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CONTENTS

INSTRUCTIONS FOR AUTHORS
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Polish Journal of Microbiology
2015, Vol. 64, No 2

Letter From the Editor

Dear Authors,

The mission of Polish Journal of Microbiology is publishing high quality research. Last year we published manuscripts authored by researchers from over 50 countries, covering various aspects of microbiology. All former and current editors and editors in chief worked very hard for many years to increase journal quality, what can be demonstrated by citation index increasing to more than 1 last year.

Our journal has been printed for many years thanks to financial support of the members of Polish Society of Microbiologist and grants from Polish Ministry of Science. To increase the journal circulation in both electronic and printed forms, we maintained open access without any publication fees.

Unfortunately, due to increasing costs of journal production, print distribution, online hosting and archiving we are forced to introduce publication fees. Articles submitted **after 1st of July 2015** are subjected to fees. It means that articles currently submitted, processed, accepted or in preparation for publication are excluded.

Publication fee for all types of articles is **250 USD**, fee for members of Polish Society of Microbiologists is reduced to **125 USD,** or equivalent in polish zloty (PLN) for authors from Poland.

All inquiries regarding fees can be directed to our editorial office editorial.office@pjmonline.org

Editor in Chief
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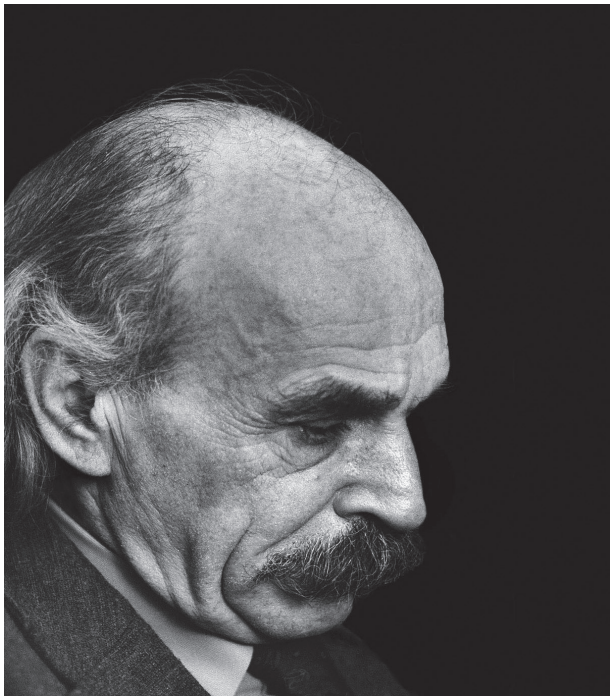

The 16th conference in the series

"*Molecular biology in the diagnostics of infectious diseases and biotechnology* – DIAGMOL 2015"

will be held on the 28th of November 2015,
in the Crystal Hall of the WULS-SGGW in Warsaw

This year's conference will be devoted to the memory of professor Władysław Kunicki-Goldfinger, on the 20th anniversary of his death.
The program of the first conference session will embrace memories about the Professor's life and accomplishments, presented by his colleagues, collaborators and students.

The faculty of the Department of Bacterial Genetics,
Institute of Microbiology, University of Warsaw

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MINIREVIEW

Acyldepsipeptide Antibiotics – Current State of Knowledge

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Abstract

The objective of this paper is to review and summarize the antimicrobial efficacy of the acyldepsipeptides and to indicate the prospects of the therapeutic values of these compounds. This work is enriched by the description of the mutations within the *clpP1clpP2* and *clpP3clpP4* operons of *Streptomyces lividans*, which are considered to be the potential mechanism of the acyldepsipeptide (ADEP) – resistance development. The researchers' conclusions demonstrated a significant impact on microorganisms including the destabilization of bacterial cell division in *Bacillus subtilis* 168, *Staphylococcus aureus* HG001 and *Streptococcus pneumoniae* G9A strains. The results of animal studies show higher bactericidal effectiveness of the acyldepsipeptides ADEP-2 and ADEP-4 compared to linezolid. ADEPs may be considered as a very important mechanism of defense against the increasing resistance of microorganisms . They also might prevent or reduce the risk of many epidemiological events.

Key words: acyldepsipeptides, caseinolytic proteases A/X, drug resistance, Hsp100 proteins, microtubule, organization, proteins FtsZ

Introduction

The appearance of pathogens resistant to presently used antibiotics is now considered an increasingly serious problem, concerning not only healthcare facilities, but also patients treated for community-acquired infections. Among the main reasons for the growing epidemiological risk are: inappropriately conducted antibiotic treatments, different therapeutic standards in individual countries, the lack of standardized procedures controlling the effectiveness of treatment, overusage of antibiotics *e.g.* in veterinary and agriculture and increased population migrations worldwide. It is estimated that the proportion of nosocomial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) strains increased in the United States' intensive care units from 2% in 1974 to 64% in 2004 (Morell and Balkin, 2010). The last decade has seen the increased spread of vancomycin-resistant enterococci (*Vancomycin-Resistant Enterococcus*, VRE) and multidrug-resistant *Streptococcus pneumoniae* strains (especially PRSP – penicillin-resistant *S. pneumoniae*) considered to be one of the main causes of prolonged hospitalization and increased mortality of patients with respiratory tract infections (Hinzen *et al.*, 2006).

The search for new molecules that exhibit different mechanisms of action than those characteristic for the presently used antibiotics are considered as the main defense line against the growing threat of microorganisms' increasing resistance. In this context, a new class of drugs with perspectives of antimicrobial efficacy may be acyldepsipeptides (ADEP), which interfere with the functioning of the protease complexes formed by ClpA and ClpP (ClpAP) and ClpX and ClpP (ClpXP), regulating the metabolism of proteins in prokaryotic cells.

It has been demonstrated that these compounds modify the enzyme's activity, switching it on uncontrolled proteolysis, resulting in degradation of key factors in terms of proper conduct of cell division. As a result, acyldepsipeptides are a group of compounds of potentially valuable therapeutic bactericidal properties.

Structure and tasks of the caseinolytic proteases (Clp) family

ClpA (Clp **A**TPase-activity subunit(s); subunit A of Clp, which possesses ATPase activities) and ClpX (Clp subunit X) belong to a diverse subfamily of AAA+ proteins (*ATPases Associated with various cellular activities*). These proteins use the energy derived from ATP for conformational changes and participate in numerous enzymatic processes in the cell, *i.e.* DNA replication, protein synthesis, degradation of biological

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membranes, microtubule organization, signal transduction or gene expression regulation (Alexopoulos *et al.*, 2012). ClpA oligomerizes as a single hexameric ring made out of the stacking of its two ATPase domains (Barreiro *et al.*, 2011; Beuron *et al.*, 1998; Truscott *et al.*, 2011). The basic core of so arranged enzyme is the protein ClpP (Clp proteolytic subunit(s); subunit P of Clp, which has the proteolytic active site), of which each of 14 analogical mobile subunits is built from an aliphatic stem, spherical domain and the N-terminal region. It is supposed that such construction of this complex provides the formation of a specific axial channel, responsible for the degradation of selected polypeptides in the interior of ClpP (Barreiro *et al.*, 2011; Beuron *et al.*, 1998; Ortega *et al.*, 2000).

The above model assumes the existence of two types of interaction between the core of this complex and ClpA or ClpX. The first one is related to the presence of a highly conservative region of IGF/L, located on the surface of both discussed cellular proteases. The second one is based on the activity of N-terminal protein loop, located near the axial channel (Lowth *et al.*, 2012; Lupas *et al.*, 1997; Marsault and Peterson, 2011). It should be emphasized that the mobility and specific distribution of subunits and axial channels of the complex is regarded as a protective barrier, which protects complex proteins and peptides containing more than 5–10 amino acids from the access to the interior of ClpP (Szyk and Maurizi, 2006; Thompson *et al.*, 1994; Truscott *et al.*, 2011).

Genetic studies have established that *clpP* gene and genes encoding the AAA+ partners are essential for virulence of *e.g.*, *S. aureus* (Frees *et al.*, 2003; 2005), *Listeria monocytogenes* (Gaillot *et al.*, 2000), *S. pneumoniae* (Kwon *et al.*, 2003; 2004; Robertson *et al.*, 2002) and for viability in *e.g. Mycobacterium tuberculosis* (Raju *et al.*, 2012b; Roberts *et al.*, 2013). According to biochemical studies, mycobacterial ClpP1 and ClpP2 form separate homoheptameric rings which gather into an active ClpP1P2 heterotetradecameric complex (Raju *et al.*, 2012b; Akopian *et al.*, 2012).

Drugs targeting ClpP are not presently in clinical use but they are worth further development.

Enopeptines – acyldepsipeptides precursors

Acyldepsipeptides antibiotics have the ability to connect to the core protein ClpP. The chemical structure of precursor molecules of this class, ADEP-1, is based on a lactone core arranged from five different amino acids with attached side chain (C_7H_9), containing three unsaturated bonds (Alexopoulos *et al.*, 2012; Szyk and Maurizi, 2006; Thompson *et al.*, 1994). The unsaturated bond in $\alpha\beta$ position is considered to be crucial in biological activity, it takes the trans configuration (Hinzen *et al.*, 2006). A similar chemical structure is typical for a group of enopeptine antibiotics, which first representatives A54556A and B, were isolated in 1982. Research showed that ADEP-1 belongs to A54556 complex (Brötz-Oesterhelt *et al.*, 2005). It has been demonstrated that enopeptins, differing from ADEP-1 by side length of chain and the presence of acetylated phenylalanine molecule as well as analogue of serine substituted by nitro group, characterize by effectiveness with regard to certain Gram-negative and Gram-positive bacteria (Koshino *et al.*, 1991; Osada *et al.*, 1991). The above-mentioned authors observed the antibacterial activity of enopeptin A, including MRSA strains, but there was no inhibition to fungi in tested concentrations (Osada *et al.*, 1991).

Acyldepsipeptide antibiotics classification

It has been shown that the activation strength of the bacterial proteolytic system is also determined by the used acyldepsipeptide type. Brötz-Oesterhelt *et al.* (2005) suggest that the precursor of this class of molecules is ADEP-1, identified as one of the products of *Streptococcus hawaiiensis* NRRL 15010 microbial metabolism. In order to receive and identify the subsequent, closely structurally related acyldepsipeptide compounds, this strain was used for the specially prepared fermentation process to yield eight acyldepsipeptides closely structurally related. Then, six of them (marked ADEP-1 – ADEP-6) were qualified for further studies.

Evaluation of *in vitro* properties, and then animal studies have shown that the strength of the bactericidal activity of ADEP-2 and ADEP-4 far exceeds the effectiveness of ADEP-1 in relation to eradication of Gram-negative bacteria, including strains resistant to multiple antibiotics. It was further observed that the described higher activity was determined by prior elimination of the active agent from the cell or addition of compounds increasing the permeability of a biological membrane to the culture medium. Other acyldepsipeptide compounds obtained, did not exhibit the desired biological activity. Brötz-Oesterhelt *et al.* (2005) passed a cell lysate of *B. subtilis* through an ADEP-5 affinity column. Reported effect was only partial, because only one protein reminded specifically bound, which the abovementioned Authors identified as ClpP by N-terminal sequencing. In turn, ADEP-3 (being R-epimer of ADEP-2), due to unfavorable conformational changes within the difluorofenyloalanine moiety shows no binding affinity with the enzyme subunit (Brötz-Oesterhelt *et al.*, 2005).

It is also suggested that the activity of selected acyldepsipeptide compounds may exceed the activ-

ity of other antibiotics showing different mechanisms of action. In *in vivo* studies in rodents infected with *Enterococcus faecalis* the effectiveness of ADEP-2 and ADEP-4 was significantly higher than the activity of linezolid – the compound which makes it impossible to connect to the 30S and 50S subunits of the bacterial ribosome, inhibiting the initial phase of protein synthesis (Colca *et al.*, 2003). Moreover, the use of ADEP-4 (12.5 mg/kg b.w.) for the treatment of sepsis caused by *S. aureus* contributed to a significant increase in survival of experimental animals and reduced the adverse effect of sepsis on parenchymal organs functioning (Brötz-Oesterhelt *et al.*, 2005). Additionally, in comparison with linezolid ADEP-4 was also significantly more effective in *S. pneumoniae* infections (Colca *et al.*, 2003). These reports seem to confirm another study conducted both *in vitro* and in animal models. Hinzen *et al.* (2006) noted that with MIC equal to 0.125 μg/ml of these antibiotics exhibit satisfactory efficacy against *S. aureus* strains. The high efficiency of this class of compounds has also been confirmed with regard to *S. pneumoniae* and *E. faecalis*.

Wolbachia is a Gram-negative bacteria causing inhibition of worm development. There is a limitation in treatment of its infections in children and pregnant/breastfeeding women because antibiotics commonly used in treatment of this infection can be dangerous for abovementioned group of patients. Fighting *Wolbachia* is hard, because although it is a Gram-negative bacterium, it has a non-classical outer membrane. This fact was explored in studies of Schiefer *et al.* (2013), who examined influence of acyldepsipeptides on inhibition of bacterial growth. These authors tested ADEP-1 and its synthetic derivatives – ADEP-2, ADEP-4, and ADEP-5 against *Wolbachia*. Comparing with doxycycline gold standard, they found that ADEP-1 removes *Wolbachia* as good as doxycycline. Less effective ADEP-5 achieved almost 80% reduction, while ADEP-2 had 30% reduction and ADEP-4 had no significant effect on bacteria removal. Furthermore, tests comparing different dilutions of ADEP-1 and doxycycline showed that even at lowest concentration, ADEP-1 was still effective allowing *Wolbachia* removal. In conclusion, the results obtained by Schiefer *et al.* (2013) showed that acyldepsipeptides dysregulated *Wolbachia* ClpP at very promising level.

Acyldepsipeptide – structural modifications

It is well-known that the conformational constraints of macrocylic molecules can be further enhanced by judicious introduction of substituents on the ring (Marsault and Peterson, 2011)

Undoubtedly acyldepsipeptides have antibacterial potential. Many scientists tried to modify their natural structures to obtain synthetic derivatives with higher efficacy. Socha *et al.* (2010) tested two strategies: one involving the replacement of the N-methyl alanine moiety in the peptidolactone with *α,α*-disubstituted amino acids, and the second, involving the replacement of this residue with a substituted pipecolic acid. Scientists decided to optimize the structure of ADEP-4 as they thought it would be a better idea than synthesis of many new compounds. In further research, they have chosen compounds with more promising activity against pathogenic *Staphylococci* and *Enterococci*.

Carney *et al.* (2014a) in their studies related to the fact, that activities of acyldepsipeptides can be improved by replacing certain amino acid constituents in peptidolactone core with more conformationally constrained counterparts. In this study, noncovalent interactions between ADEPs and ClpP are used as the object of observations. Scientists made structural changes by replacing hydrogen to deuterium in peptides taking part in bounding ADEP-ClpP complex. The main hypothesis was confirmed by the data showing that the incorporation of conformationally constrained residues in the peptidolactone has a rigidifying effect on ADEP bounding. ATPases and ADEPs are competing for connecting to ClpP and obtained data showed that ADEP-ClpP bounding is tighter and stronger than ATPase binding. In conclusion, Carney *et al.* (2014a) reported that their modification of ADEP structure led to great improvements in the antimicrobial efficacy of these agents. The authors presented data that confirm replacement of selected amino acids constituents as being a good method to stabilize a bioactive conformation of ADEPs. Furthermore, in recent studies Carney *et al.* (2014b) identified the minimal structural component of the cyclic acyldepsipeptides that exhibits antibacterial activity. This active fragment is N-acyldifluorophenylalanine and it operates *via* the same mechanism of action as ADEPs, it also needs ClpP for antibacterial activity (Carney *et al.*, 2014b). Although having the same mode of action, N-acylfluorophenylalanine fragments are much simpler in structure than the full ADEPs and are also highly amenable to structural diversification.

Structural changes of substituents on the ring are a well known method to enhance the conformational constraints among macrocylic molecules (Marsault and Peterson, 2011). These changes can be made by insertion of small methyl substituents profoundly enhancing the affinity of a large macrocycle for its biomolecular receptor as well as increasing biological activity of the molecule (Carney *et al.*, 2014a). Replacement of hydrogen atoms with methyl groups is commonly used in structure-activity relationship (SAR) studies (Barreiro *et al.*, 2011) but insertion of a methyl group into a ligand typically has deleterious effect or has no

significant influence on receptor-binding properties of modified ligand (Carney *et al*., 2014a). This statement is confirmed by Leung *et al.* (2012) who demonstrated that in 8% of cases the inclusion of a methyl group enhances bioactivity tenfold and only in 0.4% of cases resulted in 100-fold enhancement of bioactivity. Leung *et al.* (2012) revealed also that the highest improvement is made by methyl group's capacity for filling a hydrophobic environment in the receptor, and by this influencing the conformation of the ligand.

The activity of acyldepsipeptides' also differs depending on the structural differences between their particles (Carney *et al*., 2014a). As it has already been stated by Hinzen *et al.* (2006), enopeptin A is more effective than enopeptin B against *S. aureus*, *S. pneumoniae*, *E. faecalis* and *Enterococcus faecium.* Difference between enopeptin A and B is that the first one has a 4-methylproline residue and the other have an unsubstituted proline residue (Hinzen *et al*., 2006). According to study by Carney *et al.* (2014a), scientists confirmed the importance of methyl substituent to acyldepsipeptide peptidolactone. Comparing unsubstituted molecule and either 4-methyl pipecolate or *allo*-threonine substituents in acyldepsipeptide particle. Carney *et al.* (2014a) found that *allo*-threonine have the strongest influence on conformational dynamics, ClpP affinity and bioactivity. Furthermore, inclusion of *allo*-threonine in acyldepsipeptide' peptidolactone improves MIC tenfold while 4-methylproline improves MIC only twofold (Hinzen *et al*., 2006). Despite this, both 4-methylproline and *allo*-threonine substituents lead to acquisition of acyldepsipeptides with lowest MICs ever reported for antibacterial agents (Carney *et al*., 2014a). ADEP-4 is reported as the most potent acyldepsipeptide able to cure *S. aureus* infections in mice and *S. pneumoniae* infections in rats with even greater efficacy than linezolid (Hinzen *et al*., 2006). Structural optimization led to obtaining analogs which have 200-fold lower MICs than ADEP-4 against *S. pneumoniae* and *Escherichia coli*. On this basis, we can speculate that potentially lower and safer doses of modified ADEPs analogues can be as much efficacious or even better than natural acyldepsipeptides (Carney *et al*., 2014a).

The next important issue is the cost of optimized compounds preparation. As it was presented by Hinzen *et al.* (2006), cost of insertion of *allo*-threonine residues into ADEP molecule is less and synthesis is easier than in case of 4-methylproline constituents of ADEP-4. Also, which is very important, observation made by scientists show that peptides with strong transannular hydrogen bonds have enhanced oral bioavailability. All these experiments lead to the conclusion that pharmacological properties of natural products can be improved by rational design of drug particle (Carney *et al*., 2014a).

Effect of ADEP on Clp complex

X-ray studies have contributed to the development of two basic models of activating ClpP under the influence of ADEP. Biochemical studies indicate that ADEPs reprogram ClpP, converting it from a highly regulated peptidase that can degrade proteins only with the aid of its partner AAA+ to an independent and unregulated protease (Lee *et al*., 2010). It is suggested that binding the antibiotic molecule results in disorder of N-terminal structure of these fragment, which in physiological conditions are strongly stabilized by the surrounding hydrophobic groups (Lee *et al*., 2010).

ADEPs prevent formation of the complex between ClpP and ClpA ATPases and activate the independent ClpP core to degrade flexible proteins and nascent polypeptides in the course of translation (Lee *et al*., 2010). On the other hand, the alternative hypothesis says that the consequence of binding ADEP with ClpP is the formation of an open collar directly along the axis of symmetry of the complex core (Li *et al.*, 2010). The assessment of the crystal ADEP-ClpP structure lead to conclusion that ADEPs influence ClpP and reprogram its physiological function. In analysis of Kirstein *et al.* (2006; 2009), ClpP of *B. subtilis* is a monomer which, in presence of ADEP, form higher oligomeric forms, while ClpP on its own is a monomer (Kirstein *et al*., 2006). For *E. coli*, the authors examined its ClpP and drawn a conclusion, that ADEP activates isolated ClpP in the absence of Clp-ATPase to degrade polypeptides but with lower processivity (Kirstein *et al*., 2009). Results from the studies of Brötz-Oesterhelt *et al.* (2005) show that purified native *Bacillus* ClpP is not able to degrade *β*-casein, a model substance for ClpP tests. Examination showed that immediately after addition of ADEP-1 or ADEP-2 a complete casein degradation is triggered, and it occurs in absence of ATP-ases (Brötz-Oesterhelt *et al*., 2005). Furthermore, microscopic examination showed that after addition of ADEP-1 *B. subtilis* started to form long filaments instead of normal cells (Brötz-Oesterhelt *et al*., 2005). Based on above results we can say that mechanism of ADEP's action does not fall into one of the classical target areas, but involves direct or indirect inhibition of cell division (Brötz-Oesterhelt *et al*., 2005).

In the case of *B. subtilis* the conversion of ClpP into protease independent from ATP occurs, whereas in *E. coli* this process is conditioned mainly by stabilization of the whole complex, which allows the protein to penetrate to the metabolic center of the complex (Lee *et al*., 2010; Li *et al*., 2010; Dougan, 2011). Both models, however, indicate that the consequence of attaching the antibiotic is broadening the axial channel conditioned by conformational changes within the ClpP.

The studies conducted by Sowole *et al.* (2013), not only confirm previous assumptions, but also partly shed

new light on the target points for ADEP actions. The study of proteolytic activity of the crystalline ClpP isolated from *E. coli* strains showed that ADEP anchors in the hydrophobic slots of the formed core of the complex, and then stabilizes the N-terminal regions, so they take the conformation allowing broadening the axial channel and access to exposed bacterial proteins, including complex proteins and extended polypeptides that the described action may be dependent on the chemical structure. Li *et al.* (2010) suggest that ADEP long-chain alkyl residues (C_7H_9), which function as a type of action sites stabilized by strong hydrophobic interactions, are responsible for keeping the active ClpP conformation (Lee *et al.*, 2010; Li *et al.*, 2010; Dougan, 2011).

Synthetic ADEP1 derivative, ADEP-4, has been tested for possible activation of ClpP. Conlon *et al.* (2013) presented that ADEP-4 has killing potential to bacteria. Scientists compared activity of different antibiotics and ADEP-4 against *S. aureus* and found that in its ADEP-4 presence ClpP becomes a fairly nonspecific protease having abilities to stop bacterial growth. The fact, that null *clp*P mutants are resistant to ADEP-4 (Brötz-Oesterhelt *et al.*, 2005) was further explored by Conlon *et al.* (2013) and it was suggested that these mutants, despite the resistance to ADEP-4, are more susceptible to killing by many antibiotics. ADEP-4 resistant mutant seemed to be less tolerant to rifampicin and linezolid than the wild-type strain. In fact, when ADEP-4 was paired with rifampicin, this combination eradicated mutant *S. aureus* biofilm to the limit of bacteria detection (Conlon *et al.*, 2013). Even tests with different strains of *S. aureus* (in the cited study strains: SA113, USA300, UAMS-1 and 37) confirmed big efficacy of ADEP-4-rifampicin combination to biofilm reduction.

Interesting observation was made by Lowth *et al.* (2012). Scientists generated a mutant human ClpX (hClpX) in which the mutation resulted in replacing glutamate residue within the Walker B motif of hClpX by alanine. Mutated protein still had the ability to bind ATP and, surprisingly, still was able to activate hClpP for degradation of model unfolded substrates like α-casein (Lowth *et al.*, 2012). Furthermore, the activation of hClpP was also possible in the presence of ADEP. For human ClpP, further experiments are required to determine if ADEP-like molecules can cause protein degradation in mitochondria, but also examination of possible mammalian hClpP-activators existence is necessary (Lowth *et al.*, 2012).

Effect of ADEPs influence on hsp100 proteins

Hsp100 proteins (**h**eat **s**hock **p**roteins with a molecular mass 100 kDa, subfamily of AAA+ proteins) are divided into two classes. Class 1 contains proteins with two AAA+ modules and includes such proteins as Hsp104, ClpB (Clp subunit B) and their distant relatives – ClpA and ClpC (Clp subunit C). Class 2 consists of proteins with one nucleotide-binding domain such as ClpX (Doyle and Wickner, 2009). ClpP creates a proteolytic complex with Hsp100 proteins which is mandatory for the degradation of polypeptides or proteins (Horwich *et al.*, 1999; Sauer *et al.*, 2004; Wickner *et al.*, 1999). Studies conducted on isolated *E. coli* and *B. subtilis*, using fluorescein isothiocyanate-labeled casein confirmed that connecting ADEP-1 and ADEP-2 with ClpP-Hsp100 result in reprogramming of this complex (Horwich *et al.*, 1999; Kirstein *et al.*, 2006; 2008; 2009). Furthermore, ADEPs redirect the ClpP core to uncontrolled and deleterious degradation of unfolded substrates and can cause dissociation of ClpA/ClpP complex (Kirstein *et al.*, 2009). Moreover, localization of ClpC and ClpX was the same in cells treated and untreated with ADEP which suggests that acyldepsipeptides have no influence on localization of Hsp100 proteins (Kirstein *et al.*, 2008; 2009). In consequence, ADEP converts ClpP from a strongly regulated protease presenting high substrate specificity to an unrestrained and destructive proteolytic machinery (Kirstein *et al.*, 2009).

Effect of ADEP on organization of microtubules

The direct consequence of acyldepsipeptide compounds' influence on complex of proteolytic enzymes can also be significant disruption of bacterial cells division. Sass *et al.* (2011) claimed that the use of small doses of ADEP affects the structure of bacteria, which was highlighted in studies conducted on *B. subtilis* 168, *S. aureus* HG001 and *S. pneumoniae* G9A strains. In the case of *S. aureus* HG001 and *S. pneumoniae* G9A bacterial cells swelled to nearly three times larger volume than the original ones. In turn, the *B. subtilis* 168 cells took the shape of long, regular filaments under the influence of ADEP molecules. The changes occurring in the tested microorganisms, allow to assume, that, despite the partial ability to biomass creation, they are completely devoid of regulatory features. The observations using electron microscopy confirmed association of septum formation inhibition and treating cells with ADEP, which makes cells unable to build a normal cell wall (Raju *et al.*, 2012a; 2012b; Roberts *et al.*, 2013; Robertson *et al.*, 2002; Sass *et al.*, 2011). Furthermore, Sass *et al.*, (2011) investigated septum formation also in mutant of *B. subtilis* with a deletion of *clpP* gene in order to determine, whether the inhibition of septum formation was caused by ADEP-induced dysregulation of ClpP. At similar ADEP concentration Sass *et al.* (2011) observed no filamentation and normal cell division which showed crucial role of ClpP for ADEP activity.

Effect of ADEP on FtsZ proteins

Among the factors sensitive to ADEP-ClpP complex proteolytic activity, superfamily of proteins FtsZ (Filamenting temperature-sensitive mutant Z) is also mentioned. These structures, involved in formation of cytoskeleton prokaryotic cells, are homologous to tubuline and are able to hydrolyze GTP (Lan *et al*., 2009). It has been shown that the FtsZ polymerization leads to the formation of ring, which initiates bacterial cell division into two progeny structures. One of the hypothesis assumes that this ring is a specific type of scaffold for other factors, which participate in the formation and maintenance of primary partition (Sass *et al.*, 2011). However, another model suggests, that the complex formation may be related to the activity generated during the FtsZ polymerization of the peptide chains, which contribute to the creation of numerous tensions, compressing opposite cell edges (Kwon *et al*., 2003; 2004; Lan *et al*., 2009).

In vitro studies conducted by Alexopoulos *et al*. (2013), showed that destabilization of cell division leads to the formation of structures with unnatural appearance and modified properties. Additionally, it was confirmed that the exchange of proline to isoleucine within the N terminus of ClpP contributed to the complete loss of proteolytic activity against FtsZ. This confirms that ADEP's action may be closely related to a specific amino acid sequence of the complex. However, such dependencies were not observed in relation to proteins degraded by ADEP-ClpP. Sass *et al*. (2011) proved that, both bovine $\alpha\beta$-tubulin and FtsZ are metabolized in a similar way, and the only differences are associated with different length of peptide chains of final products of this process.

Effect of acyldepsipeptide on *Mycobacterium tuberculosis*

Acyldepsipeptide compounds were also tested for efficacy against *M. tuberculosis.* This bacteria has two homologs of ClpP protease, encoded by two different ClpP operons and marked successively as ClpP1 and ClpP2 (Ollinger *et al*., 2012). Both of these proteases, despite the high structural similarity, have different substrates, which may suggest that their activation entail different consequences for the vital functions of the cell. Studies performed by Compton *et al*. (2013) and Ollinger *et al*. (2012) revealed that overexpression of ClpP1 is well tolerated by *M. tuberculosis* strains in contrast to overexpression of ClpP2; the latter correlates with the appearance of toxic effects. However, on the grounds of fact that ClpP1 and ClpP2 are structural homologs. Ollinger *et al*. (2012) suggest that ADEPs would activate both mentioned ClpPs. These authors showed that acyldepsipeptides (ADEP-1, ADEP-2 and ADEP-3) and two synthetic structural antibiotics analogues of this class (IDR-10001 and IDR-10011) admittedly influence vital functions of *M. tuberculosis,* however, this effect is less distinct in comparison with the impact on more sensitive *S. aureus.* Differences in actions of studied compounds may be depending on increased amounts of lipid compounds in the structure of the mycobacterial cell wall, which also explains their specific mechanisms of resistance. Apart from that, the absence of a *clpP3clpP4* locus in *M. tuberculosis* gives hope for use of acyldepsipeptides as antibacterial drug (Compton *et al*., 2013).

Ollinger *et al*. (2012) reported that although acyldepsipeptides are effective themselves, they must be used in combination with efflux-pump inhibitors to keep low MICs in case of *M. tuberculosis*. Results obtained in their study suggests that verapamil and reserpine are good efflux pump inhibitors for increasing the effectiveness of acyldepsipeptides. It is necessary to conduct a further studies for efflux pump inhibitors development, not only in terms of breaking the resistance of microorganisms but also for the implementation of a potential combination therapy (Ollinger *et al*., 2012).

Potential mechanisms of resistance to ADEP

The first reports on acyldepsipeptides suggest that full development of effective resistance mechanisms is characterized by low probability. Nonetheless, it should be remembered that similar assumptions (as things turned out later, groundless) were put forward when implementing the quinolone treatment. Even today, some authors consider the possibility that the process of developing resistance can occur on the pharmacogenomic level.

Gominet *et al*. (2011) showed that ClpP gene expression regulation is made through the *clpP1clpP2* operon as well as single *clpP5* gene. It is suggested that mutations within these operones may lead to biosynthesis of ClpP forms insensitive to acyldepsipeptides and, in consequence, to come into existence strains resistant to ADEPs. In order to verify the above hypothesis Gominet *et al*. (2011) evaluated the effectiveness of ADEP against mutated *Streptomyces lividans* strains where the mutation of *clpP1* was made to confirm the resistance to acyldepsipeptides. It was revealed that *clpP1* mutants show ClpP1 protease activity, however, it was much weaker in comparison with the wild type strains (Gominet *et al*., 2011). To confirm that the resistance of the *S. lividans clpP1* mutant to ADEP was a consequence of the *clpP1* mutation, Gominet *et al*. (2011) transformed the strain with *pVDC742* plasmid,

carrying the wild-type *clpP1clpP* operon. This multistage process led to full restoration of sensitivity to the acyldepsipeptide compounds (Gominet *et al*., 2011).

Furthermore, it was shown that the mutation within another operon, *clpP3clpP4,* is not identical with receiving ADEP-resistant strain. In practice, *S. lividans* strain, transformed with *pJV41* plasmid that leads to overexpression of *clpP3clpP4,* is still sensitive to ADEP (Gominet *et al*., 2011). It is also assumed that ClpP3 overproduction itself, does not determine sensitivity to ADEP. In this case, the inverse relation is observed – ClpP3 is a factor insensitive to acyldepsipeptide compounds but *clpP3clpP4* mutants retain susceptibility to antibiotics of this class. Gominet *et al*. (2011) suggest also that they tried to overexpress *clpP4* under control of *erm*E promoter and they found that there is no evidence for ADEP resistance in *S. lividans* mutant. Both ClpP4 and ClpP5 proteases have atypical, so-called, catalytic triad (conditioned by the presence of serine, histidine and aspartate) and probably have regulatory, not functional, role and therefore should not be taken into account when determining the detailed mechanisms of resistance to ADEP (Gominet *et al*., 2011).

Summary. Discovering new target points for antimicrobial compounds, along with full knowledge of resistance mechanisms, is considered one of the key points of progress in modern antibiotic therapy. The achievements of researchers described in this paper, indicate that the acyldepsipeptides belonging to the enopeptine class of antibiotics, could be a class of drugs giving perspectives of antimicrobial effectiveness. Activity associated with interference in cellular activity of proteolytic enzymes of Gram-positive bacteria, *in vitro* test results, as well as results of studies carried out in animal models seem to confirm that the new, unique mechanism of action of the described group of compounds based on the impact on ClpP complexes also determine their efficacy against strains resistant to the vast majority of presently used antibiotics (*e.g.* MRSA, *M. tuberculosis*, *B. subtilis*, *S. pneumonia*, *E. faecalis*). Additionally, this mechanism also prevents the rapid emergence of resistance, which, in the optimistic variant, can translate into a significant reduction of epidemiological risk in the coming years.

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MINIREVIEW

Intestinal Microbiota, Obesity and Prebiotics

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Abstract

Over the past few decades there has been a significant increase in the prevalence of obesity in both children and adults. Obesity is a disease that has reached epidemic levels on a global scale. The development of obesity is associated with both environmental and genetic factors. Recent studies indicate that intestinal microorganisms play an important function in maintaining normal body weight. One of the objectives in the gut microbiota research is to determine the role it plays and can it be a reliable biomarker of disease risk, including the predisposition to obesity. This article discusses (1) the role of prebiotics and gut microbiota in maintaining a healthy body weight and (2) potential influence on the gut microbiota in the prevention and treatment of obesity.

Key words: microbiota, obesity, prebiotics, SCFA

Gut microbiota

The colonization of the human gastrointestinal tract begins within a few hours after birth but is not identical in all infants. The initial impact on the microbiota of the digestive system of children is determined by the impact of labor, hospital environment, food, mother/child diseases and drug use (Salminen and Isolauri, 2006). In the early years of life the gastrointestinal tract is colonized by bacteria belonging to the genus *Lactobacillus*, *Staphylococcus*, *Enterococcus*, *Escherichia*, *Enterobacter*, *Bifidobacterium*, *Bacteroides*, *Eubacterium* and *Clostridium* (Moore *et al.*, 2011; Libudzisz *et al.*, 2012). An intensive phase of colonization of bacteria in the human gastrointestinal tract usually lasts until two years of age, after which the child gut microbiota begins to resemble that of adults (Nowak and Libudzisz, 2008). Another change in the composition and quantity of microorganisms is in the elderly. There is a significant reduction in the quantity of bacteria of the genus *Bacteroides* and *Bifidobacterium*, where *Clostridium*, *Eubacterium*, and *Fusobacterium* begin to dominate. This change is related to the increase in the pH of the intestinal tract to approximately 7.0–7.5, which can cause gastrointestinal diseases in the elderly. Although the composition of the intestinal microbial changes during the human life span, in the healthy person it remains quite stable and has a "character of climax" (Nowak and Libudzisz, 2008). Strains of *Firmicutes* and *Bacteroidetes* account for more than 90% of the total population of the intestinal microbiota. At dominate genus level types are obligate anaerobes: *Bacteroides*, *Eubacterium*, *Clostridium*, *Ruminococcus*, *Peptococcus*, *Peptostreptococcus*, *Bifidobacterium* and *Fusobacterium*, as well as facultative anaerobes: *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Proteus*, *Lactobacillus* (Shen *et al.*, 2013).

The gut microbiota have many beneficial functions, among them are: help in digestion; effect on immunity; stimulates the development of microvilli; fermentation of dietary fiber and prebiotics that are very beneficial to the human body short-chain fatty acids (SCFA) (butyric, propionic and acetic acids) as well lactic acid. Microbiota may play a beneficial role in the metabolism of potentially harmful substances such as cholesterol, nitrosamines, heterocyclic amines and bile acids (Neish,

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2002; Stewart *et al.*, 2004; Alan *et al.*, 2013). Microbiota may also be a source of antigens and harmful compounds, and even pathogens. The most preferred state for a human is a state of natural balance of microbiota (Everard and Cani, 2013; Walker and Lawley, 2013). Adverse changes to human health caused by the composition of microbiota are referred to as "dysbiosis" (Tamboli *et al.*, 2004; Feng *et al.*, 2010; DuPont and DuPont, 2011). The consequence of dysbiosis may be a leakage of the intestinal barrier and the reduction of the total quantity of SCFA (Clausen *et al.*, 1991). Dysbiosis may precede the clinical manifestations of intestinal diseases and is tied to the occurrence of colorectal cancer and inflammatory bowel diseases. Dysbiosis can also lead to serious systemic disorders (Tamboli *et al.*, 2004; Feng *et al.*, 2010; DuPont and DuPont, 2011).

Influence of diet on correct development of the gut microbiota

Ridaura (Ridaura *et al.*, 2013) found that the intestinal microbiota of lean and obese people induces a similar phenotype in mice, namely, that the microbiota transplanted from a lean individual (donor) causes the decrease of fat in obese mice (recipient) where mice were fed a reduced fat diet (4 wt%) and a high content of plant polysaccharides. In addition, research was done on four pairs of adult female twins, both lean and obese, from which the microbiota was transferred to germ-free mice. In animals that received microbiota from obese people, obesity developed; whereas mice containing intestinal microorganisms from a lean person had normal body weight (Ridaura *et al.*, 2013). Research was also performed to check whether isolates from stool specimens from a slim twin would colonize the intestine of germ-free mice colonized already inhabited by microbiota derived from an obese twin. It turned out that the isolates from the slim twin prevented the development of obesity in germ-free mice with the microbiota from the obese twin. Analysis of the microbiota of these mice showed increased participation of strains of *Bacteroides* in germ-free mice colonized with samples from the slim twin. This indicates that strains of *Bacteroides* and their quantity may have a significant impact on reducing the development of obesity, but it should be noted that it is important to determine not only the genus type but also the species of a given strain. Increased abundance of *Bacteroides* has been correlated with low fat diet that contained higher levels of fruit and vegetables; however, this correlation disappeared when diet proportions of ingredients were reversed (Ridaura *et al.*, 2013; Walker and Parkhill, 2013). It has been shown that bacterial strains derived from slim persons transferred to germ-free obese mice can prevent the formation of obesity when the mice diets consist of fiber, increased amounts of polysaccharides and small amounts of fat (Ridaura *et al.*, 2013). This indicates that the composition of the intestinal microbiota, and its effect on reducing the development of obesity is closely correlated with the consumed diet (Ridaura *et al.*, 2013).

Based on the dominance of certain types of bacteria, Arumugam (Arumugam *et al.*, 2011) has isolated three bacterial enterotypes: *Bacteroides*, *Prevotella* and *Ruminococcus*. The presence of a specific enterotype is not dependent on age, gender, or ethnicity. Wu (Wu *et al.*, 2011) demonstrated that enterotype is dependent on the type of diet. Consuming large amounts of saturated fats and proteins determine the development of enterotype *Bacteroides*, while enterotype *Prevotella* reveals itself in people whose diet consists of high amounts of saccharides and fiber and is low in fats and animal proteins. The type and proportions of the microorganisms present in the gut, *i.e.*, enterotype determines the metabolic products which have important consequences for the host. These metabolites can be either beneficial or harmful. For example, short-chain fatty acids (SCFA) are formed by the fermentation of indigestible polysaccharides in the large intestine by specific groups of bacteria (Archer *et al.*, 2004; Cani *et al.*, 2004; Delzenne *et al.*, 2005; Tarini and Wolever, 2010). SCFA have numerous positive functions and these include: butyric acid that stimulates intestinal epithelial tissue, nourishes the intestinal cells and affects their proper maturation and differentiation; propionic acid has a positive effect on the growth of hepatocytes; acetic acid has a positive effect on the development of peripheral tissues. SCFA regulate glucose and lipid metabolism, stimulate the proliferation and differentiation of intestinal enterocytes, lower pH effect on the intestinal contents, and thus help out in the absorption of minerals by increasing their solubility (Blaut and Clavel, 2007; Lin *et al.*, 2012). It has been shown that in spite of SCFA as a source of energy, it contribute toward reducing the formation of obesity by inhibiting fat accumulation in adipose tissue, increased energy expenditure and increasing production increase of hormones associated with the feeling of satiety (Keenan *et al.*, 2006; Gao *et al.*, 2009; Kimura *et al.*, 2013). Influence of butyric acid on regulation of energy homeostasis of the organism may be associated with stimulation of leptin synthesis in adipocytes, induction of GLP-1 secretion by L cells of intestine and increased fatty acid oxidation (Gao *et al.*, 2009; Nicholson *et al.*, 2012). In examining the influence of metabolites of the gut microbiota on the human body, it has been confirmed that the additional source of energy to the host (human) may be propionic acid used in the synthesis of glucose and lipids (Bates *et al.*, 2007; Cani *et al.*, 2008).

The role of the intestinal microbiota in maintaining normal body weight

In 1998, the World Health Organization (WHO) classified obesity an epidemic on a global scale (WHO Report 2008, WHO Report 2009). In terms of frequency, obesity precedes the occurrence of AIDS and malnutrition. An alarming phenomenon is the growth of this obesity epidemic in children. Until just recently, adipose tissue was considered only as a reservoir of body energy substrate. Today it is known that it is an important part of the endocrine system (Fichna and Skowrońska, 2006). Pathologically increased amounts of fat in the body can result in numerous disorders in the proper functioning of the many different systems, organs and tissues. Particularly dangerous complications may occur in the cardiovascular, respiratory, endocrine, and psychosocial systems. It is estimated that 80% of the diseases in man are caused by problems associated with excessive body weight (Nowak *et al.*, 2010). Statistics predict continuous deterioration of this situation, which is a challenge for the public health sector in many countries of the world (WHO Report 2008; WHO Report 2009). The problem of obesity relates to people of all ages, and the causes have very complex character, from bad habits to environmental impact (to stress and genetic factors). A major problem is the obesity transfer from childhood to adulthood (Fichna and Skowrońska, 2006; WHO Report 2008; WHO Report 2009). Many studies have shown that obesity is also associated with significant changes in the composition and function in metabolism of the intestinal microbiota. It is recognized that a particularly important fact is to keep a correct proportion of *Bacteroidetes* and *Firmicutes* strains in the intestine (Ley *et al.*, 2006; Sanz and Santacruz, 2008). Research teams Bäckhed, Gordon and De Filippo have also indicated that obesity in humans is likely to be related to the composition of the gut microbiota (Bäckhed *et al.*, 2004; Ley *et al.*, 2006; De Filippo *et al.*, 2010). Bäckhed and colleagues determined the share of *Firmicutes* and *Bacteroidetes* in obese mice and mice with normal body weight and found that the proportion of *Bacteroidetes* is significantly lower in obese mice (20%), while in mice with normal weight the bacteria was at a larger amount – up to 40 % (Bäckhed *et al.*, 2004; Bäckhed *et al.*, 2007). In turn, Flessner demonstrated that supplying mice with high animal fat and low fiber diet results in a quantity reduction of *Bacteroidetes* strains, but conversely the growth of *Firmicutes* (Flessner *et al.*, 2010). Studies were carried out on a group of twelve obese humans, who had an increased presence of *Firmicutes* and reduced presence of *Bacteroidetes* from 1 to 5%. After supplying one group's diet with reduced fat content and for others group a diet with decreased portions of saccharides, the proportions of the major groups of microorganisms changed. In both groups' there was a gradual decline in quantity of *Firmicutes* and *Bacteroidetes* increased up to 20% (Ley *et al.*, 2006). In order to determine the relationship between the microbiota and the amount of energy, Jumpertz (Jumpertz *et al.*, 2011) conducted research on a group of 21 volunteers where an interchangeable diet of 2400 and 3400 kcal/day was administered. Fecal microbiota composition was monitored. It showed a 20% growth of *Firmicutes* strains was accompanied by a 20% reduction in the quantity of *Bacteroidetes*, and changes in the proportions of these strains were directly related to gain in body weight. It seems that an important role of gut microbiota is bifidobacteria. It showed that in overweight people and sick people with type 2 diabetes the amount of *Bifidobacterium* was significantly lower (Schwiertz *et al.*, 2010; Wu *et al.*, 2010).

