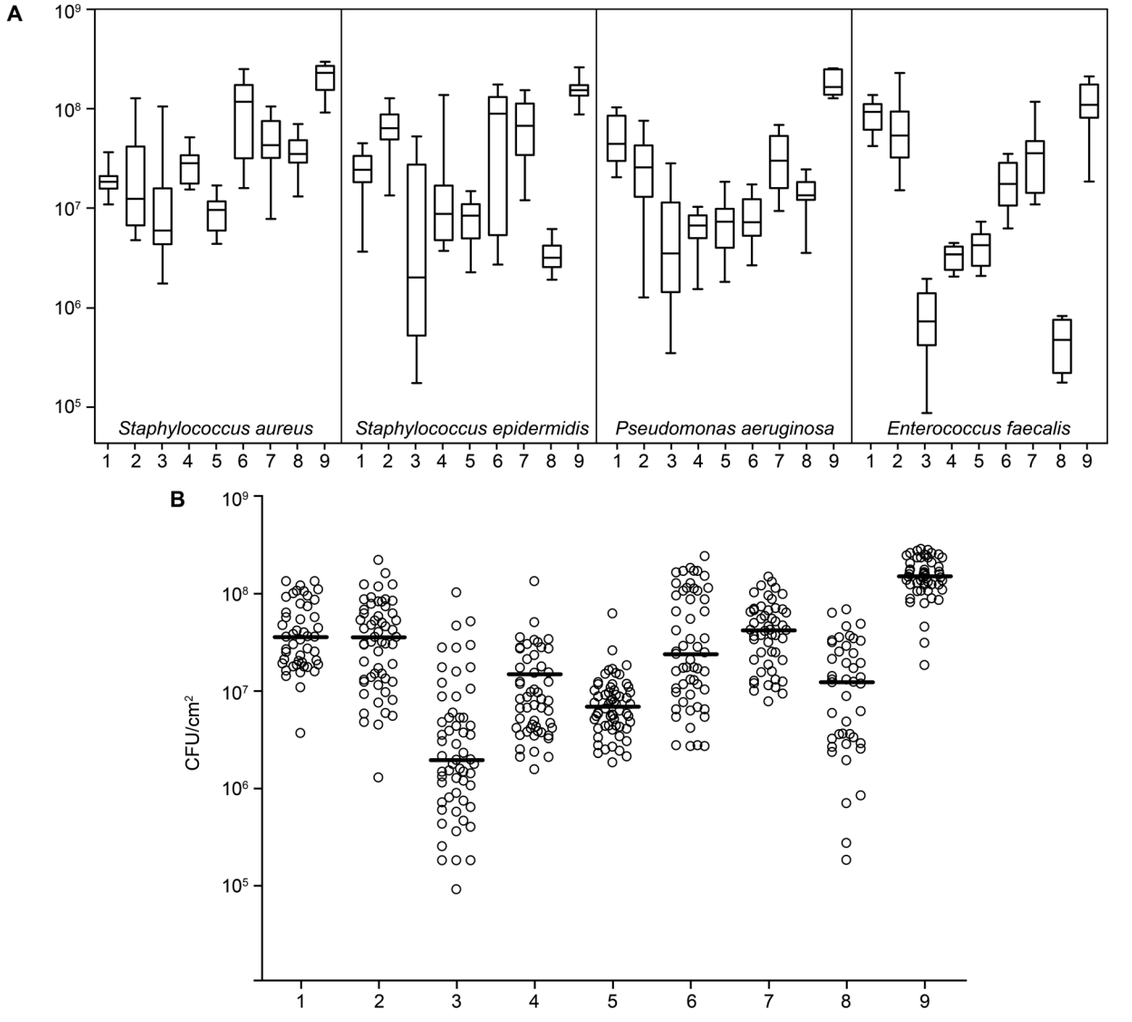

Fig. 1. Number of bacteria recovered from implants after 24 h incubation.

A. Results obtained individually for each bacterial species for implants 1–9 (description in Table 1 and in text). **B** combined data for all tested bacterial species. Each dot represents single bacterial count, from either separate implant or calculated from single dilution of bacteria. Statistical significance of observed differences in colonization was assessed using one way ANOVA with nonparametric Kruskal-Wallis test and Dunns multiple comparison post-test, * denotes $p < 0{,}05$, ** $p < 0{,}01$, *** $p < 0{,}001$. Statistically significant differences in colonization were detected for pairs 1 *vs* 3 (***), 1 *vs* 4 (***), 1 *vs* 5 (***), 1 *vs* 8 (***), 1 *vs* 9 (***), 2 *vs* 3 (***), 2 *vs* 4 (***), 2 *vs* 5 (***), 2 *vs* 8 (**), 2 *vs* 9 (***), 3 *vs* 6 (***), 3 *vs* 7 (***), 3 *vs* 8 (*), 3 *vs* 9 (***), 4 *vs* 6 (**), 4 *vs* 7 (***), 4 *vs* 9 (***), 5 *vs* 6 (***), 5 *vs* 7 (***), 5 *vs* 9 (***), 6 *vs* 8 (*), 6 *vs* 9 (***), 7 *vs* 8 (***), 7 *vs* 9 (***), 8 *vs* 9 (***)

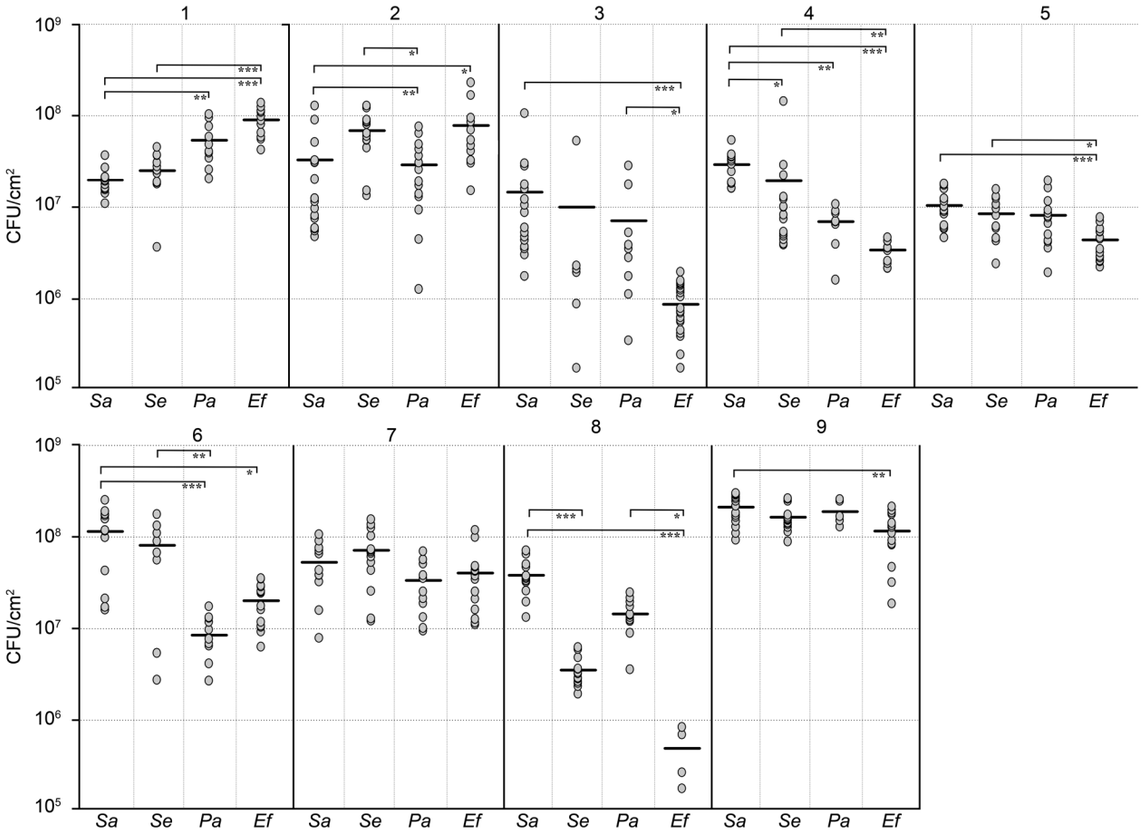

Fig. 2. The influence of bacterial species on the implant colonization.

Numerals 1–9 denote implant type (as in Table I and in text), *Sa* – *S. aureus*, *Se* – *S. epidermidis*, *Pa* – *P. aeruginosa*, *Ef* – *E. feacalis*. Each gray dot represents single bacterial count, from either separate implant or calculated from single dilution of bacteria. Statistical significance of observed differences in colonization was assessed using one way ANOVA with nonparametric Kruskal-Wallis test and Dunns multiple comparison post-test, * denotes $p < 0.05$, ** $p < 0.01$, ***$p < 0.00.1$

Table I
List of implants used in the study.

