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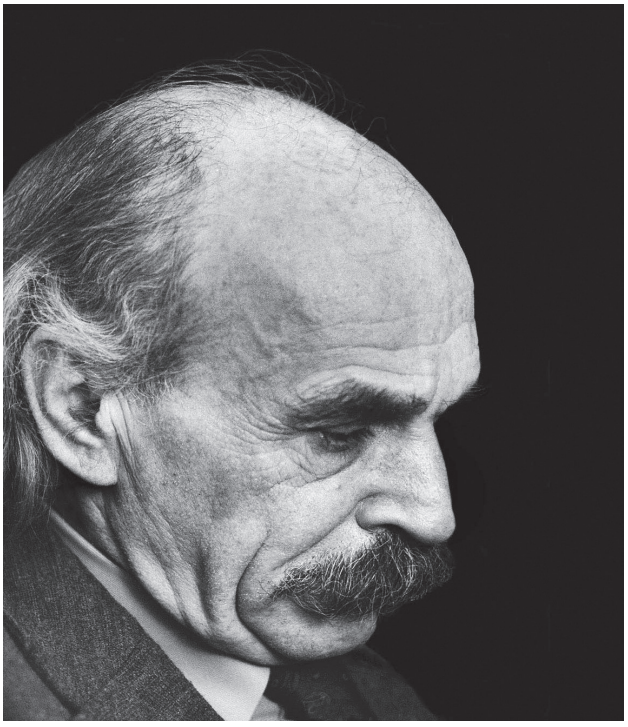
MEMORIAL TRIBUTE

To Professor Władysław J.H. Kunicki-Goldfinger (1916–1995)



This commemoration celebrates the birth one hundred years ago, on February 13, 1916, of Prof. Władysław J.H. Kunicki-Goldfinger. He was to play roles as an outstanding microbiologist, geneticist, humanist and activist. The Professor graduated from the Jagiellonian University, where, still a student, he worked in the Division of Bacteriology in the Department of Agriculture.

In 1934, Władysław J.H. Kunicki-Goldfinger enrolled in the Department of Philosophy of the Jagiellonian University, where he studied biology. He completed his master's degree dissertation in veterinary microbiology under the direction of Dr. Stanisław Śnieszko, the Director of the Independent Division of Microbiology in the Department. After completing course work, he received a master's degree in philosophy in the field of biology, the focus of his later scientific interests.

Prof. Kunicki-Goldfinger's research career in the Department of Agricultural Microbiology was interrupted by the outbreak of World War II. He was arrested in Lvov and sent to the Archangielsk Governorate, where initially he performed menial labor, then became a bacteriologist in a camp hospital. After leaving the camp, he enlisted in the Polish Army, organized by Władysław Anders in the Soviet Union, where Prof. Kunicki-Goldfinger served in a medical unit which took him throughout the entire theatre of warfare. He returned to Poland in 1947. Prof. Józef Parnas employed him in the Veterinary Department at the Maria Curie-Skłodowska University in Lublin, where he received his Ph.D. (1948) and, 2 years later, his postdoctoral degree.

In 1950, at the Maria Curie-Skłodowska University, he organized the Department of General Microbiology and became an associate professor. In 1955, he left the leadership of that Department to Prof. Zbigniew Lorkiewicz and moved with several of his coworkers to Wrocław, where he succeeded Prof. Helena Krzemieniewska in teaching microbiology in the Department of Plant Physiology. In 1957, he founded the Department of General Microbiology at the Institute of Botany of Wrocław University, leading that Department in 1957–1961. He also organized and led the Laboratory of Bacterial Genetics at the L. Hirszfeld Institute of Immunology and Experimental Therapies; this was the first Laboratory of this kind in Poland.

In 1960, Prof. Kunicki-Goldfinger, with several of his students and colleagues, left Wrocław for Warsaw. In the following year, he founded the Department of General Microbiology there, the first at Warsaw University, the precursor of the current Institute of Microbiology. A number of groups interested in basic research in microbial genetics, virology, bacterial physiology, immunology and applied microbiology originated in the Institute under his direction. Some of them later evolved into independent laboratories and departments.

Prof. Kunicki-Goldfinger authored or co-authored approximately 200 publications in various areas of microbiology, in general biology, in history of science and in philosophy of natural science. Particularly noteworthy is the cycle of 23 publications in bacterial genetics which were published under one title, *Mechanisms of Bacterial Conjugation and Recombination.*

The Professor also authored twelve books, including an extraordinary university textbook *Life of Bacteria* which went through the remarkable number of seven

editions. This textbook, brilliantly written with excellent, witty illustrations by Szymon Kobyliński, was used by generations of students.

No less important are the books on philosophy of natural science. Among them, the best known are: *Heredity and the Future – Thoughts on Molecular Biology, Evolution and Humans, In Search of Possibilities*, and the last one, *From Nowhere to Nowhere,* in which the author rendered difficult issues accessible. His profound interest in the philosophy of natural science found expression in the seminars entitled Discussions on Evolutionary and Theoretical Biology, which he organized at the Institute of Microbiology and which promoted an exchange of ideas among participants of different points of view. The Professor was also an active member of the Philosophy of Science Division of the Polish Society of Philosophy and of the Evolutionary and Theoretical Biology Committee at the Polish Academy of Sciences. In 1965, Professor Kunicki-Goldfinger became a corresponding member of the Polish Academy of Sciences, and in 1980 a full member.

In 1952, Prof. Kunicki-Goldfinger together with Prof. Jadwiga Marszewska-Ziemięcka, founded the first Polish scientific journal devoted strictly to microbiology – *Acta Microbiologica Polonica* – and served as its editor-in-chief for many years. The journal is being published to this day under the title of the *Polish Journal of Microbiology*.

In addition to scientific research and teaching, Professor Kunicki-Goldfinger was actively involved in the community at large. He participated in the so-called Flying University, which later became the Society for Studies in Humanities and Economics. In 1979, this Society established a Research Fund, under the direction of Professor Kunicki-Goldfinger, to provide aid to continue their academic efforts for students and researchers who had suffered hardships for their social activism. The Professor was one of the organizers of the Society for the Support and Promotion of Science; he also collaborated with the Committee for the Defense of Workers. On December 13, 1981 (the date of the imposition of marshal law), for these activities and his involvement in the Solidarity underground, he became the only full member of the Polish Academy of Sciences to be detained. Later still, the Professor was a member of the Lech Wałęsa's Citizen Committee, and in 1989 he participated in the "round table" negotiations in the Science and Education Section.

Later, in recognition of his contribution to the establishment of dynamic higher education bodies in the field of microbiology across Poland, Prof. Kunicki-Goldfinger received honorary doctorates from the University of Wrocław and from the Maria Curie-Skłodowska University of Lublin.

We, his senior students at the University of Warsaw, wish to remember our Teacher and Master of so many years who devoted his exemplary life to fighting for good science and for justice. He is an icon for us to follow.

Students of the Professor
at the Institute of Microbiology
in the Department of Biology, University of Warsaw

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MINIREVIEW

The Functions of Effector Proteins in *Yersinia* Virulence

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Abstract

Yersinia species are bacterial pathogens that can cause plague and intestinal diseases after invading into human cells through the Three Secretion System (TTSS). The effect of pathogenesis is mediated by *Yersinia* outer proteins (Yop) and manifested as down-regulation of the cytokine genes expression by inhibiting nuclear factor-κ-gene binding (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. In addition, its pathogenesis can also manipulate the disorder of host innate immune system and cell death such as apoptosis, pyroptosis, and autophagy. Among the *Yersinia* effector proteins, YopB and YopD assist the injection of other virulence effectors into the host cytoplasm, while YopE, YopH, YopJ, YopO, and YopT target on disrupting host cell signaling pathways in the host cytosols. Many efforts have been applied to reveal that intracellular proteins such as Rho-GTPase, and transmembrane receptors such as Toll-like receptors (TLRs) both play critical roles in *Yersinia* pathogenesis, establishing a connection between the pathogenic process and the signaling response. This review will mainly focus on how the effector proteins of *Yersinia* modulate the intrinsic signals in host cells and disturb the innate immunity of hosts through TTSS.

Key words: *Yersinia* pathogenesis, Yops, TTSS

Introduction

Yersinia species are Gram-negative bacteria in the family of *Enterobacteriaceae*, in which three *Yersinia* species, *i.e. Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, are pathogenic against humans. It was reported that the infection of *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* could cause plague, yersiniosis, and scarlatinoid fever, respectively (Viboud and Bliska, 2005). Interestingly, although these three pathogenic *Yersinia* species have different modes of transmission, they all share common virulence factors, termed Yops, and utilize the same protein export pathway, known as the TTSS. TTSS is a general secretion system in the Gram-negative bacteria like pathogenic *Escherichia coli*, *Pseudomonas*, *Salmonella*, *Shigella*, and *Yersinia* spp., which allows bacteria to inject their effector proteins from bacterial membrane into host cells cytoplasm using injectisome, a needle-like complex. Once being injected into the host cytoplasm, Yops target different proteins in the host cytoplasm, stimulating each other or performing antagonistic action in the activation of intracellular enzyme and cell death programs. In this review, we will mainly focus on how *Yersinia* spp. inhibit the host innate immune response through TTSS, in which the recent progress in studies on Yops targeting GTPase, caspase-1, and MAPK/NF-κB pathways will be emphasized.

Pathogenic effect of the effector proteins. Extensive studies have been performed to understand the virulence mediated by the effector proteins of *Yersinia*. It is believed that Yops can efficiently suppress the expression of cytokines in host cells through inhibiting the NF-κB and MAPK pathways by different means. For example, YopJ/P binds to MAPK kinase (MKK) and IκB kinase (IKK), blocking their activation (Mukherjee and Orth, 2008). Other evidence shows that YopM activates the cytoplasmic kinase RSK1, PRK2, thereby inhibiting the caspase-1 pathway to cause the activation of proinflammatory cytokines and cell death (Hentschke *et al.*, 2010). Under other circumstances, the effector proteins can perfectly co-regulate the pathogenesis of *Yersinia*, thereby inhibiting the signaling pathways, inducing the apoptosis of target eukaryotic cells, and contributing to the interference and repression of the host immune system. In the *Yersinia* infecting process, under the assistance of YoB, YopD, and LcrV, six effector proteins, including YopE, YopH, YopJ/P, YopM, YopO/YpkA,

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and YopT, are injected into the infected cells through TTSS (Swietnicki *et al.*, 2005). These Yops interfere the host cell signaling pathways and inhibit the immune responses, facilitating *Yersinia* virulence. Among Yops, YopJ and YopP share more than 95% sequence similarity, and YopO and YpkA share 98% sequence similarity, with YopJ existing in *Y. pestis* and *Y. pseudotuberculosis*, YopO existing in *Y. pestis* and *Y. enterocolitica*, YopP existing in *Y. enterocolitica*, and YpkA existing in *Y. pseudotuberculosis*, respectively (Viboud and Bliska, 2005; Aepfelbacher and Heesemann, 2001). In spite of acting on different aspects, Yops have to function jointly to accomplish pathogenesis. For example, YopE (Fallman *et al.*, 1995) and YopT together facilitate to inhibit phagocytosis and pore formation (Viboud and Bliska, 2001), albeit by distinct mechanisms. In addition, some pathological changes are caused by several effectors, in which the absence or mutation of just one effector can lead to incomplete virulence.

Delivery machinery of the effectors protein into the host cells. The effector proteins consist of over 20 proteins, which are secreted with the same conservative manner known as TTSS. In the pathogenic infection, the injectisome enables the bacteria to directly transport or inject effectors from the bacterial cytoplasm into the host cytosol. Formation of the injectisome includes three steps. Firstly, YscRSTV, YscD, YscJ, YscQ, YscNLK and YscU are assembled to form the basal body of the needle-like complex. Then, YscF joins as a needle over the basal body, and YopN is added into the complex to help the recruitment of LcrV and YopBD. In the last step, YopN is released upon cell contact, and the translocation pore is formed (Viboud and Bliska, 2005; Chen and Anderson, 2011; Jessen *et al.*, 2014; Dewoody *et al.*, 2013).

The expression of the proteins in the *Yersinia* TTSS is sophisticatedly regulated by YopD, LcrH, and LcrQ, at both transcriptional and posttranscriptional levels (Cambronn *et al.*, 2004; Rimpilainen *et al.*, 1992; Wilharm *et al.*, 2003). The increase of the LcrQ level in the bacterial cytoplasm is reported to cause constitutive repression of the TTSS (Rimpilainen *et al.*, 1992). In addition, YopD and LcrH can form a complex of YopD-LcrH, attenuating the translation of mRNA of the TTSS genes through binding to the unstructured 5' end of mRNA to prevent ribosome binding (Chen and Anderson, 2011). More interestingly, the regulation caused by YopD, LcrH, and LcrQ is environmentally responsive to calcium, as high concentration of calcium causes the repression of protein expression (Straley and Bowmer, 1986). The transcription of TTSS genes is also affected by LcrF, as activation of LcrF facilitates the formation of a protein channel through the membranes of *Yersinia*. Afterwards, it gives rise to the formation of a pore on the surface of the plasma membrane of the infected eukaryotic cells to permit the entry of the effector proteins, leading to the inhibition of the host signaling pathways and cell death.

Functional roles of effector protein as virulence factors. When *Yersinia* spp. invades mammalian cells, Yops are injected into the infected cells through the TTSS. Under the assistance of caspase-3 and caspase-7, the effector proteins cause the infected cells to exhibit the features of apoptosis, including membrane bleb, condensation of nucleus, DNA fragmentation, and large cytoplasmic vacuoles (Zheng *et al.*, 1998), finally resulting in cell death. This phenomenon is also regarded as immune silence or immunosuppression. In addition, Yops injection also leads to proinflammatory cell death called pyroptosis with the help of caspase-1 induction. Standing on *Yersinia*'s side, it is complicated and hard to control the balance of survival as the apoptosis induced by *Yersinia* leads to immune response acceleration. The effector proteins contribute to the survival of the host cells, expanding their reproduction scope and consolidating their existence, while on the other hand; they promote the positive immune response to prevent the pathogenic invasion. In the TTSS, YopB and YopD form a pore as an entrance to across the host cell cytomembrane (Montager *et al.*, 2011), from which the other six Yops are injected into the interior of host cell, and admitted to modulate the hosts' immune response. Among these Yops, YopO, YopE and YopT target the GTPase; YopJ/P causes the suppression of cytokines by modulating the NF-κB and MAPK pathway signals; YopH mainly regulates caspase-1 to induce pyroptosis (Bahta and Burke, 2012); and YopM activates the cytoplasmic kinase RSK1 and PRK2 to inhibit the caspase-1 pathway.

The virulence of YopJ/P has been well studied to reveal that barrier dysfunction and systemic disease are mainly caused by the injection of YopJ/P (Philip and Brosdky, 2012). YopJ/P-sufficient *Y. pseudotuberculos* exhibits reduced production of cell death cytokine to cause the bacterial control of the host cell immune response, while YopJ/P-deficiency results in robust cytokines production, controlled bacterial spread, and intact barrier function (Philip and Brosdky, 2012). Additionally, decreased colonization of spleen is observed when YopJ is deficient in the *Y. pseudotuberculosis* infection (Monack *et al.*, 1998), indicating YopJ's ability to manage the dissemination from mucosal tissues (Viboud and Bliska, 2005).

Recent progress on the molecular mechanism of the *Yersinia* effector proteins

YopB and YopD are hydrophobic proteins termed translocators that play important roles in the injection of Yops through the conversed TTSS. It has been

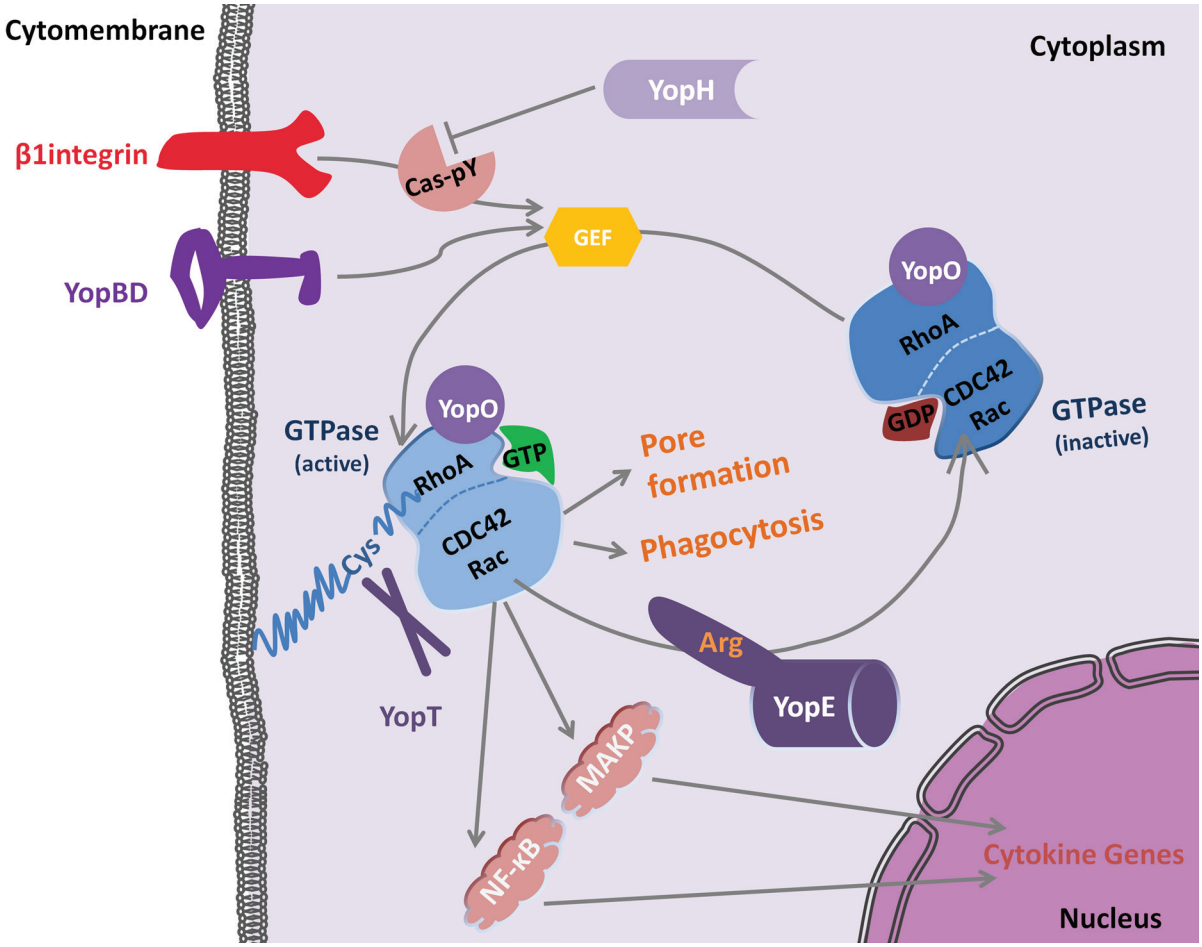

Fig. 1. GTPase-relevant Yops interact with host cell signal pathways.

YopB and YopD form a complex to let other Yops enter into the cells, stimulating the GEF. YopO binds GTPase, interferes with the Rac signals (Groves *et al.*, 2010, Prehna *et al.*, 2006), which damages the actin cytoskeleton. YopH inhibits the GEF activation signal Cas-pY emitted from β1integrin. YopT also targets the GTPase, as it cleaves the junction between cytomembrane and GTPase to repel the latter, resulting in the inactive GTPase. YopE blocks CDC42 and Rac of GTPase (Andor *et al.*, 2001), inducing the transformation of the active GTPase-GTP to inactivate GTPase- GDP to inhibit the pore formation and phagocytosis, which additionally impairs the GTPase activation signal to the MAPK and NF-κB pathways.

identified that YopB and YopD could assist the other effector proteins to enter into cytoplasm through the secretion apparatus called the needle complex (Edqvist *et al.*, 2007). During this process, YopB and YopD form the complex YopBD in the eukaryotic cell membranes with the assistance of the needle tip, constructing a pore on the target cell membrane as an entrance for other virulence effector proteins being injected in.

Interaction between GTPase and effector proteins (Fig. 1). After the virulence factors are transported into the host cell cytosols, the YopE, YopH, YopP and YopT cooperate to damage the actin cytoskeleton (Bliska, 2000) and the capacity of phagocytosis of the dendritic cells (Adkins *et al.*, 2007). YopB and YopD also activate the guanine nucleotide exchange factors (GEF), which can convert the inactive GTPase into its active form, leading to the phagocytosis and pore formation. Under normal circumstance, active GTPase with GTP bound ignites both the MAPK and the NF-κB signaling pathways, causing the genes in nucleus to be transcribed. For example, the expression of proinflammatory cytokines and other factors is enhanced by the active GTPase induced MAPK and NF-κB signaling pathways. Besides YopB and YopD, other Yops can also act on GTPase. YopH, a highly active tyrosine phosphatase (Hamid *et al.*, 1999), inhibits the contact between the β1 integrin, a transmembrane protein of the host cell, and the GEF (Viboud and Bliska, 2005).

YopE reverses the activation of GTPase, and similarly, YopO blocks the conversion between the active and inactive GTPase (Navarro *et al.*, 2007), which together with YopH down-regulate GTPase activation. Different from other Yops, YopT has a unique effect on GTPase. It inhibits phagocytosis and pore formation by scissoring RhoA in GTPase (Shao *et al.*, 2002), expelling the GTPase from the host cell membrane (Schmidt, 2011). Since the cytoskeleton is mainly regulated by GTPases, the destruction of the cytoskeleton

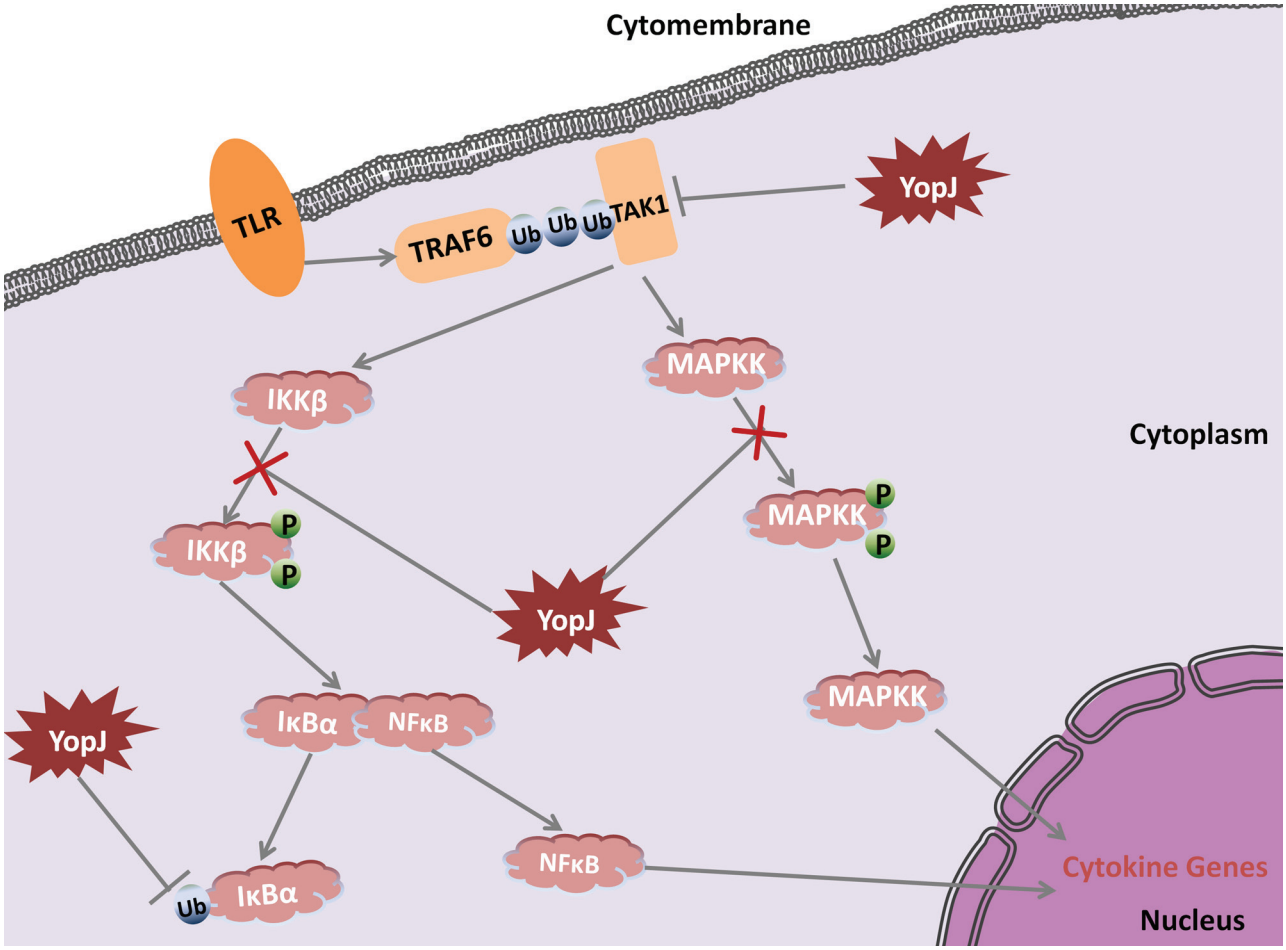

Fig. 2. YopJ inhibits MAPK and NF-κB pathways.

YopJ acetylates MAPKK and IKKβ to block the activation of MAPK and NF-κB, which in turn down-regulates the expression of proinflammatory cytokines and survival factors in cell nucleus. Additionally, YopJ removes Ub from TRAF6 and IκBα, hence leading to the inactivation of TRAF6 and TAK1. The regulation of the TAK1 is the key step to regulate the MAPK and NF-κB pathways.

will be caused by the inactive GTPases, furthermore resulting in cell death.

NF-κB and MAPK – YopJ/P (Fig. 2). Unlike other Yops, YopJ, as an important virulence factor in *Y. pseudotuberculosis*, has unique functions in *Yersinia* virulence. When macrophage or dendritic cells are infected by *Yersinia*, YopJ suppresses the MAPK/NF-κB pathways, hence deterring the formation of cytokines and survival factors. YopJ and its homologous, YopP, share 95–98% sequence similarity, and use the same secretion pathway, TTSS, to enter into the innate immune cells like membranous/microfold cells, dendritic cells, and Peyer's patches in the gut system (Viboud and Bliska, 2005).

After the infection, YopJ/P down-regulates the expression of the proinflammatory cytokine and the prosurvival proteins, and also blocks the formation of inflammasome through inhibiting the NF-κB and MAPK pathways. In addition, YopJ can activate caspase-1, and up-regulate the expression of IL-1β and IL-18 (Vladimer *et al.*, 2012; Luigi and Gabriel, 2012), giving rise to cell death in macrophages. In the interplay with the MAPK pathway, YopJ binds to MKKs (Mukherjee and Orth, 2008) and acts as a serine/threonine acetyltransferase that uses acetyl-coenzyme A (CoA) to modify the critical serine and threonine residues in the activation loop of MAPK kinasese, MKK and MAPK (Mukherjee *et al.*, 2006), thereby blocking the phosphorylation of MAPK to counteract the MAPK signaling pathway (Bliska, 2006; Paquette *et al.*, 2012). During the interaction with NF-κB, YopJ is recognized as a deubiquitinase that binds IKKβ, removing the ubiquitin moieties from critical proteins, such as TRAF6, TRAF2 and IκB (Zhou *et al.*, 2005). It is worth to note that the acetylation of TAK1 (transforming growth factor β-activated kinase 1), a member of the MAPKKK family, is the most important step for the YopJ-induced modulation of the host cell. As a downstream molecule indispensable for the nucleotide-binding and oligomerisation domain 2 (Nod2)-RICK-mediated activation of the NF-κB and MAPK pathways, it is still poorly understood whether YopJ acts directly on TAK1. However, YopJ has been indicated to restrain the NF-κB pathway at the level of TAK1 (Mukherjee and Orth, 2008; Meinzer *et al.*, 2012).

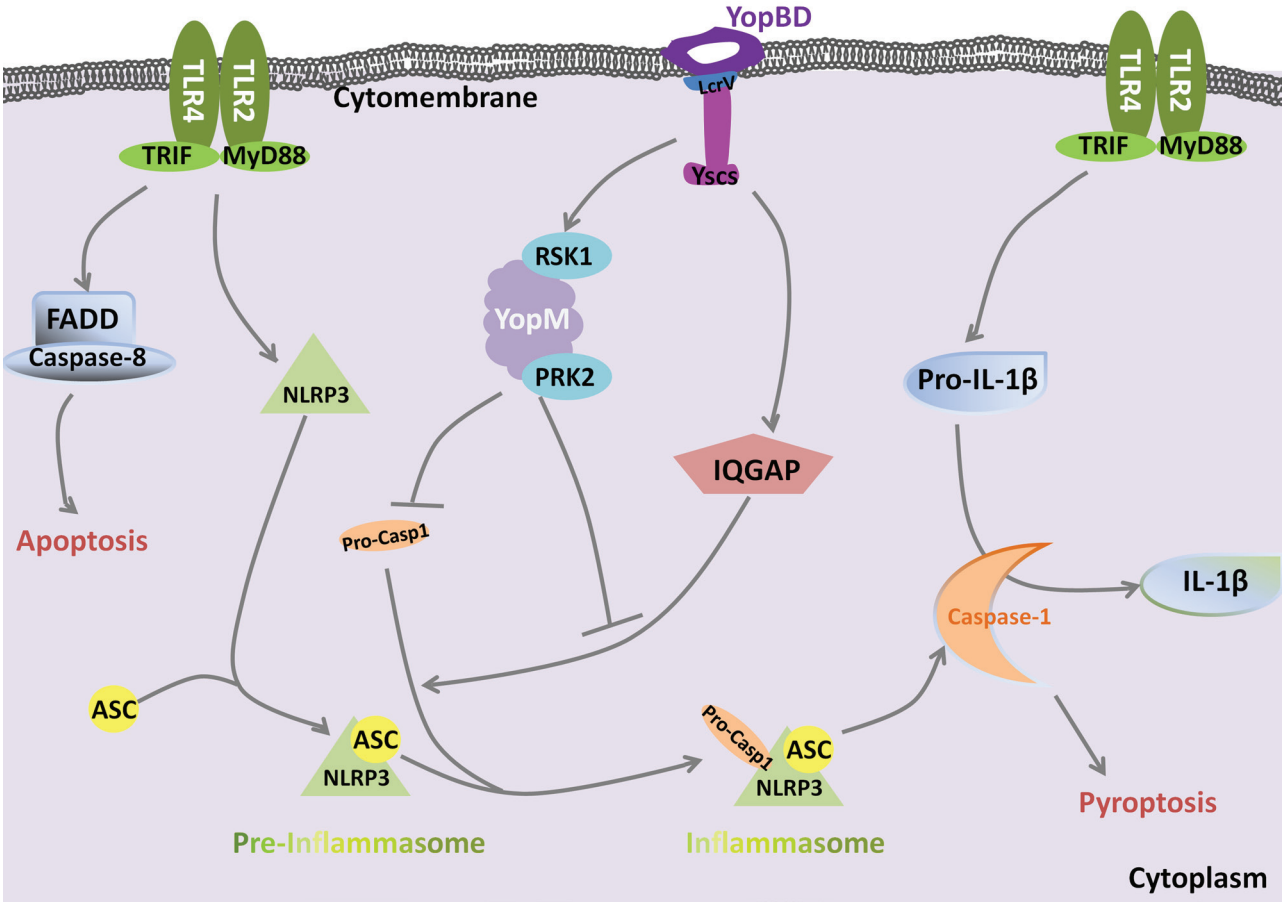

Fig. 3. Caspase-1 and other pathways in *Yersinia*-infected cell.

YopBD, LcrV and Yscs form a Needle-like injectisome across the cytomembrane, translocating the virulent Yops into the cytoplasm. The injectisome activates IQGAP, followed by induction of the caspase-1 medicated cell death, which is inhibited by the YopM-RSK1-PRK2 complex. TLRs, such as TLR2 and TLR4, stimulate FADD/Caspase-8 complex, inducing the apoptosis. In addition, TLRs can also activate the formation of pro-IL-1β and revitalize NLRP3, giving rise to the formation of pre-inflammasome. The assembly of inflammasome requires pro-caspase-1 and the activated IQGAP, which are blocked by the YopM complex. The assembled inflammasome activates caspase-1 and leads to pyroptosis. Besides, caspase-1 is also involved in the transformation from pro- IL-1β to IL-1β (von Moltke *et al.*, 2012).

Autophagy, inflammasome: caspase-1, pyroptosis – YopJ/P, YopM/K (Fig. 3). At the absence of priming, YopJ induces the NLRP3/ASC complex-dependent caspase-1 activation (Zheng *et al.*, 2012) and the NLRP12-activated inflammasome, resulting in the increase of IL-1β secretion (Broz *et al.*, 2010; Hamid *et al.*, 1999). YopJ, as a critical acetylase, acts in the activation loops of the RICK and TAK1 kinases (Meinzer *et al.*, 2012), which are the central mediators of Nod2 signaling. YopJ lowers the affinity of Nod2 for RICK, causing the unbound Nod2 to activate caspase-1, which finally results in the development of IL-1β.

Interestingly, although YopJ activates the caspase-1, the suppression of caspase-1 and inflammasome activation is carried out by YopM at the same time (McDonald *et al.*, 2003). YopM binds caspase-1 to inhibit the activation of caspase-1 (Jorgensen and Miao, 2012) and hamper the formation of the mature inflammasome, thus devastating the protective cell death program pyroptosis against pathogens (LaRock and Cookson, 2012). Additionally, the formation of RSK1 (ribosomal S6 protein kinase) and PRK2 (protein kinase C-related kinase 2) complex is relevant to YopM (Amedei *et al.*, 2011). YopM binds to RSK1 and PRK2, respectively, on its C-terminal tail and the LRR region (Viboud and Bliska, 2005). After binding, YopM blocks dephosphorylation of the activatory phosphorylation sites of RSK1 and activates RSK1-PRK2 complex (Ruckdeschel *et al.*, 2008). The complex formed by YopM and RSK1/Rsk2 blocks the formation of inflammasome through inhibi ting pro-caspase-1 and IQGAP1 signal (Chung *et al.*, 2014). YopM is also recognized as the first bacterial cell-penetrating protein, which can diminish the expression of TNF-α (tumor necrosis factor-α) and some other pro-inflammatory cytokines, such as IL(interleukin)-1, IL-12, IL-15, IL-18 (Kerschen *et al.*, 2004). Evidence also indicates that the release of bioactive IL-1β is also prevented by YopM (Bergsbaken

et al., 2009; LaRock and Cookson, 2012). Besides, the release of lysosomal exocytosis, which is an antimicrobial factor induced by caspase-1 to act on extracellular bacteria, is blocked by YopM (LaRock and Cookson, 2012; Bergsbaken *et al.*, 2011).

Similar to YopM, YopK (known as YopQ in *Y. enterocolitica*) (Holmstrom *et al.*, 1995) is functioned in down-regulating caspase-1 activation and IL-1β secretion. In the absence of all other known Yops, injection of YopK into the cells results in decreased caspase-1 activation (Brodsky *et al.*, 2010). All this evidence together indicates that YopK restricts the injection of effector proteins into the host cytoplasm and also, Caspase-1 activation (Dewoody *et al.*, 2011).

Interplay of the *Yersinia* with gut system and TLRs

Among the three species of pathogenetic bacterium to humans, *Y. enterocolitica* and *Y. pseudotuberculosis* mainly target the human gut system. Unlike *Y. Pestis*, these two enteropathogens are commonly transmitted by food or water (Trcek *et al.*, 2011). Multiple studies have indicated the involvement of Yops in the interaction of *Yersinia* and gut system.

Y. enterocolitica infection typically leads to acute enteritis, enterocolitis, mesenteric lymphadenitis and ulceration, and necrosis of the tissue. Mesenteric lymph nodes become amplified and the focal area of necrosis exhibits leukocytes infiltration (Viboud and Bliska, 2005, Ruckdeschel *et al.*, 1996). Studies of the intestine of *Y. enterocolitica* O9 infected BALB/c mice indicates that the infected mice exhibit splenomegaly, and development of $CD3^+$ total T cells, $CD4^+$ Th cells, $CD8^+$ Tc cells, and $CD11b^+$ phagocytic cells (Ruiz-Bravo *et al.*, 2001). The infection also causes the impaired response and proliferation of normal splenocytes against the mitogens, which in turn promotes the inhibition of lymphocyte responding to mitogens. In addition, the increase of IFN-γ is stimulated by the concanavalin A, but not lipopolysaccharide, which aggrandizes reactive nitrogen intermediates in macrophage. Therefore, the infection of *Y. enterocolitica* may lead to immune attenuation of spleen cells and disrupt the bacterium-pathogens-induced immune responses of host (Dessein *et al.*, 2009).

Y. pseudotuberculosis infection is typically tied to mesenteric adenitis and occasionally with terminal ileum and cecum inflammation (Galindo *et al.*, 2011). The infection normally causes the appearance of micro-abscesses or granuloma-like lesions with central necrosis. Derived from the latter tuberculosis-like lesions, *Y. pseudotuberculosis* disrupts intestinal barrier integrity through intruding the intestinal lymphoid tissue and TLR-2 signaling (Jung *et al.*, 2012). Due to anfractuous cooperation between intestinal epithelial cells and immune cells, the transcellular transport is determined by the pore size of tight junctions, which also limits the access of the luminal commensal pathogens. Studies on *Y. pseudotuberculosis* infected mice indicate that the Peyer's patches barrier dysfunction is largely associated with *Yersinia* virulence and the TLR-2 expressed by the hematopoietic cells. The TLR-2 activation determines the ideal epithelial transcript level of the anti-infective c-type lectin Reg3β, which is regarded as an intestinal resistance to *Yersinia* through controlling the bacterial load in Peyer's patches. After *Y. pseudotuberculosis* infection, TLR-2 plays a crucial role in initiating and regulating the host response due to its secretion in the intestinal epithelial cells, membranous/microfold cells, macrophages and dendritic cells. In humans, it is reported that *Y. pseudotuberculosis* disturbs epithelial gut homeostasis and may induce ileitis. All this evidence together indicates that the infection of *Y. pseudotuberculosis* may disrupt the gut system homeostasis through the interaction between bacterium lipopolysaccharide and TLRs (Dessein *et al.*, 2009; Meinzer *et al.*, 2012).

Future directions

Although the major functions of effector proteins in inhibiting the innate immune system of host cells (Amedei *et al.*, 2011) have been largely clarified, the detailed interplay of the Yops and receptors with the intracellular proteins or genes of the host cells remains unclear.

In the apoptotic pathway, YopM and YopJ both play functional roles in interfering apoptosis. Evidence has been obtained to confirm that the cell death receptor like TNF receptor and Fas could modulate the death-inducing signaling complex consisted of RIPK1, caspase-8, and Fas-associated death domain. However, how these proteins are involved in YopJ-induced cell death (Philip *et al.*, 2014), how YopJ/P inhibits mammalian TGFβ-activated kinase (Paquette *et al.*, 2012), how NOD2 interacts with caspase-1 (Meinzer *et al.*, 2012), and how Yops-induced cell death impacts the local microenvironment and host antibacterial immune responses *in vivo* are still uncertain (Philip and Brodsky, 2012).

Understanding the interactions of needle proteins and TLRs at the amino acids level is another interesting field for future study. The conclusions on how *Yersinia* recognize lipopeptides, impact host defense, and influence the TLR-2 activation on gut permeability are still controversial (Hajjar *et al.*, 2012; Galindo *et al.*, 2011). In addition, identifying the interplay between TLRs and needle proteins will enrich our knowledge of the interaction between host immune responses and TTSS. The investigation of TTSS to understand the formation of

a needle-like receptor, substrate-recognition of TTSS, and the substrate delivery is critical to further interpret the virulence of the *Yersinia*.

Yersinia invasion also significantly impacts the gut system in host cells (Persson *et al.*, 1999). Considering the strong virulence of *Y. enterocolitica* and *Y. pseudotuberculosis*, it can be believed that the understanding of the mechanisms of bacterial invasion interplaying with host gut barrier dysfunction (Zhou *et al.*, 2005) may bring useful clinic applications. All the questions mentioned above are worth being explored, and will produce effective treatment strategies towards these complicated courses of infection.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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ORIGINAL PAPER

Recovery of Gram-Positive Cocci and *Candida albicans* from Peroxygen/Silver-Based Disinfectants

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Abstract

Neutralization method evaluation is an important first step in a disinfectant validation study program. It is also crucial in assessment of the efficiency of microbial recovery media in the presence of a residual biocidal agent. In the present study, four commercially available peroxygen/silver-based disinfectant formulae – intended to be used in a pharmaceutical facility sanitization program – were tested at two dilutions against three Gram-positive cocci and one yeast; *Staphylococcus aureus* (ATCC 6538), *Kucoria rhizophila* (ATCC 9341) and *Candida albicans* (ATCC 10231) and *Staphylococcus capitis* as an environmental monitoring (EM) isolate sample (identified by miniaturized biochemical identification system). Disinfectants preparation, dilutions and the test procedure were carried on in laboratory under conditions simulating the working environment of 20–25°C and RH% 40–60. In-house made neutralizing broth was mixed with biocidal agents to make two dilutions of each disinfectant forming Peroxygen: neutralizing broth ratios of 1:10 and 1:100 (v/v). Three populations were established and two comparison ratio groups were examined, namely neutralizer efficacy and neutralizer toxicity. Two acceptance criteria were tested. One criterion showed higher rate of neutralization success than the other. *S. aureus* showed the highest rate of successful microbial recovery from neutralization process. The Disinfectant (Bixco) was exceptionally neutralized at all dilutions with all microorganisms. In conclusion, in-house made neutralizing broth effectively neutralized all disinfectants with all the tested microorganisms at 1:100 (v/v); thus, it can be used in sanitizer validation studies and EM media.