De Filippo (De Filippo *et al.*, 2010) compared the composition of intestinal microbiota in children ages 1 to 6, living in extremely different conditions. The first group of children came from rural areas of Africa (Burkina Faso); and the second group consisted of children from Italy (Florence). The intention of the study was to determine the correlation between the applied diet, and the composition of the intestinal microorganisms. The diet of children living in Africa was low in meat, but contained significant amounts of vegetables, starch and dietary fiber (about 672.2 kcal toddler ages 1–2 years old and 996 kcal children ages 2–6 years old), while nourishment to children from Europe consisted mainly of meat, and their diet contained a lot of animal fats, sugars, but poor in vegetables and fiber (about 1,068.7 kcal children ages 1–2 years old and 1,512.7 kcal children aged 2–6 years old). Regardless of the diet used in the gastrointestinal tract, this study showed that the dominant bacteria types present were *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, but their percentage was different and dependent on diet. In children coming from rural areas of Africa, *Actinobacteria* and *Bacteroidetes* dominated, respectively 10.1% and 73%; while bacteria from the phylum *Firmicutes* accounted for 10%. Within the *phylum Bacteroidetes* the dominant bacteria were *Prevotella* (53%), which indicates the microbiota of these children was mainly enterotype *Prevotella*. In the case of children coming from Florence, increased body weight was found and intestinal microbial system was different than in the case of children from Africa. The dominant bacteria of the phylum *Firmicutes* (51%), and *Actinobacteria* and *Bacteroidetes* were 6.7% and 27% respectively. A high concentration of SCFA, which has been demonstrated in children from Burkina Faso, is an additional source of energy for the host. Despite the low calorie intake, normal development was observed in these children (De Filippo *et al.*, 2010) (Fig. 1).

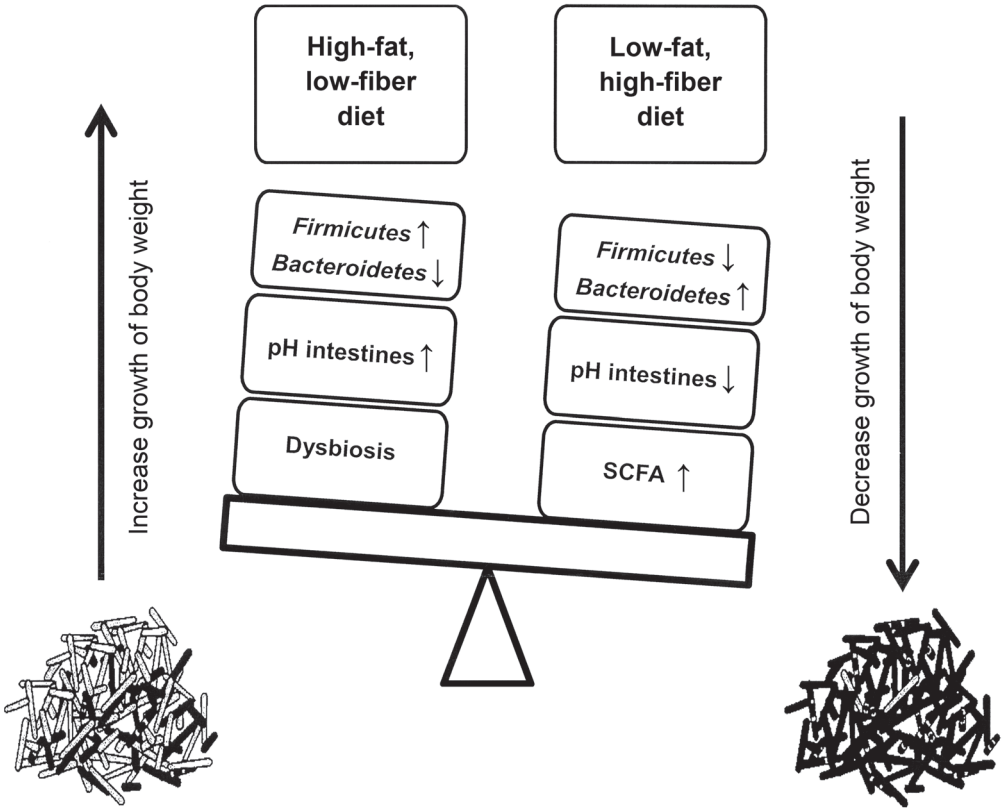

Fig. 1. Effect of diet on the development of gut microbiota and normal body weight (own layout on the basis of Archer *et al.*, 2004; Cani *et al.*, 2004; Delzenne *et al.*, 2005; Tarini and Wolever, 2010).

The gut microbiota vs. obesity – the potential mechanisms

The impact of gut microbiota on the development or slowing down of obesity is not yet fully known. It is believed that obesity is associated with elevated serum levels of lipopolysaccharide (LPS), which is a component of the cell wall of Gram-negative bacteria (Amar *et al.*, 2011a; Amar *et al.*, 2011b). LPS, due to proinflammatory properties, may be involved in the development of inflammation, present in type 2 diabetes. Intravenous administration of lipopolysaccharide in mice resulted in the development of insulin resistance and weight gain. *In vivo* correlation was observed between the increase in plasma concentrations of LPS and the implementation of a high fat diet. Cani (Cani *et al.*, 2007) concluded that fat contained in food may be an important regulator of the concentration of LPS. The introduction of four weeks of high fat diets in mice resulted in a two or even three time increase in plasma levels of LPS (Cani *et al.*, 2007; Tilg *et al.*, 2009). This phenomenon was confirmed in people diagnosed with obesity and type 2 diabetes (Cani *et al.*, 2007; Amar *et al.*, 2011a; Geurts *et al.*, 2011). In the origin of obesity a vital role may be played by intestinal alkaline phosphatase (IAP), which is involved in the degradation of lipids derived from food, and also has an important role in the detoxification of LPS (dephosphorylation of lipid part of LPS). Furthermore, increased activity of the IAP is associated with reduced endotoxemia which is caused by metabolic dysfunctions (Everard *et al.*, 2011). It has been shown that the expression of IAP may be controlled by gut microbiota (Bates *et al.*, 2007). In obese people with type 2 diabetes changes in the intestinal barrier were detected, namely an increase of cellular permeability (Everard *et al.*, 2013). The increase in intestinal permeability was observed in obese mice and can be associated with a change in the expression, localization and distribution of proteins belonging to the tight-junctions of the small intestine (Brun *et al.*, 2007; Cani *et al.*, 2008; Cani *et al.*, 2009; Everard *et al.*, 2012). Another potential factor linking gut microbiota to obesity is blocking the expression of fasting-induced adipose factor (FIAF) by the microbiota. FIAF inhibits the activity of lipoprotein lipase (LPL), an enzyme responsible for the storage of energy in fat. The decreased expression of FIAF determines increased LPL activity and enhances the process of storing energy in the form of fat (Bäckhed *et al.*, 2004). Gut microbiota modulates the activity of the endocannabinoid system and thus has an effect on the function of the intestinal barrier. These studies revealed an important role of the intestinal barrier in the etiology of obesity and Type 2 diabetes (Everard *et al.*, 2013).

Prebiotics

Since gut microorganisms to some extent are responsible for the formation of obesity, modulation of microbiota is seen as a potential tool in the prevention and treatment of disease. It was shown that the growth of beneficial microbiota, and therefore sealing the intestinal barrier and changes in the metabolism of endotoxin in the blood can be modulated by the addition of prebiotics to the diet (Everard *et al.*, 2013).

FAO/WHO defines prebiotic as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already established in the colon, and thus improve the host's health" (FAO Technical Meeting on Prebiotics, Prebiotics, 2007). Prebiotics are not hydrolyzed and absorbed in the upper parts of the gastrointestinal tract and unchanged reach the large intestine where they are nutrients for beneficial bacteria (Kowalska-Duplaga, 2003). Examples of substances having prebiotic properties are fructooligosaccharides, gluco-oligosaccharides, isomaltooligosaccharides, maltooligosaccharides, lactulose, raffinose soy oligosaccharides, stachyose, xylooligosaccharides, and inulin resistant starch (Wang, 2009; Xu *et al.*, 2009). Recently research was conducted to confirm prebiotic properties of new substances such as resistant dextrins derived from potato starch (Jochym *et al.*, 2012). These formulations have a bifidogenic effect and stimulate the growth of gut microbiota, thus limiting the growth of *Clostridium* strains (Barczynska *et al.*, 2010; Barczynska *et al.*, 2012).

Studies conducted on rats and healthy persons confirmed that prebiotics reduce hunger and increase the feeling of satiety (Cani *et al.*, 2007; Parnell and Reimer, 2009). Positive effects of modulation of gut microbiota are: the production of SCFA, increased level of PYY (this peptide is synthesized and secreted by the L-cells of the ileum and colon, and has a stimulant effect on satiety center) and GLP-1, resulting in a reduced glycemic, reduction of insulin resistance, reduced fat cells, and the perception of satiety (Delzenne *et al.*, 2011; Alvarez-Castro *et al.*, 2012; Paranel *et al.*, 2012). Adding to diets a mixture of inulin and xylooligosaccharides resulted in lowering the LPS level in blood plasma (Lecerf *et al.*, 2012).

In a study examining the effects of diet containing large amounts of polysaccharides on the composition of microbiota showed that after four week there was a fundamental change in the composition of the microbiota and its metabolic functions (Duncan *et al.*, 2007; Brinkworth *et al.*, 2009; Russell *et al.*, 2011; Walker *et al.*, 2011; Karen *et al.*, 2013). Adding resistant starch to the diet caused the number of *Ruminococcus bromii* to double (Abell *et al.*, 2008). For 17 weeks 10 volunteers were treated with diets enriched with RS4 resistant starch, and their stool samples were studied by analyzing for the presence of *Bifidobacterium*. It turned out that after a diet consisting of RS4, the amount of these bacteria increased (Abell *et al.*, 2008). Also a reduced amount of *Firmicutes* bacteria was observed, thereby increasing *Bacteroidetes and Actinobacteria* (Martinez *et al.*, 2010). The addition of fructooligosaccharides and inulin mixture (10 g/d) to the diet stimulated of the growth of bifidobacteria, in particular *Bifidobacterium adolescentis* (Ramirez-Farias *et al.*, 2010). It is proposed that the lactate produced by the bifidobacteria can be converted to butyrate by *Eubacterium hallii* and *Anaerostipes caccae* (Duncan *et al.*, 2004; Belenguer *et al.*, 2006; Falony *et al.*, 2006).

Summary. The World Health Organization (WHO) predicts that by the year 2015 the number of obese people in the world (17 years old and over) will rise above 700 million. Obesity is associated with clearly excessive caloric intake compared to low energy outflow. However, the gut microbiota have a key role in the development of adipose tissue and disorders of energy homeostasis (Everard *et al.*, 2012). An important role in maintaining a healthy body weight is to keep the proper proportion of strains of bacteria belonging to the *Firmicutes and Bacteroidetes* phylum (Bäckhed *et al.*, 2004; Bäckhed *et al.*, 2007; Turnbaugh *et al.*, 2008; Hildebrandt *et al.*, 2009; De Filippo *et al.*, 2010; Murphy *et al.*, 2010; Geurts *et al.*, 2011). It is also important not to be limited only to diversify the phylum of bacteria but also take into account the genus of bacteria within the phylum and determine the amount of these bacteria to the appropriate enterotypes of *Bacteroides* and *Prevotella*. Research is being currently being conducted to find the relationship between gut microbiota and metabolic pathways. One of the proposed mechanisms that can be relied on is the ability of the gut microbiota to increase energy from diet. It was also observed that obesity is associated with elevated levels of lipopolysaccharide (LPS) in blood plasma (Amar *et al.*, 2011a; Amar *et al.*, 2011b), but not only elevated levels of LPS in blood plasma because in obesity there is a vital role played by alkaline phosphatase (IAP) (Bates *et al.*, 2007). IAP is involved in the degradation of lipids derived from food, and it also plays an important role in the detoxification of LPS. The next potential factor linking gut microbiota to obesity is caused by blocking the expression of microbiota fasting-induced adipose factor (FIAF) (Bäckhed *et al.*, 2004). Despite extensive research on the role of the gut microbiota in maintaining a healthy body weight, the mechanisms of intestinal microbiota's influence on the development or reduction of obesity is not fully known. It is necessary to carry out research

to determine the impact of intestinal microbiota on the functioning of metabolic pathways on both animal and obese people.

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ORIGINAL PAPER

Characteristics of the *Pseudomonas aeruginosa* PA01 Intercellular Signaling Pathway (Quorum Sensing) Functioning in Presence of Porphyrins Bismuth Complexes

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Abstract

The influence of synthetic and natural porphyrins bismuth complexes on *P. aeruginosa* quorum sensing system was carried out by detection of the pyocyanin, rhamnolipids and autoinducers biosynthesis level. The highest ability to reduce pyocyanin biosynthesis showed Bi(III)-TPP. Rhamnolipids production level also decreased in the presence of studied compounds. This effect was the most expressed in presence of 40 and 80 μM of the synthetic meso-substituted porphyrins. Autoinducers biosynthesis, especially 3-oxo-C_{12}-HSL was suppressed in presence of the bismuth complexes. That suggest that the mechanisms of action of this substances is an inhibition of signaling molecules or/and receptor for them.

Key words: *Pseudomonas aeruginosa* PA01, porphyrins bismuth complexes, *quorum sensing*

Introduction

Today in connection with the high resistance of opportunistic pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *etc.* to traditional antimicrobial drugs, infections that are caused by these bacteria gain high prevalence, especially in patients with various immune deficiencies. Thus, one of the future tasks of modern pharmacology is the search for new antibacterial drugs, which may have inhibitory activity against pathogenic bacteria, especially with non-traditional mechanisms of action. One of the most promising groups of new antimicrobial agents may be compounds that break down bacterial cell-cell signaling pathways (Kociolek, 2009).

Intercellular signaling pathway, known as quorum sensing is a global regulatory mechanism based on the use of small signaling molecules that play a role in gene expression in a bacterial cell population (Bassler, 2002; Brown *et al*., 2001). This mechanism is the basis of many bacterial cell properties such as pathogenicity, and biosynthesis of secondary metabolites. Consequently, studies focused on the regulation of this system, seem to be of promise in biotechnology and medicine.

A quorum sensing system from *P. aeruginosa* (formally an autoinduction system) is based on three families of genes – *las*-, *rhl*- and *pqs*-. Each of these families activates with its own signal molecules: 3-oxo-dodecanoil-homoserine lacton (for *las*- family), butiryl homoserine lacton (for *rhl*- family) and 2-heptyl-3-hydroxy-4-quinolon (for *pqs*- family) (McKnight *et al.*, 2000). *P. aeruginosa* quorum sensing system works based on the binding of signal molecules with specific cytoplasm receptors and "signaling molecules-receptor" complexes formation. These complexes activate the expression of target genes (Winzer and Williams, 2001).

Previously, we demonstrated that synthetic porphyrins and their complexes with metals can possess antimicrobial activity; in particular inhibit bacterial biofilm formation (Galkin *et al.*, 2010). In this study we investigated *P. aeruginosa* PA01 quorum sensing system functions in the presence of synthetic and natural porphyrins bismuth complexes.

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Experimental

Material and Methods

Bacterial strains and growth conditions. *P. aeruginosa* PA01 were obtained from the collection of the microbiology, virology and biotechnology department of Odessa National University named after I.I. Mechnikov.

Bacterial strains were grown on the meat-peptone agar (MPA) and Gis media. For pyocyanin detection, bacterial were strains grown on the PB broth (g/l, peptone – 20; $MgCl_2$ – 1.4; K_2SO_4 – 10).

Chemicals. Synthetic and natural porphyrins bismuth complexes – *meso*-tetra(4-N-methyl-piridyl)porphyrin bismuth complex (Bi(III)-TPP), *meso*-tetra (6-N-methyl-quinolinil)porphyrin bismuth complex (Bi(III)-TQP) and protoporphyrine IX bismuth complex (Bi(III)-PP IX) were synthesized by method (Ishkov *et al.*, 2000) in PLMS-5 of Odessa National University named after I.I. Mechnikov (Fig. 1).

3-oxo-dodecanoyl-homoserine lactone (3-oxo-C_{12}-HSL) and butiryl homoserine lactone (C_4-HSL) standards were obtained from Sigma Aldrich.

2-heptyl-3-hydroxy-4-quinolon (PQS) was synthesized by the method of Somanathan and Smith (1981) in PLMS-5 of Odessa National University named after I.I. Mechnikov.

Cells pre-incubation with discovered compounds. To study the production of pyocyanin and ramnolipids bacterial (2×10^8 CFU/ml) cells were pre-incubated with the test substances in concentrations 0.4; 40 and 80 μM in saline buffer for 1.5 h at 37°C.

Pyocyanin production study. After incubation with porphypins bismuth complexes bacterial cells were washed three times and inoculated to 5 ml of PB broth. Bacterial cells in PB broth were incubated overnight at 37°C. After incubation bacterial cells were removed by centrifugation at 6000 × g for 10 minutes. Pyocyanin from supernatant were extracted and measured by the methods of Essar *et al.* (1990). A 5 ml of culture supernatant were extracted with 3 ml of chloroform. Chloroform layer were transferred to a fresh tubes and re-extracted with 1 ml of 0.2 N HCl. After centrifugation, the top layer was collected and its absorption at 520 nm was measured on μQuant (Bio-Rad) spectrophotometer.

Rhamnolipids production study. For rhamnolipids production study bacterial cells were inoculated to 10 ml of Gis media and were incubated overnight at 37°C. After incubation bacterial cells were removed by centrifugation at 6000 × g for 10 minutes and supernatant were concentrated as follows. The pH of 10 ml of the culture supernatant was adjusted to 6.5, and $ZnCl_2$ was added to a final concentration of 75 mM (Guerra-Santos *et al.*, 1984). The precipitated material was dissolved in 10 ml of 0.1 M sodium phosphate buffer (pH 6.5) and extracted twice with an equal volume of diethyl ether. The pooled organic phases were evaporated to dryness, and the pellets were dissolved in 500 μl of methanol.

The total amount of rhamnolipids was determined using the orcinol assay (Candrasekaran and Bemiller, 1980): 500 μl of the rhamnolipids samples were mixed with 500 μl of an orcinol reagent (0.2 g orcinol, 0.1 g $FeCl_3$ in 100 ml of the 30% HCl). After heating for 20 minutes at 100°C, the samples were cooled for 15 min at room temperature and the OD_{670} was measured on μQuant (Bio-Rad) spectrophotometer.

Autoinducers production study. Level of homoserine lactones synthesis was measured by gas chromatography/mass spectrometry method (Pearson *et al.*, 1995).

Homoserine lactones were extracted from a culture supernatant by ethyl acetate. Organic phase were collected and evaporated to dryness. The pellets were diluted in methanol and purified by HPLC on the C_{18} reverse phases columns in methanol-water gradient.

Gas chromatography/mass spectra were carried out on Hewlett-Packard 5890 with Hewlett-Packard Ultra-1

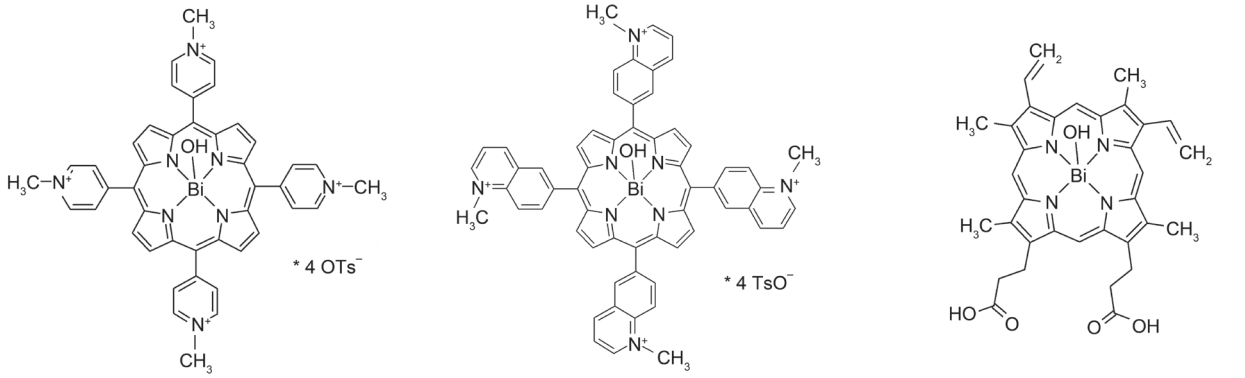

Fig. 1. Porphyrins bismuth complexes, used in the study

capillary column (25 m × 0.2 mm with film thickness of 0.33 μm). Helium as a carrier gas was used. Temperature gradient was at 70 to 240°C with increment by 10°C per minute. Mass spectra were collected by ZAB-HF mass spectrometer (VG Analytical, Manchester, UK) with homoserine lactones standards.

PQS level from culture supernatant was determined by the method of Deziel *et al*. (2004). Ethyl acetate extracts were separated by TLC in dichlormethane-acetonitryl-dioxane mixture (17:2:1) with PQS standards. PQS dotes placement was identified by UV. PQS dots were eluted from TLC plates (ALUGRAM® SIL G/UV_{254}) with ethyl acetate and luminescence of elutes were measured with LUMISTAT at 312 nm.

All experiments were carried out three times.

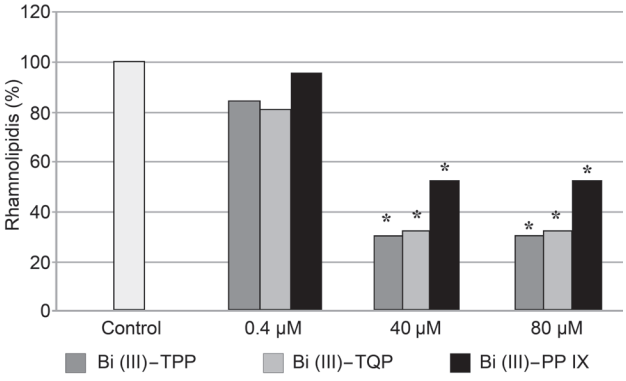

Fig. 2. Rhamnolipids biosynthesis by *P. aeruginos*a PA01 after pre-incubation with porphyrins bismuth complexes
Note: * – significantly different from the control

Results

The influence of synthetic and natural porphyrins bismuth complexes on *P. aeruginosa* quorum sensing system was carried out by detection of the pyocyanin, rhamnolipids and autoinducers biosynthesis level. For these studies bacterial cells were pre-incubated with several concentrations of the synthetic and natural porphyrins bismuth complexes (0.4; 40 and 80 μM). This was done to neutralize the inhibitory activity of used concentrations, which has been shown previously (Galkin *et al.*, 2010).

The study of the biosynthesis of pyocyanin showed that level of this pigment in supernatant of the *P. aeruginosa* PA01 overnight culture decreased in the presence of all concentrations of the compounds studied (Table I).

Determination of the basic pigment level in culture supernatant showed that *P. aeruginosa* PA01 synthesize 6.31 μg per ml of pyocyanin after overnight incubation. After treatment with a 0.4 μM of each compounds the pyocyanin level decreased by a 10%. When higher concentrations were used, the difference in the activity of the studied compounds been observed. After pre-treatment with 40 μM of the Bi(III)-TPP, pyocyanin level decreased by a 38%; Bi(III)-TQP – 30% and Bi(III)-PP IX – 18%. Maximal anti-pyocyanin activity was observed after pre-treatment of *P. aeruginosa* PA01 with an 80 μM of studied compounds. The inhibition of pyocyanin biosynthesis was in case of Bi(III)-TPP for two times, and Bi(III)-TQP and Bi(III)-PP IX – 32% and 25% respectively.

Rhamnolipids production after pre-treatment with synthetic and natural porphyrins bismuth complexes also decreased (Fig. 2). The highest ability to inhibit the synthesis of these metabolites showed Bi(III)-TPP and Bi(III)-TQP. After pre-treatment of the *P. aeruginosa* PA01 cells with 0.4 μM of each compounds the rhamnolipids level in culture supernatant were the same and 80% of the control value. When 40 μM of these compounds were used, rhamnolipids level in the culture supernatant was 32% of the control value, and after pre-treatment with 80 μM – 20 and 23%, respectively. Lowest inhibitory capacity on the rhamnolipids biosynthesis showed Bi(III)-PP IX. Rhamnolipids value after pre-treatment with 0.4; 40 and 80 μM of this compound in overnight culture supernatant was 95, 42 and 52% of the control value, respectively.

The study of the *P. aeruginosa* PA01 quorum sensing autoinducers biosynthesis after pre-treatment with synthetic and natural porphyrins bismuth complexes was conducted in a three time points – after 3, 6 and 24 hours of incubation. Obtained results showed (Table II–IV) that in the control there was a difference in appearance of autoinducers within the investigated time intervals. First the autoinducer, which appeared in the culture medium after three hours of incubation, was 3-oxo-dodecanoyl-homoserine lactone. Butiryl

Table I
Pseudomonas aeruginosa PA01 piocyanin biosynthesis level in presence of the synthetic and natural porphyrins bismuth complexes, μg/ml

Compound	Control	Porphyrins bismuth complexes concentration		
		0.4 μM	40 μM	80 μM
Bi(III)-TPP	6.31 ± 0.42	5.50 ± 0.35	3.86 ± 0.37*	2.91 ± 0.25*
Bi(III)-TQP	6.31 ± 0.42	5.62 ± 0.40	4.36 ± 0.38	4.11 ± 0.28*
Bi(III)-PP IX	6.31 ± 0.42	5.74 ± 0.51	5.17 ± 0.43	4.46 ± 0.37*

homoserine lactone appeared later and reached its maximum concentration after 6 hours of incubation. At time equal to 24 hours from the start of incubation, the levels of homoserine lactones decreased. PQS was detected first time at time point equal 6 hours of incubation and reached its maximum concentration after 24 hours.

Received data showed that after pre-treatment of *P. aeruginosa* PA01 cells with studied substances, autoinducers level in culture supernatant decreased (Tables II–IV). Autoinducers biosynthesis was more sensitive to Bi(III)-TPP. Lowest ability to inhibit an autoinducers biosynthesis showed Bi(III)-PP IX. In the case of Bi(III)-TQP, it was shown that its effects were smaller that the same effects of Bi(III)-TPP, but they were still higher than Bi(III)-PP IX. It was shown that the synthetic porphyrins bismuth complexes posses a higher activity to 3-oxo-C_{12}-HSL and PQS biosynthesis than to C_4-HSL one. In contrast, Bi(III)-PP IX showed the same effect on the biosynthesis of all studied autoinducer.

The results showed that the inhibitory effect of porphyrins bismuth complexes on the biosynthesis of autoinducer was dependent on the concentration of porphyrin. After 6 hours of incubation, *P. aeruginosa* PA01 in culture supernatant that were pre-treated with 0.4 and 40 μM of Bi(III)-TPP concentration of the 3-oxo-C_{12}-HSL was in 2.8 and 4.1 times lower than in control respectively, and after pre-treatment with 80 μM, concentration of this autoinducer in culture supernatant were practically no determinable. After pre-incubation

Table II
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-TPP

Autoinducer	Bi(III)-TPP concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0	0.65 ± 0.07	1.87 ± 0.23	1.32 ± 0.11
	0.4	traces	0.66 ± 0.14*	0.40 ± 0.15*
	40	0	0.46 ± 0.17*	Traces
	80	0	Traces	0
C_4-HSL	0	traces	12.63 ± 1.07	2.44 ± 0.20
	0.4	traces	7.09 ± 0.87*	1.15 ± 0.18*
	40	0	5.51 ± 1.08*	0.94 ± 0.23*
	80	0	3.76 ± 1.10*	0.73 ± 0.20*
PQS	0	0	2.17 ± 0.16	66.48 ± 4.75
	0.4	0	0.93 ± 0.18*	37.85 ± 4.07*
	40	0	traces	26.74 ± 5.63*
	80	0	traces	15.27 ± 3.81*

Note: * – significant different from control

Table III
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-TQP

Autoinducer	Bi(III)-TQP concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0	0.65 ± 0.07	1.87 ± 0.23	1.32 ± 0.11
	0.4	0.44 ± 0.13*	1.24 ± 0.23*	1.06 ± 0.14*
	40	0.31 ± 0.12*	0.82 ± 0.12*	0.53 ± 0.10*
	80	0	0.49 ± 0.13*	Traces
C_4-HSL	0	traces	12.63 ± 1.07	2.44 ± 0.20
	0.4	traces	9.33 ± 1.02*	2.15 ± 0.18
	40	traces	6.89 ± 0.76*	1.36 ± 014*
	80	0	4.30 ± 0.50*	1.07 ± 0.09*
PQS	0	0	2.17 ± 0.16	66.48 ± 4.75
	0.4	0	1.35 ± 0.13*	48.67 ± 5.27
	40	0	0.98 ± 0.10*	33.17 ± 4.56*
	80	0	0.71 ± 0.07*	21.83 ± 3.48*

Note: * – significant different from control

Table IV
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-PP IX

Autoinducer	Bi(III)-PP IX concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0	0.65 ± 0.07	1.87 ± 0.23	1.32 ± 0.11
	0.4	0.61 ± 0.07	1.62 ± 0.21	1.15 ± 0.12
	40	0.53 ± 0.08	1.28 ± 0.14	0.94 ± 0.08
	80	0.40 ± 0.06*	1.23 ± 0.13*	0.90 ± 0.11
C_4-HSL	0	traces	12.63 ± 1.07	2.44 ± 0.20
	0.4	traces	10.32 ± 1.11	2.24 ± 0.25
	40	traces	9.04 ± 1.02*	1.67 ± 0.17
	80	traces	7.85 ± 1.15*	1.36 ± 0.14*
PQS	0	0	2.17 ± 0.16	66.48 ± 4.75
	0.4	0	1.88 ± 0.19	55.18 ± 6.04
	40	0	1.60 ± 0.14*	49.05 ± 5.20
	80	0	1.17 ± 0.09*	40.47 ± 4.33*

Note: * – significant different from control

Table V
Autoinducers biosynthesis of *P. aeruginosa* PA01 after pre-incubation with Bi(III)-PP IX

Autoinducer	Bi(III)-PP IX concentration, μM	Autoinducers concentration, μM		
		3 hours	6 hours	24 hours
3-oxo-C_{12}-HSL	0.4	0.61 ± 0.07	1.62 ± 0.21	1.15 ± 0.12
	40	0.53 ± 0.08	1.28 ± 0.14	0.94 ± 0.08
	80	0.40 ± 0.06*	1.23 ± 0.13*	0.90 ± 0.11
C_4-HSL	0.4	traces	10.32 ± 1.11	2.24 ± 0.25
	40	traces	9.04 ± 1.02*	1.67 ± 0.17
	80	traces	7.85 ± 1.15*	1.36 ± 0.14*
PQS	0.4	0	1.88 ± 0.19	55.18 ± 6.04
	40	0	1.60 ± 0.14*	49.05 ± 5.20
	80	0	1.17 ± 0.09*	40.47 ± 4.33*

with 0.4 and 40 μM Bi(III)-TQP, 3-oxo-C_{12}-HSL a first-detected after 3 hours of incubation, but its concentration were lower than in control in 1.5 and 3.1 times, respectively.

C_4-HSL was not detected up to 6 hours of incubation in all cases (with and without porphyrins pre-treatment). After 6 hours of incubation, concentration of this autoinducer were lower after Bi(III)-TPP and Bi(III)-TQP pre-treatment than in control in 1.8–3.4 and 1.35–2.9 times respectively. After 24 hours of incubation, C_4-HSL concentration was lower in 2–3.3 and 1.1–2.3 times respectively, compared the control.

PQS biosynthesis was completely suppressed during the first 6 hours of incubation after pre-treatment with 40 and 80 μM of Bi(III)-TPP. After pre-treatment with Bi(III)-TQP, PQS were detected in all cases, but its concentration was in 1.6–3.1 times lower than in the control respectively. After 24 hours of incubation PQS concentration in the pre-treated culture were in 1.8, 2.6 and 4.4 times lower respectively in the case of Bi(III)-TPP, and 1.4, 2 and 3 times when Bi(III)-TQP were used.

Bi(III)-PP IX showed no significant effects on the biosynthesis of autoinducers. The highest level of autoinducer biosynthesis inhibition was detected after pre-treatment with 80 μM of this complex – 32–46%.

Discussion

The fact that bacterial quorum sensing system I underlies bacterial pathogenicity, makes it a promising target for novel antimicrobial drugs. Quorum sensing in *P. aeruginosa* controls the production of many virulence factors such as pyocyanin, rhamnolipids, HCN, toxin A, *etc*. Signaling molecules can also act as pathogenicity factors. It was shown that acyl-homoserin

lactones can modulate immune response, induce the death of immune cells, and affect the level of proinflammatory cytokines synthesis (Shiner *et al.*, 2005). Our study showed that synthetic and natural porphyrins bismuth complexes that were studied, could be effective inhibitors of *P. aeruginosa* quorum sensing system. Mechanisms of anti-quorum sensing action of porphyrines bismuth complexes can be linked to its ability block the synthesis of signal molecules. On the other hand, our previous results (Galkin and Ivanitsya, 2011) show that exogenous quorum sensing autoinducers can modify the anti-quorum sensing activity of these compounds. These data suggest that in some cases porphyrins bismuth complexes possibly can compete with autoinducers for binding to their receptors. The discovered ability to inhibit autoinducers biosynthesis and, as a consequence, block pathogenic factors expression (such as pyocyanin and rhamnolipids) and biofilm formation (Galkin *et al.*, 2010) make synthetic and natural porphyrins bismuth complexes very promising for future studies as a new class of antimicrobial drugs.

Acknowledgments

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ORIGINAL PAPER

Kinetic Properties of Pyruvate Ferredoxin Oxidoreductase of Intestinal Sulfate-Reducing Bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9

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Abstract

Intestinal sulfate-reducing bacteria reduce sulfate ions to hydrogen sulfide causing inflammatory bowel diseases of humans and animals. The bacteria consume lactate as electron donor which is oxidized to acetate *via* pyruvate in process of the dissimilatory sulfate reduction. Pyruvate-ferredoxin oxidoreductase activity and the kinetic properties of the enzyme from intestinal sulfate-reducing bacteria *Desulfovibrio piger* and *Desulfomicrobium* sp. have never been well-characterized and have not been yet studied. In this paper we present for the first time the specific activity of pyruvate-ferredoxin oxidoreductase and the kinetic properties of the enzyme in cell-free extracts of both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal bacterial strains. Microbiological, biochemical, biophysical and statistical methods were used in this work. The optimal temperature (+35°C) and pH 8.5 for enzyme reaction were determined. The spectral analysis of the purified pyruvate-ferredoxin oxidoreductase from the cell-free extracts was demonstrated. Analysis of the kinetic properties of the studied enzyme was carried out. Initial (instantaneous) reaction velocity (V_0), maximum amount of the product of reaction (P_{max}), the reaction time (half saturation period) and maximum velocity of the pyruvate-ferredoxin oxidoreductase reaction (V_{max}) were defined. Michaelis constants (K_m) of the enzyme reaction were calculated for both intestinal bacterial strains. The studies of the kinetic enzyme properties in the intestinal sulfate-reducing bacteria strains in detail can be prospects for clarifying the etiological role of these bacteria in the development of inflammatory bowel diseases.

Key words: kinetic analysis, inflammatory bowel diseases, pyruvate ferredoxin oxidoreductase, sulfate-reducing bacteria

Introduction

Intestinal sulfate-reducing bacteria are often isolated from the gut of healthy humans and persons with ulcerative colitis and inflammatory bowel diseases (Gibson *et al.*, 1991; Barton and Hamilton, 2010). A greater number of these bacteria is found mainly in sick people (Cummings *et al.*, 2003; Gibson *et al.*, 1991). In the presence of sulfate, lactate in human intestine contributes to the intensive bacteria growth and the accumulation of their final metabolism product, hydrogen sulfide, which is toxic, mutagenic and cancerogenic to epithelial intestinal cells (Pitcher and Cummings, 2003; Gibson *et al.*, 1991; Kushkevych, 2012a). The increased number of sulfate-reducing bacteria and the intensity of dissimilatory sulfate reduction in the gut can cause inflammatory bowel diseases of humans and animals (Cummings *et al.*, 2003; Gibson *et al.*, 1991; Kushkevych, 2012b).

Lactate is the most common substrate used by the species belonging to the sulfate-reducing bacteria (Kushkevych, 2012a). This compound is oxidized to acetate *via* pyruvate. The type of enzyme present in these microorganisms appears to be a pyruvate-ferredoxin oxidoreductase, as can be deduced from the low potential electron carriers, ferredoxin and flavodoxin, which serve as electron acceptors for the enzyme (Akagi, 1967; Hatchikian *et al.*, 1979; Guerlesquin *et al.*, 1980). In strict anaerobes microorganisms, pyruvate is oxidatively decarboxylated by pyruvate oxidoreductase (EC 1.2.7.1). Pyruvate ferredoxin oxidoreductase catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 (Akagi, 1967; Barton and Hamilton, 2010; Kushkevych, 2012a).

The reaction of this enzyme has been most extensively studied in the forward (oxidative decarboxylation) direction beginning with a series of seminal studies published in 1971 by Raeburn and Rabinowitz which have isolated and characterized pyruvate-ferredoxin oxidoreductase. They have also demonstrated that low potential electron donors, like reduced ferredoxin, can drive the reductive carboxylation of acetyl-CoA (Raeburn and Rabinowitz, 1971).

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As far as we are aware, pyruvate-ferredoxin oxidoreductase from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* sp. has never been well-characterized. In the literature there are a lot of data on pyruvate-ferredoxin oxidoreductase in various organisms as well as in sulfate-reducing bacteria isolated from environment (Akagi, 1967; Barton and Hamilton, 2010; Hatchikian *et al.*, 1979; Furdui *et al.*, 2000; Garczarek *et al.*, 2007; Guerlesquin *et al.*, 1980; Zeikus *et al.*, 1977; Raeburn and Rabinowitz, 1971; Uyeda and Rabinowitz, 1971; Ma *et al.*, 1997; Meinecke, *et al.*, 1989; Pieulle *et al.*, 1995). However, data on the activity of this enzyme from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* sp. have not yet been reported.

The aim of this work was to study pyruvate-ferredoxin oxidoreductase activity in cell-free extracts of intestinal sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 and to carry out the kinetic analysis of enzymatic reaction.

The aim was accomplished using microbiological, biochemical, biophysical methods, and statistical processing of the results; the obtained data were compared with those from the literature.

Experimental

Materials and Methods

The objects of the study were sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine and identified by sequence analysis of the 16S rRNA gene (Kushkevych, 2013; Kushkevych *et al.*, 2014).

Bacterial growth and cultivation. Bacteria were grown in a nutrition-modified Kravtsov-Sorokin's liquid medium (Kushkevych, 2013). Before seeding bacteria in the medium, 0.05 ml/l of sterile solution of $Na_2S \times 9H_2O$ (1%) was added. A sterile 10 N solution of NaOH (0.9 ml/l) in the medium was used to provide the final pH 7.2. The medium was heated in boiling water for 30 min in order to obtain an oxygen-free medium, and then cooled to +30°C. The bacteria were grown for 72 hours at +37°C under anaerobic conditions. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

Obtaining cell-free extracts. Cells were harvested at the beginning of the stationary phase, suspended in 10 mM Tris-HCl buffer in a 1/1 ratio (w/v) at pH 7.6, and disrupted using a Manton-Gaulin press at 9000 psi. The extract was centrifuged at 15,000 *g* for 1 h; the pellet was then used as sedimentary fraction, and the supernatant obtained was termed the soluble fraction (Gavel *et al.*, 1998). The soluble extract constituted by the supernatant was used as the source of the enzyme. This extract was subjected to further centrifugation at 180,000 *g* for 1 h to eliminate the membrane fraction. A pure supernatant, containing the soluble fraction, was then used as cell-free extract.

Protein concentration in the cell-free extracts was determined by the Lowry method (Lowry *et al.*, 1951).

Assays for pyruvate-ferredoxin oxidoreductase activity. The pyruvate-ferredoxin oxidoreductase was assayed and purified as described in paper (Pieulle *et al.*, 1995). The enzyme activity was routinely determined spectrophotometrically by following the reduction of methyl viologen as previously described (Zeikus *et al.*, 1977). All enzyme assays were performed under anaerobic conditions at +35°C using serum-stoppered cuvettes. Samples of enzyme were made anaerobic by flushing the solution with argon as previously reported (Fernandez *et al.*, 1985). The reaction mixture containing 50 μmol Tris-HCl (pH 8.5), 10 μmol sodium pyruvate, 0.1 μmol sodium coenzyme A, 2 μmol methyl viologen and 16 μmol dithioerythritol, in a final volume of 1.0 ml, was bubbled with argon for 20 min and the cell was then incubated at +30°C. The reaction was started by injection of pyruvate-ferredoxin oxidoreductase into the assay cuvette using a gastight syringe and the absorbance at 604 nm was followed. Rates of methyl viologen reduction were calculated using an absorption coefficient of 13.6 $mM^{-1} \times cm^{-1}$. A regenerating system was used to determine the K_m for coenzyme A as previously described (Meinecke *et al.*, 1989). One unit of enzyme activity was defined as the amount of enzyme, which catalyzes the oxidation of 1 μmol of pyruvate or the reduction of 2 μmol of methyl viologen per min under the specified conditions. Specific enzyme activity was expressed as $U \times mg^{-1}$ protein. Michaelis constant (K_m) for pyruvate-ferredoxin oxidoreductase reaction has been determined by substrate (pyruvate and coenzyme A). In order to maintain the concentration of oxidized ferredoxin, a recycling system consisting of spinach ferredoxin-NADP reductase (5 μg/assay) (Sigma) and $NADP^+$ (5 mM) was used. The overall rate was measured by the appearance of NADPH. The activity of the studied enzyme in the cell-free extracts of both bacterial strains at different temperature (from +20°C to +45°C) and pH (in the range from 5.0 to 10.0) in the incubation medium was measured. Spectral analysis of the purified enzyme was carried out as previously described (Pieulle *et al.*, 1995).

Kinetic analysis. Kinetic analysis of the enzyme reaction was performed in a standard incubation medium (as it was described above) with modified physical and chemical characteristics of the respective parameters (incubation time, substrate concentration, temperature and pH). The kinetic parameters characterizing the pyruvate-ferredoxin oxidoreductase reaction are the initial (instantaneous) reaction velocity (V_0),

maximum velocity of the reaction (V_{max}), maximum amount of the reaction product (P_{max}) and characteristic reaction time (time half saturation) were determined. The amount of the reaction product was calculated stoichiometrically. The kinetic parameters characterizing pyruvate-ferredoxin oxidoreductase reactions such as Michaelis constant (K_m) and maximum reaction velocity of substrate decomposition were determined by Lineweaver-Burk plot (Keleti, 1988). For analysis of the substrate kinetic mechanism of pyruvate-ferredoxin oxidoreductase, initial velocities were measured under standard assay conditions with different substrate concentrations. The resulting data were also analyzed by global curve fitting in SigmaPlot (Systat Software, Inc.) to model the kinetic data for rapid equilibrium rate equations describing ordered sequential, $V=(V_{max}\ [A]\ [B])/(K_A\ K_B+K_B\ [A]+[A]\ [B])$, and random sequential, $V=(V_{max}\ [A]\ [B])/(\alpha\ K_A\ K_B+K_B\ [A]+K_A\ [B]+[A]\ [B])$, kinetic mechanisms, where V is the initial velocity, V_{max} is the maximum velocity, K_A and K_B are the K_m values for substrates A and B, respectively, and α is the interaction factor if the binding of one substrate changes the dissociation constant for the other (Segal, 1975).

Statistical analysis. Kinetic and statistical calculations of the results were carried out using the software MS Office and Origin computer programs. The research results were treated by the methods of variation statistics using Student *t*-test. The equation of the straight line that the best approximates the experimental data was calculated by the method of least squares. The absolute value of the correlation coefficient *r* was from 0.90 to 0.98. The significance of the calculated parameters of line was tested by Fisher's *F*-test. The accurate approximation was when $P \leq 0.05$ (Bailey, 1995).

Results and Discussion

Specific activity of pyruvate-ferredoxin oxidoreductase, an important enzyme in the process of organic compounds oxidation in sulfate-reducing bacteria, was measured in different fractions obtained from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cells (Table I).

Results of our study showed that the highest specific activity of the enzyme was detected in cell-free extracts (1.24 ± 0.127 and 0.48 ± 0.051 $U \times mg^{-1}$ protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively). The slightly lower values of activity of pyruvate-ferredoxin oxidoreductase were determined in the soluble fraction compared to cell-free extracts. Its values designated 1.11 ± 0.114 $U \times mg^{-1}$ protein for *D. piger* Vib-7 and 0.37 ± 0.033 $U \times mg^{-1}$ protein for *Desulfomicrobium* sp. Rod-9. The enzyme activity in sedimentary fraction was not observed.

The effect of temperature and pH of the reaction mixture on pyruvate-ferredoxin oxidoreductase activity in the cell-free extracts of the sulfate-reducing bacteria was studied (Fig. 1). The maximum specific activity for both bacterial strains was determined at +35°C. The highest enzyme activity of pyruvate-ferredoxin oxidoreductase for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was measured at pH 8.5.

Thus, temperature and pH optimum of this enzyme was +35°C and pH 8.5, respectively. An increase or decrease in temperature and pH led to a decrease of the activity of studied enzyme in the cell-free bacterial extracts of the sulfate-reducing bacteria. The enzyme activity exhibited typical bell-shaped curves as a function of temperature and pH.