No.	Implant type	Material	Manufacturer
1	DALLON H	conventional warp knitted polyester vascular graft	TRICOMED SA
2	Gelsoft Plus	gelatin impreganted, conventional warp knitted polyester vascular graft	VASCUTEC Ltd. a TERUMO Company
3	IMPRA	expanded polytetrafluoroethylene (ePFTE)	BARD Peripheral Vascular, Inc.
4	VIABAHN ENDOPROSTHESIS	self-expanding endoluminal endoprosthesis consisting of an expanded polytetrafluoroethylene lining with an external nitinol support extending along its entire length	W.L.GORE &ASSOCIATES, Inc.
5	FLUENCY PLUS	self-expanding Nitinol Stent encapsulated with expanded polytetrafluoroethylene	BARD Peripheral Vascular, Inc.
6	Zenith Flex AUI AAA Endovascular Graft	full-thickness woven polyester fabric sewn to self-expanding stainless steel Cook-Z stents with braided polyester and monofilament polypropylene suture	WILLIAM COOK EUROPE ApS
7	Silver Graft	warp-knitted, double-velour vascular polyester prosthesis, impregnated with modified bovine gelatine (Polygelin) and coated with a layer of silver on its surface	B. Braun Melsungen AG Vascular Systems
8	NO-REACT PATCH	porcine pericardial patch	BioIntegral Surgical, Inc
9	Omniflow II Vascular Prosthesis	a composite biosynthetic material comprised of polyester mesh incorporated within a cross-linked ovine fibrocollagenous tissue matrix	Bio Nova International Pty Ltd

$1{,}56 \times 10^8$ CFU/cm^2 for Omniflow II, based on combined data recorded for all tested species ($p < 0.0001$). The lowest overall colonization was detected for implants no. 3 (Impra), 5 (Fluency) and 8 (porcine patch), regardless of the species (Fig. 1A and B). It confirms claims of porcine patch manufacturer that No-React® proprietary treatment with glutaraldehyde significantly lowers infection rate after implantation. Low colonization rate for both PFTE implants is also not surprising as lower attachment to hydrophobic surface can be expected.

The highest overall colonization rate was observed for Omniflow II (9) implant (Fig. 1), as shown by Bozoglan *et al.* (2016).

Increased susceptibility of Dacron to colonization *in vitro* was usually caused by its greater porosity than that of PTFP. Smoothing the Dacron surface with gelatin makes it less susceptible to infections in a mouse model (Yasim *et al.*, 2006). For polyester implants, we did not observe the influence of the treatment with gelatin or gelatin and silver ions on the number of implant associated bacteria (1 *vs* 2, and 1 *vs* 7, Fig. 1B).

Two way ANOVA analysis of the whole dataset revealed that the major source of variation, *i.e.* primary factor influencing colonization, is the implant type (56,22% of total variation, $p < 0.0001$) and bacterial species is responsible for only 1,81% of total variation ($p < 0.0001$). Interaction of those two factors is a source of 13,09% of variation ($p < 0.0001$).

In a conclusion, a factor that the most influences colonization is the type and the material of the implant. We show that PFTE (No. 3) is colonized to much less extent than Omniflow II. The number of bacteria of different species associated with various implants varies. For example in our experiment, the best colonizer is *S. aureus.* Aseptics and a perioperative antibiotic therapy still remain the most common methods of combating infections of vascular prostheses (Giacometti *et al.*, 2000).

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SHORT COMMUNICATION

Antiviral Resistance of Splenocytes in Aged Mice

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Abstract

We compared the susceptibility to viral infection of splenocytes, isolated from young versus old CBA mice, and evaluated the antiviral actions of lactoferrin in splenocytes infected with *Encephalomyocarditis virus* (*EMCV*). Recombinant mouse lactoferrin (rmLF) and bovine lactoferrin (bLF) were used. There were no differences in the susceptibility to *EMCV* infection in the studied age categories. Both types of lactoferrins were protective in young and old mice. The study confirmed the undisturbed viral resistance in old mice and the protective actions of lactoferrin in viral infection. The antiviral action of the homologous mouse lactoferrin was demonstrated for the first time.

Key words: immune ageing, lactoferrin, mice, splenocytes, viral resistance

Senescence is associated with gradual impairment of physiological processes, although the functions of the immune cell types may not be uniformly suppressed (Kogut *et al*., 2012; Sansoni *et al*., 2008; Scholz *et al*., 2013). The immune ageing is correlated with preferential diminution of the T-cell compartment, increased ratio of the memory cell phenotype and lower CD28 expression (Simioni *et al*., 2007). Although B cells from aged mice show decreased antigen-induced expansion, the ability of aged B cells to respond appropriately to T-dependent antigens and differentiate into antibody-secreting cells seems to be intact (Dailey *et al*., 2001). In aged humans, the antigen-presenting function of peripheral blood cells in response to staphylococcal enterotoxin is poor in comparison to young individuals (Castle *et al*., 1999). The susceptibility to viral infections in old animals has been a subject of consideration. Twenty two to twenty four month old mice, infected with respiratory syncytial virus, demonstrated diminished virus specific $CD8^+$ cytolytic response and IFN γ production (Zhang *et al*., 2002). In elderly human donors, the majority of the clonally expanded, virus-specific $CD8^+$ cells, was dysfunctional (Ouyang *et al*., 2003). However, the frequency of viral antigen-specific $CD8^+$ T cells was high in the majority of subjects older than 85 years and serologically positive for the viral epitopes (Scholz *et al*., 2013). These data indicate importance of a chronic antigenic stimulation, induced by persistent viral infections during a lifetime that may result in enhanced resistance to viral infections.

Lactoferrin is a multifunctional protein, present in excretory fluids and circulating neutrophils, involved in iron metabolism (Zimecki and Kruzel, 2007). The protein interferes with viral infection by means of several mechanisms, such as inhibition of virus replication and direct interaction with viruses (Välimaa *et al*., 2009; Picard-Jean *et al*., 2014) or blocking cell receptors for viruses (Pietroantoni *et al*., 2015; Zheng *et al*., 2012). The aim of this study was to compare the susceptibility of splenocytes from young versus aged organisms to viral infection and to investigate antiviral actions of a homologous, recombinant mouse and native bovine lactoferrin.

Three and 13 months old CBA mice, provide by the institute of Laboratory Medicine, Łódź, Poland, were used for the study. The local ethics committee approved the study. Low endotoxin (< 1.0 EU/mg) recombinant mouse lactoferrin (rmLF) produced by Chinese hamster ovary cells was obtained from PharmaReview Corporation, Houston, USA, and bovine milk-derived lactoferrin

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(bLF) was generously provided by Morinaga Co., Japan. The culture media were purchased from Cytogen and fetal calf serum (FCS) from Gibco. L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics were from Sigma. *Encephalomyocarditis virus* (*EMCV*) was obtained from The Laboratory of Virology, Institute of Immunology and Experimental Therapy, Wrocław, Poland. L929 cells (ATCC CCL 1) was purchased from ATCC. We followed the procedures of preparation of splenocyte suspension and fractionation of splenocytes on glass wool columns which were described elsewhere (Russo *et al.*, 1979). Such a procedure allowed to remove from the splenocyte suspension strongly adherent cells (macrophages), erythrocytes, debris and dead cells, as well as a part of T cell population, resulting in a B-cell enriched population. The determination of the cell phenotype of this fraction indicated (FACS measurement, not shown) that it was about 65–72% CD19 positive (a pan B cell marker). One of the reasons, we have chosen such a model, was because the activity of B cell compartment is well preserved in aged mice.

For the infection of cells we used *EMCV*, which was propagated and titrated in L929 cells. The titer of the virus was expressed in reference to the value of TCID50 (tissue culture infectious dose), based on the cytopathic effect caused by the virus in 50% of infected cells. In the present study we applied two approaches to evaluate the protective effects of lactoferrins. In the first model, the cells were infected with the virus, followed by the addition of lactoferrins (50 μg/ml). The viral replication was determined after 24 h and 5 days of cell culture. In the second model, the cells (2×10^6/ml), re-suspended in the culture medium consisting of RPMI-1640 medium, 10% of FCS, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics, were incubated for 1 h at 37°C with the studied lactoferrins (50 μg/ml). Then, the cells were washed twice with Hanks' medium, re-suspended in the culture medium and infected with *EMCV*. The