Key words: disinfectant validation, neutralization method, peroxygen/silver, recovery media, sanitization program

Introduction

The harmonized methods in the compendia (United States Pharmacopoeia [USP], European Pharmacopoeia [EP], and Japanese Pharmacopeia [JP]) provide detailed tests for the detection of specified organisms, including *Staphylococcus aureus* and *Candida albicans*. These organisms are considered objectionable if present in certain types of products, taking into consideration their known pathogenicity as well as contamination potential during pharmaceutical manufacturing. However, the presence of other organisms that might be considered objectionable to a product or manufacturing process must not be overlooked (Clontz, 2008).

Commercial disinfectants are usually composed of a mixture of biocidal agents to provide synergistic activity against a wide of microorganisms. In one form or another, silver and its compounds have long been used as antimicrobial agents (Brown and Anderson, 1968; Russell and Hugo, 1994). The most important silver compound currently in use is silver sulfadiazine (AgSD), other examples of silver-based compounds possessing antimicrobial activities include silver metal, silver acetate, silver nitrate, and silver protein, which have been listed in Martindale, The Extra Pharmacopoeia (McDonnell and Russell, 1999).

The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl, SH) groups (Fuhrmann and Rothstein, 1968; Bragg and Rainnie, 1974; Belly and Kydd, 1982; Furr *et al.*, 1994), although other target sites remain a possibility (Richards, 1981; Thurman and Gerba, 1988). The study of Liau *et al.* (1997) demonstrated that amino acids such as cysteine and sodium thioglycollate containing thiol groups possess the ability to neutralize the activity of silver nitrate against *Pseudomonas* aeruginosa. In contrast, amino acids containing disulfide (-S-S-) bonds, non-sulfur-containing amino acids, and sulfur-containing compounds such as cystathione, cysteic acid, l-methionine, taurine, sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag^+ activity. These and other findings imply that the

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interaction of Ag^+ with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation, although other cellular components may be involved.

Hydrogen peroxide (H_2O_2) is a common biocide which is widely used for disinfection, sterilization, and antisepsis. It is a clear, colorless liquid that is commercially available in a variety of concentrations ranging from 3 to 90%. H_2O_2 is considered environmentally friendly, because it can rapidly degrade into the innocuous products; water and oxygen. H_2O_2 demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores. In general, greater activity is seen against Gram-positive than Gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance at lower concentrations. On the other hand, higher concentrations of H_2O_2 (10 to 30%) and longer contact times are required for sporicidal activity (Russell, 1991), although this activity is significantly increased in the gaseous phase. Mechanistically, H_2O_2 acts as an oxidant by producing hydroxyl free radicals (·OH) which attack essential cell components, including lipids, proteins, and DNA. It has been proposed that the accessible sulfhydryl groups and double bonds are particularly targeted (Block, 1991).

Peracetic acid (PAA) (CH_3COOOH) is considered a more potent biocide than H_2O_2, being sporicidal, bactericidal, virucidal, and fungicidal at low concentrations (< 0.3%) (Block, 1991). PAA also decomposes into safe by-products (acetic acid and oxygen) but has the extra advantages of being not susceptible to decomposition by peroxidases, unlike H_2O_2, and remaining active in the presence of organic loads (Lensing and Oei, 1984; Malchesky, 1993). Interestingly, its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (Crow, 1992; Malchesky, 1993). Similar to H_2O_2, PAA probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulfhydryl (SH) and sulfur (SS) bonds (Baldry and Fraser, 1988; Block, 1991).

In order to ensure the validity of data derived from a test, two parameters must be checked, namely: "accuracy" and "integrity" of the output readings. Minimum and maximum ranges for accuracy of plate count readings have been addressed previously (Sutton, 2011). A measurement that is clearly questionable because of a failure in the assay procedure should be rejected, whether it is discovered during the measuring or tabulation procedure. The arbitrary rejection or retention of an apparently aberrant measurement can be a serious source of bias. Each suspected potency measurement, or outlier, may be tested against a criterion which is based on the variation within a single group of supposedly equivalent measurements from a normal distribution (Pharmacopeial Forum Number 34, 2010).

This study aimed to evaluate and optimize a method of neutralizing peroxygen-based biocides with in-house made neutralizer for the recovery of environmentally and pharmaceutically important Gram-positive cocci and *C. albicans* (as an example of yeast) at two dilution levels. This aim would serve as a part of an improvement program for the detectability of selected microbial species in clean room environment in the presence of residual tested sanitizers, and to evaluate a recovery broth from disinfectants in biocidal agents' evaluation study. Such an optimized improvement program of quality could be applied in healthcare facility generally and a pharmaceutical plant specifically.

Experimental

Materials and Methods

Preparation of microbial suspension. Standard strains were purchased from the American Type of Culture Collection (ATCC; Manassas, Virginia) and handled according to standard procedure while bacterial environmental monitoring (EM) isolates were isolated and identified using miniaturized biochemical identifications kits of BBL™ Crystal™ Identification System purchased from BD (Becton Dickinson Microbiology Systems, Cockeysville, Md.). All media were purchased from OXOID (Basingstoke, Hampshire) and chemicals from Sigma-Aldrich (St. Louis, MO 63103). Table I shows the list of microorganisms used in the current study, source, family and general characteristics.

Standardized stable suspensions of test strains were used and prepared as stated by the supplier. Seed-lot culture maintenance techniques (seed-lot systems) were used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot. All organisms were kept and preserved at –80°C in validated –86°C Ultra low temperature freezer (–86 Degree ULT Freezers, Qingdao, Shandong, China) in controlled cryogenic environment and reactivated only prior to conduction of the study using standard method determined by the supplier. All media were sterilized by autoclaving in steam sterilizer (FEDEGARI FOB3, Fedegari Autoclavi SpA, SS 235 km 8, 27010 Albuzzano (PV), Italy). All pH measurements and weighing procedures were done using Mettler-Toledo S20 SevenEasy™ pH Meter and XPE Analytical Balance respectively (Mettler-Toledo, LLC 1900 Polaris Parkway Columbus, OH 43240).

Suspensions were quantified by making serial dilutions and performing duplicate plate counts using conditions and media suitable for each tested microorganism

Table I
List of microorganisms challenged in neutralizer validation study with the source, family and general characteristics of them.

Challenged microorganisms	Source	Family	General characteristics
Staphylococcus aureus	ATCC6538	Staphylococcaceae	Gram-positive cocci and represent a large percentage of microorganisms isolated from environmental samples in pharmaceutical facilities
Staphylococcus capitis	EM isolate*		
Kucoria rhizophila	ATCC9341	Micrococcaceae	
Candida albicans	ATCC10231	Saccharomycetaceae	Diploid fungus (grows as yeast and filamentous cells)

* = Environmental Monitoring isolate identified mainly from air samples (active and passive) in clean area using BBL CRYSTAL GP miniaturized biochemical system kits.

to choose suspensions of concentration 300–1000 CFU/50–100 µl were chosen as working suspensions. Microbial test suspensions were used as soon as results of serial dilutions could be enumerated using digital colony counter (Digital Colony Counter Model: 361, Laxman Mahtre Rd. Navagaon, Dahisar West, Mumbai).

Neutralization validation study of peroxygen-based biocidal agents. The purpose of this study was to ensure that the assumed contact time is valid, *i.e.* the neutralizing agent can efficiently stop the action of the tested sanitizer after being mixed with it and to ensure that the neutralizing agent does not possess any inhibitory or toxic effect on microorganisms. It was suggested that two comparisons among three populations be performed. The first comparison is Neutralizer Efficacy (NE) which could be determined by evaluating survivors in the neutralizing broth in the presence and the absence of the biocide. The ability of the neutralizing broth alone to allow survival is a second important consideration in this analysis. The second comparison was Neutralizer Toxicity (NT); an aspect of neutralization that was determined by comparing survivors in the neutralizing medium without the biocide with the viability (growth) control (Sutton *et al*., 2002; Eissa *et al*., 2012).

Test solutions were freshly prepared and diluted under conditions simulating the actual usage environment of biocidal agents at the highest concentration of recommended by the manufacturer (5%; v/v). These commercial disinfectants were denoted Bixco (Hyrogen Peroxide/Ag^+), BafD 50 (Hyrogen Peroxide/Ag^+), Pury (Hyrogen Peroxide/Peroxyacetic Acid/Ag^+) and Mil (Hyrogen Peroxide/Ag^+). The peroxide concentration equivalents to H_2O_2 in the four disinfectants, was determined by conventional chemical titration method. Using neutralizing broth as a diluent 1:10 and 1:100 (v/v) dilutions of the test solution – *i.e.* disinfectant final concentrations per 10 ml of the neutralizer were 5% and 0.5% (v/v) respectively – were made at working concentration, then an aliquot of one ml of each dilution was transferred to each of a duplicate petri dishes; this was the test group. The neutralizer exposed group was prepared in parallel in the same manner as the test group but using sterile saline or buffer instead of the test solution. The viability control group was prepared using peptone water without the solutions or neutralizing broth. Organisms were prepared so that the required inoculums did not exceed 0.5–1.0% of the total volume in the tubes. In-house neutralizer of the study was double strength Fluid Thioglycollate Medium Thiosulfate (FTMT) which was supplemented with resazurine dye (redox indicator) to detect oxygen diffusion and used fresh or reheated once if there was a considerable diffusion of oxygen in the reservoir tube.

Inoculums of each used microorganisms were added to each of the above described tubes so that the final count per plate of positive control ranged between 30 to 100 CFU per plate. Then about 20 ml of the molten suitable medium at 45°C was added; allowed to solidify, then incubated at suitable temperature for 30–35°C for three days for bacteria and 20–25°C for at least three to five days for fungi in incubators (Hotpack 175 series; model 417532, Dutton Rd., Philadelphia, USA). After that, duplicate plate counts were done for the 3 groups. Negative control for each media containing the same volume of diluents or neutralizers was prepared and similarly treated to ensure the sterility of all used materials. All tests and control groups were performed in triplicate for each microorganism, disinfectant and dilution combination.

Testing of aberrant observation was done using the following formula: $G_1 = (y_2 - y_1) / (y_N - y_1)$ when N = three to seven. If G_1 exceeded the critical value of 0.987 for outlier measurements for the observed triplicate results, there was a statistical basis for omitting the outlier measurement. In this case, the test was repeated to replace the rejected values (Pharmacopeial Forum Number 34, 2010). At least three independent replicates of the experiment should be performed, and each should demonstrate that the average number of CFU recovered from the challenge product is not less than 70% of that recovered from the inoculum control (USP < 1227 >, 2014). Another criterion was selected from Sutton *et al.* (2002) for comparison which is the geometric means of results must not be less than 75%. If there was suspect results in the group,

Log_{10} transformation of recovered CFU were done then One-Way Analysis of Variance (ANOVA) was performed for transformed results groups to confirm the significance followed by Dunnett's Multiple Comparison Test at $p < 0.001$ which was used to confirm the success or failure of the test. Tukey's Multiple Comparison Test at $p < 0.001$ was used to perform comparisons between groups. Finally, all statistical analysis was performed using GraphPad Prism version 5.00.288 for Windows. Any interpretation of complex calculation and programming of equations was performed using Microsoft Excel 2007.

Results

NT study revealed that FTMT did not possess any adverse effects on the tested Gram-positive cocci and *C. albicans*, accordingly the neutralizing broth passed toxicity test at both USP and Sutton *et al.* (2002) criteria. The recovery ratio of the four microorganisms was equal or better than one and no statistical analysis was required. The currently applied procedure used to detect the presence of aberrant observations revealed that none of the results in each group was unusual and required omitting as $G_{1calc.} < G_{1tab.}$ either from NT or NE populations. This finding is illustrated in Table II in addition to the geometric recovery mean recovery ratio. Total number of samples that passed NE study was 21 and 23 per 32 for USP < 1227 > (2014) and Sutton *et al.* (2002) criteria respectively and all failing results came from 1:10 (v/v). The difference between both criteria was *S. aureus* recovery from BafD 50 and Mil 1:10 (v/v) dilutions which failed in the first and passed the second. Six results out of 32 did not show any microbial recovery from 1:10 (v/v) dilution of BafD 50, Mil and Pury with both *Staphylococcus capitis* and *C. albicans*. This finding is demonstrated in Table II and normally is evident in Fig. 2 and 4 as missing bars of the 3 previously mentioned biocidal agents. These two microorganisms were sensitive to the dilution of the 3 tested biocidal agents namely: BafD 50, Mil and Pury.

The concentration of peroxide equivalent to H_2O_2 for Bixco, BafD50, Mil and Pury was 23.0, 49.2, 49.2 and 32.8% (w/w) respectively. Table III shows that five results – all from 1:10 (v/v) – did not pass the NE tests, thus the total rate of microbial recovery success was 5 and 7 per 16 using USP<1227> (2014) and Sutton *et al.* (2002) criteria, respectively, while it was 16/16 with 1:100 (v/v) from all disinfectants in both criteria. If both acceptance criteria were taken into consideration the descending order of microbial recovery success was: *S. aureus* (14 successful recovery per 16) > *S. capitis* = *C. albicans* = *Kucoria rhizophila* (10 successful recovery per 16). However, the comparison in recovery in terms of recovered colonies in culture media regardless passing acceptance criteria or not gave the following order: *S. aureus* > *K. rhizophila* > *C. albicans* > *S. capitis*. This finding is demonstrated in Table II.

The total rate of neutralization success of the tested disinfectants was in the following decreasing order: Bixco > Pury > Mil = BafD 50. Both Tables II and III demonstrated these findings. Bar graphs of Fig. 1, 2, 3 and 4 illustrate the average recovery of Log_{10} transformed CFU with standard deviations and compared with both reference control of USP<1227> (2014) crite-

Table II
NT and NE ratios and G_1 calculated values for determination of outliers.

Organism	NT ($G_{1cal.}$)	NE ($G_{1cal.}$) of selected biocidal agents at 2 dilutions levels in FTMT neutralizer							
		Bixco		BafD 50		Mil		Pury	
		1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v
S. aureus	1.06 (0.20)	1.08 (0.97)	1.33 (0.89)	0.47* (0.62)	1.20 (0.94)	0.62* (0.88)	1.08 (0.84)	0.75* (0.45)	0.98 (0.17)
S. capitis	1.38 (0.42)	0.78 (0.82)	1.15 (0.68)	0.00	0.95 (0.70)	0.00	0.89 (0.50)	0.00	0.87 (0.86)
K. rhizophila	1.40 (0.22)	0.73* (0.13)	0.88 (0.50)	0.54‡ (0.50)	1.02 (0.75)	0.61‡ (0.11)	0.80 (0.79)	0.50‡ (0.33)	1.19 (0.97)
C. albicans	1.20 (0.32)	1.06 (0.74)	1.20 (0.75)	0.00	0.89 (0.84)	0.00	0.90 (0.78)	0.00	0.94 (0.41)

If $G_{1cal.}$ at ($P = 0.02$) < 0.987 (when N = 3) the suspect data are not aberrant results but if $G_{1cal.} \geq G_{1tab.}$ they are considered true outlier measurements.
* = Results that were subjected to One-Way ANOVA followed by Dunnett's test to confirm success or failure after being transformed to Log_{10} values and were found to pass the test at $P < 0.001$.
‡ = Results that were subjected to One-Way ANOVA followed by Dunnett's test to confirm success or failure after being transformed to Log_{10} values and were found to not pass the test at $P < 0.001$.
NT and NE ratios were derived utilizing the geometric mean of the recovery in the different populations. Acceptable NT and NE ratios are defined as ≥ 0.75. Statistical analysis was performed in all groups. Below geometric means between parentheses were G_1 computed to determine the relative gap by comparing the reference critical value with the calculated ones.

Table III
Rate of microbial recovery from the neutralization process and the ease of neutralization process of biocidal agents at the two dilutions level against the two selected reference criteria namely US Pharmacopoeia <1227> and Sutton *et al.*

Organism	Criteria of comparison	NT	Pass/Fail score according to test criteria for 4 selected Peroxygen/Ag^+-based disinfectants at 2 dilutions levels in FTMT neutralizer								Total rate of microbial recovery success
			Bixco		BafD 50		Mil		Pury		
			1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	1:10 v/v	1:100 v/v	
S. aureus	USP<1227>	+	+	+	–	+	–	+	+	+	6/8
	Sutton *et al.*	–	–	–	–	–	–	–	–	–	8/8
		+	+	+	+	+	+	+	+	+	
S. capitis	USP<1227>	+	+	+	–	+	–	+	–	+	5/8
	Sutton *et al.*	–	–	–	–	–	–	–	–	–	5/8
		+	+	+	–	+	–	+	–	+	
K. rhizophila	USP<1227>	+	+	+	–	+	–	+	–	+	5/8
	Sutton *et al.*	–	–	–	–	–	–	–	–	–	5/8
		+	+	+	–	+	–	+	–	+	
C. albicans	USP<1227>	+	+	+	–	+	–	+	–	+	5/8
	Sutton *et al*	–	–	–	–	–	–	–	–	–	5/8
		+	+	+	–	+	–	+	–	+	
Total rate of disinfectant neutralization success			4/4	4/4	0/4	4/4	0/4	4/4	1/4	4/4	21/32
			4/4	4/4	1/4	4/4	1/4	4/4	1/4	4/4	23/32

rion for acceptance. When statistical analysis was performed using One-Way ANOVA followed by Tukey's Multiple Comparison Test – to compare between different test groups was conducted at $p < 0.001$ ($F = 6.761$ – $R_{squared} = 0.765$) on data obtained from bar graphs – of all microorganisms the results showed that there is no significant impact of Bixco dilution from 1:10 to 1:100 (v/v) on microbial recovery from FTMT. None of the 1:100 (v/v) dilution of any tested disinfectant-microorganism combination differed significantly from one to another. The obvious failure (no CFU recovered) of both *S. capitis* and *C. albicans* with 1:10 (v/v) of BafD 50, Mil and Pury excluded them from any statistical analysis or comparison. *S. aureus* and *K. rhizophila* recoveries from BafD 50 and Pury 1:100 v/v respectively were significantly higher than that of *S. aureus* and *K. rhizophila* recovered from BafD 50 1:10 (v/v) and *K. rhizophila* recovered from Pury 1:10 (v/v). On the other hand, *S. aureus* recovery from Mil 1:100 (v/v) and Bixco 1:10 and 1:100 (v/v) was significantly different in recovery from that recovered from BafD 50 1:10 (v/v). While *S. aureus* recovered from BafD 50 1:10 (v/v) was

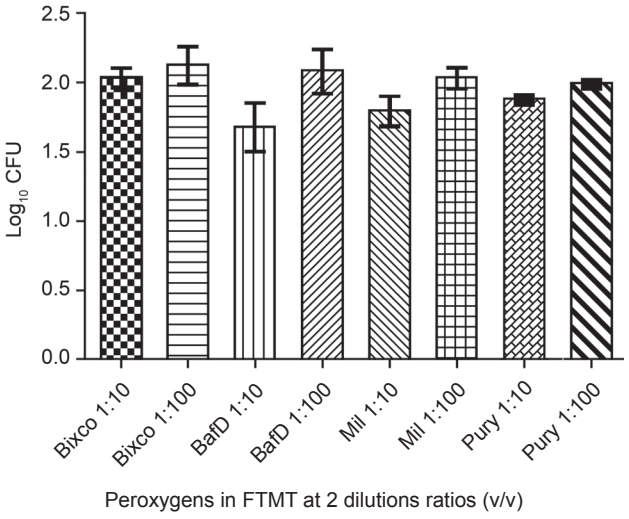

Fig. 1. *S. aureus* vs. 4 Peroxygens in FTMT. NE of FTMT on *S. aureus* (ATCC 6538) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

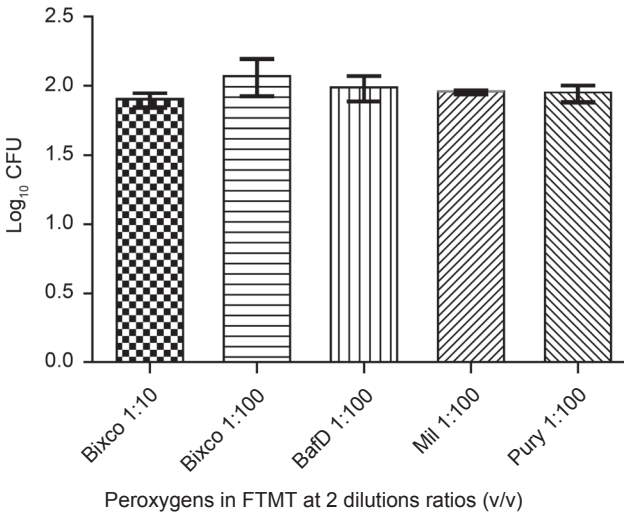

Fig. 2. *S. capitis* vs. 4 Peroxygens in FTMT. NE of FTMT on *S. capitis* (EM isolate) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

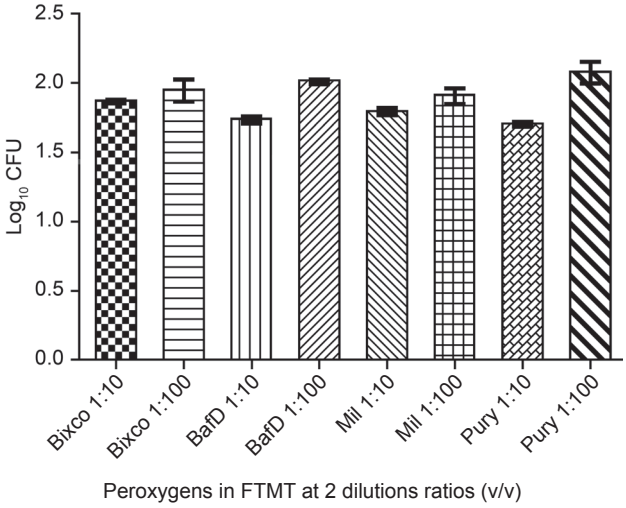

Fig. 3. *K. rhizophila* vs. 4 Peroxygens in FTMT. NE of FTMT on *K. rhizophila* (ATCC 9341) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

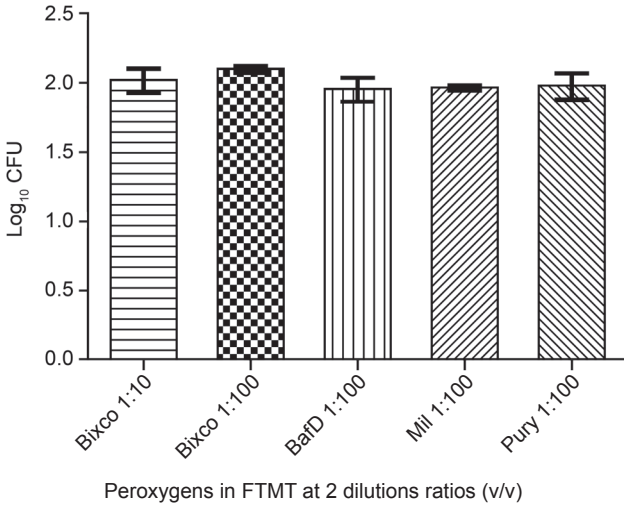

Fig. 4. *C. albicans* vs. 4 Peroxygens in FTMT. NE of FTMT on *Candida albicans* (ATCC 10231) with Bixco, BafD 50, Mil and Pury at concentrations 0.5% and 5% per 10 ml of neutralizing broth. All results are expressed in means of Log_{10} CFU ± S.D.

significantly lower in recovery from *C. albicans* and *S. capitis* from Bixco 1:10 and 1:100 (v/v) respectively. In the same line, *K. rhizophila* recovered BafD 50 1:10 (v/v) was significantly lower than *C. albicans* and *S. aureus* from Bixco 1:10 and 1:100 (v/v) respectively. Finally, *K. rhizophila* recovery from Pury 1:10 (v/v) was considerably lower than *C. albicans* recovery from Bixco 1:10 (v/v), *S. aureus* and *S. capitis* recovered from Bixco 1:100 (v/v) and *S. aureus* from BafD 50 1:100 (v/v). Fig. 1, 2, 3 and 4 illustrate this difference.

Discussion

This study fulfilled two major aims as a part of sanitization validation program to test index microorganisms, either standard strains or selected environmental isolates, against certain commercially-available disinfectants to compare between their efficacies. The first aim was testing in-house neutralizer for suitability of recovering organisms from EM samples. As for the second aim, it was to provide proper chemical neutralization for commercial peroxygen-based disinfectants (which are usually fortified and stabilized with silver compounds) for assessment of their potency. Gram-positive cocci were chosen because they were generally found mainly in both active and passive air samples and *K. rhizophila* was found to contribute to large populations of aerially distributed microorganisms (Eissa, 2014). *S. aureus* and *C. albicans* are among objectionable microorganisms that should not be present in pharmaceutical products (USP<62>, 2014). *S. capitis* is an EM isolate and was subjected to testing in current validation program as being frequently isolated in different EM samples at critical processing points in clean area. Consequently, it could contribute to either environmental samples failure and/or compromising drug quality thus it was important to include it in risk assessment program.

Effective neutralization of a biocidal agent is critically important to the accuracy of the information obtained from any disinfectant efficacy study (Langsrud and Sundheim, 1998). The determination of NT and NE should be a comparison between a test and a control population. NT was determined as the ratio of recovery between a viability population, and a population exposed to the neutralizer. This comparison directly examined the toxicity of the individual neutralizing media for the different microorganisms. The efficacy of a particular neutralizer was defined as the ratio of recovery between the neutralizer and the biocide, and the neutralizer exposed populations. Therefore, only the effect of the biocide in the system was measured. These ratios allowed for a threshold value (≥ 0.75) as the first test. The second test was a statistical one to confirm success or failures (Sutton *et al*., 2002). Another criterion of comparison was USP < 1227 > (2014) of three independent replicates of recovery ≥ 70% in agar medium.

According to USP < 1072 > (2014): Biocidal activity reduction folds = (Dilution folds)$^{\eta}$, where η is the concentration exponent. Thus, the reduction of activity of hydrogen peroxide (η = 0.5) is about three to ten-fold the reduction in activity only for 1: 10 and 1:100 respectively while for $AgNO_3$ (η = 0.9 – 1) it is about eight and ten to 63 and 100. This indicates that for hydrogen peroxide, the dilution has minor effect in abolishing the antimicrobial properties although peracetic acid is neutralized effectively by dilution (Russell, 1990). It is the neutralizer capacity that played the role in the chemical neutralization as 1:100 (v/v) dilution passed the test

while all of the failure of neutralization process were from 1:10 (v/v), *i.e*. at lower dilution the neutralizer capacity was insufficient to scavenge the residual peroxides released from disinfectants. However, these values of concentration exponents were for each component alone and not in synergistic combinations. Further investigation is needed to determine if the concentration exponents are skewed in these formulae or not.

Another important consideration is the byproduct of the chemical neutralization reaction and its probable toxicity to microorganisms. Some investigators demonstrated that tetrathionate is produced from reaction of hydrogen peroxide and thiosulfate. Their work showed that at pH range six to seven (the range of neutralization in the current study) tetrathionate is produced during the course of the reaction till reaching a steady state while the thiosulfate is continuously depleted. Some researchers, also, showed that thiosulfate: tetrathionate at three-to-one ratio is inhibitory to many microorganisms at certain concentrations (Palumbo and Alford, 1970). If during the course of the reaction this ratio is attained at specific concentration, even transiently, possible toxicity to microorganisms could occur. This is a point that needs more investigation on the reaction mechanism in this neutralizer to elucidate if there is a true impact on microbial recovery or not from the residual biocidal agent declining in concentration and reaction by product accumulating.

Cystine $(SCH_2CH(NH_2)CO_2H)_2$ is the amino acid formed by the oxidation of two cysteine molecules that covalently link *via* a disulfide bonds and is cleaved rapidly at higher temperatures (Aslaksena *et al*., 2006) (such as these of media preparation and sterilization). Cystine is found in NIH Thioglycollate (NIH) in half amount of that in FTMT. Cysteine amino acid has reducing properties similar to thioglycollate (mercaptoacetate) with peroxygens. However, the autooxidation of the peroxide scavenging components in the neutralizing broth is expected on standing from the atmospheric oxygen in the head space of the reservoir tube thus neutralizing broth must be either prepared fresh, heated once directly before use and/or incorporation of thickening agent in the neutralizing broth (*eg.* small portion of agar to render the media thick but not solid) which retard atmospheric oxygen diffusion as sodium thioglycollate in the medium consumes oxygen (Madigan *et al*., 2010). The cysteine thiol group is nucleophilic, highly reactive and easily oxidized. Moreover, the reactivity is enhanced when the thiol is ionized, and has pK_a values close to neutrality, so are often in their reactive thiolate form (Bulaj *et al*., 1998). This property is more prominent in FTMT than NIH. Consequently, redox indicators such as resazurin is a good monitor for such situation to judge visually the presence and the degree of oxygen penetration into the media for either reheat or discard of.

Neutralizer toxicity study performed for FTMT used in this study revealed that it was non-toxic and could be used in the validation program. NIH investigated by other researchers gave similar outcome. NIH was non toxic or of low toxicity against microorganisms. In-house made neutralizer FTMT is in between DEB and NIH in composition. The other important subsequent aspect is NE. The combination of microorganism, neutralizer and disinfectant is unique and thus the success of one combination with one microorganism does not mean that same combination with other microorganisms will do accordingly (Sutton *et al*., 2002). This is in agreement with our findings in NE which showed different outcomes for microbial recovery from neutralization process.

Microorganisms may become sublethally injured after exposure to many chemical and physical stresses (Busta, 1976; Hurst, 1980). Injury may be measured by the difference in counts when stressed cells are simultaneously enumerated on selective and nonselective media. Only uninjured cells are recovered on selective medium, whereas the nonselective medium is assumed to recover both injured and uninjured cells (Busta, 1976; Hackney *et al*., 1979; Hurst, 1980). However, agents lethal to injured cells may be formed spontaneously in either selective or nonselective medium (Barry *et al*., 1956; Baird-Parker and Davenport, 1965; Carlsson *et al*., 1978). Media containing manganese, citrate, or both will autooxidize and form peroxides in concentrations lethal to bacterial cells stressed by ionization (Barry *et al*., 1956). Carlsson *et al*. (1978) reported the formation of superoxide radicals and hydrogen peroxide in anaerobic broth media exposed to oxygen. When thioglycollate is present in the media, H_2O_2 formation is inhibited but superoxide radicals are still formed. Supplementation of media with compounds which degrade H_2O_2 has been studied (Baird-Parker and Davenport, 1965; Martin *et al*. 1976; Rayman *et al*., 1978). On the other hand, Baird-Parker and Davenport (1965) reported that incorporation of pyruvate into selective media enhances the recovery of *S. aureus*. However, Martin *et al*. (1976) noted an improved recovery of injured *S. aureus*, *Pseudomonas fluorescens*, *Salmonella typhimurium* and *Escherichia coli* when the media selective for these microorganisms are supplemented with either catalase or sodium pyruvate. Rayman *et al*. (1978) reported that the addition of pyruvate to nonselective tryptic soy agar greatly increases the recovery of heat-injured *Salmonella senftenberg*. Thus, it is recommended to include a supplement to scavenge superoxide from general non-selective media. This supplement is made of collection of wide range of "repairing compounds" to cover most types of injuries caused to a broad index of microorganisms. Then, this media are tested against standard unsupplemented media

to confirm the absence of toxicity to microbial cells. This will improve detectability of microorganisms and hence furtherly decrease the risk of getting false negative results (McDonald *et al*., 1983).

Although the commercially studied disinfectants were similar in the composition of the main active biocidal agents, the manufacturers have incorporated about 20 to 25 other constituents in their formulae that they keep confidential. Nevertheless, these components may have antimicrobial properties and thus may impact biocidal agent true activity and efficiency. Example of these components but not limited to are stabilizers, anticorrosives, surfactants, *etc*. The importance of disinfectant neutralization procedure validation generally resides on two facts: first of all it is considered important preliminary step in the determination of disinfectant true activity. The second aspect is that it could be incorporated in EM media such as contact plates and as broth for surface swabbing technique to detect low level of attenuated microbial contamination in hostile environment. A successful neutralization program is assessed by its ability to detect attenuated microorganisms – especially those having deleterious effect in the manufacturing process and product quality – in environmental samples in the presence of residual disinfectants. The last consideration is that if NE could not be estimated correctly the result will be improper biocidal agent potency determination which may be reflected in the failure of sanitization program of clean rooms and crippling microbes that may pass undetected in EM samples causing serious trouble in pharmaceutical manufacturing and other healthcare settings which impair patient health safety, cost companies huge financial loss and finally their reputability. The future plan of implementing of Rapid Microbiological Methods (RMMs) in this field is dependent to a large extent on intelligent approach of neutralization procedure so that the sensitivity of RMM instrumentation is not affected adversely. It can be concluded that in-house made neutralizing broth is a suitable candidate for neutralizing commercial peroxygen/Ag^{+}-based biocidal agents by combined chemical and dilution (at 1:100 (v/v)) inactivation to recover the tested environmentally important cocci and *C. albicans* as an objectionable yeast.

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ORIGINAL PAPER

Effectiveness of Antipseudomonal Antibiotics and Mechanisms of Multidrug Resistance in *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a leading human pathogen that causes serious infections at various tissues and organs leading to life threatening health problems and possible deadly outcomes. Resistance patterns vary widely whether it is from hospitals or community acquired infections. Reporting resistance profiles to a certain antibiotics provide valuable information in a given setting, but may be extrapolated outside the sampling location. In the present study, *P. aeruginosa* isolates were screened to determine their susceptibilities against antipseudomonal antimicrobial agents and possible existing mechanisms of resistance were determined. Eighty-six isolates of *P. aeruginosa* were recovered. Isolates representing different resistance profiles were screened for the existence of three different resistance mechanisms including drug inactivation due to metallo-β-lactamases, drug impermeability by outer membrane proteins and drug efflux. All tested isolates showed uniform susceptibility (100%, n = 86/86) to piperacillin, meropenem, amikacin, and polymyxin B. A single isolate was found to be imipenem resistant (99%, n = 85/86). The possible mechanisms of resistance of *P. aeruginosa* to imipenem involve active drug efflux pumps, outer membrane impermeability as well as drug inactivating enzymes. These findings demonstrate the fundamental importance of the *in vitro* susceptibility testing of antibiotics prior to antipseudomonal therapy and highlight the need for a continuous antimicrobial resistance surveillance programs to monitor the changing resistance patterns so that clinicians and health care officials are updated as to the most effective therapeutic agents to combat the serious outcomes of *P. aeruginosa* infections.

Key words: *Pseudomonas aeruginosa*, antibiotics, antimicrobial, carbapenems, efflux pump, mechanisms, metallo-β-lactamases, polymyxins, mutidrug, resistance

Introduction

Pseudomonas aeruginosa is a ubiquitous opportunistic Gram-negative non fermentative bacterium of clinical significance and preferentially causes severe infections in patients with diseases including cancer, diabetes, cystic fibrosis, deliberate immunosuppression, and major surgery (Osman *et al*., 2010). The bacterium can colonize implanted devices, catheters, heart valves, ventilators or dental implants resulting in device-associated hospital acquired infections which are of major concern globally (El-Kholy *et al*., 2012). *P. aeruginosa* is associated with different types of infections which cause morbidity and mortality (Driscoll *et al*., 2007; Suárez *et al*., 2010). The high prevalence of *P. aeruginosa* in developing countries and resource-limited parts of the world as well as other parts of the world owes much to its battery of secreted virulence factors as well as to its high resistance to antimicrobial and various chemical agents (Van Delden and Iglewski, 1998).

Much evidence on its prominence and emergence as a life threatening pathogen is attributed to its high intrinsic and acquired resistance to diverse classes of antimicrobial agents including antipseudomonal agents (Wolter *et al*., 2009). The resistance rates of *P. aeruginosa* are escalating globally posing a serious public health threat (Jones *et al*., 2003). *P. aeruginosa* is characterized by increased resistance to antipseudomonal agents (Strateva *et al*., 2007). *In vitro* sensitivity tests are used as a guide for appropriate antimicrobial therapy prior to antibiotic treatments.

Geographical variations and differences in the resistance rates of *P. aeruginosa* usually correlate with the prescription patterns of antimicrobial agents prescribing habits, overuse of antimicrobial agents in different parts of the world, and the selective pressure of certain

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antibiotics (El Zowalaty *et al.*, 2015). The literature is rich in surveillance studies from all over the world reporting varying resistance rates among *P. aeruginosa* against different antimicrobial agent. Recently, studies have focused on the decreased susceptibility of *P. aeruginosa* to currently used antipseudomonal agents, including β-lactams, aminoglycosides, and fluoroquinolones (Al-Tawfiq, 2007) since resistance of *P. aeruginosa* to carbapenems, piperacillin, and other highly active antibiotics has emerged and is increasing which makes treatment of *P. aeruginosa* infections troublesome (Strateva *et al.*, 2007).

Recently, resistance rates of *P. aeruginosa* clinical isolates recovered from patients admitted to Zagazig University hospitals in Egypt against different classes of antimicrobial agents were reported (El Zowalaty, 2012). The current study further examined the susceptibilities and possible resistance mechanisms of *P. aeruginosa* isolates collected from hospitalized patients against selected antipseudomonal agents that are available in the Egyptian pharmaceutical market and are frequently prescribed to patients.

Experimental

Materials and Methods

Study site. The specimens were collected from Zagazig university-affiliated hospitals as well as outpatient clinics. Meropenem, polymyxin B, and piperacillin have not been previously prescribed while imipenem was sometimes prescribed (depending on socioeconomic factors). Other antimicrobial agents including ceftazidime, ceftriaxone, ciprofloxacin, amikacin, gentamicin, cefotaxime, are first line frequently prescribed antibiotics to all patients regardless of the pathogen antimicrobial sensitivity profile.

Ethics statement. Ethical approval to perform the study was obtained from all patients. Consent was obtained from each patient included in the study as well as from Zagazig University hospital and the Department of Microbiology ethical committee. All samples were de-identified and analyzed anonymously.

Bacterial isolates. Eighty-six non-repeat clinical isolates of *P. aeruginosa* were collected from hospitalized patients with urinary tract infections, respiratory tract infections, cystic fibrosis, wounds, ear infections, and septicaemia. All patients were under antimicrobial clinical protocol treatment consisting of cefotaxime, ceftazidime, ceftriaxone, gentamicin, or ciprofloxacin. Specimens were collected as urine, purulent discharge or sputum according to the type of infection. The isolates were collected, identified, and confirmed to be *P. aeruginosa* by routine conventional biochemical tests. *P. aeruginosa* isolates were cultured aerobically in Muller-Hinton broth for 16–24 hours at 37°C. The isolates were Gram stained, and first inoculated into brain heart infusion medium, then cultured on cetrimide agar. Gram-negative bacilli were further confirmed to be *P. aeruginosa* using conventional biochemical characteristics. The isolates were further tested for the presence of cytochrome oxidase enzyme using oxidase reagent (bioMérieux, Marcy-l'Etoile, France), oxidative fermentation, and ability to grow at 42°C. All isolates were stored in Mueller-Hinton broth (Difco Laboratories, Maryland, USA) with 30% glycerol (Merck, Darmstadt, Germany) at −20°C until additional tests were performed as described below. The standard laboratory reference strain *P. aeruginosa* ATCC 90271 (Manassas, VA, USA) was used as control in this study.

Antibiotics. The following antibiotics were obtained from the corresponding supplier: amikacin (Bristol Myers Squibb, Cairo, Egypt), imipenem (Merck Sharp and Dohme, Hertfordshire, U.K.), meropenem (AstraZeneca, Cheshire, U.K.), ticarcillin and piperacillin, (Sigma-Aldrich, Saint Louis, Missouri, USA), and polymyxin B (Novo Industry A/S, Copenhagen, Denmark).

Antimicrobial susceptibility testing. The minimum inhibitory concentrations (MICs) (μg/ml) of different antibiotics were determined on Muller-Hinton agar dilution method as previously described (Andrews, 2001) and in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015) .

Detection of metallo-β-lactamases (MβLs). Detection of MβLs in imipenem resistant *P. aeruginosa* isolate was performed using EDTA-disc diffusion synergy test as described previously (Jesudason *et al.*, 2005). An overnight broth culture of the carbapenem resistant isolate was adjusted to 0.5 McFarland opacity standards and was used to inoculate plates of Mueller-Hinton agar. After drying the plates by incubation at 37°C for one to 2 h, a 10 μg imipenem disc (Oxoid Ltd., Basingstoke, Hampshire, England) and a blank filter paper disc were placed 10 mm apart from edge to edge, 5 μl of 0.5 M EDTA disodium. Aqueos solution, prepared by dissolving 186.1 g in 1000 ml of distilled water and adjusting it to pH 8.0 using NaOH and sterilized by autoclaving, was then applied to the blank disc, which resulted in a concentration of approximately 750 μg EDTA per disc. After overnight incubation, the presence of an enlarged zone of inhibition was interpreted as EDTA synergy positive. *P. aeruginosa* ATCC 90271 was used as negative control microorganism.