Next task of this study was to carry out a spectral analysis of the purified pyruvate-ferredoxin oxidoreductase from the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. The absorption maxima were 317 and 423, 316 and 425 nm for pyruvate-ferredoxin oxidoreductase from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively (Fig. 2). Ten-minute incubation of the enzyme with 0.75 mM sodium pyruvate led to a slight decrease in absorption maxima. The same peaks of absorption as without addition of sodium pyruvate were observed. However, the significant decrease in absorption spectra after the addition of 0.75 mM sodium pyruvate and 0.1 mM coenzyme A

Table I
Pyruvate-ferredoxin oxidoreductase activity in different fractions obtained from the bacterial cells

Sulfate-reducing bacteria	Specific activity of the enzyme ($U \times mg^{-1}$ protein)		
	Cell-free extract	Individual fractions	
		Soluble	Sedimentary
Desulfovibrio piger Vib-7	1.24 ± 0.127	1.11 ± 0.114	0
Desulfomicrobium sp. Rod-9	0.48 ± 0.051**	0.37 ± 0.033***	0

Comment: The assays were carried out at a protein concentration of 43.57 μg/ml (for *D. piger* Vib-7) and 41.94 μg/ml (for *Desulfomicrobium* sp. Rod-9). Enzyme activity was determined after 20 min incubation. Statistical significance of the values $M \pm m$, $n = 5$; **$P < 0.01$, ***$P < 0.001$, compared to *D. piger* Vib-7 strain

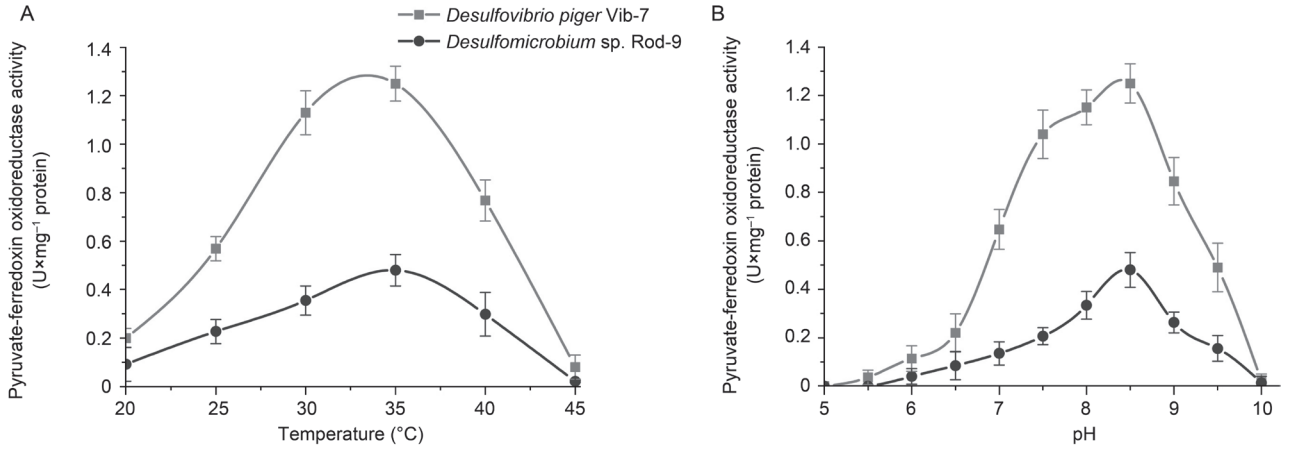


Fig. 1. The effect of temperature (A) and pH (B) on the pyruvate-ferredoxin oxidoreductase activity in the cell-free extracts of the sulfate-reducing bacteria

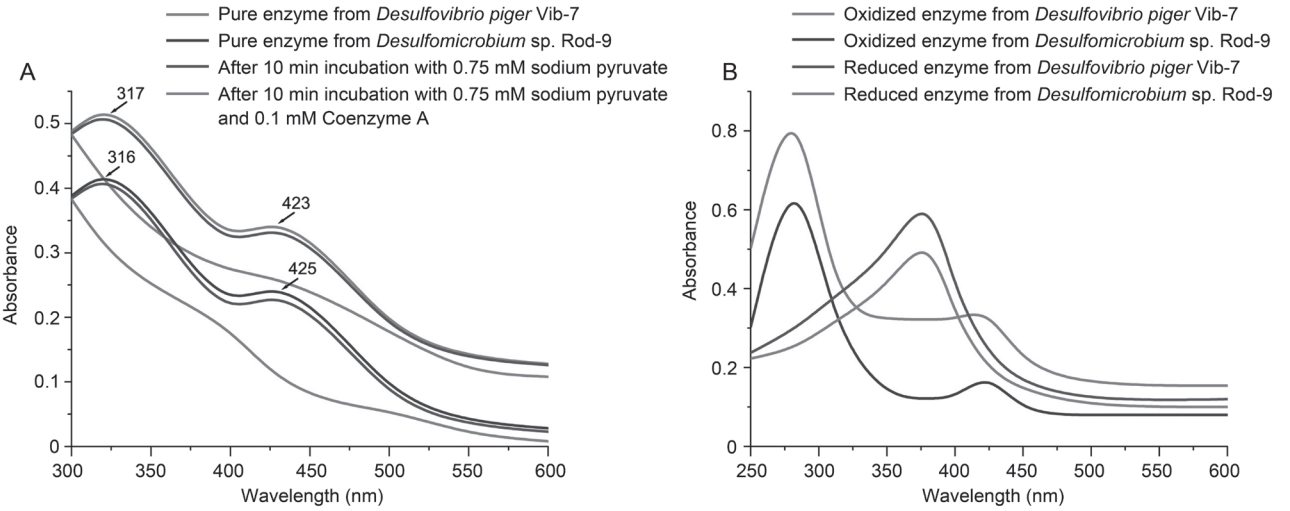


Fig. 2. Absorption spectra of pyruvate-ferredoxin oxidoreductase from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. The serum-stoppered cuvette contains 3 μM of pure enzyme in 50 mM Tris-HC1 (pH 8.5) under argon at +35°C, final volume, 1 ml (A). The spectra were recorded in a final volume of 1 ml in a serum-stoppered cuvette of path length 1 cm under argon. Spectrum of the oxidized enzyme and spectrum of the reduced enzyme after injection of 2 μl dithionite (150 mM) (B)

in the incubation medium was registered. The absorption peaks was no observed (Fig. 2A). The spectroscopic analyses of oxidized and reduced pyruvate-ferredoxin oxidoreductase from *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains were also carried out (Fig. 2B).

Similar data on the absorption spectra of pyruvate-ferredoxin oxidoreductase from *Desulfovibrio africanus* were obtained by Pieulle *et al.* (1995). The authors described the ultraviolet-visible spectrum of studied enzyme which was typical of an iron-sulfur protein with a broad absorbance band around 400 nm and a shoulder in the 315 nm region (Pieulle *et al.*, 1995). Iron and acid-labile sulfide content, as well as the absorption coefficient at 400 nm suggest the presence of six [4Fe-4S] clusters per molecule of enzyme. The absorption band at 400 nm was partially bleached after addition of dithionite; this indicates only partial reduction of the protein, if one considers that full reduction of iron-sulfur clusters should lead to about 50% decrease of the absorption band. Pyruvate reduced the enzyme slightly, whereas pyruvate and CoASH produced a more pronounced reduction of the protein than that obtained with dithionite (Pieulle *et al.*, 1995).

To study the characteristics and mechanism of pyruvate-ferredoxin oxidoreductase reaction, the initial (instantaneous) reaction velocity (V_0), maximum velocity of the reaction (V_{max}), maximum amount of reaction product (P_{max}) and reaction time (τ) were defined. Dynamics of reaction product accumulation was studied for investigation of the kinetic parameters of pyruvate-ferredoxin oxidoreductase (Fig. 3).

Experimental data showed that the kinetic curves of pyruvate-ferredoxin oxidoreductase activity have a saturation tendency (Fig. 3A). Analysis of the results allows to reach the conclusion that the kinetics of pyruvate-ferredoxin oxidoreductase activity in the

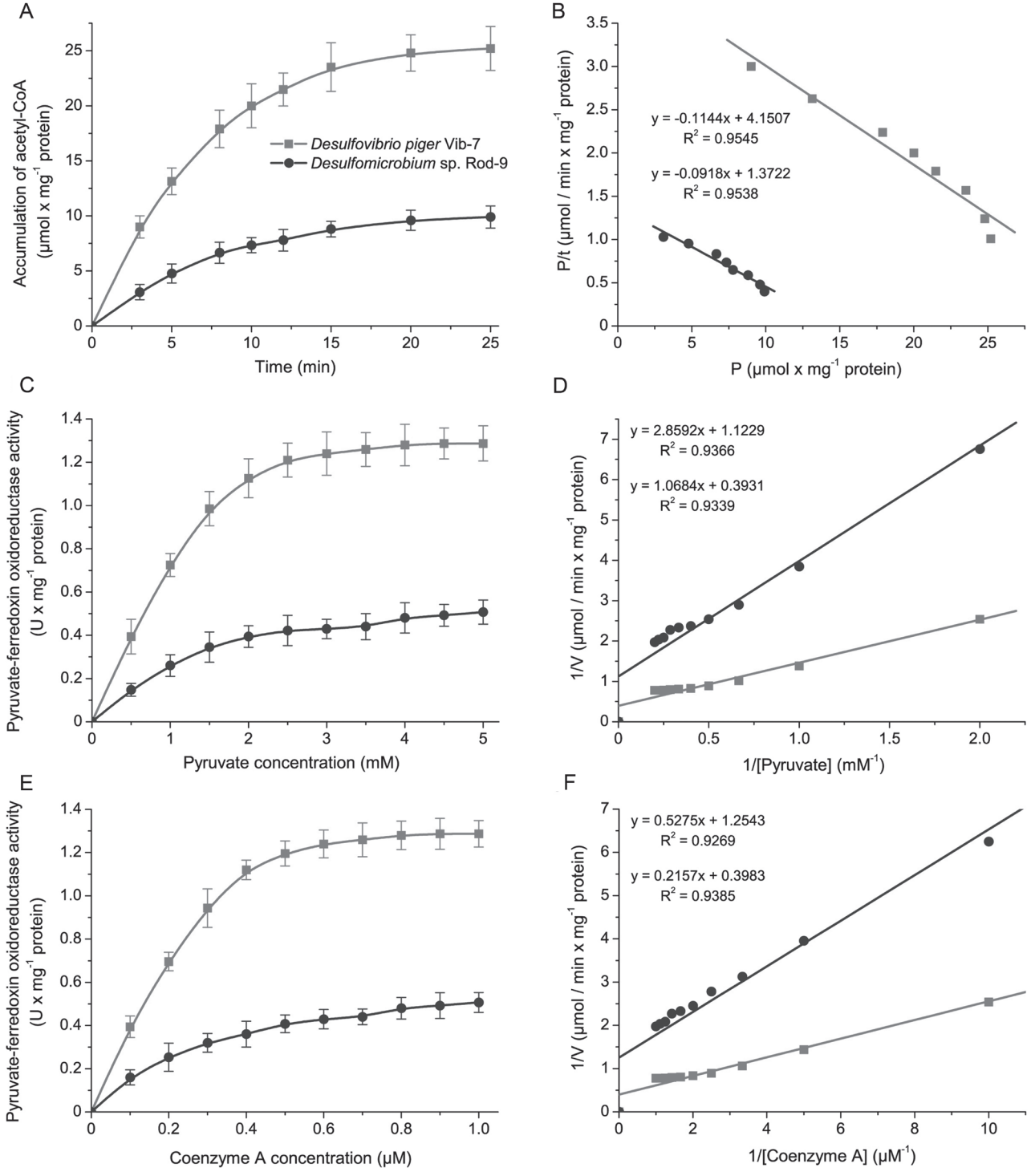


Fig. 3. Kinetic parameters of pyruvate-ferredoxin oxidoreductase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9: A – dynamics of product accumulation (M ± m, n = 5); B – linearization of curves of product accumulation in {P/t; P} coordinates (n = 5; $R^2 > 0.95$; $F < 0.02$); C, E – the effect of different concentrations of substrate (pyruvate and coenzyme A) on the enzyme activity (M ± m, n = 5); D, F – linearization of concentration curves, which are shown in fig. 3C, E, in the Lineweaver-Burk plot, where V is velocity of the enzyme reaction and [Pyruvate] or [Coenzyme A] is substrate concentration (n = 5; $R^2 > 0.9$; $F < 0.005$)

sulfate-reducing bacteria was consistent to the zero-order reaction in the range of 0–10 min (the graph of the dependence of product formation on the incubation time was almost linear in this interval of time). Therefore the duration of the incubation of bacterial cells extracts was 10 min in subsequent experiments.

The amount of the product of pyruvate-ferredoxin oxidoreductase reaction in the *D. piger* Vib-7 was the higher (36.28 ± 3.59 μmol × mg^{-1} protein) compared to the *Desulfomicrobium* sp. Rod-9 (14.95 ± 1.48 μmol × mg^{-1} protein) in the entire range of time factor. The basic kinetic properties of the reaction in the

Table II
Kinetic parameters of the pyruvate-ferredoxin oxidoreductase from intestinal sulfate-reducing bacteria

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
V_0 (μmol × min^{-1} × mg^{-1} protein)	4.15 ± 0.43	1.37 ± 0.12***
P_{max} (μmol × mg^{-1} protein)	36.28 ± 3.59	14.95 ± 1.48**
τ (min)	8.74 ± 0.88	10.89 ± 1.11

Comment: V_0 is initial (instantaneous) reaction velocity; P_{max} is maximum amount (plateau) of the product of reaction; τ is the reaction time (half saturation period). Statistical significance of the values M ± m, n = 5; **P < 0.01, ***P < 0.001, compared to the *D. piger* Vib-7 strain.

Table III
Kinetic parameters of pyruvate-ferredoxin oxidoreductase reaction

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
$V_{max}^{Pyruvate}$ μmol × min^{-1} × mg^{-1} protein)	2.54 ± 0.261	0.89 ± 0.092***
$K_m^{Pyruvate}$ (mM)	2.72 ± 0.283	2.55 ± 0.245
V_{max}^{CoA} (μmol×min^{-1}×mg^{-1} protein)	2.51 ± 0.248	0.81 ± 0.076***
K_m^{CoA} (μM)	0.54 ± 0.052	0.42 ± 0.044

Comment: V_{max} is maximum velocity of the enzyme reaction; K_m is Michaelis constant which was determined by substrate (pyruvate and coenzyme A). Statistical significance of the values M ± m, n = 5; ***P < 0.001, compared to the *D. piger* Vib-7 strain.

sulfate-reducing bacteria were calculated by linearization of the data in the {P/t; P} coordinates (Fig. 3B, Table II).

The kinetic parameters of pyruvate-ferredoxin oxidoreductase from both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different. Values of initial (instantaneous) reaction velocity (V_0) for the enzyme was calculated by the maximal amount of the product reaction (P_{max}). As shown in Table II, V_0 for pyruvate-ferredoxin oxidoreductase reaction was slightly higher (4.15 ± 0.43 μmol × min^{-1} × mg^{-1} protein) in *D. piger* Vib-7 compared to *Desulfomicrobium* sp. Rod-9 (1.37 ± 0.12 μmol × min^{-1} × mg^{-1} protein). In this case, the values of the reaction time (τ) were more similar for the studied enzyme in both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains. Based on these data, it may be assumed that the *D. piger* Vib-7 can consume lactate ion much faster in their cells than a *Desulfomicrobium* sp. Rod-9. Moreover, this hypothetical assumption can be also confirmed by obtained data on maximal velocities of accumulation of the final reaction products, where V_{max} for enzyme reaction in *D. piger* Vib-7 were also more intensively compared to *Desulfomicrobium* sp. Rod-9 (Table III).

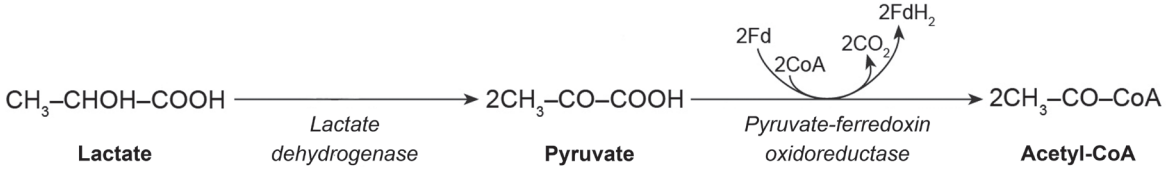
The kinetic analysis of pyruvate-ferredoxin oxidoreductase reaction depending on concentration of substrate (pyruvate and coenzyme A) was carried out. The increasing pyruvate concentrations from 0.5 to 5.0 mM and coenzyme A concentrations from 0.1 to 1.0 μM caused a monotonic rise of the studied enzyme activity and the activity was maintained on unchanged level (plateau) under substrate concentrations over 5.0 mM and 1.0 μM, respectively. (Fig. 3C, E). Curves of the dependence {1/V; 1/[S]} were distinguished by the tangent slope and intersect the vertical axis in one point (Fig. 3D, F). The basic kinetic parameters of pyruvate-ferredoxin oxidoreductase activity in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were identified by linearization of the data in the Lineweaver-Burk plot (Table III).

Calculation of the kinetic parameters of enzyme activity indicates that the maximum velocities (V_{max}) of pyruvate and coenzyme A in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were significantly different from each other. However, it was observed a correlative relationship between $V_{max}^{Pyruvate}$ and V_{max}^{CoA} in both intestinal bacterial strains. Michaelis constants (K_m) of pyruvate-ferredoxin oxidoreductase reaction were identified for pyruvate and coenzyme A. The values of K_m were quite similar for pyruvate (2.72 ± 0.283, 2.55 ± 0.245 mM) and coenzyme A (0.54 ± 0.052, 0.42 ± 0.044 μM) in both *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 strains, respectively.

The obtained parameters of pyruvate-ferredoxin oxidoreductase reaction in *D. piger* Vib-7 are consistent with previously described data by Pieulle *et al.* for the activity of pyruvate-ferredoxin oxidoreductase from *D. africanus.* The apparent K_m for pyruvate and coenzyme A were also 2.5 mM and 0.5 μM, respectively and the V_{max} values were 10240 min^{-1} and 5890 min^{-1}, respectively. The apparent K_m for methyl viologen was found to be 0.5 mM in the presence of 10 mM and 0.1 mM of pyruvate and CoASH, respectively. Kinetics studies done with the enzyme and a slight decrease in the affinity for pyruvate and in the catalytic activity (K_m of 5.5 mM and V_{max} of 4810 min^{-1}) were reported (Pieulle *et al.*, 1995).

Furdui and Ragsdale (2000) have described the pyruvate-ferredoxin oxidoreductase from the *Clostridium thermoaceticum.* The Michaelis-Menten parameters for pyruvate synthesis by the enzyme were: V_{max} 1.6 unit/mg, $K_m^{Acetyl\text{-}CoA}$ 9 μM. The intracellular concentrations of acetyl-CoA, CoASH, and pyruvate were also measured (Furdui and Ragsdale, 2000).

Pyruvate-ferredoxin oxidoreductatse, an important enzyme in process of dissimilatory sulfate reduction and organic compounds oxidation in sulfate-reducing bacteria, carries out the central step in oxidative decarboxylation of pyruvate to acetyl-CoA (Kushkevych, 2012a):



Garczarek *et al.* (2007) have purified this enzyme from *Desulfovibrio vulgaris* Hildenborough as part of a systematic characterization of as many multiprotein complexes as possible for this organism (Garczarek *et al*., 2007).

Thus, based on the obtained studies results and according to the kinetic parameters of pyruvate-ferredoxin oxidoreductatse reaction for both bacterial strains, we have concluded that the enzyme activity, V_0 and V_{max} were significantly higher in the *D. piger* Vib-7 cells than *Desulfomicrobium* sp. Rod-9. However, Michaelis constants were quite similar for pyruvate (2.72 ± 0.283, 2.55 ± 0.245 mM) and coenzyme A (0.54 ± 0.052, 0.42 ± 0.044 μM) in both bacterial strains. The maximum enzyme activity for both strains was determined at +35°C and at pH 8.5. These data correspond to conditions which are present in the human large intestine from where the bacterial strains were isolated. Perhaps such conditions favor intensive development of the *D. piger* and *Desulfomicrobium* sp. bacterial strains in the gut. The kinetic parameters of enzyme reaction are depended on the substrate concentration. The studies of the pyruvate-ferredoxin oxidoreductatse in the process of dissimilatory sulfate reduction and kinetic properties of this enzyme in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 intestinal strains, their production of acetate in detail can be a perspective for clarification of their etiological role in the development of the humans and animals bowel diseases. These studies might help in predicting the development of diseases of the gastrointestinal tract, by providing further details on the etiology of bowel diseases, which are very important for the clinical diagnosis of these disease types.

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ORIGINAL PAPER

The Application of Impedance Microsensors for Real-Time Analysis of *Pseudomonas aeruginosa* Biofilm Formation

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Abstract

Biofilms formed by nosocomial pathogens represent a major threat to patients undergoing invasive procedures. As prophylaxis remains the most efficient anti-biofilm option, it is of paramount importance to develop diagnostic tools able to detect biofilm at the early stage of formation. The present study investigates the ability of impedance microsensors to detect *Pseudomonas aeruginosa* biofilm presence using the impedance spectroscopy method. The measured data were analyzed using Electrical Equivalent Circuit modelling (EEC). It allowed to recognize conduction and polarization phenomena on the sensors surface and in its environment. The impedance assay results, confirmed by means of electron microscopy and quantitative cultures, indicate that specific EEC parameters may be used for monitoring the development of pseudomonal biofilm.

Key words: *Pseudomonas aeruginosa*, biofilm, impedance sensors

Introduction

The majority of bacteria exist in settled communities, referred to as biofilms. Contrary to their planktonic (free-swimming) counterparts, bacterial cells in biofilm are embedded within an extracellular matrix, which serves the bacteria as a shelter and a shield protecting from antimicrobials and immune system components. Biofilms develop easily on tissues (most preferably damaged) and on abiotic surfaces (*i.e.* dressings, catheters, intubation tubes (Flemming *et al*., 2008; Pradeep *et al*., 2013; Bjarnsholt *et al*., 2008).

Biofilms are responsible for up to 80% of nosocomial infections (James *et al*., 2008). Presently, there are neither biofilm-resistant medical implants nor procedures guaranteeing biofilm eradication. The only exception, to some extent, are non-specific actions such as surgical removal of infected tissue or removal of a colonized implant. However, the above mentioned procedures are not always possible or safe for the patient.

Therefore, there is an urgent need to develop diagnostic tools for an early detection of biofilm presence. Sensors, measuring physical value referred to as impedance, display promising properties that may be used for this purpose (Ben-Yoav *et al*., 2013; Zhenga *et al*., 2013; Padresa *et al*., 2013). Impedance describes the potential of natural objects to resist the electric current flow and to store energy in the form of electric or magnetic field. All physicochemical changes occurring within biological systems have an impact on their electrical properties and affect the impedance value. Also such important biological phenomena as fluctuation of ionic concentration, cell division or adhesion to the surface, may be potentially detected by impedance sensors (Ge *et al*., 2008; Hakki and Bozkurt, 2011). The most important advantage of using impedance microsensors is the possibility of real-time monitoring of these changes. Other advantages are non-invasiveness, label-free detection (no markers needed) and provision of data concerning electrical properties of the environment.

The detection of bacteria by means of impedance microsensors may be helpful in virtually all flow systems endangered by the development of microbes. Among the examples of such systems in nosocomial

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settings are indwelling catheters, nutrition accesses or hospital water distribution pipes (Taeyoung *et al*., 2011; Munoz *et al*., 2006).

As the majority of impedance-related research on biofilm is still at the preliminary level (Dominguez-Benetton *et al*., 2012), the aim of this study was to contribute to the exploration of this issue. Thus, specially designed impedance microsensors were applied for the real-time analysis of biofilm growth of *Pseudomonas aeruginosa*, a recognized nosocomial pathogen.

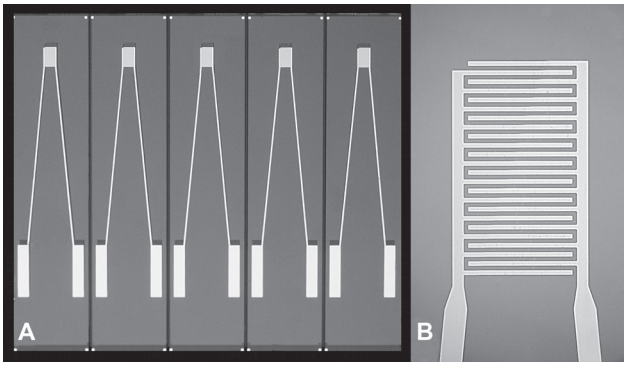


Fig. 1. Left side: Impedance microsensors on a glass substrate
Right side: IDE structure of sensor. Digits of electrodes are visible

Experimental

Materials and Methods

Impedance microsenors with interdigitated electrodes (IDE) were used in the experiment. The electrodes were made of vacuum-evaporated gold on PYREX glass with a titanium adhesion layer. The distance between the electrodes and the width of the electrode digits were 20 μm. The area of the electrodes was 0.6×1 mm. The shape and contact pads placement of the sensors allowed to mount them manually to the micro USB plug and to place them vertically in the wells of a 24-well plate (CellStar, Germany). The sensors used for the experiment are presented in Fig. 1. To prepare electrodes, sensors were rinsed in distilled water, then in acetone and in isopropanol, finally.

GW InstekLCR-8101G was used as Impedance Analyser (IA). The measurement system was designed to handle 8 sensors. It consisted of a suitable 8-channeled switch and the above-mentioned IA (Fig. 2).

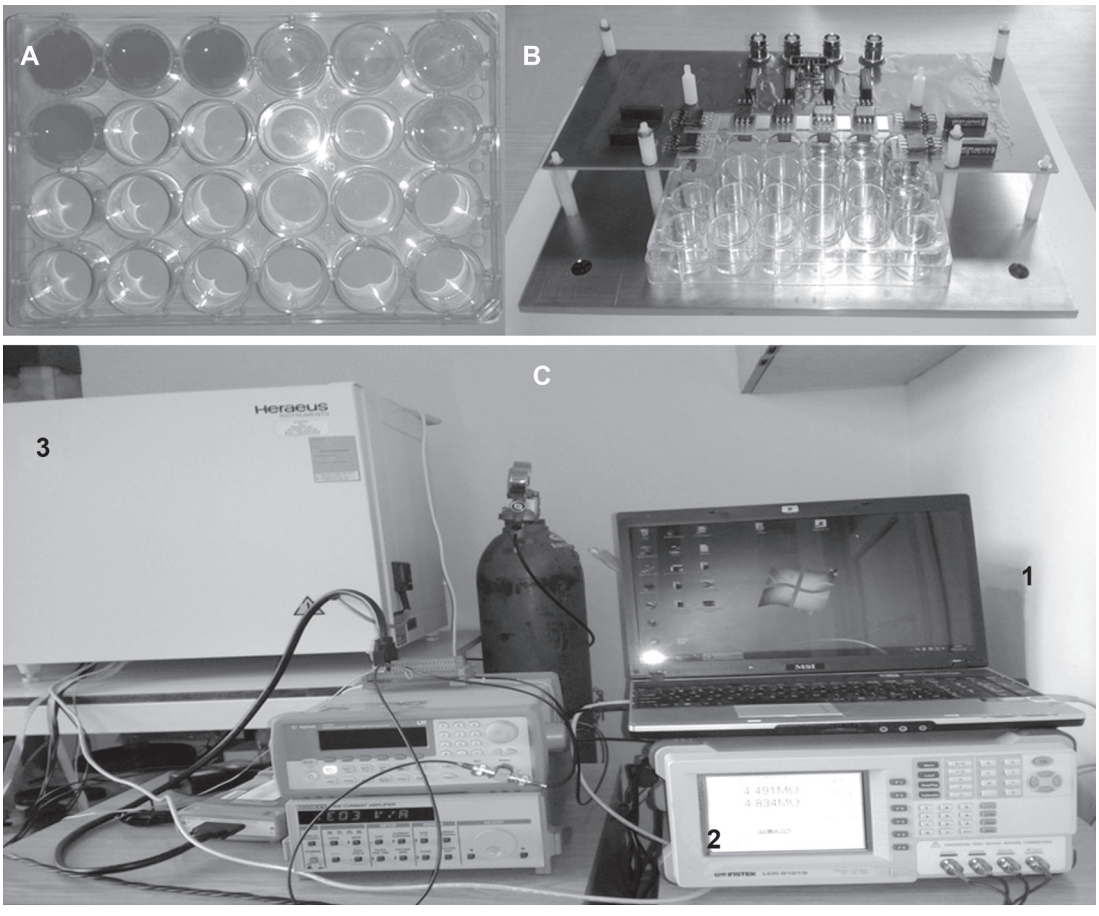


Fig. 2. Upper left side: A 24-well plate filled with *P. aeruginosa* culture (left side) and a sterile medium (right side)
Upper right side: 8-channel switch with sensors dipped in the wells. After connecting to the impedance analyser, the switch is placed in the incubator.
Bottom. Work station for impedance biofilm assays: 1 – PC with ImpeDancer software; 2 – Impedance Analyser; 3 – incubator of 24-well plates

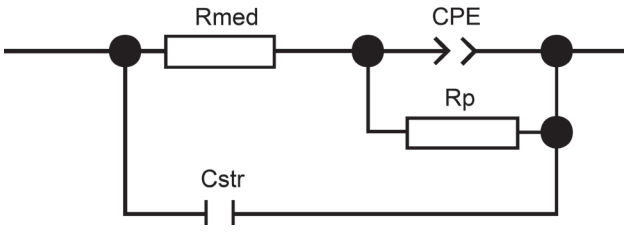


Fig. 3. Electrical equivalent circuit (EEC) of the impedance sensor in bacteria-free and bacteria-containing medium

Particular parts of the EEC represent the following electrical properties of measured object: Rmed – medium resistance, Cstr – sensor's structure parasitic capacitance, CPE – capacitance of electrodes-environment interface, Rp – parallel resistance to CPE

Such a setting allows to perform quasi-simultaneous measurement of the sensors placed in different wells of a microtitre plate. The measurement system was controlled by a PC computer with a customized software described in more detailed manner in (Piasecki *et al.*, 2012) (Fig. 2).

Real-time impedance measurement of biofilm formation. A reference *P. aeruginosa* ATCC 14454 was used for experimental purposes. The ability of the aforementioned strain to form biofilm on abiotic surfaces has been already recognized in the previous work of the authors (Junka *et al.*, 2013). An overnight culture of the investigated strain was diluted to 1 McFarland using a densitometer (Biomerieux, Poland) and subsequently to 10^3 cfu/ml using dilution method. Two ml of the strains' culture was introduced to the wells of the plate. Next, the sensors were mounted aseptically in a 8-channel switch and placed in the wells. Impedance spectra of each sensor were measured for 24 hours/37°C using the impedance spectroscopy method with four minute intervals. Pure Tryptic Soya Broth (TSB, BioMerck, Poland) was used as a negative control of the experiment.

The obtained impedance spectra were analysed using the Electrical Equivalent Circuit (EEC) method. A special EEC was created for this experiment (Fig. 3). It allowed to model conductivity and polarization processes occurring in the impedance sensor area (medium and biofilm). Two EEC components describe the phenomena taking place on the electrodes of the sensors. These are CPE and Rp (capacitance of electrodes-environment interface and parallel to it resistance, respectively). The admittance of CPE is given by $Y_{CPE}(\ )$ where Q and n are the parameters and ω is a radial frequency (Barsoukov and Macdonald, 2005). The values of these parameters were calculated for each of the time-points using ZView software by Scribner. Rp, Q and n were analysed with regard to the process of *P. aeruginosa* biofilm formation.

Quantitative cultures. The *P. aeruginosa* strain and the impedance apparatus were prepared as described above. The strain was incubated in the presence of sensors for 2, 6, 12 and 24 hours. Next, the sensors were aseptically removed from the apparatus, rinsed with saline, and transferred to 1ml of mild detergent – 0.5% saponine (Sigma Aldrich, Poland). The sensors were vortex mixed for 1 minute to remove biofilm from their surface. Subsequently, the obtained suspensions were diluted 10–10^7 times. 100µl of each dilution was cultured on a McConkey Agar Medium (Merck, Poland) and incubated at 37°C for 24 hours. Next, the bacterial colonies were counted and the number of bacterial cells forming biofilm was assessed.

Electron microscopy. The strains were allowed to form biofilm on the sensors' surface under the conditions described above. Subsequently, the sensors were aseptically removed from the apparatus, rinsed 3 times with saline to remove non-adherent bacteria and dried at 37°C/4 hours. The dried samples were covered with Au/Pd (60:40, sputter current: 40 mA, sputter time: 50 sec) using QUORUM machine (Quorum International Forth Worth, USA) and examined on Scanning Electron Microscope Zeiss EVO MA25.

Results

Biofilm dynamics. After two hours of incubation, pseudomonal cell clusters were found on the sensors' surface (Fig. 4, upper left side). Quantitative cultures revealed the presence of *ca* 10^4 colony forming units of bacteria on the sensor. During the next four hours, the number of bacteria increased to 10^8. Therefore, this period may be identified as an intensive growth phase. The surface of the sensors was partially covered with a dense multi-layered bacterial biofilm. Although slower than previously, after 12 hours from the beginning of the experiment the number of cells was still increasing. This suggests the phase of biofilm maturation. After 24 hours, virtually the entire surface of the sensor was covered with biofilm in different stadia of development (Fig. 4, lower right side).

Electrical measurements. Changes of the environment's physicochemical properties detected by the sensors are presented as a set of impedance spectra in the form of serial capacitance and dissipation factor (Fig. 5). The measured data were analysed using Electrical Equivalent Circuit (EEC) modelling.

Such approach allows to obtain qualitative information about the measured object from impedance spectra. As can be seen in Figure 6, all three analysed parameters – Rp, Q and n, were informative for the purposes of analysing *P. aeruginosa* biofilm formation. The Q parameter was found to be positively correlated with increasing number of cells on the sensors, whereas the n value decreased along with biofilm development (Fig. 6A-B). From the 6th hour of the experiment, the

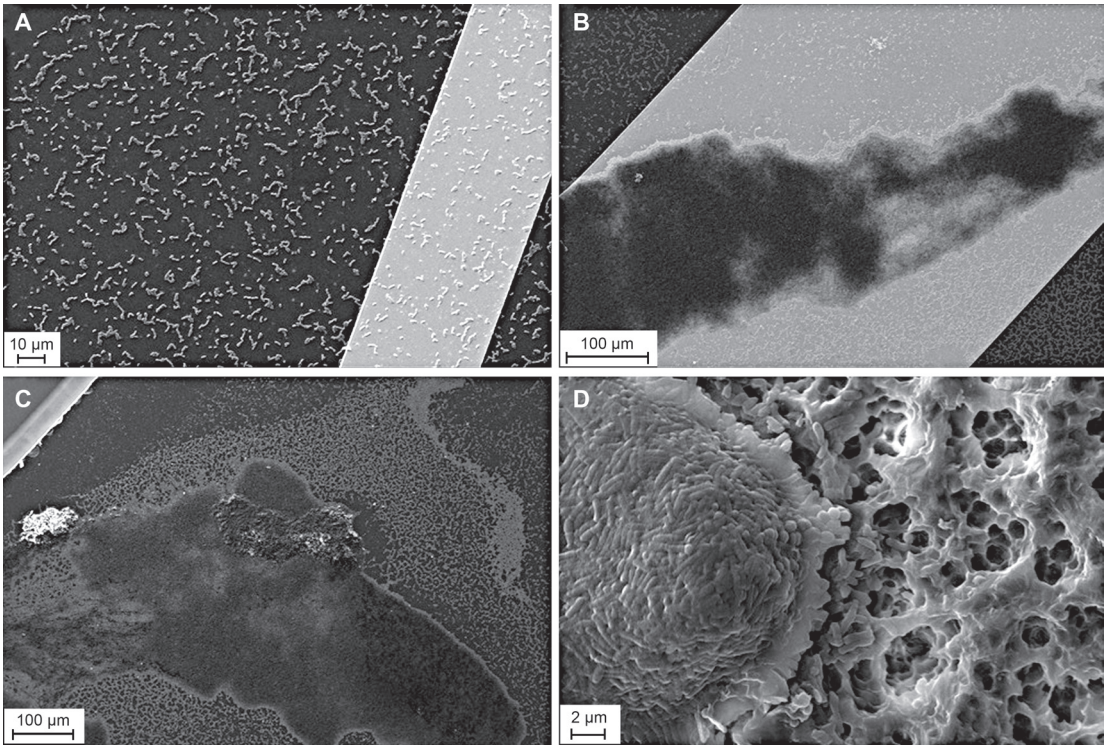


Fig. 4. Upper left side: Pseudomonal cells on the surface of impedance sensor after 2 hours of incubation Magn.x1450. Upper right side: Pseudomonal cells on the surface of impedance sensor after 6 hours of incubation. Different stadia of biofilm formation are visible. Magn.x441.Lower left side: Pseudomonal cells on the surface of impedance sensor after 12 hours of incubation. Different stadia of biofilm formation are visible. Magn.x325. Fig Lower right side: Multi-layer pseudomonal biofilm on the surface of impedance sensor after 24 hours of incubation. Both the extracellular matrix and pseudomonal cells are visible. Magn.x9070. All pictures presented were taken using Electron Microscope Zeiss Evo MA 25

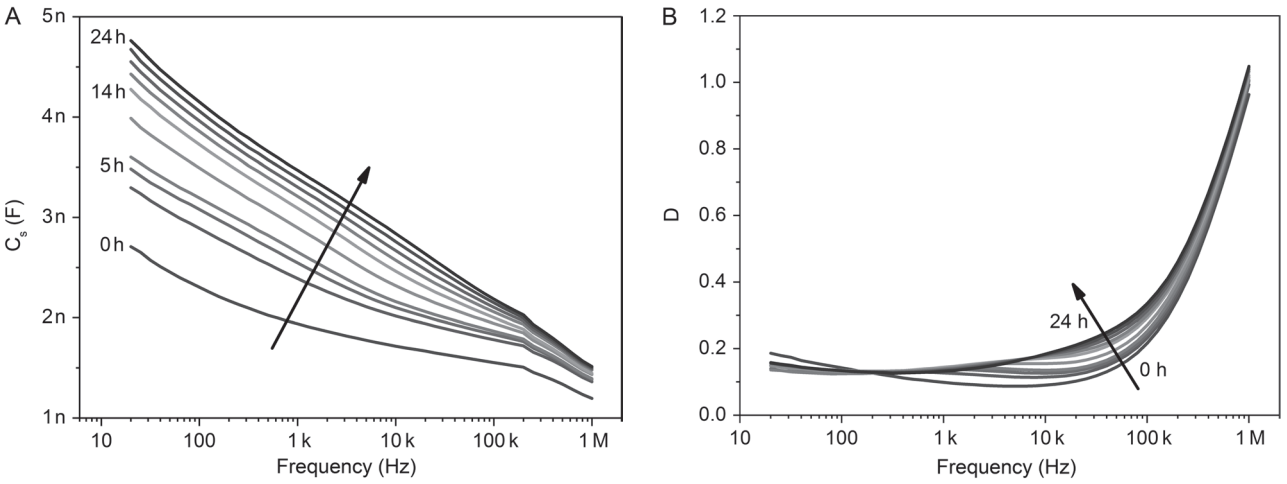
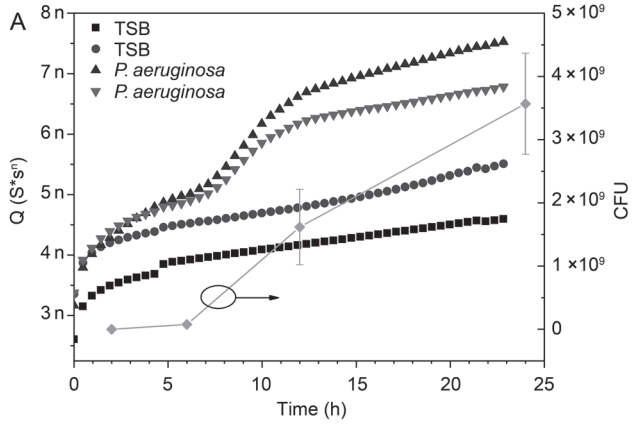


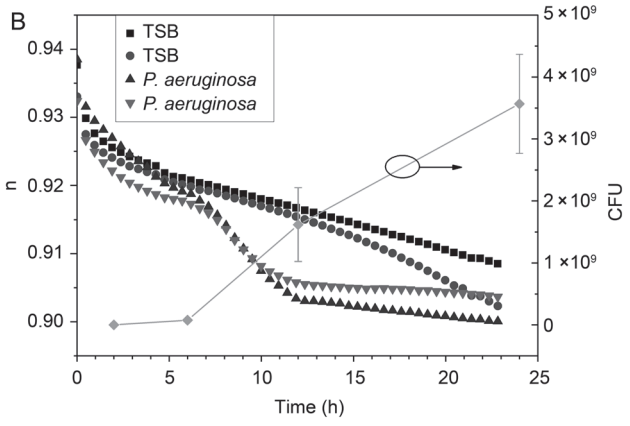
Fig. 5. Typical spectra of serial capacitance C_s and dissipation factor D in time registered in *P. aeruginosa* filled wells

differences in Q measured in the control and tested well started to be visible and such a state continued for another 5 hours. Afterwards, the value of the Q parameter in the tested sample resembled that of the control again. A different type of results was obtained for Rp. The value of this component was slowly and uniformly increasing until the 11-th hour of the experiment and increased rapidly afterwards, reaching the value of 10^{15} ohm (Fig. 6C).

Discussion

In the *in vitro* setting applied, pseudomonal biofilm underwent all specific stages of development, including adhesion, matrix synthesis and maturation (Fig. 4). As it was shown, the impedance sensors used were able to distinguish between bacteria-free and bacteria-containing environment. This ability offers application potential, especially as the value of one of the param-





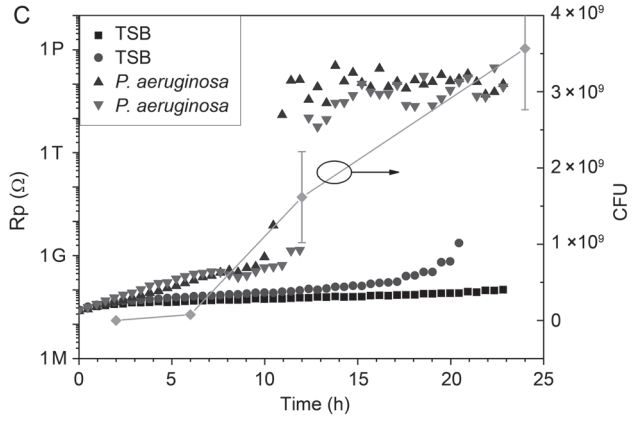


Fig. 6. Upper left side: Values of the Q parameter of EEC component CPE, correlated with changes of *P. aeruginosa* cfu number Each plot represents different series of measurement. The ellipse with the arrow points out right scale of the graph describing values of cfu. Upper right side Values of the n parameter of EEC component CPE, correlated with changes of *P. aeruginosa* cfu number. Each plot represents different series of measurement. The ellipse with the arrow points out right scale of the graph describing values of cfu. Bottom: Values of EEC component Rp correlated with changes of *P. aeruginosa* cfu number. Each plot represents different series of measurement. The ellipse with the arrow points out right scale of the graph describing values of cfu

eters analysed, namely Q, started to rise as early as after 2 hours from the start of the experiment, along with an increasing bacterial cell number. Moreover, Q values seemed to correlate with the phases of bacterial growth on the surface of the sensor (Fig. 6A).

On the other hand, during the first 6 hours, the differences of the n value measured for control and bacteria-containing environment were not as distinct as in the case of the Q parameter. Thus, it may limit the potential applicability of the n parameter for biofilm detection. Further experiments should be performed to interpret the results obtained for n, with special attention to the phenomena taking place between 6^{th}–11^{th} hour from the beginning of the experiment.

Puzzling results were obtained for Rp, the third parameter analysed. After the 11^{th} hour of incubation, the value of this component increased significantly. According to the electrical model applied, such a phenomenon may be explained by a loss of DC conductivity. It might be the result of biofilm development on the entire surface of the electrodes. However, the discussed rise of the Rp value took place late in the course of the experiment. At that time, vast biofilm clusters formed on the sensors were seen not only by means of electron microscopy (Fig. 4) but also with the naked eye. Therefore, Rp for biofilm detection is useful only in systems where optical detection is impossible.

Still, at least one of three tested parameters, namely Q, may be useful for the analysis of bacterial biofilm formed on the sensors' surface. This finding, confirmed by electron microscopy and microbiological techniques, may be helpful in future development of clinical applications. Biofilm-related infections represent a major threat to patients undergoing invasive nosocomial procedures. The presented study aims to be another brick in the wall built for the patients' protection.

Summary

- The applied impedance microsensors are able to detect bacterial presence
- Electrical Equivalent Circuit modelling is useful for analysing sensor response
- The applicability of Rp and n parameters for the detection of biofilm presence seems to be limited.
- The value of Q changes along with biofilm phase of growth. The suitability of this parameter for biofilm detection is promising, although further experiments need to be performed.

Acknowledgments

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ORIGINAL PAPER

Chemical Composition and Antibacterial Activity of Essential Oils of Two Species of *Lamiaceae* against Phytopathogenic Bacteria

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Abstract

In this study, we aimed to determine chemical composition and antibacterial activities of *Satureja hortensis* and *Calamintha nepeta* against to 20 phytopathogenic bacteria causing serious crop loss. The essential oils of *S. hortensis* and *C. nepeta* were isolated by the hydrodistillation method and the chemical composition of the essential oils were analyzed by GC-MS. The antibacterial properties of the essential oils were evaluated against 20 phytopathogenic bacteria through Disc diffusion assay and micro dilution assay. The results revealed that the essential oils of *S. hortensis* and *C. nepeta* have significant antibacterial activity. Furthermore, the findings of the study are valuable for future investigations focusing on the alternative natural compounds to control plant diseases.