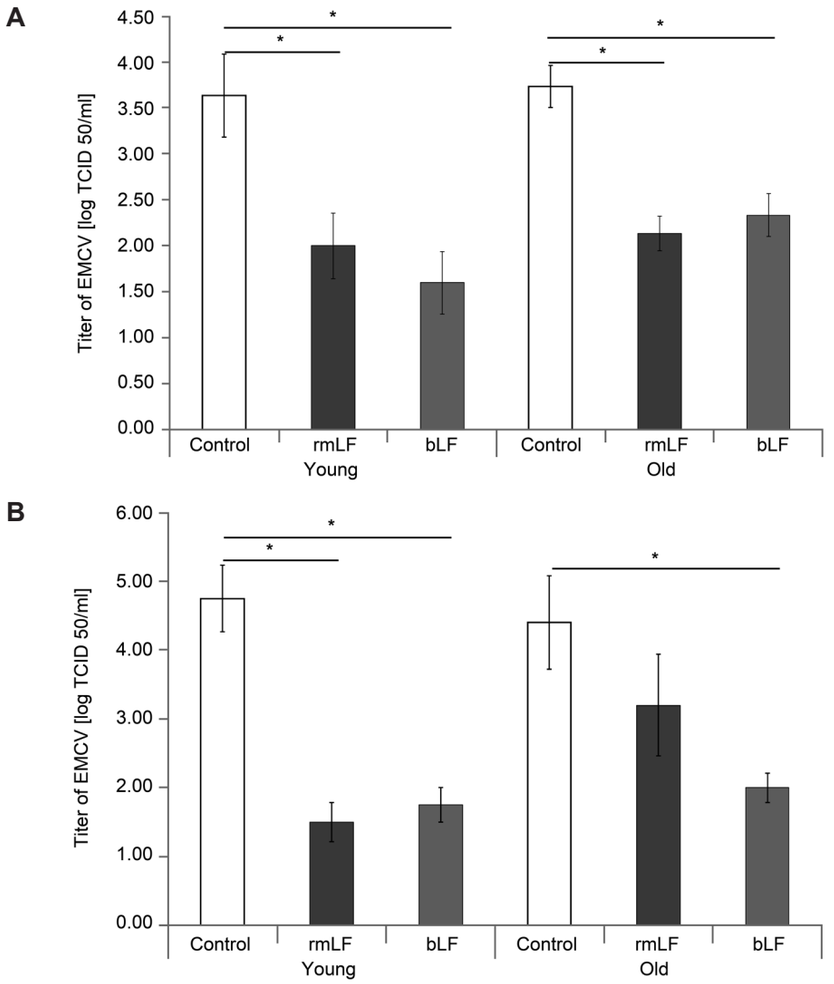

Fig. 1. The effect of lactoferrins on *EMCV* replication in the B-cell enriched splenocyte population from young and old mice.
Lactoferrins (50 μg/ml) were added to the cells after *EMCV* adsorption. Samples of culture medium from LFs-treated and non-treated cells, incubated at 37°C, were collected after 24 h (**A**) or 5 days (**B**) and titrated in L929 cells.

viral replication was determined after 5 days of culture. The cells were infected with a dose of 100 $TCID_{50}$/ml of *EMCV* in a presence or absence of lactoferrins. After 45 min of adsorption at 37°C, the virus was disposed by 5 times wash of the cells in Hanks' medium. The infected cells were then re-suspended in the culture medium and transferred to a 96-well plate. After 24 h or 5 days of incubation at 37°C, samples of the supernatants were collected and frozen at –20°C. The supernatants were then thawed, serially diluted and plated in the presence of target L929 cells to determine the viral titer, indicated as the TCID50. For the statistical evaluation of the data, the analysis of variance (one-way ANOVA) was applied due to a homogeneity of variance between the groups, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of differences between the groups. The significance was determined at $p < 0.05$ and indicated as*. The results are presented as mean values ± standard error (SE). The result of one representative experiment was shown, out of three experiments performed.

The splenocytes from young and old mice were investigated for their susceptibility to viral infection. The effects of lactoferrins on these parameters were also determined. In one model, the cells were infected with the virus, washed and incubated with the lactoferrins for 24 h or 5 days (Fig. 1A-B), followed by determination of the virus titer. Thus, in this model, lactoferrin was designed to act solely as an inhibitor of virus replication. It appeared that after 24 h culture the rate of infection in cells from both young and old mice was the same (Fig. 1A). Both types of lactoferrins significantly reduced the virus titer in cells derived from young and old mice. The inhibitory effects of lactoferrins were stronger in young mice, particularly in the case of bLF. After 5-day exposure to the virus and the lactoferrins, the infection rates of cells from young and old mice were comparable (Fig. 1B). The inhibitory effects of lactoferrins were stronger in young mice, both lactoferrins being equally active. On the other hand, the virus replication in old mice was significantly inhibited only by bLF. In the second model the cells were incubated for 1 h with lactoferrins, washed and exposed to the virus for 5 days (Fig. 2) in order to test an ability of this protein to interfere with virus binding to the cells. The susceptibility of cell populations from both age categories was comparable. While the suppressive effects of bLF on the virus titers were statistically significant in both splenocyte populations, the action of rmLF was weaker and not significant.

Although we demonstrated more potent actions of bovine lactoferrin on resistance of splenocytes to viral infection, as compared to recombinant mouse lactoferrin, the efficacy of the homologous lactoferrin in the antiviral resistance was shown here for the first time. Small differences in the efficacy of bovive and mouse lactoferrin in the antiviral resistance of splenic B cells could be due to different structures of glycan moieties in the two types of lactoferrins. These structures are responsible for blocking CD21 receptors on B cells (Zheng *et al.*, 2012) which are also used for viruses to enter cells. The heparan sulfate receptors for viruses are, in turn, blocked by a highly cationic N-terminal end of lactoferrin (Pietroantoni *et al.*, 2015).

Our results on the suceptibility to viral infection in old versus young individuals are, in part, consistent with a hypothesis (Scholz *et al.*, 2013) that the viral resistance in the elderly may be even enhanced due to a cumulative, frequent contacts with viral antigens

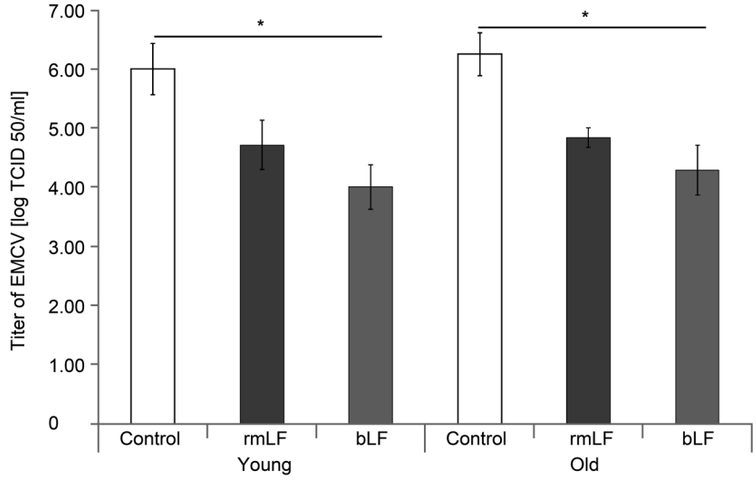

Fig. 2. Effect of preincubation of the B-cell enriched splenocytes from young and old mice, with lactoferrins on viral replication.
The cells were re-suspended in the culture medium and cultured with lactoferrins (50 µg/ml) for 1h at 37°C. After the incubation, the cells were washed twice with Hanks' medium, re-suspended in the culture medium and infected with *EMCV*. Samples of culture medium were collected after 5 days and titrated in L929 cells.

resulting in a presence of a bigger pool of antigen-specific memory cells. Although mouse (Scholz *et al*., 2013; Zhang *et al*., 2002) and human studies (Labeur *et al*., 1991) indicated impaired susceptibility of aged organisms to viral infection, it is also clear (Dailey *et al*., 2001; Castle *et al*., 1999) that the activation status and functional capacity of B cells from old mice are higher or at least not lower than those in young ones.

EMCV infects several cell types beside lymphocytes and the mechanisms of interference with virus replication by LF are probably common for most cell types. We also think that interference of other cell types and cytokines on the susceptibility of B cells to viral infection may be negligible. Besides, we obtained similar results with a T-cell-enriched cell fraction (not shown).

In view of the literature on the protective role of lactoferrin in viral infection (Pietroantoni *et al*., 2015; Välimaa *et al*., 2009; Zheng *et al*., 2012) it was not unexpected that also in this study lactoferrins demonstrated antiviral properties in the applied models, *i.e.* potential block of virus receptors on target cells and interference with virus replication. We also suggest still another mechanism of the antiviral activity of lactoferrin, since the protein is an enhancer of COX-1 expression (Kruzel *et al*., 2013), important in maintaining antiviral resistance (Carey *et al*., 2010). In addition, a lack of age-related difference in COX-1 protein levels found by others (Hayek *et al.*, 1997) further supports our findings on the comparable susceptibility to viral infection in both age categories. Our results provided also the evidence that the homologous (mouse lactoferrin) may play a physiological role in the antiviral resistance in mice and that the protective effect of lactoferrin is not species specific. It was also encouraging to find that cells from old individuals were at least equally resistant to viral infection as those derived from young ones. In conclusion, we showed that the resistance to viral infection was preserved in the senescent mice. We also demonstrated, for the first time, the antiviral actions of the homologous mouse lactoferrin. Based on the results, regarding the protective effect of bovine lactoferrin on virally infected splenocytes from old mice, we also suggest a potential benefit of diet supplemented with bovine lactoferrin for the elderly in enhancing antiviral resistance.

Statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Acknowledgements

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Polskie Towarzystwo Mikrobiologów
Zarząd Główny
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NIP 521-11-21-855

Warszawa, 17.03.2017 r.