β-lactam hydrolysis assays. The β-lactamase activity was determined by spectrophotometric assay using β-lactam antibiotics (ampicillin and imipenem) as substrates in the presence and absence of β-lactamase inhibitors (clavulanic acid and *p*-chloromercuriben-

zoate; *p*-CMB). The effects of crude β-lactamase extract on various β-lactam antibiotics were determined as previously described (Danel *et al*., 1999; Ayala *et al*., 2005). Briefly, the hydrolytic activity of crude β-lactamase extracts of *P. aeruginosa* isolates to degrade β-lactam antibiotics was assayed using UV spectrophotometry at 37°C in the presence of phosphate bufferd saline at pH 7.0. The following wavelengths were used: ampicillin, 235 nm; cefotaxime, 260 nm; ceftazidime, 260 nm; and imipenem, 299 nm. Inhibition of enzymatic activity of crude extract was performed using different concentrations of clavulanic acid, 2 µg/ml; tazobactam, 4 µg/ml; oxacillin, 1 µM; EDTA (2 µM and 5 µM); and sodium *p*-chloromercuribenzoate ($_p$-CMB), 1 µM and assayed following the incubation of the crude extract for 20 minutes at 25°C in presence of the previously mentioned concentrations of the inhibitor. Each of the crude β-lactamase extracts or cell lysates of isolates, at a fixed volume of 200 µl aliquot of crude extract, was mixed with the antibiotic solution at zero time in 0.1 M phosphate buffer (pH 7.0) at 37°C and the change in the concentration was monitored by measuring the absorbance at the corresponding wavelength. The crude extract or cell lysate was pre-incubated with the inhibitor for 20 minutes at 37°C. A control without the inhibitor was used.

Detection of efflux pumps activity. The existence of efflux mechanism in *P. aeruginosa* isolates was determined by detection of the accumulation of ethidium bromide in the presence or absence of efflux inhibitors as described previously with modifications (Nishino and Yamaguchi, 2004). Overnight cultures were adjusted to approximately 10^5 cfu/µl. Washed cells were resuspended in 20 µl of 1 µg/ml ethidium bromide with or without either 100 µM dinitrophenol (DNP, Steinheim, Germany), 0.4% glucose or 0.1% of toluene and were incubated at 37°C for 15 min. Cells were collected by centrifugation at 1200 × g for 5 minutes and re-suspended in 10 µl of PBS. Five microliters aliquots of cell suspensions were spotted onto the surface of 1% agarose gel and examined over ultraviolet transilluminator. Drug accumulation in *P. aeruginosa* cells was observed as bright fluorescence of ethidium bromide. To further confirm the presence of efflux system of *P. aeruginosa* resistant isolate, the MICs of antimicrobial agents for the resistant isolate were determined in the presence and absence of 100 µM of the efflux pump inhibitor DNP and dicyclohexylcarbodiimide (DCCD, Steinheim, Germany).

Molecular detection of antimicrobial resistance determinants. Chromosomal DNA template was extracted and conventional PCR was performed. Resistant isolates were screened for resistance genes using sets of specific oligonucleotide primers as follows: $bla_{\text{IMP-1}}$ forward (CTACCGCAGCAGAGTCTT TGC) and $bla_{\text{IMP-1}}$ reverse (GAACAACCAGTTTTG CCTTACC) (Poirel *et al*., 2000), $bla_{\text{VIM-1}}$ forward (TCTACA TGACCGCGTCTGTC) and $bla_{\text{VIM-1}}$ reverse (TGTGCTTTGACAACGT TCGC) (Poirel *et al.,* 2000), $bla_{\text{OXA-50}}$ forward (AATCCGGCGCTC ATCCATC) and $bla_{\text{OXA-50}}$ reverse (GGTCGGCGACTGAGGC GG) (Girlich *et al*., 2004), $bla_{\text{IBC-2}}$ forward (CGTTCCATACAGAAGCTG) and $bla_{\text{IBC-2}}$ reverse (AAGCAGACTTGCCTGA) (Mavroidi *et al*., 2001), *mex*R forward (AACCAATGAACTACCCCGTG) and *mex*R reverse (ATCCTCAAGCGGTTG CGCGG) (Dubois *et al*., 2001) were used to amplify $bla_{\text{IMP-1}}$, $bla_{\text{VIM-1}}$, $bla_{\text{OXA-50}}$, $bla_{\text{IBC-2}}$, and *mex*R genes, respectively. The isolates were inoculated into 5 ml of trypticase soy broth and incubated for 16 hours at 37°C with shaking. Cells from 1.5 ml of an overnight culture were harvested by centrifugation for 10 minutes at 15 000 × g. The supernatant was decanted and chromosomal DNA from cell pellets was extracted. Whole-cell genomic DNA of *P. aeruginosa* isolates was extracted using a QIAamp DNA Mini Kit (Qiagen, Maryland, USA) according to manufacturer's instructions with one hour incubation at 56°C using 20 µl proteinase K solution. DNA was purified using Qiagen DNeasy Mini spin column protocol. DNA was hydrated in 150 µl of DNA elution solution to increase the final DNA concentration in the eluate. Extracted DNA was aliquoted, stored at –20°C until use. PCR analysis was performed using DNA thermal cycler Biometra Tpersonal Combi (Whatman Biometra, Goettingen, Germany) in a reaction mixture of 100 µl volume containing 10 µl (final concentration of 1 µM or 1 picomole per µl) of each upstream primer, 10 µl (final concentration of 1 µM or 1 picomole per µl) of each downstream primer, 5 µl (final concentration of 250 nanogram) of DNA template, 50 µl PCR Master Mix, 2X (containing 50 units/ml *Taq* DNA polymerase, 400 µM deoxynucleotides triphosphate [dATP, dGTP, dCTP, dTTP] and 3 mM $MgCl_2$ and nuclease-free water was added to complete the volume of the reaction to 100 µl. PCR conditions for the amplification were as follows: an initial incubation of 10 min at 37°C and an initial denaturation step at 94°C for 5 min, followed by 30 cycles of DNA denaturation at 94°C for 1 min, primer annealing at 54°C for 1 min, and primer extension at 72°C for 1.5 min. After the last cycle, the products were stored at 4°C. The PCR amplification products were analyzed and revealed using 2% agarose gel electrophoresis in 1X trisacetate buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]). Ten microlitres of each PCR product were mixed with 2 µl of blue/orange 6X loading dye and were subjected to electrophoresis for 45 min at 80 V using horizontal apparatus. After electrophoresis, the ethidium bromide-stained PCR amplification products were visualized under UV light transilluminator. The size of each

PCR products was determined by comparing of PCR products with DNA molecular size marker (1 kb/100 bp ladder; Promega, WI, USA).

Electrophoretic separation of outer membrane proteins. The outer membrane proteins were analyzed using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), as previously reported (Laemmli, 1970), with 10.7% (wt/vol) acrylamide and 0.3% (wt/vol) *N*, *N*9-methylenebisacrylamide in the running gel. Samples for SDS-PAGE were treated with 2% SDS w/v – 5% w/v 2-mercaptoethanol at 100°C for 5 min or at 37°C for 10 min, and then subjected to electrophoresis at a constant current of 25 mA at 4°C. The gel was stained using coomassie brilliant blue to visualize the protein bands. The size of the proteins was determined as compared to size of a protein marker (Bio-Rad protein ladder).

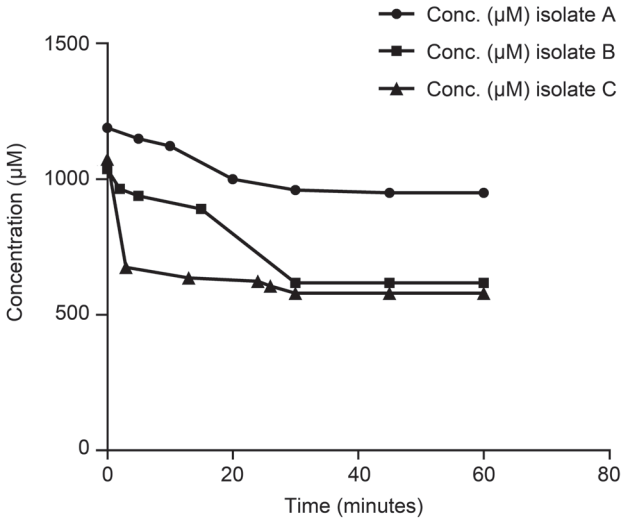

Fig. 1. Effect of crude β-lactamase extracts of *P. aeruginosa* isolates on ampicillin concentration in absence of β-lactamase inhibitors.

Results

Antimicrobial susceptibility testing. Antimicrobial susceptibility results were interpreted using the CLSI breakpoints (CLSI, 2015). It was reported previously that *P. aeruginosa* isolates were highly resistant to commonly prescribed antibiotics (El Zowalaty, 2012). The resistance rates of *P. aeruginosa* clinical isolates to one or more antimicrobial agents were shown in Table I. The respective MIC_{90} distributions of different antibiotics for 86 isolates of *P. aeruginosa* were shown. All tested isolates of *P. aeruginosa* were susceptible to the antibiotics piperacillin, meropenem, amikacin, and polymyxin B. A single isolate was found resistant to imipenem. For other antibiotics tested namely ticarcillin, ciprofloxacin, ceftazidime, and gentamicin the susceptibility rates were shown in Table I. *P. aeruginosa* isolated strains were highly resistant to all other antibiotics tested. In addition, all of the 86 clinical isolates of *P. aeruginosa* were resistant to more than three classes and were defined as MDR. The resistance rates of *P. aeruginosa* isolates to one or more antimicrobial agents were shown in Figure 1 and Table II. In total, forty-two out of eighty-six isolates were found to be resistant to three or more antimicrobial agents and the rate of multidrug resistant (MDR) *P. aeruginosa* isolates was 47.1% (El Zowalaty, 2012).

In order to explore the possible existing antimicrobial resistance mechanisms in the as-found MDR *P. aeruginosa* isolates, the detection of MβLs, spectrophotometric β-lactamase assays, efflux pump activity, outer membrane protein profiling, and molecular detection of resistance determinants were performed. A single isolate was found to be imipenem resistant as determined using the disk susceptibility testing and had

Table I
Susceptibility of *P. aeruginosa* isolates ($n = 86$) to different antimicrobial agents classes.

Antibiotic	MIC_{50}	MIC_{90}	Susceptible[a]	Resistant[a]	Intermediate[a]
Meropenem	2	2	100	0	0
Imipenem	4	4	98.9	0	1.1
Piperacillin	8	32	100	0	0
Ticarcillin	64	128	80.9	0	19.1
Polymyxin B	2	2	100	0	0
Amikacin	8	8	100	0	0
Ceftriaxone	32	256	0	70.8	29.2
Ceftazidime	8	32	59.5	28.1	12.4
Cefotaxime	64	256	0	43.8	56.2
Gentamicin	128	512	12.3	7.9	79.8
Ciprofloxacin	1	128	60.7	6.7	32.6

[a] Percentage of all isolates. MICs were determined and isolates were defined as resistant, intermediate resistant, and sensitive according to CLSI guidelines.

Table II
Profiles of *P. aeruginosa* antibiotic resistance.

No. of agents to which isolates were resistant	Frequency	Percent
0	3	3.4
1	20	22.5
2	24	27
3*	23	25.8
4*	14	15.7
5*	4	4.5
6*	1	1.1

* Forty-two out of 89 (47.1%) isolates were resistant to three or more antimicrobials and were defined as MDR.

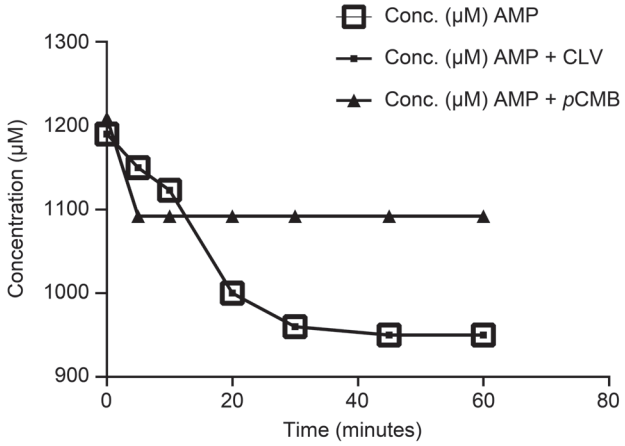

Fig. 2. Effect of crude β-lactamase extract of *P. aeruginosa* (isolate C) on ampicillin concentration in presence and absence of β-lactamase inhibitors (CLV: clavulanic acid, *p*CMB: *p*-chloromercuribenzoate).

a zone diameter of 10 mm. In presence of EDTA disc, the zone diameter of imipenem increased to 21 mm.

The spectrophotometric β-lactamase assays showed a decrease in the concentration of ampicillin due to the effect of the crude β-lactamase extract activity Figure I. The crude β-lactamase extract activity was not inhibited by clavulanate, tazobactam or oxacillin while in presence of *p*-chloromercuribenzoate ($_p$CMB) the crude β-lactamase extract activity was inhibited as shown in Figure 2. The crude β-lactamase extract activity had no effect on the concentration of cefotaxime and ceftazidime.

As shown in Figure 3, the crude β-lactamase extract activity of IMP-sensitive isolate (B) had no effect on the concentration of imipenem while there was a decrease in the concentration of imipenem that might be attributed to the effect of the crude β-lactamase extract activity of IMP-resistant isolate (C). The crude β-lactamase extract activity of IMP-resistant isolate (C)

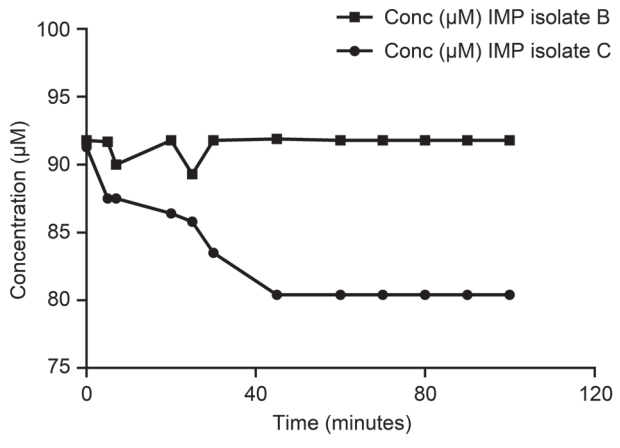

Fig. 3. Effect of crude β-lactamase extracts of *P. aeruginosa* isolates (B: imipenem sensitive and C: imipenem resistant) on imipenem concentration.

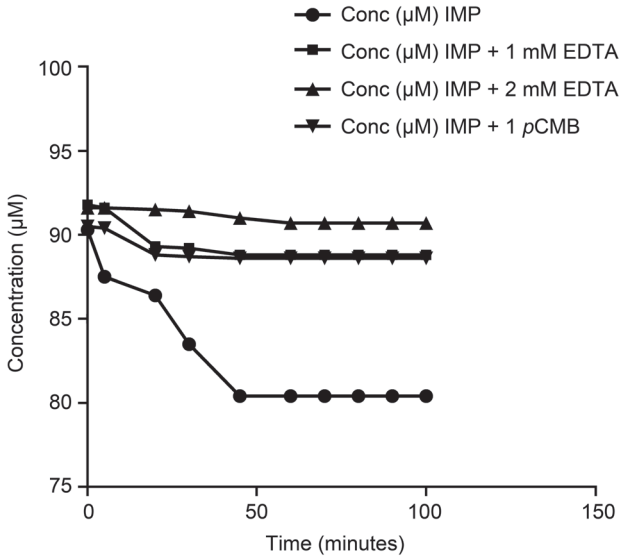

Fig. 4. Effect of crude β-lactamase extract of *P. aeruginosa* isolate C (imipenem resistant) on imipenem concentration in presence and absence of enzyme inhibitors (EDTA and *p*CMB).

was inhibited in presence of either EDTA or $_p$-CMB as shown in Figure 4.

Resistance through the efflux pump. It was found that IMP-resistant isolate was positive for efflux pump activity as shown in Figure 5. The reduction in fluorescence intensity was observed in the absence of efflux pump inhibitor and in the presence of glucose which is an efflux pump energizer. In the presence of efflux pump inhibitor or toluene, the latter is a membrane permeabilizer; there was an increase in the fluorescence intensity. *P. aeruginosa* ATCC 90271 was used as negative control. The effect of efflux pump inhibitors (DNP, and DCCD) on the MIC of imipenem resistant isolate was determined. The MICs of ticarcillin, imipenem,

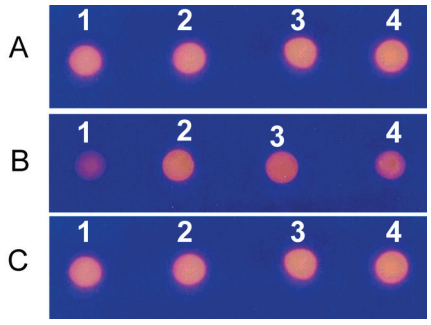

Fig. 5. Detection of efflux pump activity using ethidium bromide accumulation test showing the effect of efflux pump inhibitor (dinitrophenol) and glucose (pump energizer) on the accumulation of ethidium bromide in cells of *P. aeruginosa* (A) *P. aeruginosa* ATCC 90271, (B) *P. aeruginosa* imipenem resistant isolate and (C) *P. aeruginosa* imipenem sensitive isolate. Cells loaded with ethidium bromide in presence of glucose (1), cells loaded with ethidium bromide in presence dinitrophenol (DNP) (2), cells loaded with ethidium bromide in presence toluene (3), and cells loaded with ethidium bromide (4).

meropenem, and norfloxacin in the presence and absence of the efflux inhibitors was determined. The reduction in MIC of a certain antibiotic with DNP or DCCD is indicative of resistance against this antibiotic mediated by an efflux system.

The addition of DNP and DCCD enhanced the activities of selected antibiotics by lowering the MIC as observed in the reduction of MIC. In the presence of DNP and DCCD, the largest effect was observed with ticarcillin and norfloxacin (a 32-fold decrease in MIC) followed by aztreonam (16-fold decrease in MIC). An intermediate effect was obtained with meropenem (8-fold decrease in MIC). These results emphasized the existence of efflux-mediated resistance in the tested isolates.

Polymerase chain reaction. The tested isolates carried the *mex*R gene as was determined using PCR analysis. In addition, PCR analysis revealed the absence of the screened $bla_{\text{IMP-1}}$, $bla_{\text{VIM-1}}$, $bla_{\text{OXA-50}}$, and $bla_{\text{IBC-2}}$ genes in the tested isolates; however this does not exclude the presence of other resistance determinants.

Analysis of outer membrane proteins. The outer membrane protein profiles of *P. aeruginosa* isolates representing different resistance profiles showed the presence of a protein band of approximate weight of 50 kDa, in addition to several bands of approximate weights of 17, 23, 35, 38 and 49 kDa.

Discussion

P. aeruginosa gains specific concern among health care officials especially in resource limited settings (RLS) and developing countries. There are only few recent reports on the antimicrobial resistance of *P. aeruginosa* isolated from patients in Egypt (Abdel *et al.*, 2010). The present study reported the *in vitro* activity of antipseudomonal drugs against *P. aeruginosa* clinical isolates. Antibiotic treatment guidelines recommended for *P. aeruginosa* are not similar due to different resistance profiles among isolates from different sources.

The current study showed that all tested *P. aeruginosa* clinical isolates were uniformly susceptible to meropenem, piperacillin, imipenem, amikacin, and polymyxin B. In this study, antimicrobial susceptibility testing of eighty-six clinical isolates of *P. aeruginosa* was performed using the agar dilution method according to the guidelines of the CLSI (CLSI, 2015). The MIC_{50} and MIC_{90} were 2 and 2 μg/ml for polymyxin and meropenem; 8 and 8 μg/ml for amikacin and 8 and 32 μg/ml for piperacillin, respectively.

On the contrary, carbapenem resistance among *P. aeruginosa* have been increasing in other parts of the world posing a continuous threat and possible looming emergence of highly serious pandrug resistant *P. aeruginosa*, which may be explained in parts by several factors including the intensive use of carbapenems which enhanced the emergence of carbapenem-resistant isolates (Walsh, 2010).

It has been reported that extensive use and consumption of carbapenems forced the emergence of resistance to these antimicrobial agents (Benčić and Baudoin, 2001). This probably will present a particular challenge and could results in a major global problem since carbapenems are the final choice in the treatment of the difficult-to-treat pseudomonal infections and they are often the last resort for treating infections due to multidrug resistant isolates (Nordmann, 2010). The emerging carbapenem resistance will be very dangerous and of serious complications resulting in pan drug resistant strains leading to increased mortality rates (Hong *et al.*, 2015; Liu *et al.*, 2015).

In the present study, the resistance rate to imipenem was relatively low and accounted for only 1%. Except for a single isolate which was found to be imipenem resistant with an MIC of 16 μg/ml, all isolates were sensitive to imipenem with MIC of 4 μg/ml, MIC_{50} and MIC_{90} were equal to 4 μg/ml. Contrary to the present findings, higher resistance rate to imipenem were reported where it was found that 29% and 14.3% (Hassan *et al.*, 2010) of *P. aeruginosa* clinical isolates were resistant to imipenem. In another study from Egypt, 11.9% out of 261 clinical isolates of *P. aeruginosa* isolated from Zagazig University hospital between 2003 and 2004 were resistant to imipenem as determined by disc diffusion method (El-Behedy *et al.*, 2002).

Contrary to the current study, the overall resistance rates of *P. aeruginosa* to imipenem are on continuous increase globally. In a Saudi Arabian hospital between 1998 and 2003, rates were 2.6% and 5.8%, respectively (Al-Tawfiq, 2007). In a study from California, USA, the annual imipenem resistance rates against *P. aeruginosa* isolates increased from 2% in 1996 to 18% in 1999 (Huang *et al.*, 2002).

The susceptibility rate to imipenem in clinical isolates of *P. aeruginosa* in a study in Spain was 89.7% from 2005 to 2010 (Casal *et al.*, 2012). The susceptibility and resistance rates of *P. aeruginosa* to imipenem in USA were reported to be 24% and 70%, respectively.

In the present study, all tested isolates of *P. aeruginosa* were sensitive to meropenem with MIC_{50} and MIC_{90} of 2 and 2 μg/ml. Contrary to the present results, a study in Egypt reported a resistance rate of 37.7% to meropenem among *P. aeruginosa* isolated from hospitalized cancer patients (Decousser *et al.*, 2003). This is explained by the differences in the pattern of antibiotic prescription and usage between the two studies. The susceptibility rate to meropenem in clinical isolates of *P. aeruginosa* in a study in Spain was 92.98% from 2005 to 2010 (Casal *et al.*, 2012).

According to our findings, the susceptibility rate of ticarcillin was found to be 80.1%. Similarly, the susceptibility rate to ticarcillin was reported to be 81% in a study in France (Decousser *et al*., 2003). The resistance rate of *P. aeruginosa* in the present study to ticarcillin was found to be 19.9% which was much lower than the resistance rate reported in a study in Egypt, where the resistance rate of *P. aeruginosa* isolated from hospitalized cancer patients to ticarciilin was found to be 91.7% (Ashour and El-Sharif, 2009) which is much higher than the resistance rate in the present study which was found to be 19.9%.

All *P. aeruginosa* isolates, in the present study, were susceptible to piperacillin with MIC_{50} and MIC_{90} of 8 and 32 μg/ml, respectively. On the other hand, only 53% of 303 clinical isolates of *P. aeruginosa* collected from patients in five hospitals in the greater Cairo region between July 1999 and June 2000, were susceptible to piperacillin (El Kholy *et al*., 2003).

In line with the literature (Landman *et al*., 2008), the present data revealed that polymyxin B had *in vitro* activity against the isolates tested, with susceptibility rates of 100% for *P. aeruginosa.* In contrast to the present findings recent studies showed resistance of *P. aeruginosa* to polymyxin B. While *P. aeruginosa* are typically susceptible to polymyxins, resistance has been known to occur as polymyxin usage increases, the emergence of resistance to this agent of last resort becomes an obvious concern (Landman *et al*., 2005).

All *P. aeruginosa* isolates in the present study were susceptible to amikacin with MIC_{50} and MIC_{90} of 8 and 8 μg/ml, respectively. In agreement with the current amikacin susceptibility results were data in studies in Turkey where the susceptibility rate of *P. aeruginosa* strains to amikacin was 100% (Gerçeker and Gürler, 1995) and in Jamaica (Brown and Izundu, 2004).

To determine the possible mechanisms of resistance of *P. aeruginosa* isolates to antibiotics, the isolates were tested for β-lactamase production and efflux pumps-mediated resistance. *P. aeruginosa* is known to possess β-lactamase-mediated resistance to antibiotics (Walsh, 2010). In the present study, 48.8% of isolates showed β-lactamase production activity. The reduction in MICs of ticarcillin, aztreaonam, and meropenem in the presence of an efflux pump inhibitors (DNP or DCCD) suggested the contribution of an efflux-mediated mechanism in tested *P. aeruginosa* isolates to different antibiotics. This finding was consistent to other reports which showed a major contribution of efflux as the major resistance mechanism in *P. aeruginosa* (Drissi *et al*., 2008).

The possible mechanisms of low-level imipenem resistance in the imipenem resistant isolate were investigated. First, the effect of EDTA on the zone of inhibition by imipenem disc was performed. The addition of EDTA increased the inhibition zone from 11 mm to 21 mm, which might suggest a MβL-mediated imipenem resistance (Jesudason *et al*., 2005). Therefore, PCR analysis of the isolate was performed to detect IMP and VIM MβLs, which was supported by the full sensitivity of the isolate to meropenem. Although, the present PCR results excluded the presence of the presence of the aforementioned metallo-β-lactamase genes, several types of MBL enzymes including IMP-type, VIM-type, SPM-1, GIM-1, SIM-1 – have been reported in *P. aeruginosa* (Queenan and Bush, 2007). In the present study, imipenem resistance may be explained by the presence of efflux pump-mediated mechanism using the constitutively expressed MexAB-OprM efflux system which extrudes most β-lactams in its broad substrate spectrum including imipenem (Quale *et al*., 2006) and the MexEF-OprN system although not contribute to β-lactam efflux, its overexpression indirectly affects the efficacy of carbapenems through a concomitant reduction of the carbapenem-specific OprD porin protein (Rodriguez-Martinez *et al*., 2009). Another possibility is the overproduction of chromosomal AmpC β-lactamase as shown in the spectrophotometric hydrolysis of imipenem. The inducible effect of some β-lactamases slowly hydrolyses imipenem as shown in several studies which demonstrated the role of cephalosporinase in imipenem resistance among *P. aeruginosa* (Farra *et al*., 2008).

Other mechanisms of carbapenem resistance have been identified such as class Clavulanic acid inhibited ESBLs with hydrolytic activity that encompasses imipenem such as GES-2 (Poirel *et al*., 2001). Thus, imipenem resistance in the present study is probably due to several interplay mechanisms including AmpC overproduction, efflux pumps, and loss of OprD rather than due to the production of specific MBLs, although a novel MBLs may be involved (Shehabi *et al*., 2011). In agreement to the present study, *P. aeruginosa* isolates were reported negative to $bla_{VIM1a,b}$ and $bla_{IMP1,2}$ genes, however isolates were found positive to class 1 integrons (Kouda *et al*., 2009). Contrary to the absence of integron mediated MBLs in the present study, class 1 integron containing MBL-mediated resistance was reported elsewhere (Tawfik *et al*., 2012). *P. aeruginosa* can very often accumulate different resistance mechanisms leading to increased resistance to carbapenems as well as other antimicrobial agents (Farra *et al*., 2008).

ESBLs were reported in *P. aeruginosa* isolates (Strateva and Yordanov, 2009) and ESBLs and MBLs were detected at high prevalence rate in neighbouring regions (Woodford *et al*., 2008). In addition, ESBLs are on the rise globally as resistant determinants among *P. aeruginosa* isolates (Livermore, 2002). A possible resistance mechanism of these isolates could be due to the loss of porin (OprD) (Quale *et al*., 2006). The discrepancy

between the results of the EDTA-disc diffusion synergy test, spectrophotometric assay of imipenem and the PCR might be explained by the presence of carbapenemases other than IMP- or VIM-type MBLs. This is consistent with the other findings that in the absence of specific carbapenemases, the mechanisms leading to carbapenem resistance are usually multifactorial and it has been recently implicated to involve the interplay among various contributory factors as augmented antibiotic extrusion efflux pumps, increased chromosomal cephalosporinase or AmpC activity, and reduced OprD porin expressions (Rodriguez-Martinez *et al*., 2009).

In summary, the results of the present study demonstrate the effectiveness of carbapenems against the problematic *P. aeruginosa*. Independent on the geographical location, meropenem, piperacillin, amikacin, polymyxin B, and imipenem were the most active agents against *P. aeruginosa*. Monotherapy with polymyxin B may be adequate to control *P. aeruginosa* infections. Although data presented in this study revealed that no resistance of clinical isolates of *P. aeruginosa* against piperacillin, meropenem, polymyxin B, and amikacin was detected, the importance of the results is indicating that escalating rates of MDR among isolates still pose a clinical problem for patients and health officials. The prevalence of multi-drug-resistant *P. aeruginosa* (MDR-PA) in many parts of the world is concerning and will jeopardize the current antimicrobial agents because efficacious antimicrobial therapeutic options are limited (Song, 2008).

Conclusions. One of the major scientific concerns in the medical community is that the antibiotic clinical protocol for the treatment of bacterial infections in private and governmental clinics and hospitals in developing countries is inappropriate. Antibiotics are prescribed without prior recommendation and knowledge of the *in vitro* antimicrobial susceptibility testing. In addition, over-the-counter (OTC) antimicrobial prescription among pharmacists and self-antibiotic medication among the public is a present ongoing phenomenon. The patterns of antibiotic usage in developing countries such as overuse, underuse, or inadequate dosing contribute to a great extent to the emergence of antimicrobial resistance in Gram negative bacteria (Barbosa and Levy, 2000; Essack *et al*., 2008).

The misuse of antibiotics will contribute to the failure of treatment as well as the emergence of new resistant bacterial strains. Furthermore, the present study highlights the importance of improvement or amendment of antibiotic drug policies and antibiotic stewardship in developing countries as well as globally (Essack *et al*., 2008). In addition, this alarming trend of resistance deserves attention and concern among health care providers and requires the continuation of antimicrobial surveillance studies worldwide and reduction in antibiotic use to control antibiotic resistance (Hamilton-Miller, 2004). Furthermore, search for new antimicrobial agents including nanoantimicrobial antibiotics and alternative therapeutic agents will help control the challenging and spreading resistance of *P. aeruginosa* to antimicrobial agents.

In developing countries, high proportion of patients in hospital and outpatient clinics receive antibiotic without prescription and the inappropriate antibiotic may be prescribed without prior antimicrobial sensitivity testing as well. One more issue is that, little data about the endemic antimicrobial resistance is available from developing countries, where over-the-counter antibiotic usage is a common phenomenon. Further studies are recommended to thorouglhy understand the different resistance machanisms, interactions among bacteria as well as to continue global surveillance studies to monitor the emerging resistance trends. This will help find appropriate and effective measures to restore the balance of coexistence between humans and bacteria. We are currently investigating antimicrobial resistance among zoonotic *P. aeruginosa* isolates.

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ORIGINAL PAPER

Streptococcus anginosus (*milleri*) Group Strains Isolated in Poland (1996–2012) and their Antibiotic Resistance Patterns

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Abstract

Streptococcus anginosus, *Streptococcus intermedius* and *Streptococcus constellatus* form a group of related streptococcal species, namely the Streptococcus Anginosus Group (SAG). The group, previously called "milleri" had been rarely described until 1980/1990 as source of infections. Nowadays SAG bacteria are often described as pathogens causing predominantly purulent infections. The number of infections is highly underestimated, as SAG strains are often classified in the microbiology laboratory as less virulent "viridans streptococci". Epidemiological situation regarding SAG infections in Poland has been unrecognized, therefore we performed a retrospective analysis of strains isolated between 1996 and 2012. Strains suspected of belonging to SAG were re-identified using an automated biochemical approach (Vitek2) and MALDI-TOF MS. We performed first analysis of antibiotic resistance among SAG strains isolated in Poland using automated methods (Vitek2), disk diffusion tests and E-Tests. We also performed PCR detection of resistance determinants in antibiotic resistant strains. Clonal structure of analyzed strains was evaluated with PFGE and MLVF methods. All three species are difficult to distinguish using automated diagnostic methods and the same is true for automated MIC evaluation. Our analysis revealed SAG strains are rarely isolated in Poland, predominantly from purulent infections. All isolates are very diverse on the genomic level as estimated by PFGE and MLVF analyses. All analyzed strains are sensitive to penicillin, a substantial group of strains is resistant to macrolides and the majority of strains are resistant to tetracycline.

Key words: *Streptococcus anginosus* group, *Streptococcus constellatus*, *Streptococcus intermedius*, *Streptococcus milleri*, antimicrobial resistance, MLVA, MLVF, PFGE

Introduction

Streptococci are a diverse group of Gram positive bacteria divided into multiple clusters based on mixed and constantly changing criteria. Recent classification reflects genetic relationships between groups and separates them into six divisions: (i) pyogenic, (ii) anginosus (formerly milleri), (iii) mitis/oralis, (iv) salivarius, (v) mutans, (vi) bovis and species of unknown evolutionary position such as *Streptococcus suis*. The genus includes species belonging to physiological flora, human and animal pathogens and opportunistic pathogens (Kohler, 2007). In the past, classification of Streptococci was based mostly on phenotypic traits such as carbohydrate fermentation and later on 16S rDNA sequencing. Unfortunately, even with the use of 16S rDNA sequencing, some of the strains cannot be clearly classified to previously described streptococcal species (Olson *et al.*, 2013; Thompson *et al.*, 2013). There are some methods available to discriminate streptococcal strains to the species and sub-species level (Poyart *et al.*, 1998; Picard *et al.*, 2004; Glazunova *et al.*, 2010; Zbinden *et al.*, 2011; Obszanska *et al.*, 2015a; Takao *et al.*, 2004). However, sequencing methods are not often used in diagnostic laboratory as the routine species identification and not all microbiology diagnostic laboratories have PCR set up.

Streptococcal groups that include the most pathogenic species are predominantly pyogenic (*Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* subsps. *equisimilis* and *dysgalactiae*) and mitis (includes *Streptococcus pneumoniae*) divisions.

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However, in recent years, an increasing number of streptococcal species is being considered as pathogenic to humans. Three of the species (*Streptococcus anginosus*, *Streptococcus intermedius* and *Streptococcus constellatus*, SAG – streptococcus anginosus group), described until 1980/1990 as rare sources of infections are currently described as pathogens causing predominantly purulent infections (Asam and Spellerberg, 2014).

The classification of *S. anginosus*, *S. constellatus* and *S. intermedius* is especially confusing as they are described in literature under multiple names. In 1991 Whiley and Beighton proposed a revision of systematic relationships between SAG strains previously described mostly as *Streptococcus milleri* (Whiley and Beighton, 1991). The proposed classification was recently confirmed by genome analysis using next generation sequencing (Olson *et al.*, 2013) and tandem repeat analysis (Obszanska *et al.*, 2015a).

Unfortunately, SAG are rarely considered as pathogens as they represent the natural flora of the upper respiratory, digestive and reproductive tracts, however, epidemiological data suggest the need to consider SAG an etiological factor of infections ranging from mild skin infections to severe purulent and life threatening infections (Giuliano *et al.*, 2012; Whiley *et al.*, 1992; Asam and Spellerberg, 2014). A correlation between *S. intermedius* and brain and liver abscesses and *S. constellatus* and infections of respiratory tract has been detected (Whiley *et al.*, 1992). The majority of published material about SAG is closely related to case reports and suggests that the number of infections caused by SAG is underestimated (Reissmann *et al.*, 2010). Case reports usually describe otherwise healthy patients without any previous medical history and describe the role of SAG in the formation of various kinds of abscesses, which are probably caused by high resistance to phagocytosis and inhibition of chemotaxis (Wanahita *et al.*, 2002). Possible molecular mechanisms involved in that process may include production of hydrogen sulfide from L-cysteine by L-cysteine desulfhydrase (Takahashi *et al.*, 1995). The other postulated possibility draws similarity from increased survival of bacteria upon phagocytosis (Wanahita *et al.*, 2002; Hoe *et al.*, 2002; Voyich *et al.*, 2004).

On the contrary to other streptococcal pathogens, the pathogenicity mechanisms of SAG are often unknown or poorly described (Sitkiewicz and Hryniewicz, 2010; Szczypa *et al.*, 2012; Obszanska *et al.*, 2014). Only recently genomic analyses of SAG genomes were published (Olson *et al.*, 2013; Thompson *et al.*, 2013). Experimental reports describing SAG virulence factors are scarce.

The epidemiological situation in Poland regarding infections with non β-hemolytic streptococci that are not *Streptococcus pneumoniae* has been not described so far. Therefore, we decided to investigate strains collected between 1996 and 2012 by reference centers (KORLD, KOROUN and Polmicro) in Poland and during nationwide viridans streptococci surveillance causing invasive infections (2008–2009).

Experimental

Materials and Methods

Bacterial strains. Bacterial strains were collected between 1996 and 2012 by KORLD, KOROUN and Polmicro reference centers during their routine performance and the data about infected patients was stripped from all identifiers, except age, sex and the source of isolation (*i.e.* blood, wound, puss *etc.*). Ethics approval was not required. The collected strains were analyzed retrospectively.

Collected strains were stored at –80°C and prior to all microbiological tests or DNA isolation were plated on Columbia agar plates (Becton Dickinson) with 5% sheep blood and incubated 24–48 h at 37°C, 5% CO_2. Prolonged incubation was required because of the slow growth of these bacteria.

Strains re-identification. To confirm species identification all strains sent to our reference centers were re-identified using Vitek2 (bioMerieux) automated system with GP card. For the identification we used higher initial inoculum (0.5 McFarland) than recommended by the manufacturer. All strains were also identified using IDStrep (bioMerieux) that allows to grow bacteria under optimal O_2/CO_2 conditions for time long enough to read the strip and MALDI-TOF MS (Brücker and bioMerieux) according to manufacturer's instructions. All strains were also tested for Lancefield antigen using Strep Plus test (Oxoid).

Antibiotic resistance. Screen for antibiotic resistance was performed using ST101 card (bioMerieux) dedicated to streptococci on Vitek2 system. The card allows detection of ampicillin, benzylpenicillin, cefotaxime, ceftriaxone, clindamycin, erythromycin, levofloxacin, linezolid, tetracycline, trimethoprim/sulfamethoxazole and vancomycin resistance. In some cases only subset of assays from the ST101 card was assayed by the instrument, so to confirm MIC values, for erythromycin, clindamycin, tetracycline, penicillin and linezolid we used E-test (bioMerieux) or M.I.C. Evaluator (Oxoid) or microdilution reference method and applied interpreted according to EUCAST (EUCAST, 2014). For strains with detected resistance to erythromycin, we performed double disc synergy test with erythromycin (15 μg) and clindamycin (2 μg) according to EUCAST (EUCAST, 2014).

Antibiotic resistance determinants. Chromosomal DNA was isolated as described by Obszanska and coworkers (Obszanska *et al.*, 2015a). Starters and reaction conditions used to detect antibiotic resistance determinants are presented in Table I.

Table I
Starters and conditions used for detection of antibiotic resistance determinants

Gene	Starter name	Sequence (5'-3')	Reaction	Expected product size	Source
*erm*A	ermA_F_IS3 ermA_R_IS3	TGGGTCAGGAAAAGGACATTTTACCAAGG ACATTCGCATGCTTCAGCACCTGT	35 cycles, Annealing 67°C, 25 sec. Elongation 72°C , 30 sec.	551 bp	This work
*erm*B	ermB_F_IS3 ermB_R_IS2	CGACGAAACTGGCTAAAATAAGTAAACAGG ATTGGAACAGGTAAAGGGCATTTAACG	30 cycles, Annealing 67°C, 25 sec. Elongation 72°C, 30 sec.	600 bp	This work
*mef*A	mef_F_IS mef_R_IS	CGTATTGGGTGCTGTGATTG AACCCAATTGGCATAGCAAG	30 cycles, Annealing 65°C, 25 sec. Elongation 72°C, 30 sec.	566 bp	This work
*tet*O	TetO_F_IS TetO_R_IS	CCATCCACATAGAAGTCCCG GTGGAACATATGCCGAACCT	35 cycles, Annealing 57°C, 25 sec. Elongation 72°C, 30 sec.	616 bp	This work
*tet*40	Tet 40 up Tet 40 dn	CTACCTGCTGTTCCGATTTGTC TGATGAAGGTATCACCGCAACC	35 cycles, Annealing 67°C, 25 sec. Elongation 72°C, 30 sec.		This work
*tet*M	tetM-Forward tetM-Reverse	AGTTTTAGCTCATGTTGATG TCCGACTATTTGGACGACGG	30 cycles, Annealing 55°C, 1 min Elongation 72°C, 1.5 min	1861 bp	(Doherty *et al.*, 2000)
*tet*W	TetW-FW TetW-RV	GAGAGCCTGCTATATGCCAGC GGGCGTATCCACAATGTTAAC	25 cycles, Annealing 64°C, 30 sec. Elongation 72°C, 30 sec.	168 bp	(Aminov *et al.*, 2001)
*tet*S	TetS-FWT 1 TetS-RVT 2	ATCAAGATATTAAGGAC TTCTCTATGTGGTAATC	30 cycles, Annealing 55°C, 1 min Elongation 72°C, 1 min	573 bp	(Charpentier *et al.*, 1993)
*tet*T	TetT-FW TetT-RV	AAGGTTTATTATATAAAAGTG AGGTGTATCTATGATATTTAC	25 cycles, Annealing 46°C, 30 sec. Elongation 72°C, 30 sec.	169 bp	(Aminov *et al.*, 2001)
*tet*K	tetK-up tetK-rev	TATTTTGGCTTTGTATTCTTTCAT GCTATACCTGTTCCCTCTGATAA	35 cycles, Annealing 50°C, 1 min Elongation 72°C, 1.5 min	1159 bp	(Trzcinski *et al.*, 2000)
*tet*L	tetL-up tetL-rev	ATAAATTGTTTCGGGTCGGTAAT AACCAGCCAACTAATGACAATGAT	35 cycles, Annealing 50°C, 1 min Elongation 72°C, 1.5 min	1077 bp	(Trzcinski *et al.*, 2000)
*tet*Q	MR6 MR7	CTGTCCCTAACGGTAAGG TTATACTTCCTCCGGCATCGG	35 cycles, Annealing 46°C, 30 sec. Elongation 72°C, 2 min	658 bp	(Chung *et al.*, 1999)

Restriction Fragment Length Polymorphism – Pulsed Field Gel Electrophoresis. RFLP-PFGE analysis and results interpretation were performed as described previously (Obszanska *et al.*, 2015b).