Key words: *Calamintha nepeta*, *Satureja hortensis*, antibacterial activity, biopesticide, chemical composition

Introduction

In recent years, one of the most popular subjects is the increase of yield production because of starvation that threats millions of people (Fletcher *et al.*, 2006). Every year, substantial part of the yield has been lost due to plant diseases caused by fungi, bacteria and viruses. Bacteria can also cause undesirable effects on quality, reliability and preservation of crop. To solve these problems, synthetic chemicals have been mostly used for many years. However, due to indiscriminate use of antimicrobial synthetic chemicals in the treatment of infectious diseases, both human and plant pathogenic microorganisms have developed resistance to multiple drugs/chemical substances (Sahin *et al.*, 2003; Gormez *et al.*, 2012). In addition, these chemical compounds can cause undesirable effects on environment because of their slow biodegradation and several serious side effects on mammalian health because of toxic residues in agricultural products (McManus *et al.*, 2002; Horvath *et al.*, 2009; Kotan *et al.*, 2010). This situation forced the researchers to discover new natural antimicrobial substances from various sources like medicinal plants (Clark, 1996; Cordell, 2000). Among many plant products, essential oils are the most studied plant secondary metabolites. Essential oils such as biopesticide have some advantages, where pathogenic microorganisms are not likely to develop resistance against them, little or no mammalian toxicity and not accumulated in soils (Heisey and Heisey, 2003; Singh *et al.*, 2003, 2005; Cardile *et al.*, 2009; Grosso *et al.*, 2010; Tian *et al.*, 2011). Therefore, the present study was conducted to investigate alternative antimicrobial agents among essential oils of *Lamiaceae* species that can be used as biopesticide.

The *Lamiaceae* is a family of plants having about 233 genera and 6900 species (Heywood *et al.*, 2007). The phenolic compounds, such as rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, luteolin, apigenin, genkwanin, quercitrin, rutin, epicatechin and catechin are rich in *Lamiaceae* (Moreno *et al.*, 2006; Ben Farhat *et al.*, 2009; Castro-Vazquez *et al.*, 2009). Due to its rich contents of plants, they have many biological activities, such as anti-inflammatory, anticancer, antifungal,

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antimicrobial activity (Sarer and Pancali, 1998; Cheung and Tai, 2007; Figueiredo *et al.*, 2008; Takaki *et al.*, 2008; Quave *et al.*, 2008). *C. nepeta* and *S. hortensis* are well known aromatic and medicinal plants which belong to *Lamiaceae*. They have been used in folk medicines to treat many illnesses because of their antispasmodic, expectorant, diuretic, antimicrobial activities as it is stated in the relevant literature (Sarer and Pancali, 1998; Baser *et al.*, 2000; Sahin *et al.*, 2003). However, there is no report concerning the antibacterial activity of these essential oils against these many phytopathogenic bacteria.

In the study, we aimed to determine chemical compositions of hydro-distilled essential oils of *S. hortensis* and *C. nepeta* by GC-MS system as their biological activities were connected to their chemical compositions and to evaluate their antibacterial potentials against plant pathogen bacteria which have not been evaluated in the previous studies.

Experimental

Materials and Methods

Plant materials. *C. nepeta* and *S. hortensis* were collected at the flowering stage in July 2010, from the eastern part of Erzurum in Turkey. Identification of the plant materials was confirmed by a plant taxonomist, Assoc. Prof. Dr. Ozkan AKSAKAL, in the Department of Biology, Ataturk University, Erzurum, Turkey. Plants herbarium samples were stored in the herbarium of the Science Faculty, Ataturk University, Erzurum.

Isolation of the Essential Oils. Plant samples were dried in a canopy room. The aerial parts (leaves, flowers and steams) of the plants were powdered with blender and then subjected to water distillation for 2–3 h in a Clevenger-type apparatus (Thermal Laboratory Equipment, Turkey). The essential oils were stored at +4°C for further studies.

GC-MS Analysis Conditions. The essential oils were analyzed by using a Thermofinnigan Trace GC/Trace DSQ/A1300, (E.I Quadrapole) equipped with a SGE-BPX5 MS capillary column (30 m X 0.25 mm *i.d.*, 0.25 µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was the carrier gas at a flow rate of 1 ml/min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. The program was used at 50–150°C at a rate of 3°C/min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 µl were injected manually and in the splitless mode. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, Wiley 7N library data of the GC-MS system and literature data. The results were also confirmed by the comparison of the compounds elution order with their relative retention indices on non-polar phases reported in the literature.

Table I
Plant pathogenic bacterial species used in the study

Bacteria	Strain No	Host
Agrobacterium tumefaciens	Apricot	AA-685
Bacillus pumilus	Apricot	AA-479
Clavibacter michiganensis subsp. *michiganensis*	Tomato	AA-703
Enterobacter intermedius	Cherry	AA-184
Erwinia caratovora subsp. caratovora	Tomato	AA-687
Erwinia chrysanthemi	Apricot	AA-58
Pseudomonas cichorii	Peach	AA-234
Pseudomonas corrugate	Tomato	AA-684
Pseudomonas fluorescens	Apricot	AA-616
Pseudomonas syringae pv. *syringae*	Cherry	AA-218
Pseudomonas syringae pv. *syringae*	Apricot	AA-637
Pseudomonas syringae pv. *syringae*	Apricot	AA-638
Pseudomonas syringae pv. *syringae*	Apricot	AA-647
Pseudomonas syringae pv. *phaseolicola*	Beans	AA-652
Pseudomonas syringae pv. *pisi*	Peach	AA-237
Pseudomonas syringae pv. *tabaci*	Apricot	AA-704
Pseudomonas syringae pv. *tomato*	Cherry	AA-220
Ralstonia solanacearum	Apricot	AA-116
Xanthomonas axonopodis pv. *campestris*	Pepper	AA-705
Xanthomonas vesicatoria	Tomato	AA-683

Plant pathogenic bacterial strains. The essential oils of the plants were tested against 20 plant pathogenic bacterial strains which were shown in the Table I. All the bacterial strains were isolated from some fruits and vegetables exhibiting typical bacterial disease symptoms on their respective host plants. They were identified by using conventional methods such as morphological, biochemical, pathogenicity tests and microbial identification system (MIS) (Miller and Berger, 1985). The isolated and identified bacterial cultures were preserved in Luria broth and 30% glycerol solutions at –80°C prior to use.

Antimicrobial activity: *2 Disc diffusion assay*. Twofold serial dilutions of the essential oils were made by diluting %10 DMSO to prepare a decreasing concentration range from 500 µg/ml to 7.81 µg/ml. Antimicrobial tests were carried out by disc diffusion assay using 100 µl of suspension containing 10^8 cfu/ml of bacteria spread on tryptic soy agar (TSA) medium by a sterile swab (Murray *et al.*, 1995). The discs (6 mm in diameter) were individually impregnated with 10 µl of essential oils at all the prepared concentrations and placed on the inoculated agar. Negative controls were prepared using the same solvents employed to dilute the essen-

Table II
Antibacterial activities of the essential oils of *S. hortensis* and *C. nepeta*

Bacteria	*S. hortensis*			*C. nepeta*			Negative control	Positive control
	DD		MIC	DD		MIC	DMSO	Standart antibiotic discs
	500 µg	7.81 µg		500 µg	7.81 µg			
Agrobacterium tumefaciens	48	8	7.81	48	8	7.81	–	28 (SCF)
Bacillus pumilus	47	7	7.81	47	7	7.81	–	23 (OFX)
Clavibacter michiganensis ssp. *michiganensis*	48	9	7.81	48	8	7.81	–	26 (SCF)
Enterobacter intermedius	16	8	7.81	35	7	7.81	–	26 (SCF)
Erwinia caratovora ssp. *caratovora*	48	9	7.81	45	7	7.81	–	29 (OFX)
Erwinia chrysanthemi	48	8	7.81	46	8	7.81	–	25 (SCF)
Pseudomonas cichorii	10	–	31.25	36	8	7.81	–	25 (OFX)
Pseudomonas corrugate	48	10	7.81	48	7	7.81	–	26 (OFX)
Pseudomonas fluorescens	48	7	7.81	43	8	7.81	–	11 (OFX)
Pseudomonas syringae pv. *syringae****	48	9	7.81	48	8	7.81	–	25 (OFX)
Pseudomonas syringae pv. *syringae*	8	–	31.25	33	8	7.81	–	21 (OFX)
Pseudomonas syringae pv. *syringae*	8	–	31.25	39	8	7.81	–	20 (OFX)
Pseudomonas syringae pv. *syringae*	8	–	31.25	42	10	7.81	–	21 (OFX)
Pseudomonas syringae pv. *phaseolicola*	14	–	15.63	44	7	7.81	–	24 (OFX)
Pseudomonas syringae pv. *pisi*	14	–	15.63	41	8	7.81	–	24 (OFX)
Pseudomonas syringae pv. *tabaci*	10	–	31.25	45	9	7.81	–	23 (OFX)
Pseudomonas syringae pv. *tomato*	11	8	7.81	45	8	7.81	–	25 (OFX)
Ralstonia solanacearum	48	10	7.81	48	8	7.81	–	24 (SCF)
Xanthomonas axonopodis pv. *campestris*	48	–	31.25	47	10	7.81	–	23 (SCF)
Xanthomonas vesicatoria	47	7	7.81	47	8	7.81	–	21 (SCF)

DD, Inhibition zone in diameter (mm/sensitive strains) around the disks (6 mm); MIC, minimal inhibitory concentration; * DMSO; Dimethyl sulfoxide (%10); ** OFX, ofloxacin (10 µg/disc); SCF, sulbactam (30 µg/disc) + cefoperazone (75 µg) (105 µg/disc) were used as positive reference standart antibiotic discs (oxoid); *** from different host (cherry).

tial oils. Positive controls were prepared using the antibiotics as indicated in Table II. The bacterial cultures were incubated at 27°C for 48 h. Antimicrobial activities of the essential oils were evaluated by measuring the zone of inhibition against the bacteria. Each test assays were repeated in triplicate.

Micro dilution assay. The minimal inhibition concentration (MIC) values studied for the bacteria were determined as sensitive to the essential oils in disc diffusion assay. The inocula of the bacteria were prepared from 12 h broth cultures and cultures were adjusted to 0.5 McFarland Standard turbidity. The essential oils were prepared by diluting 10% DMSO to prepare a decreasing concentration range from 500 µg/ml to 7.81 µg/ml to be tested in 10 ml sterile test tubes containing tryptic soy broth. MIC values of the essential oils against bacterial strains were determined based on a micro-well dilution method (Zgoda and Porter, 2001). The 96-well plates were prepared by dispensing into each well 95 µl of tryptic soy broth and 5 µl of the inoculum. Then 100 µl from essential oils from all the prepared concentrations were individually added into the wells. A negative control was prepared as the last well containing 195 µl tryptic soy broth without essential oil and 5 µl of the inoculum. Maxipime (Bristol-Myers Squibb) at the concentration range of 500–7.81 µg/µl was prepared in tryptic soy broth and used as standard drug for positive control. The plate was covered with a sterile plate sealer, mixed on plate shaker at 300 rpm for 20 s, and then incubated at 27°C for 24 h. Bacterial growth was determined by absorbance at 600 nm using the EL × 800 universal microplate reader and confirmed by plating 5 µl samples from clear wells on tryptic agar medium. The essential oils were tested against all the bacteria for three times. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms.

Results and Discussion

Chemical composition of the essential oils. The essential oil compositions of Turkish *Satureja*, *Calamintha* and the relative amounts of the components are shown in the Table III. This table shows that the Turkish *Satureja* contains carvacrol (79.17%), γ-terpinene (9.05%), *p*-cymene (3.14%), thymol acetate (2.24%), β-caryophyllene (1.48%); *Calamintha* contains

Table III
Essential oil contents of *S. hortensis* and *C. nepeta*

RI*	*S. hortensis*			*C. nepeta*			Identification methods
	RT**	Components	(%)	RT	Components	(%)	
983	11.84	β-Pinene	0.33	–	–	–	GC, MS, RI
995	–	–	–	13.21	3-Octanol	0.70	GC, MS, RI
1023	13.75	α-Terpinene	0.55	–	–	–	GC, MS, RI
1034	14.24	*p*-Cymene	3.14	–	–	–	GC, MS, RI
1037	–	–	–	14.29	Limonene	13.51	GC, MS, RI
1067	15.72	γ-Terpinene	9.05	–	–	–	GC, MS, RI
1106	–	–	–	18.07	Linalool	0.51	GC, MS, RI
1172	21.61	Borneol	0.64	21.59	Borneol	0.14	GC, MS, RI
1178	21.99	Terpinen-4-ol	0.96	21.87	Terpinen-4-ol	4.55	GC, MS, RI
1190	–	–	–	22.82	α-Terpineol	0.38	GC, MS, RI
1255	–	–	–	25.65	cis-Piperitone epoxide	48.66	GC, MS, RI
1289	26.97	Thymol	0.10	–	–	–	GC, MS, RI
1296	27.43	Carvacrol	79.17	27.46	Carvacrol	2.13	GC, MS, RI
1313	–	–	–	28.33	Dihydrocarveol acetate	1.24	GC, MS, RI
1347	29.39	Thymol acetate	2.24	–	–	–	GC, MS, RI
1369	–	–	–	30.43	Piperitenone oxide	22.08	GC, MS, RI
1419	32.25	β-Caryophyllene	1.48	32.24	β-Caryophyllene	2.21	GC, MS, RI
1442	33.04	Aromadendrene	0.30	–	–	–	GC, MS, RI
1478	34.60	γ-Muurolene	0.25	–	–	–	MS, RI
1486	–	–	–	34.87	Germacrene D	0.42	GC, MS, RI
1494	35.24	Viridiflorene	0.35	–	–	–	GC, MS, RI
1513	36.33	γ-Cadinene	0.51	–	–	–	MS, RI
1574	39.40	Spathulenol	0.92	–	–	–	MS, RI
1579	–	–	–	39.59	Caryophyllene oxide	0.80	GC, MS, RI

RI*; Retention index relative to *n*-alkanes on SGE-BPX5 capillary column, **RT****; retention times, **GC**; identification was based on retention times of authentic compounds on SGE-BPX5 capillary column, **MS**; tentatively identified based on computer matching of the mass spectra of peaks with Wiley 7N and TRLIB libraries and published data, **RI**; identification was based on comparison of retention index with those of published data

cispiperitone epoxide (48.66%), piperitenone oxide (22.08), limonene (13.51%) and terpinen-4-ol (4.55%) as major components.

According to the previous studies, essential oil compositions of *S. hortensis* and *C. nepeta* from different origins showed varieties in terms of quality and quantity. The compositions of essential oils of *S. hortensis* were reported as γ-terpinene (40.9%) and carvacrol (39.3%) with 4.46% oil content by Gora *et al.* (1996); carvacrol (40–49%) and γ-terpinene (36–45%) by Svoboda (2003); carvacrol (46%), γ-terpinene (37.7%) and oil content of 0.93% by Sefidkon *et al.* (2006); carvacrol (42.0–83.3%), γ-terpinene (0.5–28.5%) and *p*-cymene (1.0–17.1%) with twenty nine components in the oils by Hadiana *et al.* (2010); γ-terpinene (35.5%), thymol (18.2%) and carvacrol (29.7%) from extracted oil through supercritical fluid extraction by Khajeh (2011). It can be concluded from all the previous studies that carvacrol, thymol, and their precursors, *p*-cymene and γ-terpinene are major components of *S. hortensis* oil. Carvacrol and thymol were determined as the major components in all *Satureja* from Turkey, too. The compositions of essential oils of *S. hortensis* from Turkey were reported: thymol (29.0%), carvacrol (26.5%), a total 22 constituents consisted of γ-terpinene (22.6%), and *p*-cymene (9.3%) by Gulluce *et al.* (2003); carvacrol (42.0–63.0%) with oil content ranged from 1.30% to 2.67% by Baser *et al.* (2004); *p*-cymene (40.6% and 35.9%), thymol (39.9% and 43.4%), carvacrol (5.7% and 16.0%) and γ-terpinene (3.7% and 3.2%) with oil content of 0.5% and 0.7% by Azaz *et al.* (2005); thymol (40.54%), γ-terpinene (18.56%), carvacrol (13.98%), and *p*-cymene (8.97%) by Adiguzel *et al.* (2007).

The main constituents of *C. nepeta* oils were determined in the previous studies as pulegone (about 50%); menthone (9.4%), limonene (7.0%), menthol (4.6%), piperitenone oxide (4.6%), piperitone oxide (3.9%), and piperitenone (3.4%) by Flamini *et al.* (1999); pulegone (41.0%), menthone (32.0%), piperitone (7.3%) and piperitenone (7.0%) by Couladis and Tzakou (2001);

pulegone (75.5%), piperitenone oxide (6.0%), menthone (5.3%) and menthol (4.3%) by Kitic *et al.* (2005); pulegone (76.5%) and piperitone (6.1%) by Schulz *et al.* (2005); pulegone, piperitenone oxide and piperitenone by Marongiu *et al.* (2010). According to previous studies, the essential oils of *S. hortensis* and *C. nepeta* contain similar major compounds in spite of differences in their quantity. These differences might have been derived from local, climatic, seasonal and experimental factors. Our results have generally confirmed the findings of the previous studies.

Antibacterial activities of essential oils. In this study, the essential oils at 7.81–500 µg/disk concentrations were also tested for antibacterial activities against 20 phytopathogenic bacterial strains isolated from fruit and vegetables origins (Table II). The inhibition zone above 7 mm in diameter was regarded as positive result. As shown in this table, the oils of *S. hortensis* and *C. nepeta* exhibited considerable antibacterial activities against most of the tested bacteria (7–48 mm inhibition zone). Both gram-positive and Gram-negative bacteria were sensitive to the tested essential oils. No significant difference in susceptibility was found between Gram-negative and Gram-positive bacteria. It was interesting to find that most of the essential oils had stronger MIC values than standard antibiotic. 10% DMSO was used as a negative control, it exhibited no inhibition zone (Table II).

In various studies, although the extracts or essential oils of *S. hortensis* and *C. nepeta* were tested for their antimicrobial activity, there are no satisfactory reports against plant pathogenic bacteria. There is only a few data about the antibacterial effectiveness of the essential oil of *S. hortensis* against to phytopathogenic bacteria, which were provided by Gulluce *et al.* (2003), Sahin *et al.* (2003), Kizil and Uyar (2006), Kotan *et al.* (2007), Mihajilov-Krstev *et al.* (2009). The findings of those studies are supported by our findings demonstrating strong antimicrobial activity of essential oil of *S. hortensis*. To our knowledge, there is no report about the antibacterial properties of essential oil of *C. nepeta* against phytopathogenic bacterial strains. So, this study is the first report on the antibacterial effectiveness of the essential oil of *C. nepeta* against phytopathogenic bacteria.

According to our results the antibacterial effect of oil of *S. hortensis* was found to be lower than the essential oil of *C. nepeta* according to inhibition zone. But, generally, it is clear that both of the essential oils have strong antibacterial activity against tested phytopathogenic bacteria. Furthermore, in our study, we detected bactericidal activity against the tested bacteria, especially at high concentrations of essential oil of SH. In our study, generally most of the tested organisms were also sensitive to many of the essential oils. The maximal inhibition zones and MIC values of *S. hortensis*, *C. nepeta* showed a significant difference in the range of 7–48 mm and 7.81–31.25, 7.81 µg/ml, respectively (Table II). *A. tumefaciens*, *B. pumilus*, *C. michiganensis* subsp. *michiganensis*, *E. intermedius*, *E. chrysanthemi*, *P. fluorescens*, *P. syringae* pv. *syringae* (from cherry), *P. syringae* pv. *tomato*, *R. solanacearum* and *X. vesicatoria* were the most sensitive organisms against to both of essential oils (MIC value 7.81 µg/ml). *P. cichorii*, *P. syringae* pv. *syringae* (isolated from apricot), *P. syringae* pv. *tabaci* and *X. axonopodis* pv. *campestris* were the most resistant microorganisms to the essential oil of *S. hortensis* with the MIC value (31.25 µg/ml). The other resistant microorganisms to essential oil of *S. hortensis* were *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *pisi* (15.63 µg/ml). It is thought that the sensivity can be caused by the differences in host, virulent of pathogens, toxins produced by these pathogens. For example, although *P. syringae* pv. *syringae* isolated from cherry was determined as sensitive (7.81 µg/ml), *P. syringae* pv. *syringae* isolated from apricot was the most resistant microorganism to *S. hortensis* oil with MIC value (31.25 µg/ml). As shown in the Table II, *C. nepeta* showed promising inhibitory activity especially even at low concentration. All of the tested bacteria were sensitive against to the essential oil of *C. nepeta*, too.

According to these results, it is clear that the essential oils have a potential antibacterial effect on the tested bacteria. Many of the previous studies demonstrated that essential oils show a considerable antimicrobial activity due to the presence of chemical compounds containing mainly aromatic oxygenated monoterpenes and high phenolic contents; carvacrol, thymol, ketones, pulegone, piperitone and piperitenone. For example, the antimicrobial activity of the essential oil of *C. nepeta* can be explained with the high contents of ketones, pulegone, piperitone and piperitenone (Panizzi *et al.*, 1993). This claim is further supported by our findings (Table III). Therefore, in our study; a high antibacterial effect of essential oil of *C. nepeta* can be associated with the presence of many components. In addition, according to studies made very recently, the antibacterial effect against the microorganisms were associated with the main constituents of the oil. According to Flamini *et al.* (1999), pulegone among constituents of *C. nepeta* only showed antimicrobial activity. It is also reported that some components such as carvacrol and thymol have potentials for controlling certain important plant pathogenic bacteria and seed disinfectant (Kotan *et al.*, 2007, 2010). So, the high antimicrobial activity of *S. hortensis* essential oil could be explained through the high level of carvacrol, well known for having antibacterial activity; *C. nepeta* have cis-piperitone epoxide, piperitenone oxide. Furthermore, the synergistic and antagonistic effects of these chemicals and

minor components can also affect the antibacterial activity of essential oils. In this regard, it is very important to stimulate systemic resistance mechanisms of the plants through the natural stimulators, use of healthy seeds, and seed disinfection through natural antimicrobial substances. Therefore, it is necessary to test several different combinations in commercial formulations of volatile oils and extracts and to determine bio-formulations according to the results obtained from these tests. It showed that essential oils of these plants are more effective than the antibiotics produced commercially against many bacteria. So; these essential oils are alternative components for defeating plant diseases. High level of antimicrobial activity of certain species in the Eastern Anatolia Region in Turkey put forward the necessity to take their gene sources under control and to research the possibility to cultivate them before dying out. Furthermore, it is necessary to carry out serious studies on their cultivatability.

In conclusion, the development of natural antimicrobials will help to decrease the negative effects (residues, resistance, and environmental pollution) of synthetic drugs. In this respect, natural antimicrobials may be also effective, selective, biodegradable, and less toxic to environment. In conclusion, according to the results presented in this study, we suggest that the essential oil of these plants can be used as antimicrobial agents in the management of plant diseases. However, the safety and toxicity of these compounds will need to be addressed.

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ORIGINAL PAPER

Investigation of the Actual Causes of Hip Joint Implant Loosening Classified as Aseptic – Analysis of Microbiological Culture Results and Levels of Inflammatory Markers

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Abstract

Loosening of the hip joint prosthesis is considered as one of the most significant postoperative complications in recent years. The laboratory diagnostic procedure used to differentiate periprosthetic infection from aseptic loosening is very difficult because of the biofilm which microorganisms form on the implant surface. The purpose of this research was to evaluate the level of concordance between clinical classification of implant loosening among 50 patients subjected to reimplantation procedure and laboratory investigation of PJI including microbiological culture results and the levels of inflammatory markers assessed in the patients' synovial fluid samples, serum, and full blood. The synovial fluid was collected for leukocyte count, differential cell count, and culture on standard media. The levels of systemic inflammation markers such as the ESR and CRP concentration were determined in serum and full blood. Tissue samples were collected for microbiological studies. Components from endoprostheses were exposed to ultrasound in a process called sonication. Among the parameters measured in serum and full blood the levels of ESR and CRP were higher in the septic group of patients. Cytologic analysis of synovial fluid was in correlation with microbiologic identification. The most frequent isolated bacteria was *Staphylococcus epidermidis*. Culture results from materials such as synovial fluid, sonicate and tissues are crucial to establish the infectious aetiology of the loosening. Microscopic analysis of synovial fluid represents a simple, rapid and accurate method for differentiating PJI from aseptic failure. Sonication increases detection of the infectious process, and culture results are in correlation with the cytologic analysis of synovial fluid.

Key words: aseptic loosening, biofilm, hip joint, implant failure, leukocyte cell count

Introduction

Hip joint implantations have represented one of the most frequent orthopaedic surgery procedures within the last years. Loosening of the hip joint prosthesis is considered as one of the most significant postoperative complications (Schinsky *et al*., 2008). It may follow an aseptic or septic course. Aseptic loosening is associated with activation of macrophages caused by wear debris from implant biomaterial which can occur in the periprosthetic space during its normal activity. Septic loosening is a result of the activation of neutrophils and production of inflammatory mediators induced by bacteria. Bacteria which are isolated most frequently include coagulase-negative staphylococci, *Staphylococcus aureus*, *Propionibacterium* spp. The causative microorganisms may exist on the implant surface or in its close proximity in forms which significantly hamper their detection by routine cultivation techniques. These forms include the biofilm, SCV (*small colony variants*) subpopulations or intracellular localization (Marculescu and Cantey 2008; Moran *et al*., 2010).

Prosthetic joint infections (PJIs) are classified as: early (<3 months after implantation) and late (>3 months after implantation). The early infections are a result of intraoperative bacterial infection whereas the late infections develop as a result of hematogenous bacterial translocation from distant sites of infection. In both cases bacteria can produce biofilm on the implant surface which is considered as one of their most important causes of difficulties associated with treatment of PJIs. Risk factors predisposing to the development of PJIs include previous joint surgeries including their types and numbers, accompanied diseases, age, obesity, rheumatoid arthritis, infections such as urinary tract infections, periodental infections or purulent

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skin changes (Górecki *et al.*, 2008; Moran *et al.*, 2010; Zimmerli, 2006).

It should be mentioned that aseptic loosening commonly considered among the most frequent causes of implant dysfunction, has been increasingly reported to be associated with infection. Moreover, aseptic, biomechanical loosening is difficult to differentiate from oligosymptomatic chronic infectious process. It also complicates treatment strategy, which becomes more expensive and not effective, despite reimplantation procedure.

The laboratory diagnostic procedures used to differentiate periprosthetic infection from aseptic loosening include:

- analysis of the levels of CRP, ESR, WBC in patient's serum and full blood,
- white blood cell count and its differential cell counts measured in synovial fluid,
- histopatological examination of periprosthetic tissues based on the determination of the count of neutrophils and macrophages in tissues bioptate,
- microbiological culture of sonicate fluid, synovial fluid, periprosthetic tissue fragments,
- image research – radiography, computer tomography, USG, magnetic resonance, nuclear medicine methods (Bauer *et al.*, 2006; Bedair *et al.*, 2011; Cipriano *et al.*, 2012; Górecki *et al.*, 2008; Moran *et al.*, 2010; Parvizi *et al.*, 2011; Zimmerli, 2006).

According to guidelines published by Parvizi *et al.* (2011) a definite diagnosis of PJI can be made when the following conditions are met:

1. a sinus tract communicating with the prosthesis or
2. a pathogen is isolated by culture from two separate tissue or fluid samples obtained from the affected prosthetic joint or
3. four of the following six criteria exist:
   a. elevated serum erythrocyte sedimentation rate (ESR) or serum C-reactive protein (CRP) concentration,
   b. elevated synovial white blood cell (WBC) count,
   c. elevated synovial neutrophil percentage (PMN%),
   d. presence of purulence in the affected joint,
   e. isolation of a microorganism in one culture of periprosthetic tissue or fluid,
   f. greater than five neutrophils per high-power field in five high-power fields observed from histologic analysis of periprosthetic tissue at 400 times magnification (Parvizi *et al.*, 2011).

It should be mentioned that no single routinely used clinical or laboratory test has been shown to achieve ideal sensitivity, specificity, and accuracy for the diagnosis of PJI. Hence, a combination of laboratory, histopathology, microbiology, and imaging studies is frequently necessary (Schinsky *et al.*, 2008; Trampuz and Zimmerli, 2005). The diagnostic significance of the examination of the synovial fluid should be highlighted. Valuable information useful to distinguish between aseptic implant failure and PJI can be provided by the analysis of synovial fluid both macroscopically (its colour, transparency, viscosity) and microscopically (white blood cell count and the polymorphonuclear leukocytes percentage). The obtained results should be interpreted in the context of microbiological culture results (Bedair *et al.*, 2011; Dougados, 1996; Górecki *et al.*, 2008; Zmistowski *et al.*, 2012).

The purpose of our work was to evaluate the level of concordance between clinical classification of implant loosening among patients subjected to reimplantation procedure and laboratory investigation of PJI including microbiological culture results and the levels of inflammatory markers assessed in the patients' synovial fluid samples, serum, and full blood.

Experimental

Materials and Methods

Materials were collected from 50 patients (mean age: 71.8+/−10.3 years) who underwent prosthetic hip joint revision due to loosening between April 2009 and December 2011. The patients were treated at the Orthopaedic Ward of the Clinical Hospital No. 4 in Lublin, Poland. The blood samples were collected in the direct preoperative period. The synovial fluid samples, tissue fragments, and the elements of explanted prostheses were collected intraoperatively. All diagnostic procedures were taken in laboratory of Medical Microbiology Department in Medical University of Lublin, Poland.

In all patients enrolled in the study the loosening was clinically classified as aseptic due to the absence of fistula and/or the purulence in the affected joint. The mean period to the onset of the loosening symptoms was 95 months ±62.4. Eleven patients had undergone previous revision procedures. Among them two patients had a history of two reimplantations whereas the remaining nine experienced one reimplantation. Eight patients suffered from comorbidities such as diabetes, hyperthyroidism, rheumatoid arthritis, chronic renal insufficiency, chronic obstructive pulmonary disease. All investigation was conducted in accordance with the ethical standards of the bioethical committee on human experimentation.

Intraoperatively, tissue samples from the close proximity of the implant and demonstrating the most obvious inflammatory changes were collected for microbiological studies. At least two tissue samples were collected from each patient. The synovial fluid was collected intraoperatively for leukocyte count, differential cell count, and culture from patients with "asep-

tic" loosening. The prosthetic components were placed in 1-liter, straight-sided, wide-mouthed polypropylene jars that had been autoclaved at 132°C for 15 minutes. The specimens were processed by the microbiology laboratory within 2 hours.

The levels of systemic inflammation markers such as the ESR and CRP concentration were determined in the direct preoperative period.

Synovial fluid analysis: Synovial fluid was aspirated intraoperatively using a sterile syringe and transferred to the test-tube containing EDTA. One portion of synovial fluid was analysed in the Fast Read 102 chamber (40 × magnification) in order to calculate the leukocyte count, the remaining portion was centrifuged (2500 × g/min for 5 minutes at room temperature). The supernatant was frozen at –80°C. The sediment was used to prepare a May-Grunwald-Giemsa (MGG) stained smear. The MGG smear was used to establish a percentage of neutrophils, monocytes and limfocytes in the synovial fluid sample (Zimmermann-Górska *et al*., 1997).

Conventional microbiological methods: Synovial fluid was inoculated in 100 µl aliquots onto aerobic blood agar, chocolate blood agar, and anaerobic blood agar. The aerobic and anaerobic blood agar plates were incubated at 35°C – 37°C in 5–7% carbon dioxide aerobically and anaerobically for 7 days.

Tissue specimens were inoculated into thioglycollate broth and incubated at 35°C – 37°C for up to 7 days. Cloudy thioglycollate broth was subcultured onto conventional bacteriologic media.

Sonication of removed prostheses: Components from endoprostheses were exposed to ultrasounds in process called sonication. Five hundred milliliters of sterile saline were added to each container. The container was vortexed for 30 seconds using a Vortex-Genie and subsequently subjected to sonication for 7 minutes at the temperature of 20°C (Monsen *et al*., 2009). Sonication was followed by additional vortexing for 30 seconds. The resulting sonicate fluid was removed under aseptic conditions and placed into 50-ml sterile Falcon tubes. Samples were then centrifuged at 4200 × g for 20 minutes. One hundred µl of the sedimented sonicate fluid were inoculated onto a set of routine aerobic and anaerobic bacteriologic media. The culture result was considered positive if there were at least 5 colony-forming units of the same organism on either plate.

WBC, ESR and ER analysis: The levels of inflammatory markers in serum and full blood were determined 24 hours before the operation. The WBC counts, serum erythrocyte sedimentation rate in full blood, as well as the CRP concentration in serum were analysed.

Statistical analysis: Statistical analysis was conducted using Statistica programme. In the non-parametric test (U Mann-Whitney) the p value < 0,05 was considered statistically significant.

Results

Positive culture results were obtained from 12 patients. The cultivated microorganisms were represented by the following species: *Staphylococcus epidermidis*, *S. aureus*, *Staphylococcus warneri*, *Enterobacter cloacae*, *Streptococcus mitis*, *Propionibacterium acnes* (Table I).

Based on the positive and negative culture results all patients were classified into two groups: aseptic (38 patients) and septic (12 patients). The analysis of the white blood cell count in the synovial fluid and the percentage of neutrophils revealed statistically important difference between aseptical and septical group of patients (Table II). In all septic cases, the white blood cell count and the percentage of neutrophils exceeded the cut-off value: > 1700 cells/µl and > 65% of polymorphonuclear leukocytes (Trampuz *et al*., 2007).

There was a statistically significant difference concerning the values of inflammatory markers (ESR, CRP) between aseptic and septic group of patients. The values of these parameters were almost two-fold higher in patients classified as septic than in the group of patients with aseptic implant failure. The mean count of WBC was similar in both groups (Table III).

Table I
The number of aseptical and septical cases of hip joint implant loosening in the aspect of cultured microorganisms

Microorganism	No. of cases
Aseptic loosening of the hip	38
Staphylococcus epidermidis	6
Staphylococcus warneri	2
Staphylococcus aureus	1
Streptococcus mitis	1
Enterobacter clocae	1
Staphylococcus epidermidis, *Propionibacterium acnes*	1

Table II
Comparison of parameters measured in synovial fluid with the type of implant loosening

Mean Value (SD) / Type of loosening	Synovial fluid volume (ml)	White blood cell count (cell/µl)	%N	PMN amount (cell µl)	%M	Monocytes cell count (cell/µl)	%L	Lymphocytes cell count (cell/µl)
Septic loosening (n = 12)	6.08 ± 4.5	22.359.3 ± 39.868.5	82.3 ± 22.9	18.401.7 ± 39.493	10.3 ± 14.8	2.303 ± 1.846	5.8 ± 10.5	1.296.8 ± 2.183.6
Aseptic loosening (n = 38)	4.35 ± 2.9	321.3 ± 295.2	38.5 ± 19.8	123.7 ± 144.8	44.5 ± 18	142.9 ± 171.1	16.7 ± 11.3	53.65 ± 68
p	p > 0.05	0.000001	0.000025	0.000001	0.000014	0.00001	0.001	0.0001

Table III
Comparison of gender, time to loosening and inflammatory markers in serum and full blood of patients with septic and aseptic type of implant loosening

Parameter (SD) / Type of loosening	Age (years)	Time to loosening (months)	ER (mm/h)	CRP (mg/l)	WBC (tys/mm^3)
Septic loosening (n = 12)	68.1 ± 13.8	82±70.5	45.7 ± 24.4	18.49 ± 20.8	7.67 ± 3.3
Aseptic loosening (n = 38)	71.5 ± 10.8	78.6 ± 57.1	27.8 ± 19.4	10.5 ± 23.1	6.73 ± 1.9
p	p > 0.05	p > 0.05	0.004	0.004	p > 0.05

Table IV
Time to loosening and inflammatory markers in serum and full blood of patients with different bacterial species

Microorganism (number of cases)	Time to loosening (months) SD	ER (mm/h) SD	CRP (mg/l) SD	WBC (tys/mm^3) SD
CNS n = 8	79 ± 72	43.6 ± 20.4	12.5 ± 13.1	7.1 ± 3.5
S. aureus n = 1	17	13	13.29	13.3
E. cloacae n = 1	169	52	36.67	7.52
S. mitis n = 1	129	91	72.99	7.95
S. epidermidis i *P. acnes n = 1*	18	19	0.74	7.78

Table V
The synovial fluid inflammatory parameters in the context of cultivated bacterial species

Microorganism (number od cases)	Synovial fluid volume (ml) SD	White blood cells count (cell/ul) SD	PMN amount (cell/ul) SD	%N SD	%M SD	%L SD
CNS n = 8	4.7 ± 3.2	5.586 ± 3.905	4.581 ± 3.721.8	75 ± 25.7	14 ± 16.9	7.7 ± 12.3
S. aureus n = 1	7	149.600	142.120	95	0	5
S. mitis n = 1	6.5	29.915	28.120	94	6	0
S. epidermidis i *P. acnes n = 1*	3.6	37.600	36.470	97	3	0
E. cloacae n = 1	17	6.503	6.170	95	2	3

In the next step of the research we investigated the relationship between all parameters measured in the synovial fluid, serum and full blood and the microbial species isolated from synovial fluid, sonicate fluid and periprosthetic tissue cultures (Table IV, Table V).

Discussion

Despite significant advance in the laboratory and image diagnostics there is still a need to find a simple and fast method of identification of the type of loos-

ening, soon enough to prevent its progression and to protect the implant. The major criteria used to clinically classify the type of loosening include the RTG image and the results of ER, CRP, WBC analysis in serum and full blood. Microbiologically, culture results from materials such as synovial fluid, sonicate and tissues are crucial to establish the infectious aetiology of the loosening. It should also be mentioned that microscopic analysis of synovial fluid (leukocyte count and differential) represents a simple, rapid and accurate method for differentiating PJI from aseptic failure. The crucial limitation of this diagnostic approach, however, is the invasive (intraoperative) way of collection of this material.

Ideally, the infection is diagnosed (or excluded) before surgery, which enables starting antimicrobial treatment preoperatively and allows planning of the most appropriate surgical management (Trampuz and Zimmerli, 2005).

Synovial fluid, tissues and elements from prosthesis (mandrel, pan) were subjected to microbiological culture. Mandrel and pan were subjected to ultrasounds in a simple and useful process called sonication. The use of sonication increases the chance of dislodging microorganisms adhered to the surface of explanted devices which, in turn, may increase the sensitivity of the culture. Sonication is innovatory method which is very helpful in diagnostic of orthopaedic implant loosening (Monsen *et al*., 2009; Trampuz *et al*., 2007).

The results of microbiological culture enabled to classify patients enrolled in the study into two groups: septic (12 cases) and aseptic (38 cases). Bacteria isolated most frequent included *S. epidermidis* which is an evidence to intraoperatively infection of this skin bacteria (in patients with early type of loosening) or exogenic infection during an operations (in patients with late type of loosening).

The results of bacteriological culture were confronted with cytologic analysis of synovial fluid. This analysis provided early (before the culture results were available) information on the type/nature of the loosening. As mentioned, septic loosening is suspected if at least 1700 white blood cells/μl and more than 65% of neutrophils are detected in the synovial fluid sample (Trampuz *et al*., 2007). Among 11 patients the above mentioned parameters significantly exceed the cut-off values which was indicative of the septic type of loosening. The preliminary diagnosis was confirmed by the presence of microorganisms in 10 synovial fluid microscopic smears and positive culture results from synovial fluid, sonicate fluid and tissues in 12 patients. In 3 out of 50 cases compatibility between the results of microbiological culture and cytological analysis of synovial fluid was not confirmed. In case of one patient from septic group the white blood cell count in synovial fluid exceed the cut-off value whereas the percentage of PMN was below the cut-off. Nevertheless, this patient was considered as infected due to the positive culture result. In another patient cytological analysis of synovial fluid was not indicative of inflammation associated with infection which stood in contradiction to a positive culture result obtained from the sonicate. We assume that the positive result of culture was a result of material contamination during analysis; therefore, the patient was considered as false-positive. Similarly, in aseptic group, in one patient we cytological analysis of synovial fluid was suggestive of the infectious type of loosening, but the culture result was negative. It could be associated with the short period between the primary surgery and revision – only a few days. The concentration of CRP in the patient's serum was elevated whereas the WBC count remained within the physiological range.

Leukocytosis measured in the synovial fluid seems to be the more useful for the diagnosis of the late type of loosening since high amount of granulocytes shortly after the surgery can be associated with the surgical intervention itself and can remain elevated even for several days (Cipriano *et al*., 2012).

Taking into account bacterial species and cytologic analysis of synovial fluid important differences concerning polymorphonuclear cells amount between isolated species, which was indicative of the immunological strength of response of macroorganism depending on the pathogen, were observed. The strongest response was generated by *S. aureus* (149.600 cells/μl), which represented almost 90-fold increase of the cut-off value. Other bacteria like *S. mitis* and *S. epidermidis* with *P. acnes* also induced strong granulocytic response, equal to 29.915 cells/μl and 37.600 cells/μl, respectively. Granulocytic response probably depends on the number and types of bacterial virulence factors, that's why it's not a surprise that *S. aureus* causes the strongest reaction of immunological cells.

Synovial fluid analysis includes macroscopic and microscopic observation. The sterile fluid is transparent, clear, yellow, and viscous as an egg white. The cell count is below 100–200 cells/mm^3, among which monocytes are predominant; other types of cells include neutrophils, lymphocytes, macrophages, synoviocytes and crystals – cholesterol, hydroxyapatites, *etc*. (Cipriano *et al*., 2012, Courtney and Doherty, 2009; Zimmermann-Górska *et al*., 1997). The septic fluid, in turn, is opaque, bloody or purulent, rich in leukocytes (Courtney and Doherty, 2009; Dougados, 1996; Zimmermann-Górska *et al*., 1997). During the macroscopic analysis of synovial fluid samples collected from patients enrolled in the study opacity and cloudiness was detected in all fluid specimens obtained from infected individuals. Synovial fluid polymorphonuclear granulocytes (PMN) also called the "first line of defense" are characteristic of the septic implant loosening. In addition to their

accumulation at the site inflammation and antimicrobial effect PMNs send signals to other immune cells in order to control/eradicate the infection. Antimicrobial activity is a result of the presence of granules within neutrophils which contain antimicrobial compounds such as lactoferrin, myeloperoxidase, proteases, elasthases, kathepsin G (Kumar and Sharma, 2010; Leszczyńska-Gorzelak and Poniedziałek-Czajkowska, 2009).

According to literature data, the ESR, CRP and WBC are the inflammatory markers used most frequently to investigate PJIs. Chevillotte and co-workers, Berbari and co-workers or Piper and co-workers also analysed these blood markers. Chevillotte research group reported the above mentioned markers to be non-specific and respresenting a weak diagnostics value. Levels of these parameters may only suggest further directions of the diagnosic proceedings, like the synovial fluid aspiration, blood culture, radiography. Similar conclusions were drawn by KE Piper research group. These authors also detected elevated levels of both ESR and CRP in the septic group of patients compared with aseptic one. According to Berbari's research group the levels of both ESR and CRP remaining within the physiological ranges give the evidence of non-inflammatory nature of the loosening of hip joint prostheses (Berbari,. *et al*., 2010; Chevillotte *et al*., 2009; Piper *et al*., 2010). Our investigations confirmed it in only 17 out of 38 aseptic patients. As mentioned, CRP is a non-specific marker, its concentration can remain elevated during the course of many diseases, *eg*. heart attack, cancer, and after surgical operations (Leszczyńska-Gorzelak and Poniedziałek-Czajkowska, 2009; Piper *et al*., 2010; Schinsky *et al*., 2008). The WBC count, the multifunctional diagnostic parameter, also cannot be associated only with the implant loosening process, which was confirmed in our research. The ESR and CRP also considered as non-specific, can be useful at the first stage of the diagnosis. Patients suffering from PJI had two-fold elevated ESR level compared to the aseptic group of patients. It should also be emphasized that differences in the septic group probably depend on the time to the loosening, because the ESR level is almost 2-fold higher in patients with early type of implant loosening compared to the late one. It was also observed in case of the WBC count, but it can be an effect of short period from the operation.

Comparison of both groups of patients – septic and aseptic revealed that parameters like age, time to loosening, and the WBC count were similar in both groups, with the exception of ESR and CRP, which were statistically significant between both groups noticed. Significant relation between the analysed parameters and bacterial species has not been observed. None of the mentioned markers is not specific only for prosthetic joint infection, additionally, their determination has very low sensitivity. The elevated levels of ESR and CRP may be suggestive of not only PJI but also other health problems like diabetes, kidney disease, heart disease, obesity (Cipriano *et al*., 2012; Chevillotte *et al*., 2009; Piper *et al*., 2010; Schinsky *et al*., 2008; Shah 2009).

The local immunological response (based on the white blood cell count and the percentage of PMN's) in synovial fluid has higher predictive value in the diagnosis of PJIs. It has been observed that in the septic group these parameters were 70-fold and 2.5-fold, respectively, higher than in the aseptic group of patients.

Among the measured parameters bacteriological culture result interpreted in the context of cytological analysis of synovial fluid and the levels of ESR and CRP in serum and full blood of patient has the highest diagnostics value for the PJIs identification.