Szanowni Mikrobiolodzy i wszyscy Państwo
zainteresowani badaniami mikrobiologicznymi w Polsce

We wrześniu ub. roku ukonstytuowało się nowe Prezydium Zarządu Polskiego Towarzystwa Mikrobiologów (PTM). Głównym celem naszego Stowarzyszenia jest propagowanie rozwoju nauk mikrobiologicznych i popularyzowanie osiągnięć mikrobiologii wśród członków PTM oraz szerokich kręgów społeczeństwa.

Zarząd PTM stara się zaprosić do współpracy i zjednoczyć w ramach Towarzystwa wszystkie osoby wnoszące istotny wkład w rozwój rozmaitych gałęzi mikrobiologii: klinicznej, weterynaryjnej, środowiskowej, przemysłowej, farmaceutycznej, mikrobiologii żywności, wody, osoby prowadzące badania podstawowe, rozwojowe, przeglądowe, kontrolne, genetyczne i inne.

Wiele jest zagadnień wymagających wnikliwych badań i oceny przez mikrobiologów, np.: problem narastającej lekooporności drobnoustrojów zwłaszcza chorobotwórczych, mikrobiologiczne zanieczyszczenie środowiska, GMO, poprawa systemu jakości i metod kontroli żywności, kosmetyków, leków, wyrobów medycznych i produktów biobójczych. Do realizacji tych planów konieczna jest szeroka współpraca nie tylko mikrobiologów: naukowców, nauczycieli akademickich, lekarzy, specjalistów pracujących w przemyśle, rolnictwie, wykonujących mikrobiologiczne badania kontroli w rozmaitych obszarach, ale także firm diagnostycznych, farmaceutycznych, kosmetycznych, produkujących żywność, środki przeciwdrobnoustrojowe, odczynniki oraz aparaturę kontrolną i badawczą.

W 2017 r. z okazji 90-lecia działalności Towarzystwa planujemy zorganizować Konferencję „90 lat PTM wczoraj – dziś – jutro", która odbędzie się w dniach 22–23 września w Krakowie i jak zakładamy zgromadzi kilkaset osób związanych z mikrobiologią.

W związku z tak szeroką działalnością, zwracamy się do Państwa z prośbą o wsparcie naszego Towarzystwa poprzez:

– darowizny na cele statutowe
– udział w Konferencji z okazji 90-lecia Towarzystwa. Planujemy w trakcie jej trwania udostępnienie powierzchni na organizację stoisk firm, pragnących reklamować swoje produkty.
– przystąpienia do Polskiego Towarzystwa Mikrobiologów jako **członek wspierający PTM**. Prezydium PTM podjęło decyzję o minimalnej wysokości składki rocznej członka wspierającego:

Członek Zwyczajny – 3.000 zł; Członek Srebrny – 5.000 zł; Członek Złoty – 10.000 zł

Członkowie wspierający (informacje o nich: nazwa firmy, logo i adres) uzyskują następujące przywileje:

- wszyscy wymienieni są na stronie PTM, na facebooku oraz w każdym numerze 3 czasopism – kwartalników PTM: Medycyna Doświadczalna i Mikrobiologia, Postępy Mikrobiologii i Polish Journal of Microbiology, w danym roku;.
- wszyscy wymienieni są w materiałach konferencji organizowanych i współorganizowanych przez PTM. Możliwe jest dołączenie materiałów informacyjnych firm do materiałów konferencyjnych;
- wszyscy otrzymują w danym roku wszystkie numery 3 czasopism (łącznie 12 numerów – zeszytów);
- otrzymują zniżki 15% (**Członek Srebrny**) 25% (**Członek Złoty**) na stoisko, jeżeli takie będą na konferencjach organizowanych przez PTM;
- możliwe jest nieodpłatne umieszczanie reklam czarno-białych i kolorowych z 50% zniżką, w kwartalnikach: Postępy Mikrobiologii oraz Polish Journal of Microbiology (oba czasopisma z IF):

Członek Srebrny – 1 reklama całostronicowa lub 2 reklamy po pół strony raz w roku, w jednym numerze jednego z ww. wymienionych czasopism

Członek Złoty – 2 reklamy całostronicowe lub 3 reklamy po pół strony raz w roku, w jednym numerze obu ww. wymienionych czasopism.

Niniejszy list jest informacją i jednocześnie prośbą o współpracę z Towarzystwem.

W przypadku Państwa zainteresowania, możliwości udziału Państwa firmy w działalności PTM będziemy omawiać bezpośrednio (ptm.zmf@wum.edu.pl)

PREZES
Polskiego Towarzystwa Mikrobiologów
prof. dr hab. Stefan Tyski

INFORMACJA O SKŁADKACH CZŁONKOWSKICH PTM W 2017 ROKU

Zarząd Główny Polskiego Towarzystwa Mikrobiologów uprzejmie informuje, iż wysokość składki członka zwyczajnego PTM w roku 2017 wynosi 100 zł/rok. Wysokość składki dla emerytowanych członków zwyczajnych PTM wynosi 50 zł/rok.

W przypadku zmiany danych wcześniej przekazanych do ZG PTM (np. zmiana miejsca pracy, adresu, e-maila, przejście na emeryturę itp.), prosimy o aktualizację i wypełnienie „Formularza aktualizacji danych osobowych" (dostępny na stronie PTM). Z opłacania składki członkowskiej zwolnieni są członkowie honorowi Polskiego Towarzystwa Mikrobiologów.

SKŁADKI PROSIMY OPŁACAĆ DO KOŃCA I KWARTAŁU KAŻDEGO ROKU KALENDARZOWEGO

Statut PTM §14 ust 3. „Członek zwyczajny jest zobowiązany do regularnego opłacania składki członkowskiej w wysokości ustalonej przez Walne Zgromadzenie Delegatów Towarzystwa".
Statut PTM §15 ust 3. „ Członkostwo zwyczajne ustaje na skutek skreślenia z listy członków Towarzystwa z powodu niezapłacenia składki członkowskiej przez dwa kolejne lata…"

W związku z porządkowaniem listy członków zwyczajnych PTM, osoby, które nie zapłaciły składki za lata 2015, 2016 uprzejmie są proszone o uregulowanie tych składek.

Osoby, które nie opłaciły składek za lata 2014, 2013 i wcześniejsze, proszone są o uregulowanie tych składek (można to uczynić w miesięcznych ratach w ciągu I półrocza 2017 r.), a w przypadku nie uregulowania składek do 30.06.2017 r. osoby te zostaną skreślone z listy członków zwyczajnych PTM. W przypadku, gdy dysponujemy aktualnym adresem, osoby te zostaną wcześniej powiadomione listem wysłanym z Oddziału Terenowego PTM, o możliwości skreślenia ich z listy członków PTM w sytuacji nie uregulowania zaległych składek PTM do dnia 30.06.2017 r.

Jednocześnie informujemy, że tylko członkowie z opłaconą składką za dany rok otrzymają nieodpłatnie kwartalnik *Postępy Mikrobiologii* lub *Polish Journal of Microbiology* (prosimy o podanie wybranego czasopisma przy opłacaniu składki członkowskiej). W przypadku braku deklaracji przesyłany będzie kwartalnik *Postępy Mikrobiologii*. Istnieje możliwość rocznej prenumeraty drugiego kwartalnika koszt – 40 zł/rok (dla członków PTM).

Również tylko członkowie z opłaconą składką za dany rok mogą starać się o stypendia FEMS, niższe opłaty wpisowe na konferencje współorganizowane przez PTM oraz występować jako osoby rekomendujące: kandydatów na nowych członków PTM oraz kandydatów na stypendia FEMS.

NUMER KONTA

Składki członkowskie należy wpłacać na konto:

Polskie Towarzystwo Mikrobiologów, ul. Banacha 1 b, 02-097 Warszawa
Bank: BGŻ BNP Paribas, nr konta: 57 2030 0045 1110 0000 0261 2550

Na przekazie prosimy umieścić informacje:
imię, nazwisko, oddział PTM, rok za który dokonywana jest opłata składki.
Otrzymaliśmy kilkanaście wpłat składek, bez określenia, kto i za jaki rok wnosi opłatę.

BIURO Polskiego Towarzystwa Mikrobiologów

ul. Banacha 1 b, 02-097 Warszawa WUM Budynek CePT, tel: 22 116 61 77,
fax: 22 116 61 96, e-mail ptm.zmf@wum.edu.pl, www.microbiology.pl
https://www.facebook.com/Polskie-Towarzystwo-Mikrobiolog%C3%B3w-246114759137556/

CZŁONKOWIE WSPIERAJĄCY
POLSKIE TOWARZYSTWO MIKROBIOLOGÓW

Członek Złoty Wspierający PTM
– od 27.03.2017 r.

HCS Europe – Hygiene & Cleaning Solutions

ul. Warszawska 9a, 32-086 Węgrzce k. Krakowa
tel: (12) 414 00 60, 506 184 673, fax (12) 414 00 66
www.hcseurope.pl; NIP 675-12-63-252
Dyrektor Generalny: Mateusz Jurczyk

Firma projektuje profesjonalne systemy utrzymania czystości i higieny dla klientów o szczególnych wymaganiach higienicznych, m.in. kompleksowe systemy mycia, dezynfekcji, osuszania rąk dla pracowników służby zdrowia, preparaty do dezynfekcji powierzchni dla służby zdrowia, systemy sterylizacji narzędzi.