Multi Locus Variable Tandem Repeat Fingerprinting. MLVF analysis and results interpretation were performed as described previously (Obszanska *et al.*, 2015a).

Results and Discussion

During 16 years of surveillance by reference centers in Poland, we collected only 78 isolates (about 5 isolates a year) confirmed as SAG. Nationwide invasive viridans streptococci strain collection did not significantly increase the number of SAG strains sent to reference centers compared with previous years. It can demonstrate either low level of infections caused by SAG, or poor recognition of SAG as pathogens. It is more likely poor recognition of SAG by health personnel and/or authorities as surveillance reports published worldwide describe much higher incidence reaching 8.65/100,000 population for SAG invasive disease (Laupland *et al.*, 2006; Siegman-Igra *et al.*, 2012; Arinto-Garcia *et al.*, 2015). One of the reports describes a higher incidence of invasive SAG infections than those caused by groups A or B streptococci (Laupland *et al.*, 2006). Unfortunately, in Poland recognition of etiological microbiological factors is rather poor. Epidemiological reports regarding streptococcal infections are usually

based on the physician's reports and strain collection is not mandatory, reports are then collected by epidemiological authorities and sent to the National Institute of Public Health (NIZP-PZH) and published as bi-weekly reports (NIZP-PZH, 2015). In case of streptococcal infections only *S. pyogenes* infections such as scarlet fever (Lp. 46 in the cited report (NIZP-PZH, 2015)) erysipelas (considered invasive disease, Lp. 55), STSS (Lp. 56), puerperal fever (Lp. 57) and undefined invasive infections (Lp. 58) are reported. Using this data, incidence of invasive *S. pyogenes* is estimated to be 0.03/100,000 population for STSS or 0.2/100,000 population for undefined *S. pyogenes* invasive infections, what can be an example of dramatically underestimated streptococcal infections. We receive streptococcal strains in reference centers usually in cases when diagnostic laboratories have problems with identifications or there are problems with treatment.

Usually identification of non β-hemolityc streptococci such as anginosus group is quite a challenge; strains are often misidentified by automated systems because of their slow growth under oxygen conditions. On the other hand manual, and semi-automated system such as ID strips (bioMerieux) often identify species only to anginosus group. To verify systematic classification into species, all strains sent to reference centers as anginosus group, or either Lancefield group F or non β-hemolytic Lancefield C and G, were initially re-identified using Vitek2 automated system with GP card (bioMerieux). The results given by IDStrep and MALDI-TOF MS were usually consistent while results from Vitek2 system often misidentified strains as closely related *Streptococcus gordonii* or called 50%/50% identification either as *S. anginosus*/*S. constellatus* or *S. anginosus*/*S. intermedius*. The problems with identification of streptococci using MALDI-TOF MS have been also reported by other groups (Woods *et al.*, 2014; Chen *et al.*, 2015). So, even with advanced techniques used for strain identification, we experienced multiple problems with proper species identification. In laboratories that routinely perform identification using PCR methods, detection and differentiation of SAG can be performed using molecular methods (Takao *et al.*, 2004). From the clinical point of view, identification to anginosus group is enough to consider an isolate as an etiological factor of infection, however, identification as *S. gordonii*, which is a natural oral flora, may lead to misdiagnosis and not considering the strain as source of the infection.

Of all strains collected during that time, 48 isolates were consistently identified as *S. anginosus*, 27 as *S. constellatus* and only one as *S. intermedius*. One isolate could not be identified to the species level using neither IDStrep, VITEK2 nor MALDI-TOF MS system, another isolate was identified as *S. anginosus*/*constellatus*. The most common Lancefield antigen detected among SAG isolates was C, followed by F and G. In case of 20 isolates, we did not detect any of the A, B, C, G and F Lancefield antigens, what is consistent with group classification (Kohler, 2007). Two isolates of *S. constellatus* carried antigen A.

Strains from the analyzed collection were isolated from patients of all age groups, from young children to elderly over 65 years. Of all strains with provided information about isolation source, thirteen strains were isolated from invasive infections (blood and cerebrospinal fluid), 23 from pharyngitis and lower respiratory tract infections and 33 from purulent soft tissue infections, including swabs taken during surgeries. Large number of soft tissue isolates is consistent with large number of described purulent infections caused by SAG bacteria (Asam and Spellerberg, 2014). Mean age of patients with blood/CNS infections *vs* soft tissue *vs* respiratory tract isolates was different (54 vs 44 vs 35 years of age, respectively), however, only the difference between age of patients with invasive and respiratory tract infections was statistically significant ($p = 0.03$).

Initial screening for resistance to antibiotics usually used against streptococcal infections was performed using automated Vitek2 system. Unfortunately, similarly to experiences with strain identification, automated minimal inhibitory concentration (MIC) evaluation failed for ~10% of strains and was performed manually for erythromycin, clindamycin, tetracycline, penicillin and linezolid using E-test (bioMerieux), M.I.C. Evaluator (Oxoid) or reference microdilution method and interpreted according to EUCAST criteria (EUCAST, 2014). In some cases only a subset of assays from the ST101 card was assayed by the instrument.

We did not detect any ampicillin/benzylpenicillin, cefotaxime, ceftriaxone, levofloxacin, linezolid, and vancomycin, resistance and MIC_{50} and MIC_{90} values were within "sensitive" range (Fig. 1 and Table II). Unfortunately, clear interpretation criteria for anginosus streptococci cannot be easily applied. In case of some antibiotics such as benzylpenicillin, according to EUCAST (EUCAST, 2014), different breakpoints should be applied for β-hemolytic streptococci (such as *S. pyogenes*), different to *S. pneumoniae* and different to a large group of species named viridans streptococci (Fig. 1 and Table II). Based on the slowly increasing knowledge about anginosus streptococci, it is not clear which group of breakpoints should be applied for the analysis.

Among the analyzed strains, several of them were resistant to erythromycin and/or clindamycin, however MIC_{50} and MIC_{90} values for the population were still within "sensitive" range. In case of detected erythromycin resistance, double disc diffusion test was performed to determine inducible or constitutive mechanism of the resistance. Among 5 strains with detected *erm*A gene, 3 strains exhibited inducible clindamycin resistance.

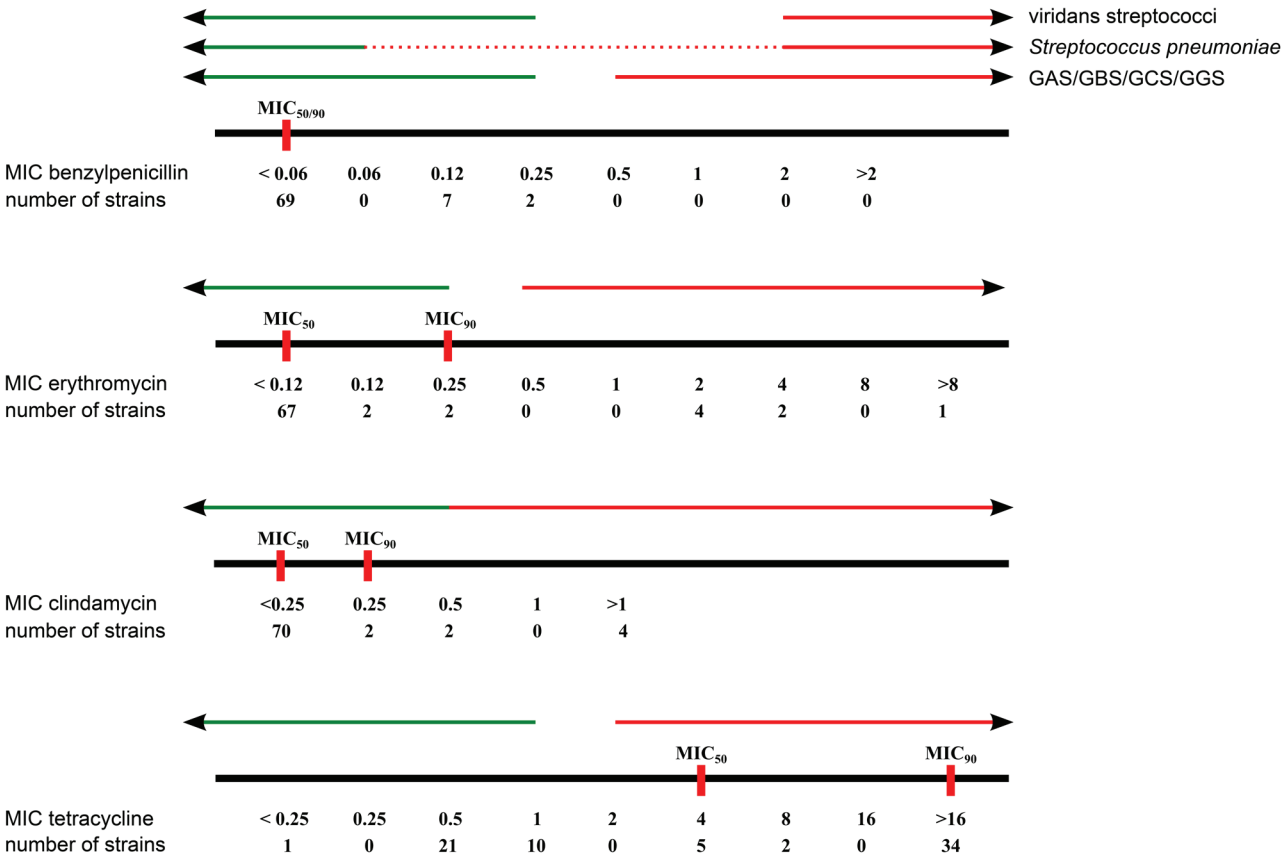

Fig. 1. MIC values detected for benzylpenicillin, erythromycin, clindamycin and tetracycline. Horizontal green and red arrows denote sensitive/resistant breakpoints according to EUCAST (EUCAST, 2014). In case of benzylpenicillin, breakpoints for GAS/GBS/GCS/GGS, *S. pneumoniae* and viridans streptococci are shown. Red rectangles on the horizontal axis denote MIC50 or MIC90. For each antibiotic dilution number of strains with detected MIC is given.

Contrary to detected sensitivity to the majority of tested antibiotics, over 50% of strains were resistant to tetracycline, with MIC_{50} and MIC_{90} values within "resistant" range according to Eucast (Fig. 1).

To identify genes responsible for the resistance phenotypes, we performed PCR detection of resistance determinants using chromosomal DNA isolated from all analyzed strains with starters that allow detection of

Table II
MIC_{50} and MIC_{90} values estimated for group of analyzed SAG strains.

Antibiotic	MIC_{50}	MIC_{90}	Breakpoints for sensitive S≤	Breakpoints for resistant R>
Ampicillin	<0.25	<0.25	ND / 0.5 / 0.5	ND / 2 / 2
Benzylpenicillin	<0.06	<0.06	0.25 / 0.06 / 0.25	0.25 / 2 / 2
Cefotaxime	<0.12	0.25	ND / 0.5 /0.5	ND / 2 / 0.5
Ceftriaxone	0.25	0.5	ND / 0.5 / 0.5	ND / 2 / 0.5
Clindamycin	<0.25	0.25	0.5 / 0.5 /0.5	0.5 / 0.5 /0.5
Erythromycin	<0.12	0.25	0.25 / 0.25 / –	0.5 / 0.5 / –
Levofloxacin	0.5	1	1 / 2 / –	2 / 2 / –
Linezolid	<2	<2	2 / 2 / –	4 / 4 / –
Tetracycline	4	>16	1 / 1 / –	2 / 2 / –
Trimethoprim /sulfamethoxazole	<10 (0.5/9.5)	40 (2/38)	1 / 1 / – *	2 / 2 / – *
Vancomycin	0.5	0.75	2 / 2 / 2	2 / 2 / 2

MIC values for sensitive (S) or resistant (R) classes are given for β-hemolytic streptococci / *S. pneumoniae* / viridans streptococci, respectively, according to EUCAST, "–" insufficient data supporting S vs R call or antibiotic not recommended for use, ND not determined and sensitivity for β-hemolytic streptococci inferred from sensitivity to benzylpenicillin. * EUCAST breakpoints for trimethoprim/sulfamethoxazole are expressed as trimethoprim concentration. trimethoprim/sulfamethoxazole tested with ST-101 card in the ratio 1:19.

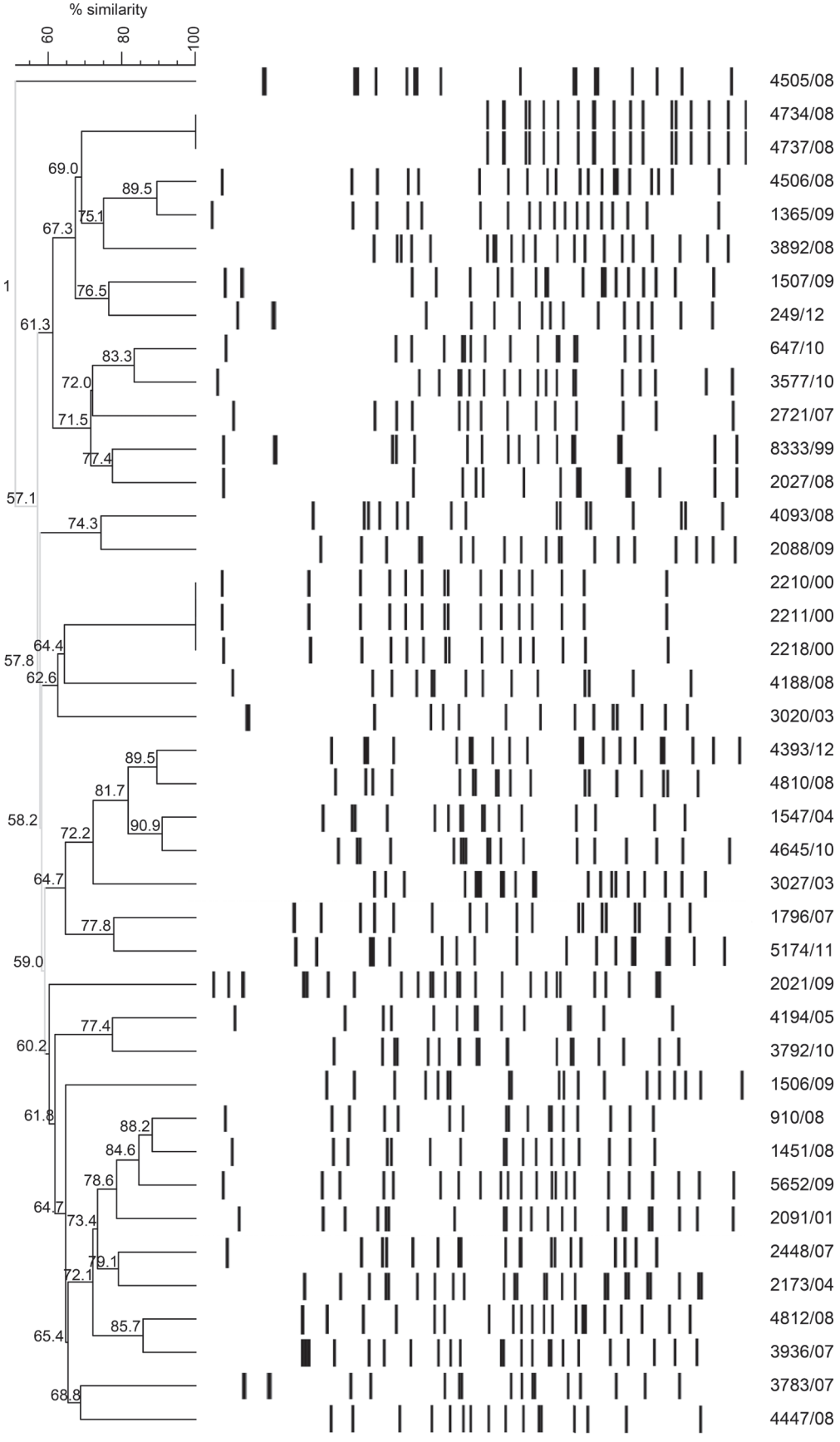

Fig. 2. RFLP-PFGE analysis of SAG strains resistant to tetracycline or macrolide antibiotics. Analysis was performed as described in (Obszanska *et al.*, 2015b), and DNA in plugs was digested with *Eag*I. The similarity coefficient was calculated using Dice algorithm (optimization and tolerance parameters set to 1%), followed by clustering analysis using UPGMA method (BioNumerics). Numbers on the dendrogram show percent of similarity between branches.

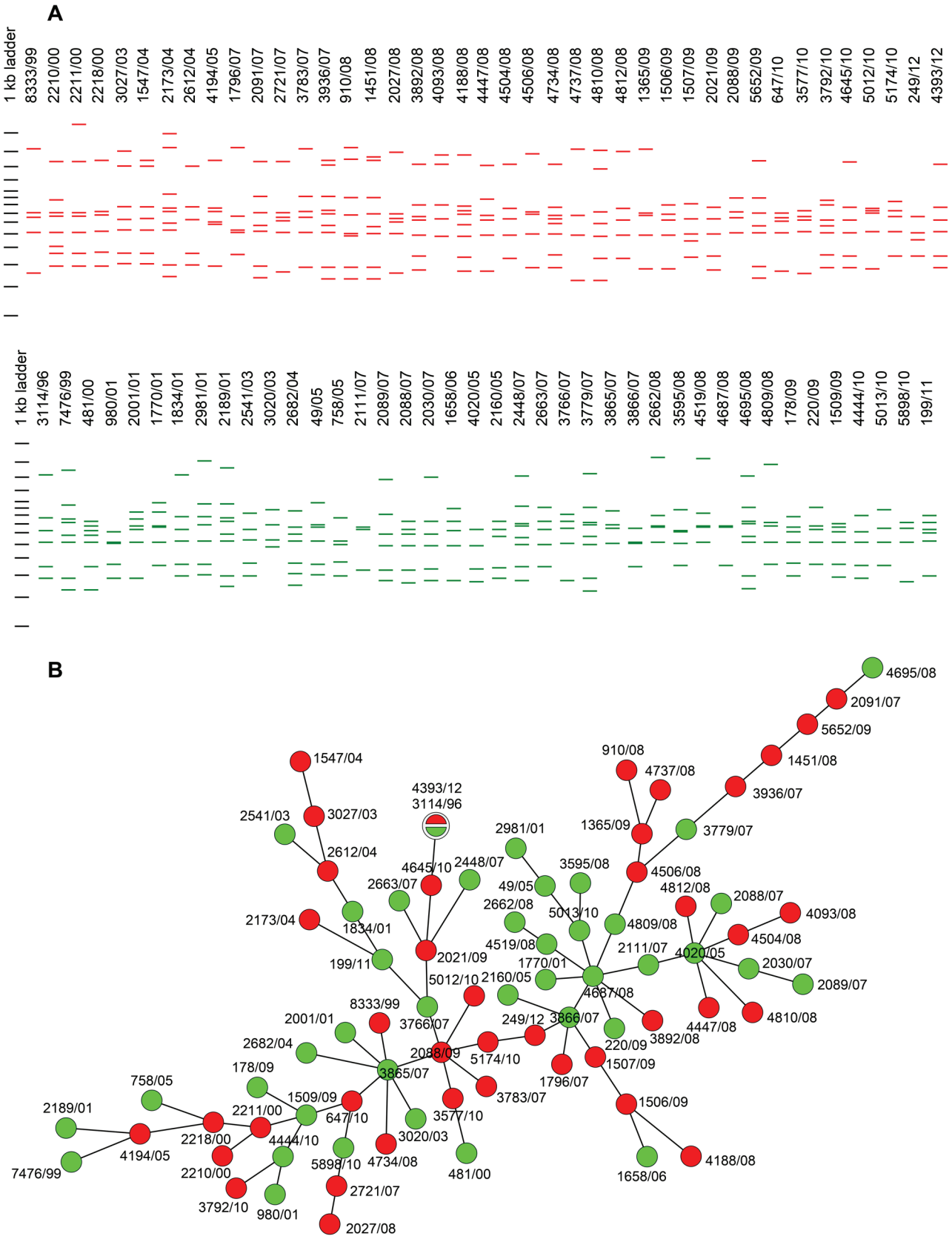

Fig. 3. MLVF analysis of SAG collection.

A. Unclustered patterns obtained using MLVF typing (Obszanska *et al.*, 2015a), patterns in red represent strains resistant to tetracycline, erythromycin and clindamycin, patterns in green represent strains sensitive to all tested antibiotics.

B. Minimum spanning tree generated using MLVF patterns, similarity coefficient was calculated based on the number of different bands with optimization and tolerance set to 0.3%. Red circles represent strains resistant to tetracycline, erythromycin and clindamycin, green circles represent strains sensitive to all tested antibiotics

Table III
Antibiotic resistance determinants detected for analyzed population

Antibiotic	Number of resistant strains	Number of strains	Resistance determinant
Tetracycline	40/78 (51%)	30	*tet*M
		1	*tet*O
		1	*tet*W
		8	Not found
Erythromycin	7/78 (9%)	4	*erm*A
		1	*erm*B
		1	*mef*A
		1	*mef*A + *erm*A

erythromycin and tetracycline determinants in streptococci (Table III). For all strains resistant to erythromycin, we were able to detect the gene responsible for the phenotype, however in case of tetracycline resistance we could not detect any resistance determinant in 8 strains with any of the starters listed above. This suggests the presence of a novel variant of the resistance gene/genes that cannot be amplified with the used starters.

To test whether resistant strains are similar to each other, we performed strain comparison on the genomic level using modified PFGE (Fig. 2). The analysis shows that the group of resistant strains is highly diverse, except for two small clusters that exhibit the same patterns. Strains 2210/00, 2211/00 and 2218/00 were sent to the reference center from the same hospital to confirm species identification. Unfortunately, we do not have any additional epidemiological records, nor access to them. The strains may be as well isolates from multiple patients or sequential isolates from the same person. Based on the PFGE pattern they can be considered almost identical. Two other strains (4737/08 and 4734/08) were sent to the reference center during the survey from the same hospital, but were isolated from two different patients and different infections (blood and lower respiratory tract, respectively).

To further investigate the relationships between strains, we performed multi locus analysis of variable number of tandem repeats (MLVF) (Fig. 3). The analysis was performed for strains that belonged to both "sensitive" and "resistant" groups to see whether the "resistant" group shows any unique traits in comparison with the "sensitive" group. The analysis showed very similar patterns detected for 3 strains 2210/00, 2211/00 and 2218/00, confirming the common source. However, strains 4737/08 and 4734/08 with identical PFGE pattern, shown different MLVF profiles, consistent with two different sources. We did not observe any significant clustering of MLVF patterns for resistant or sensitive strains (Fig. 3) and MLVF analysis confirms that all SAG strains isolated in Poland are diverse on the genetic level.

Conclusions. Streptococci belonging to anginosus group are rarely isolated in Poland presumably because of poor recognition by epidemiological and health authorities, predominantly from purulent infections. All analyzed isolates are very diverse on the genomic level as estimated by PFGE and MLVF analyses. All analyzed strains are sensitive to penicillin, a substantial group of strains is resistant to macrolides and the majority of strains are resistant to tetracycline.

Abbreviations

CNS – central nervous system; EUCAST – The European Committee on Antimicrobial Susceptibility Testing; KORLD – Krajowy Ośrodek Referencyjny d.s. Lekowrażliwości Drobnoustrojów (National Reference Center for Antimicrobial Resistance); KOROUN – Krajowy Ośrodek Referencyjny d.s. Zakażeń Ośrodkowego Układu Nerwowego (National Reference Center for Central Nervous System Infections); MALDI-TOF MS – Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometer; MIC – Minimal Inhibitory Concentration, MLVF – Multi Locus Variable tandem repeats Fingerprinting; RFLP-PFGE, Restriction Fragment Length Polymorphism – Pulsed Field Gel Electrophoresis; SAG – Streptococcus anginosus group

Competing interests

The authors have no competing interests to declare

Authors contribution

KO performed strain re-identification, MLVF and PFGE analyses and wrote the manuscript, IKZ performed PFGE analysis, AK performed MIC analysis, KM performed MIC analysis and detection of antibiotic resistance determinants, ES coordinated strain collection, performed strain re-identification, WH analyzed the data, IS coordinated the study, analyzed the data and wrote the manuscript

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ORIGINAL PAPER

Comparison of Free and Immobilized L-asparaginase Synthesized by Gamma-Irradiated *Penicillium cyclopium*

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Abstract

Gamma irradiation is used on *Penicillium cyclopium* in order to obtain mutant cells of high L-asparaginase productivity. Using gamma irradiation dose of 4 KGy, *P. cyclopium* cells yielded L-asparaginase with extracellular enzyme activity of 210.8 ± 3 U/ml, and specific activity of 752.5 ± 1.5 U/mg protein, which are 1.75 and 1.53 times, respectively, the activity of the wild strain. The enzyme was partially purified by 40–60% acetone precipitation. L-asparaginase was immobilized onto Amberlite IR-120 by ionic binding. Both free and immobilized enzymes exhibited maximum activity at pH 8 and 40°C. The immobilization process improved the enzyme thermal stability significantly. The immobilized enzyme remained 100% active at temperatures up to 60°C, while the free asparaginase was less tolerant to high temperatures. The immobilized enzyme was more stable at pH 9.0 for 50 min, retaining 70% of its relative activity. The maximum reaction rate (V_{max}) and Michaelis-Menten constant (K_m) of the free form were significantly changed after immobilization. The K_m value for immobilized L-asparaginase was about 1.3 times higher than that of free enzyme. The ions K^+, Ba^{2+} and Na^+ showed stimulatory effect on enzyme activity with percentages of 110%, 109% and 106% respectively.

Key words: *Penicillium cyclopium*, Amberlite IR-120, gamma irradiation, ionic binding immobilization, L-asparaginase

Introduction

The enzyme L-asparaginase (ASN) (EC.3.5.1.1; L-asparagine aminohydrolase) catalyzes the deamination of L-asparagine (Asn) to L-aspartate and ammonia. The enzyme is isolated from several sources: animal and plant cells, yeast, fungi, and bacteria, with a wide variety of microbial strains producing it (Gulati *et al*., 1997). L-asparaginase received increased attention in recent years as a food processing aid which reduces the formation of acrylamide in starch-based food that is baked, roasted or fried (Tareke *et al*., 2002).

It has been identified as an effective agent in the treatment of certain types of lymphoma and leukemia. Its use in anti-cancer therapy is based on its ability to cleave L-asparagine, an amino acid essential for the growth of lymphoblasts, to ammonia and L-aspartic acid in serum and cerebrospinal fluid. Since lymphoblasts are unable to produce endogenous L-asparagine, starvation for this amino acid leads to the death of these cells (Kotzia and Labrou, 2007).

The enzyme produced by *Escherichia coli* and *Erwinia carotovora* was clinically used to treat patients suffering from asparaginase-dependent leukemia (acute lymphoblastic leukemia) and lymphomas (Keating *et al*., 1993). A number of undesirable side effects were observed in cases of acute lymphoblastic leukemia, which were attributed to the contamination of enzyme preparations with bacterial endotoxins. The search for other asparginase sources such as eukaryotes, can lead to an enzyme with less adverse effects. It has been observed that eukaryote microorganisms such as yeast and filamentous fungi *e.g. Aspergillus*, *Penicilliumm* and *Fusarium* have a potential for asparaginase production (Sarquis *et al*., 2004).

Immobilization of enzymes is one of the important trends in biotechnology. The use of immobilized enzymes lowers production costs as these can be readily separated from the reaction mixture and can hence be used repeatedly and continuously (Maysa *et al*., 2010). Native L-asparaginase is often chemically modified and physically embedded with various kinds of soluble

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and insoluble biocompatible polymers in order to produce immobilized L-asparaginase. It has been reported that the immobilized enzyme not only reduces toxicity, but also greatly improved resistance to proteolysis compared to native L-asparaginase (Zhang *et al.*, 2004; Ghosha *et al.*, 2011). Attempts were made for the preparation of insoluble matrix supports such as collagen (Jefferies *et al.*, 1977), carboxy methyl cellulose (Hasselberger *et al.*, 1970), polyacrylamide and poly (2-hydroxyethyl methacrylate) gels (O'Driscoll *et al.*, 1975) derivatives bioconjucated with L-asparaginase for use in cancer therapy. That said, enzyme immobilization has attracted great interest by chemists and biochemists for its wide application in academic research and industrial processes (Mahmoud and Helmy, 2009; Shafei *et al.*, 2015).

This research applied partial purification and immobilization of gamma-irradiated *Penicillium cyclopium* in order to improve the stability of L-asparginase for the effective mitigation of acrylamide formation in industrially processed and home-cooked high heat-treated potato products. The work was extended to compare between the kinetic parameters such as pH, thermal stability, K_m and V_{max} of L-asparginase in its native and immobilized forms.

Experimental

Materials and Methods

Chemicals. All the chemicals used in this study are of analytical grade unless otherwise stated. Dowex, duolite, chitosan, cellulose, silica gel were purchased from Fluka company, Switzerland; Amberlite IR-120 from Fine-Chem Ltd. Boisar; and L-asparagine from Merck Company.

Microorganism. *P. cyclopium* was obtained from the culture collection of the National Research Center, Dokki, Cairo, Egypt. The stock culture was maintained on agar slopes that contained potato dextrose agar medium (PDA) at 30°C and preserved at –80°C in 50% (v/v) glycerol with regular monthly transfer.

Enzyme production. *P. cyclopium* was incubated at 30°C for 3 days on a rotary shaker (200 × g) using a medium of the following composition (g/l): sucrose, 2; L-asparagine, 10.0; $NH_4(SO_4)_2$, 8.77; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4 \cdot 7H_2O$, 0.52; and $Cu(NO_3)_2 \cdot 3H_2O$, $ZnSO_4 7H_2O$ and $Fe SO_4 \cdot 7H_2O$ as trace elements (El Refai *et al.*, 2014). Fresh fungal spores were used as inoculums and 1 ml spore suspension (containing around 10^6 spores/ml) was added to the sterilized medium and incubated at 30°C containing 100 ml of the sterile medium. The pH of the medium was initially adjusted at pH 6.2 in reciprocal shaker. At the end of the incubation period, the mycelia were removed by centrifugation (5000 × g) for 20 min at 4°C. Proteins in the filtrate were precipitated by 60% acetone. This partially purified enzyme (specific activity 2120.8 U/mg protein) was used for the determination of L-asparaginase activity.

Enzyme assay. L-asparaginase activity of culture filtrate was determined by quantifying the ammonia formation using Nessler's reagent (Usha *et al.*, 2011). One unit (IU) of L-asparaginase activity is defined as the amount of enzyme which liberates 1 μmol of ammonia per minute (μmole/ml/min) under the standard assay conditions.

Protein determination. The protein content was determined according to Lowry method (1951) using bovine serum albumin as standard.

Mutagenesis of *P. cyclopium* isolates by gamma irradiation

Effect of γ-irradiation on *P. cyclopium*. *P. cyclopium* was cultivated on potato dextrose agar plates and incubated at 30°C for 7 days. The developed colonies were scraped off and suspended in sterile saline solution. Spore suspension in triplicates was irradiated using Co^{60} gamma source according to Iftikhar *et al.* (2010), and radiation doses of 0.5–6.0 KGy in 0.5 KGy intervals were applied. As control, the number of colony-forming unit (CFU/ml) prior to irradiation was determined upon culturing on potato dextrose agar plates, as well as the number of survivors after exposure to different radiation doses.

Viable count determination. Ten-fold serial dilutions of irradiated spore suspension of *P. cyclopium* along with the control (non-irradiated) were prepared, and 0.1 ml of each appropriate dilution was plated onto sterile potato dextrose agar media. The plates were incubated at 30°C for 24 h, and the count of survived colonies as well as the initial one were determined. A dose response curve was plotted using the resulting counts. The sublethal dose and the D_{10} value for the strain were also calculated.

Effect of γ-irradiation on L-asparaginase activity. After exposure of spore suspension of *P. cyclopium* to gamma radiation, each test tube was inoculated in the basal medium and the flasks were incubated at 30°C for 3 days. L-asparaginase activity for each irradiated dose was estimated as explained above, and the results were compared to those of the control (irradiated).

Immobilization methods

Physical adsorption. Enzyme immobilization using physical adsorption was done using alumina, polyvinyl alcohol and silica gel, prepared according to Abdel-Naby *et al.* (1998). One hundred milligrams of the carriers were incubated with 2.545 U of 60% acetone enzyme fraction

dissolved in 1 ml of 0.1 M Tris-HCL buffer (pH 8.0) at 4°C overnight. The unbound enzyme was removed by washing with Tris-HCl buffer (0.1 M, pH 8.0).

Ionic binding. Ionic binding was used to achieve enzyme immobilization using 0.2 g of the cation (Dowex-50w, Amberlite IR-120) or anion exchanger (DEAE-cellulose) equilibrated with 0.1 M acetate buffer (pH 8) or 0.1 M Tris-HCL buffer (pH 8) respectively, and incubated with 5 ml partially purified enzyme containing 2545 U in the same buffer at 40°C for 24 h. The unbound enzyme was removed by washing with the same buffer.

Covalent binding. For enzyme immobilization using covalent binding, 0.4 g of chitosan was shaken in 5 ml of Tris-HCL and 0.1 M buffer (pH 8) containing 2.5% (V/V) glutaraldehyde (GA) for 24 h at 30°C. The solubilized chitosan was precipitated by the addition of 1 ml of 0.1 M NaOH. The precipitate was collected by filtration and washed with distilled water to remove excess GA. The wet chitosan was mixed with 1 ml partially purified enzyme (2545 U). After being shaken for 1 h at 30°C, the unbound enzyme was removed by washing with distilled water.

Immobilization yield (U/g carrier). Immobilization yield was calculated according to the following equation:

Properties of the free and immobilized L-asparaginase

Optimum pH. The effect of pH on the free and immobilized L-asparaginase was studied using citrate-phosphate buffer (0.1 M pH 3.0–7.0), and Tris-HCL buffer (0.1 M, pH 8.0–10.0).

Optimum temperature. The effect of temperature was studied by incubating both the free and immobilized enzymes in their respective optimum pH at different temperatures (ranging from 25 to 70°C), with different controls, for 20 min using 2.0% L-asparagine as substrate.

Thermal and pH stability of L-asparaginase. The thermal stability of L-asparaginase was investigated by incubating the free and immobilized enzyme at various temperatures (30–80°C) with different incubation periods (15, 30, 45 and 60 min) in absence of the substrate. The relative activities were then determined. For pH stability, the free and immobilized enzymes were incubated using different pH buffers for different time intervals (30 and 60 min) after which the residual enzyme activity was determined.

Activation energy (Ea). The activation energy was determined using the slope of a linear plot of the log of the enzyme activity (v) versus 1/T. The enzyme activity (v) was expressed in U (μg protein)$^{-1}$, the temperature (T) in Kelvin (K), the gas constant (R) = 1.987 cal K^{-1} mol^{-1} and the activation energy (Ea) in kcal mol^{-1}.

Substrate concentration and determination of K_m and V_{max}. The effect of incubating different concentrations of L-asparagine (0.02–0.12 mM) with the free or immobilized enzyme at 40°C for 30 min, was investigated by estimating the residual enzyme activity. The initial velocity was measured as a function of substrate concentration and plotted as double reciprocals in accordance with the line-weaver-Burk analysis (Lineweaver and Burk, 1934). The K_m and V_{max} values were 0.0259 mM and 757.6 U/mg protein for the free enzyme, and 0.033 mM and 581 U/mg protein for the immobilized enzyme.

Effect of metal ions and EDTA on L-asparaginase activity. For determining the effect of some metal ions on L-asparaginase activity, the partially purified and immobilized enzyme were preincubated with 10^{-3} M of Na^+, K^+, Ba^{2+}, Ca^{2+}, Fe^{2+}, Hg^{2+}, Mn^{2+} and EDTA for 2 h at 30°C and the residual activity was determined.

Statistical analysis. All experiments were repeated three times. Data provided in the corresponding tables and figures represent the mean values of the results obtained, along with the relative standard deviations.

Results and Discussion

Effect of γ-radiation on the survival of *P. cyclopium*. *P. cyclopium* strain was exposed to different doses of γ-radiation ranging from 0.5 to 6.0 KGy at 0.5 KGy interval. The number of viable cells decreased exponentially with increasing radiation dose. The D_{10} value was 2.5 KGy and the sublethal dose was found to be 6.0 KGy.

Production of L-asparaginase by mutant gamma-irradiated *P. cyclopium*. The potential improvement of L-asparaginase production in γ-irradiated *P. cyclopium* was studied. Table I shows that L-asparaginase activity

Table I
Influence of Co^{60} gamma irradiation at various doses on L-asparaginase activity

Recovered activity (%)	Specific activity (U/mg protein)	L-asparaginase activity (U/ml)	Dose (KGy)
100	400 ± 0.6	160 ± 1	Control
33 ± 1.5	294.4 ± 3	53 ± 2.5	0.5
46 ± 1	389.5 ± 0.5	74 ± 2.6	1
49.7 ± 0.9	397.5 ± 4	79.5 ± 1.6	1.5
57.9 ± 0.9	421.4 ± 2.5	92.7 ± 2.5	2
62.9 ± 1	437.8 ± 2	100.7 ± 0.5	2.5
93.7 ± 1.5	441.4 ± 3.3	150 ± 2.9	3
122.2 ± 0.8	651.7 ± 1.3	195.5 ± 1.4	3.5
131.6 ± 0.9	752 ± 1.5	210.8 ± 3	4
65.9 ± 0.4	363.4 ± 1.8	105.4 ± 2.8	4.5
34.8 ± 0.5	289.5 ± 2.4	55 ± 2.9	5

and specific activity increased with increasing the dose of gamma radiation. They both reached a maximum 210.8 ± 3 U/ml and 752.5 ± 1.5 U/mg protein at dose level 4 KGy. This was 1.75 times more for asparaginase activity, and 1.53 times more for specific activity of the wild strain. Similar results were obtained by El-Batal *et al.* (2000) and Fadel and El-Batal (2000). This enhancement by gamma radiation may be either due to an increase in gene copy number or gene expression or both (Rajoka *et al.*, 1998). Several studies recorded that low doses of gamma radiation may stimulate microbial growth and metabolic activities. Meanwhile, high doses of gamma radiation were proved to be inhibitory for both growth and enzymatic activities of microorganisms. The exposure of cells to ionizing radiation sets off a chain of reactions giving rise to chemical and then to metabolic or physiological changes. The irradiation presents an additional stress to the cells which tends to disturb their organization. Irradiation effects have been shown to occur with proteins, enzymes, nucleic acid, lipids and carbohydrates, all of which may have marked effects on the cell (Ismail *et al.*, 2010).

Partial purification of *P. cyclopium* L-asparaginase. The crude enzyme of the γ-irradiated *P. cyclopium* was subjected to purification (Table II). Both the enzyme activity and the specific activity of the partially purified asparaginase were increased to 3.0 fold and 2120.8 U/mg protein respectively.

Immobilization of *P. cyclopium* L-asparaginase using different carriers. The partially purified enzyme was dialysized, lyophilized and immobilized. Different immobilization techniques were investigated: physical adsorption, covalent binding and ionic binding. Results are presented in Table III. The highest loading efficiency (1200 ± 1.1 U/g carrier) and immobilization yield (80 ± 0.2%) were detected with ionic binding technique using Amberlite IR-120 which is a strongly acidic cation exchange resin suitable for a wide variety of chemical process applications. The least enzyme activity (200 U/g carrier) and immobilization yield (11%) were detected with covalent binding using Duolite 147 as a carrier. Therefore, Amberlite IR-120 proved to be the most appropriate carrier and was used in the succeeding work. Abdel-Naby *et al.* (1998) found that Amberlite IR-120 was a good carrier for *Bacillus mycoides* alkaline protease ionic binding. Also, Spinelli *et al.* (2013) stated that the use of low-cost matrices such as Amberlite IR-120 for enzyme immobilization represents a promising product for enzymatic industrial applications. On the contrary, Sundaramoorthi *et al.* (2012) showed that the highest enzyme immobilization activity and highest immobilization yield were achieved by the cross-linking technique using silica gel.

Optimum pH for free and immobilized enzymes. Both free and immobilized *P. cyclopium* L-asparaginase preparations exhibited maximum relative activity at pH 8.0 (Fig. 1). The immobilized form showed better relative activities across different pH levels ranging from 3.0 to 10.0. Dramatic decrease in relative activity (55–45%) was observed for the free enzyme at pHs 9

Table II
A profile of partial purified *P. cyclopium* L-asparaginase.

Purification steps	Protein of fraction (mg/F)	Recovered protein (%)	Total activity (U/ml)	Recovered activity (%)	Specific activity (U/mg protein)	Purification Fold
Culture filtrate	28 ± 0.3	100.00	210.8 ± 3.7	100.0	750 ± 2.8	1.0
Precipitation by 40–60% acetone	1.2 ± 0.2	4.3 ± 0.7	2545 ± 4.5	12 ± 0.05	2120.8 ± 0.9	3 ± 0.1

Table III
Different immobilization methods of *P. cyclopium* L-asparaginase.