In conclusion we can claim that:

1. Synovial fluid is the most sensitive diagnostic material. It is useful for the preliminary differentiation between septic and aseptic type of implant loosening.
2. Sonication increases detection of the infectious process, and culture results are in correlation with the cytologic analysis of synovial fluid
3. Determination of the levels of ESR and CRP has the highest predictive diagnostic value among inflammatory parameters measured in serum and full blood.

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ORIGINAL PAPER

In Vitro Studies of Antibacterial and Antifungal Wound Dressings Comprising H_2TiO_3 and SiO_2 Nanoparticles

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Abstract

The incidence rate of the infected and complex wound is established at approximately 40 000/1 million of the world's adult population. The aim of this study was to assess the efficiency of three novel types of wound dressings comprising sodium chloride, metatitanic acid and silicon dioxide nanoparticles. The study design was to prove their antimicrobial properties against the microorganisms most commonly causing wound infections. The study evaluated the antimicrobial effect of tested dressings on referenced strains of bacteria (ATCC collection, Argenta, Poland) and strains of fungi species (our own collection of fungi cultured from patients). The dressings were tested with both bacterial and fungal strains on solid media (Mueller-Hinton, Sobouraud, bioMerieux, France) in the standard method. The results confirmed the inhibition of growth of bacteria and revealed zones of inhibition for *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*. Significant zones of inhibition were established for *Staphylococcus aureus* and for fungi species of the *Candida* sp. These results would be crucial due to the fact of the low availability of antifungal therapeutics for both systemic and topical usage. Moreover, the current standard of antifungal treatment is associated with high costs and high toxicity in general. The preliminary results are very promising but further studies are necessary. Based on the obtained results, the tested dressings may contribute to the development of the surgical armamentarium of complex wound management in the near future.

K e y w o r d s: *Candida* sp., antibacterial wound dressing, antifungal wound dressing, metatitanic acid, silicon dioxide nanoparticles

Introduction

The incidence rate of the infected and complex wound is established an approximately 40 000/1 million of adult population (Pelka, 1997). Moreover, a constant increase of this rate is expected, considering the aging population, increased number of civilization diseases, as well as the prolonged survival rate of chronically ill patients. According to recent data approximately 1.5% of the population of developed counties and 3% of the population aged over 60 years will complain of a chronic and complex wound in the near future (Pelka, 1997; Gottrup *et al*., 2010). Impaired wound healing is commonly associated with the infection of the wound and inadequate treatment, usually based only on empirical antibiotic therapy without any antibiograms performed previously. Currently, the availability of antibiotics has contributed to their overuse and unreasonable usage in various clinical situations. Antibiotics administered not in accordance with the activity spectrum, reduce the ability of antibiotic tissue penetration (Kowalska-Krochmal, 2012). Antibiotics regimen of complex wound therapy is usually used without taking into consideration the appropriate dose and duration of the therapy. It is important to note that antibiotics constitute only one element of the complex approach that should be implemented. Topical antimicrobial agents utilized routinely for complex wounds

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are frequently ineffective. Thus prompted the research for new methods of dressing augmented with agents with topical antimicrobial action.

According to the latest report of the United States Centers for Disease Control National Nosocomial Infections Surveillance System (CDC NNIS), the profile of microorganisms isolated from surgical site infections (SSI) in the recent years has not changed. The most common are: *Staphylococcus aureus*, coagulase-negative bacteria of the *Enterococcus* spp. (*Enterococcus faecalis, Enterococcus faecium*), Gram-negative bacteria of the *Enterobacteriaceae* spp. (*Escherichia coli*) and non-fermentative rods (*Pseudomonas aeruginosa*, *Acinetobacter baumannii*). Currently there is an increase of multidrug-resistant bacteria causing SSI. On the other hand, *Candida* spp. (especially *Candida albicans*) are increasingly recognized as causes of SSI (Sikora and Kozioł-Montewka, 2010).

In recent years there has been tremendous progress of research regarding the potential use of nanotechnology in medicine, especially the application of nanocarriers as a controlled drug release to facilitate the delivery of therapeutics to the site of action (Moghimi *et al.*, 2005). According to this technology, therapeutics may themselves constitute a nano-scale, and thus can be simultaneously used as a carrier (De Jong and Borm, 2008). Therapeutics in this form have various advantages such as high stability and carrying capacity. Both the hydrophilic and hydrophobic agents may be utilized with the availability of various routes of administration (oral, inhalation, *etc.*) greatly facilitating their use. Therapeutics form as nanoparticles may be also designed to be released in a controlled way from enmeshed matrix. All of these properties would contribute to the improvement of the bioavailability of the drugs. It is believed that the use of nanoparticles as drug carriers may undeniably reduce the toxicity of therapeutics (Fadel and Garcia-Bennett, 2010). Silicon dioxide widely exists in the environment, usually in the form of quartz as a component of rocks, sand and minerals. In an active form (SiO_2) as a result of its activation reactive oxygen species (ROS) may be produced, which can lead to DNA defragmentation and thus to subsequent apoptosis of the cells. Entering the cells, nanoparticles induce the lysis of the cell structures succeeding in cell death (Chu *et al.*, 2011; 2012).

Sodium chloride (NaCl) is a highly hygroscopic property substance. As a component of dressing materials it plays an important role as absorptive particles. Titanium dioxide (H_2TiO_3) is a substance with varying spectrum of usage. It acts as a catalyst in many chemical reactions. It may be also utilized as an antibacterial agent. As a component of various types of filters it may counteract the harmful nitrogen oxides emitted by cars (Bai *et al.*, 2012). Due to its activation induced with ultraviolet (UV) light or hydrogen peroxide (H_2O_2), the biological activity of titanium oxide significantly increases. This property is considered to improve its antimicrobial activity as a component of wound dressing (Bagriche *et al.*, 2012; Charpentier *et al.*, 2012; Zhukova *et al.*, 2012; Roguska *et al.*, 2012).

Application of the above mentioned compounds for dressing materials aims to create a composite dressing for wound usage, including complex and infected wounds. We hypothesize that a dressing impregnated with suspension of 5% silicon dioxide and 1% metatitanic acid would increase the antimicrobial capability. Moreover, a dressing composed of 5% silicon dioxide and 5% sodium chloride would possess significantly greater absorption capacity. Thus the dressings would play a crucial role as a comprehensive management assisting complex wound therapy.

Experimental

Materials and Methods

The aim of this study was to compare the antimicrobial efficiency of three tested dressings. Radiation was used as an established method for dressing sterilization. The dressings were denoted as number 1, 2 and 3. The study evaluated the antimicrobial effect on bacteria and fungi most commonly causing wound infection. The study used reference strains of bacteria (ATCC collection, Argenta, Poland) and fungi (a collection of our own laboratory). Strains cultured from patients were applied anonymously.

As a representative of the *Staphylococci* family, *S. aureus* was selected and for the *Enterococci* family, *E. faecalis* was designated. As a representative of Gram-negative non-fermenting pathogen, *P. aeruginosa* was chosen. As representatives of the *Enterobacteriaceae* family, *E. coli*, *Klebsiella oxytoca*, *Citrobacter freundii* and *Proteus mirabilis* were selected. As a representative of fungi, five strains of *Candida* spp. were selected based on patients' pathogens culture.

The study was performed obtaining the acceptance number 186/12 of the Bioethics Committee of the Poznan University of Medical Sciences. The study was only designed as an in vitro phase. This research did not include any studies on animal models, as well as humans. There were no medical and biological incidents with the agents, as well as no dangerous conditions related to contact with contaminated dressing.

The study was performed at the Central Laboratory of Microbiology in H. Swiecicki Hospital of Poznan University of Medical Sciences.

Composition of dressings:

Dressing No 1. This was a gas-soaked dressing with 5% suspension of silicon dioxide, A-300. Silicon dioxide was dispersed in an aqueous medium using a high speed blender, as well as the ultrasound technique. Following this fabrication, the excess amount of the medium was removed with a specifically designed device.

Dressing No 2. This dressing was impregnated with a medium composing of 5% silicon dioxide and 5% sodium chloride. Sodium chloride particles displayed an osmotic effect, thus intentionally improving the antimicrobial effect. The sodium chloride concentration in the medium was less than usually used in the hypertonic solution (9%) due to the fact of the pH range of silicon dioxide in aqueous solution (pH 3.6–4.2).

Dressing No 3. This dressing was impregnated with a medium composing of 5% silicon dioxide and 1% metatitanic acid. It needed to be activated with 3% hydrogen peroxide solution before application. Metatitanic acid accelerated the decomposition of hydrogen peroxide into active OH^- and O_2H^- ions that effectively decontaminate the wound.

The tested dressings constituted a complex novel pattern of dressing material, based on silicon nanostructures. The unique dressings' composition facilitated both their bacteriostatic, bactericidal and absorptive action.

Processing and sterilization of the dressing. All dressings were designed in a round shape with radius 1 cm. They were packed in *TYWEK* sleeves and were sterilized using radiation. According to the study protocol, all procedures as well as the storage of the dressings were made using aseptic techniques. The sterility of the dressings was confirmed by microbiological examination prior to the implementation in the study.

Antimicrobial activity of all dressings was tested with the referenced strains of pathogens: seven standardized bacterial strains (Argenta, Poland): *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *K. oxytoca* ATCC 13182, *C. freundii* ATCC 43864, *P. mirabilis* ATCC 25933, and five fungal strains from our own laboratory collection cultured from patients (*Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida krusei*, *Candida famata*).

All dressings tested for bacterial and fungal strains were verified in microbiological growth medium (Mueller-Hinton, Sobouraud, bioMerieux, France). The study was based on a suspension of 0.5 on the McFarland scale. This parameter was preferred as commonly used for antibiotic susceptibility testing. Following preparation of the bacterial suspensions, they were placed on the microbiological growth medium of Mueller-Hinton agar using a swab moistened with a 3-fold inoculating bacterial culture. For such a prepared substrate, dressings were applied aseptically. Dressing No 3 was activated with 5 drops of hydrogen peroxide.

Three samples of tested dressings were evaluated for each strain of bacteria and fungi measuring the average size of the zones of inhibition. Dressings applied on well-established growth medium with pathogens were incubated at 37°C for 24 hours. Following this period of incubation zones of inhibition were assessed. Fungi of *Candida* strains were cultured on Sabouroud agar. Following incubation at 30°C for 24 hours, zones of inhibition were assessed. Because of required prolonged cultivation time for some fungi strains, cultures were extended for a further 24 hours. Disk diffusion method was used in this study. A tissue-paper disc impregnated with the antibiotic was applied on the agar medium with a cultured bacterial strain. The inoculum density of 0.5 McFarland scale was used for the disk diffusion method (Zabicka *et al*., 2009).

Results

Evaluation of the antibacterial activity. A zone of inhibition was observed (measured in mm). Due to the irregular structure of the gauze the dressings were made from, the exact result of zone of inhibition was difficult to assess. To overcome this disadvantage, the average measurement of zone of inhibition was used as the most reliable feature. There was no zone of inhibition obtained for both dressings No 1 and 2. This result might indicate the lack of antimicrobial potential against the seven strains of bacteria the dressings were tested with.

For dressing No 3 the following zones of inhibition were evaluated (Table I).

Table I
Dressing No 3 inhibition zones for Bacterial strains

Bacterial strain	Inhibition zone
Staphylococcus aureus	3 mm +/– 1 mm
Enterococcus faecalis	no zone of inhibition
Pseudomonas aeruginosa	2.5 mm +/– 1 mm
Escherichia coli	about 1 mm, questionable result (zone assessed as +/–)
Klebsiella oxytoca	8.5 mm +/– 1 mm
Citrobacter freundii	4 mm +/– 1 mm
Proteus mirabilis	7 mm +/– 1 mm

In addition, some tests were performed with selected strains (*E. coli*, *S. aureus* and *E. faecalis*) at 0.1 McFarland scale. No zones of inhibition were evaluated for *E. coli* and *E. faecalis*.

Comparable zones of inhibition for *S. aureus* were assessed for both 0.5 and 0.1 McFarland scale. These results might indicate that the dressing possessed the same antimicrobial potential, regardless of the inoculum (density) of the bacteria at the site of infection. Therefore, since hydrogen peroxide is well established antimicrobial substance, an additional test was performed to test the zone of inhibition of hydrogen peroxide alone for *E. coli*, *E. faecalis* and *S. aureus*. It was confirmed in all these three cases, that using hydrogen peroxide alone inhibited the growth of these bacterial strains. However, using both hydrogen peroxide with dressing did not inhibit the growth of *E. faecalis* and *E. coli*. The antibacterial effects occurred only for *S. aureus*. According to this experiment, it was established that using hydrogen peroxide alone did not prevent the growth of bacterial strains. Moreover, the antimicrobial effect was confirmed for the dressing No 3 activated with hydrogen peroxide.

Evaluation of the antifungal activity. Zones of inhibition were not observed near the applied dressings No 1 and 2 for any of the tested strains. This result may indicate the absence of antifungal activity of these types of dressings tested for five species of fungi. The zones of inhibition were perceived for dressing No 3 for all tested fungi species. Following zones of inhibition were revealed near the applied dressing (Table II).

Dressing No 3 was the only one presenting zones of inhibition for all fungi species tested in this study. There was a significant zone of inhibition for tested dressing No 3 suggesting a stronger antifungal effect than the antibacterial one. This outcome seems to be a crucial because of *Candida* spp. as a common etiological agent of wound infections. Because of the low availability of systemic or topical antifungal therapeutics, high cost of therapy, as well as their high toxicity, this would have a substantial impact on current management of complex wound (Figure 1).

Table II
Dressing No 3 inhibition zones for Fungal strains

Fungial strain	Inhibition zone
Candida albicans	6 mm +/– 1 mm
Candida parapsilosis	2,5 mm +/– 1 mm
Candida glabrata	16 mm +/– 1 mm
Candida krusei	7,5 mm +/– 1 mm
Candida famata	1,5 mm +/– 1 mm

Discussion

Wounds, both acute and chronic, are susceptible to be infected because of the loss of the inherent ability of the skin to act as a sterile barrier. The lack of the natural skin barrier facilitates easily the migration and colonization of the wound by the pathogens. Microorganisms within the wound form some kind of "biofilm" which is believed to be responsible for the delay in wound healing. It is probably caused by decreasing immune

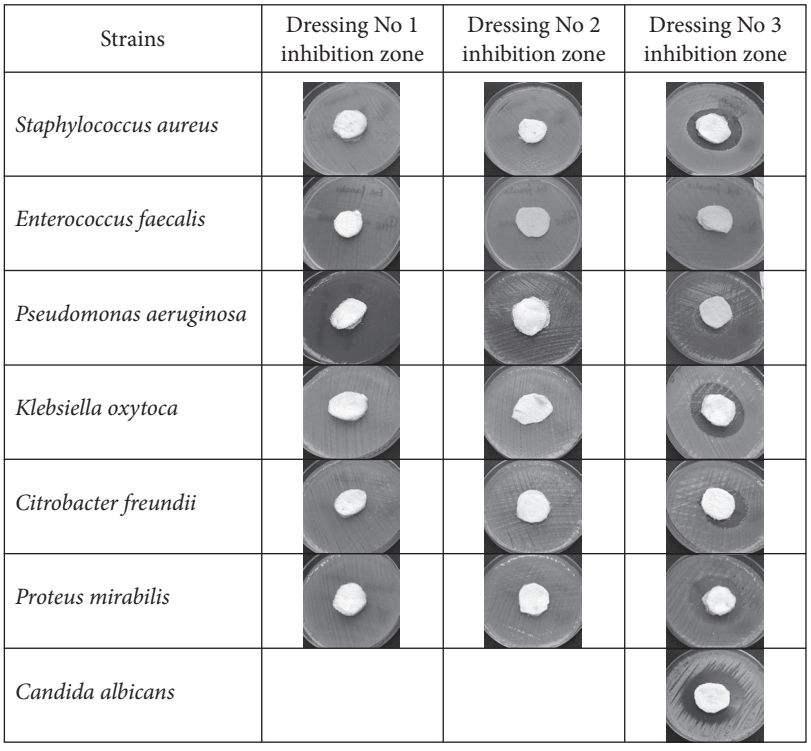

Fig. 1. Inhibition zones for bacterial and fungal strains

response of the patient and low penetration rate of the antibiotic.

Surgical site infection may be caused by varying strains of pathogens. This depends on many factors such as an anatomy region, surgical approach, general condition of the patients, comorbidities and others. The most frequently isolated microorganisms of surgical site infection are: *S. aureus*, *coagulase-negative staphylococci*, *Enterococcus* spp., Gram-negative bacilli of the family *Enterobacteriaceae* (*E. coli*) or *P. aeruginosa* (Dierzanowska, 2008; Percival *et al.*, 2012; Kim and Steinberg, 2012; Walter *et al.*, 2012; Schweizer and Herwaldt, 2012).

Regular debridement of the wound is considered to be the efficient and preferred management of chronic wounds, especially as a preventive method for pathogen biofilm development (Davis *et al.*, 2006; Wolcott *et al.*, 2009). Unfortunately, such a procedure seems to be usually insufficient.

In the purpose of overcoming this disadvantage, systemically and topically antibiotics, as well as antiseptics and mechanical debridement are used simultaneously. This kind of management may easily generate multidrug-resistant strains (MDR-multi drug resistant).

Based on not fully satisfactory results of many antimicrobial therapeutics used systemically, gradually researchers focus their attention on other methods, including dressings that would act topically. The antimicrobial properties of silver usage have been described for many years, with the well-established high efficiency of the impregnated dressings (Thomas and Mccubbin, 2003; Kostenko *et al.*, 2010). Another method giving great hope in the treatment of complex wounds is negative wound pressure therapy. Remarkable progress in the field of therapy has still some limitations associated with its availability and price.

Both chronic and acute wounds are susceptible to be simply infected due to their imbalanced property to maintain a sterile environment secured by intact skin barrier. A complex wound facilitates the development of new microorganisms within it (Percival and Bowler, 2004; Cooper and Okhira, 2006; Saye, 2007). Thus, there is an usual consequence of being infected with fungi, especially in patients treated with antibiotics for a long period of time. Therefore, it is important to introduce a new model of comprehensive antimicrobial dressings having both antibacterial and antifungal activity. Dressings No 1 and 2 were characterized by lack of antimicrobial activity. Growth of bacteria and fungi rate in the control groups was the same as in the experimental dressing. Dressing No 3 was characterized by a strong and specific antibacterial activity and, importantly, antifungal activity as well. The selective action of the tested dressing containing a silicon dioxide, A-300 and metatitanic acid against bacterial strains was related to the diameter of bacterial cells, this seems to be associated with nanostructured silicon dioxide used. This effect mentioned above was observed utilizing H_2O_2 as a catalyst, resulting in the effect of the active form of metatitanic acid. A silicon dioxide, A-300 nanostructure has an excellent absorption property. This fact would greatly benefit the absorption capacity of the dressing and allows its use in wounds with large amount of exudate.

Currently, there is no description in the literature of dressings based on silicon nanostructures (there is only a very promising scientific report using nanostructures in general). The unique properties of silicon nanostructures allow them to be used as redox catalysts, thus enhancing their antimicrobial action. Metatitanic acid $TiO(OH)_2$ is a potent antibacterial agent with a very simple mechanism of activation (contact with hydrogen peroxide). It has a strong and controlled cytotoxic effect, thus eliminating pathogens without affecting the healing wound process.

The presented study is a preliminary report. It is required to use the dressings in further clinical trial studies. Their application seems to be an effective method for the treatment of complex wounds, especially infected ones. The dressings also require the safety evaluation of their usage. All these aspects determine the subsequent steps of trial phases of this type of dressing in the future. Numerous reports have been described regarding the toxic effects of titanium and silicon nanostructures due to increased intracellular ROS levels and DNA damage.

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ORIGINAL PAPER

Escherichia coli and *Salmonella* spp. Early Diagnosis and Seasonal Monitoring in the Sewage Treatment Process by EMA-qPCR Method

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Abstract

Numerous studies have recently shown that molecular biology tools can allow for early diagnosis of pathogens and can substitute existing cost and time-taking traditional methods. One of them, the qPCR, is successfully used in microbiology and its utility has been assessed for many different biological materials. The aim of this study was to: 1) determine, optimize and apply qPCR as a method to detect *Escherichia coli* and *Salmonella* spp. in primary influents and final effluents from municipal wastewater treatment plant 2) define if addition of ethidium bromide monoazide (EMA) before DNA extraction can allow to distinguish between alive and dead bacteria, 3) quantify *E. coli* and *Salmonella* spp. in wastewater during four seasons by qPCR and traditional spread plate method and determine the correlation between the indicator and pathogenic microorganisms. The obtained results has shown that qPCR can be used as a quantitative method in the diagnosis of investigated bacteria in wastewater with EMA pretreatment as a crucial step for a proper quantitative analysis of the presence of these bacteria in wastewater. Both *E. coli* and *Salmonella* spp. bacteria species were present in all samples of primary influents and final effluents. Our study shown that the quantity of investigated bacteria is strictly correlated with the season that they were obtained in.

Key words: *Escherichia coli*, *Salmonella* spp., ethidium bromide monoazide, qPCR, sewage treatment

Introduction

Municipal wastewater effluents may constitute a significant contribution to drinking and recreational water pollution. To assess the danger to the environment from the microbiological point of view, Total and Fecal Coliform Assays are used to indicate the hazard of the presence of pathogens associated with fecal material. Generally, it is expected that a reduction in their presence is equal to a reduction in quantity of all pathogens in the analyzed material (Hagendorf *et al.*, 2005). However this conventional method does not indicate the actual state of a microbiological pollution (Shannon *et al.*, 2007). Traditional methods are time consuming and are limited by proper media content and culture conditions. Some microorganisms may also divide faster on media causing the incorrect interpretation of obtained data (Gilbride *et al.*, 2006). In this case, the detection of indicator bacteria, like fecal coliforms or fecal streptococci is not sufficient, because inactivation of pathogens depends strongly on their nature and applied system for sludge treatment in the wastewater treatment plant (Sidhu and Toze, 2009).

Escherichia coli is one of facultative anaerobes of human colonic flora, and some strains, like *E. coli* O157:H7 may cause very dangerous enteric diseases, including hemorrhagic diarrhea, abdominal cramps and hemolytic uremic syndrome. *E. coli* is the main indicator organism used commonly in the evaluation of microbiological contamination. This enterohaemorrhagic *E. coli* may give rise to infections in the gastrointestinal tract of mammals and cross contaminate humans. In 1993 the first report on the potential transfer of bacterial pathogens from land applied biosolids to humans was published. Besser *et al.* (1993) described cases after the consumption of cider made from apples collected from field treated with cow manure. *Salmonella typhimurium* is a pathogen which causes foodborne infections and salmonellosis and in general is present in raw and treated biosolids (Sahlstorm *et al.*, 2004). According to the FDA (Food and Drug Administration), the infective dose of *Salmonella* can be lower than 20 cells depending on the age and health of the host organism (FDA, 2003).

Pathogens may be easily detected with molecular biology methods in water, food and in samples coming

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from wastewater treatment plants. Since 1990 the polymerase chain reaction (PCR) has been used as a method allowing for a completely culture-independent analysis of all microbial communities of bacteria (Giovannoni *et al.*, 1990). In qPCR technique, an increasing fluorescence signal is measured in real time, which enables the direct analysis of the results after PCR without additional detection steps. However, DNA is isolated not only from live, but also from dead bacteria, so a positive PCR result can be obtained irrespective of the viability of cells (Fijałkowski *et al.*, 2014). To avoid this problem ethidium bromide monoazide (EMA) may be used. This substance is a dye, which intercalates the DNA only of damaged bacteria after photoactivation (Nogva *et al.*, 2003). DNA with covalently bounded dye will not react in PCR reaction, thus completely eliminating the problem of live/dead cell differentiation.

The primary objective of the study discussed in this paper was to optimize the qPCR method for detection and identification of *Salmonella* spp. and *E. coli* in wastewater. We examined the SYBR® Green I dye, specific primers and EMA cross-linking to create an easy protocol for determining the quantity of investigated bacteria in samples derived from wastewater treatment plants.

Experimental

Materials and Methods

Wastewater samples. Wastewater (primary influents and final effluents) samples were collected from a municipal wastewater treatment plant with secondary treatment facilities based on an activated sludge process in southern Poland (unit per capita loading PE = 315 000, wastewater treatment plant capacity Q = 90 000 m^3/d) during four different time periods (December-February = winter, March – May = spring, June – August = summer, September-November = autumn). Samples were obtained in biological triplicates according to the Standard Methods for the Examination of Water and Wastewater (APHA, 1995). Samples were transported in 5 l sterile plastic bottles in 4°C and analyzed within 6 h. Due to the varying amount of solid biomass contained in each type of wastewater samples, different volumes of wastewater were used for biomass collection by centrifugation at 5000 × g for 10 minutes at 4°C, respectively: 10–100 ml for primary influents, 100–300 ml for final effluents.

Bacterial strains (for standard curve in real time PCR reaction). *Salmonella enterica* subsp. *enterica serovar Typhimurium str. LT2* (DSM 50912, DSMZ Germany) and *E. coli* (DSM 10235, DSMZ, Germany) bacterial strains were aseptically plated on Brain Heart Infusion broth (BHI) and incubated at 37°C with shaking at 350 rpm in thermomixer (Eppendorf, Germany) overnight. The actively growing cells were harvested by centrifugation 10000 × g for 5 minutes and used for genomic DNA extraction and bacteriological analysis. DNA was isolated using Qiagen DNeasy Blood & Tissue Kit (QIAGEN, Dusseldorf, Germany) according to the manufacturer's protocol. The quantity and purity of the isolated DNA was determined spectrophotometrically at an absorbance of 260 nm and A260/A280, respectively. DNA was stored at a temperature of minus 80°C.

Genomic DNA isolation from wastewater. Wastewater samples were first filtered on an cellulose acetate membrane filter 0.45 µm (Whatman, USA) with a vacuum filtration system (Merck KGaA, Germany). DNA was isolated from the filter using MO BIO PowerWater® DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, USA) according to the manufacturer's protocol. DNA purity was confirmed by spectrophotometry (BioPhotometer, Eppendorf, Hamburg, Germany) as a A260/A280 ratio, which was in range 1.7–2.0 for all analyzed samples. Isolated DNA was stored at a temperature of minus 80°C (Innova range U101, New Brunswick Scientific co., Inc., New Jersey, USA).

EMA cross-linking. EMA dye was dissolved in water to a stock concentration of 5 mg/ml and stored at minus 20°C in the dark. EMA cross-linking was carried out according to Nocker and Camper (2006) for all studied wastewater samples. Membrane filters were placed in tubes containing 1 ml of sterile water and EMA was added. Final concentration of EMA in samples was 100 µg/ml. After a 5-min incubation in the dark, samples were light exposed for 1 min using a 650-W halogen light source placed 20 cm from the samples. Through whole procedure samples were placed on ice to avoid excessive heating. After EMA cross-linking, DNA was isolated from the filters as described above.

Real time quantitative PCR. Purified genomic DNA and primers were added to PCR reaction tubes containing 2 × PCR mastermix (Power SYBR® Green PCR Master Mix, Life Technologies Corp., USA) to final volume 20 µl. The final PCR solution contained 900 nM of each foward (reverse) primer and 10 to 100 ng of DNA. PCR was carried out in a Mastercycler Ep Realplex^2 (Eppendorf, Germany). The PCR reaction with all reaction components using sterile Milli-Q water (Merck KGaA, Germany) in place of DNA template was always run as a negative control in order to rule out any carry over contamination. Primers were as follows: forward 5'-GGTCTGCTGTACTCCACCTTCAG-3' and reverse 5'-TTGGAGATCAGTACGCCGTTCT- 3' (Calvó *et al.*, 2008) for *Salmonella* spp. bipA gene and EcoF 5'-GTCCAAAGCGGCGATTTG-3' and EcoR 5'-GAGGCCAGAAGTTCTTTTTCCA-3' for uidA gene for *E. coli* (Lee *et al.*, 2006). A standard curve was obtained by analysing 10-fold serial dilutions of DNA isolated

from *S. enterica* subsp. *enterica* serovar *Typhimurium str. LT2*. Log-linear regression analysis showed a significant correlation ($R^2 = 0.995$) between the CT values and the copy numbers of *bipA* gene of *S. typhimurium*. A similar standard curve was prepared for *E. coli uidA* gene analysis. The quantity of target gene copy number in analyzed samples was calculated from those standard curves. The sample was considered negative if: the fluorescent signal did not increase within 40 cycles or its peak in melting curve was out of 80.7–82.0°C for *Salmonella* spp. and 76.1–77.6°C for *E. coli*.

The PCR program was as follows: initial denaturation at 95°C for 10 min and then 40 cycles at 95°C for 15 s, respectively: 64°C for 35 s for *Salmonella* spp. and 56°C for *E. coli*, followed by 72°C for 30 s. The melting curve program was: 95°C for 1 min, 60°C for 5 min then linear increase of temperature to 95°C in 30 min.

Traditional spread plate technique. The bacteriological analyses were conducted immediately after samples were transferred to the laboratory. Appropriate sample volumes in triplicate were diluted (10^{-2}–10^{-6} dilution) in a phosphate saline buffer (140 mM NaCl, 10 mM phosphate buffer, and 3 mM KCl, pH 7.4) and varied according to sample source to ensure obtaining plates with 30–300 colonies. Inoculated plates were incubated for: 48 h at 37°C on Bismuth sulfite agar acc. to WILSON-BLAIR (Merck KGaA, Germany) for *S. typhimurium* detection and for 24 h at 37°C on Chromocult® Coliform Agar (Merck KGaA, Germany) for *E. coli* detection.

Statistical analysis. All results concerning the target gene copy number and colony forming units were expressed as means ± standard errors. Differences between means were determined by the Tukey's test, with the level of significance established at $P < 0.05$. The Pearson's correlation was used to test the relationship between *E. coli* and *Salmonella* spp. concentrations in raw and treated waste water samples.

Results and Discussion

QPCR and EMA-qPCR analyses of *Salmonella* spp. and *E. coli*. Both *Salmonella* spp. and *E. coli* were present in primary influents samples during all analyzed time periods (Table I). EMA pretreatment resulted in statistically important decrease in detected target gene copies number for *E. coli* in all analyzed samples and for *Salmonella* spp. in samples taken in autumn and spring. The major differences among target gene copy numbers were observed between final effluents samples. This is caused by the influence of the wastewater treatment process, in which microbiological contamination is partly reduced by chemical and biological treatment. Similar observations were done by Soejima *et al.* (2008) who discriminated live and heat-treated *Listeria monocytogenes* cells by EMA-qPCR. EMA-qPCR was also used to study *E. coli O157*, *Salmonella* spp. and *L. monocytogenes* survival under decontamination and antibiotic treatments (Rudi *et al.*, 2005). Those authors suggested the usage of this method for complex samples with mixed food borne bacterial communities, especially present in food. In this study, authors confirmed that this method may be useful in the case of wastewater samples and may help to evaluate the effectiveness of the wastewater treatment process because of elimination of false positive results caused by detection of DNA from dead cells.

On the other hand, many authors have been critical of EMA treatment as a viability assay for qPCR-based

Table I
Enumeration of *Salmonella* spp. and *E. coli* in primary influents and final effluents by qPCR (target gene copy/100 ml) and traditional Petri dish method (C.F.U./100 ml). Results shown as means + SE n = 6

	Primary influents			Final effluents		
	Target gene copy/100 ml × 10^4		C.F.U/100 ml × 10^4	Target gene copy/100 ml × 10^4		C.F.U/100 ml × 10^4
	qPCR	EMA-qPCR		qPCR	EMA-qPCR	
Salmonella typhimurium						
Summer	110.22 ± 26.95 a	74.29 ± 27.71 ab	25.00 ± 7.07 b	27.98 ± 1.7 a	6.28 ± 0.28 b	2.00 ± 0.00 b
Autumn	5810.00 ± 438.41 a	9300 ± 989.95 b	66.50 ± 31.82 c	0 a	0 a	0 b
Winter	365.03 ± 41.26 a	254.95 ± 22.31 a	64.50 ± 6.36 b	280.06 ± 31.5 a	3.01 ± 0.33 b	0 c
Spring	3427.53 ± 7.88 a	1668.88 ± 34.33 b	143.50 ± 4.95 c	49.87 ± 3.22 a	2.95 ± 0.21 b	14.75 ± 1.06 c
Escherichia coli						
Summer	30.34 ± 2.96 a	14.22 ± 0.93 b	1160.00 ± 84.85 c	4.88 ± 2.07 ab	1.12 ± 0.14a	0 c
Autumn	1206.64 ± 135.26 a	491.35 ± 31.44 b	626.5 ± 2.12 a	2.75 ± 0.48 a	0 b	3.50 ± 0.71 b
Winter	85.81 ± 13.98 a	38.45 ± 4.33 b	105.00 ± 22.63 ab	46.22 ± 4.87 a	8.43 ± 0.6 b	0 c
Spring	391.35 ± 39.10 a	215.55 ± 40.88 b	140.50 ± 2.2 c	27.73 ± 2.92 a	5.06 ± 0.36 b	7.5 ± 0.71 c

a, b, c – shows statistically significant differences within experimental groups in rows ($p < 0.05$)

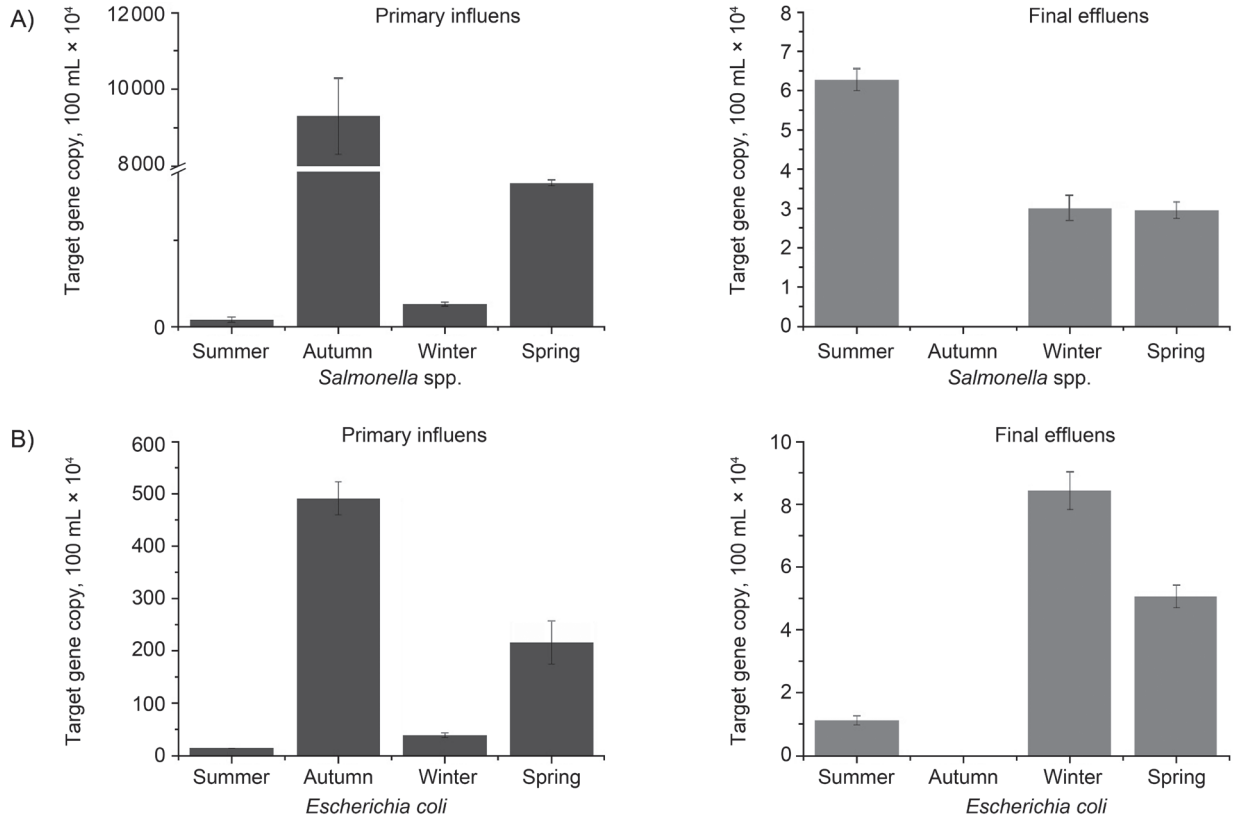

Fig 1. Evaluation of effectiveness of wastewater treatment process by EMA-qPCR method: A) *Escherichia coli uid*A target gene copies/100 ml × 10^4 analyzed in primary influents (left) and final effluents (right) through four seasons; B) *Salmonella* spp. *bip*A target gene copies/100 ml × 10^4 analyzed in primary influents (left) and final effluents (right) through four seasons

methods, because at high concentrations it is able to penetrate viable cells resulting in lower qPCR results and reduced total DNA recovery (Gedalanga and Olson, 2009). Moreover, authors have shown how many factors can influence EMA treatment regarding not only specific conditions of environmental samples (such as turbidity), but also specific cell membrane composition or cell physiology of target organisms that naturally occur in wastewater.

Viable bacteria quantification by the spread plate method. Results obtained by qPCR method were compared with the traditional spread plate method. For all analyzed samples, the target gene copy number was higher than the number of colony forming units per 100 ml of sample (Table I). Similar conclusions were stated by Morio *et al.* (2008) while comparing traditional and qPCR method for detection of *Legionella pneumophila* in environmental water samples. Lee *et al.* (2006) also explained that direct comparison between gene copy numbers and colony forming units is inappropriate because of DNA isolation from dead bacterial cells. Moreover, even though we assume that there is only single copy of a gene in one bacterial cell, it may appear in other bacterial strains in multiple copies.

Evaluation of the effectiveness of wastewater treatment process by EMA-qPCR method. *E. coli* and *Salmonella* spp. were present in primary influents during all four seasons (Fig. 1). According to Shannon (2007) the number of *Salmonella* spp. population ranges from 10^2–10^4 cells per 100 ml, which corresponds to the results obtained in this study. *Salmonella* spp. gene copies number was the highest in autumn and spring, medium in winter and the lowest in summer. *E. coli* was present mostly during spring and winter and was slightly detected during summer and autumn. Statistically important differences between all samples were observed. Similar results were obtained by Ulrich *et al.* (2005) who indicated that *E. coli O157:H7* is sporadically present or absent in primary influents in warm seasons.

Quantification of bacteria in final effluents has shown very good effectiveness of treatment of wastewater in the analyzed plant. Reduction in *Salmonella* spp. cells varied from 1.07 log units during summer, 1.92 log units in winter, 2.75 log units during spring, to more than 2.75 in autumn. Similar results were obtained for *E. coli* – 0.65 log units reduction in winter, 1.1 in summer, 1.63 in spring and 1.92 reduction of viable bacteria in autumn, prove that the process leads to reducing the risk of contamination from pathogens.

Correlation between microorganisms. Pearson's correlation analysis was used to identify whether any correlation existed between the concentration of indica-

Table II
Relationships between indicators concentrations measured as target gene copy number/100 ml by qPCR and EMA-qPCR method and colony forming units by traditional spread plate technique

Salmonella spp.	*E. coli*		
	qPCR	EMA-qPCR	CFU
Primary effluents	0.95*	0.98*	–0.72*
Final effluents	0.88*	0.09	0.85*

* Significant correlation for $P < 0.05$; qPCR – without ethidium ethidine monoazide; EMA-qPCR – with ethidium ethidine monoazide; CFU – colony forming units by traditional spread plate technique

tor and pathogenic microorganisms in the dependence on the used identification method. The significant correlations were obtained in the case of primary effluents (Table II) independently on the used method. However for final effluents the values were lower, moreover no significant correlations were observed between *E. coli* and *S. typhimurium* presence when EMA was used. In the literature contradictory data can be found on the relationship between microorganisms occurring in water and wastewater. Some authors suggest that fecal coliforms (FC) are good indicators of the presence in water of such microorganisms as *Salmonella, Shigella*, *Klebsiella*, *E. coli*, *Vibrio* or *Pseudomonas* (Patra *et al*., 2009). Others suggest no significant correlation between the concentration of *E. coli* and *Salmonellae* in wastewater (Song *et al*., 2010).

Conclusions. In this study, EMA-qPCR was successfully used for determining the degree of contamination of wastewater with *E. coli* and *Salmonella* spp. cells. The analysis made in four different time periods allows to assess the rate of occurrence of pathogens in the municipal wastewater treatment plant in Poland.

The qPCR reaction is a highly sensitive molecular tool which allows for the quantification of bacterial cells in wastewater. Application of EMA – pre-treatment eliminates the problem of false-positive results in standard PCR reaction and may become an effective tool to assess the effectiveness of a wastewater treatment process. Compared to the traditional spread plate method, it is more precise and can give information about the presence of specific microorganism, not only the indicator bacteria. The most important advantage of using qPCR for diagnosis of pathogens is the possibility of obtaining the result within 5 hours, which is sometimes crucial for a proper reaction.

The concentrations of *E. coli* and *Salmonella* spp. were higher when molecular methods (including EMA treatment) were used. In most cases significant correlations were observed between the concentration of the fecal indicator and pathogen. However taking into consideration negative results, *E. coli* could not be reliably used to predict the presence of pathogens in wastewater.

Moreover, in our opinion, further research is required to understand the persistency of the traditional fecal indicators in environmental water samples in relation to other pathogenic microorganisms. Hence, quantitative PCR data could be required to assess the survival of traditional fecal indicators along with the other pathogens depending on wastewater treatment and time.

Acknowledgments

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ORIGINAL PAPER

Biologically-Induced Precipitation of Minerals in a Medium with Zinc Under Sulfate-Reducing Conditions

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Abstract

Sulfate-reducing microbial communities were enriched from soils collected in areas with crude-oil exploitation. Cultures were grown in modified Postgate C medium and minimal medium, with ethanol or lactate as an electron donor. The batch cultures were grown with addition of zinc in concentrations of 100–700 mg/l. A lack of increased protein concentration in the solutions compared with the control batch, was noted in cultures containing over 200 mg Zn^{2+}/l. The 16S rRNA method was applied to determine the specific composition of the selected microorganism communities. The analysis indicated the presence of *Desulfovibrio* spp., *Desulfobulbus* spp. and *Desulfotomaculum* spp. in the communities. Diffractometric analysis indicated the presence of biogenic sphalerite in cultures with 100 and 200 mg Zn^{2+}/l and elemental sulfur in cultures with 200 mg Zn^{2+}/l. Other post culture sediments (300–700 mg Zn^{2+}/l) contained only hopeite [$Zn_3(PO_4)_2 \cdot 4H_2O$] formed abiotically during the experiment, which was confirmed by studies of the activity of sulfate-reducing microbial communities.

Key words: biogenic precipitation, biomineralization, 16S rRNA, sulfate-reducing bacteria, zinc

Introduction

In the topmost part of the lithosphere, zinc is a trace element that poses a serious hazard to the environment. Heavy metals, including zinc, migrate in the hypergenic zone during various petrogenetic and geochemical processes, resulting in their concentration but equally often in their dispersion. The environmental issue caused by the migration of heavy metals, including zinc, is complex. On the one hand, such migration causes disappearance of the metals from ore beds, but on the other hand, the metals appear in increasing concentrations in the exploitation area. Ore exploitation and further treatment processes linked with the utilization of the raw ore deposit result in the formation of post-exploitation and technological wastes containing significant amounts of metals. Industrial wastes containing zinc are generally formed during the production of batteries, paints, plastics, polymer stabilizers as well as in printing enterprises (Fosmire, 1990). Environments contaminated by zinc may be harmful. Effluents that are naturally generated in waste dumpsites often discharge to surface water reservoirs, watercourses and soil, causing potential hazard to the environment. There are also areas with concentrations of selected metals, *e.g.*, in areas of crude-oil exploitation. At present, there is an urgent need to apply processes that will allow recycling of heavy metals from wastes and poor ores and at the same time will minimize their negative influence on living organisms. Working out of an effective method of zinc recycling, *e.g.*, from spoil tips, requires knowledge of the geochemical and mineral-forming processes taking place in the environment that will allow determination of the stability of the resulting mineral phases. Densification of heavy metals and their forms in soils depends on numerous factors of the physical and chemical environment, *e.g.*, the magnitude of adsorption, the presence of humic acids and other soil components, the pH, the redox potential and others. Additionally, studies should include the role of microorganisms, especially sulfate-reducing bacteria (SRB), in the formation of secondary metal sulfides. SRB are a diverse group of anaerobic microorganisms that have the ability to reduce oxidized sulfur compounds and to oxidize organic compounds (Postgate, 1984; Hao *et al.*, 1996). They are considered to be the main producers of

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hydrogen sulfide in the biosphere. Sulfides that are produced during dissimilatory sulfate reduction may react with metals, including zinc, creating various secondary sulfides. In recent years, SRB have been applied to neutralize acid mine water, which may contain heavy-metal cations; and sparingly soluble metal sulfides are formed under these conditions (Jonson and Halleberg, 2003; Luptakova and Kusnierova, 2005; Kaksonen and Puhakka, 2007; Ong *et al.*, 2010). Application of SRB to acid mine drainage may remove heavy metals such as zinc from this environment. A few authors have asked why different kinds of minerals are formed during biological processes, and when and what influences these processes. Knowledge of biogeochemical interactions in the environment allows us to describe the processes and to predict them. It was long considered that the high toxicity of many heavy metals affects microbiological precipitation of sulfides by SRB. This opinion changed in 1961 when Becking and Moore (1961) carried out an experiment in which salts of selected metals were added to a medium with a composition close to that of marine water. The results explicitly pointed to SRB participation in the formation of secondary mineral phases because the post-culture sediments contained sphalerite (ZnS), a product of SO_4^{2-} and $ZnCO_3$ biotransformation. Taking into account the possible geochemical reactions, it can be assumed that in sedimentary settings, heavy-metal ions are largely adsorbed by clay minerals and form metal-organic compounds. All these processes lead to decrease of the toxicity level of a given metal by decreasing its concentration in the solution, although the metal is still capable of reactions with hydrogen sulfide and sulfide formation. On the other hand, the products of biochemical processes may include extracellular polymeric substances (EPS), which are mixtures of polysaccharides, mucopolysaccharides and proteins produced by microorganisms. The composition of EPS produced by SRB may be modified by the presence of different forms of organic matter in the environment, which may influence the increase of the metal-binding capacity (Zinkevich *et al.*, 1996) So far, the microbiological, geochemical and mineralogical processes leading to the formation of ZnS in environments impacted by human activities remain unrecognized. Spherical aggregates of sphalerite are commonly observed in biofilm structures where anaerobic conditions prevail. These zones are usually dominated by SRB that are relatively tolerant to oxygen, representing the families *Desulfobacteraceae* and *Desulfovibrionaceae* (Labrenz *et al.*, 2000; Vainshtein *et al.*, 1992). The precipitation of sphalerite at low temperatures may takes place in mine-water environments (Ledin and Pedersen, 1996; Drury, 1999; Moreau *et al.*, 2004). In order to determine the effect of zinc concentration on the activity of selected groups of microorganisms, experiments were performed in which zinc chloride was added at various concentrations to SRB cultures. There are only a few reports that describe both the influence of SRB on the formation of mineral phases that contain zinc and its effect on SRB activity.