INFORMACJA O KONFERENCJACH WSPÓŁORGANIZOWANYCH PRZEZ POLSKIE TOWARZYSTWO MIKROBIOLOGÓW ORAZ Z PATRONATEM PTM W 2017 r.

Konferencja Naukowa
90 lat Polskiego Towarzystwa Mikrobiologów,
PTM wczoraj – dziś – jutro

Kraków, 22–23 września 2017 r.

Komunikat I

Konferencja Naukowa Polskiego Towarzystwa Mikrobiologów organizowana jest przede wszystkim z okazji 90. rocznicy powołania naszego Stowarzyszenia. Jednocześnie w 2017 roku przypada rocznica 160-lecia urodzin Ojca mikrobiologii polskiej profesora Odona Bujwida oraz 130-lecia wygłoszenia przez niego w Krakowie słynnych pięciu „odczytów o bakteryjach". W 2017 roku upływa także 90 lat od powołania ogólnoświatowego stowarzyszenia towarzystw mikrobiologicznych International Society of Microbiology, obecnie International Union of Microbiological Societies (IUMS), którego współzałożycielem było PTM**.**

Konferencji towarzyszyć będzie Nadzwyczajne Walne Zgromadzenie Delegatów Polskiego Towarzystwa Mikrobiologów, które podejmie uchwały w sprawie wprowadzenia zmian do Statutu PTM.

Tematyka konferencji, poza częścią poświęconą historii polskiej mikrobiologii ze szczególnym uwzględnieniem roli prof. O. Bujwida oraz działalności PTM, będzie również dotyczyła przeglądu osiągnięć naukowych różnych dyscyplin mikrobiologii.

Planuje się zorganizowanie sesji wykładowej, na którą zostaną zaproszeni najwybitniejsi polscy naukowcy z różnych dyscyplin mikrobiologii oraz kilku sesji plakatowych, na których wszyscy mikrobiolodzy, a zwłaszcza Delegaci na Walne Zgromadzenie PTM będą mogli przedstawić wyniki swoich prac.

Planuje się również stworzenie warunków umożliwiających zorganizowanie stoisk wystawowych przez różne firmy działające w obszarze mikrobiologii.

Spotkanie członków Polskiego Towarzystwa Mikrobiologów będzie okazją do poznania początków polskiej mikrobiologii i serologii, do dyskusji, przeglądu sesji plakatowych, do integracji pokoleń mikrobiologów, do rekreacji w czasie spotkań koleżeńskich w uroczym klimacie dawnej stolicy Polski.

Konferencja organizowana jest przez Polskie Towarzystwo Mikrobiologów Oddział w Krakowie i Uniwersytet Jagielloński.

Termin nadsyłania streszczenia plakatu: 01.05.2017 r.
Termin powiadomienia o akceptacji streszczenia: 21.05.2017 r.

Opłaty zjazdowe:

Osoby do 35 roku życia (w tym studenci) członkowie PTM
W terminie do dnia 15.06.2017 r.: 100 zł; w terminie po 15.06.2017 r.: 150 zł

Osoby do 35 roku życia (w tym studenci) nie będący członkami PTM – udział czynny
W terminie do dnia 15.06.2017 r.: 200 zł; w terminie po 15.06.2017 r.: 250 zł

Członkowie PTM
W terminie do dnia 15.06.2017 r.: 300 zł; w terminie po 15.06.2017 r.: 400 zł

Osoby nie będące członkami PTM
W terminie do dnia 15.06.2017 r.: 400 zł; w terminie po 15.06.2017 r.: 500 zł

VI Konferencja Naukowo-Szkoleniowa
MIKROBIOLOGIA FARMACEUTYCZNA 2017
Gdańsk 31.05–2.06.2017

Komunikat I

Szanowni Państwo,

W imieniu Rady Naukowo-Programowej serdecznie zapraszamy do udziału w **Konferencji – Mikrobiologia Farmaceutyczna 2017.** Tegoroczna Konferencja jest organizowana przez **Polskie Towarzystwo Mikrobiologów** i firmę Transpharmacia i jest kontynuacją rozpoczętego w 2009 roku cyklu spotkań naukowo szkoleniowych, którego celem jest stworzenie Forum Specjalistów zainteresowanych mikrobiologią farmaceutyczną, służącego wymianie wiedzy, poglądów i doświadczeń w obszarach oceny i zapewnienia jakości, badań mikrobiologicznych oraz bezpieczeństwa mikrobiologicznego produktów leczniczych..

Podczas naszego spotkania przedstawimy szereg zagadnień istotnych dla Mikrobiologów pracujących w wytwórniach farmaceutycznych, laboratoriach mikrobiologicznych współpracujących z przemysłem farmaceutycznym, wydziałach farmaceutycznych uczelni, a także dla Osób Wykwalifikowanych, Specjalistów Zapewnienia i Kontroli Jakości, Audytorów oraz Osób odpowiedzialnych za współpracę z kontraktowymi laboratoriami mikrobiologicznymi.

Do udziału w konferencji zapraszamy również mikrobiologów z wytwórni kosmetycznych.

Tematem wiodącym konferencji będzie Jakość Badań Mikrobiologicznych, ale również jakość mikrobiologiczna produktów leczniczych i wyrobów medycznych obecnych na rynku. Tradycyjnie już przedstawimy aktualne i planowane zmiany w przepisach, wymaganiach farmakopealnych, oraz nowości w temacie Woda i problemy z nią związane.

Szczegółowe informacje i program zostaną zamieszczone w następnym komunikacie.

Organizatorzy konferencji Mikrobiologia Farmaceutyczna 2017 – Transpharmacia:

Tel. +48 601382182; +48 509710103
e-mail: transpharmacia@transpharmacia.pl

KONFERENCJA BIOMILLENIUM 2017
„Trendy i rozwiązania w biotechnologii i mikrobiologii"
Gdańsk, 6–8 września 2017 r.

Komunikat I

Szanowni Państwo,

W imieniu Komitetu Organizacyjnego mamy zaszczyt zaprosić Państwa do udziału w **Konferencji BioMillenium 2017 – „Trendy i rozwiązania w biotechnologii i mikrobiologii", która odbędzie się w dniach 6–8 września 2017 na terenie Politechniki Gdańskiej w Gdańsku.**

Głównym organizatorem Konferencji jest Katedra Biotechnologii Molekularnej i Mikrobiologii Politechniki Gdańskiej oraz **Gdański Oddział Polskiego Towarzystwa Mikrobiologów.** Współorganizatorami są również Katedra Mikrobiologii i Katedra Immunologii Wydziału Biologii Uniwersytetu Szczecińskiego oraz Gdański Oddział Polskiego Towarzystwa Epidemiologów i Chorób Zakaźnych.

Prezentowane zagadnienia będą podzielone na pięć tematycznych sesji: Biotechnologia: w medycynie, w przemyśle, w ochronie środowiska, Mikrobiologia: kliniczna oraz molekularna

Konferencja rozpocznie się wykładem przedstawiającym sylwetkę, zainteresowania i osiągnięcia naukowe zmarłego w tym roku **Pana Prof. dr hab. Józefa Kura**, założyciela Katedry Biotechnologii Molekularnej i Mikrobiologii Politechniki Gdańskiej oraz naukowca, który swoimi pracami nie tylko wiele wniósł wiele do mikrobiologii i biotechnologii, ale był ich wielkim popularyzatorem.

Przewidujemy wykłady plenarne, prezentacje ustne, sesję plakatową oraz warsztaty praktyczne prowadzone przez firmy. Mamy nadzieję, że prezentacje wyników Państwa badań naukowych pokażą, że biotechnologia nie jest wciąż dziedziną „nauki przyszłości", ale już dziś przyniosła rozwiązania stosowane w wielu gałęziach przemysłu

i w medycynie oraz że mikrobiologia nie jest „skostniałą dziedziną", ale obszarem nauki, w którym zarówno tworzy się, jak i wykorzystuje nowoczesne technologie.

Wierzymy, że Konferencja będzie okazją do nawiązania kontaktów umożliwiających efektywną współpracę.

Wszystkim, którzy wezmą udział w Konferencji, życzymy miłego pobytu w Gdańsku. Szczegółowy program i informacje zostaną przesłane w następnym komunikacie.

Organizatorzy Konferencji BioMillenium 2017
Kontakt: 58 347 24 17 – sekretariat
oraz 58 347 23 83; 58 347 64 12; 58 347 24 06; 58 347 23 02

Mikrobiologia w Ochronie Zdrowia i Środowiska
– MIKROBIOT 2017,
Łódź, 19–21 września 2017 r.

Microbiology in Health Care and Environmental Protection
– MIKROBIOT 2017
Lódź, Poland, September 19–21, 2017.