Method of immobilization	Carrier	Added Enzyme (U/g carrier) A	Unbound Enzyme (U/g carrier) B	Immobilized enzyme (U/g carrier) I	Immobilization yield (I/A-B) x100
Physical adsorption	Alumina	2545	603.8 ± 0.9	700 ± 2	36.1 ± 0.2
	P.V.A	2545	241.5 ± 1.3	328.1 ± 2	14.2 ± 0.1
	silica gel	2545	633.9 ± 2.1	800 ± 3	41.8 ± 1.2
Covalent binding	Chitosan	2545	682.5 ± 1	500 ± 1.5	26.8 ± 3
	duolite-147	2545	301.9 ± 1	200 ± 2	11 ± 0.25
	duolie-C280	2545	300 ± 2.5	450 ± 2.6	36.1 ± 0.3
Ionic binding	Amberlite 120	2545	1045 ± 2.6	1200 ± 1.1	80 ± 0.2
	Dowex 40–50	2545	656.3 ± 2.4	350 ± 2.8	18.5 ± 1.5
	Dowex 50	2545	780 ± 0.6	250 ± 0.6	14 ± 0.2
	Cellulose	2545	600 ± 1.5	360 ± 2.8	18.5 ± 0.3

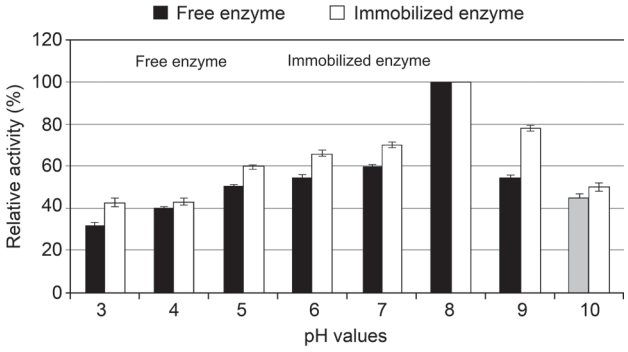

Fig. 1. Effect of pH values on the activities of the free and immobilized *P. cyclopium* L-asparaginase.

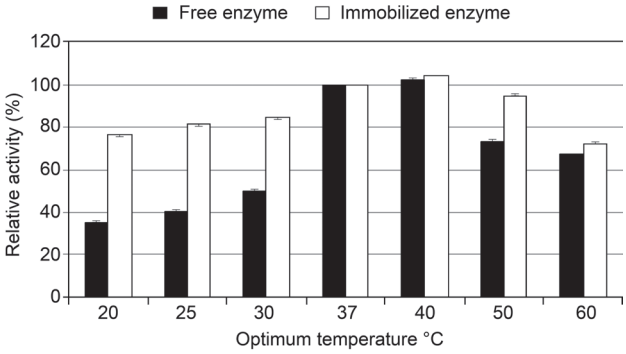

Fig. 2. Effect of temperature on the activities of the free and immobilized *P. cyclopium* L-asparaginase.

and 10. This clearly reflects the suitability of Amberlite IR-120 as a carrier for this enzyme as pH varies. Tabandeh and Aminlari (2009) reported that the optimum activity of L-asparaginase is at the alkaline pH of 8. This is probably due to the production of L-aspartic acid which acts as a competitive inhibitor for the enzyme under acidic conditions. Changes in pH activity behavior may be due to the immobilization of the enzyme, which could be explained by the unequal distribution of hydrogen and hydroxyl ions between the polyelectrolyte phase on which the enzyme is immobilized and the external solution (Kojima and Shimizu, 2003).

pH stability of free and immobilized L-asparaginase activity. The profile of pH stability (Table IV) showed that the immobilized L-asparaginase was more stable in a wider range of pH (3.0–6.0) during 30 and 60 min incubation compared to the free enzyme (pH 3.0–4.0), where it retained 100% of its relative activity. This result means that immobilized L-asparaginase would be more resistant to pH changes, hence could be used at the industrial level. The ionic binding technique using Amberlite IR-120 stabilizes the enzyme in acidic pH, making it suitable for a wide variety of chemical process applications.

Table IV
The pH stability of the free and immobilized *P. cyclopium* L-asparaginase.

pH value	Relative activity (%)			
	Time of exposure			
	Free		Time of exposure	
	30 min	60 min	30 min	60 min
control	100	100	100	100
3	100	100	100	100
4	100	100	100	100
5	100	80 ± 0.5	100	100
6	80 ± 0.7	75 ± 1	100	100
7	72 ± 0.5	67.8 ± 1.4	95.7 ± 0.3	90 ± 0.5
8	70 ± 0.3	60 ± 1.1	88 ± 1.1	80 ± 1
9	60 ± 0.5	40 ± 0.6	86 ± 1.5	70 ± 1.7
10	30 ± 1.5	20.5 ± 0.5	45 ± 1.3	28 ± 0.5

Optimum temperature. When profiling relative activity vs. reaction temperature, the optimum reaction temperature for maximal activity was apparently shifted to 40°C for the free and immobilized enzymes. Even though the reaction temperature rose to 60°C, the relative activity of the immobilized enzyme was still above 72%, while that of free L-asparaginase was 66.7% (Fig. 2). The temperature data were replotted in the form of Arrhenius plots. The plots of the immobilized and free enzymes were found to be linear, and the calculated values of Ea were 1.75 and 3.46 Kcal/mol, respectively. The E_a of asparaginase binding to Amberlite IR-120 was much lower than that of the free one, suggesting that the enzyme had significantly higher affinity to the Amberlite IR-120 active sites (Su *et al.*, 2010). That said, the immobilization of the enzyme widened the optimum reaction temperature range (Zhang *el al.*, 2004). Also, Youssef and Al-Omair (2008) reported that the optimum reaction temperature of immobilized asparaginase produced by *E. coli* was 60°C while that of free L-asparaginase was 50°C.

Thermal stability for the free and immobilized *P. cyclopium* L-asparaginase. Heat stability of the free and immobilized L-asparaginase in terms of the residual activities was compared (Table V). The immobilized enzyme remained 100% active at temperatures up to 60°C for 30 min while the free asparaginase was 100% active up to 50°C for 30 min. On the other hand, the effect of high temperatures (50–80°C) was more pronounced in case of the free enzyme than the immobilized one, the later was more resistant to high temperatures compared to the free one. On the contrary, Zhang *et al.* (2004) reported that the thermostability of the immobilized L-asparaginase was very similar to that of the native enzyme, and that there were no obvious changes in the activities. On plotting log of the relative activity against time at different tested temperatures (60, 70 and 80°C), both the free and the immobilized enzyme preparations gave straight lines (Fig. 3 and

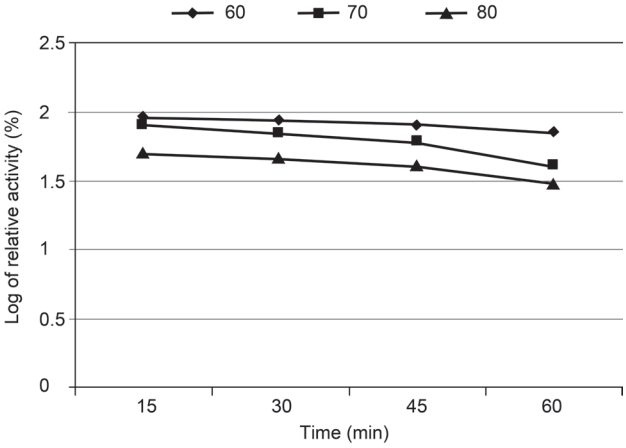

Fig. 3. First-order plots of thermal inactivation for the free *P. cyclopium* L-asparaginase activity.

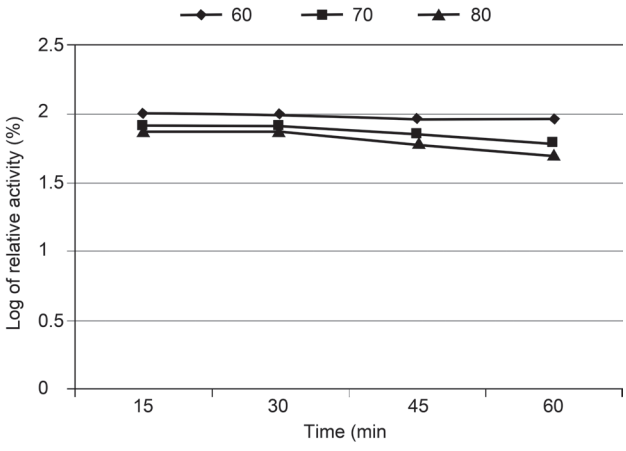

Fig. 4. First -order plots of thermal inactivation for the immobilized *P. cyclopium* L-asparaginase activity.

Fig. 4 respectively). This means that the thermal inactivation process of both enzyme forms corresponded to the theoretical curves of the first order reaction. The results showed that the immobilized enzyme was more thermostable than the free one. For example, the calculated half-lives of the free enzyme at 60, 70 and 80°C were 5.2, 3.6 and 2.47 hrs, respectively, proving to be lower than those of the immobilized enzyme, which were 10.4, 4.26 and 3.06 hr, respectively. The values of the deactivation rate constant (the slope of the relative activity) at increasing temperatures showed that the immobilized enzyme is highly stable compared to the free one. The calculated deactivation rate constants at 60, 70 and 80°C for the free enzyme were 2.2×10^{-3}, 3.7×10^{-3} and 5.3×10^{-3}, compared to 1.1×10^{-3}, 2.7×10^{-3} and 3.8×10^{-3}, respectively, for the immobilized enzyme.

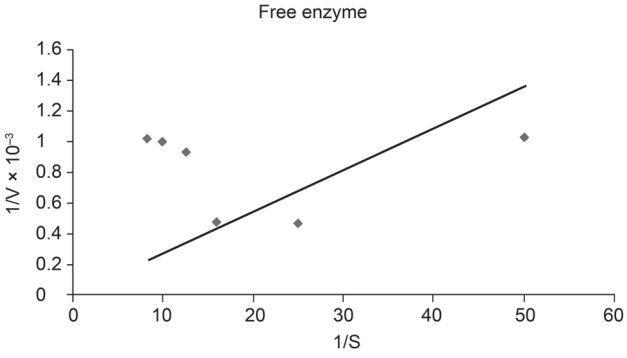

Fig. 5. Lineweaver – Burk plots for the free *P. cyclopium* L-asparaginase activity.

Kinetics and hydrolysis. The Michaelis-Menten Kinetics of the hydrolytic activity of the free and immobilized L-asparaginase were investigated using varying initial concentrations of asparagine as a substrate. There was a parallel increase in the relative enzyme activities of the free and immobilized treatment and the substrate concentrations (0.02–0.04 mM/ml). The initial velocity was measured as a function of substrate concentration for both free and immobilized enzyme and plotted (Fig. 5 and Fig. 6 respectively) as double reciprocals in accordance with the line-weaver-Burk analysis (Lineweaver and Burk, 1934). The K_m and V_{max} values were 0.0259 mM and 757.6 U/mg protein, respectively, for the free enzyme, and 0.033 mM and

Table V
Thermal stability of the free and immobilized *P. cyclopium* L-asparaginase activity.

Temperature °C	Residual activity (%)							
	Free				Immobilized			
	Time of exposure (min)							
	15	30	45	60	15	30	45	60
Control	100	100	100	100	100	100	100	100
30	100	100	100	100	100	100	100	100
40	100	100	100	100	100	100	100	100
50	100	100	90 ± 1.2	80 ± 1	100	100	100	100
60	90 ± 1	85.5 ± 0.8	80 ± 1.2	70 ± 1.5	100	100	90 ± 0.5	90 ± 0.5
70	80 ± 1.1	70 ± 0.6	60 ± 1	40 ± 0.5	80 ± 0.6	80 ± 0.6	70 ± 0.6	60 ± 1.7
80	50 ± 1.1	45.9 ± 0.5	39.9 ± 0.5	30 ± 2.3	73.9 ± 1	73.7 ± 0.9	60 ± 1.7	50.0 ± 0.5

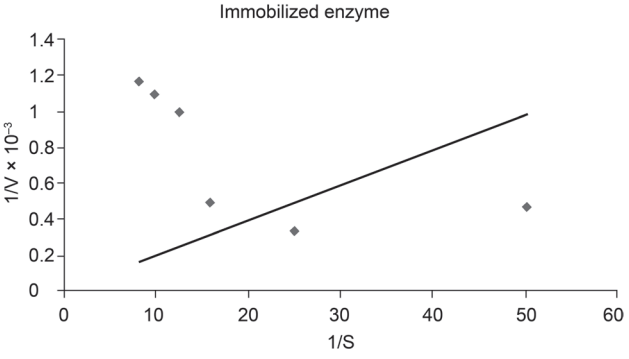

Fig. 6. Lineweaver – Burk plots for the immobilized *P. cyclopium* L-asparaginase activity.

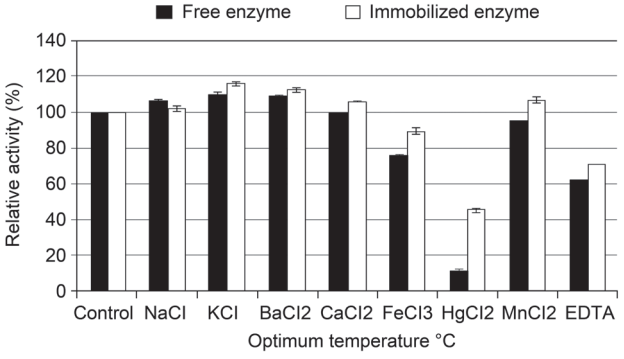

Fig. 7. Effect of different additives on activity of L-asparaginase of *P. cyclopium.*

581 U/mg protein, respectively, for the immobilized enzyme. The K_m value for immobilized L-asparaginase was about 1.3 times higher than that of the free enzyme, indicating a lower affinity towards the substrate. This increase in K_m might be either due to the structural changes in the enzyme induced by the immobilization method, or due to the lower accessibility of the substrate to the active sites (Su *et al*., 2010). On the other hand, fixation of the enzyme on the immobilization matrix could lead to a decrease in the flexibility of the enzyme molecule, which is commonly reflected by a decrease in the catalytic activity (Cao, 2005). Consequently, the maximum rate of the reaction V_{max} catalyzed by the immobilized enzymes was lower than that of the free enzyme, which was in agreement with Su *et al.* (2010). The ratio V_{max}/K_m is a measurement of the catalytic efficiency of an enzyme-substrate pair. The catalytic efficiency of L-asparaginase was decreased by about 40% upon immobilization. Another important aspect to take into consideration in order to evaluate the immobilization process is the efficiency factor. This factor can be calculated from the higher reaction rates of the immobilized enzyme divided by that of the free counterpart. That said, the efficiency factor = $v_{immobilized}/v_{free}$, where $v_{immobilized}$ is the reaction rate of the immobilized enzyme, and v_{free} is that of the free enzyme. For L-asparaginase, the efficiency factor was calculated to be 0.766.

Effect of metal ions on L-asparaginase activity. In this experiment, both the free and immobilized enzymes were incubated with different metal ions in their salt solutions at room temperature for 30 minutes. Following this, the relative activity was measured at optimum conditions. The results show that K+, Ba2+ and Na+ enhanced the enzyme activity with 110%, 109% and 106% respectively (Fig. 7). Considerable loss of activity was observed with Mn^{2+} and Fe^{3+}. Inhibition of enzyme activity with EDTA by 61.9% possibly suggest that the purified L-asparaginase might be a metaloenzyme (Elshafei *et al*., 2012). However, the highest inhibition value was recorded with Hg^{2+} which inhibited the enzyme to 11%. This might be indicative of essential vicinal sulfhydryl groups (SH-group) of the enzyme for productive catalysis (Elshafei *et al*., 2012). Similar results were reported by Basha *et al.* (2009) and Moorthy *et al.* (2010).

Conclusions. Our aim in this study was using gamma irradiation, purification, immobilization and characterization of L-asparaginase from *P. cyclopium* and to do a comparative study based on temperature, pH, inhibitor and activation concentration between free and immobilized enzyme. Gamma irradiation proved to be effective in the production of L-asparaginase with a total increase in enzyme activity of 1.75 folds and specific activity of 1.53 folds over the wild strain. L-asparaginase was partially purified then immobilized onto Amberlite IR-120. The immobilized enzyme showed optimal activity over a wide range of temperature and pH values. The immobilized L-asparaginase widened the optimum reactive temperature range and showed higher thermostability as well as higher affinity to the substrate. Considering all these characteristics, the production of L-asparaginase from gamma irradiated immobilized *P. cyclopium* may be recommended for industrial production.

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ORIGINAL PAPER

Characterization and Optimization of Biosynthesis of Bioactive Secondary Metabolites Produced by *Streptomyces* sp. 8812

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Abstract

The nutritional requirements and environmental conditions for a submerged culture of *Streptomyces* sp. 8812 were determined. Batch and fed-batch *Streptomyces* sp. 8812 fermentations were conducted to obtain high activity of secondary metabolites. In the study several factors were examined for their influence on the biosynthesis of the active metabolites-7-hydroxy-6-oxo-2,3,4,6-tetrahydroisoquinoline-3-carboxyl acid ($C_{10}H_9NO_4$) and N-acetyl-3,4-dihydroxy-L-phenylalanine ($C_{11}H_{13}NO_5$): changes in medium composition, pH of production medium, various growth phases of seed culture, amino acid supplementation and addition of anion exchange resin to the submerged culture. Biological activities of secondary metabolites were examined with the use of DD-carboxypeptidase 64–575 and horseradish peroxidase. *Streptomyces* sp. 8812 mycelium was evaluated under fluorescent microscopy and respiratory activity of the strain was analyzed. Moreover, the enzymatic profiles of the strain with the use of Api®ZYM test were analyzed and genetic analysis made. Phylogenetic analysis of *Streptomyces* sp. 8812 revealed that its closest relative is *Streptomyces capoamus* JCM 4734 (98%), whereas sequence analysis for 16S rRNA gene using NCBI BLAST algorithm showed 100% homology between these two strains. Biosynthetic processes, mycelium growth and enzyme inhibitory activities of these two strains were also compared.

Key words: *Streptomyces* sp. 8812, biologically active compounds, media optimization, submerged cultures

Introduction

Streptomyces are Gram-positive bacteria with a remarkably complex developmental cycle. These bacteria are isolated from soil and water in all ecosystems. *Streptomyces* are producers of many secondary metabolites with a wide range of activities, *e.g.* antimicrobial, antitumor and immunosuppressive (Hopwood, 2007). Bioactive secondary metabolites are mostly isolated from submerged cultures, often in discreet amounts. One of the strategies to improve production of secondary metabolites is optimization of chemical and physical conditions of the submerged culture. This process involves defining the composition of the production medium, temperature and pH value (Genilloud *et al.*, 2011).

In this work, the authors present results of a set of experiments performed in submerged cultures for *Streptomyces* sp. 8812 isolated from Brazilian soil. The described research is a continuation of the work on the strain *Streptomyces* sp. 8812 and its metabolites. Previously, two bioactive metabolites with antibacterial activity were isolated and characterized (Solecka *et al.*, 2009a; 2009b; 2012a). One of them had a novel structure. The chemical structure and biological and physico-chemical properties of two secondary metabolites produced by *Streptomyces* sp. 8812 have been determined. The first metabolite is an isoquinoline alkaloid, 7-hydroxy-6-oxo-2,3,4,6-tetrahydroisoquinoline-3-carboxyl acid ($C_{10}H_9NO_4$), with molecular mass of 207.06 Da. The second *Streptomyces* sp. 8812 metabolite is a protoalkaloid, N-acetyl-3,4-dihydroxy-L-phenylalanine ($C_{11}H_{13}NO_5$) with molecular mass of 239.07 Da. Both compounds exhibit antibacterial properties, have the ability to inhibit DD-carboxypeptidase 64–575 activity and are stable to β-lactamase activity. Their chemical structures may serve as lead compounds for modifications enhancing their biological activities (Solecka *et al.*, 2009a; 2009b; 2012a; 2012b).

In the present study, the authors defined the optimum composition and pH value of the production medium for bioactive secondary metabolites biosynthesis. The seed cultures at various growth phases were tested. A fed-batch strategy was used to study the effect of different amino acids on secondary metabolites

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activities. The authors examined whether addition of a resin into the production medium has an effect on the biosynthesis of biologically active compounds. Biological activity of the metabolites was studied under the guidance of DD-carboxypeptidase 64–575 and horseradish peroxidase inhibition reaction. Selected methods were chosen due to their high sensitivity which is significant in the case of secondary metabolites produced in very small quantities (hundredths of permille).

In further steps, genetic classification of *Streptomyces* sp. 8812 using 16S rRNA analysis was performed. Mycelium viability and respiratory activity of *Streptomyces* sp. 8812 during submerged culture was observed and characterized by fluorescent microscopy. The Api® ZYM test was used for biochemical characterization of mycelium during fermentation.

Experimental

Materials and Methods

Microorganism. *Streptomyces* sp. 8812 is a strain isolated from Brazilian soil. The strain is deposited in the Polish Collection of Microorganisms in Wroclaw, with an accession number B/00017. The 16S rRNA gene sequence of *Streptomyces* sp. 8812 has GenBank accession number KT951721.

Morphological and carbon utilization properties of *Streptomyces* sp. 8812 were described in previous paper (Solecka *et al*., 2009a). Spores of *Streptomyces* sp. 8812 were stored at –70°C.

Media and growth conditions. *Streptomyces* sp. 8812 was maintained on yeast-malt agar (ISP2) slants (Shirling and Gottlieb, 1966). The seed medium and initial production medium (M) consisted of (g/l): lactose 10.0, yeast extract 5.0, corn steep liquor (CSL) 10.0, Bacto™ peptone 4.0, Bacto™ tryptone 17.0, $MgCl2 \times 7H_2O$ 0.5, KH_2PO_4 2.0, K_2HPO_4 4.0, $CaCO_3$ 3.0 and microelements: $MnSO_4 \times H_2O$ 0.002, $FeSO_4 \times 7H_2O$ 0.01, $ZnSO_4 \times 7H_2O$ 0.01, $CoCl_2$ 0.0008, $CuSO_4 \times 5H_2O$ 0.001. After sterilization, pH was adjusted to 6.7 value.

In pH-optimization experiment pH of medium M after sterilization was adjusted to several values: 5.5, 5.9, 6.3, 6.7, 7.0, 7.4, 7.8 and was measured every 24 hours during fermentation.

Slants with *Streptomyces* sp. 8812 were incubated at 28°C for 10 days. Spores from fresh solid culture were transferred into 35 ml of the medium M in 500 ml shake flasks. The seed cultures were incubated in a rotary shaker at 220 rpm for: 24, 48 or 72 hours at 28°C. Then, 3.5 ml of seed cultures were transferred to 35 ml of production medium. The biosynthesis process was conducted at 220 rpm for 120 h at 28°C. Samples were taken every 24 h. Yeast-malt broth was used for mycelium growth for genetic analysis. Cultures for genetic analysis were incubated in a rotary shaker at 220 rpm for 24 h at 28°C.

Optimization of the production medium. Several nitrogen sources, like soybean flour, yeast extract, Bacto™ peptone, Bacto™ tryptone, neopeptone, were tested in a set of experiments (Table I). Medium M was modified in three ways: deletion or replacement, and adjustment of the component(s) concentration. Concentrations of macroelements, such as K^+, Mg^{2+}, Ca^{2+}, and microelements were constant in all media. The percentage of C and N values in all tested media was estimated on the basis of Becton Dickinson (BD) tables (BD Biosciences, 2006), for CSL (Keller and Heckmann LPP, 2006). The initial C/N values varied from 0.11 to 2.07.

Statistical method. The most significant component of the production medium was estimated using a statistical method. Each parameter (carbon and nitrogen source in the production medium) was characterized by mass percentage composition. Correlation between the share of each substrate in overall mass of medium and the DD-carboxypeptidase 64–575 inhibitory activity at 120 h was assessed using Spearman correlation coefficient and test of its significance. Calculations were performed using R 3.0.1 statistical software (R Foundation for Statistical Computing, 2013).

Determination of biomass growth. One ml of submerged cultures was taken every 24 h. Probes were centrifuged and the biomass was washed twice with distilled water. Dry biomass was obtained after incubation at 110°C in dryer (Pol-Eco). The drying process was finished when the biomass weight was stable.

Determination of secondary metabolites activities. To determine the optimum conditions for conducting the submerge culture of *Streptomyces* sp. 8812, activity of secondary metabolites was measured. DD-carboxypeptidase 64–575 from *Saccharopolyspora erythraea* 64–575 (Solecka and Kurzątkowski, 1999; Solecka *et al*., 2003) and horseradish peroxidase (HRP, Sigma-Aldrich) were used for enzymatic assays to determine the activity of secondary metabolites.

Inhibition of DD-carboxypeptidase 64–575 activity. The assay was done according to the method previously described by Frère *et al.* (1976), with modifications (Adam *et al.*, 1990; 1991; Solecka *et al*., 2003). The reaction mixture consisted of: 5 µl of DD-carboxypeptidase 64–575, 5 µl of 10 mM 2-((2-benzamidopropanol) thio)acetic acid, 5 µl of supernatant and 85 µl of 0.1 M phosphate buffer pH 8.0. All samples were triplicated. Absorbance was measured at 250 nm for 1200 s at 37°C (Jasco V-630). DD-carboxypeptidase 64–575 inhibition was calculated using the formula shown below:

$$DD - carboxypeptidase\ 64–575\ inhibition\ [\%] = 100 - \left(\frac{A_2 - A_3}{A_0 - A_1} \times 100\right)$$

The initial value of the absorbance of the enzyme control is A_0 whereas final value of the absorbance of the enzyme control is A_1. The initial value of the absorbance of the supernatant sample is A_2 and final value of the absorbance of the supernatant sample is A_3. Due to the method limitation, results were measured with 5% error.

Inhibition of HRP activity. The assay was done according to the method of Lehmann *et al*. (1974) with modifications (Chance and Maehly, 1955). The reaction mixture consisted of: 37.5 µl of 0.01 M phosphate buffer pH 8.0, 2.5 µl of *Streptomyces* sp. 8812 submerged culture supernatant, 5 µl of 0.025 mg/ml HRP, 5 µl of 1.26 mg/ml o-dianisidine and 10 µl of 0.480 mM hydrogen peroxide in 0.01 M phosphate buffer pH 8.0. After 10 min incubation at 37°C, 100 µl of water-methanol-sulfuric acid (5:5:6 v/v) was added and incubated for further 10 min at room temperature. The absorbance was measured at 540 nm (Fluostar Omega, LABTECH). HRP inhibition was calculated using the formula shown below:

$$HRP\ inhibition\ [\%] = \frac{A_{\text{control}} - A_{\text{supernatant}}}{A_{\text{control}}} \times 100$$

The absorbance for the control is A_{control}, whereas the absorbance in the presence of a supernatant is $A_{\text{supernatant}}$. The positive control for enzymatic activity measurements consisted of 2.5 µl of water instead of supernatant and an analogous blank contained phosphate buffer instead of hydrogen peroxide. Sample, control and related blanks were triplicated and carried out under the same condition on the same microliter plate.

Culture supplementation with amino acids. Different concentrations: 0.1, 1, 10 and 100 mM of L-tyrosine, L-tryptophan and L-phenylalanine were separately used in secondary metabolites activity tests. One ml of each concentration was added to shake-flasks cultures every 24 h till the end of fermentation.

Addition of anion exchange resin to production medium. Two g of anion exchange resin IRA-400 (OH) (Supelco) was equilibrated twice with 50 ml of 2.0 M acetic acid and rinsed with sterile water till pH value reached 7.0. Resin IRA-400 (2.0 g) was added to 24-h or 48-h old shake-flask cultures. All probes were incubated for 24 h. Next, the resin was removed from flasks and cultivation was continued till 120 h. The resin was rinsed with distilled water and eluted using 100 ml of 2.0 M acetic acid. Material eluted from the resin was lyophilized and compound activity was measured (7 µl of 1 mg lyophilizate in 100 µl of 0.01 M phosphate buffer pH 8.0).

Genetic analysis. Genomic DNA of *Streptomyces* sp. 8812 was obtained using the Wizard® Genomic DNA Purification Kit (Promega). Nearly complete 16S rRNA gene (~ 1500 nucleotides [nt]) was amplified using universal primers: pHr and pAf (Edwards *et al.*, 1989). The amplified product was purified with Wizard® SV Gel and PCR Clean-Up System (Promega). The 16S rRNA gene was sequenced with an automated DNA sequencing system (ABI 3730) and BigDye terminator cycle sequencing kit (Applied Biosystems). Primers used for sequencing are listed by Coenye *et al.* (1999).

A BLAST search service of the GenBank database was used to establish bacterial species most similar to *Streptomyces* sp. 8812 based on the 16S rRNA gene sequence. A phylogenetic tree was constructed using the neighbor-joining, maximum-likelihood, maximum-parsimony tree-making algorithms. An evolutionary distance matrix was generated. Confidence values of branches of the phylogenetic tree were determined in a bootstrap analysis based on 1.000 resampling of the neighbor-joining dataset.

Mycelium observation under fluorescent microscopy. *Streptomyces* sp. 8812 mycelium viability was determined with LIVE/DEAD® *Bac*-Light™ Bacterial Viability Kit (Invitrogen). Centrifuged mycelium samples from submerged cultures were washed gently two times with sterile 0.2% NaCl solution. Then, the harvested mycelium was stained according to the Molecular Probes protocol (15 min at 37°C in dark). The SYTO 9 green fluorescent stain labels cells with intact and damaged membranes. Propidium iodide (PI) only enters bacteria with damaged membranes and affects SYTO 9 fluorescence. Thus, live bacteria appear fluorescent green whereas dead bacteria appear red. The stained mycelium was observed with fluorescent microscope OPTA-TECH MN-800FL immediately after preparation.

The respiration activity of *Streptomyces* sp. 8812 was determined with 5-cyano-2,3-bis(4-methylphenyl)-2*H*-tetrazolium chloride (CTC) (Sigma-Aldrich). During cell respiration CTC is reduced intracellularly to the red fluorescent formazan crystals (CTF). The final CTC concentration in the probe was 5.0 mM. Samples were incubated for 3 h at 37°C in the dark, fixed with 2% formalin (final concentration) and washed twice with sterile 0.2% NaCl solution. Afterwards, probes were observed under the fluorescent microscope OPTA-TECH MN-800FL. Observed pellets were documented on merged photos showing the whole mycelium (light microscopy) and red fluorescent formazan crystals (fluorescent microscopy).

Analysis of enzyme profiles using the api® ZYM test. Mycelium probes from seed and production cultures of *Streptomyces* sp. 8812 conducted in medium M were characterized with api® ZYM tests (bioMérieux SA, France). This semi-quantitative micromethod is designed for determining enzymatic activities. It consists of api® ZYM stripes with enzymatic substrates, incubation boxes, ZYM A and ZYM B reagents. The tested enzymes include phosphatases, esterases, lipases, arylamidases, trypsin, α-chymotrypsin, phosphohydrolase,

α-, β-galactosidases, β-glucuronidase, α-, β-glucosidases, N-acetyl- β-glucosaminidase, α-mannosidase and α-fucosidase. Mycelium from the submerged culture was harvested by centrifugation and washed twice with sterile 0.2% NaCl. Mycelium suspension adjusted to 5–6 McFarland turbidity standard was added to the wells. After 4–4.5-h incubation at 37°C one drop of ZYM A and ZYM B were added to each well. Intensity of the colors that appeared in the wells was estimated on a 1–5 scale, according to the protocol. *Pseudomonas aeruginosa* ATCC 27853 was used as a quality control strain.

Results and Discussion

Medium optimization for bioactive metabolites production by *Streptomyces* sp. 8812

Utilization of nitrogen sources. To establish the optimal conditions of *Streptomyces* sp. 8812 fermentation for secondary metabolites biosynthesis, the production medium M was modified in 17 manners (Table I), with respect to nitrogen sources. The DD-carboxypeptidase 64–575 inhibitory activities of secondary metabolites produced in the different media were compared to those synthesized during *Streptomyces* sp. 8812 fermentation in medium M (Table I). Positive values of correlation coefficients were achieved for soybean flour, Bacto™ peptone, yeast extract, CSL, neopeptone and soytone, indicating that high contents of these nitrogen sources in the medium lead to enhanced production of DD-carboxypeptidase 64–575 inhibitors (Table II). Addition of soybean flour to the medium (medium 11 and 17) had a positive impact on the level of the produced bioactive metabolites. Bacto™ peptone was added to each medium, except medium 4 and 13, and its increasing amounts had a favorable effect on the production of active metabolites. Similar influence was noted for yeast extract, which was present in all media, except 4 and 12; its influence on production of biologically active substances was proportional to its content in the medium.

A negative value of correlation coefficient was obtained for Bacto™ tryptone (Table II), which was present in all tested media. High amounts of this nitrogen source in the medium, impaired enzyme inhibitory activities. For media 2 and 3, containing 5 g of Bacto™ tryptone, which were additionally enriched with 5 g of neopeptone and 5 g of soytone, respectively, secondary metabolites activities were as high as after fermentation in medium M. However, Bacto™ tryptone, when used as the only source of nitrogen in the production medium, was not sufficient for secondary metabolites production (medium 4). A decrease in inhibitory activity of bioactive metabolites was also noticed when the amounts of Bacto™ tryptone, Bacto™ peptone and yeast extract in the production medium were reduced by 50% (medium 5). In comparison to the composition of medium M, increasing the amounts of these three components by 50% (medium 6) did not enhance biosynthesis of bioactive metabolites. Additionally, when the yeast extract and Bacto™ peptone were separately removed from the initial medium (medium 12 and 13, respectively), a decrease in secondary metabolites activity was also observed.

Further on, the influence of CSL in the production media on the biosynthesis of enzyme inhibitors was examined. CSL (10 g/l) was found to be an important component of medium M in obtaining biologically active secondary metabolites. Elimination of CSL (medium 7) resulted in low inhibitory metabolites activities. As a rich source of amino acids, vitamins and glucose, CSL is often used as a component of microbial fermentation broth (De Azeredo *et al.*, 2006; Zou *et al.*, 2009). It is produced in the maize steeping process. The low costs of CSL production are an important factor standing in favor for its utilization in pharmaceutical industry (De Azeredo *et al.*, 2006).

DD-carboxypeptidase 64–575 inhibitory activities were comparable to the levels observed for medium M cultures after fermentation in medium 11, which contained a reduced amount of Bacto™ tryptone (from 17 g to 5 g), 20 g of soybean flour and which was deprived of CSL. It was shown that soybean flour is more nutritional for bacteria in submerged cultures than other soya extracts (Ortiz *et al.*, 2007). Overall, the conducted studies show clearly the great importance of complex nitrogen sources on production of bioactive metabolites by *Streptomyces* sp. 8812. Moreover, statistical analysis revealed that higher amounts of each nitrogen source (except Bacto™ tryptone) tested had a positive impact on the production of bioactive metabolites.

Utilization of carbon sources. The influence of α-lactose in the production media on the biosynthesis of enzyme inhibitors was examined. Statistical analysis showed that higher DD-carboxypeptidase inhibitory activity was observed when the percentage of α-lactose ($rho = -0.489$, $P = 0.040$, $n = 18$) was lower in the production medium (Table II). Therefore, increase of the lactose content to 15 g (medium 15) did not improve the activity of bioactive metabolites.

The relationship between the C/N ratio values in the different media and the activity of secondary metabolites (Table I) was analyzed. Efficient production of bioactive metabolites during fermentation processes was determined to occur at moderate C/N ratio values for some *Streptomyces* strains (Zhinan and Peilin, 1999). Statistically significant correlation for the value of C/N ($rho = -0.508$; $P = 0.031$, $n = 18$) (Table II) was observed. In spite of similar initial C/N ratio values

Table I
The 17 modifications of initial medium M.

	α-Lactose	Soybean flour	Bacto™ Peptone	Yeast Extract	Bacto™ Tryptone	Corn Steep Liquor (CSL)	Neopeptone	Soytone	C/N*	Activity at 120 h (%)***
M	10	–	4	5	17	10	–	–	1.27	100
1	10	–	4	5	5	10	–	–	2.04	65.2
2	10	–	4	5	5	10	5	–	1.62	100
3	10	–	4	5	5	10	–	5	1.92	100
4	10	–	–	–	17	–	–	–	1.87	50.2
5	10	–	2	2.5	8.5	10	–	–	2.07	39.1
6	10	–	6	7.5	25	10	–	–	0.93	100
7	10	–	4	5	17	–	–	–	1.34	72
8	10	–	4	5	17	15	–	–	1.24	87.5
9	10	–	4	5	17	10	–	5	1.27	79.1
10	10	–	4	5	17	10	5	–	1.09	87.5
11	10	20	4	5	5	–	–	–	nc**	100
12	10	–	4	–	17	10	–	–	1.36	67.2
13	10	–	–	5	17	10	–	–	1.49	65.5
14	–	–	4	5	17	10	–	–	0.26	100
15	15	–	4	5	17	10	–	–	1.77	95.5
16	–	–	4	5	17	–	–	–	0.11	66.7
17	–	10	4	5	5	10	–	–	nc	100

* C/N means percentage contribution of C and N in medium
** nc means not calculated
*** activity is a percent of DD-carboxypeptidase 64–575 inhibition

of medium M (1.27), 7 (1.34), 8 (1.24), 9 (1.27) and 12 (1.36), medium M gave the highest activity of secondary metabolites after fermentation. In the light of the above, it seems that for *Streptomyces* sp. 8812 nitrogen sources were more important than α-lactose for biosynthesis of bioactive metabolites.

Characterization of mycelium growth and biosynthesis of secondary metabolites – DD-carboxypeptidase 64–575 inhibitors. The most intensive *Streptomyces* sp. 8812 mycelium growth rate was observed during the first 24 hours of fermentation (0.38 mg/ml/h). Mycelium biomass concentration after 24 h was maintained at a similar level until the end of fermentation (to 144 h). Production of DD-carboxypeptidase 64–575 inhibitors began at the same time as biomass formation (Table III) and reached the maximum level after 48 h. The overlap of trophophase (intensive growth) and idiophase (secondary metabolites formation) resulted in an uniphasic culture. It was shown in other

Table II
Correlation between the percentage share of each substrate in overall mass of medium and the DD-carboxypeptidase 64–575 inhibitory activity at 120 h.

	N	Spearman correlation coefficient (rho)	P-value
α-Lactose	**18**	**–0.489**	**0.040**
Soybean flour	18	0.386	0.114
Bacto™ Peptone	18	0.338	0.170
Yeast extract	18	0.302	0.224
Bacto™ Tryptone	18	–0.136	0.590
Corn Steep Liquor	18	0.014	0.956
Neopeptone	18	0.204	0.418
Soytone	18	0.154	0.542
C/N	**18**	**–0.508**	**0.031**

Table III
DD-carboxypeptidase 64–575 inhibitory activity of *Streptomyces* sp. 8812 metabolites and biomass concentration during 144 h of cultivation.

Time of cultivation (h)	DD-carboxypeptidase 64–575 inhibition (%)	Mycelium biomass concentration (mg/ml)
24	70	9.14
48	82.6	6.3
72	87.4	5.5
96	90	5.6
120	92.4	5.4
144	90	5.7

studies that when defined media support the rapid growth of *Streptomyces coelicolor* A3(2), the strain produced actinorhodin in a fully biphasic fermentation profile. Many environmental factors (media composition, temperature, *etc.*) determine the correlation between biomass growth and bioactive metabolite production (Liao *et al.*, 1995).

Optimization of pH value of the production media. The pH value during the fermentation process affects bacterial cell growth and bioactive product formation, as well as stimulates or inhibits the activity of enzymes (Chen *et al.*, 2011). HRP inhibitory activities of secondary metabolites were tested during *Streptomyces* sp. 8812 fermentation in medium M at different initial pH values. Highest metabolites activities were obtained when initial pH values of the media did not exceed 7.4 (Supplementary Fig. 1(A)S).

During the first 24 hours of fermentation the pH value rose from 6.7 to 8.2, and maintained at similar level to the end of the cultivation (Supplementary Fig. 1(B)S). Due to the glycolysis process in bacterial cells and the release of pyruvate, the pH value typically decreases during the first 24 h of fermentation (Desai *et al.*, 2002; Chen *et al.*, 2011). In the present studies, the pH value increased during the first 24 h of fermentation. Possibly, *Streptomyces* sp. 8812 showed a low intensity of glycolysis process under submerged culture conditions. Perhaps, this strain intensively utilizes peptides and amino acids as nitrogen and carbon sources, generating ammonia as a side product which causes the increase of pH value. Furthermore, *Streptomyces* sp. 8812 biomass growth and bioactive secondary metabolites biosynthesis were not affected by high pH values resulting from the 24-hour fermentation (Table III).

Optimization of the age of the seed culture. The optimum age of the seed culture and its physiological state, including the morphological form and metabolic activity, are important factors for efficient production of secondary metabolites (Zou *et al.*, 2011). Inhibitory activities of *Streptomyces* sp. 8812 metabolites during fermentation cultures that were initiated with seed cultures at various age (at 24, 48 or 72 h) are presented on Supplementary Fig. 2S. The 24- and 48-hour seed cultures, at late logarithmic phase and middle stationary growth phase, respectively, contributed short lag phases of the submerged cultures. So, the optimal age of seed culture for the production of bioactive metabolites was 24 h or 48 h. The 72-hour seed culture needed more time to adapt to new conditions and entered once more the logarithmic growth phase. This resulted in a delay in the reaching the maximum activity of secondary metabolites.