The present study focused on the role of SRB isolated from soils with increased zinc concentrations, collected in areas of crude-oil exploitation, in the formation of zinc sulfide at high concentrations of the metal. It also focused on the toxicity of zinc in relation to sulfate reducing microbial communities in batch cultures containing easily accessible carbon sources for SRB, *i.e.*, lactate or ethanol.

Experimental

Materials and Methods

Selection and isolation of sulfate-reducing microbial communities. The microorganisms were enriched from soil polluted by crude oil and oil-derived products from SE Poland. SRB are commonly found in soils contaminated by crude oil due to the ability to metabolize the oil derived products (Feio *et al.*, 2004). In tested soils samples, C total was 3.2%, total S was about 120 mg/kg dry weight, total N was about 700 mg/kg, and Zn^{2+} was 92 mg/kg. In the tested soil samples the Zn^{2+} ions were only determined. First, an Easycult S test (Orion Diagnostica Espoo of Finland) was made to check for the presence of sulfidogenic microorganism communities; next, the microorganisms were selected using the microcosm method. Soil samples (10 g) were inserted in 100-ml flasks and covered with 80 ml of the particular medium. Two types of media were applied: a modified Postgate C medium (without yeast extract and sodium citrate) and a minimal medium with lactate or ethanol as electron donors. The flasks were tightly closed and incubated in darkness for 6 weeks at room temperature (about 22°C) in order to select anaerobic, sulfidogenic microorganism communities capable of simultaneous biodegradation of the applied carbon sources and sulfate reduction. The obtained SRB community was the inoculum to the SRB cultures in main experiment.

Cultures of sulfate-reducing microbial communities. Anaerobic batch cultures in modified liquid Postgate C medium were carried out in 0.5 l glass bottles filled to 0.25 l volume. The bottles were tightly sealed with rubber stoppers pierced with needles connected permanently to syringes, which were used to introduce the inoculum and to collect samples under N_2. The inoculum-to-medium ratio was 1:10. The anaerobic conditions in the cultures were controlled by addition of resazurin as the oxygen-level indicator. Violet

Table I
Experimental setup

Source of carbon	SRB cultures		Biotic control		Abiotic control (for diffractometric analysis)	
	Ethanol	Lactate	Ethanol	Lactate	Ethanol	Lactate
Zn^{2+} concentration [mg/l]	100	100	0	0	100	100
	200	200			200	200
	500	500			500	500
	700	700			700	700
SRB inoculum	10%	10%	10%	10%	without inoculum	without inoculum
Repetitions	2 ×	2 ×	2 ×	2 ×	1 ×	1 ×

colour indicated that the culture contained oxygen, and its absence pointed to anaerobic conditions. The experiment was carried out in two variants, one with ethanol and the other with lactate as the sole carbon source (4000 mg/l). The control batch consisted of SRB cultures without zinc (biotic control) and cultures with zinc and without SRB (abiotic control). The abiotic controls were conducted in order to analyze of mineral phases formed without microbial activity. All the SRB cultures were stationary and were conducted in the modified Postgate C medium. The experiment and the chemical determinations in cultures were made in duplicate. The experimental setup is shown in Table I.

Media. A modified liquid Postgate C medium (Postgate, 1984), composed of: KH_2PO_4 (500 mg/l), NH_4Cl (1000 mg/l), $CaCl_2$ (60 mg/l), $MgSO_4$ (60 mg/l), $FeSO_4$ (100 mg/l), Na_2SO_4 (4500 mg/l) without yeast extract and citrate, and a minimal medium (Wolicka and Kowalski, 2006), composed of NH_4Cl (1000 mg/l) and Na_2SO_4 (4500 mg/l), were used in the experiment. Lactate (4000 mg/l) or ethanol (4000 mg/l) were added to both media as the sole carbon sources. Resazurin (1 mg/l) was added to all cultures in order to control the level of oxidation. The medium did not contain yeast extract or sodium citrate. Zn^{2+} was added in concentrations of 100, 200, 500 and 700 mg/l to a modified Postgate C medium. Zn^{2+} was added as zinc (II) chloride.

Sulfate determinations were made using the turbidimetric method after reaction with barium chloride in a Thermo spectrophotometer at $\lambda = 400$ nm wavelength (Greenberg *et al*., 1985).

Protein determinations in the cultures were made using the Lowry method after a biuret test enhanced by the Folin-Ciocalteau reagent in a Thermo spectrophotometer at $\lambda = 670$ nm (Genesys 10Vis, Thermo). The samples (5 ml) for determination were previously sonicated (30 kHz, 30 s) in order to determine total protein from cultures. The measurement was performed as follows. The 1 ml of sonicated sample was put into glass tubes and 5 ml of reagent (49 ml 2% Na_2CO_3 in 0.1 M NaOH + 0.5 ml 2% potassium sodium tartrate + 0.5 ml 1% $CuSO_4$) was added. After 5 min., 0.2 ml of Folin-Ciocalteau reagent (POCH, Gliwice, Poland) was added and immediately mixed. After 5 min, 0.2 ml 6 M NaOH was added and mixed, then the absorbance of the colored solution was immediately measured spectrophotometrically. Protein measurements were used as an indicator of biomass.

Zinc determinations in stationary cultures. The determinations were made using the Thermo spectrophotometer with application of available kits for determining zinc concentrations (Merck Zinc Test with a pyridylazo naphthol derivative).

Analysis of the post-culture sediments. After incubation, the cultures were centrifuged at 10 000 × g, and the obtained post-culture sediment was dried at 30°C under N_2. The samples were next ground in an agate mortar, and their mineral composition was determined using X-ray powder diffraction in a diffractometer (Panalytical X' Pert PRO MPD). The diffractometric analysis was conducted on 10 samples of post-culture sediments; five were taken from cultures that used ethanol as the sole carbon source, and five were taken from cultures with lactate as the sole carbon source. Additionally, the analysis of sediments from abiotic control with Zn^{2+} (500 and 700 mg/l) were also conducted.

Molecular analysis of the selected sulfidogenous microorganism communities. The taxonomic composition of the sulfate-reducing bacterial communities was obtained using molecular analysis. Isolation of chromosome DNA and analysis of gene 16S rRNA fragments were carried out according to commonly applied procedures of Collins *et al.* (1991). Bacterial DNA was isolated from a fluid culture of microorganisms with a commercial kit for chromosomal DNA isolation (A&A Biotechnology). The purity and concentration of the resulting DNA preparation were determined spectrophotometrically at 260 nm. Primers specific for bacterial 16S rRNA (27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-GGTTACCTTGTTACGACTT-3') were used to amplify a 1540-bp segment from the 16S rRNA gene. The PCR reaction was made

using the GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Invitrogen). Amplification products were purified using the Wizard Purification System (Promega) and analyzed by electrophoresis. After amplification, the material was sequenced using the ABI 3730 Genetic Analyzer with application of the Perkin Elmer sequencing kit. The resulting nucleotide sequences were compared with gene 16S rRNA sequences available in the National Centre for Biotechnology Information (NCBI) database using NCBI's Blast 2.0 program and showed 99% homology with the corresponding sequences among different anaerobic species.

Results and Discussion

The influence of zinc concentrations on the activity of selected SRB communities is presented in Figs. 1 and 2 and the changes of concentration of sulphate and protein in control cultures are presented in Fig. 3. The initial concentration of protein at the level of about 2000 mg/l was derived from the inoculum. A slight increase of protein concentration and decrease of sulfate concentration was observed in cultures where the Zn concentration was 100 mg/l on a medium with ethanol as the sole carbon source (Fig. 1). Such trends were not observed in the remaining cultures, regardless of the applied carbon source. A slight increase of protein concentration and decrease of sulfate concentration was noted in the control batch. Based on the obtained results, it can be stated, that the zinc concentration tolerated by the isolated SRB community was 100–200 mg/l.

The results may partially confirm existing literature data. Zinc is a metal that may hampers in higher concentrations the metabolic activity of various microorganisms, including SRB (Utgikar *et al.*, 2002), but on the other hand many mechanisms responsible for metal-ion resistance in bacteria have been described (Brocklehurst and Morby, 2000). SRB are effective in reducing the sulfate concentration and neutralizing its acidity. Furthermore, most of the heavy metals present in acidic mine drainage can be precipitated as insoluble sulfides using biogenic sulfide produced by sulfate reduction (Barton and Tomei, 1995; Costa *et al.*, 2008; Martins *et al.*, 2009a). The reported toxic concentrations of heavy metals to sulfate reducers range from a few mg/l to 100 mg/l (Loka Bharathi *et al.*, 1990; Poulson *et al.*, 1997; Utgikar *et al.*, 2001). Data in the literature indicate that various concentrations of zinc inhibit SRB activity. Radhika *et al.* (2006) estimated that the concentration of zinc lethal to SRB is about 210 mg/l. Castillo *et al.* (2012) isolated communities from sediments in two acid streams draining the Iberian Pyrite Belt, in which zinc occurred at con-

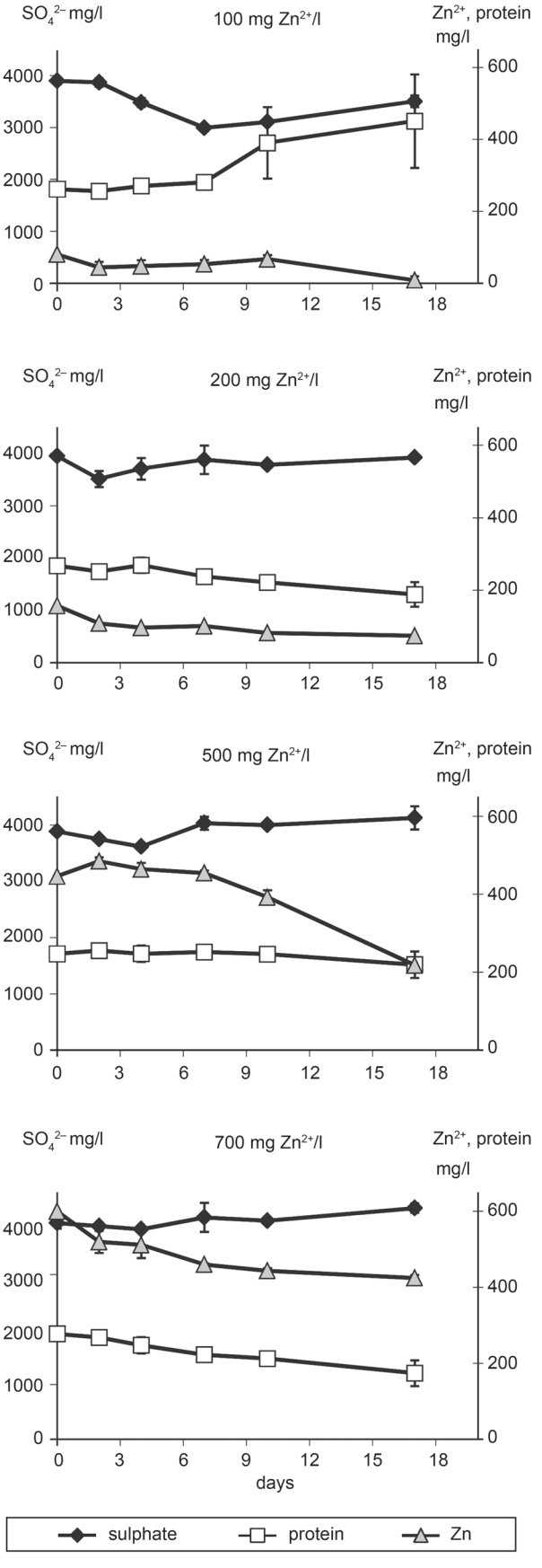

Fig. 1. Concentration changes of sulfate, protein and zinc in sulfidogenic microbial cultures with ethanol as the sole organic carbon source at variable initial concentrations of Zn^{2+} (100, 200, 500, 700 mg/l). Standard deviation has been marked

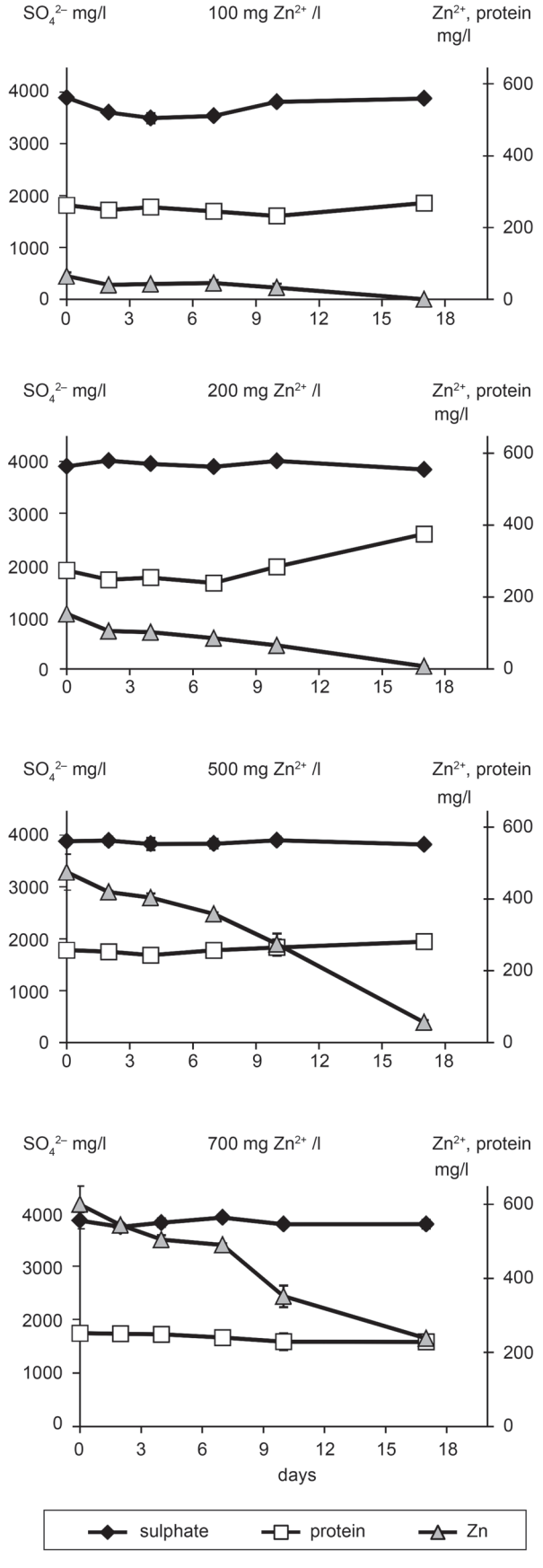

Fig. 2. Concentration changes of sulfate, protein and zinc in sulfidogenic microbial cultures with lactate as the sole organic carbon source at variable initial concentrations of Zn^{2+} (100, 200, 500, 700 mg/l). Standard deviation has been marked

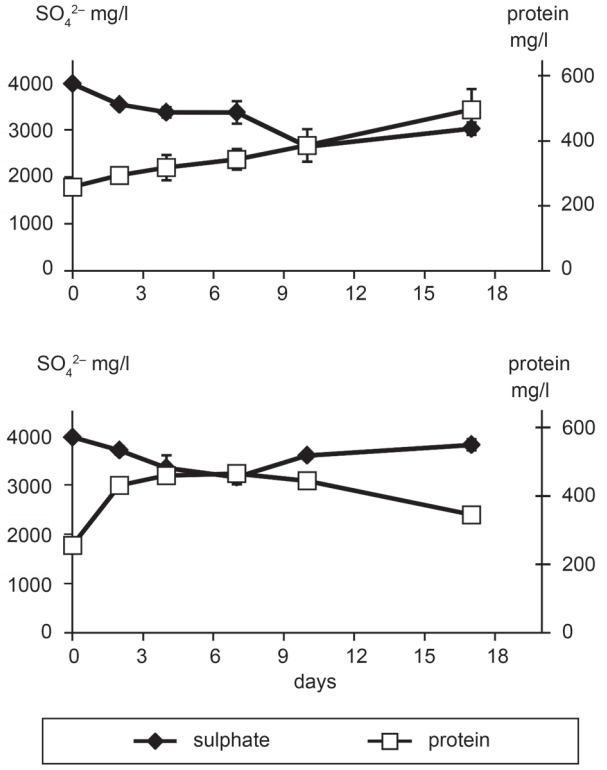

Fig. 3. Concentration changes of sulfate and protein in control batches (without addition of Zn^{2+}) of sulfidogenic microbial cultures with ethanol (above) or lactate (below) as the sole organic carbon source. Standard deviation has been marked

centrations of 400 and 30 mg/l. According to Azabou *et al.* (2007), a zinc concentration of 400 mg/l is toxic for SRB and inhibits their activity; SRB can carry out metabolic processes at concentrations of up to 150 mg/l (Martins *et al*., 2009b). Zinc inhibits electron transport in the respiration cycle of microorganisms, and its toxicity in comparison to such metals as Hg, Cd, Cu, Ni, Co and Pb is rather low. There is data in the literature on the influence of zinc on SRB activity, but there are no reports that simultaneously discuss the influence of zinc concentration on SRB activity and the types of the resulting mineral phases.

In the present study, the determination of the influence of Zn^{2+} on the mineral composition of post-culture sediments was conducted. The presence of zinc sulfide was determined in the post-culture sediments from cultures with ethanol as the sole carbon source and 100 mg Zn/l (Fig. 4). In the culture, where the zinc concentration was 200 mg/l, sphalerite (ZnS) as well as elemental sulfur were observed. It could be that elemental sulfur is formed by sulfidogenic bacterial communities. In natural ecosystems, the sulfur cycle should be in balance, meaning that the amount of sulfide that is oxidized should correspond to the amount of sulfate that is reduced. Such a balance can be found in a sulfuretum. This is a syntrophical bacterial community in which H_2S produced by sulfate-reducing bacteria is reoxidized

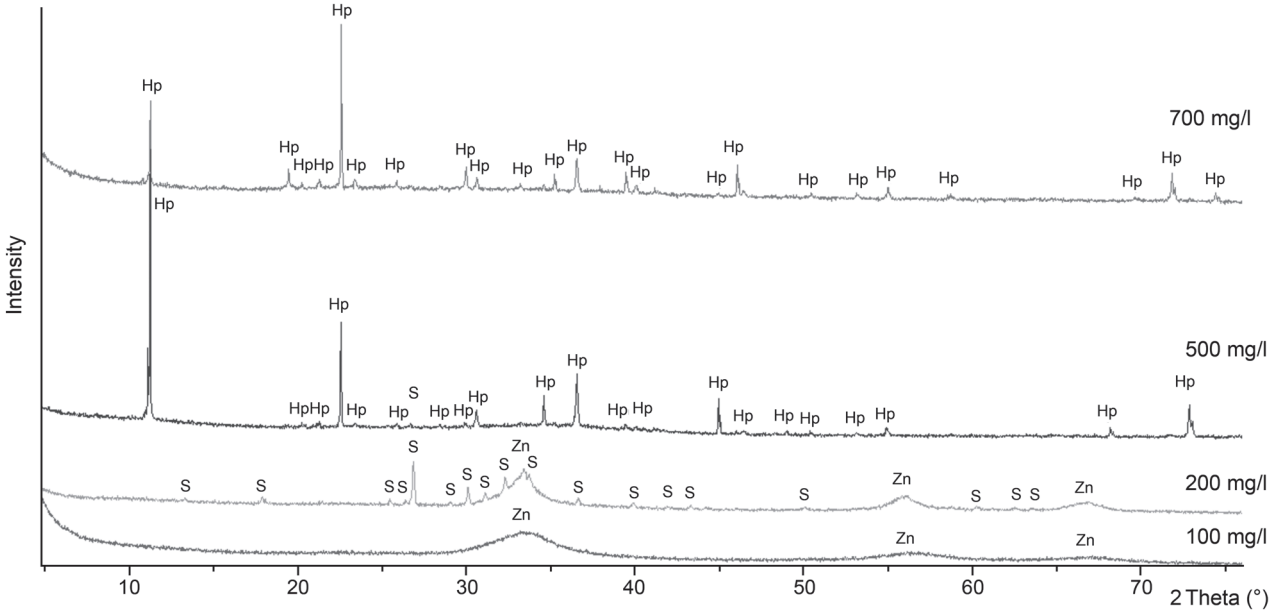

Fig. 4. X-ray powder diffractograms of post-culture sediments in cultures of selected SRB communities on a modified Postgate medium with ethanol as the sole carbon source and with addition of Zn^{2+} in concentrations of 100–700 mg/l. Symbols: Zn, sphalerite ZnS; Hp, hopeite $Zn_3(PO_4)_2 \cdot H_2O$; S, sulfur

by the sulfur-compound-oxidizing bacteria. This process is not as common in the natural environment as dissimilation sulfate reduction, but it should not be excluded (Roy and Trudinger, 1970; Hedderich *et al.*, 1999). The remaining cultures containing more than 200 mg Zn^{2+}/l contained hopeite $[Zn_3(PO_4)_2 \cdot H_2O]$, which was probably formed by abiotic processes because the medium used for SRB growth contained phosphates (KH_2PO_4). The abiotic control batch did not contain any mineral phases except hopeite. Hopeite was detected in abiotic controls containing 500 and 700 mg Zn^{2+}/l.

On a medium with lactate as the sole carbon source (Fig. 2), significant SRB activity could not be observed, but a slight increase of protein content in the cultures worth noting, pointing to the development of microorganisms accompanying SRB that were capable of activity in the presence of high zinc concentrations. The significant decrease of zinc concentration may have been the effect of this microflora activity, although this fact cannot be unambiguously confirmed. The effect of inoculum can be appear only at initial stage of experiment, when it can be observed the slight decrease of zinc concentration. A considerable impediment in the analysis of the results is also the lack of determination of the minerals in post-culture sediments that could not be obtained using X-ray diffraction. This lack probably was caused by the low number of mineral phases in the post-culture sediment and precipitation of amorphous or nanostructured chemical compounds, which often accompany EPS organic compounds. Moreau *et al.* (2007) suggest that formation of mineral aggregates is induced by extracellular metal-binding polypeptides and proteins. Disordered morphological features of zinc sulfides have been described by Gramp *et al.* (2007), who tested for formation of Zn sulfides n cultures of SRB.

The 16S rRNA method was applied in order to determine the specific composition of the selected microorganism communities. The analysis indicated the presence of *Desulfovibrio* spp., *Desulfobulbus* spp. and *Desulfotomaculum* spp. in the communities. Some of these microorganisms are capable of sulfate reduction to elemental sulfur, which was confirmed by diffractometric analysis of the post-culture sediments. Moreover, removal of hydrogen sulfide from the environment by formation of sulfides results in a decrease of the reducing conditions that are indispensable for SRB activity (Labrenz *et al.*, 2000).

Conclusions. The results supplement the knowledge of the mineral-forming processes taking place in cultures with various concentrations of zinc and indicate a possibly significant participation of SRB in the processes taking place in the natural environment under hypergenic conditions. Moreover, they indicate the possible application of SRB in the treatment of acid mine drainage, but the high concentrations of metals potentially can limit the SRB activity. Cultures with zinc showed strong inhibition of the activity of selected sulfidogenic communities in cultures containing 200 mg Zn^{2+}/l. The post-culture sediments of cultures in a Postgate medium with ethanol as the sole carbon source contained sphalerite (cultures containing 100 mg Zn^{2+}/l) and elemental sulfur (cultures contain-

ing 200 mg Zn^{2+}/l). In the remaining cultures, in which the content of zinc ions was much higher, the post-culture sediments contained zinc phosphate formed by abiotic processes. The results confirm the participation of SRB in mineral-forming processes in environments containing zinc.

Acknowledgments

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ORIGINAL PAPER

Role of Antagonistic Microorganisms and Organic Amendment in Stimulating the Defense System of Okra Against Root Rotting Fungi

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Abstract

Without application of chemical pesticides control of soilborne diseases is a great challenge. Stimulation of natural plant's defense is considered as one of the most promising alternative strategy for crop protection. Organic amendment of soil besides direct suppressing the pathogen, has been reported to have an influence on phytochemicals in plants. In the present study, *Pseudomonas aeruginosa*, a plant growth promoting rhizobacterium and *Paecilomyces lilacinus,* an egg parasite of root knot and cysts nematodes were examined individually and in combination in soil amended with cotton cake for suppressing the root rotting fungi and stimulating the synthesis of polyphenols and improving the antioxidant status in okra. Application of *P. aeruginosa* and *P. lilacinus* in soil amended with cotton cake significantly ($P < 0.05$) suppressed *Macrophomina phaseolina*, *Fusarium oxysporum*, and *Fusarium solani* with complete reduction of *Rhizoctonia solani.* Combine use of biocontrol agents in cotton cake amended soil showed maximum positive impact on plant growth, polyphenol concentration and antioxidant activity in okra.

K e y w o r d s: antagonistic microorganisms, okra, organic amendment, root rotting fungi

Introduction

Vegetable crops are vulnerable to a range of pathogenic organisms that reduce yield by killing the plant or damage the product and make it unmarketable. Plant diseases on average are responsible for up to 26% yield loss to global agriculture and sometimes there may be complete crop failure leading to 100% yield loss in a locality or a field (Khan *et al*., 2009). Okra [*Abelmoschus esculentus* (L.) Moench] is an important vegetable crop and is grown worldwide including Pakistan (Athar and Bokhari, 2006). Okra is a warm, rainy season crop and requires high temperature. However, diseases are the limiting factor in okra production. In Pakistan okra crop is attacked by various soil borne plant pathogenic fungi like *Macrophomina phaseolina, Rhizoctonia solani, Fusarium* spp. and the root knot nematodes *Meloidogyne* spp. (Afzal *et al.,* 2013; Ehteshamul-Haque *et al.,* 1996). Without application of chemical pesticides control of soilborne diseases is a great challenge. Among the new biological approaches, the stimulation of natural plant's defense is considered as one of the most promising alternative strategies for crop protection (Anderson *et al*., 2006; Walters and Fountaine, 2009; Walters *et al*., 2005). This original biological approach does not exert direct effects on the pathogen (Walters and Fountaine, 2009) but stimulates natural defenses in plants, leading to a systemic acquired resistance (Vallad and Goodman, 2004).

Plants produce a wide range of secondary metabolites in response to biotic stress that are toxic to pathogens and herbivores. Phenolic phytochemicals are secondary metabolites that are common constituents of fruits and vegetables that function in the defense against insect and animal herbivory (Stevenson *et al*., 1993). These phenolic metabolites protect the plants against biological and environmental stresses and therefore are synthesized in response to pathogenic attack

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such as fungal or bacterial infection or high energy radiation exposure such as prolonged UV exposure (Briskin, 2000). Phenolic phytochemicals, because of their important protective biological functions, are ubiquitous in all plants and therefore find their place in almost all food groups. In resistant plants, phenolic based defense responses are characterized by the early and rapid accumulation of phenolics at the infection site resulting in the effective isolation of the pathogen (Chérif *et al*., 1991).

Farmers and agricultural scientists have long understood that organic amendments applied to field soils improve soil functions such as infiltration, water holding capacity, nutrient retention and release, and resistance to wind and water erosion and can suppress soilborne diseases (Bonanomi *et al*., 2007; Stone *et al*., 2003). Beside a wide variety of organic matters that have been tested as organic amendments for managing plant pathogens, oil seed cakes can decrease the population of soil borne pathogens (Ehteshamul-Haque *et al*., 1995; Sharma *et al*., 1995). It has been observed that several antimicrobial by-products (*e.g*. organic acids, hydrogen sulfide, phenols, tannins and nitrogenous compounds) are released during the decomposition of organic amendments or synthesized by microorganisms involved in such degradation (Rodriguez--Kabana *et al*., 1995). Furthermore organic fertilizers enhance the antioxidant content in plants and consequently improve plant defense against pests and diseases (Dumas *et al*., 2003).

The root colonizing bacteria that have a beneficial effect on plants are termed as plant growth promoting rhizobacteria (PGPR) and have been reported to improve plant growth either through direct stimulation of the plant by producing growth regulators or by suppression of pathogens (Inam-ul-Haq *et al*., 2012; Weller *et al*., 2002). Of the various rhizospheric bacteria, the bacteria belonging to the fluorescent *Pseudomonas* which colonize roots of a wide range of crop plants are reported to be antagonistic to soil-borne plant pathogens (Siddiqui and Ehteshamul-Haque, 2001). PGPR may induce plant growth promotion by direct or indirect modes of action (Kloepper, 1993). Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones, lowering of the ethylene level in plant, improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources; non-symbiotic nitrogen fixation) and stimulation of disease-resistance mechanisms (Antoun and Prévost, 2005). The present report describes the role of soil amendment and application of PGPR on the suppression of okra root diseases and polyphenol content and antioxidant activity in okra alone or with *Paecilomyces lilacinus*, an egg parasite of root knot and cyst nematodes.

Experimental

Materials and Methods

Biological antagonist. Cultures of *P. aeruginosa* and *P. lilacinus,* used in this study were obtained from Karachi University Culture Collection (KUCC).

Experimental design / Screen house experiment. Dry powder of cotton cake was mixed in sandy loam soil, pH 8.0, @ 1.0% w/w. The soil had natural infestation of 5–11 sclerotia of *M. phaseolina* g^{-1} of soil, as determined by wet sieving and dilution technique (Shiekh and Ghaffar, 1975), 4–13% colonization of sorghum seeds was used as bait for *R. solani* (Wilhelm, 1955), and 3000 $cfu.g^{-1}$ of soil of a mixed population of *Fusarium solani* and *F. oxysporum* as determined by a soil dilution technique (Nash and Synder, 1962). One kg of amended soil was transferred to 12 cm diameter clay pots. The pots were watered daily to allow the decomposition of the organic substrate. After two weeks, aqueous suspensions of *P. aeruginosa* (10^8 cfu/ml) grown on KB broth and *P. lilacinus* (10^7 cfu/ml) grown on potato dextrose broth were drenched onto each pot at 25 ml per pot. Pots without amendment/antagonists or fungicides served as control. Aqueous suspension (100 ppm) of a fungicide, carbondazim at 25 ml per pot served as positive control. Six seeds of okra were sown in each pot and pots were kept randomized on a screen house bench of Department of Botany at 50% water holding capacity with four replicates of each treatment. After germination, only four seedlings were kept and excess were removed.

Determination of fungal infection and growth parameter. To assess the efficacy of *P. aeruginosa* and *P. lilacinus* in suppression of root disease, plants were uprooted after 45 days of growth. To determine the incidence of fungi, roots were washed with running tap water then surface disinfested with 1% $Ca(OCl)_2$ and 1 cm long root pieces from tap roots, (5 from each plant) were plated onto potato dextrose agar plates supplemented with penicillin (100,000 units/litre) and streptomycin (0.2 g/litre). After incubation for 5 days at 28°C, the incidence of root infecting fungi was recorded. Infection percentage for each pathogen was calculated using the formula:

$$\text{Infection \% of a pathogen} = \frac{\text{Number of plants infected by a pathogen}}{\text{Total number of plants}} \times 100$$

Plant growth parameters, such as plant height and fresh weight of shoot, root length and root weight were also recorded.

Determination of polyphenol. Okra leaves were oven-dried at 80°C for 24 hours. Dried leaves were ground into fine powder using a clean pestle mortar and finally crushed samples were suspended in etha-

nol. Samples were collected in screw capped centrifuge tubes. The extracts were centrifuged for 20 minutes at 3,000 rpm. The supernatants were collected and used for analyzing phenolic content and antioxidant activity.

The estimation of polyphenol was done by Folin-Ciocalteu phenol reagent as describe by Chandini *et al.*, (2008). For estimation 100 µl aliquots of ethanolic leaves extract were mixed with 2 ml of 2% Na_2CO_3 and allowed to stand for 2 minutes at room temperature. After incubation 100 µl Folin-Ciocalteu phenol reagent was added and mixture was mixed thoroughly and allowed to stand for 30 minutes at room temperature in dark. Absorbance of samples was recorded at 720 nm using spectrophotometer and phenolic content was expressed as gallic acid equivalents.

DPPH radical scavenging activity. Antioxidant activity in okra was determined using DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay (Zubia *et al.*, 2007) with some modification. An aliquot of 200 µl of ethanolic leaves extract (0.2 mg/ml of ethanol) was mixed with 800 µl of 100 mM Tris-HCl buffer (PH 7.4). The mixture was added to 30 µM DPPH (dissolved in DMSO) and vortex, then left to stand at room temperature in the dark. The absorbance was measured at 517 nm after 1 minute and 30 minute of incubation, using UV-visible spectrophotometer against ethanol, used as blank. One ml ethanol with 1 ml of DPPH was used as control. Synthetic BHT was used as positive control. The ability to scavenge the DPPH radical was calculated using the follow equation:

$$\% \text{ of inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where the $A_{control}$ is the absorbance of the control (DPPH solution without sample), the A_{sample} is the absorbance of the test sample (DPPH solution plus test sample).

Statistical analysis. The experiment was conducted twice and data were subjected to analysis of variance (ANOVA) and means were separated using the least significant difference (LSD) according to (Gomez and Gomez, 1984).

Results

Influence of cotton cake and antagonistic microorganism on plant growth and development of root rot infection. Application of cotton cake 1% alone or with PGPR and *P. lilacinus* showed positive impact on plant growth by improving plant height, fresh shoot weight and root length. PGPR and *P. lilacinus* with cotton cake 1% w/w significantly ($P < 0.05$) increased plant growth and caused maximum reduction in diseases severity. Greater plant height was observed in mixed treatment of cotton cake with PGPR and *P. lilacinus* followed by *P. lilacinus* together with cotton cake. A significant increase in fresh shoot weight was found where cotton cake 1% w/w was used followed by cotton cake with carbendazim or cotton cake with *P. lilacinus* (Table I). Highest root length was observed by the application of carbendazim used in amended soil, whereas greater fresh root weight was recorded in cotton cake with *P. lilacinus* treatment (Table I). Application of PGPR and *P. lilacinus* in soil amended with cotton cake 1% significantly suppressed infection of *M. phaseolina*, *Fusarium oxysporum*, *F. solani* with complete inhibition of *R. solani* (Table II). Use of carbendazim with cotton cake also significantly inhibited root rotting fungi as compare to the control. Soil application of cotton cake alone also showed reduction of *M. phaseolina*, *R. solani*, and *F. solani. P. lilacinus* in cotton cake amended soil also showed suppressive effect on root rotting fungi (Table II).

Table I
Effect of *P. aeruginosa* and *P. lilacinus* on growth of okra plants in soil amended with cotton cake (1% w/w)

Treatments	Shoot length (cm)	Fresh shoot weight (g)	Root length (cm)	Root weight (g)
Control	27.84	4.05	9.4	0.60
Carbendazim	28.08	3.65	11.58	0.31
Cotton cake (1% w/w)	26.68	6.51	7.98	0.49
P. aeruginosa	29.09	3.01	9.51	0.25
P. lilacinus	30.62	2.82	6.75	0.17
P. aeruginos + *P. lilacinus*	28.56	2.77	8.65	0.26
Cotton cake + carbendazim	28.05	6.40	11.16	0.54
Cotton cake + *P. aeruginosa*	28.28	5.30	7.19	0.44
Cotton cake + *P. lilacinus*	32.12	6.79	7.78	1.63
Cotton cake + *P. aeruginosa* + *P. lilacinus*	33.01	5.59	9.64	0.64
$LSD_{0.05}$	5.78[1]	1.54[1]	2.91[1]	ns

[1] Mean values in column showing differences greater than LSD values are significantly different at $p < 0.05$

Table II
Effect of *P. aeruginosa* and *P. lilacinus* on the infection of *M. phaseolina*, *R. solani*, *F. solani* and *F. oxysporum* in soil amended with cotton cake (1% w/w)

Treatments	*F. oxysporum*	*F. solani*	*M. phaseolina*	*R. solani*
Infection %				
Control	43.7	18.7	62.5	31.2
Carbendazim	37.5	6.2	43.7	62.5
Cotton cake (1% w/w)	50	12.5	25	18.7
P. aeruginosa	50	6.2	43.7	43.7
P. lilacinus	43.7	25	43.7	37.5
P. aeruginosa + *P. lilacinus*	50	18.7	25	25
Cotton cake+ carbendazim	25	18.7	43.7	25
Cotton cake + *P. aeruginosa*	18.7	18.7	37.5	18.7
Cotton cake + *P. lilacinus*	12.5	18.7	12.5	6.2
Cotton cake + *P. aeruginosa* + *P. lilacinus*	6.2	12.5	18.7	0

$LSD_{0.05}$ = Treatments = 18.09[1], Pathogens = 11.44[2]
[1] Mean values in column showing differences greater than LSD values are significantly different at $p < 0.05$
[2] Mean values in rows showing differences greater than LSD values are significantly different at $p < 0.05$

Table III
Effect of *P. aeruginosa* and *P. lilacinus* on antioxidant activity and polyphenol contents in okra in soil amended with cotton cake (1% w/w)

Treatments	Antioxidant activity (% inhibition)		Phenolic contents (mg% gallic acid)
	1 minute	30 minutes	
Standard (BHT)	80.32^{a}	62.66^{a}	– – –
Control	20.92^{e}	31.5^{de}	37.25^{b}
Carbendazim	29.75^{cde}	31.05^{de}	34.25^{b}
Cotton cake (1% w/w)	49.95^{b}	52.30^{abc}	45.25^{ab}
P. aeruginosa	24.72^{de}	33.58^{de}	50.0^{ab}
P. lilacinus	8.25^{f}	25.33^{e}	39.15^{b}
P. aeruginos + *P. lilacinus*	25.13^{de}	36.94^{cde}	42.5^{ab}
Cotton cake + carbendazim	39.82^{bc}	53.88^{abc}	35.5^{b}
Cotton cake + *P. aeruginosa*	38.62^{bcd}	46.2^{abcd}	45.75^{ab}
Cotton cake + *P. lilacinus*	33.20^{cde}	42.99^{bcde}	48.25^{ab}
Cotton cake + *P. aeruginosa* + *P. lilacinus*	33.19^{cde}	57.66^{ab}	57.75^{a}
$LSD_{0.05}$	12.64[1]	16.13[1]	14.09[1]

Mean values in column bearing same superscript letters are not significantly ($P < 0.05$) different according to Duncan's multiple range test
[1] Mean values in column showing differences greater than LSD values are significantly different at ($P < 0.05$)

Polyphenols content. In this study, phenolic content was measured in terms of mg% gallic acid equivalent (mg% GAE) using the Folin Ciocalteu reagent (Table III). Cotton cake 1% alone or with PGPR and *P. lilacinus* showed phenolic content ranged from (45.25 mg% GAE to 57.75 mg% GAE). Combined soil application with cotton cake + PGPR + *P. lilacinus* showed significantly ($P < 0.05$) higher polyphenols (57.75 mg% GAE) as compared to the control and chemical fungicide, carbendazim treatments *i.e.*, 37.25 mg% and 34.25 mg% GAE respectively. Application of PGPR alone also showed higher phenolic content (50 mg% GAE) as compared to the *P. lilacinus* (39.15 mg% GAE) used alone but with cotton cake showed (48.25 mg% GAE).

DPPH radical scavenging activity. DPPH Radical Scavenging Assay-1, 1-diphenyl-2-picryl hydrazyl (DPPH) was used in this study to determine the free-radical scavenging activity of the plant samples. This is a stable free radical whose color changes from violet to yellow when it is reduced by hydrogen donation. Butylated hydroxytoluene (BHT) was used as a stand-

ard. The activity of leaves extract at two different time intervals *i.e*. at 1 minute and 30 minute were observed. The activity of extracts increased with the time of incubation as compare to BHT as shown in (Table III). The antioxidant activity initially was weaker than standard but increased with time. The activity was significantly ($P < 0.05$) higher (57.66%) in PGPR + *P. lilacinus* treated plants in amended soil. Okra plants with chemical fungicide and without any treatment showed lowest antioxidant activity (31.5%, 31.05%). It was also observed that extracts which show highest polyphenols, showed highest antioxidant activity (Table III). Furthermore antioxidant activity was reached more than 50% in plants grown in cotton cake amended soil alone or with carbendazim or with PGPR + *P. lilacinus* (Table III).

Discussion

The biological control of soil-borne pathogens with mixture of biocontrol agents, organic amendments and micronutrients is a new approach in crop protection to reduce the disease damage level in economically important crops (Bharathi *et al*., 2004). In this study soil amendment with cotton cake caused significant control of root rotting fungi and improved growth of okra. Organic amendments are generally used for improving crops, increasing agricultural productivity and suppressing soil borne diseases (Stone *et al*., 2003; Sultana *et al*., 2011). Among the wide variety of organic matters tested as organic amendments for managing plant pathogens oil seed cakes significantly suppressed the soilborne pathogens (Ehteshamul-Haque *et al*., 1995; Sharma *et al*., 1995).

In this study application of PGPR or *P. lilacinus* in amended soil showed promising results by reducing the soilborne pathogens, producing the healthier plants and improving the antioxidant status of the okra plants. There are reports that microbiota, *e.g.* rhizobacteria, *Trichoderma*, and *Pseudomonas* spp., present in decomposing organic matter may enhance growth and yield of crops (Sylvia, 2004) by producing plant growth hormones and chemical compounds (*e.g.* siderophores, tannins, phenols) which are antagonistic to various soilborne pathogens (Antonio *et al.*, 2008). In this study plants grown in cotton cake amended soil and received both PGPR and *P. lilacinus* showed maximum amount of polyphenols as compared to other treatments. Organic fertilization has been reported to have larger impact on the phyto-nutritional quality of crops. Phenolic compounds are important plant secondary metabolites that can help plants tide over oxidative stress working as antioxidants (Grassmann *et al*., 2000; Urquiaga and Leighton, 2000). Toor *et al.* (2006) reported that organic fertilizers increased the content of ascorbic acid and total phenolics in tomato. Similarly Dumas *et al.* (2003) reported that inorganic fertilizers reduce the antioxidants while organic fertilizers were proved to enhance the antioxidant content in plants. It is also known that phenolic compounds are potential antioxidants and free radical- scavengers. Kumar *et al*. (2008) reported that there should be a close relation between the content of phenolic compounds and antioxidant activity.

Fertilizer and pesticide affect the human health and cause damage to the environment. Application of organic amendment and biocontrol agents are environmental friendly and an alternative strategy to the prevalent use of synthetic pesticides. Combination of introduced biocontrol agents with oil cake was more consistent against disease suppression. The results of the present study show that mixed application of oil cake with PGPR and *P. lilacinus* enhance plant growth and suppress the infection of soil borne root rotting fungi via increasing the polyphenols and antioxidant activity in okra plants.

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ORIGINAL PAPER

Biodiversity of Dominant Cultivable Endophytic Bacteria Inhabiting Tissues of Six Different Cultivars of Maize (*Zea mays* L. ssp. *mays*) Cropped under Field Conditions

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Abstract

Endophytic bacteria (EnB) play a crucial role in plant development. This study was an attempt to isolate and identify dominant cultivable EnB inhabiting young seedlings germinated *in vitro* and leaves of six maize cultivars grown under field conditions at temperate climate zone with culture-dependent approach. We isolated bacteria from field cropped maize only. Strains were identified based on 16S rRNA gene sequencing. In particular, members of *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and α- and γ-*Proteobacteria* were found. Species of two genus *Pseudomonas* and *Bacillus* were dominant among them. Higher diversity of EnB was found in plants collected from Kobierzyce, where we identified 35 species from 16 genera with 22 species uniquely found at this field. On the contrary, from maize leaves collected at Smolice we identified 24 species representing 10 genera with 10 species uniquely isolated from this field. However, none of species was common for all cultivars at both locations. Among isolated EnB six species only, *Pseudomonas clemancea*, *Pseudomonas fluorescens*, *Bacillus megaterium*, *Bacillus simplex*, *Arthrobacter nicotinovorans* and *Arthrobacter nitroguajacolicus*, were found in aboveground parts of the same cultivar grown on both tested fields. The fact that the same cultivars, sown from the same lots of seeds, under field conditions on two different locations were colonized with noticeably different associations of cultivable EnB suggest that cultivar genotype is an important factor selecting endophytic bacteria from local agro-environment. To our knowledge this is first report about the significant variation of diversity of cultivable endophytic bacteria inhabiting aboveground parts of the same maize cultivars grown at different locations.