Komunikat I

W imieniu Organizatorów, Instytutu Mikrobiologii, Biotechnologii i Immunologii Uniwersytetu Łódzkiego oraz **Polskiego Towarzystwa Mikrobiologów** mamy przyjemność serdecznie Państwa zaprosić do wzięcia udziału w **IV edycji konferencji naukowej „Mikrobiologia w Ochronie Zdrowia i Środowiska" – MIKROBIOT 2017**, która odbędzie się **w Łodzi w dniach 19–21 września 2017 r.**

On behalf of the Organizing Committee, The Institute of Microbiology, Immunology and Biotechnology at the University of Lodz in Poland, together with Polish Society of Microbiologists, it is our great honor and pleasure to invite you to attend the 4th edition of scientific conference "**Microbiology in Health Care and Environmental Protection" – MIKROBIOT 2017**. This event will take place **in Lodz, Poland, September 19–21, 2017**.

The goal of the meeting is to provide opportunities for exchange ideas and research experience in the fields of microbiology, immunology and biotechnology, including microbial virulence factors, human immune response to infections, epidemiology, structure and physiology of environmental microorganisms, their use in biotechnological processes and in the removal of environmental pollution, as well as the role of microorganisms in their habitats and their relationships with other organisms.

As in the previous edition of the conference MIKROBIOT, which enjoyed great interest of the scientific community from all over Poland and met with appreciation from foreign visitors, during the upcoming 4th edition of MIKROBIOT 2017 plenary lectures will be given by eminent scientists, among others from Germany, Portugal, France or Italy. The official conference language will be English. During the conference MIKROBIOT 2017 four scientific sessions are provided for:

1. Clinical microbiology and immunology
2. Microbial biotechnology
3. General and environmental microbiology
4. Genetics and genomics of microbes

Konferencja pod patronatem PTM

IX OGÓLNOPOLSKA KONFERENCJA HYDROMIKROBIOLOGICZNA

HYDROMICRO 2017:
DROBNOUSTROJE – OSIĄGNIĘCIA I WYZWANIA

17–19 września 2017, Olsztyn

Szanowni Państwo!

Bardzo serdecznie zapraszamy na IX Ogólnopolską Konferencję Hydromikrobiologiczną, organizowaną w dniach od 17 do 19 września 2017 roku przez Katedrę Mikrobiologii Środowiskowej Wydziału Nauk o Środowisku Uniwersytetu Warmińsko Mazurskiego w Olsztynie. Będzie to dziewiąte ogólnopolskie spotkanie środowisk naukowych związanych z mikroorganizmami ekosystemów wodnych. Poprzednie spotkania odbywały się w: Słupsku-Ustce (2000), Toruniu (2002), Zielonej Górze-Łagowie (2004), Mikołajkach (2006), Warszawie-Wierzbie (2008), Gdańsku (2010), Wrocławiu (2013) oraz Gliwicach (2015).

Tematyka konferencji obejmuje: mikroorganizmy wód śródlądowych, podziemnych i morskich, mikroorganizmy w inżynierii środowiska, sanitarno-bakteriologiczne aspekty oczyszczania ścieków, transmisję mikroorganizmów patogennych drogą wodną, zanieczyszczenia biologiczne w systemach wodnych biobezpieczeństwo i bioremediację wód, diagnostykę organizmów wodnych, mikrobiologię przemysłową i biotechnologię.

Językiem konferencji jest język polski lub angielski Zgłoszenia (w postaci wypełnionej karty zgłoszenia uczestnictwa – do pobrania ze strony www.hydromicro2017.pl należy przesłać do 30 maja 2017 roku na adres: hydromicro@uwm.edu.pl

Streszczenia w języku polskim lub angielskim przygotowane wg schematu zamieszczonego na stronie Konferencji prosimy przesyłać droga mailową do 30 czerwca 2017 roku. Konferencja odbędzie się w Olsztynie, w atrakcyjnie usytuowanym hotelu Omega.

W imieniu Komitetu Organizacyjnego
Dr hab. Zofia Filipkowska, prof. UWM

Konferencja pod patronatem PTM

IV EDYCJA KONFERENCJI
„WEKTORY I PATOGENY – W PRZESZŁOŚCI I PRZYSZŁOŚCI"

Wrocław, 24 listopada 2017

Szanowni Państwo,

Instytut Genetyki i Mikrobiologii Uniwersytetu Wrocławskiego oraz **Wrocławski Oddział Polskiego Towarzystwa Mikrobiologów** i Wrocławski Oddział Polskiego Towarzystwa Parazytologicznego zapraszają na IV edycję konferencji pt. *„Wektory i patogeny – w przeszłości i przyszłości"*.

Konferencja ma na celu prezentację badań z zakresu mikrobiologii i parazytologii jakie są prowadzone aktualnie w krajowych jak i zagranicznych jednostkach naukowych. Pragniemy również udokumentować historyczny dorobek polskich naukowców w tych dziedzinach. W tym roku szczególną uwagą objęty będzie problem uwarunkowanych środowiskowo chorób infekcyjnych i inwazyjnych, których czynnikami etiologicznym są patogeny transmitowane przez stawonogi (wektory), głównie hematofagiczne kleszcze i komary, a także ukazanie skutecznych sposobów zapobiegania i monitorowania tych zagrożeń.

Podczas konferencji planowana jest prezentacja praktycznych osiągnięć 20-letniej współpracy Instytutu Genetyki i Mikrobiologii UWr z Wydziałem Środowiska i Rolnictwa Urzędu Miasta Wrocławia w zakresie biologicznego (mikrobiologicznego) zwalczania komarów na terenie Aglomeracji Wrocławskiej. Ważnym celem konferencji jest także integracja środowiska naukowego oraz ukazanie osiągnięć naukowych młodych adeptów nauki.

Szczegółowe informacje zamieszczone są na stronie: http://www.mikrobiologia.uni.wroc.pl

Organizator: Uniwersytet Wrocławski Instytut Genetyki i Mikrobiologii

Miejsce: Uniwersytet Wrocławski Instytut Genetyki i Mikrobiologii
ul. Przybyszewskiego 63/77, 51-148 Wrocław

Konferencja pod patronatem PTM

7 MIĘDZYNARODOWA KONFERENCJA WEIGLOWSKA

Lwów, 26–29 września 2017 r.

http://cellbiol.lviv.ua/2017/

Rudolf Weigl był wybitnym polskim mikrobiologiem austriackiego pochodzenia. Pracował w czasach międzywojennych we Lwowie (dzisiejsza Ukraina) w dziedzinach mikrobiologii lekarskiej, parazytologii, immunologii oraz biotechnologii, opracowując przy tym pierwszą skuteczną szczepionkę przeciwko durowi plamistemu.

Mikrobiolodzy polscy i ukraińscy od 2003 r. organizują naprzemiennie w Polsce i Ukrainie dwustronne (później międzynarodowe) konferencje Weiglowskie w dziedzinie mikrobiologii i dyscyplinach pokrewnych. Poprzednie konferencje Weigowskie odbywały się we Lwowie (2003), Warszawie (2007), Odessie (2009), Wrocławiu (2011), Czerniowcach (2013) oraz w Gdańsku (2015). Kolejną, **7 konferencję Weiglowską** zaplanowano zorganizować we Lwowie, mieście, gdzie R. Weigl pracował.

Główne tematy konferencji to mikrobiologia ogólna, mikrobiologia lekarska, mikrobiologia środowiskowa, immunologia oraz biotechnologia. Konferencja odbędzie się w przepięknym Lwowskim Budynku Uczonych. Głównymi uczestnikami konferencji będą mikrobiolodzy ukraińscy i polscy, jednak przewiduje się także udział naukowców z innych krajów (Austria, Francja, Niemcy, Szwecja, Włochy, USA, Japonia). W konferencji weźmie udział około 250 uczestników. Oprócz programu naukowego, przewidziane są: wycieczka po Lwowie, koncert, recepcja oraz na życzenie – bankiet, spektakl w Operze Lwowskiej, a także wycieczka do pobliskich zamków. Konferencja przyczyni się do nawiązania bliższych kontaktów z naukowcami z Ukrainy oraz wzmocnieniu przyjacielskich stosunków między naszymi narodami.

Planuje się, ze opłata konferencyjna wyniesie 140 Euro oraz 110 Euro dla młodych naukowców. Koszty obejmą 3 obiady, recepcję, 5 poczęstunków podczas przerw na kawę, wycieczka po mieście oraz materiały konferencyjne. Dodatkowo płatne będą bankiet (około 40 Euro) oraz spektakl w operze Lwowskiej (10 Euro). Po konferencji można będzie odwiedzić zamek Oleski (1 dzień), Poczajow i Krzemieniec (1 dzień) lub Kamieniec Podolski (2 dni), wycieczki te są płatne dodatkowo.