Influence of amino acid supplementation on secondary metabolites activities. Fed-batch culture conducted by supplementation amino acids to the fermentation broth is a process aiming at efficient biomolecule production (Zhang *et al.*, 2012). In the present work, the authors studied the stimulatory effect of amino acids on secondary metabolites production by *Streptomyces* sp. 8812 (Supplementary Fig. 3(A)S-(C)S). Among bioactive metabolites isolated during *Streptomyces* sp. 8812 fermentation was a novel isoquinoline alkaloid (Solecka *et al.*, 2009a; Solecka *et al.*, 2009b). Biosynthesis of isoquinoline alkaloids in nature starts from three amino acids: L-phenylalanine, L-tyrosine and L-tryptophan (Kegg Pathway Maps, 2014). Thus, these three amino acids were chosen for evaluating their influence on bioactive metabolites activities (HRP inhibition). The rising concentrations of L-tryptophan in the media led to a slight decrease of metabolites activities (Supplementary Fig. 3(A)S). Cultures supplemented with 1 mM L-tyrosine were characterized by high secondary metabolites activities at 24 h proceeded by a sudden drop (Supplementary Fig. 3(B)S). L-tyrosine at 100 mM concentration had a stimulatory effect on secondary metabolites production. Cultures containing 10 mM L-tyrosine (Supplementary Fig. 3(B) S) as well as 0.1, 1, 10 and 100 mM L-phenylalanine (Supplementary Fig. 3(C)S) had comparable secondary metabolites activities to control cultures. In summary, the authors observed a concentration-dependent stimulation of metabolites activities during L-tyrosine feeding. Below the optimal 100 mM concentration of L-tyrosine the activity was equal or lower in respect to the control fermentation.

In bacteria, the three tested amino acids are involved in biosynthesis of phosphorylated nicotinamide adenine dinucleotide [NAD(P)] and melanin pigment (Arai and Mikami, 1972). Biosynthesis of bioactive metabolites appeared to be closely related to melanin production. *Streptomyces* sp. 8812 colonies obtained from regenerated protoplasts lacking the ability to produce melanin showed no bioactive metabolites activities (data not shown). The progressive concentration of L-tyrosine clearly influenced melanin production in *Streptomyces* sp. 8812 (data not shown). It seems that bioactive metabolites have similar precursors as melanin. Probably, when L-tryptophan is added to the *Streptomyces* sp. 8812 culture, the primary metabolism pathway tends more toward NAD(P) biosynthesis than secondary metabolites production. In comparison to the tested strain, *S. coelicolor* produces blue antibiotic-actinorhodin. *S. coelicolor* mutants, defective at different stages of actinorhodin biosynthesis pathway were all impaired in blue antibiotic production (Hopwood, 2007).

Effect of anion exchange resin on bioactive metabolites production. Bioactive metabolites produced by *Streptomyces* sp. 8812 were shown to bind to a strong anion exchange resin added to the fermentation broth.

Substances eluted from the resin added to 24-hour old cultures showed 46.6% of HRP enzyme inhibition. When resin was added at 48 h to the fermentation broth, inhibition of the HRP enzyme by eluted substances reached 56.6%.

The authors examined the supernatant from submerged cultures of *Streptomyces* sp. 8812 supplemented with the anion exchange resin. Addition of the resin to the 24- and 48-hour cultures resulted in a decrease of metabolites activities by 14.5% and 21.4%, respectively (Supplementary Fig. 4S). In both cases, the microorganism did not produce more bioactive metabolites after the resin was removed from the fermentation broth. Thus, it seems that reduction of the amount of bioactive metabolites in the culture broth is not a stimulating factor for further biosynthesis. An opposite effect of adding resin was observed for cultures which result in pristinamycin (streptogramin family), teicoplanin (glycopeptide family) and proteasome inhibitor NPI-0052 production (Lee *et al.*, 2003; Jia *et al.*, 2006; Tsueng and Lam, 2007). In all mentioned cases, synthesis of bioactive secondary metabolites was shown to increase.

The choice of resin type was made based on its ionic and hydrophobic/hydrophilic properties. Environmental conditions, such as temperature and pH value, are also important factors for active substance absorption (Casey *et al*., 2007). It has been well documented that addition of resin to the fermentation broth can reduce feedback repression, eliminate the toxic effect of antibiotics on growth, stabilize the structure of active compounds (prevention from hydrolysis) and, consequently, increase the production of secondary metabolites (Lee *et al.*, 2003; Jia *et al.*, 2006; Tsueng and Lam, 2007). What more, addition of the resin to the medium facilitates further recovery of bound metabolites.

Phylogenetic study of *Streptomyces* sp. 8812. Sequence analysis for 16S rRNA gene using NCBI BLAST algorithm showed 100% homology of *Streptomyces* sp. 8812 to *Streptomyces capoamus* JCM 4734. Phylogenetic analysis of *Streptomyces* sp. 8812 indicated that its closest relative is *Streptomyces capoamus* JCM 4734 (98%) (Supplementary Fig. 5S). *S. capoamus* is a known producer of antitumor and antifungal compounds (Goncalves da *et al.*, 1968; Hayakawa *et al.*, 1985; Singh *et al*., 2008).

Mycelium observation under fluorescent microscopy. Mycelium viability and development of *Streptomyces* sp. 8812 cell clumps are shown in Fig. 1(A)-(F). The clumps are irregular in shape, unlike pellets, and smaller (Denser Pamboukian *et al.*, 2002). In 24-hour seed cultures, single live cells were observed at the periphery of the clumps (Fig. 1(A)-(B)). After 17 h of production culture live and dead cells were observed in the clumps. At 24 h, the edge of the clumps consisted of thick live mycelium and a decaying center (Fig. 1(D)). The hyphae inside older clumps were dying, probably due to its high density and low permeability to nutrient substances. At 48 h and 72 h dead cells occupied most of the clump area (fluorescent red) (Fig. 1(E)-(F)). Remaining of live cells was seen in the center of the clumps.

Fig. 2(A)-(F) depicts changes in the respiratory level of *Streptomyces* sp. 8812 under submerged culture. The intensity of red fluorescence is indicative of the respiratory level of bacterial cells. Red areas on the figures represent high respiratory level. Mycelium which formed clumps showed the highest respiratory activity at 17 h (Fig. 2(C)). High respiratory activity is correlated with high biomass production and active uptake of nutritious substances from the culture broth. After 17 h of cultivation, cultures enter the stationary phase and a drop in respiratory activity was observed (Fig. 2(D)). At 48 h, there were single red spots seen inside the clumps (Fig. 2(E)). No detectable respiratory activity was observed after 72 h of cultivation. The primary metabolism of the clumps at 72 h was low and could not be detected by the CTC fluorescent method (Fig. 2(F)). The CTC assay allows precisely establishing the logarithmic growth phase as well as early and late stationary phase. Using both fluorescent staining methods, the authors determined that the most intensive growth of *Streptomyces* sp. 8812 mycelium under submerged culture occurs during the first 17 h of cultivation.

In summary, mycelium observations allowed to establish that the intensity of production of secondary metabolites with DD-carboxypeptidase 64–575 and HRP inhibitory activity by *Streptomyces* sp. 8812 was correlated with high respiratory level and mycelium viability.

Mycelium enzymatic profiles. Results of the api® ZYM test for *Streptomyces* sp. 8812 revealed the highest activity of two enzymes: leucine arylamidase and α-chymotrypsin. Both enzymes confirmed that *Streptomyces* sp. 8812 assimilates nitrogen during the whole fermentation process. Other enzymes from the aminopeptidase group: valine and cystine arylamidases, and trypsin showed low activity at the initial stages of cultivation. Arylamidases and trypsin were active till 48 h and 120 h, respectively. The api® ZYM test revealed low activity of hydrolases, such as esterase or esterase lipase (color intensity 3). The activity of esterase and esterase lipase was noted at 0 h and 24 h of cultivation, respectively. There was no esterase activity observed after 72 h. Esterase lipase was active till the end of fermentation. Acid phosphatase showed a high level of activity during the first 17 h, between 24 h and 120 h its activity was reduced. The api® ZYM results revealed that acid phosphatase could be an enzymatic marker to precisely determine the logarithmic phase of *Streptomyces* growth. The most intensive phase of

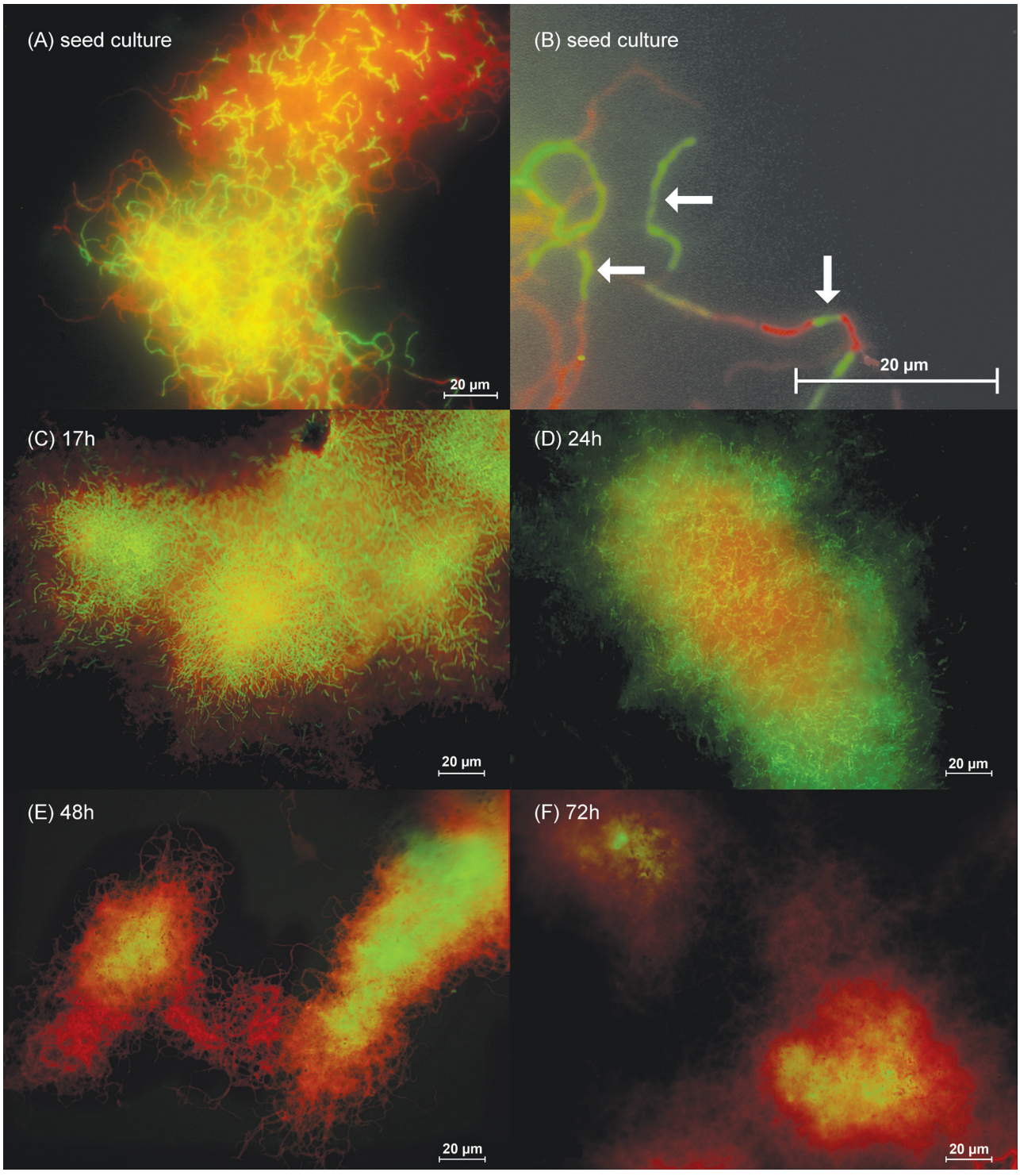

Fig. 1 (A)-(F). Analysis of the development and cell death processes of mycelium in clumps of *Streptomyces* sp. 8812 in submerged cultures. Images correspond to mycelium stained with SYTO 9 and PI. (B) Single live cells in hyphae from seed culture are indicated with arrows.

Streptomyces sp. 8812 growth was between 17 h and 24 h of cultivation. An alkaline phosphatase was active at low level from 48 h to 120 h. The activity of naphthol-AS-BI-phosphohydrolase was high only at 120 h and could result from release of this enzyme from the cytosol of dead bacterial cells. No activity was detected for lipase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase during the whole process of *Streptomyces* sp. 8812 fermentation.

Comparison of *Streptomyces* sp. 8812, *S. capoamus* and their metabolites. *S. capoamus* and *Streptomyces* sp. 8812 cultured for 14 days on ISP2 agar showed similar phenotypic characteristics of aerial mycelium.

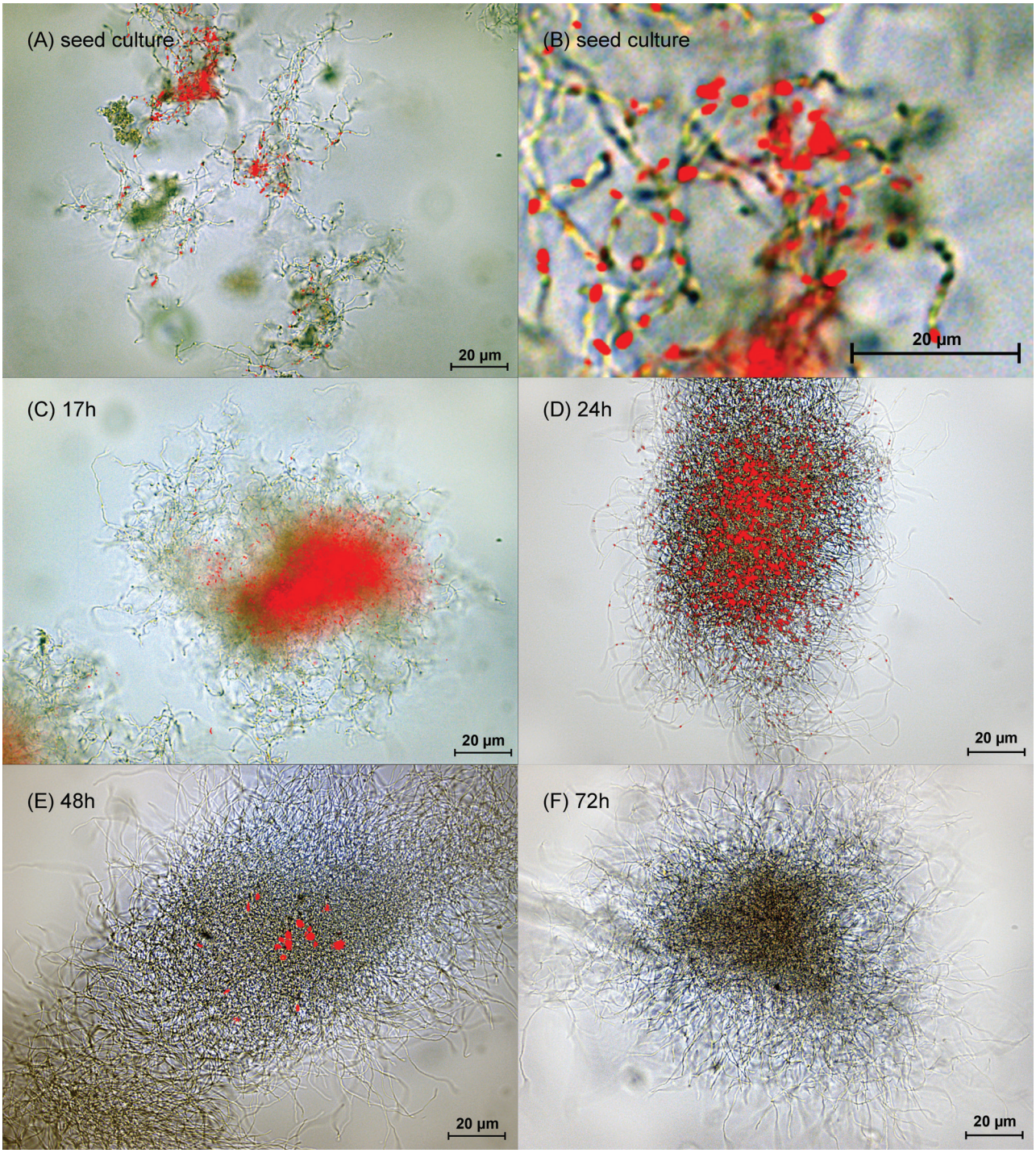

Fig. 2 (A)-(F). Analysis of respiratory processes of mycelium in clumps of *Streptomyces* sp. 8812 in submerged cultures. Images correspond to mycelium stained with CTC.

When both strains were cultivated in medium M, their secondary metabolites exhibited very similar DD-carboxypeptidase 64–575 inhibitory activities (Supplementary Fig. 6S). Until now, the only antitumor and antifungal metabolites isolated from *S. capoamus* fermentation broth were capoamycin and ciclacidine (Goncalves *et al.*, 1968; Hayakawa *et al.*, 1985). In the present study, it was determined that *S. capoamus* produces also DD-carboxypeptidase 64–575 inhibitors.

Conclusions. Conducted studies confirmed that biosynthesis of bioactive metabolites by *Streptomyces* sp. 8812 is a bioprocess influenced by various external factors (pH, medium composition, temperature, feeding). Statistical analysis showed that for *Streptomyces* sp. 8812 increasing amounts of complex nitrogen sources (except Bacto™ tryptone) in the tested media was important for biosynthesis of DD-carboxypeptidase 64–575 inhibitors. Higher DD-carboxypeptidase

64–575 inhibitory activities of secondary metabolites were achieved when the C/N ratio was lower. Production of DD-carboxypeptidase 64–575 inhibitors began at the same time as biomass formation (Table III) and reached the maximum level after 48 h. Production of secondary metabolites was not affected by pH value of the production medium in the measured pH range of 5.5 to 7.4. The optimal age of seed culture for the production of bioactive metabolites was 24 h or 48 h. The authors also observed a concentration-dependent stimulation of metabolites activities during L-tyrosine feeding. Phylogenetic analysis of *Streptomyces* sp. 8812 indicated that its closest relative is *S. capoamus* JCM 4734 (98%), whereas sequence analysis for 16S rRNA gene using NCBI BLAST algorithm showed 100% homology between these two strains.

Development studies of *Streptomyces* sp. 8812 in submerged culture were performed by means of LIVE/DEAD® Bac-Light™ Bacterial Viability Kit, respiratory activity CTC test and api® ZYM test. Obtained results allowed to separate precisely the logarithmic and stationary phase of growth. Intensive production of secondary metabolites with DD-carboxypeptidase 64–575 and HRP inhibitory activity ceased when the respiratory level and viability of the mycelium dropped. Overall, it was determined that vital functions determine the production of secondary metabolites in *Streptomyces* sp. 8812.

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ORIGINAL PAPER

Isolation and Characterization of α-Endosulfan Degrading Bacteria from the Microflora of Cockroaches

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Abstract

Extensive applications of organochlorine pesticides like endosulfan have led to the contamination of soil and environments. Five different bacteria were isolated from cockroaches living in pesticide contaminated environments. According to morphological, physiological, biochemical properties, and total cellular fatty acid profile by Fatty Acid Methyl Esters (FAMEs), the isolates were identified as *Pseudomonas aeruginosa* G1, *Stenotrophomonas maltophilia* G2, *Bacillus atrophaeus* G3, *Citrobacter amolonaticus* G4 and *Acinetobacter lwoffii* G5. This is the first study on the bacterial flora of *Blatta orientalis* evaluated for the biodegradation of α-endosulfan. After 10 days of incubation, the biodegradation yields obtained from *P. aeruginosa* G1, *S. maltophilia* G2, *B. atrophaeus* G3, *C. amolonaticus* G4 and *A. lwoffii* G5 were 88.5% , 85.5%, 64.4%, 56.7% and 80.2%, respectively. As a result, these bacterial strains may be utilized for biodegradation of endosulfan polluted soil and environments.

Key words: α-endosulfan, biodegradation, cockroaches, isolation

Introduction

Endosulfan is a chlorinated cyclodiene and broad-spectrum insecticide used in a wide variety of food and non-food crops against many insect pests. Technical grade endosulfan is a mixture of two stereoisomers, *i.e.*, α and β-endosulfan in a ratio of 7:3. Due to its extensive use, endosulfan residues are commonly found in the environment such as atmosphere, soils, sediments, surface and ground waters and foodstuffs (Hussain *et al*., 2007; Kataoka and Takagi, 2013; Kong *et al*., 2014; Kumar *et al*., 2014). Its abundant use poses a threat to environmental quality and public health. Endosulfan affects the central nervous system, kidney, liver, blood chemistry, parathyroid gland and has teratogenic and mutagenic effects (Lu *et al*., 2000).

Several microorganisms, both bacteria and fungi, have been isolated from different sources which have the capability to degrade endosulfan (Goswami and Singh, 2009; Bajaj *et al*., 2010; Bhattacharjee *et al*., 2014). These microorganisms can use endosulfan either as carbon or sulfur source or both (Siddique *et al*., 2003). Such organisms can later be investigated as source of enzymes for further enzymatic reactions in detoxification of endosulfan (Thangadurai and Suresh, 2014). The use of endosulfan as a sulfur source and the subsequent removal of sulfur from the compound considerably reduced its toxicity to mammals (Dorough *et al*., 1978; Goebel *et al*., 1982).

Invertebrates host numerous microorganisms with interactions ranging from symbiosis to pathogenesis, but the microflora of insects is unexplored as yet. The gut microflora plays an important role in pheromone production, pesticide degradation, vitamin synthesis, enzyme synthesis and pathogen prevention (Reeson *et al*., 2003). Cockroaches have been searched as transmitters of pathogenic bacteria and carrries of multiple antibiotic resistance strains (Fotedar *et al*., 1991; Pai *et al*., 2005). No information is available about the intestinal microbes of cockroaches, which live in a pesticide environment. The insect gut provides ideal conditions for gene transfer between bacteria (Dillon and Dillon, 2004). Microorganisms adapt to changing environmental conditions by horizontal gene transfer (De Boever *et al*., 2007; De Gelder *et al*., 2008), conjugative plasmids (De Boever *et al*., 2007) and simple mutations which provides them with new traits (antibiotic-resistance, xenobiotic degradation) so they can survive and colonize their new environment. Considering these points, we suggest that isolated bacterial strains may have

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developed resistance to pesticides. If this is true, we can isolate new and effective α-endosulfan degrading bacteria from cockroaches' microflora and these isolates can be used for the biological treatment of waters and soils polluted with endosulfan and other insecticides.

Experimental

Material and Methods

Chemicals. α-endosulfan and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). All solvents used were of the highest analytical grade and were employed without further purification. The stock α-endosulfan solution was prepared in acetone and used for all the experiments. The chemical structure and the most important physical parameters of endosulfan are summarized in Fig. 1 and Table I, respectively.

Isolation of endosulfan degrading microorganisms. Cockroaches (*Blatta orientalis* Linnaeus, 1758; Dictyoptera) were collected from stables contaminated with pyrethroid and chlorinated organochlorine pesticide in Samsun (Turkey). Three insects were surface sterilized using 70% ethanol for 1 min and crushed in a sterile mortar containing 10 ml of 0.85% (w/v) saline solution (Okay *et al.*, 2013). Dilutions of 10^{-4} of each sample (1 ml) were suspended in 50 ml liquid non sulfur medium (NSM) containing 100 mg/l α-endosulfan in a 250 ml Erlenmeyer flask, and incubated at 30°C with shaking (150 rpm). The NSM was autoclaved at 121°C for 20 min after which it was aseptically spiked with α-endosulfan dissolved in acetone to yield a final concentration of 100 mg/l. The NSM consisted of (g/l): K_2HPO_4, 0.225; KH_2PO_4, 0.225; NH_4Cl, 0.225; $MgCl_2 \cdot 6H_2O$, 0.845; $CaCO_3$, 0.005; $FeCl_2 \cdot 4H2O$, 0.005; D-glucose, 1.0; and 1 ml of trace element solution per litre. The trace element solution prepared for NSM contained (mg/l): $MnCl_2 \cdot 4H_2O$, 198; $ZnCl_2$, 136; $CuCl_2 \cdot 2H_2O$, 171; $CoCl_2 \cdot 6H_2O$, 24; and $NiCl_2 \cdot 6H_2O$, 24 (Siddique *et al.*, 2003). After 7 days, 5 ml of each culture was re-inoculated into new α-endosulfan-NSM medium and further incubated at 30°C for 7 days. This subculture was repeated under the same culture conditions, and then an aliquot (0.2 ml) from each culture was applied to solid α-endosulfan-NSM for isolation of single colonies.

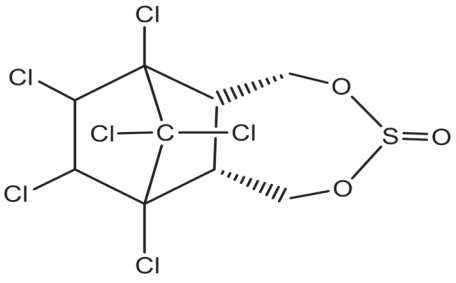

Fig. 1. Molecular structure of α-endosulfan (Fan, 2007).

Table I
Physical parameters of α endosulfan (Fan 2007)

Molecule formula	$C_9H_6Cl_6O_3S$
Molecular weight	406.95 g/mol
Melting point	108–110°C
Water solubility	0.33 µg/L
Density	1.745 g/cm³
Boiling point	<300°C
Color	Yellowish, brown

Identification of endosulfan degrading microorganisms. α-endosulfan degrading bacterial isolates were identified by various tests, such as pigment formation, Gram staining, nitrate reduction, catalase and oxidase tests, and starch hydrolysis. Biochemical reactions were conducted according to Benson (2001). Biochemical activities were determined according to the recommended scheme of Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Identification was confirmed by the fatty acid analysis for all bacterial isolates. Fatty acid methyl ester (FAME) profiles of each bacterial strain were identified by comparing the commercial databases (Tripticase Soy Broth Agar 40) with the MIS software package. The identity of bacterial strains was revealed by computer comparison of FAME profiles of the unknown test strains with those in the library. FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m × 0.2 mm) with cross-linked 5% phenyl methyl silicone.

Preparation of bacterial inoculum for biodegradation studies. Bacterial strains were grown in Nutrient Broth (Merck) to mid log phase of growth. The cells were then centrifuged at 10.000 rpm for 10 min, washed three times with sterile 0.85% saline solution and diluted with sterile water to a uniform optical density (OD_{600}) of 0.75.

Analytical methods. Samples were taken at intervals of 3, 5 and 10 days to determine the levels of bacterial growth and α-endosulfan degradation. The optical densities of the culture media were determined spectrophotometrically by measuring the absorbance at 600 nm. α-endosulfan was measured by HPLC. In the cultures α-endosulfan was extracted using ethyl acetate. The sample was dried with anhydrous Na_2SO_4, and concentrated with a rotary evaporator. Samples were detected by HPLC using ODS Hypersil Column (250 × 4.6 mm) as the stationary phase and acetonitrile: water (70:30, v/v) as the mobile phase. The solutes were determined utilizing UV-VIS detector at 214 nm (Hussain *et al.*, 2007). All experiments were performed in triplicate.

Results

Isolation and identification of α-endosulfan degrading bacteria. Five different bacteria which were able to use α-endosulfan as the sole sulfur source at 100 mg/l concentration were successfully isolated and identified. They were identified based on their morphological, biochemical characteristics (Table II) and the cellular fatty acid compositions (Table III). Different species had various cellular membrane compositions and all strains contained 16:0 fatty acids. Strain *Pseudomonas aeruginosa* G1 was gram negative, catalase and oxidase positive, nitrate reductase negative. Its major fatty acids were 10:0 3OH, 18:1 w7c and 16:0. Strain *Stenotrophomonas maltophilia* G2 was gram negative and catalase negative, oxidase and nitrate reductase positive. The results of fatty acid methyl esters (FAMEs) analysis identified G2 as *S. maltophilia*. As shown Table III, totally 22 different fatty acids were detected in G2 strain. Isopentadecanoic (15:0 iso;13-Methyltetradecanoic) had higher relative mass comparing to remaining FAMEs. Its major fatty acids were 15:0 iso, 16:1 w7c/15:0 iso2OH, 15:0 antesio, 16:0. Strain *Bacillus atrophaeus* G3 was gram positive and catalase, oxidase, nitrate reductase positive. Its major fatty acids were 15:0 antesio, 15:0 iso, 17:0 antesio. Strain *Citrobacter amolonaticus* G4 was gram negative and catalase, oxidase, nitrate reductase positive. Its major fatty acids were 16:0, 18:1 w7c, 16:1 w7c/16:1w6c and 14:0. Strain *Acinetobacter lwoffii* G5 was gram negative and catalase and oxidase negative, nitrate reductase positive. Its major fatty acids were 16:0, 16:1 w7c/15:0 iso2OH, 18:1 w9c and 12:0. *A. lwoffii* possesses fatty acids with almost identical characteristics. The most significant fatty acid was 16:1 w7c/15:0 iso 2OH, with an average proportion of approximately 38.81%. The other three major fatty acids were 12:0 (10.26%), 16:0 (20.58%) and 18:1 w9c (22.85%).

Biodegradation of α-endosulfan. The bacterial growth and α-endosulfan degradation were determined after 3, 5 and 10 days of incubation. The growth performances of test bacteria in liquid NSM+ α-endosulfan were spectrophotometrically monitored by measuring the optical density of the cultures, and the results are shown in Fig. 2. Bacterial strains showed similar growth on the third day of incubation and the cell density increased exponentially with time. At the end of 10th day, the highest optical densities for G1 (1.05), G2 (0.93) and G5 (0.85) were detected in NSM+ α-endosulfan.

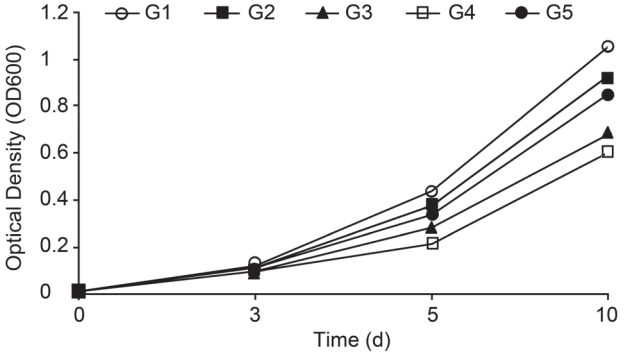

Fig. 2. Bacterial growth monitered by measuring optical densities at 600 nm.

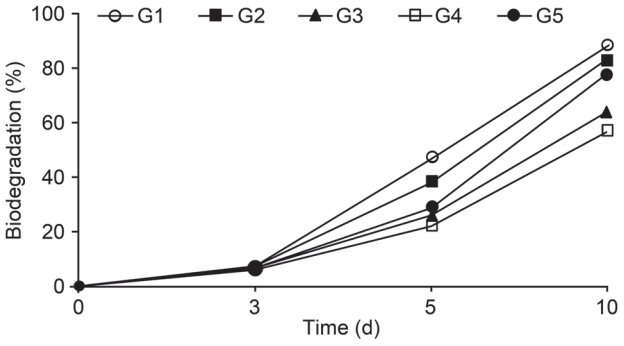

Fig. 3. Biodegradation of α-endosulfan in the broth by isolated bacteria.

As shown in Fig. 3, three strains identified as *P. aeruginosa* G1, *S. maltophilia* G2 and *A. lwoffii* G5 showed the highest potential to degrade α-endosulfan. *P. aeruginosa* G1 exhibited the highest degradation rate of 88.5%. The lowest percentage of degradation (56.7%) was observed with *C. analyticus* G4 strain. The result also demonstrated thatwhen growth of the bacterial cell increased, α-endosulfan degradation also

Table II
The morphological and biochemical characteristics of bacterial isolates of *Blatta orientalis*

Tests	*P. aeruginosa* G1	*S. maltophilia* G2	*B. atrophaeus* G3	*C. amolonaticus* G4	*A. lwoffii* G5
Pigment	Blue-green	Bright yellow	–	Yellow	–
Shape	Bacilli	Bacilli	Bacilli	Bacilli	Coccobacilli
Gram stain	–	–	+	–	–
Catalase	+	–	+	+	–
Oxidase	+	+	–	–	–
Nitrate reduction	–	+	+	–	+

Table III
The fatty acid profiles of bacterial isolates using Microbial Identification System

Fatty acids	*P. aeruginosa* G1	*S. maltophilia* G2	*B. atrophaeus* G3	*C. amalonaticus* G4	*A. lwoffii* G5
10:0		0.59			
11:0 iso		2.87			
11:0 iso 3OH		1.51			
10:0 3OH	3.75				
12:0	4.7			3.79	10.26
12:0 2OH	3.70				
12:0 3OH	3.64	2.93			3.55
13:0 iso		0.39			
14:0 iso		0.67			
14:0		4.23		5.46	
13:0 iso 3OH		2.52			
13:0 2OH		0.36			
15:1 iso F		1.22			
15:0 iso		32.27	39.21		
15:0 Antesio		12.33	46.70		
15:0		0.47			
16:0 iso		0.88			
16:1 w7c			0.96		
16:1 w11c			0.79		
16:0	22.84	7.58	0.72	25.84	20.58
16:1 w9c		3.87			
17:1 iso w9c		4.35			
17:0 cyclo				1.88	
17:1 iso w10c			2.00		
17:0 iso		2.63	1.09		
17:0 antesio		0.31	4.77		
18:1 w7c	44.46	0.99		26.18	3.94
18:1 w9c		1.89			22.85
18:0	0.87				
17:1 iso/antesio B			3.76		
16:1 w7c/15:0 iso2OH		14.06			38.81
16:1w7c/16:1w6c				29.51	
14:0 3OH/16:1 iso 1				6.42	

relatively increased. All isolates were capable of utilizing α-endosulfan as a sulfur source and degraded 56.7–88.5% of α-endosulfan after 10 days.

Discussion

Invertebrates are known to harbour a rich and complex community of microorganisms in their guts and other body regions (Dillon and Dillon, 2004; Ozdal *et al*., 2012). Insect gut symbiotic microbiota allow us to find novel biocatalysts. Many researchers have reported pesticide degrading bacteria isolated from pesticide contaminated soil (Goswami and Singh, 2009; Thangadurai and Suresh, 2014). The present study describes the isolation and identification of bacterial strains capable of degrading α-endosulfan from cockroaches body microflora. Kikuchi *et al*. (2012) and Werren (2012) showed that infection with an insecticide degrading bacterial symbiont immediately establishes insecticide resistance in pest insects as chemical detoxifying agents. Also, although our livers get much of the credit as a toxin degrading organ, our gut microbiome is likely a major player as well (Gill *et al*., 2006). Therefore, the chance of isolating novel microorganisms capable of degrading α-endosulfan from the insect microflora is very high.

A total of five bacterial isolates were isolated and screened for their ability to utilize α-endosulfan as

a sole source of sulfur. These isolates were identified as *P. aeruginosa* G1, *S. maltophilia* G2, *B. atrophaeus* G3, *C. amolonaticus* G4 and *A. lwoffii* G5. Mainly, different applied methods are used for the identification of unknown microorganisms. The first method is based on biochemical, physiological and morphological criteria. The second method is based on chemotaxonomy. The chemotaxonomic approach for obtaining bacterial fatty acid profiles is based on analysis of the methyl esters of their fatty acids by gas chromatography (Basile *et al*., 1998; Fang *et al*., 2000). Whole cellular fatty acid methyl esters content is a stable bacterial profile, the analysis method is rapid, cheap, simple to perform and highly automated, and has long been used for microbial diagnostics and taxonomy (Giacomini *et al*., 2000). *Pseudomonas* species have 3-OH 10:0, 3-OH 12:0 and 12:0, in addition to 16:1 w9c, 16:0 and 18:1 w7c, as their cellular fatty acids, as well as the Q-9 ubiquinone (Ikemoto *et al*., 1978; Vancanneyt *et al*., 1996). *S. maltophilia* has a complex fatty acid profile and different OH-acids detected in the sample. Similiar results were reported by David *et al*. (2008). The predominance of terminally methylbranched iso and anteiso fatty acids having 12 to 17 carbons is a characteristic observed in all *Bacillus* species. The normal fatty acids such as 14:0 and 16:0, the most common fatty acids in the majority of organisms, are generally minor constituents in the genus *Bacillus* (Kaneda, 1977). The major cellular fatty acids of *Citrobacter* are C16:0, C14:0, C18:1 w7c, C16:1 w7c/16:1 w6c and 14:0 3OH/16:1 iso (Whittaker *et al*., 2007). The major fatty acids of *Acinetobacter* strains were 18:1 w9c, 16:1 w7c, 16:0. Each strain of *Acinetobacter*, except *A. lwoffii*, also contained a small amount of 12:0 2-OH (Moss *et al*., 1988).

Previous researchers have reported that endosulfan can be used as the sole sulfur source (Hussain *et al*., 2009; Kataoka and Takagi, 2013; Kumar *et al*., 2014) or the sole carbon source for microbial growth (Sutherland *et al*., 2000; Kumar *et al*., 2007; Castillo *et al*., 2011). In this study, the isolates were screened for their ability to utilize α-endosulfan as the sole sulfur source. Endosulfan is a poor biological energy source, as it contains only six potential reducing electrons. However, it has a relatively reactive cyclic sulfite diester group and can serve as a good sulfur source (Sutherland *et al*., 2000). The removal of sulfur moiety from the endosulfan substantially reduced its vertabrate toxicity and therefore helped in detoxification of the compound (Goebel *et al*., 1982; Singh and Singh, 2011).

The isolated bacterial strains could degrade about 56.7–88.5% of 100 mg/l of α-endosulfan in 10 days of incubation. The difference in degradation capability of various strains may be due to difference in enzyme system and/or difference in their growth rate (Bhattacharjee *et al*., 2014).

There was a very little α-endosulfan biodegradation during the first 3 days, because there was possibly a lag phase. As the incubation continued, the biodegradation rates of α-endosulfan then accelerated. This positive effect may be due to the induction/activation of the enzymes required for degradation (Verma *et al*., 2011). Optical density of the isolates increased with time. This demonstrated that α-endosulfan was degraded by the isolates for their growth. This effect is due to a greater number of organisms that can more easily tolerate the toxic compounds (Hussain *et al*., 2007; Gur *et al*., 2014).

To the best of our knowledge, G3 and G4 have not yet been reported as α-endosulfan degraders. However, in this study, we demonstrated that our strains could degrade α-endosulfan much higher or similiar than of previously reported bacterial strains. *S. maltophilia* E4, *Rhodococcus erythropolis* E5 (Kumar *et al*., 2007), *P. aeruginosa* MN2B14 (Hussain *et al*., 2009) degraded 46% (15 days), 24% (15 days) and 95% (14 days) of α-endosulfan, respectively. In the case of the experiment done by Yu *et al*. (2012), *Stenotrophomonas*. sp. LD-6 completely degraded of α-endosulfan and β-endosulfan (100 mg/l) after 10 days.

Conclusions. Strains of *P. aeruginosa* G1, *S. maltophilia* G2, *B. atrophaeus* G3, *C. analyticus* G4 and *A. lwoffii* G5, able to use α-endosulfan as the only sulfur source, were isolated from cockroaches. Pesticide tolerance in these bacteria may be important in the bioremediation of polluted environments by the pesticides. In this study, we showed that pesticide degrading bacteria can be isolated from insects. Other insects (Colorado potato beetle, fleas and termites) which lives in pesticide contaminated environments should be researched to isolate different pesticide degrading bacteria as well. We demonstrated that the α-endosulfan degrading bacterial strains establish a particular and beneficial symbiosis with the cockroaches.

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ORIGINAL PAPER

Bacterial Diversity and Composition in Oylat Cave (Turkey) with Combined Sanger/Pyrosequencing Approach

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Abstract

The microbiology of caves is an important topic for better understanding subsurface biosphere diversity. The diversity and taxonomic composition of bacterial communities associated with cave walls of the Oylat Cave was studied first time by molecular cloning based on Sanger/pyrosequencing approach. Results showed an average of 1,822 operational taxonomic units per sample. Clones analyzed from Oylat Cave were found to belong to 10 common phyla within the domain Bacteria. *Proteobacteria* dominated the phyla, followed by *Actinobacteria, Acidobacteria* and *Nitrospirae.* Shannon diversity index was between to 3.76 and 5.35. The robust analysis conducted for this study demonstrated high bacterial diversity on cave rock wall surfaces.

Key words: bacterial diversity, Oylat Cave, Sanger/pyrosequencing, subsurface biosphere

Introduction

Diversity, composition, and functional roles of microorganisms in the habitable extreme environments have been intensively studied in the last decades. Caves are one of the habitable extreme habitats, and microorganisms have been determined in these ecosystems based on independent culture techniques in late 1990s such as in Nullarbor Cave, Australia (Holmes *et al*., 2001); Wind Cave, United States (Chelius and Moore, 2004); Niu Cave, China (Zhou *et al*., 2007); Altamira Cave, Italy (Portillo *et al*., 2009); Kartcher Caverns, United States (Ortiz *et al*., 2014).

Turkey has a great geothermal potential due to a high degree of orogenic, magmatic, and volcanic activity, as part of the Alpine-Himalayan orogenic belt (Ketin, 1966). Due to active faults and volcanism, there are more than 600 terrestrial hydrothermal vents, mainly in the Aegean Region, Northwest, Middle Anatolia, East and Southeast Anatolia regions. In addition, there are more than 20,000 caves in Turkey; however, a limited study has been conducted on the microbial diversity in these cave systems.