Key words: endophytic bacteria, maize, plant growth promoting bacteria

Introduction

Maize was domesticated in south-western Mexico from wild grasses (teosintes) about 9,000 years ago (Matsuoka *et al*., 2002) and now is one of the most important crops, cultivated across the world. In Central Europe it started to be cultivated about 200 years ago. However as a grain crop it got the wider attention of breeders and farmers in the temperate climate zone, including Poland, about 30 years ago. The relations between maize and nonpathogenic microbes, especially inhabiting maize tissues, in Central Europe's temperate climate zone are practically not described in the literature. The interrelationships between plant development and microorganisms are related to local ecological factors such as soil, climate as well as agronomic practice. The nonpathogenic relationships between plants and microbes vary from neutral to commensal or symbiotic. Harmless microbes residing in plant tissues are defined as endophytic bacteria (EnB), which form commensal or mutualistic relationships with theirs host (Hallman *et al*., 1997; Zinniel *et al*., 2002). Endophytes are assumed to originate from seeds, rhizosphere and/or the aerial portions of plants from which they are isolated (Segher *et al*., 2004). The diversity of EnB was found to be dependent on several biotic and abiotic factors like soil type, agriculture practice, as well as plant physiology (Hallmann *et al*., 1997; Hardoim *et al*., 2008). Moreover, diverse communities of endophytic bacteria are related to plant organs from which isolation is done (Bodenhausen *et al*., 2013; Pal and Paul, 2013).

The study of bacterial communities associated with plants is indispensable in agricultural systems because the application of beneficial bacteria can dramatically increase production and improve plant health (Xu *et al*., 2013) as well as reduce agricultural production costs through more efficient use of agrochemicals.

Several papers described the endophytes of maize tissues (McInroy and Kloepper, 1995; Chelius and Triplett, 2001; Zinniel *et al*., 2002) but these have not

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focused on maize grown in the European temperate climate zone, where abiotic factors are drastically different from the ones in regions of long lasting history of maize cultivation. The origin of endophytic bacteria in aboveground tissues of maize is still discussable. Johnston-Monje and Raizada (2011) pointed out that endophytic bacteria of maize cultivars and breeding lines originated from Central America and cultivated in North America are transmitted with the seeds from one generation to another. However, Rijavec *et al.* (2007) isolated sporadically bacteria from germinated seeds of maize harvested in Slovenia. Rijavec *et al.* (2007) among isolated species from maize seedlings identified *Pantoea anantis* as the predominating bacteria. This species recently was described as pathogenic for maize in America and in Central Europe (Pérez-y-Terrón *et al*., 2009; Krawczyk *et al*., 2010). Furthermore, a study in southern China revealed significant modification and simplification of diversity of EnB in seeds during maturation, especially in the case of hybrid cultivars (Liu *et al*., 2012; 2013). Also, Liu *et al.* (2012; 2013) did not identify stable core associations of bacterial species inhabiting seeds of parent lines as well as seeds of hybrid cultivars originated from these breeding lines. Still little is known about diversity of endophytic bacteria associated with different hybrid maize cultivars in the temperate climate zone cropped under field conditions. Better knowledge could help us to search new potential Plant Growth Promoting Bacteria (PGPB) for agricultural applications since cultivable bacteria are potentially good candidates for commercialization (Xu *et al*., 2013).

In this paper we focus on a comparative study of biodiversity of cultivable dominant EnB of different hybrid cultivars of maize. For this purpose we isolated bacteria from plant tissues of six maize cultivars sown from the same lots of seeds in two locations on different soils as well as grown *in vitro*. The aims of the study were to evaluate the hypotheses that core associations of EnB cultivable species in aboveground parts of maize are similar or are connected with specific cultivars independently of environmental factors.

Experimental

Material and Methods

Plant material and cultivation condition. Plant material was collected from two experimental fields of Małopolska Plant Growing Company – HBP LLC at Kobierzyce (50°58'N, 16°55'E Kobierzyce, Poland) and at Smolice (51°42'N, 17°10'E Smolice, Poland). Six cultivars of maize (*Zea mays* L. spp. *mays*) (single hybrids; KB1902, KB1903 and triple hybrids; KB2704, Kosmo230, Cyrkon, Król) listed in Table I were sown in May 2009. The tested 6 maize cultivars were released from Kobierzyce Seed Centre (Kobierzyce, Poland) of Małopolska Plant Growing Company – HBP LLC (Poland). Complete cultivar vouchers are available from the Central Laboratory for Studies of Cultivable Plants "COBORU" (Slupia Wielka, Poland). Plants (three per cultivar per location) were collected at the BBCH 18–19 stage. At both locations ~85 000 seeds ha^{-1} of tested cultivars were sown. They were sown in Kobierzyce on gleyic chernozemic (siltic) soil (pH_{KCl} 7.1; $C_{org.}$ 1.94 %). The contents of plant available macro nutrients in the soil were as follows; phosphorus ~250 mg P 1 kg $d.m.^{-1}$, potassium ~460 mg K 1 kg $d.m.^{-1}$ and magnesium ~57 mg Mg 1 kg $d.m.^{-1}$. Winter wheat was a pre-crop and before sowing plots was fertilized with nitrogen, phosphorus and potassium at the level of 130, 40 and 82 kg ha^{-1}, respectively. In Smolice maize plants were cultivated on haplic luvisols (loamic) soil (pH_{KCl} 6.2; $C_{org.}$ 1.45 %) and the contents of plant available macro nutrients were as follows; phosphorus ~250 mg P 1 kg $d.m.^{-1}$, potassium ~230 mg K 1 kg $d.m.^{-1}$ and magnesium ~62 mg Mg 1 kg $d.m.^{-1}$. Maize was a pre-crop and before sowing plots was fertilized with nitrogen, phosphorus and potassium at the level of 138, 45 and 95 kg ha^{-1}, respectively.

Seedlings of tested maize cultivars grown *in vitro* were used in experiments as well. Seeds of six cultivars were hand-shelled from cobs harvested in October 2009 from both sampling sites and surface-sterilized with

Table I
Information about seeds, their parental lines and area of application

Zea mays cultivar	Type	FAO	Grain type	Registry form		Area of use
				mother	father	
KB1902	SC *	190	flint-dent	S, I.D**	D	grain
KB1903	SC	190	flint-dent	L.D, D	D	grain
KB2704	TC	270	flint-dent	S, D × S, I.D.	F	grain; livestock feed
KRÓL	TC	270	flint-dent	D	F	livestock feed
KOSMO230	TC	240	flint-dent	D	D	grain; livestock feed
CYRKON	TC	250	flint-dent	D	F	livestock feed

* SC – single cross, TC – triple cross
** S – semident; I.D. – lodent; L.D. – light dent; D – dent; F – flint

1% $NaClO^-$ for 30 minutes. Then they were washed three times with sterile dH_2O (2×5 min, 1×90 min.) and placed in test tubes with 0.8% agar supplemented with 21 ppm Hoagland solution (Hoagland and Arnon, 1950). Test tubes plants were incubated in a growth chamber at 14 hours daylight, temperature of 22°C, 16°C night temperature until the third leaf appears. We analyzed 2–3 seeds from each cultivar collected from both locations separately.

Endophytes isolation and cultivation. Aboveground parts of maize were collected from both testing locations and immediately after cutting were put into sterile bags and kept at +4°C during transfer to the laboratory. Isolations were done about 2–3 hours later. Plants leaves were surface-sterilized with 70% C_2H_5OH and subsequently washed 3-times with sterile distilled water. Field samples of leaves for endophyte isolation were cut out of bigger sterilized leaves fragments into ~6 cm^2 pieces from the center part of the leaf with a sterile blade. The whole plants grown i*n vitro*, at the stage of third true leaf were separated into three parts (roots, stems, leaves), and after mentioned above surface-disinfection used for analysis as well.

Each sample after surface-sterilization was macerated in aseptic 0.1 M $MgSO_4$ and ten-fold diluted suspensions were transferred onto solid 1/3 TSA (Difco, USA) medium for field and *in vitro* samples. Additionally, macerated plant samples of *in vitro* plants were transferred onto Rojo Congo (RC) medium (Rodríguez Cáceres, 1982) for enumeration of cultivable nitrogen-fixing bacteria. Plates were incubated at 28°C by 7 days. After incubation from each sample 10–12 dominating, morphologically differentiated bacterial colonies were randomly picked and used for further tests. Three consecutive transfers onto 1/3 TSA medium were done to obtain single colony cultures. Pure strains were maintained as frozen stocks at –70°C in a storage medium (Bactotryptone 10 g; yeast extract 5 g; NaCl 0.5 g; K_2HPO_4 6.3 g; KH_2PO_4 1.8 g; sodium citrate 0.45 g; $MgSO_4 \times 7H_2O$ 0.09 g; $(NH_4)_2SO_4$ 0.9 g; 1000 ml dH_2O; pH 7.0). Surface sterilization parameters like selection of disinfectant; its strength and time were optimized prior experimentation with pot cultivated maize plants (data not shown).

DNA isolation and 16S rRNA gene sequencing. Selected single isolates were cultivated on solid King B or PDA medium. Genomic DNA was extracted using the Genomic Mini AX Bacteria kit (AA Biotechnology, Poland) according to the instructions of the manufacturer. Amplified fragments of the 16S rRNA gene were generated by PCR with the primers FAM27f (5'-GAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTTACGACTT-3') and 5x Hot FIREPol Blend Master Mix (Solis Biodyne, Estonia). The PCR products were purified with the PureLinkTM Genomic DNA Mini Kit (Invitrogen, USA) or ExoSAP-IT (GE Healthcare Life Sciences, USA) in accordance with the manufacturer's protocol. Sequencing of the amplified 16S rRNA gene fragments was carried out with primers FAM27f and 1492r, which annealed to either end of the gene, as well as with primers 704f (5'-TGTGTAGCGGTGAAATGCGTAGA-3') and 765r (5'-CTGTTTGCTCCCCACGCTTTC-3'), which annealed to the central portions of the gene. Sequencing was performed on ABI 3730X1 DNA Analyzer (Applied Biosystems, Foster, USA). The obtained sequences were aligned using the ClustalW method of the DNAStar software package (DNAStar Lasergene Inc., USA). A BLASTN search of the NCBI database and search of the RDP database were performed to compare the 16S rRNA gene sequences of isolates with those available online.

Statistical analysis. Data were analyzed statistically using Statistica V.9.0PL (StaSoft Inc., USA) software. Duncan's multiple range test was used to assess the differences among the means (three replicates for each) at 95% level of significance ($P = 0.05$).

Results

Isolation of endophytic bacteria. We did not isolate bacteria able to grown on 1/3 TSA or RC medium from any of twenty-eight 15-20-d old seedlings of examined maize cultivars germinated from surface sterilized seeds and cultivated *in vitro*.

The enumeration of CFU of cultivable endophytic bacteria inhabited leaves of tested six maize cultivars revealed significant differences among them as well as between both cultivation plots (Table II). The CFU number enumerated in leaves collected from maize cultivated in Smolice ranged from 5.43 to 6.26 log10 CFU $(1 \text{ g d.w})^{-1}$ and from 4.63 to 5.73 log10 CFU $(1 \text{ g d.w.})^{-1}$ in leaves collected from maize cultivated in Kobierzyce. The number of log10 CFU in leaves of all cultivars was significantly higher in plants cultivated in Smolice than in Kobierzyce (Table II). The highest log10 CFU numbers were found in leaves of Kosmo 230 cv. in comparison with other maize cultivars (Table II), where the log10 CFU numbers were not statistically different.

Isolates identification. The dominant, morphologically differentiated bacterial colonies, in total, 125 isolates, representing six maize cultivars planted in two sampling sites were identified based on partial 16S RNA gene sequence. Overall, the isolates represented five taxonomic divisions: *Actinobacteria*, *Bacteroidetes*, *Firmicutes* as well as α- and γ-*Proteobacteria*. The most common phyla were γ-*Proteobacteria* and *Actinobacteria*, 40% and 26.4% of all isolates, respectively. Phyla γ-*Proteobacteria* was detected in all tested plants, in contrary to α-*Proteobacteria* detected only in five (out

Table II
Number of colony forming units of bacteria grown on 1/3 TSA (log10 CFU per g d.w. of leaf tissue) in leaf tissue of six maize cultivars

Zea mays cultivar	Number of selected isolates			Number of selected isolates
	Smolice	Kobierzyce	Means for cultivar	
KB1902	5.79^{a-b}	4.20^{b-d}	5.30^{B}	21
KB1903	5.45^{a-d}	5.02^{b-d}	5.23^{B}	19
KB2704	5.43^{a-d}	4.72^{c-d}	5.07^{B}	23
KRÓL	5.57^{a-d}	4.63^{d}	5.10^{B}	22
KOSMO230	6.26^{a}	5.73^{a-c}	5.99^{A}	20
CYRKON	5.81^{a-b}	4.79^{b-d}	5.30^{B}	20
Means for localization	5.72^{α}	4.95^{B}	– – –	– – –
Number of selected isolates	64	61	– – –	125

Values for cultivars at both locations followed by the same small letter, means for cultivar followed by the same capital letter and means for localization followed by the same Greek letter are not significantly different according to Duncan's multiple range test ($P < 0.05$)

of six) cultivars grown in Kobierzyce location, only. Phyla *Actinobacteria* was detected in ten out of twelve plants, except Kosmo 230 cv. and Król cv. from Kobierzyce and Smolice sampling sites respectively (Fig. 1). Altogether, identified bacteria represented 46 species from 18 genera. Higher diversity of EnB was found in plants collected from Kobierzyce, where we identified 35 species from 16 genera and among them 22 species were uniquely found at this field, only. In the contrary, from maize leaves collected at Smolice we identified 24 species representing 10 genera and among them 10 species were uniquely isolated from this field (Table III). The most frequently encountered genera were *Pseudomonas* (33.6%), *Bacillus* (17.6%), *Arthrobacter* (11.2%) and *Microbacterium* (9.6%). Strains belonging to *Pseudomonas* genus were isolated from all tested plants except KB2704 cv. grown in Kobierzyce. Strains of *Pseudomonas fluorescens* were most frequently isolated in leaves tissues of five cultivars expect Cyrkon cropped at Smolice. Aforementioned, species was found in leaves of two cultivars, Król and Kosmo230, cultivated at Kobierzyce, only. Among *Bacillus*, a second dominated genus, strains of *Bacillus megaterium* were most frequently isolated. This species was found in leaves of five cultivars cropped at Smolice, expect of cultivar KB1902, but was isolated only from tested tissues of Cyrkon grown at Kobierzyce. Among *Arthrobacter* genus, *Arthrobacter. nicotinovorans* was the most frequently isolated species. They were found in leaves of KB2704 and Król collected at Kobierzyce, KB1903 and Cyrkon collected at Smolice as well as in tissues of KB1902 collected from both

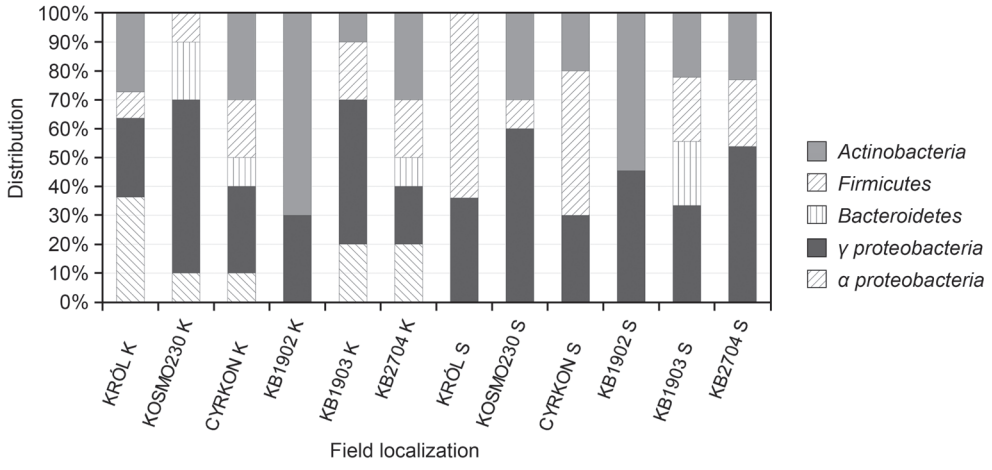

Fig. 1. Phylogenetic distribution of endophytic bacteria at phyla/class level expressed per host-plant species per localization. Bacteria were classified based on *16S rRNA* gene data with ClustalW method of the DNAStar software package (DNAStar Lasergene Inc., USA). A BLASTN search of the NCBI database and search of the RDP database were performed to compare the *16S rRNA* gene sequences of isolates with those available online. Samples are labeled with letters indicating sampling site (S – Smolice, K – Kobierzyce)

Table III
Identification of endophytes isolated from leaves of six maize cultivars grown on two different fields.

Phylum	Genus	Closest match according to the 16S rRNA gene sequence		Number of isolates	Field localization and *Zea mays* cultivar											
		Nearest relative strains	Acces. No. of nearest relative strain (% of the match*)		Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice
					KB1902		KB1903		KB2704		Król		Kosmo230		Cyrkon	
α-proteoabcteria	*Methylobacterium*	*M. aminovorans*	AB175629 (99.1)	2	0	0	0	0	0	0	2	0	0	0	0	0
		M. extorquens	D32224 (99.6)	2	0	0	0	0	0	0	2	0	0	0	0	0
	Rhizobium	*Rh. radiobacter*	AB247615 (97.0)	3	0	0	2	0	0	0	0	0	1	0	0	0
		Rh. larrymoorei	Z30542 (98.7)	1	0	0	0	0	1	0	0	0	0	0	0	0
	Sphingomonas	*Sph. glacialis*	GQ253122 (98.0)	1	0	0	0	0	1	0	0	0	0	0	0	0
		Sph. paucimobilis	U37337 (99.5)	1	0	0	0	0	0	0	0	0	0	0	1	0
γ-proteobacteria	*Acinetobacter*	*Ac. calcoaceticus*	AJ888983 (98.6)	2	0	0	0	0	2	0	0	0	0	0	0	0
		Ac. lwoffi	X81665 (99.1)	3	0	0	0	0	0	0	0	0	3	0	0	0
		Ac. schindleri	AJ278311 (98.3)	1	0	0	0	0	0	0	0	1	0	0	0	0
	Erwinia	*E. persicina*	U80205 (99.2)	1	0	0	0	0	0	0	0	0	0	1	0	0
	Pseudomonas	*Ps. clemancea*	AM419155 (98.7)	4	0	0	0	1	0	0	0	0	0	0	1	2
		Ps. extremaustralis	AJ583501 (99.9)	1	0	0	0	0	0	0	0	1	0	0	0	0
		Ps. fluorescens	AF094729 (99.9)	1	0	0	0	0	0	0	0	0	0	0	0	1
		Ps. fluorescens	AJ308307 (99.5)	16	0	3	0	2	0	4	1	2	2	2	0	0
		Ps. graminis	Y11150 (99.8)	1	0	0	0	0	0	0	0	0	0	1	0	0
		Ps. grimontii	AF268029 (99.9)	3	2	0	0	0	0	0	0	0	0	0	1	0
		Ps. lurida	AJ581999 (99.9)	3	0	2	0	0	0	1	0	0	0	0	0	0
		Ps. marginalis	Z76663 (99.4)	4	1	0	2	0	0	0	0	0	0	1	0	0
		Ps. migulae	AF074383 (99.7)	1	0	0	0	0	0	0	0	0	0	0	0	1
		Ps. orientsalis	AF064457 (99.6)	4	0	0	0	0	0	1	2	0	1	0	0	0
		Ps. poae	AJ492829 (99.9)	2	0	0	0	0	0	1	0	0	0	0	1	0
		Ps. thievervalensis	AF100323 (99.5)	2	0	0	2	0	0	0	0	0	0	0	0	0
		Ps. viridiflava	AY180972 (99.8)	1	0	0	1	0	0	0	0	0	0	0	0	0
	Shigella	*S. flexneri*	X96963 (98.1)	1	0	0	0	0	0	0	0	0	0	1	0	0
Actinobacteria	*Arthrobacter*	*Ar. nicotinovorans*	X80743 (99.6)	10	2	2	0	1	1	0	2	0	0	0	0	2
		Ar. nitroguajacolicus	AJ512504 (99.8)	4	1	0	1	1	0	0	0	0	0	0	1	0
	Brahybacterium	*Br. conglomeratum*	AB537169 (100.0)	1	1	0	0	0	0	0	0	0	0	0	0	0
	Kocuria	*K. rhizophila*	Y16264 (97.3)	1	0	0	0	0	0	1	0	0	0	0	0	0
		K. kristinae	X80749 (99.4)	1	0	0	0	0	1	0	0	0	0	0	0	0
	Microbacterium	*M. phyllosphere*	AJ277840 (98.4)	1	0	0	0	0	0	0	0	0	0	1	0	0
		M. testaceum	X77445 (98.0)	11	0	4	0	0	0	2	1	0	0	2	2	0
	Micrococcus	*M. yunnanensis*	FJ214355 (99.9)	1	0	0	0	0	1	0	0	0	0	0	0	0
	Rhodococcus	*Rh. qingshengii*	DQ090961 (99.6)	1	1	0	0	0	0	0	0	0	0	0	0	0
	Rothia	*R. amarae*	AY043359 (99.4)	2	2	0	0	0	0	0	0	0	0	0	0	0
Bacteroidetes	*Chryseobacterium*	*Chr. indoltheticum*	AY468448 (99.1)	1	0	0	0	0	0	0	0	0	1	0	0	0
		Chr. jejuense	EF591303 (97.3)	3	0	0	0	2	0	0	0	0	0	0	1	0
	Pedobacter	*P. borealis*	EU030687 (98.8)	2	0	0	0	0	1	0	0	0	1	0	0	0
Firmicutes	*Bacillus*	*B. aerophilus*	AJ831844 (99.4)	3	0	0	0	0	0	0	0	2	1	0	0	0
		B. circulans	AY724690 (99.5)	4	0	0	0	0	0	2	0	2	0	0	0	0
		B. idriensis	AY904033 (99.9)	1	0	0	0	0	1	0	0	0	0	0	0	0
		B. megaterium	D16273 (98.7)	10	0	0	0	1	0	1	0	2	0	1	1	4
		B. methylotrophicus	EU194897 (99.5)	1	0	0	1	0	0	0	0	0	0	0	0	0

Table III
Continued

Phylum	Genus	Closest match according to the 16S rRNA gene sequence		Number of isolates	Field localization and *Zea mays* cultivar											
		Nearest relative strains	Acces. No. of nearest relative strain (% of the match*)		Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice	Kobierzyce	Smolice
					KB1902		KB1903		KB2704		Król		Kosmo230		Cyrkon	
Firmicutes	*Bacillus*	*B. pumilus*	AY876289 (97.9)	1	0	0	0	0	0	0	0	0	0	0	0	1
		B. simplex	AJ439078 (98.6)	2	0	0	0	0	0	0	1	1	0	0	0	0
	Staphylococcus	*St. haemolyticus*	X66100 (99.2)	2	0	0	0	1	1	0	0	0	0	0	0	0
		St. pasteuri	AB009944 (99.3)	1	0	0	0	0	0	0	0	0	0	0	1	0
		St. saprophyticus	AP008934 (99.3)	1	0	0	1	0	0	0	0	0	0	0	0	0
	Number of species			46	7	4	7	7	9	8	7	7	7	8	9	6
	Number of species unique for cultivar in Kobierzyce			X	3		4		6		2		2		2	
	Number of species unique for cultivar in Smolice			X		0		0		1		2		4		2
	Number of species unique for cultivar at both locations				0		0		0		1		0		0	
	Number of species common for cultivar at both locations				1		1		0		2		1		2	
	Number of species uniquely isolated from maize grown on field Kobierzyce			22												
	Number of species uniquely isolated from maize grown on field in Smolice			11												

* gave for the lowest value

locations. Strains of Microbacterium testaceum, which were most frequently isolated among Microbacterium genus, were found in leaves of two cultivars cropped at Kobierzyce (Król, Cyrkon) and three cultivars cropped at Smolice (KB1902, KB2704 and Kosmo230).

However, none of species was common for all cultivars at both locations (Table III). Among isolated EnB six species only, *Pseudomonas clemancea*, *P. fluorescens, B. megaterium, Bacillus simplex, Ar. nicotinovorans* and *Arthrobacter nitroguajacolicus,* were found in aboveground parts of the same cultivar grown on both tested fields (Table III).

There were differences in number of unique species based on cultivar source. Cultivar KB1902 was inhabited by three unique species (*Brachybacterium conglomeratum*, *Rhodococcus qingshengii*, *Rothia amarae*) in Kobierzyce but we did not found any unique one in cultivar cropped in Smolice. Likewise cultivar KB1903 was inhabited by four unique species (*Bacillus methylotrophicus*, *Pseudomonas thievervalensis*, *Pseudomonas viridiflava*, *Staphylococcus saprophyticus*) in Kobierzyce but we did not found any unique one in cultivar cropped in Smolice. On the contrary, other four cultivars were inhabited by unique species in both locations (Table III). For example, Król cultivar was inhabited by two unique species in Kobierzyce (*Methylobacterium aminovorans*, *Methylobacterium extorquens*) and by two unique species in Smolice (*Acinteobacter schindlerii*, *Pseudomonas extremaustralis*).

Discussion

Genotypic diversity of endophytes. This study was an attempt to isolate and characterize endophytes of six maize cultivars inhabiting young seedlings germinated from surface-sterilized maize seeds *in vitro* as well as from leaves of these cultivars cropped at two locations with culture-dependent approach. Our approach did not result in the isolation of cultivable endophytes from seedlings germinated from second generation of seeds of tested cultivars on organic medium (1/3 TSA) as well as on RC medium used for isolation of nitrogen-fixing bacteria. This may suggest that seeds did not harbour cultivable endophytic bacteria colonizing seedlings. However, Johnston-Monje and Raizada (2011) isolated cultivable bacteria and identified several non-cultivable bacteria in seeds of wild ancestors (teosinte) and domesticated varieties of maize. They pointed out that bacteria are transmitted with the seeds from one generation to another. Among investigated seeds Johnston-Monje and Raizada (2011) tested seeds of two commercial cultivars, dent inbred B73 and hybrid Pioneer 3751, which in our opinion are similar to seeds of commercial cultivars tested in our studies. They were able to isolate on three diverse media cultivable bacteria from the genus *Bacillus* and *Enterobacter* from the second generation of seeds of hybrid Pioneer 3751 but not from the seeds of inbred B73. Also Rijavec *et al.* (2007) did not isolate cultivable

bacteria from most of tested germinated kernels of different maize cultivars collected in Slovenia (16 strains from 195 tested germinated seeds). They were able to isolate bacteria from 11% to 20% of tested germinated seeds of different cultivars and the lowest number of germinated seeds inhabited by EnB was found in the case of non-named Pioneer inbreed. Among isolated bacteria, 5 out of 15 isolates Rijavec *et al.* (2007) identified as *Pantoea anantis,* which recently was described as a pathogenic for maize species in America and in Central Europe (Pérez-y-Terrón *et al.*, 2009; Krawczyk *et al.*, 2010). A similar study on the diversity of endophytic bacteria in seeds with non-culture method reported by Liu *et al.* (2012) also revealed a generally lower number of species in seeds of hybrid than in seeds of parental lines. Lack of cultivable endophytic bacteria in young seedlings of tested maize cultivars grown *in vitro* could reflect the effect of inbreeding similar like in the case of seeds of inbreed B73 (Johnston-Monje and Raizada, 2011). Noticeable smaller biodiversity of cultivable and non-cultivable EnB and even lack of cultivable EnB in self-pollinated seeds of second generation of wild ancestors as well as Mexican maize landraces collected on field near Guelph (Canada) was described also by Johnston-Monje and Raizada (2011).

The influence of different environmental condition, especially temperature during maturation of seeds is another factor that should be taken into consideration, explaining the lack of cultivable endophytic bacteria in young seedlings maize hybrid cultivars tested in our studies. Harvest of maize seeds for our studies in October 2009 was done after night frost, which probably did not support the survival of endophytic bacteria in our seeds. Liu *et al.* (2013) also reported significant decrease of biodiversity of EnB communities in seeds of two cultivars from proembryo-forming stage to dough stage. Mentioned above research and our results support hypotheses that environmental conditions like fertilization, soil properties, weather conditions during maturation as well as maturation stage of seeds are important factors limiting the survival and biodiversity of EnB communities in seeds of maize regard inbreed effect.

The results of the study of dominant endophytic bacteria isolated from leaves tissues of six maize cultivars grown under field condition show different composition of cultivable bacteria and noticeable cultivar and location dependent diversity between these associations. However, among EnB isolated from leaves, bacteria from the phylum γ-*Proteobacteria* with the genus *Pseudomonas* were the most often encountered. Several authors also reported that among endophytic bacteria *Pseudomonas* were frequently isolated and were found to be present in tissues of sugar beet (Jacobs *et al.*, 1985), carrot (Surette *et al.*, 2003), soybean (Kuklinsky-Sorbal *et al.*, 2004), ginseng (Cho *et al.*, 2007), maize (McInroy and Kloepper, 1995; Rai *et al.*, 2007; Rijavec *et al.*, 2007) as well as in maize kernels (Johnston-Monje and Raizada, 2011; Liu *et al.*, 2012). Frequently encountered genera were also *Bacillus*, *Arthrobacter* and *Microbacterium.* Aforementioned genera have been previously described as maize endophytes (McInroy and Kloepper, 1995; Rai *et al.*, 2007; Rijavec *et al.*, 2007; Liu *et al.*, 2013). Studies of Johnston-Monje and Raizada (2011) showed that TRFLP method predicted *Clostridium* and *Paenibacillus* species as conserved across all studied *Zea* genotypes. In contrary, studies by Liu *et al.* (2012) on four Chinese maize hybrids and their parental lines showed that the genus *Paenibacillus* was present only in two parental lines (Ye478; Chang 7–2) but its hybrid Yuyu 23 did not harbour them. Moreover, second conserved genus *Clostridium* has not been identified among Chinese cultivars. In this study we did not reported genus *Paenibacillus* or *Clostridium* harbouring maize leaf tissues. Species common to the Polish and Chinese maize hybrids were *Acinetobacter lwoffii*, *Acinetobacter schindlerii*, *Pseudomonas poae* and *Shigella flexneri*, only. In contrary to Rijavec *et al.* (2007) we did not isolate *Panotea ananatis* species.

This study shows more diverse EnB communities in leaves than described in steams of commercial cultivars maize by Johnston-Monje and Raizada (2011). Similar more various communities of bacterial endophytes in leaves than in stem and root segments were described in the case of medicinal herb *Hygrophila spinosa* (Pal and Paul, 2013). Among 46 identified species from 18 genera, 16 have been previously reported as maize endophytes, except for the genera *Brachybacterium* and *Pedobacter*. The genera *Brachybacterium* and *Pedobacter* have recently been described as endophytes in the leaves of young radish (Seo *et al.*, 2010) and in *Diapensa lapponica* plants (Nissinen *et al.*, 2012).

This study indicates the colonisation of different maize cultivars by noticeable various associations of EnB and important effect of local agro-environmental conditions. Higher diversity of EnB was found in plants collected from Kobierzyce, where we identified 35 species from 16 genera, than in plants from Smolice, where among 10 genera we identified 24 species. The most common phyla were γ-*Proteobacteria* and *Actinobacteria*, 40% and 26.4% of all isolates respectively, however there were 20.8% of isolates represented phylum *Firmicutes,* 8% represented phylum γ-*Proteobacteria* and 4.8% represented phylum *Bacteroidetes* as well. At the genus level, *Pseudomonas* (33.6%), *Bacillus* (17.6%), *Arthrobacter* (11.2%) and *Microbacterium* (9.6%) were dominant among them.

Among EnB isolates only strains identified as *Ps. fluorescens* were isolated from all tested six cultivars. Other most common species was *M. testaceum*, *B. megaterium*

and *Ar. nicotinovorans*. Among isolated EnB six species only, *Ps. clemancea*, *Ps. fluorescens*, *B. megaterium*, *B. simplex*, *Ar. nicotinovorans* and *Ar. itroguajacolicus,* were found in aboveground parts of the same cultivar grown on both tested fields (Table III).

To the best of our knowledge, this study is the first to focus on the isolation and identification of endophytic bacteria of maize grown at temperate climate zone, sown from the same lots of seeds in two locations on different soils as well as grown *in vitro* using culture-dependent method. Our research provides partial information about significant differentiations of associations of dominant cultivable EnB inhabiting different hybrid maize varieties.

Moreover, our studies based on species identification did not support the hypotheses that core associations of cultivable endophytic bacteria inhabiting maize tissues in temperate climate zone are cultivar dependent and are transmitted with the seeds from one generation to another. Nevertheless, each cultivar is inhabited by similar genera, but by different species. The fact that the same cultivars, sown from the same lots of seeds, under field conditions on two different locations were colonised with noticeably different associations of cultivable EnB suggest that cultivar specific metabolites are an important factor selecting endophytic bacteria from local agro-environment.

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SHORT COMMUNICATION

Characterisation of Yersinia Secretion Apparatus – Pathogenicity Island (Ysa-PI) of *Yersinia enterocolitica* 1B/O8 in Poland: an Idle Ysa is a Specific Hallmark of the Epidemic Sensu Stricto Strain

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Abstract

Yersinia secretion apparatus (Ysa), the chromosomal type three secretion system (T3SS) is considered to contribute to virulence of high-pathogenicity Yersina *enterocolitica* biovar 1B. DNA-sequence of Ysa pathogenicity island was determined for clinical isolate DM0110 of *Y. enterocolitica* 1B/O8 with origin in Poland. We found a premature stop-codon in the regulatory gene *ysrR* (mutation at position 269). Altered *ysrR* was detected in all tested 78 isolates of *Y. enterocolitica* 1B/O8 collected from clinical samples in Poland from 2004 to 2013. Since aberrations in YsrR are considered to inactivate Ysa, our findings may suggest Ysa is not indispensable for *Y. enterocolitica* 1B/O8 to infect humans.

Key words: *Y. enterocolitica* 1B/O8, T3SS, virulence, Ysa, Ysa-PI

Yersina enterocolitica is a causative agent of gastrointestinal disorders in humans with a variety of clinical manifestations, including reactive arthritis and erythema nodosum (Bottone, 1997). Pathogenic strains of *Y. enterocolitica* harbour a suit of virulence factors encoded on virulence plasmid pYV and the chromosome. Strains of bioserotype 1B/O8 are considered lethal to mouse and highly pathogenic to humans (Aulisio *et al*., 1983). *Y. enterocolitica* 1B/O8 was originally reported in northern America in the 70s and 80s of the past century. In the early 90s bioserotype 1B/O8 disappeared in America and emerged in Japan (Ichinohe *et al*., 1991). The first case of human *Y. enterocolitica* 1B/O8 infection in Europe was reported in Germany (Schubert *et al*., 2003). However, this bacterium is nowadays most numerously isolated in Poland, where 224 human clinical isolates were collected from 2004 to 2013. *Y. enterocolitica* 1B/O8 isolates collected were tightly clonal (Gierczyński *et al*., 2009; Zacharczuk, 2012). The bacterium is an important causative agent of yersiniosis in Poland as shown by serological studies (Rastawicki *et al*., 2013).

High pathogenicity of *Y. enterocolitica* 1B/O8 is attributed to virulence factors encoded in the chromosome. Chromosomal type III protein secretion system (T3SS) named Yersinia secretion apparatus (Ysa) is considered a potential virulence factor of the bacterium. Ysa was found to have specific induction conditions – nutrient rich medium supplemented with a high salt concentration (190–400 mM NaCl or KCl) at a moderate temperature (26–28°C) (Haller *et al*., 2000, Venecia and Young, 2005). Therefore, Ysa activity was initially considered as limited to the gastrointestinal tissues. The T3SS was shown to translocate some specific proteins named Yersinia secreted proteins (Ysps) (Haller *et al*., 2000, Venecia and Young 2005). Moreover, Ysa was found to secrete some of pYV-encoded Yersinia outer proteins (Yops) (Venecia and Young 2005). In recent studies, Ysa was proposed to act also at the systemic phase of the disease (Bent *et al*., 2013). All the aforementioned findings were derived from investigations conducted on laboratory reference strainsof *Y. enterocolitica* 1B/O8 and their mutants. However, little is known about Ysa activity in clinical isolates. Therefore we decided to investigate the Ysa activity in clinical isolates of *Y. enterocolitica* 1B/O8 collected in Poland.

Clinical strain DM0110 of *Y. enterocolitica* 1B/O8 originated in Poland in 2005 and was selected for DNA-sequencing of the complete Ysa pathogenicity-island (Ysa-PI). DNA-sequencing was conducted by Sanger method using a BigDye Terminator v3.1 (Applied Biosystem). A library of 1.5–2.5 kbp fragments of Ysa-PI

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Table I
Y. enterocolitica 1B/O8 reference strains and clinical isolates tested in this study

Strain ID (no. isolates)	Source[a]	Year of isolation	Ysps secretion[b]	*ysrR* type[c]
WA-314	Laboratory, MPI	NA	+	Wild
1105	Laboratory, IP	NA	+	Wild
13804	Laboratory, IP	NA	+	Wild
17451	Laboratory, IP	NA	+	Wild
20167	Laboratory, IP	NA	+	Wild
20169	Laboratory, IP	NA	+	Wild
20175	Laboratory, IP	NA	+	Wild
20176	Laboratory, IP	NA	+	Wild
20178	Laboratory, IP	NA	+	Wild
20189	Laboratory, IP	NA	+	Wild
20232	Laboratory, IP	NA	+	Wild
DM0099	Clinical sample	2008	–	Altered
DM0102	Clinical sample	2008	–	Altered
DM0110	Clinical sample	2005	–	Altered
DM0147	Clinical sample	2006	–	Altered
DM0149	Clinical sample	2005	–	Altered
DM0150	Clinical sample	2004	–	Altered
DM0209	Clinical sample	2009	–	Altered
DM0249	Clinical sample	2009	–	Altered
(n = 70)	Clinical samples	2004–2013	Not tested	Altered

a MPI (Max Von Pettenkofer Institut, Munich, Germany); IP (Pasteur Institute, Paris, France); b (+) for Ysps secretion (–) for no Ysps secretion; c result of PCR-RFLP assay for position 269 in the *ysrR* gene; NA – not applicable

of DM0110 was cloned in plasmid pJet1.2/blunt Cloning Vector (Thermo Scientific, Lithuania) prior to DNA sequencing. Sequencing results were read on automated capillary sequencers in a biotechnological company (Genomed, Poland). Reviewed complete 30379 bp – long YSA-PI cluster was deposited in GenBank (www.ncbi.nlm.nih.gov) database under no. KC784374.1.

Further examinations were performed on a number of 78 clinical isolates and 11 reference strains (Table I). PFGE typing (data not shown) showed that all the isolates were tightly-clonal and belonged to the previously described *Xba*I genotype II (Gierczyński *et al*., 2009). Ysa and Ysps proteins were visualised by SDS-PAGE assay. The assay was performed in accordance with the procedure described by Matsumoto and Young (2006). Eight *Y. enterocolitica* 1B/O8 clinical isolates originating in Poland were tested by SDS-PAGE together with DM0110 and the 11 reference strains listed in Table I. Bands specific for Ysps and Ysa proteins were detected for the reference strains only. In contrast to the reference strain WA-314 no Ysps could be detected for clinical isolate DM0110 in duplicate experiments (Fig. 1A). Ysps were also lacking in seven randomly selected clinical isolates (Fig. 1B).

The Ysa-PI sequence of DM0110 was aligned to the homologous loci deposited in commonly accessible databases using CLC Sequence Viewer 7 (CLC Inc, Denmark). Comparison of nucleotide and deduced amino-acid sequences of Ysa-PI of *Y. enterocolitica* 1B/O8 reference strains: 8081 (NC_008800.1), A127/90 (AF369954.1 and AY100449.2) and WA314 (AKKR01000083) was conducted to trace amino-acid substitutions and other alterations.

DM0110 revealed 99% homology to Ysa-PI of reference strains 8081, A127/90 and WA314. No structural aberrations were observed. The only significant mutation specific for tested DM0110 was found in *ysrR* gene that is part of the YsrRST phosphorelay system (Venecia and Young 2005, Walker *et al*., 2010). Single nucleotide substitution (C to A) at position 269 of *ysrR* resulted in a premature stop-codon that terminates translation of YsrR at position 90. No such mutation was found in the reference *Y. enterocolitica* 1B/O8 strains examined in this study.

To verify whether the stop-codon prematurely terminates the expression of *ysrR*in DM0110, the complete *ysrR* gene (711 bp length) from DM0110 was cloned to expressing vector and examined in *Escherichia coli*. Expression system pET-30 Ek/LIC Vector Kit (Novagen, USA) was used according to the manufacturer's instruction. The unaltered *ysrR* gene from strain WA-314 was used as a reference. Recombinant proteins of both

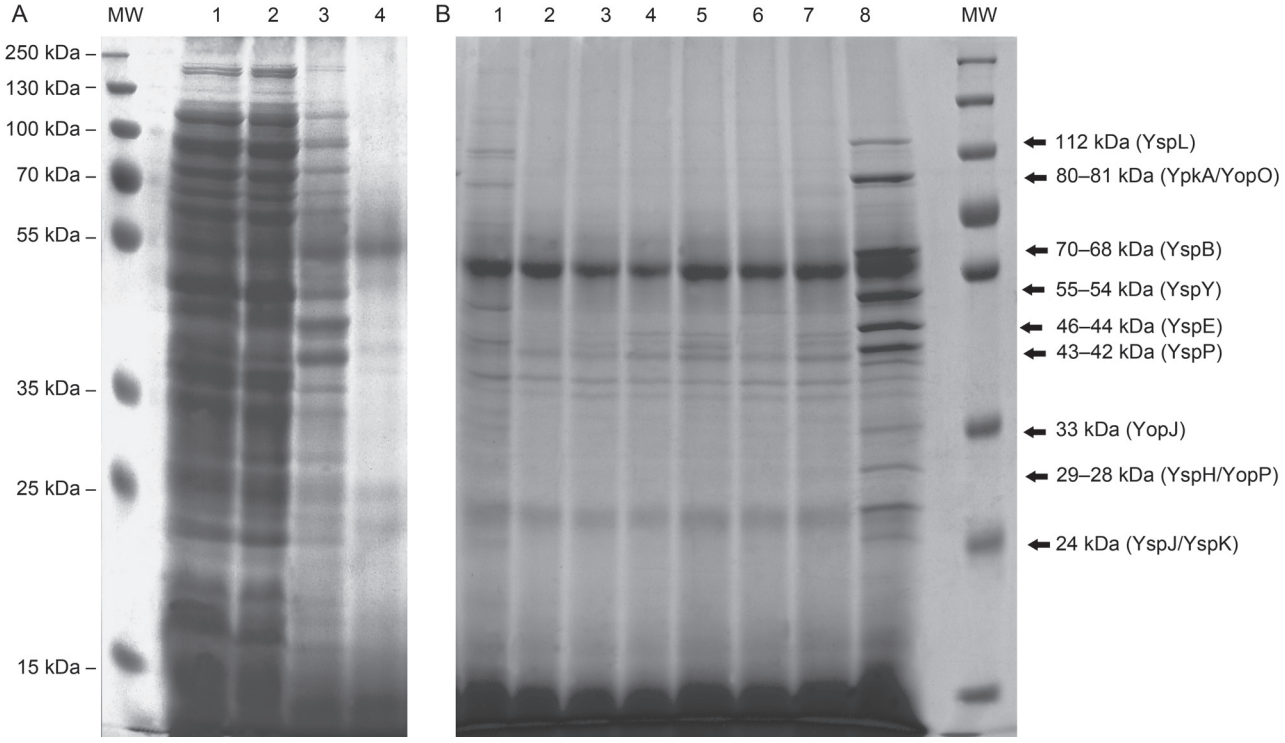

Fig. 1. SDS-PAGE profiles of secreted proteins Ysa-Ysp of *Y. enterocolitica* 1B/O8 reference strains and clinical isolates from Poland
A: lines 1 and 2 – strain WA-314; 3 – strain 20175; 4 – clinical isolate DM0110. B: lines 1 to 7 – clinical isolates: DM0150, DM0147, DM0099, DM0102, DM0149, DM0249, DM0209 respectively; line 8 – strain WA-314; MW – molecular weight standard

DM0110 and WA-314 were purified on column with Ni^{2+}-ISA His-Binding Resin (Novagen, USA). Molecular weight of the expressed recombinant proteins was determined by SDS-PAGE according to Laemmli (1970).The recombinant YsrR protein of DM0110 was about 10 kDa, while the YsrR recombinant of the unaltered *ysrR* gene from WA-314 was 27 kDa. These results confirmed that the premature stop-codon in *ysrR* is active *in vitro*. Further analysis *in silico* performed with Domain Enhanced Lookup Time Accelerated BLAST (DELTA-BLAST) algorithm showed that YsrR protein REC domain is only half-expressed in DM0110 while the LuxR-C-like domain is lacking.