7TH INTERNATIONAL WEIGL CONFERENCE (LVIV, SEPTEMBER 26–29, 2017)

Rudolf Weigl was the outstanding Polish microbiologist of Austrian descent. He worked in the interwar period in Lviv (contemporary Ukraine) in the fields of medical microbiology, parasitology, immunology and biotechnology and created the first successful anti-typhus vaccine. Polish and Ukrainian microbiologists started in 2003 joint initiative to organize in turn in Ukraine and Poland bilateral (later, international) Weigl conferences in the fields of microbiology and related disciplines. Previous Weigl conferences took place in Lviv (2003), Warsaw (2007), Odessa (2009), Wroclaw (2011), Chernivtsi (2013) and Gdansk (2015). The next, 7th International Weigl conference is planned to organize again in Lviv, the city, where he did his discoveries. The main topic of the conference will be general microbiology, medical microbiology, environmental microbiology, immunology and biotechnology. The conference is planned to be held in the beautiful House of Scientists. Main participants of the conference will be Polish and Ukrainian microbiologists, cell biologists and immunologists; however, participation of the scientists from many other countries is expected. Size of the conference is limited by 250 participants. In addition to scientific program, Lviv city tour, concert, get-together and optional banquet, visiting Lviv Opera House and the tours to neighboring castles are planned. Conference has to develop existing contacts and establish the new ones between Polish and Ukrainian scientists.

http://www.cellbiol.lviv.ua/2017
Ukrainian Society of Cell Biology

7th International Weigl Conference
September 26-29, 2017
Lviv, Ukraine

For the attention of specialists in the field of microbiology, biotechnology, immunology.

Institute of Cell Biology National Academy of Sciences of Ukraine, All-Ukrainian Public Organization "Ukrainian Society of Cell Biology" inform you about:

7th International Weigl Conference that will be held on September 26–29, 2017 in Lviv, Ukraine, in the main building of Ivan Franko Lviv National University.

List of planned sessions:

1. Microbial cell biology.
2. Microbial biotechnology.
3. Environmental microbiology.
4. Metabolism and regulation.
5. Medical microbiology.
6. Immunology.
7. Microbe-host cell interaction.
8. Microbial genetics.

Participation of leading foreign and ukrainian scientists is expected in the Conference.
Working language – English.

Conditions of participation:
Deadline for Abstract submission – **May 31, 2017**.
Registration form and abstracts should be filled and sent to the conference e-mail:
weigl2017@gmail.com

Early bird registration & payment (till May 31, 2017): 110 € / 90 €.
Materials received after the deadline will not be accepted. Each person may submit up to 3 abstracts.
Invitation letters to the participants of the conference will be sent by email before 1st September 2017.

CONTACT INFORMATION OF ORGANIZING COMMITTEE REPRESENTATIVES

Address:
Institute of Cell Biology, NAS of Ukraine, Drahomanov Street, 14/16, Lviv, 79005 Ukraine.
Dmytruk K.V., Head of Secretariat PhD, Senior Scientist
Phone: 00 380 32 261 21 63;
FAX: 00 380 32 261 2108,
e-mail: dmytruk@cellbiol.lviv.ua // dmytruk77@gmail.com

Barska M.L., Executive Secretary of Ukrainian Society of Cell Biology, PhD,
Phone: 00 380 32 261 2142;
FAX: 00 380 32 261 2108,
e-mail: barska@cellbiol.lviv.ua

Polish Journal of Microbiology
2017, Vol. 66, No 1

Instruction for Authors

SCOPE

Polish Journal of Microbiology (*PJM*) publishes original research articles describing various aspects of basic and applied microbiological research. We are especially interested in articles regarding

- basic biological properties of bacteria and archaea, viruses, and simple eukaryotic microorganisms
- genetics and molecular biology
- microbial ecology
- medical bacteriology and public health
- food microbiology
- industrial microbiology
- bacterial biotechnology

A manuscript of the original publication (full length paper and short communication) submitted to *PJM* must present reports of original research that have not been previously published and are not being considered for publication elsewhere. All authors of manuscripts are responsible for their content and it is assumed that they all have contributed substantially to the presented research, read and approved the manuscript. Mini-reviews in areas of particular interests and importance in microbiology are also published.

HOW TO SUBMIT A MANUSCRIPT

Manuscripts should be submitted at http://pjm.indexcopernicus.com/. The system requires one-time registration. The selected login and password allows future submission of new articles as well as checking the status of the editorial processing of a manuscript already submitted. Manuscript will be assigned a reference number, that should be used in all future correspondence with the editorial office. After manuscript registration, all correspondence to the editorial office should be directed via internal messaging system available throughhttp://pjm.indexcopernicus.com web page. In case of technical problems with the electronic submission, please contact directly Index Copernicus at office@ indexcopernicus.com

ARTICLE PAYMENT CHARGES

All articles require the payment of an Article Publication Charges (APC)

Publication fee for all types of articles is 250 USD, or equivalent in polish zloty (PLN) for authors from Poland. Fee for members of Polish Society of Microbiologists is reduced to 125 USD. For reduced fee, at least corresponding author must be a member of Polish Society of Microbiologists, what should be stated in a cover letter and Polish Society of Microbiologists membership fee must be paid in full.

When the article is accepted for publication, corresponding author will receive pro forma invoice, and manuscript will be processed for printing and online publication only after payment.

FORMAL REQUIREMENTS (BASIC INFORMATION)

To ensure fast and efficient cooperation with the editorial office and editors, the submitted manuscript should be formatted according to details listed below and accompanied by cover letter.

Manuscript files

Manuscript text, tables and figures should be submitted as separate files. Manuscript should be prepared in editable text files (.doc, .docx or .rtf). Literature should be formatted in author-date format described below in details. Figures must be prepared in ready to print size, format (.tiff with LZW compression) and required resolution.

The editorial office does not accept figures embedded in text, only as separate .tiff files, resolution 300 (for photographs), 600 dpi for combination art (lettering and images) and 1200 dpi for line art (graphs, vectors). That means that figure must be at least 1800/3600/7200 points wide depending on the graphic type.

For instructions on creating acceptable .tiff files, refer to the Cadmus digital art website Cadmusdigital art.

As an alternative our graphics department offers professional service and can prepare figures. However, the additional service is 50 USD for single panel figure.

There is no charge for figures' publication in greyscale, but there is for color illustrations.

Cover letter

The manuscript must be accompanied by a cover letter that should include:

- A title, a running title (about 50 characters in length, including spaces), author's names (format: Name, Initial, Last name – for example Anna E. Smith) and key words (maximum 5 in alphabetical order)
- Impact of the presented research
- Brief description of the results and major conclusions of the manuscript
- Postal address, phone and fax numbers and e-mail of the corresponding author who is responsible for all correspondence regarding the submitted paper
- To ensure rapid manuscript review authors should present list of 5 putative reviewers, with their full affiliations. Authors should choose specialists in their field, however they should not be co-authors within last five years or share affiliation

- Statement that all authors read and accepted the manuscript and the statement about conflict of interests.
- Statement that the manuscript has not been published elsewhere, or is not in processing by any other journal

Submissions without cover letter will be not processed.

Language editing

The manuscript should be written in English. Grammar, syntax and spelling must be carefully checked before submission of the paper. Authors who are unsure of proper English usage should have their manuscript checked by someone proficient in both the English language and biological terminology. Manuscripts may be rejected on the basis of poor English or lack of conformity to accepted standards of style before the review process.

Manuscript processing

All manuscripts are subjected to pre-screening by the Editor in Chief and can be rejected at submission stage or returned for corrections before evaluation (if they do not meet the criteria given in the Instruction to Authors, including language quality or are out of the scope of *PJM*). After passing the pre-screening stage the manuscripts are assigned to one of the section editors and sent to at least one qualified outside referee, but the editors themselves may also act as reviewers if they are experts in the presented topic.

When the manuscript is accepted for publication the transfer of copyright to the Publisher takes effect. The articles are generally printed in no more than three months after returning the corrected version and obtaining its final acceptance. The articles are usually printed within three months after final acceptance or assigned DOI number and published online ahead of print while waiting for print.

The entire process of review and manuscript preparation is carried out electronically. All messages to the editor must be sent via internal messaging system available on the http://pjm.indexcopernicus.com/ web page after login. The author can also track the status of the manuscript using journal submission system.

Preparation of Manuscripts – Regular paper/Minireview

The manuscript of the full length original paper in general:

- Should not exceed 30 typed pages (up to 1800 characters per page) including tables and figures
- Be typed in 12 points Times New Roman font
- Every portion of the manuscript should be double-spaced

No part of the manuscript should be underlined and written using capital letters. Acceptable text formatting should be only restricted to:

All caps:

- authors names, for example ANNA SMITH, JOHN BROWN and ANDREW SHEPARD

Bold:

- manuscript title, for example **The Oral Microbiome in Dental Caries**
- Subheadings **(Abstract, Introduction, Experimental, Materials and Methods, Results, Discussion, Acknowledgments, Literature)**
- Authors names in the literature list for example **Robert Y. and A. Sheiham.** 2002. The burden of restorative dental treatment for children in Third World countries. *Int Dent J* 52: 01–09.