Oylat Cave (39°56'36"N, 29°35'26"E) is located 17 km south of the town Inegol, which is 80 km southeast of Bursa city, Turkey. Oylat Cave has formed due to karstification within the Permian-Triassic recrystallized limestone, develops along two fault zones in WNW-ESE and NE-SW directions. In the three parts creating the cave, debris and carbonate sediments have accumulated. In the first part, there are debris stores including pebble stone, sand stone and silt stone and carbonate things including stalactite, stalagmite, column, cave pearls. In the second part, carbonate formations are present and these are cave breaches, stalactite, stalagmite, column, macaroni structures, curtain stalactites, cave pearls, giant stalactites pools. In the third part, mudstone, siltstone, sand stone and thick cave breaches are present (Atabey *et al*., 2002).

The purpose of this work was to determine the bacterial diversity and composition at Oylat Cave (Bursa, Turkey) using a combined Sanger and 454 pyrosequencing approaches. Even though, previous studies have documented distinct bacterial communities in limestone caves in the world (Barton *et al*., 2007; Legatzki *et al*., 2012), the current work is first effort to document bacterial diversity and taxonomic composition for Oylat Cave (Bursa, Turkey).

Experimental

Materials and Methods

Sample collection and DNA extraction. Cave wall samples were collected using sterile spatula and stored in sterile Whirl-Pak bags (Nasco, Ft. Atkinson, WI, USA) in September 2013, Oylat Cave (Fig. 1 and 2).

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After collection, the samples were frozen on dry ice on site, and stored at –20°C upon return to the laboratory (Groth *et al.*, 1999). Environmental DNA was extracted from samples using Fast DNA Spin kit for soil (MP biomedicals, Solon, OH USA).

Clone library construction and Sanger sequence analysis. Total genomic DNA was used as a template for 16S rRNA PCR amplification using bacteria 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') universal primers (Weisburg *et al.*, 1991). Each 20 µl reaction mixture contained: l µl environmental DNA template, 2.25 mM $MgCl_2$, 2 µl GeneAmp 10X PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 100 µM dNTPs (Sigma-Aldrich, Saint Louis, MO, USA), 0.2 µM each primer, 2.5 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Carlsbad, CA, USA). Thermal cycling was as follows: initial denaturation 5 min at 94°C, 25 cycles of 94°C for 1 min, hybridization at 50°C for 25 s and elongation at 72°C for 2 min followed by a final elongation at 72°C for 20 min. PCR products were purified using a QIAquick kit (QIAGEN, Valencia, CA, USA), and were cloned into *Escherichia coli* hosts using the TOPO TA Cloning kit with the pCR 2.1 Vector (Invitrogen Corporation, Carlsbad, CA, USA). Plasmid DNA was extracted and purified using the Ultra Clean Standard Mini Plasmid Prep Kit (MoBio Laboratories). Cloning products were sequenced by TUBITAK MAM DNA Services Facility at Gebze, Turkey, using standard M13 primers.

Partial sequences were assembled with CodonCode Aligner v.1.2.4 (CodonCode, USA) and manually checked. Assembled sequences were checked for chimera by Bellerophon server (Huber *et al.*, 2004) and Chimera_Check v 2.7 (Cole *et al.*, 2005). Sample sequences were aligned by BioEdit (Ibis Biosciences, Carlsbad, CA, USA). Phylogenetic analysis was performed in PAUP (Sinauer Associates, Sunderland, MA) using parsimony, neighbor-joining, and maximum likelihood analyses. The 16S rRNA gene sequences were submitted to the NCBI Gen Bank database under accession numbers JQ065958-JQ065959 and JQ219081-JQ219137.

454 pyrosequencing and sequence analysis. For the pyrosequencing, the V6 region of the 16S rRNA gene was amplified using PCR with a bacterial primer set 967f (5'-MWACGC GAR GAA CCT TAC C-3') and 1070r (5'-AGC TGACGA CAR CCA T-3') (Baker *et al.*, 2003). A single-step 30 cycle PCR using HotStar Taq Plus Master Mix Kit (Qiagen, Valencia, CA) were used under the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 40 s, and 68°C for 40 s and final extension at 68°C for 5 min was performed. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). The samples were sequenced with a Roche FLX 454 (Roche, Mannheim, Germany) in a commercial facility (Research and Testing Laboratories, Lubbock, TX).

Raw results 454 pyrosequencing analyzed with combining the QIIME (Quantitative Insights into Microbial Ecology), Mothur and RDP (ribosomal database project) programs as described in Nakayama *et al.* (2013). The obtained sequence data were depleted of barcodes and primers then short sequences < 200 bp are removed by using the QIIME split library. Sequences with ambiguous base calls removed, and sequences with homopolymer runs exceeding 6 bp removed. Sequences were then denoised and chimeras removed using the Chimera.uchime program in Mothur (http://www.mothur.org/wiki/Download_mothur) (Edgar *et al.*, 2010; Wright *et al.*, 2012). Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity) by using the pick_otus_through_otu_table.py script of QIIME. OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis and Hugenholtz, 2006) and compiled into each taxonomic level into both "counts" and "percentage" files. Counts files contain the actual number of sequences while the percent files contain the relative (proportion) percentage of sequences within each sample that map to the designated (Cole *et al.*, 2005; Wang *et al.*, 2007) taxonomic classification using the RDP Classifier (http://rdp.cme.msu.edu). Sequences were deposited in the NCBI Sequence Read Archive under accession number PRJNA258221.

SEM (Scanning electron microscopy) with EDS. Scanning electron microscopy was used to examine the morphological structure of the samples in high resolution. The sample was fixed in glutaraldehyde for 3–4 hours. Fixed samples were then dried, mounted and coated with gold (Borsodi *et al.*, 2012). The samples were examined using a Jeol JSM – 7001FA scanning electron microscope at an accelerating voltage of 15 kV. For the microanalyses of the chemical element composition of the samples were accomplished by EDS using.

Results

Field observation and SEM investigations. Samples were collected from three locations inside the cave, fossil part, section II and section III (Fig. 1 and 2). Scanning electron microscopy (SEM) images revealed that sample were composed of a mass of calcite crystals (Fig. 3a). Solid samples on walls at Oylat Cave is also presented in Fig. 3b and Fig. 3c. Elemental analysis of solids at fossil part sample indicates that the highest

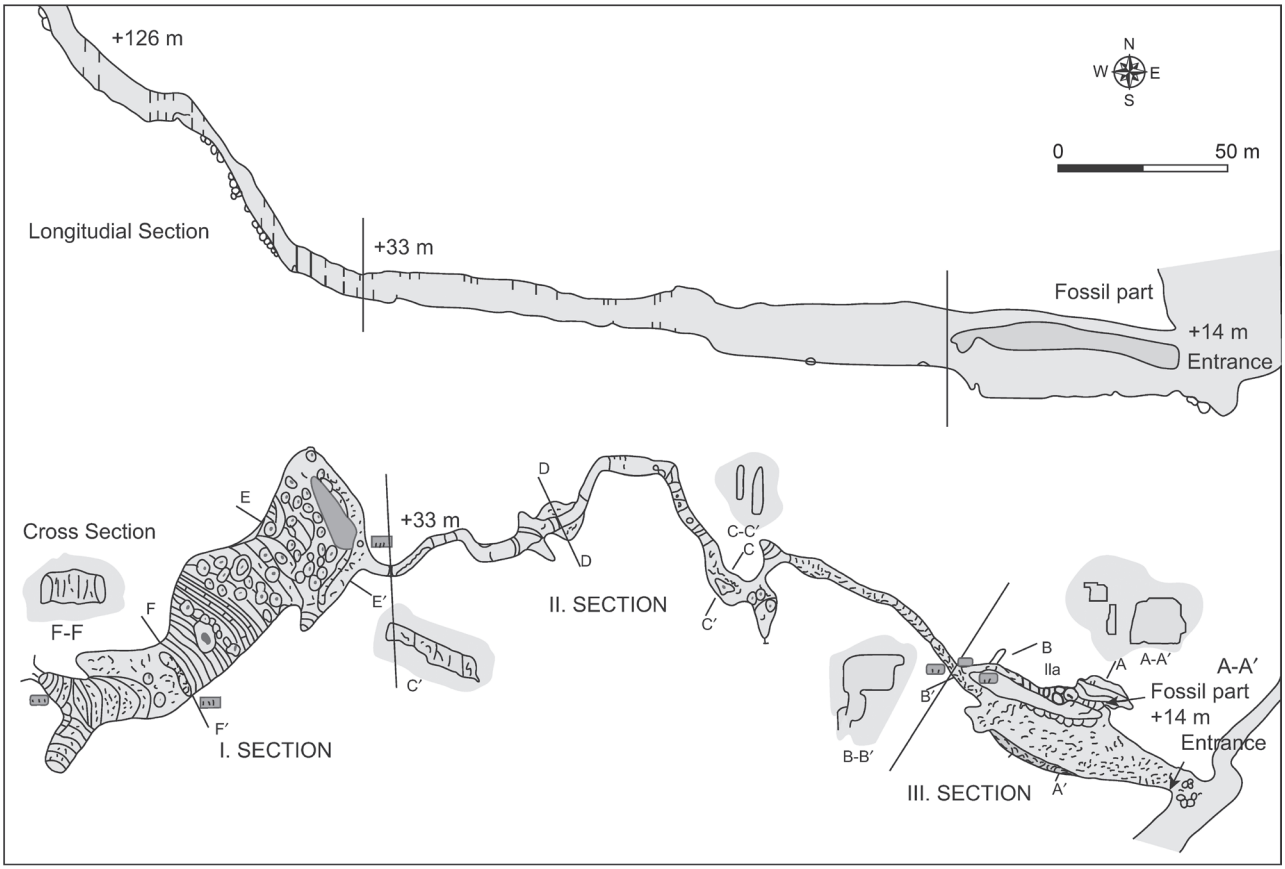

Fig. 1. Map of Oylat Cave (Atabey *et al.*, 2002).

(wt. %) of calcium (Fig. 3d). The highest elemental wt. % iron is observed in wall rock sample collected from the section III (Fig. 3e).

16 S rRNA gene library. To investigate the microbial diversity in Oylat Cave (Bursa, Turkey), a 16S rRNA clone library for bacteria was constructed and 87 clones were randomly selected and analyzed. The majority of the sequences identified from the clone libraries belonged to the *Proteobacteria* taxonomic division, specifically the *Gammaproteobacteria*, *Betaproteobacteria,* and *Alphaproteobacteria*, as well as from other bacterial divisions, including *Actinobacterium*, *Acidobacterium*, *Bacteroides*, *Gemmatimonodates*, *Verrucomicrobia*, *Firmucutes*, *Chloroflexi Planctomycetes* and *Nitrospirae* divisions. As shown in Figures 4a, uncultured *Solitalea* sp. clone OYLT, a *Bacteriodetes* that clustered in a lineage with a *Solitalea korensis* strain had 99% similarity. Putative *Actinobacteria*, clustered with a clone from Pajsarjeva jama cave, Slovenia (FJ535083) and Oylat sample clone have ≥ 99 similarity. For *Chloroflexi* phyla, uncultured chloroflexi bacterium clone OYLT show similarity ≥ 95 similarity with uncultured chloroflexi bacterium clone (FJ535096). The unique OTUs belonged to a diverse group of phyla including *Acidobacteria*, *Nitrospirae*, *Planctomycetes*, *Firmicutes* and *Gemmatimonadetes* (Fig. 4a). *Proteobacteria* clones were phylogenetically associated with 3 classes of *Proteobacteria* with similarities between 85%–95 (Fig. 4b). *Alphaproteobacteria* clone is uncultured *Sphingomonas* sp. OYLT clone was 95% similar to uncultured *Sphingomonas* sp. (KC172197), a soil heterotrophic bacterium. The majority of the Sanger OTUs analyzed were associated with heterotrophic bacteria in the phylogenetic analysis (Fig. 4b).

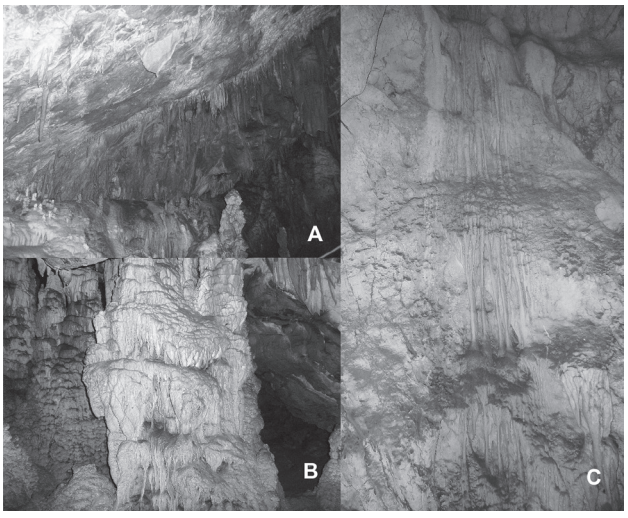

Fig. 2. Sampling locations at Oylat Cave (2a, 2b, 2c).

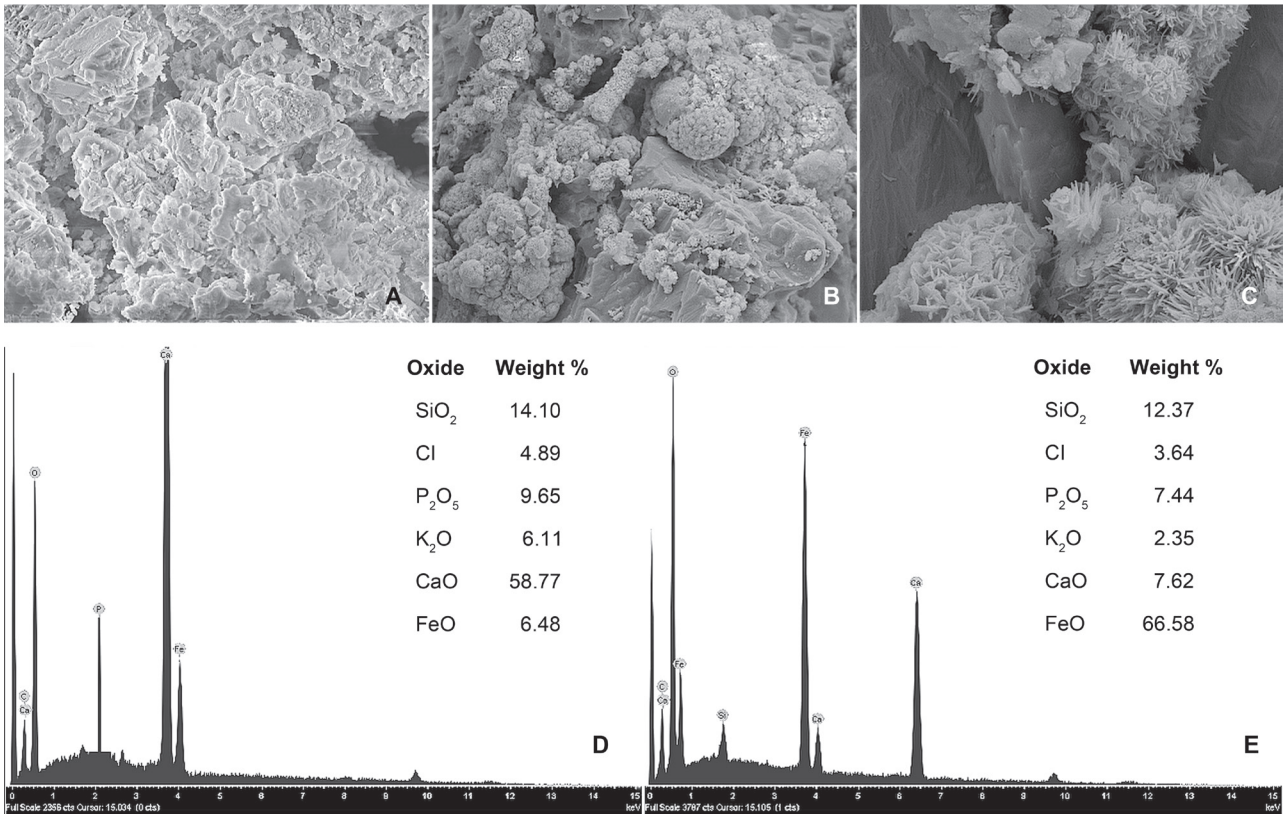

Fig. 3. Scanning electron micrograph of from Oylat Cave and EDS results (3a, 3b, 3c, 3d, 3e).

Diversity and taxonomic analysis of bacterial communities with 454 Pyrotag. The 454-pyrosequencing of three samples generated a total of 152,629 sequence reads after quality filtering and contaminant removal, representing 85% of the original dataset. Sample library size ranged from 3276 to 20,653 sequence reads. Alpha diversity index at Oylat Cave samples (Table I) presented the high biodiversity in all diversity metrics. Number of OTU was determined between 1428 and 2457. Chao1 was calculated as 2,507 and 3,944. The range in the Shannon diversity index for the each sample was 3.76 to 5.35.

Bacterial community structures were determined for each sample based on analysis of 454 pyrosequencing. A total of 10 bacterial phyla were identified from Oylat Cave. The bacterial communities were dominated by *Proteobacteria*, with abundances ranging from 42–63% (Fig. 5a) for all sampling sites. The *Proteobacteria* was composed of *Deltaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria* and *Betaproteobacteria* in the samples (Fig. 5b). Second dominated phyla is *Actinobacteria* in the (OYLT1) sample, followed by *Acidobacteria*, *Nitrospirae*, *Firmicutes*, *Bacteriodetes*, *Planctomycetes*, *Gemmatimonadete*, *Verrucomicrobia* and *Chloroflexi* (Fig. 5a). In the other sample is (OYLT2), *Proteobacteria* (48%) dominated, followed by *Acidobacteria*, *Actinobacteria*, *Planctomycetes* and *Nitrospirae*. Third sample is (OYLT3) dominated *Proteobacteria* (63%), followed by *Actinobacteria*, *Nitrospirae*., *Acidobacteria*, *Planctomycetes*, and *Firmicutes.*

Table I
Summary of 454 – pyrotaq OTUs and diversity and richness estimates

Sample	Number of OTUs	Chao1	ACE	Shannon
OYLT1	1,583	2,670	3,429	4.58
OYLT2	2,457	3,944	4,328	5.35
OYLT3	1,428	2,507	3,317	3.76

[a] Calculated using ACE richness estimator

Discussion

The deep subsurface remains one of the least explored microbial habitats on Earth, despite the increasing number of investigations in the past decade. The microbiology of caves is an important topic for better understanding subsurface biosphere diversity.

Limestone caves, such as Altamira Cave in Italy (Portillo *et al.*, 2009), the Niu Cave in China (Zhou *et al*., 2007), the Pajsarjeva jama cave in Slovenia (Pasic *et al*., 2010), Kartchner Caverns in the United States of America (Ortiz *et al*., 2012), and Jinjia Cave in western Loess Plateau of China (Wu et al., 2015) have been

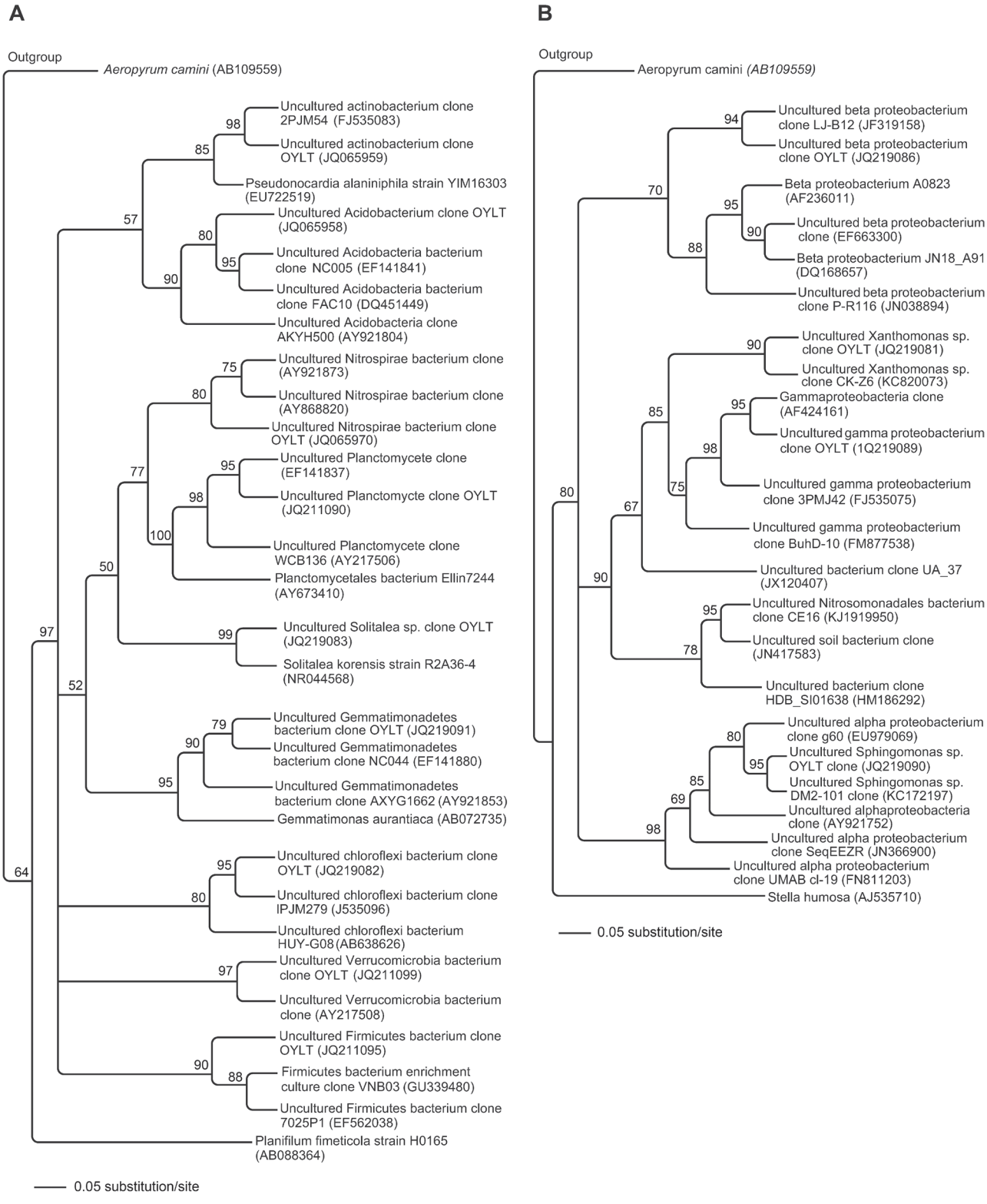

Fig. 4. Maximum parsimony phylogenetic inference of 16S rRNA sequences obtained from environmental clones at Oylat Cave.
4a. Phylogenetic tree of gene sequences associated with non-*Proteobacteria* phyla;
4b. Phylogenetic tree of gene sequences associated with *Proteobacteria* classes.

microbiologically and geochemically studied in last decade. In this study, bacterial phylogenetic diversity and composition observed in Oylat Cave (Bursa, Turkey) using Sanger and 454 pyrosequencing.

In other limestone cave studies, *Proteobacteria* was identified as the dominant phylum, with *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* classes being most common (Schabereiter-Gurtner

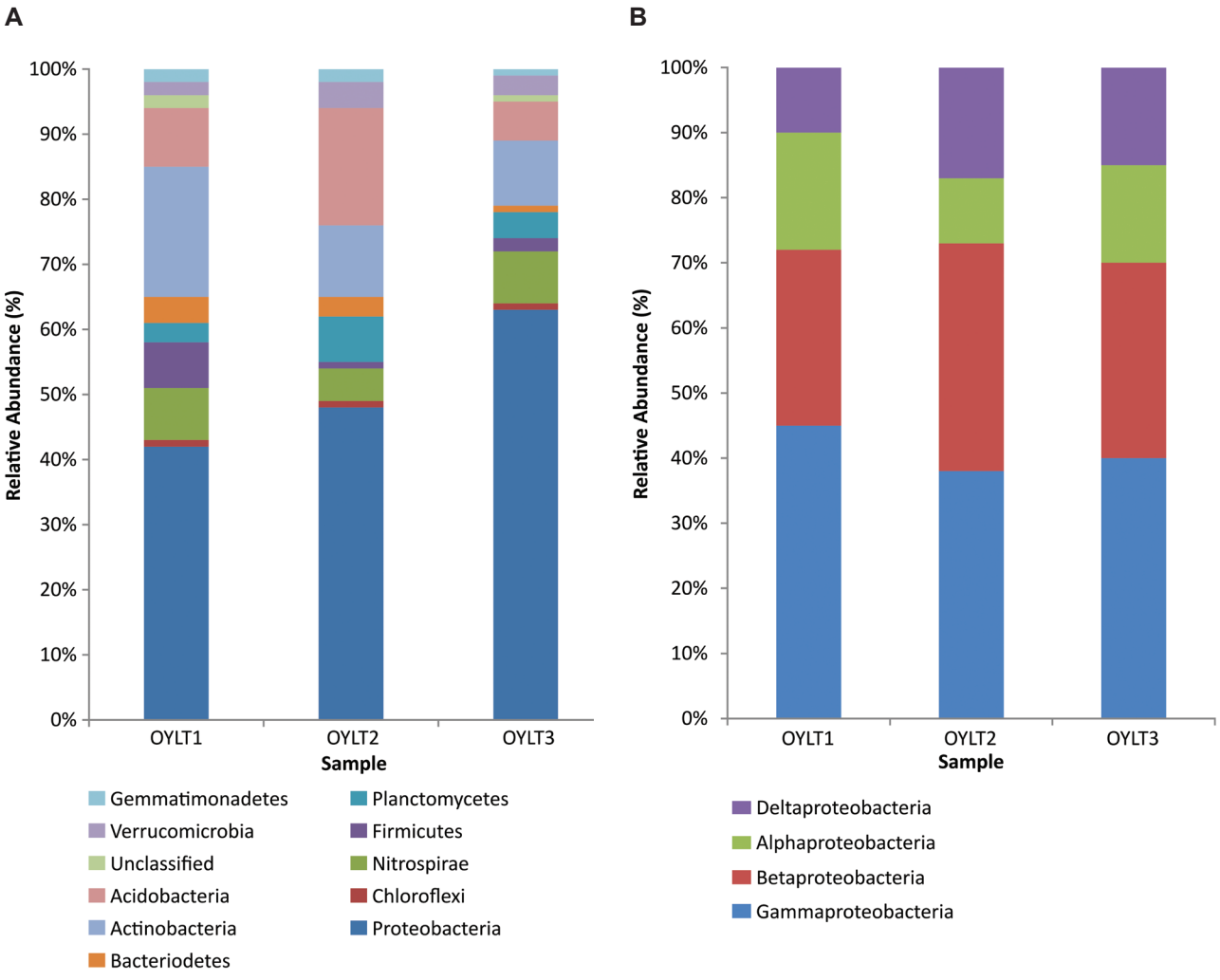

Fig. 5. Bacterial community composition of 454 sequence libraries.
5a. Distribution of dominant phyla in Oylat Cave samples: OYLT1 represent fossil part; OYLT2 represent Section II; OYLT3 represent Section III;
5b. Distribution of *Proteobacteria* (classes) in 454 samples.

et al., 2004; Barton *et al.*, 2006; Ortiz *et al.*, 2012). Similarly, the results of this study revealed that *Proteobacteria* phyla is dominant, also *Gammaproteobacteria* are dominant classes in the structure of community. Both the Sanger and pyrotag OTUs for *Gammaproteobacteria* related to sequences from different habitats (soil, hot spring, sewage, ground water). From comparing the distributions of *Proteobacteria* phyla, a core limestone microbiome becomes apparent.

Actinobacteria and *Acidobacteria* were second and third dominating phylogenetic groups in Oylat Cave respectively. Barton *et al.* (2007) found *Actinobacteria* to be the dominant phylum, representing 60% of the bacterial community of an oligotrophic limestone rock surface, in Carlsbad Caverns, New Mexico. *Actinobacteria* members are typical heterotrophs, actively participate in the carbon cycle by degradation of organic wastes (Ivanova *et al.*, 2013). Also, they have functional role on the biomineralization in the cave ecosystems (Zhou *et al.*, 2007). *Acidobacteria* found abundantly in several karstic cave environments, however their functional role unknown at present (Pasic *et al.*, 2010).

Nitrospirae members are likely to occur in different cave ecosystems. For instance, *Nitrospirae* clones observed from the extremely acidic Frasassi Cave (Macalady *et al.*, 2006). Members of *Nitrospirae* also observed in the limestone caves, Pajsarjeva jama Cave and Tito Bustillo Cave (Schabereiter-Gurtner *et al.*, 2002; Pasic *et al.*, 2010). *Nitrospirae* were identified as the fourth most abundant phylum in the overall Oylat Cave microbial community. *Nitrospirae members* especially *Nitrospirales* order's play role on nitrogen cycling such as nitrite oxidation in cave environment (Ortiz *et al.*, 2012).

Additional components of the bacterial cave wall microbial community belonged to the phyla *Firmicutes*, *Planctomycetes*, *Bacteroidetes*, *Verrucomicrobia*, *Gemmatimonadetes* and *Chloroflexi,* respectively in Oylat

Cave. These phyla reported from different limestone caves worldwide (Schabereiter-Gurtner *et al*., 2004; Zhou *et al*., 2007; Barton *et al*., 2007; Pasic *et al*., 2010; Ortiz *et al*., 2012; Wu *et al*., 2015).).

In conclusion, this diversity and taxonomic analysis conducted in Oylat Cave provides key information about these microbial communities. Both the Sanger and pyrosequencing clone library, robust analyses results to provide clues to potential energy sources in the cave, such as carbon and nitrogen cycling. In future studies, focus on functional metagenomics effort in Oylat Cave to determine the presence of clones closely associated with bacteria that have carbon- and nitrogen-fixing capabilities.

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ORIGINAL PAPER

Biochemical and Molecular Characterization of Carotenogenic Flavobacterial Isolates from Marine Waters

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Abstract

Carotenoids are known to possess immense nutraceutical properties and microorganisms are continuously being explored as natural source for production of carotenoids. In this study, pigmented bacteria belonging to *Flavobacteriaceae* family were isolated using kanamycin-containing marine agar and identified using the molecular techniques and their phenotypic characteristics were studied along with their potential to produce carotenoids. Analysis of random amplification of polymorphic DNA (RAPD) banding patterns and the fragment size of the bands indicated that the 10 isolates fall under two major groups. Based on 16S rRNA gene sequence analysis the isolates were identified as *Vitellibacter* sp. (3 isolates), *Formosa* sp. (2 isolates) and *Arenibacter* sp. (5 isolates). Phenotypically, the isolates showed slight variation from the reported species of these three genera of *Flavobacteriaceae*. Only the isolates belonging to *Vitellibacter* and *Formosa* produced flexirubin, a typical yellow orange pigment produced by most of the organisms of the family *Flavobacteriaceae*. *Vitellibacter* sp. and *Formosa* sp. were found to produce higher amount of carotenoids compared to *Arenibacter* sp. and zeaxanthin was found to be the major carotenoid produced by these two species. The study indicated that *Vitellibacter* sp. and *Formosa* sp. can be exploited for production of carotenoids, particularly zeaxanthin.

Key words: *Arenibacter* sp., *Flavobacteriaceae*, *Formosa* sp., *Vitellibacter* sp., carotenoid, zeaxanthin

Introduction

Carotenoids are very widely distributed in nature, where they play an important role in protecting cells and organisms against the harmful effects of radicals. The primary mechanism of action is the ability of carotenoids to quench excited sensitizer molecules and quench singlet oxygen. Carotenoids can also serve as antioxidants under conditions other than photosensitization (Krinsky, 1989). Carotenoids from marine resources are known to possess various health beneficial activities (Sowmya and Sachindra, 2011).

Bacteria belonging to the phylum Cytophaga-Flavobacterium-Bacteroides (CFB) are common inhabitants of marine environments. Several *Flavobacterium* species are pathogenic to fish and invertebrates. *Flavobacterium columnare* causes columnaris disease (Decostere, 2002), and *Flavobacterium psychrophilum* causes infections in salmonid fish (Nematollahi *et al*., 2003). Flavobacteria are characterized by their pigmentation properties, which is due to production of carotenoids. Zeaxanthin is the predominant carotenoid synthesized biologically by few species of the genus Flavobacterium (Johnson and Schroeder, 1995). Among the carotenoids present in the body, only lutein, zeaxanthin and meso-zeaxanthin, are found in the macula lutea of the eye. Lutein and zeaxanthin provide significant protection against the potential damage caused by light as reported in many studies (Bone *et al*., 2007; Roberts *et al.*, 2009). It is also suggested that zeaxanthin is protective against age-related increase in lens density and cataract formation (Sajilata *et al*., 2008). Liver carcinogenesis in C3H/He male mice was suppressed when fed with zeaxanthin mixed as an emulsion (Nishino *et al*., 1999). Zeaxanthin plays an important role in the inhibition of macrophage-mediated LDL oxidation suggesting that it might help in slowing atherosclerosis progression (Carpenter *et al*., 1997).

Much emphasis has been laid on the pathogenic Flavobacteria, but very little importance is given to the beneficial aspects of other bacteria belonging to *Flavobacteriaceae*. It has been reported that Flavobacteria produces not only zeaxanthin but also others carotenoids. Rählert *et al*. (2009) identified three carotenogenic

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genes from *Flavobacterium* P99-3 that produces myxol, a monocyclic carotenoid and reported that *crt*A gene encodes for a novel carotenoid 2-hydroxylase involved in myxol biosynthesis which is highly homologous to *crt*A from purple bacteria encoding an acyclic carotenoid 2-ketolase. Another marine myxol-producing bacteria *Robiginitalea myxolifaciens* strain YM6-073 belonging to the *Flavobacteriaceae* have been isolated (Manh *et al*., 2008). Saproxanthin and myxol isolated from *Flavobacteriaceae* are monocyclic carotenoids rarely found in nature and have antioxidative activities against lipid peroxidation in the rat brain homogenate model and neuro-protective effect from L-glutamate toxicity (Shindo *et al*., 2007).

Eventhough, many new genera of CFB are described in the literature, their biochemical and molecular characterization is lacking, particularly the aspect of carotenoid production. In this study, the emphasis was given to the characterization of bacteria of the *Flavobacteriaceae* family from the Indian marine environment and carotenoid produced by them.

Experimental

Materials and Methods

Media and chemicals. Zobell marine broth, Zobell marine agar, Hi-carbokit and other media components were procured from Hi-Media Pvt. Limited. Mumbai, India. PCR components, lysozyme and primers were from Sigma-Aldrich, India. Solvents and analytical grade chemicals were from SRL, India. *Flavobacterium* sp. (MTCC 4664) was obtained from IMTECH, Chandigarh, India.

Selective isolation of bacteria of *Flavobacteriaceae* family. Samples of marine sediment, water from different depths, seaweeds and sponges were collected from different regions of East and west coast of India (Tuticorin, Mandappam, Rameshwaram and Mangalore). A selective marine agar medium containing kanamycin was used for isolation and enumeration of yellow-pigmented colonies by modified method of Flint (1985). Zobell marine broth containing kanamycin at 50 micrograms/ml concentration was used for isolation of bacteria from marine water and sediments samples. After incubation for 24 hrs, a loopful of sample was streaked onto marine agar plates containing kanamycin (50 µg/ml). Colonies showing the yellow-orange pigmentation were isolated and purified by repeated streaking.

Biochemical characterization. Biochemical tests performed include gram staining, motility test, oxidase activity, catalase production, deamination of L-tryptophan, decarboxylation of indole, MR-VP, oxidation and fermentation of sugars. Utilization of glucose, mannitol, inositol, sucrose and other 31 sugars was assessed by using Hi-carbo kit. Hydrolysis of esculin, gelatin and starch were also performed using standard techniques. Growth in marine broth at different temperatures of 10°C, 25°C, 37°C and 50°C, at different pH of 4,7 and 10 were performed to characterize the growth of organisms. Flexirubin production by the isolates was tested by the method of Fautz and Reichenbach (1980). Haemolytic activity of the cultures was tested using blood agar. Antibiotic sensitivity of the isolates was tested by the method of Bauer *et al*. (1966).

Scanning Electron Microscopic (SEM) analysis. Morphological analysis of the culture isolates were performed according to the method of McDougall *et al*. (1994). Briefly, selected isolates grown in marine broth were centrifuged, washed thrice with phosphate buffer saline (pH 7.0) to remove salts, fixed with glutaraldehyde (2%) and subjected to gradual alcoholic dehydration. The processed samples were then analyzed on SEM (Leo-435 VP, Leo Electron Microscope, Zeiss Ltd., Cambridge, UK).

Random Amplification of Polymorphic DNA (RAPD). Genomic DNA was extracted from the bacterial isolates using the standard DNA isolation protocol (Sambrook and Russell, 2001). Random amplification of polymorphic DNA (RAPD) was performed using M13 primer (5'-GAGGGTGGCGGTTCT-3') for microbial typing according to Schillinger *et al*. (2003) and the product was run on 1.8% agarose gel. RAPD banding pattern was further analyzed using GeneSys® software (SYNGENE, UK) and the similarity of the band profiles and the grouping of the RAPD-PCR patterns were calculated based on the Pearson's coefficient and agglomerative clustering with unweighted pairs group matching algorithm (UPGMA), and the dendrogram was constructed using GeneSys® software.

16S rRNA gene sequencing and phylogenic analysis. 16S rRNA gene was amplified using the universal bacterial forward and reverse primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (51-GGTTACCTTGTTACGACTT-31) respectively. Each PCR mixture of 25 µl contained template DNA (20 ng/µl), 0.2 µM of each primer, 0.25 mM of each deoxynucleoside triphosphate (dNTP), and 2.5 U of *Taq*DNA polymerase in a final concentration of 10 mM Tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$. PCR was performed under the following cycle conditions: an initial denaturation step at 94°C for 5 min and 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 2 min, and extension at 72°C for 2 mins. A final extension step at 72°C for 10 min was performed. The PCR product of 1.4 kb was purified and sequenced at Amnion Biosciences, Bangalore, India. The 16S rRNA gene sequences obtained was subjected to nBLAST

for similarity identification. Multiple alignment and sequence similarity with that of available sequences of reference strains from GenBank database were analyzed using CLUSTAL X. The phylogenetic tree was constructed using the MEGA 4 program (Biodesign Institute, Tempe, AZ, USA) by the method of neighbor-joining (Kumar *et al*., 2008). The sequences have been deposited at NCBI GenBank.

Whole-cell protein analysis. Whole-cell protein extracts were prepared by suspending the culture pellet in phosphate buffer saline (pH 7.0) and sonicating at 150 W power until the suspension is clear (Chart, 1994). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the whole-cell protein extracts was performed with a 12% gel by the procedure of Laemmli (1970) and run at a constant current of 16 mA, stained overnight with coomassie brilliant blue R-250 and following destaining the electrophoretic patterns were scanned with a gel documentation system (G-Box, SYNGENE, UK) and cluster analysis of electrophoretic patterns was done by the software GeneSys®. A dendrogram representing the relationship between all strains tested was derived from the TREE option. The whole-cell protein patterns of the 10 isolates and standard culture were compared.

Extraction and characterization of carotenoids. The bacterial isolates were inoculated into 50 ml marine broth and grown for 5 days at 37°C under shaking condition (200 rpm) and the cells were harvested by centrifuging at 8000 rpm for 15 min. Cells were suspended in sterile saline and lysed by adding the lysozyme (20 mg/ml). Carotenoids were extracted by homogenizing the lysed cell suspension with acetone, filtered, and cells re-extracted with fresh acetone. The acetone extract was pooled and subjected to phase separation with equal quantity of hexane. The hexane extract was dried with sodium sulphate to remove traces of water if any, filtered and then evaporated under vacuum at 40°C using a rotary flash evaporator and flushed with nitrogen to obtain the total carotenoid extract. The yield of recovered carotenoid was determined.

Thin layer chromatography (TLC) of carotenoid extract from selected isolates was performed using Silica Gel 60 plates (MERCK). The samples along with standard carotenoids and plates were developed using the mobile phase of acetone: hexane (75:25). Then, HPLC of the extracted carotenoids was done by C18 TSK gel ODS-80TS column (4.6 mm ID × 25 cm) with gradient run of 35 min with solvent A as methanol and water (95:5) and solvent B as methanol and tetrahydrofuran (7:3) with a flow rate of 1.25 ml/min at 470 nm using a diode array detector.

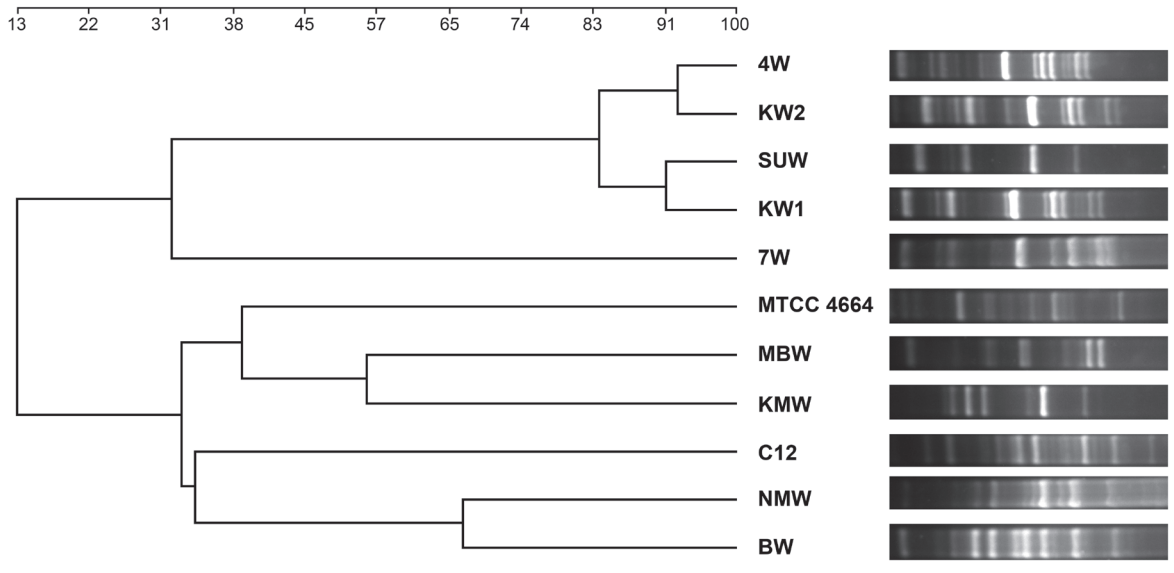
Statistical analysis. Mean values of yield of crude carotenoid extract and zeaxanthin content in the carotenoid extract were subjected to ANOVA to determine the effects, and mean differences observed were subjected to Duncan's multiple range test using the software STATISTICA (Statsoft, 1999).