To determine whether other clinical isolates of *Y. enterocolitica* 1B/O8 in Poland carry unaltered or prematurely-terminated *ysrR*, PCR-RFLP assay targeting the unaltered (wild-type) *ysrR* gene was developed. Primers (ysrR-F 5'ATGACACAAACGAAAACGCTCAAT and ysrR-R 5'TTATAGAGAAATTTCATGAGCAT) were used to amplify the 711 bp PCR-product of the *ysrR* gene. PCR was conducted as described previously (Wołkowicz *et al*., 2014). The *ysrR* PCR-product was further digested by *Bts*I endonuclease (New England BioLabs) as recommended by the manufacturer. The PCR-product of the unaltered, wild-type *ysrR* gene yielded two fragments of expected size 268 and 445 bp. In the case of mutation in position 269 of *ysrR*, the PCR-product remained undigested. In each PCR-RFLP experiment the reference strain WA-314 was used as a control for the wild-type *ysrR*. DM0110 and all the tested clinical isolates listed in Table I were shown by the PCR-RFLP assay to bear *ysrR* gene altered in position specific for the premature stop-codon. No alterations were detected in ten reference strains of *Y. enterocolitica* 1B/O8 (Table I) previously found to secrete Ysps (Rokosz-Chudziak *et al*., 2013).

In the past two decades *Y. enterocolitica* 1B/O8 emerged in Japan, Germany and Poland. Since 2004 this bacterium has been isolated in Poland from humans with clinically confirmed yersiniosis. Noteworthy, all clinical isolates of *Y. enterocolitica* 1B/O8 in Poland were found to be tightly clonal (Gierczyński *et al*., 2009, Zacharczuk, 2013). *Y. enterocolitica* 1B/O8 was isolated from a variety of clinical samples including stool and blood. We, therefore, assumed our collections of clinical isolates constitute an excellent material to study Ysa activity in human yersiniosis.

The results obtained in this study are, however, surprising when compared with other reports on Ysa T3SS published to date (Venecia and Young 2005, Matsumoto and Young 2006, Mildiner-Earley *et al*., 2007, Bent *et al*., 2013). First of all we showed that clinical isolates of *Y. enterocolitica* 1B/O8 collected in Poland from 2004 to 2009 were unable to secrete Ysps. Moreover, clinical isolate DM0110 was unable to produce Ysps under a variety of inducible conditions described elsewhere (Mildiner-Earley *et al*., 2007, Witkowski *et al*., 2008) (data not shown). These findings together have

prompted us to search Ysa-PI of DM0110 for possible reasons for Ysa inactivation. One particularly interesting mutation in the *ysrR* gene of YsrRST phosphorelay system was detected. This mutation triggers the premature stop-codon that early terminates the translation of regulatory gene *ysrR*.

The YsrRST phosphorelay system is considered the major regulatory system for YSA-PI (Venecia and Young, 2005, Walker *et al*., 2010). Consequently, the premature stop-codon in *ysrR* may play a key role in Ysa T3SS silencing in *Y. enterocolitica* 1B/O8 circulating in Poland. Noteworthy, disruption of the *ysrR* gene has been already reported to inactivate Ysps secretion by Ysa T3SS. Similarly to strain DM0110, a *ysrR* mutant of *Y. enterocolitica* 1B/O8 strain JB580v, constructed by Venecia and Young (2005), was also found unable to secrete Ysps.

Our results showed that expression of *ysrR* in the clinical strain of *Y. enterocolitica* 1B/O8 is terminated at one third of the functional protein. Important REC and LuxR-C-like domains of the functional YsrR are, therefore, lacking or partially expressed in *Y. enterocolitica* 1B/O8 circulating in Poland. Interestingly, PCR-RFLP test showed that all investigated clinical isolates from Poland bear the altered *ysrR* gene. This finding corresponds with the strong clonality of clinical isolates of *Y. enterocolitica* 1B/O8 in Poland revealed by PFGE. Therefore, we conclude that the premature stop-codon in the *ysrR* gene may serve as a hallmark of the epidemic sensu stricto strain of *Y. enterocolitica* 1B/O8 in Poland.

To the best of our knowledge, dysfunctional YSA-PI has not been yet reported in *Y. enterocolitica* 1B/O8 from patients with clinically confirmed yersiniosis. Our findings together may, however, suggest that Ysa T3SS is not indispensable for *Y. enterocolitica* 1B/O8 to cause infection in human.

Preliminary results of this study were, in part, presented during the 11th International Symposium on Yersinia, Suzhou, China, 24–28 June 2013 (Rastawicki W., Szych J., Rokosz N., Zacharczuk K., Wołkowicz T. and Gierczyński R. The emergence of high-pathogenicity Yersinia enterocolitica bioserotype 1B/O8 infections in Poland. Final Program *Yersinia* 11th, pp. 74).

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SHORT COMMUNICATION

Immunomodulatory Effect of β-Glucan on Peritoneal Macrophages of Babl/c Mice

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Abstract

We assessed the effect of β-Glucan on macrophages by Griess reagent and viability by MTT assay and cytotoxicity. Assay of macrophages culture supernatants were carried out on WEHI-164 fibrosarcoma cell line as tumor necrosis factor-α bioassay were done. NO release was increased at the dose of 10 μg/ml (P = 0.001) of β-Glucan while the viability of macrophages in all concentrations was the same. In TNF-α bioassay, the supernatant of macrophages stimulated with β-Glucan had a significant cytotoxic effect on WEHI-164 cells (P = 0.023). β-Glucan had a positive effect on increasing tumoricidal activity of macrophages which may help in anti-cancer immune responses.

K e y w o r d s: β-Glucan, macrophage, nitric oxide, TNF-α

One of the most promising alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses (Nemunaitis, 1997). Several types of immunomodulators have been identified, including mammalian mediators such as interferon-gamma (IFN-γ), granulocyte colony-stimulating factor (Hamilten and Anderson, 2004) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Plaulsen, 2001), as well as the substances isolated and purified from microorganisms (Nemunaitis, 1997). Recently, polysaccharides isolated from botanical sources (mushrooms, algae, lichens and higher plants) (Wasser, 2002) have also attracted a great deal of attention in the biomedical arena because of their broad spectrum of therapeutic properties and their relatively low toxicity (Chihara, 1992). Several major substances with immunomodulatory and/or antitumor activity such as β-D-glucans, polysaccharopeptides (PSP), polysaccharide proteins and proteins have been isolated from mushrooms (Cristina *et al.*, 2005). β-Glucan is a major structural component of fungi and it has reported that fungi have a β-Glucan rich cell wall compromised of glucose residues arranged in β (1–3) D glucopyranosyl polymers with β (1–6) D glucopyranosyl side chains of varying length and frequency distribution that can activate macrophage for release inflammatory agent (Lebron *et al.*, 2003). These extracts exert their biological effect through different mechanisms. One of the active compounds responsible for the immunomodulatory effects of natural products is in the form of complex polysaccharides known as β-Glucans (Chang *et al.*, 2009). Indeed, some the basic mechanisms of the immunostimulatory, anti-tumor, bactericidal and other therapeutic effects of botanical polysaccharides is thought to occur via macrophage stimulation (Wang *et al.*, 1997) and modulation of the complement system (Beutler, 2004). Macrophages represent the first line of host defense. In addition, macrophages can function as antigen-presenting cells and interact with T lymphocytes to modulate the adaptive immune responses (Lingen, 2001). Furthermore, macrophages are involved in tissue remodeling during embryogenesis, wound repair (Klimp *et al.*, 2002), clearance of apoptotic cells and hematopoiesis (Gruchalla and Jones, 2005). Activated macrophages play a critical role in infections by eliminating microbial pathogens through the generation of nitric oxide (NO) (Fang and Vazquez-Torres, 2002). NO as a critical effector molecule of macrophages can be released upon stimulation of macrophages with a variety of stimuli such as bacterial

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products or cytokines. TNF-α also is a main cytokine of macrophages with defined activities (Lorsbach and Russel, 1992). In the present study, we evaluated the immunomodulatory effects of β-Glucan on peritoneal macrophages as NO production and cytotoxic effect of β-Glucan on cancer cell line with emphasis on coordinate macrophage effect on tumor cells and its function as TNF-α bioassay. With this background, β-Glucan was purchased from Sigma as a powder that was purified from *Saccharomycess servisiae*. In this study, we prepared different concentrations of β-Glucan by dissolving the powder in distilled water and these were used in our study. Female inbred BALB/c mice (at 8 to 10 weeks of age) were obtained from Pasteur Institute (Tehran, Iran). All mice were maintained in a specific pathogen-free animal facility at Tarbiat Modares University sterilized water and autoclaved standard mouse pellet throughout the study. The animal study was approved by a local ethics committee. Peritoneal exudates macrophages were harvested by peritoneal lavage from 5 mice by i.p. injection of 10 ml sterile ice cold RPMI-1640 (Sigma Chemical Co). The pooled cells were centrifuged at 200 × g, washed with PBS (pH = 7.2) and resuspended in complete RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 u/Penicillin and 100 µg/ml streptomycin and 3×10^5 cells/well were added onto 96-well flat-bottomed plates (Nunc) followed by incubation at 37°C for 4 h under humidified 5% CO_2 atmosphere. The non-adhering cells were then removed by washing the wells three times with PBS. The adherent cells were incubated for desired time cultured in complete RPMI medium and different concentration of β-Glucan (1, 10, 100, 200 µg/ml) was added to macrophage culture as triplicate wells with the final volume of 200 µl/well. Unstimulated macrophages and also macrophages stimulated with 50 IU/ml IFN-γ were considered as negative and positive controls, respectively (Ribeiro-Dias *et al.*, 1998). The cultured cells were incubated at 37°C for 48 h under humidified 5% CO_2 atmosphere. Supernatant fluids from macrophage cultures were collected at the end of incubation time and stored at –20°C for further assays. For MTT assay Macrophage viability was evaluated by the MTT (3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) (Sigma) assay. After 48 h of macrophage culture, 20 µl of MTT (5 mg/ml in PBS) was added to wells and the plates were incubated for 4 h. Formazan crystals developed from MTT reduction by living cells. The supernatants were then gently removed, 100 µl of isopropanol in 0.04% HCl (Sigma, USA) was added in order to dissolve the formazan crystals. The plates were incubated overnight and the absorbance of each well was measured by ELISA reader (Multiskan MS, England) at wavelength of 540 nm (Ribeiro-Dias *et al.*, 1998). Stimulation Index (SI) was determined as: Absorbance at 540 nm of Test /absorbance at 540 nm of Control. In case of Measurement of Nitrite Concentration; NO released into the supernatants of cultured macrophages. NO is unstable and rapidly converts to nitrite and nitrate. Accordingly, we estimated the level of NO synthesis by macrophages via measuring the amount of nitrite accumulating in the cultures, using the method of Stuehr and Nathan (1989). Briefly, nitrite concentration was determined with the standard Griess reaction, by adding 50 µl of test solution (supernatants of macrophage culture) to 96-well flat-bottomed plates containing 50 µl of Griess reagent [1% sulfanilamide/0.1% N-(1-naphthyl) ethylenediamine dihydrochloride/2.5% H_3PO_4] (Merck). The samples were assessed in triplicate. After 15 min at room temperature, the absorbance of each well was measured using a ELISA reader (Multiskan MS, England) microplate reader at 540 nm and the nitrite concentration was determined from a standard curve of sodium nitrite (Stuehr and Nathan, 1989). Mean of nitrite concentration (µM) was expressed. For TNF-α bioassay; Effect of β-Glucan on tumoricidal activity of macrophages in cell-free specimens of each macrophage culture was determined by means of the viability of WEHI 164 mouse fibrosarcoma cell line through MTT assay (Arora *et al.*, 2005). Briefly, macrophages treated with β-Glucan (1, 10, 100 and 200 µg/ml) for 48 h and the supernatant was stored. Then WEHI 164 target cells (2×10^4 cells in 50 µl) cultured in 96-well microtiter plate together with 50 µl of stored culture fluid of treated macrophages treated with β-Glucan (1, 10, 100 and 200 µg/ml). WEHI cultures were incubated for 20 h at 37°C and 5% CO_2 in a humidified incubator. After this time, 10 µl of MTT (5 mg/ml in PBS) was added to each well and incubated for a further 4 h. Then, supernatants were removed from each well and replaced with 100 µl of 0.04 N HCl in isopropanol. The plates were then stored overnight in the dark at room temperature. After dissolving the dark blue formazan, the optical density of each well was measured with an ELISA reader (Multiskan MS, England), using the wavelength of 540 nm. The percentage of target cells death was calculated as Suppression Index (SuI) as: Absorbance at 540 nm of Test /absorbance at 540 nm of Control (Espevik and Nissen-Meyer, 1987). After test were done for Statistical analysis; the cells were harvested from 5 mice and assays were done as triplicate manner. Data was expressed as mean ± SD. The results of MTT test were analyzed, using One-Way Analysis of Variance (ANOVA) followed by Tukey tests, and a value of $P < 0.05$ were used as the significance levels. After preparation of different concentrations of β-Glucan with dissolving in distilled water doses of 1, 10, 100 and 200 µg/ml of β-Glucan were added to each well of microplate containing macrophage cultures in complete

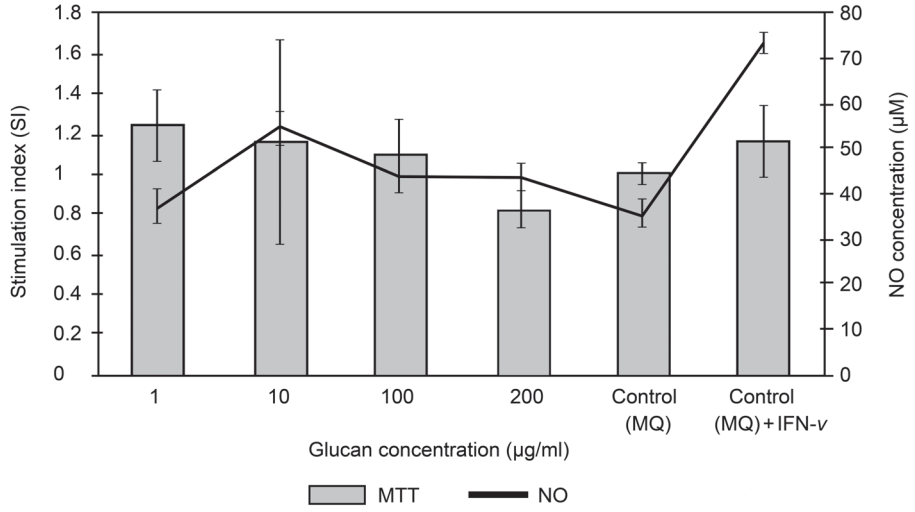

Fig. 1. Effect of β-Glucan on NO release and macrophages viability

Isolated peritoneal macrophages from BALB/c mice were incubated in the absence or presence of β-Glucan for 48 h. The cell supernatants were collected and nitrite production was evaluated. NO production by macrophages was increased at the dose of 10 μg/ml ($p = 0.0017$) compared to controls. MTT reduction ability of macrophages, as a criteria of cell viability for all concentration of β-Glucan was the same as control ($p > 0.05$). MQ: Macrophages, IFN: Interferon-gamma. Data represent mean ± S.D

medium. NO amount was evaluated in the collected supernatants of culture. As shown in Fig. 1, NO production was significantly increased ($P = 0.0017$) at the dose of 10 μg/ml compared to the negative control (only macrophages). Results of MTT assay indicated that viability of macrophages in all concentration of Glucan was the same as untreated macrophages ($P > 0.05$). For evaluate the cytotoxic effect of the supernatant of macrophages stimulated by β-Glucan on the tumor cell line WEHI-164 murine fibrosarcoma as TNF-α bioassay, colorimetric MTT cytotoxicity assay was done. The results (Fig. 2) indicated that the supernatant of treated macrophage contained cytotoxic activity in all examined doses ($P < 0.05$). These effects were declared by reduced Suppression Index in β-Glucan-treated group compared to negative control. Cytotoxic effect in IFN-γ positive control group also was significant ($P < 0.05$). For description of the findings; Glucans are a heterogeneous group of glucose polymers with naturally polysaccharides component consisting of a backbone of β(1,3)-linked β-D-glucopyranosyl units with β(1,6)-linked side chains of varying distribution and length that are produced by a variety of plants, such as oat, barley, and fungi (Lebron *et al.*, 2003). β-Glucans are the constituents of the cell wall of certain pathogenic bacteria and fungi. The main components of the

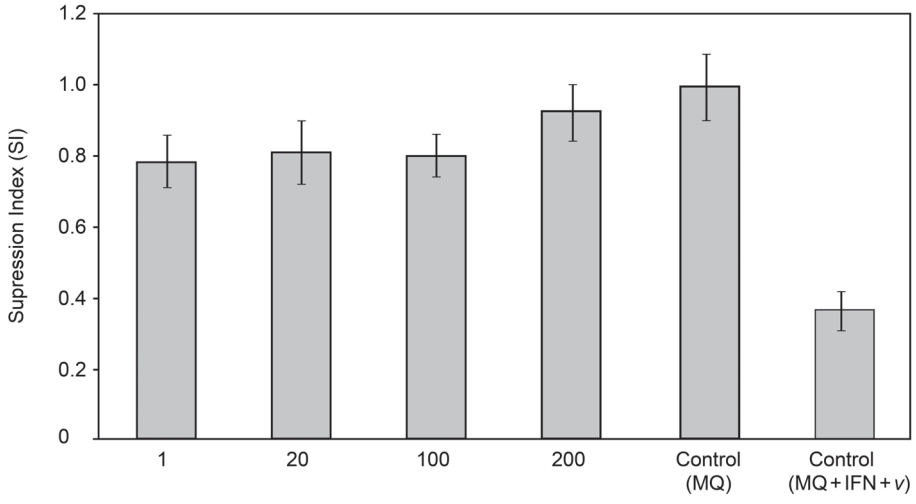

Fig. 2. Mean ±SD of Suppression Index (SuI) for cytotoxic effect of macrophage supernatant on WEHI-164 fibrosarcoma cells

WEHI-164 cells death was calculated as Suppression Index (SuI) as: Absorbance at 540 nm of Test /absorbance at 540 nm of Control. Increase of Supernatant TNF-α results in decreased amount of SuI. All concentrations of 1, 10, 100 and 200 μg/ml of β-Glucan significantly ($P = 0.023$) increased the ability of macrophage supernatants to kill the cancer cell line

fungal cell wall are polysaccharides and glycoproteins. β-Glucan has been purified from brewer's and backer's yeast (Tokunakak *et al*., 2000), from oats and barley bran (Baursk and Geisler., 1996). The healing and immunostimulatory properties of β-Glucan have been suggested for years. β-Glucan may be a powerful immune stimulant and be a beneficial antagonist to both benign and malignant tumors (Chang *et al*., 2009). Glucans are thought to mediate their effects via interaction with membrane receptors on macrophages, neutrophils and NK cells. Macrophages play a critical role in all phases of host defense that are both innate and adaptive immune responses in case of an infection. macrophage can produce cytokine and inflammatory mediators such as nitric oxide, NO, and hydrogen peroxide, H_2O_2. Thus activation of macrophage functions by β-Glucans increases host immune responses. Some previous studies evaluated the effects of β-Glucan on macrophages in vitro and in the present study, we evaluated in vitro effects of β-Glucan on macrophage functions as NO release as a marker of inflammatory responses and anti-cancer potential of macrophage cells as a major component of the anti tumor immune responses. Our results showed that β-Glucan in all examined concentrations had no cytotoxic effect on macrophages and imply probable use of β-Glucan without toxic effects on the cells. β-Glucan at dose of 10 μg/ml significantly increased the NO production by peritoneal macrophages. A number of studies have indicated that polysaccharides stimulate NO but not in reactive oxygen intermediate production in a dose-dependent manner from peritoneum macrophages (Ohno *et al*., 1996). Therefore, β-Glucan has a direct anti-cancer effect (Chang *et al*., 2009) and can inhibit tumor development as well as reducing its proliferation. (Akramiene *et al*., 2007). In order to evaluate the anti-tumor properties of Glucan, macrophage culture supernatants were affected on WEHI-164 fibrosarcoma cells. WEHI-164 cells are susceptible to the release of TNF-α from macrophages (Arora *et al*., 2005). The results indicated that in all examined concentrations, tumoricidal TNF-α bioactivity which was compatible with IFN-γ positive control was enhanced. Increased cytotoxicity of macrophage supernatant culture on WEHI fibrosarcoma cell line suggests that β-Glucan could elevate the tumoricidal activity of macrophages as one of its indirect anti tumor activities. Moreover, fungal cell wall component has been found to be a major element in the activation of macrophages and the production of inflammatory mediators from these cells. (Chan *et al*., 2009; Fernandesda *et al*., 2010). These results indicate that β-Glucan could enhance proinflammatory agents production from macrophages and also increase the tumoricidal activity of macrophages on tumor cell line. These results are coordinate with other investigations which have disclosed inhibitory effect of β-Glucan on tumor growth. In addition, the findings proved that β-Glucan increased the number of peritoneal macrophages receptors in molecular host-mediated mechanisms (Ukaway *et al*., 2000). Natural components regulate macrophages functions. In particular, these compounds have been shown to increase macrophage cytotoxic activity against tumor cells and microorganisms, activate phagocytic activity, increase reactive oxygen species (ROS) and nitric oxide (NO) production and to enhance the secretion of cytokines and chemokines, such as tumor necrosis factor (TNF-α), interleukin (IL)-1, IL-6, IL-8, IL-12, IFN-γ (Brummer *et al*., 2007). The resulting antitumor effect of β-Glucan could be due to each of these mechanisms or a combination of several mechanisms. The discovery and identification of new and safe drugs without adverse side effects, is the ultimate goal of research in the biomedical science. Medicinal applications have been demonstrated for many traditionally used mushrooms, with large differences in immunomodulatory properties. The species studied so far represent a vast source of immunomodulating and anti tumor extracts and metabolite (Cristina *et al*., 2005) . Finally, the results of the present study indicate that β-Glucan could enhance NO production from macrophages and increase the tumoricidal activity of macrophages on WEHI-164 fibrosarcoma cells. The results point to β-Glucan as a natural immunostimulatory agent with tumoricidal activity as well as its application in the treatment of cancer. Further studies are needed to clarify the advantages of this component in clinical applications.

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SHORT COMMUNICATION

Bacterial Diversity and Abundance in Shell Biofilms from the Freshwater Snail *Pleurocera canaliculatum* (Cerithioidea: *Pleuroceridae*)

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Abstract

Mollusk shells provide a hard substrate for aquatic biofilm colonization. While most work has focused on bivalve shells and grazing, little work has focused on gastropod shells and the microbes growing on them. We sampled biofilms from 14 *Pleuroceracanaliculatum* and analyzed them using a metagenomic approach. Microbial diversity varied between individuals, and rarefaction suggested that 63 snails would need to be sampled to capture all of the estimated genus-level diversity. *Cyanobacteria* and species of *Novosphingobium* and *Methylosoma* were the most abundant taxa across all shells.

Key words: bacterial diversity on snail shell, biofilms on gastropod shells, metagenomics approach

Mollusk shells can make up a significant portion of the available hard substrate in aquatic systems (Gutiérrez *et al*., 2003). Shells can harbor a diversity of prokaryotic and eukaryotic organisms enclosed in an extra-cellular matrix growing in multicellular biofilms (Lopez *et al*., 2010). Biofilms often promote both species abundance and richness locally, and impose benefits and costs to shells carrying epibionts. In terms of biofilms, research on snail interactions with freshwater biofilms generally focuses on grazing (*e.g.* Sheldon and Walker, 1997; Lopez-Doval *et al*., 2010; Hladyz *et al*., 2011; Lundqvist *et al*., 2012), including snails grazing on other snails' shells (Abbott and Bergey, 2007). Work on biofilms growing on mollusk shells has focused on bivalves (Gillanand De Ridder, 1997; Gillan *et al*., 1998; Ivanov *et al*., 2006; Bischoff and Wetmore, 2009). For snails, Abbott and Bergey (2007) reported that freshwater snail shells are generally free of algal coverings and may harbor diatoms, but no mention of prokaryotic biofilm diversity on snail shells was given in this or any other reference.

Given the importance of both snails (Johnson, 2009) and biofilms (Besemer *et al*., 2012) in the ecology of freshwater systems, we aimed to examine the diversity of bacteria in biofilms growing on snail shells. We sampled snails and biofilms from Bayou Bartholomew, a slow-moving eutrophic waterway in Arkansas and Louisiana. The bayou has a long history of habitat degradation, from bank erosion due to agriculture, to heavy metal and other contaminant inputs from industry (Layher, 2005). We focused on the siltyhorn snail, *Pleurocera canaliculatum* (Pleuroceridae), an oviparous algal grazer that can be found on soft and hard substrates (Dillon, 2000) and that is common in Bayou Bartholomew (Minton *et al*., 2008).

Fourteen *P. canaliculatum* (Fig. 1) were collected by hand from Bayou Bartholomew in Bastrop, Louisiana (32.8017°N, 91.9495°W) in September 2013. Shells were covered in dark oxides along with visible biofilms, and snails were crawling on either the bayou bottom or submerged hard surfaces (*e.g.* bridge pilings, woody debris). Nitrile gloves were worn to avoid human contamination. Biofilms were scraped from individual shells using sterile razor blades and placed directly into bead-beating tubes. For comparison, we collected two sediment samples from the site. DNA was extracted from biofilms using the Power Soil DNA isolation kit (Mo Bio Laboratories), according to manufacturer's

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Fig. 1. Photograph of *Pleurocera canaliculatum* shell

directions. For metagenomic analysis, DNA samples were sent to MrDNALab (Shallowater, Texas). The *16S rDNA* gene variable region V4 was amplified from the bacterial DNA samples using the 515/806 primer pair (Caporaso *et al*., 2011). A single-step, 30 cycle PCR using HotStarTaq Master Mix kit (Qiagen, USA) was run (94°C for three minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for one minute, followed by a final elongation step at 72°C for five minutes). Sequencing was performed on an Ion Torrent PGM following the manufacturer's guidelines. Sequence data were processed using MrDNALab's proprietary analysis pipeline for depletion of barcodes and primers, followed by removal of sequences less than 150 bp, sequences with ambiguous base calls, and homopolymer runs exceeding 6 bp. Sequences were de-noised, chimeras were removed, and operational taxonomic units (OTUs) defined by clustering at 97% similarity. Final OTUs were taxonomically classified using nBLAST against a curated GreenGenes database (DeSantis *et al*., 2006).

Using the species-level counts, we performed sample-based rarefaction with extrapolation in EstimateS 9 (Colwell, 2013) to determine if our sample size was large enough to capture the bacterial diversity on the shells. We used EstimateS to calculate Chao's abundance-based Jaccard indices between snails and sediment samples to determine how similar the bacterial communities were between shells and the environment. Chao-Jaccard indices are based on the probability that two randomly chosen individuals, one from each of two samples both belong to species shared by both samples. This approach reduces the negative bias that undermines the usefulness of traditional similarity indices, especially when incompletely sampling rich communities (Chao *et al*., 2005). Finally, we used principal coordinates analysis of genus-level counts in STAMP (Parks *et al*., 2014) to visualize differences in diversity between snails and the sediment.

Metagenomic analysis yielded an average of over 68,500 sequences per snail shell that could be matched to 1,258 bacterial genera in 64 phyla. Proteobacteria and Cyanobacteria were the two most abundant phyla on *P. canaliculatum* shells. The most abundant genus on shells was *Novosphingobium*, followed by *Leptolyngbya* and *Methylosoma*. Sample-based rarefaction indicated that 14 shells sufficiently captured phylum-level bacterial diversity but were insufficient to capture the genus-level diversity on them. Rarefaction extrapolation suggested that sampling 63 shells would be needed to accurately describe bacterial communities at the genus level, estimated to be 2,014 genera. Chao-Jaccard indices for genus-level pairwise comparisons of shells to shells and sediment to sediment ranged from 0.873 to 0.987, where a value of 1.0 indicated identical samples. Chao-Jaccard indices comparing shells to sediment were much lower, ranging from 0.656 to 0.707. Principal coordinates analysis suggested that the shell and sediment bacterial communities possessed a high degree of overlap (Fig. 2).

Our data suggest that individual snails have diverse bacterial communities growing on their shells, and that these communities differ between snails. The most abundant bacteria found on *P. canaliculatum* shells reflected the biology of the bayou and the life history of the snail. Species in the genus *Novosphingobium* are gram-negative bacteria that break down aromatic compounds including phenol, nitrobenzene, and carbofuran. They are frequently isolated from aquatic environments exposed to high anthropogenic activities (Gan *et al*., 2013). The bayou watershed regularly receives agricultural, residential, and industrial inputs that likely include the aromatics used by *Novosphingobium* (Kresseand Fazio, 2002). *Leptolyngbya* species are cyanobacteria commonly found in soils and in periphyton and metaphyton of freshwater and marine systems. *Leptolyngbya* also live epiphytically in the extra-cellular matrix produced by other organisms, explaining their presence in the biofilm (Komárek and Hauer, 2013). Cyanobacteria are also common in eutrophic systems like the bayou, and the shells of *P. canaliculatum* remain exposed in the water column. Other cyanobacteria occurring in high abundance were *Chamaesiphon*, *Phormidium*, and *Pleurocapsa*. Finally, species of *Methylosoma* are aerobic methanotrophs, active in sediments where methane and oxygen meet (Rahalkar*et al*., 2007). *Pleurocera* spend time crawling on the benthos, likely bringing them in proximity to *Methylosoma*.

McClean (1983) showed the importance of snail shells in shaping benthic community structure. Our

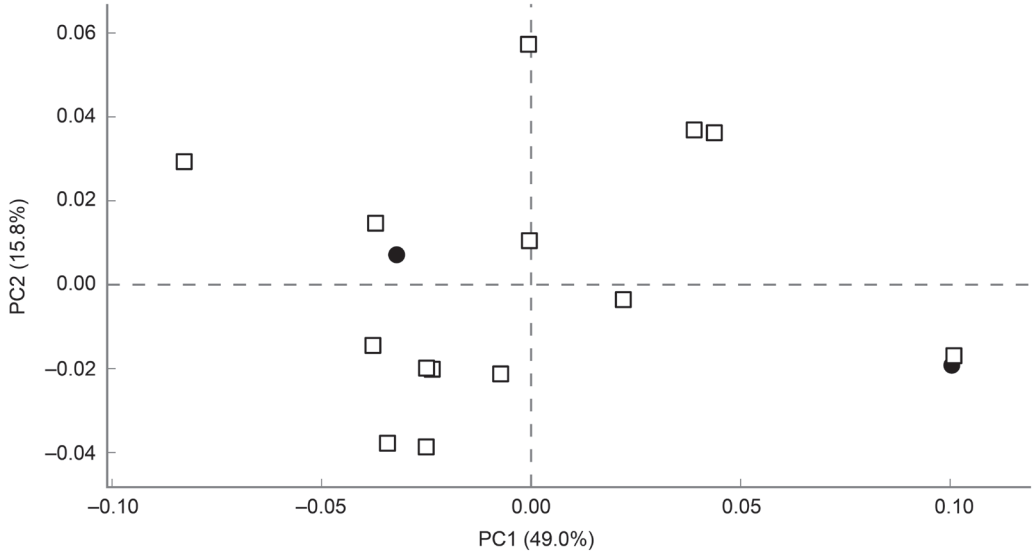

Fig. 2. Principal coordinates analysis of genus-level diversity counts showing variation among bacterial communities on snail shells (open squares) and in the sediment (filled circles). Data for the first two coordinates and the percent variation explained by each are shown

findings represent the first study of snail shell microbes and we suggest that snail shells play a role influencing microbial diversity and abundance in freshwater systems. Aquatic biofilms show seasonal variation (Olapade and Leff, 2005) and succession (Tien and Chen, 2013). We hope our data can serve as a baseline data for other shell biofilm studies and represent a starting point for future research on how shell biofilms change temporally.

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SHORT COMMUNICATION

Isolation and Antimicrobial Testing of *Aeromonas* spp., *Citrobacter* spp., *Cronobacter* spp., *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp., and *Trabulsiella* spp. from the Gallbladder of Pigs

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Abstract

The presence of Gram-negative bacteria species, other than *Salmonella* spp., in the gallbladder of pigs was examined. Isolated Gram-negative bacteria were assigned to species using the Microgen™ GnA+B-ID Systems. Of the 64 isolated strains 43 were identified as *Escherichia coli*, seven as *Enterobacter* spp., three each as *Klebsiella* spp., *Citrobacter freundii*, *Aeromonas hydrophila* and *Cronobacter sakazakii* and one each as *Escherichia fergusonii* and *Trabulsiella guamensis*. Their antibiograms showed very high resistance to ampicillin, amoxicillin, tetracycline, chloramphenicol and sulfamethoxazole/trimethoprim. It was concluded that the pigs' gallbladder is a reservoir of potentially pathogenic Gram-negative bacteria for pork consumers.

Key words: aztreonam, cholecystitis, doripenem, microflora of gallbladder, potentially pathogenic Gram-negative bacteria

Enteric bacteria have acquired the genetic ability to resist the defence mechanisms of the digestive system, some of which are gastric secretions, hydrochloric acid and bile, variations in pH, low oxygen levels, nutrient limitations and elevated osmolarity (Chowdhury *et al*., 1996). By this ability, they are either pathogenic or potentially pathogenic for their host. If such bacteria colonize the gallbladder of pigs, they could become pork contaminants and a risk to consumers (Gunn, 2000). Bile is for some bacteria the regulating factor of their survival in the intestinal tract, thus a regulator of gut colonization. They survive the killing effect of bile, but also antibiotics and the host's immune response by forming protective biofilms (Begley *et al*., 2005; Jensen *et al*., 2010). Hence, the microflora of the gallbladder is evidence of an animal's intermittent gut colonization by potential pathogens.

Therefore, the objective was to asses Gram-negative bacteria of the pig's gallbladder, as risks to pork consumers.

Swab samples collected at slaughter from 145 randomly selected pigs' gallbladders, originating from 15 finishing farms located in Central Greece were bacteriologically examined.

Samples were enriched in Buffered Peptone Water (BPW Oxoid, England) for 18 ± 2 h at 37°C and subcultured on Columbia Blood Agar (CBA) and MacConkey agar (Oxoid, England). All morphologically different colonies were subcultured on CBA and after 24 h at 37°C, they were examined by Gram's stain. Gram-negative rods were tested for oxidase production (Bactident Oxidase Merck, Germany) and further examined as recommended by Quinn *et al*. (1994). In total, 79 Gram-negative isolates were selected for speciation using the Microgen™ GnA+B-ID (Microgen Bioproducts Ltd, UK) System.

Sixty four non *Salmonella* species were tested against 24 antimicrobial agents using the disk diffusion method, as described elsewhere (Evangelopoulou *et al*., 2014a). The selection of the antimicrobials was based on their use for treating animal and human infections. They were amoxicillin (30 μg), amoxicillin/clavulanic acid (20/10 μg), ampicillin (10 μg), ampicillin/sulbactam (10/10 μg), aztreonam (30 μg), cefotaxime (30 μg),

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cefoxitin (30 μg), ceftazidime (30 μg), ceftiofur (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), chloramphenicol (30 μg), colistin (50 μg), doripenem (10 μg), enrofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), penicillin G (10 μg), rifampin (30 μg), sulfamethoxazole/trimethoprim (23.75/1.25 μg), tetracycline (30 μg) and tigecycline (15 μg). Isolates exhibiting resistance to at least three antimicrobial agents belonging to different antimicrobial classes were considered multidrug resistant (MDR) (Schwarz *et al*., 2010).

Of the 93 (64.1%) bacteria positive gallbladders, 79 Gram-negative bacteria were selected for speciation. Of them 15 were identified as *Salmonella* spp. and 64 were *Escherichia coli* (43), *Enterobacter* spp. (7), three each *Klebsiella* spp., *Citrobacter freundii*, *Aeromonas hydrophila* and *Cronobacter sakazakii* and one each *E. fergusonii* and *T. guamensis* (Table I). The three *A. hydrophila* isolates exhibited beta-haemolysis after 48 hours of incubation.

High antimicrobial resistance was observed for ampicillin, amoxicillin, tetracycline, chloramphenicol and sulfamethoxazole/trimethoprim (Table I). Somewhat lower was resistance to cephalosporins, quinolones and aminoglycosides, as groups. All isolates, except one (*E. fergusonii*), were resistant to penicillin G and all were susceptible to aztreonam and doripenem used for human treatments. Sixty isolates were considered MDR (Table I).

Of the 79 Gram-negative 15 were *Salmonella* spp. published elsewhere (Evangelopoulou *et al*., 2014b). Of the remaining, *E. coli* forming the normal intestinal microbiota was the predominant aerobic microorganism identified by the Microgen System. *E. coli* is helpful

Table I
Cumulative results of antimicrobial resistance of Gram-negative isolates recovered from pigs' gallbladder

	Escherichia coli	*Enterobacter aerogenes*	*Enterobacter gergoviae*	*Citrobacter freundii*	*Klebsiella pneumoniae*	*Klebsiella oxytoca*	*Cronobacter sakazakii*	*E. fergusonii*	*Trabulsiella guamensis*	*Aeromonas hydrophila*	Total No of resistant strains
AML	37	3	3	2	1	1	1	0	1	3	52
AMC	12(I)	3	0	1, 1(I)	0	1	0	0	1	3	9
AMP	37	3	3	2	1	1	2	0	1	3	53
SAM	4, 8(I)	1(I)	0	1	0	1	1	0	0	1	8
AZT	0	0	0	0	0	0	0	0	0	0	0
CTX	2(I)	0	0	0	0	0	0	0	0	3	3
FOX	1(I)	0	1(I)	2(I)	0	1	1(I)	0	1	3	5
CAZ	1(I)	0	0	1	0	0	0	0	0	0	1
EFT	1(I)	0	0	1(I)	0	0	0	0	0	3	3
CRO	1	0	0	1	0	0	0	0	0	3	5
CXM	1, 13(I)	3(I)	1(I)	3(I)	0	1(I)	2(I)	0	0	3	4
CT	0	0	0	1	0	1	0	0	0	0	2
C	31	2	0	2	0	2	0	0	0	3	40
DOR	0	0	0	0	0	0	0	0	0	0	0
ENR	2, 2(I)	0	0	1(I)	0	0	0	0	0	1(I)	2
E	39	3	4	3	1	2	3	1	1	3	60
CN	2, 1(I)	0	0	1	0	0	0	0	0	0	3
K	4, 7(I)	0	1(I)	1	0	1	0	0	0	2	8
NA	5, 4(I)	0	0	1	0	1	0	0	0	3	10
P	43	3	4	3	1	2	3	0	1	3	63
RD	21,19(I)	2, 1(I)	4	3	1	2	2, 1(I)	0	1	3	39
SXT	39	2	0	2	0	2	1	0	1	3	50
TE	39	2	0	2	1	2	2	0	1	3	52
TGC	9(I)	2(I)	1(I)	0	1(I)	1(I)	0	0	0	3	3
Total N.	**43**	**3**	**4**	**3**	**1**	**2**	**3**	**1**	**1**	**3**	**64**

AML Amoxicillin, *AMC* Amoxicillin-clavulanic acid, *AMP* Ampicillin, SAM Ampicillin-sulbactam, *AZT* Aztreonam, *CTX* Cefotaxime, *FOX* Cefoxitin, *CAZ* Ceftazidime, *EFT* Ceftiofur, *CRO* Ceftriaxone, CXM Cefuroxime, *C* Chloramphenicol, *CT* Colistin, *ENR* Enrofloxacin, *E* Erythromycin, *CN* Gentamycin, *K* Kanamycin, *NA* Nalidixic acid, *P* Penicillin G, *RD* Rifampin, *SXT* Sulfamethoxazole/Trimethoprim, *TE* Tetracycline, *TGC* Tigecycline-(I)= Intermediate Resistance

in the development of a normal mucosal immune system, the suppression of harmful bacteria by overtaking their attachment sites and the production of essential nutrients (Canny and McCormick, 2008). However, pathogenic strains could invade the gallbladder and lead to its inflammation (Gunn, 2000). These strains and *Klebsiella pneumonia*e, *Enterobacter* spp. and *Enterococcus* spp. are associated with infectious cholecystitis (Wang *et al*., 2003; Abeysuriya *et al*., 2008; Carpender and Gilpin, 2014). Thus, enteric bacteria have developed sophisticated mechanisms not only for resisting the effects of bile salts, but also using favorably this unique environment for their survival, thus becoming under certain circumstances potential pathogens.

Among them could also be *T. guamensis* and *C. freundii*. The two could be confused, due to their phenotypic and antigenic behaviour, with *Salmonella* spp. or *E. coli* (McWhorter *et al*., 1991; Delgado *et al*., 2013), if a variety of methods for their differentiation are not used. Specifically, *T. guamensis* does not rapidly ferment lactose or sucrose and produces abundantly H_2S, resembling phenotypically *Salmonella enterica* subspecies *diarizonae* and subspecies *hountanae* (McWhorter *et al*., 1991). Although its clinical significance for animals is unknown, it could cause diarrhoea, as it does in man, resembling mild salmonellosis. Furthermore, carrier animals could be the source for these occasional human enteric problems.

C. freundii, a commensal microorganism of undocumented clinical importance to animals, is isolated from serious nosocomial infections in man (Nayar *et al*., 2014). Its pathogenicity is attributed to multidrug resistance (Pepperell *et al*., 2002), a property observed also in the present investigation (Table I). Evolutionary acquisition of resistance genes affects the course of an infectious disease, the evasion of the immune response and the events of host-pathogen interactions (Delgado *et al*., 2013).

The same events could be important in human infections caused by multiresistant *Enterobacter* spp., as observed here, a bacterium easily acquiring resistance genes, mainly to beta-lactams, quinolones, tetracycline and chloramphenicol, thus emerging as a public health risk (Thiolas *et al*., 2005; Boban *et al*., 2011). The two species isolated here, *Enterobacter aerogenes* and *Enterobacter gergoviae,* are associated to infections of immunocompromised individuals (Boban *et al*., 2011), as is also *Klebsiella* spp. showing phenotypic and DNA relatedness to *E. aerogenes* (Brisse *et al*., 2006). *K. pneumoniae,* causing sporadic disease in individual pigs (AHVLA, 2012), is implicated in human pneumonia, urinary tract infections, neonatal septicemia and liver abscesses (Chang *et al*., 2000; Bleich *et al*., 2008).

C. sakazakii, previously a species of the genus *Enterobacter*, is reclassified as a new genus within the family of Enterobacteriaceae (Iversen *et al*., 2007). Although its pathogenic importance is unknown in animals and adult man, it has been recently implicated in fetal fatal meningitis, neurologic damage, brain abscess, septicemia, etc. (Healy *et al*., 2010; Joseph and Forsythe, 2011). This association raises questions as to its role with immunocompromised pork consumers.

A. hydrophila, incriminated in a variety of human clinical conditions, such as gastroenteritis, septicemia, cellulitis, myonecrosis, peritonitis, hepatitis, pancreatic abscesses, respiratory, urogenital and eye infections of immunocompromised individuals, is also implicated in infections of poikilothermic animals (amphibians, reptiles and fish) (Janda and Abbott, 2010). However, it is more often isolated from food of plant and animal origin than animal disease (Queiroga *et al*., 2012). *A. hydrophila* is producing haemolysins, enterotoxin (Ljungh *et al*., 1981) and is multidrug resistant (Queiroga *et al*., 2012), as it was observed here. Its isolation from pig gallbladders and its resistance to most of the antibiotics tested here, support its classification as an emerging pathogen for animals and man. Although the above microbes are opportunistic pathogens, their high resistance to commonly used antimicrobials makes them potential pathogens for man, and perhaps, unrecognized causes of reduced animal productivity.

Additionally, the multidrug resistance observed here indicates that pigs are a primary reservoir of multi-resistant bacteria. The use of antimicrobials in food producing helps faecal excretion of highly pathogenic Gram-negative enteric bacteria, such as *Salmonella*, eventually making pork carcasses the source of pork product contamination, thus consumer infections (Friendship *et al*., 2009).

Observed high resistance above 60% to chloramphenicol, used in the treatment of human salmonellosis, but no longer used in animals in the EU (EVMP, 1994) could result from a variety of reasons. Although non-compliance of farmers is one reason, the number of farms participating (15 farms) is not supportive. Thus it could result from transfer of resistance genes between different bacteria species coding for similar classes of antimicrobials and/be residual caused by the persistence of resistance genes encoded in a microbial resistome (Sommer and Dantas, 2011). Persistent antimicrobial resistance is transferrable not only to pathogens, but also commensal bacteria or opportunistic pathogens, like the above, helping the development of a "superbug" (Thiolas *et al*., 2005; Shailesh *et al*., 2012; Frye and Jackson, 2013).

Such events in natural bacteria populations may have important implications in the evolution of bacteria and the means of evading the immune system, thus the outcome of infectious diseases (Delgado, *et al*., 2013). Hence, pathogenic microorganisms can reside

transiently or permanently in the gallbladder of pigs, making it a reservoir of multidrug resistant Gram-negative bacteria contaminating pork products and infecting consumers.

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