Italics:

- Microorganisms names, for example *Escherichia coli*, *E. coli*
- Latin expressions, for example *in vivo*, *in vitro*, *et al.*, *etc.*, *e.g.*, *via*
- journal title abbreviations, for example *J. Bacteriol.*, *Int Dent J*;

Others:

- genetic *loci*, for example *rep*A, *car*O;
- antibiotic resistance determinants such as $bla_{\text{OXA-23}}$, $bla_{\text{OXA-51}}$, $bla_{\text{OXA-66}}$;
- Proteins: OXA-23, RecA;
- Others, for example LD_{50}, LC_{90};

The full length paper should be divided into the following sections written in sequence: **Title, Abstract, Introduction, Experimental: Materials and Methods, Results, Discussion, Acknowledgments, Literature.**

Title

The title should briefly describe the contents of the paper. Below the title, the manuscript should include full name (including first name and middle initial) of each author and affiliations of all authors. Street address, telephone number and e-mail address should be given only for the corresponding author and placed in the footnote at the bottom of the first page. Under the authors affiliations proposed running title should be included (50 characters including spaces)

Abstract

Limit the abstract to 250 words or fewer. Because the abstract will be published separately by abstracting services, it must be complete and understandable without reference to the text. It should be written in an impersonal form. Abbreviations, diagrams and references are not allowed.

Key words

Five keywords or short phrases should be given below the abstract. If names of microorganisms are used, they should precede the key words, and be followed by the latter in alphabetical order. All keywords should be relevantly connected with the subject matter (avoid common terms like: bacteria, medium, soil, temperature *etc.*) as they will be used for indexing purposes.

Introduction

The introduction should provide background information to allow the reader to understand and evaluate the

results of the present study and describe the purpose of the undertaken research. However, broad "academic lectures" on the subject and extensive literature reviews should be avoided.

Experimental

Materials and Methods

This section should contain description of materials (biological and others) used and sufficient technical information so that the experiments can be repeated. For commonly used materials and methods (*e.g.* commonly used media, protein determination) a simple reference is sufficient. Novel or modified procedures should be described in detail.

When a large number of microbial strains or mutants were used in a study, include strain tables identifying the sources and properties of the strains, mutants, bacteriophages, plasmids, etc.

In the unit description, the space should be put between the number and the unit (2 mM NOT 2mM). Units should be given in SI system, however, for practical reason:

- For a liter designation "l" not "L" should be used (1 l NOT 1 L, 20 mg/ml NOT 20 mg/mL)
- In the description of centrifuging conditions, the value should be presented rather in "g" not in "rpm" (for example 20 000 × g)

Latin species names are written in full the first time the name appears in text; subsequently, only use the first letter of the genus name followed by the species epithet (*e.g. Escherichia coli*, then *E. coli*).

Results

In the **Results** section, only the results of the experiments should be include; reserve extensive interpretation of the results for the **Discussion** section. When justified by the nature of the paper the **Results** and **Discussion** sections may be combined into **Results and Discussion** chapter. The results should be presented as concisely as possible and illustrated with tables or figures if applicable (presentation of the same results in both tables and figures is not acceptable). The use of graphs to present data that might be more concisely presented in the text should be avoided and illustrative materials must be limited to those that are absolutely necessary to demonstrate the original experimental findings. Number figures (Arabic numerals) and tables (Roman numerals) in the order in which they are cited in the text, and be sure to cite all figures and tables.

Tables. Each table must be typed on a separate page and numbered with a Roman numeral (Table I, *etc.*) in the order it is cited in the text. The headings should be sufficiently clear so that the data will be understandable without reference to the text. Explanatory footnotes are acceptable. Prepare your tables as simple as possible in text file, not in Excel. No "decorative" frames should be made.

Figures (graphs, diagrams etc.) should be submitted ready for reproduction, each in a separate file. Files should have size and resolution as intended by authors and must be saved in tiff format (with LZW compression). Proper resolution is 300 dpi for greyscale and colour, 600 dpi for combination art (lettering and images), and 1200 dpi for line art. Instruction how to prepare figures is available at Cadmusdigital art.

Figures should be numbered as in the text (Arabic numerals, Fig. 1., *etc.*) and marked with the name of the first author. Figures should be understandable without referring to the text. Original recorder tracing (outprints) of NMR, IR, ESR spectra *etc.* are not acceptable for reproduction; they should be redrawn. Figure titles and legends must be listed on a separate page.

Black and white figures are free of charge. For current colour pages charge, please contact editorial office at editorial.office@pjmonline.org.

New nucleotide sequences must be accompanied by the accession number obtained from proper databases and should be included in the text.

Discussion

The **Discussion** should provide an interpretation of the obtained results in relation to previously published work and to the experimental system at hand and should not contain extensive repetition of the **Results** section or reiteration of the introduction. In some papers the Results and Discussion can be combined into one section as mentioned above.

Acknowledgements

Acknowledgements for financial support and for a personal assistance (with the permission of person named) are given in two separate paragraphs below the main text.

Literature

In text, references should be cited by the names of the authors and the year of publication, e.g. "Nowak and Kowalski (1999) stated that..."; "as previously described (Nowak and Kowalski, 2000; Nowak, 2005)". When a paper has more than two authors, the first author's name should be followed with *et al.* and the year of publication, *e.g.* Nowak *et al.*, 2002. While references occur that are not identified by the authors' names and year, use a, b *etc.* after the year (Nowak *et al.*, 2002a; 2002b).

The list of the papers cited (Literature) must be arranged alphabetically according to the last name of the first author and not numbered. Papers with one only author are listed in chronological order (the earliest first); papers with two authors are listed alphabetically according to the last name of the first author and by the last name of the second author; paper with three or more authors appear as those with two authors and are listed chronologically. When the paper has more than ten authors, give the names of the first ten, followed by "and others". For citations of books, books chapters, thesis, printed conference proceedings etc. see examples given below. Please follow the punctuations, brackets, capital letters etc. exactly as shown. Put "and", not "&" before the last author's name.

Examples

Books and bookchapters

- **Last name Initial., Initial. Last name and Initial. Last Name**. Year. *Title*, edition. City

For example:

- **Sambrook J., E.F. Fritsch and T. Maniatis.** 1989. *Molecular Cloning; a Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- **Snyder L. and W. Champness.** 2003. *Molecular Genetics of Bacteria*. 2nd ed. ASM Press, Washington, D.C.
- **Funnel B.E. and G.J. Phillips** (eds). 2004. *Plasmid Biology*. ASM Press, Washington, D.C.
- **Belfort M., V. Derbyshire, M.M. Parker, B. Cousineau and A.M. Lambowitz**. 2004. Mobile introns: pathways and proteins, pp. 761–783. In: Funnel B.E. and G.J. Philips (eds). *Plasmid Biology*. ASM Press, Washington, D.C.

Journal articles

For one author:

- **Last name Initial.** Year. Title. *Journal Name* volume: pages.
  For example **Eckhardt T.** 1978. A rapid method for identification of plasmid desoxyribonucleic acid in bacteria. *Plasmid* 1:584–588.

For two authors:

- **Last name Initial. and Initial.Last name**. Year. Title. *Journal Name* volume: pages.
  For example **Sołyga A. and D. Bartosik.** 2004. Entrapment vectors – how to capture a functional transposable element. *Pol J. Microbiol* 53: 139–144.

For 3–10 authors:

- **Last name Initial., Initial. Last name and Initial. Last name**. Year. Title. *Journal Name* volume: pages.
  For example **Bartosik D., M. Szymanik and J. Baj**. 2004. Identification and distribution of insertion sequences of *Paracoccus ventivorans*. *Appl. Environ. Microbiol*. 69: 7002–7008.

For morethan 10 authors:

- **Last name Initial., Initial. Last name**, **Initial. Last name**, **Initial. Last name**, **Initial. Last name**, **Initial. Last name**, **Initial. Last name**, **Initial. Last name**, **Initial. Last name**, **Initial. Last name and others**. Year. Title. *Journal Name* volume: pages.
  For example **Roberts R.J., M. Belford, T. Bestor, A.S. Bgagwat, T.A. Bickle, J. Bitinaite, R.M. Blumenthal, S.K. Degtyarey, D.T. Dry den, K. Dybyig and others.** 2003. A nomenclature for restriction enzymes. DNA methyl-transferases, homing endonucleases and their genes. *Nucleic Acids Res*. 31: 1805–1812.

Articles published in other language than English – title should be translated to English, the original language should be stated in parenthesis.

- **Last name Initial.** Year. Title (in original language) *Journal Name* volume: pages.
  For example **Bartosik D.** 2001. Bacterial plasmids stability (in Polish). *Post. Biochem.* 47: 138–145.

Thesis

- **Last name Initial.** Year. PhD Thesis (optional title) Affiliation. City. Country.
  For example **Szymanik M.** 2006. Ph.D. Thesis. Warsaw University. Warsaw. Poland

Conference proceedings (selected cases)

- **Last name Initial., Initial. Last name and Initial. Last name.** Year. Title. Abstracts of *Conference name*. City, Country, page(s).
  For example **Dziewit L., M. Jazurek, L. Drewniak, J. Baj and D. Bartosik.** 2006. Identification of a novel family of addiction systems. Abstracts of *International Plasmid Biology Conference. Plasmid Biology 2006*. Fallen Leaf Lake, South Lake Tahoe California. USA. p. 163.

Internet articles

- **Names.** Year. Title. WWW address. Access date.
  For example **Pais V.M., T. Santora and D.B. Rukstalis.** 2013. Fournier gangrene. http://emedicine.medscape.com/article/2028899, 2014.08.20.

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