Results and Discussion

Isolation and characterization of carotenoid-producing bacteria. Pigmented colonies were selected from the marine agar plates containing the kanamycin and were purified. In all 10 isolates from different location and source were selected for further study based on pigmentation, source and location (Table I). The color of the selected isolates was yellow-orange to dark orange, and all the isolates had a slimy surface. The 10 selected cultures along with the standard culture *Flavobacteria* sp. MTCC 4664 had distinct RAPD banding patterns, and the fragment size of the bands ranged from 250 to 2370 bp. According to the dendrogram (Fig. 1) and cluster analysis, the isolates were grouped into two major groups, wherein isolates KW1, KW2, SUW, 4W and 7W were in one group and the other 5 isolates along with MTCC 4664 were in the other group.

Table I
Details of isolates

Isolate	Source	Location	Colony characteristics
BW	Sea water (beach side)	13.87°N, 74.63°E	Dark orange, slimy
C12	Sea water (12 meter depth)	12.57°N, 74.47°E	Dark orange, slimy
NMW	Mangrove water	12.80°N, 74.85°E	Dark orange, slimy
MBW	Sea water (beach side)	13.35°N, 74.70°E	Orange, slimy
KMW	Mangrove water	13.21°N, 75.02°E	Orange, slimy
4W	Sea water (beach side)	08.53°N, 78.36°E	Yellow orange, slimy
7W	Sea water (beach side)	08.48°N, 78.12°E	Yellow orange, slimy
KW1	Sea water (beach side)	13.49°N, 74.70°E	Yellow orange, slimy
KW2	Sea water (beach side)	13.49°N, 74.70°E	Yellow orange, slimy
SUW	Sea water (beach side)	12.98°N, 74.78°E	Yellow orange, slimy



	7W	NMW	MBW	BW	4664	SUW	KW1	KMW	C12	4W	KW2
7W	1.000										
NMW	0.230	1.000									
MBW	0.028	0.157	1.000								
BW	0.228	0.669	0.495	1.000							
4664	0.154	0.333	0.361	0.214	1.000						
SUW	0.501	0.025	0.000	0.209	0.312	1.000					
KW1	0.500	0.115	0.000	0.176	0.318	0.934	1.000				
KMW	0.062	0.481	0.566	0.358	0.448	0.000	0.000	1.000			
C12	0.172	0.404	0.526	0.300	0.153	0.082	0.166	0.244	1.000		
4W	0.140	0.281	0.007	0.278	0.172	0.839	0.879	0.004	0.116	1.000	
KW2	0.086	0.320	0.000	0.174	0.191	0.829	0.870	0.065	0.118	0.936	1.000

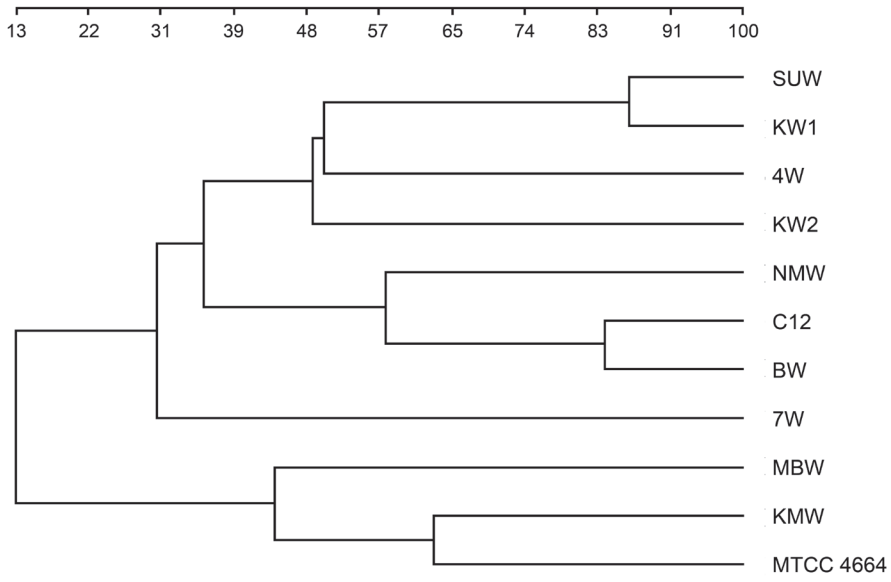
Fig. 1. Dendrogram drawn using RAPD profile of bacterial isolates.
UPGMA bootstrapping values of this clade were 95–99. Culture isolates were grouped by considering the significances in bootstrap values, interior branch lengths, and diversification rate.

The second group consisted of three subgroups having isolates NMW and BW in one subgroup and C12, KMW and MBW in another subgroup, with MTCC 4664 being a separate subgroup.

The whole-cell protein patterns of the 10 isolates with the standard culture had a major protein band patterns ranging from 18 kDa to 116 kDa. Heterogeneity in SDS-PAGE protein patterns among different isolates was clearly observed. A dendrogram was created based on the electrophoretic pattern and is depicted in Fig. 2. Two major groups were formed based on SDS-PAGE patterns of whole cell protein, where the isolates MBW, KMW along with MTCC 4664 isolates formed one group and the rest being in the other group. Among the 8 isolates in the second group, a high similarity of 0.891 was observed between the isolates SUW and KW1.

The selected isolates were identified by 16S rRNA gene amplification. The 16S rRNA gene products were sequenced, and the sequences were used for database query. The nucleotide sequence data has been deposited at the National Center for Biotechnology Information (NCBI). The 16S rRNA gene sequences were subjected for BLAST using the megablast tool of GenBank (http://www.ncbi.nlm.nih.gov/), which revealed that the isolates belonged to three genera of *Flavobacteriaceae*, namely, *Formosa*, *Vitellibacter* and *Arenibacter*. The isolates under genus *Arenibacter* along with their GenBank accession number are *Arenibacter* sp. SUW (KJ658267), *Arenibacter* sp. KW1 (KJ658268), *Arenibacter* sp. 4W (KJ658269), *Arenibacter* sp. KW2 (KJ658270) and *Arenibacter* sp. 7W (KJ658274). The three *Vitellibacter* strains are *Vitellibacter* sp. C12 (KJ658271), *Vitellibacter* sp. NMW (KJ658275), *Vitellibacter* sp. BW (KC888001) and the two *Formosa* strains were *Formosa* sp. KMW (KC888002) and *Formosa* sp. MBW (KC888003).

Representatives of maximum homologous (98–99%) sequences of each isolate and other members of the Flavobacteria family like *Aequorivita, Olleya, Yeosuana, Lacinutrix and Flexibacter* were obtained from NCBI GenBank and were used for the construction of a phylogenetic tree which is rooted where a common ancestor is defined. All the 5 *Arenibacter* isolates were found to be grouped along with *Arenibacter latericius, Arenibacter certessi* and *Flexibacter aggregans*. All three *Vitellibacter* isolates were in a group with *Vitellibacter vladivostokensis* and *Aequorivita* sp., while *Formosa* isolates were in the group comprising of *Oleya* sp., *Yeosuna* sp., *Gaelibulibacter* sp., *Gelidibacter* sp. and *Bizionia* sp. (Fig. 3).



	7W	4W	C12	NMW	BW	SUW	KW1	KW2	MBW	KMW	4664
7W	1.000										
4W	0.390	1.000									
C12	0.517	0.275	1.000								
NMW	0.389	0.304	0.685	1.000							
BW	0.471	0.362	0.869	0.628	1.000						
SUW	0.546	0.593	0.582	0.538	0.634	1.000					
KW1	0.440	0.599	0.525	0.578	0.636	0.891	1.000				
KW2	0.300	0.576	0.395	0.502	0.460	0.579	0.609	1.000			
MBW	0.232	0.067	0.539	0.481	0.320	0.193	0.169	0.178	1.000		
KMW	0.405	0.033	0.470	0.603	0.201	0.142	0.154	0.156	0.501	1.000	
4664	0.404	0.014	0.649	0.537	0.475	0.290	0.243	0.263	0.598	0.704	1.000

Fig. 2. Dendrogram derived from the UPGMA clustering of correlation coefficients based on the SDS-PAGE of whole-cell protein patterns of 10 Flavobacterial isolates along with standard culture (MTCC 4664).

The phenotypic characteristics of all the isolates are given in Table II. All the isolates were gram-negative rods with yellow-orange colored slimy colonies. The selected isolates were non-motile, asporogenic, indole-, citrate-, malonate- and MRVP-negative and catalase-positive. Except three isolates of *Vitellibacter* all the other isolates were positive for oxidase. All strains were negative for fermentation and hence, they are strictly aerobic. The isolates belonging to *Vitellibacter* and *Formosa* were not able to produce acids from any sugars while those belonging to *Arenibacter* genus were able to produce acid from many of the sugars. *Vitellibacter* sp. BW and two *Formosa* isolates were able to hydrolyse gelatin. The optimum growth of these marine organisms was at pH 7.0. However, some of the isolates could grow at pH 4.0. *Arenibacter* sp. 4W showed weak growth at pH 10.0. All the isolates were able to grow at 37°C; however the isolates belonging to *Vitellibacter* showed better growth and pigmentation at 25°C. All the isolates were found to be nonpathogenic as tested by haemolytic activity. With respect to antibiotic sensitivity of isolates, all the isolates were sensitive to most of the antibiotics tested. However, 5 of the isolates were resistant to tobramycin (data not shown) indicating that tobramycin could also be used in the media for selective isolation and to prevent contamination from other bacteria when used for carotenoid production.

SEM images of the bacteria give a clear image of the structural features of the bacteria. The SEM images of the different *Flavobacteriaceae* isolates are shown in Fig. 4. Isolates belonging to *Vitellibacter* were thin elongated rod-shaped bacteria without flagella or fimbriae. The isolates belonging to *Formosa* were short rods, while those belonging to *Arenibacter* were moderately long rods.

Production of yellow or orange pigments on agar plates has been used as one of the criteria for isolation of the members of the family *Flavobacteriaceae*. However, the overgrowth of non-pigmented bacteria, particularly from soil samples necessitates use of selective media for isolation of flavobacteria. As flavobacteria are known to be resistant to kanamycin, Flint (1985) suggested the use of kanamycin-containing medium for selective isolation of flavobacteria. In the present study when kanamycin-containing marine agar was used for isolation of bacteria from marine sources, the dominance of pigment producers in the media was observed. The pigment producing isolates were purified for further

Table II
Phenotypic characteristics of the isolates.

	Vitellibacter sp. C12	*Vitellibacter* sp. BW	*Vitellibacter* sp. NMW	*Formosa* sp. KMW	*Formosa* sp. MBW	*Arenibacter* sp. 4W	*Arenibacter* sp. 7W	*Arenibacter* sp. KW1	*Arenibacter* sp. KW2	*Arenibacter* sp. SUW
Gram reaction	–	–	–	–	–	–	–	–	–	–
Morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Oxidase	–	–	–	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
MR/VP	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
Indole	–	–	–	–	–	–	–	–	–	–
O/F	–/–	–/–	–/–	–/–	–/–	+/–	+/–	+/–	+/–	+/–
Flexirubin production	+	+	+	+	+	–	–	–	–	–
Acid from										
Lactose	–	–	–	–	–	+	+	+	+	+
Xylose,	–	–	–	–	–	+	+	+	–	+
Maltose	–	–	–	+	+	+	+	+	+	+
Fructose	–	–	–	–	–	+	+	+	+	+
Dextrose	–	–	–	+	+	+	+	+	+	+
Galactose	–	–	–	–	–	+	+	+	+	+
Raffinose	–	–	–	–	–	+	+	+	+	+
Trehalose	–	–	–	+	+	+	+	+	+	+
Melibiose	–	–	–	–	–	+	+	+	+	+
Sucrose	–	–	–	w	w	+	+	+	+	+
L-Arabinose	–	–	–	–	–	–	–	–	–	–
Mannose	–	–	–	+	+	–	–	–	–	–
Inulin	w	w	w	w	w	+	+	+	+	+
Sodium gluconate	–	–	–	–	–	+	+	–	+	+
Glycerol	–	–	–	–	–	–	–	–	–	–
Salicin	–	–	–	+	+	–	–	–	+	–
Dulcitol	–	–	–	–	–	+	+	+	+	+
Inositol	–	–	–	w	w	–	–	–	+	–
Sorbitol	–	–	–	–	–	–	–	–	–	–
Mannitol	–	–	–	–	–	–	–	–	–	–
Adonitol	–	–	–	–	–	–	–	–	–	–
Arabitol	–	–	–	–	–	–	–	+	–	+
Erythritol	–	–	–	–	–	–	–	–	–	–
α-Methyl-D-glucoside	–	–	–	–	–	–	–	–	+	–
Rhamnose	–	–	–	–	–	+	+	+	+	+
Cellobiose	–	–	–	w	w	–	–	–	–	–
Melezitose	–	–	–	–	–	+	+	+	+	+
α-Methyl-D-Mannlside	–	–	–	–	–	+	+	+	–	+
Xylitol	–	–	–	–	–	+	+	+	+	+
D-arabinose	–	–	–	–	–	+	+	+	+	+
Sorbose	–	–	–	–	–	–	–	–	–	–
β-galactosidase	–	–	–	–	–	–	–	–	–	–

Table II. Continued.

	Vitellibacter sp. C12	*Vitellibacter* sp. BW	*Vitellibacter* sp. NMW	*Formosa* sp. KMW	*Formosa* sp. MBW	*Arenibacter* sp. 4W	*Arenibacter* sp. 7W	*Arenibacter* sp. KW1	*Arenibacter* sp. KW2	*Arenibacter* sp. SUW
Hydrolysis of										
Esculin	–	–	–	–	–	–	–	–	–	–
Gelatin	–	+	–	+	+	–	–	–	–	–
Starch	–	–	–	–	–	–	–	–	–	–
Citrate Utilization	–	–	–	–	–	–	–	–	–	–
Malonate Utilization	–	–	–	–	–	–	–	–	–	–
Growth at pH										
4.0	–	w	–	+	–	w	–	w	w	+
7.0	+	+	+	+	+	+	+	+	+	+
10.0	–	–	–	–	–	w	–	–	–	+
Growth at temperature										
25°C	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+
50°C	–	–	–	w	w	–	–	–	–	–
Haemolysis	–	–	–	–	–	–	–	–	–	–

+: Positive; –: Negative; W: Weak

characterization using phenotypic and molecular techniques like Random Amplified Polymorphic DNA (RAPD) finger printing 16S rRNA gene sequencing.

RAPD is a relatively rapid PCR-based genomic fingerprinting method and is a useful tool for genome analysis in bacterial identification where isolates can be compared and grouped. Polymorphism between strains is detected as the difference between the patterns of amplified DNA fragments. In the present study the analysis of RAPD banding patterns of the isolates by dendrogram and cluster analysis indicated that the 10 isolates were in two major groups. Five isolates, which were later identified as *Arenibacter* sp. formed one group and the other 5 isolates later identified as either *Vitellibacter* sp. or *Formosa* sp. formed another group. The results indicated that genetic homogeneity between *Vitellibacter* sp. and *Formosa* sp. and their diversity from *Arenibacter* sp. under the *Flavobacteriaceae* family.

Whole-cell protein pattern analysis by SDS-PAGE and dendrograms obtained from numerical analysis is one of the techniques used for typing of bacterial species and grouping (Walia *et al*., 1988). In the present study analysis of whole cell protein from the different isolates using SDS-PAGE indicated that based on banding pattern the isolates can be clubbed into two major groups. However, grouping was not similar to grouping based on RAPD fingerprinting. Even the isolates belonging to same genera were in different groups, indicating that SDS-PAGE of whole cell protein is not a reliable tool for grouping of selected isolates.

The phenotypic characteristics of the bacterial isolates provide information about nutrient and environmental requirement for the growth of the organisms, and such information will be useful when the organisms used for exploiting their beneficial role. The phenotypic characteristics of the isolates indicated that they are able to use different sugars and optimum growth conditions were found to be pH 7.0 and a temperature range of 25–37°C.

Analysis of 16S rRNA gene sequences of the selected isolates indicated that they belong to three genera of *Flavobacteriaceae*, namely, *Formosa*, *Vitellibacter* and *Arenibacter*. Isolation and characterization of organisms belonging to these three genera from different sources has been reported. Two species of *Formosa* genus, *Formosa algae* KMM3553 (Ivanova *et al*., 2004) and *Formosa agariphila* (Nedashkovaskaya *et al*., 2006a) has been reported so far. *F. algae* isolated from degrading thallus of the brown alga *Fucus evanescens* was found to be light yellow pigmented and positive for gliding motility, catalase, urease and utilization of lactose and glycerol and oxidase negative (Ivanova *et al*., 2004), while *F. agariphila* isolated from green alga *Acrosiphonia sonderi* and from sea water was positive for gliding motility, oxidase, catalase and for sugar utilization.

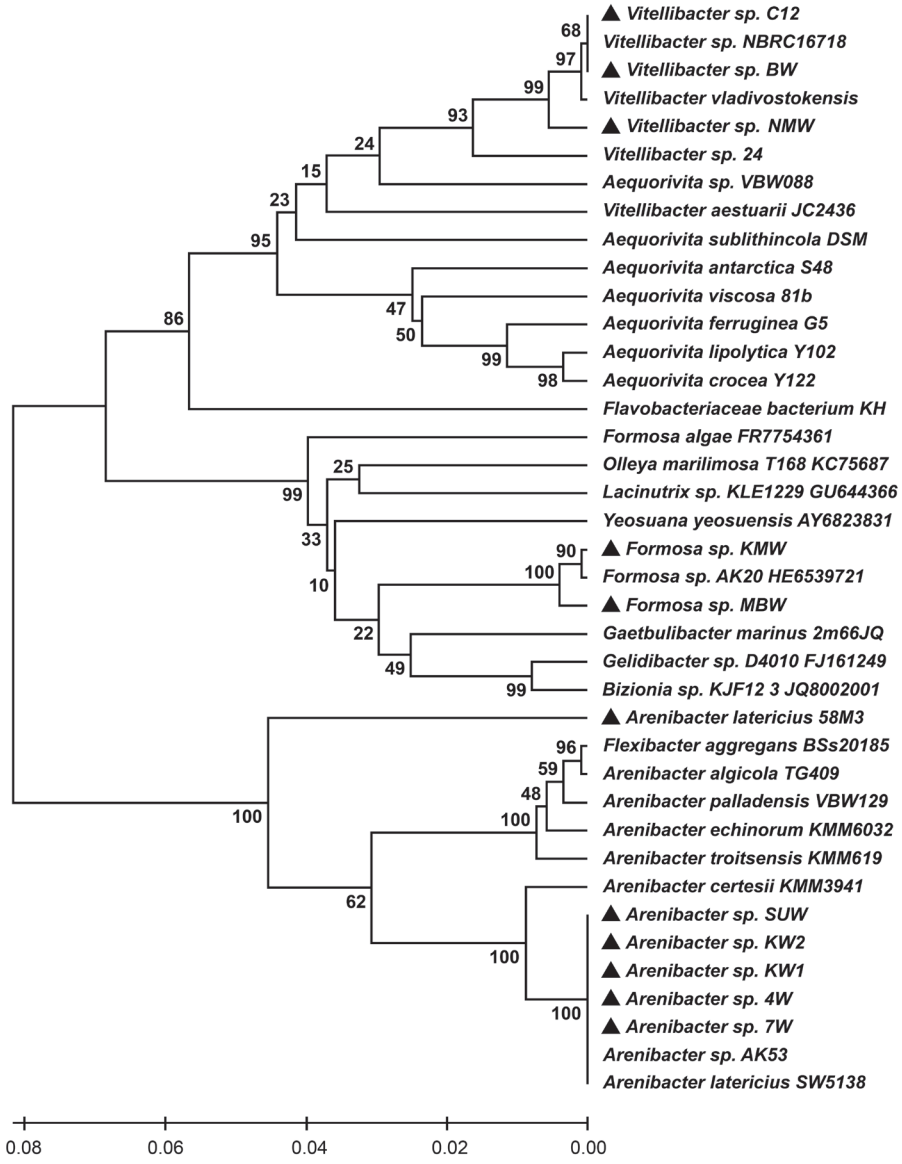


Fig. 3. Phylogenetic analysis of isolated cultures based on 16S rRNA gene sequence.

The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analyses was conducted by MEGA4 software. Representative sequences were obtained from GenBank and accession numbers given in parentheses. Flavobacterial cultures isolated are represented by

However, the two isolates of *Formosa* identified in the present study are phenotypically different indicating that they may be different species of *Formosa*.

The three species of *Vitellibacter* genus reported are *Vitellibacter vladivostokensis* KMM3516 (Nedashkovskaya *et al*., 2003a), *Vitellibacter aestuarii* JC2436 (Kim *et al*., 2010), and *Vitellibacter soesokkakensis* (Park *et al*., 2014). *V. vladivostokenisis* isolated from holothurian *Apostichopus japonicus* collected from Troitsa Bay in the Sea of Japan were yellow-orange pigmented, non-motile, asporogenic, and strictly aerobic, gram-negative, oxidase- and catalase-positive bacteria. *V. aestuarii* isolated from tidal-flat sediment of Oi Island in Korea was found to be closely affiliated to *V. vladivostokensis*, with 96% sequence similarities to the type strain. The difference between the two strains was found to be *V. vladistokensis* being catalase-negative and susceptible to benzylpencillin but not to ampicillin and lincomycin (Kim *et al*., 2010). The *Vitellibacter* sp. isolated in the present study does not match exactly with the known *Vitellibacter* species reported and hence may be different species of *Vitellibacter*.

Presently six species of *Arenibacter* have been reported. They are *Arenibacter latericius* isolated from marine sediments (Ivanova *et al*., 2001), *Arenibacter troitensis* isolated from marine bottom sediments

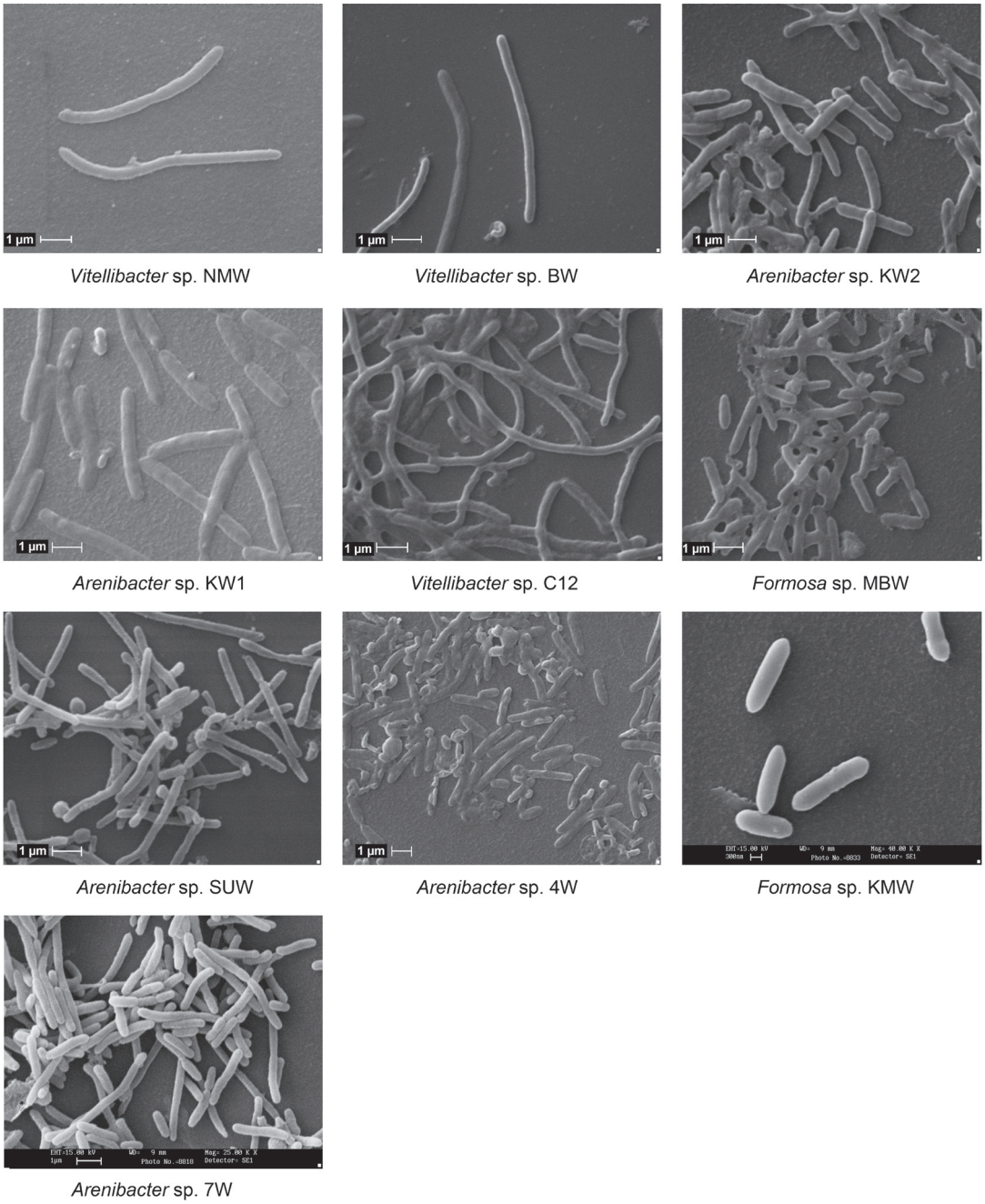


Fig. 4. SEM image of Flavobacterial isolates depicting their structural morphology.

(Nedashkovskaya *et al*., 2003b), *Arenibacter certessi* isolated from green algae (Nedashkovskaya *et al*., 2004), *Arenibacter palledensis* isolated from green algae (Nedashkovskaya *et al*., 2006b), *Arenibacter echinorum* isolated from sea urchin (Nedashkovskaya *et al*., 2007) and *Arenibacter nanhaiticus* from marine sediment (Sun *et al*., 2010). Nedashkovskaya *et al.* (2007) compared the phenotypic characteristics of five species of *Arenibacter* and reported that they vary with respect to motility, production of acids from sugar, and gelatin hydrolysis. Three species (*A. echinoum*, *A. latericius* and *A. palledensis*) were able to produce acid from many of the sugars, and *A. echinorum* was able to utilize malonate and citrate, but not the other isolates. In the present study also all the *Arenibacter* isolates were able to produce acids from many of the sugars, but many other phenotypic characteristics did not match with the known isolates.

Extraction and characterization of carotenoids. The total carotenoid yield from different Flavobacterial isolates varied (Fig. 5). Significantly ($p < 0.05$) higher carotenoid yield (115.7 ± 5.0 mg/g biomass) was observed in *Vitellibacter* sp. BW, followed by *Arenibacter* sp. 4W (99.3 ± 21.9 mg/g biomass). There was no specific dependency between carotenoid yield and the specific genus of the isolate.

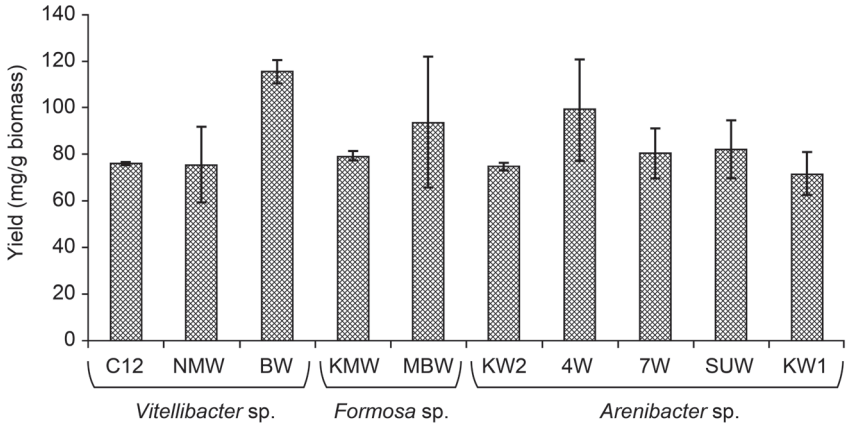


Fig. 5. Yield of crude carotenoid extract from different isolates.

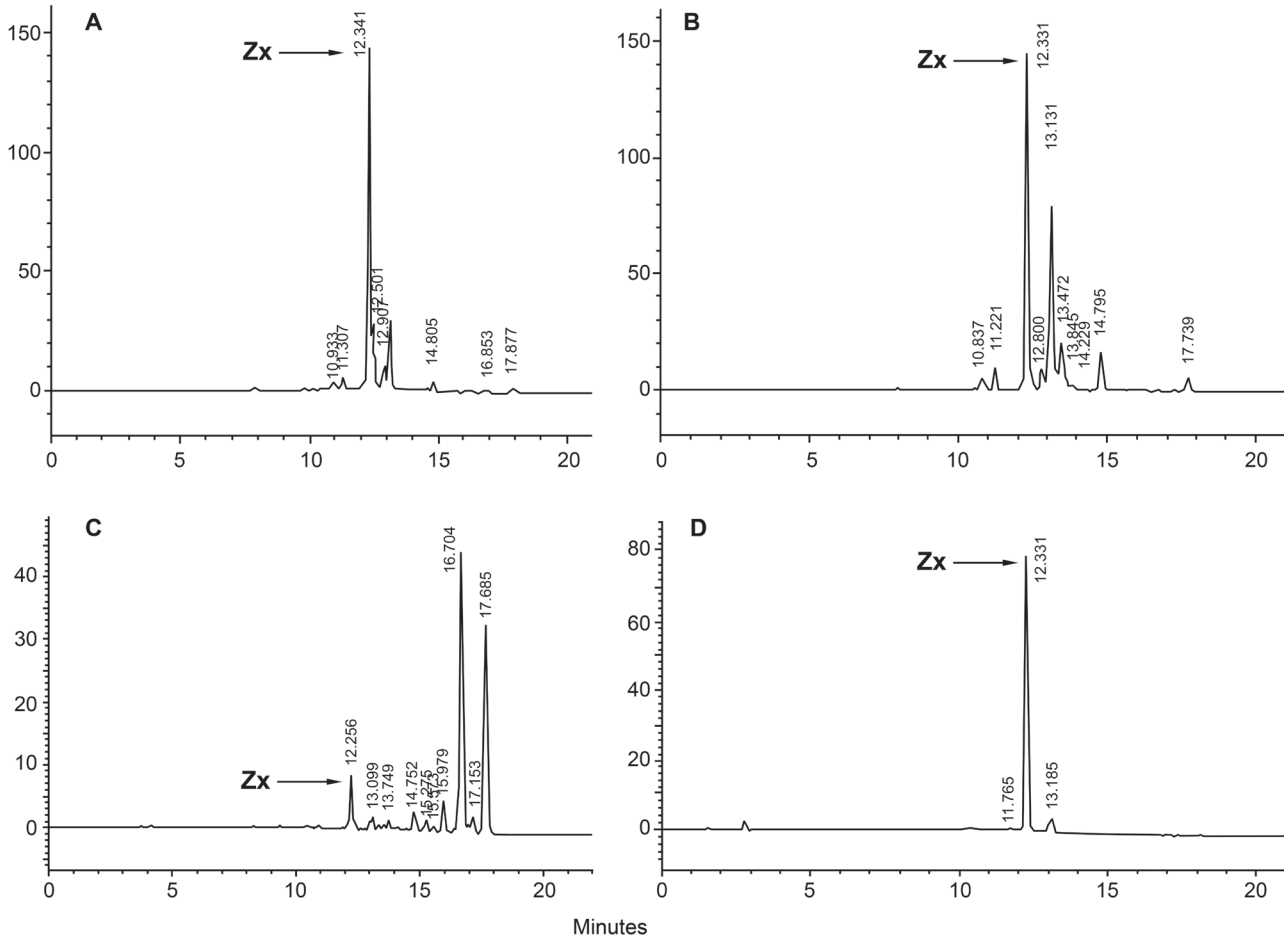


Fig. 6. HPLC profile of carotenoid extract of: (A) *Vitellibacter* sp. NMW; (B) *Formosa* sp. MBW; (C) *Arenibacter* sp. 4W; (D) Zeaxanthin (Zx) standard.

The preliminary test for the identification of carotenoids in the crude carotenoid extract by TLC showed the presence of β-carotene and zeaxanthin. HPLC analysis of crude carotenoid extract from all isolates confirmed the presence of zeaxanthin and β-carotene, however, the concentration of two major carotenoids differed with the isolates. The typical HPLC profiles of one isolate from the three genera *Vitellibacter* NMW, *Formosa* MBW and *Arenibacter* 4W are given in figure 6A, 6B and 6C respectively and the HPLC profile of zeaxanthin standard in figure 6D. Significantly higher ($p < 0.05$) zeaxanthin concentration of $61.4 \pm 8.3\%$ was observed in the crude carotenoid extract from *Formosa* sp. KMW, followed by 59.6 ± 18.8 and $52.2 \pm 20.2\%$ in carotenoid extract from *Vitellibacter* sp. BW and C12 (Fig. 7). The zeaxanthin content in the carotenoid extract

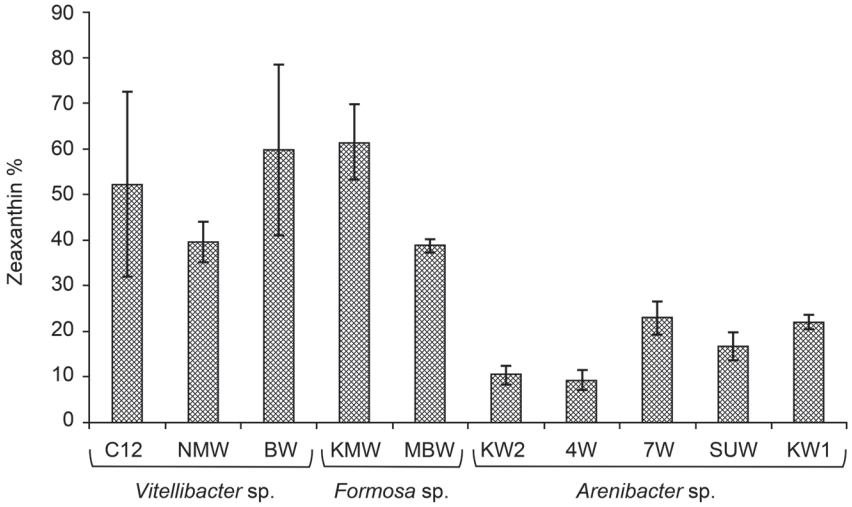


Fig. 7. Percentage of zeaxanthin in carotenoid extract from different Flavobacterial isolates.

from *Arenibacter* sp. was comparatively lower and was in the range of 9.2 to 23.0% of total carotenoid content.

Organisms belonging to *Flavobacteriaceae* are well known for the production of pigments. Flavobacterial isolates from soil and fresh water are known to produce flexirubin, a yellow-orange pigment, which is used as a taxonomic marker (Reichenbach *et al*., 1980). In the present study, only the isolates belonging to *Vitellibacter* and *Formosa* were found to produce flexirubin pigment (Table II). However, yellow pigmentation of marine isolates has been mainly attributed to carotenoids, particularly zeaxanthin (Stafsnes and Bruheim, 2013). Studies on the carotenoid production by *Vitellibacter* sp., *Formosa* sp. and *Arenibacter* sp. is lacking. As carotenoids have been shown to provide various health benefits, the isolates in the present study were tested for production of carotenoids with an aim to select a suitable isolate for further investigation on carotenoid production.

Studies on carotenoid production by *Flavobacterium multivorum* indicated that the organism produces zeaxanthin as the major carotenoid (Alcantara and Sanchez, 1999; Bhosale *et al*., 2004; Masetto *et al*., 2001). However, under specific nutrient condition such as the presence of inorganic salts and urea in the growth media interferes with the zeaxanthin biosynthesis pathway and results in the accumulation of intermediates such as canthaxanthin and β-carotene (Bhosale and Bernstein, 2004). The present study indicated that the isolates *Vitellibacter* sp. BW and C12 and *Formosa* sp. KMW can be successfully exploited as zeaxanthin producers.

In conclusion, the study resulted in selective isolation and identification of 10 isolates of carotenoid producing *Flavobacteriaceae* belonging to three genera of *Arenibacter*, *Formosa* and *Vitellibacter*. Since, not much information is present on these organisms and on the carotenoid production aspects of *Vitellibacter* sp., *Formosa* sp. and *Arenibacter* sp. an attempt has been made to study them. *Vitellibacter* sp. and *Formosa* sp. was found to produce higher amount of carotenoids compared to *Arenibacter* sp. Further, zeaxanthin was found to be the major carotenoid produced by *Vitellibacter* sp. and *Formosa* sp. These isolates have potential to be used for production of zeaxanthin in large scale that needs further studies on factors influencing zeaxanthin production by these isolates.

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SHORT COMMUNICATION

Serotype-Specific Pneumococcal Status prior to PCV 13 Administration in Children and Adolescents with Inflammatory Bowel Disease

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Abstract

The aim of this study was to evaluate the serotype-specific pneumococcal status of children and adolescents with inflammatory bowel disease (IBD) who were naïve to pneumococcal vaccination before administering the 13-valent pneumococcal conjugate vaccine (PCV 13). This was an open, prospective study on children and adolescents aged 5–18 years who had IBD and were naïve to pneumococcal vaccination. A single dose of PCV 13 was administered to each patient. The geometric mean concentrations (GMCs) were measured for all 13 serotypes. A total of 122 subjects completed the study. Prevaccination GMCs ranged from 0.55 μg/ml (serotype 4) to 4.26 μg/ml (serotype 19A). Prior to the administration of PCV 13, high GMCs were detected in older children and adolescents who had IBD and were naïve to pneumococcal vaccination.

Key words: autoimmune disease, Crohn's disease, PCV, ulcerative colitis, vaccine

Since 2013, the 13-valent pneumococcal conjugate vaccine (PCV 13) has been recommended in children and adolescents aged 6–17 years, regardless of whether they are healthy (Rubin *et al*., 2014) or suffer from immunocompromising conditions such as IBD (CDC, 2013). At the time of this study very little research has been conducted to determine the serological status of older children and adolescentswith respect to the 13 PCV serotypes. Only two studies have been conducted to date: one in healthy young Americans (Frenck Jr. *et al.*, 2014) and one in children and adolescents with perinatally acquired HIV infection in Africa (Bamford *et al*., 2014). Differences in preimmunization pneumococcal vaccine-specific serological status were observed between these studies. This could be explained by worldwide differences in pneumococcal serotype distribution modifying the natural response and by the impaired serological response of HIV-infected children. A recently published study assessed the prevaccination serological status of older Polish infants and toddlers who were naïve to pneumococcal vaccination (Wysocki *et al*., 2015). The aim of this report was to assess the pneumococcal serological status of older Polish children and adolescents with IBD prior to the administration of one dose of PCV 13.

This report was part of an open, prospective study that was conducted between April 2013 and March 2014 across six hospitals in Poland (cities of Warsaw, Poznan, Wroclaw, Katowice, Gdansk and Cracow) to compare the immunogenicity and safety of using PCV 13 in subjects with IBD versus healthy controls (grant of the Polish Ministry of Science and Higher

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Education no. N404 077140 0771/B/P01/2011/40). Our part of the study focused on subjects between 5 and 18 years of age who had been diagnosed with IBD based on clinical signs and symptoms in addition to endoscopic, histologic and radiologic results, according to the Porto criteria (Levine *et al*., 2014). Additionally, the subjects had not been previously vaccinated with any of pneumococcal vaccines and had no history of IPD. IBD patients were included from any stage of the disease and treatment process. All participants received one dose of 0.5 ml 13-valent PCV (Diphtheria CRM197 Protein) from Wyeth Pharmaceuticals, Inc. Blood samples were collected immediately prior to vaccination. Sera were stored at –20°C to –70°C until analysis was performed. Each serum sample was tested for serotype-specific anti-pneumococcal polysaccharide IgG antibodies to the 13 polysaccharides of PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19A, 19F and 23F) using a standardized enzyme linked immunoassay according to an adapted WHO protocol (WHO, 2005). To enhance the specificity of the serotype-specific antibodies, the assay included the preabsorption of test sera from the pneumococcal C polysaccharide and the pneumococcal polysaccharide from the non-vaccine 22F serotype (Wernette *et al*., 2003; Concepcion *et al*., 2001). All parents and children ≥ 16 years old provided written informed consent. Geometric mean concentrations (GMCs) of prevaccination antibody levels were used as the measure of central tendency. Confidence intervals at $1 - \alpha = 0.05$ were computed (CI 95%). This study was approved on Jan 26th 2010 by the Clinical Research Ethics Committee of the Medical University of Warsaw, Poland. This study was supported by grant no. N404 077140 0771/B/P01/2011/40 from the Polish Ministry of Science and Higher Education. The project was approved Febr. 4th, 2010.

This study included 122 older children and adolescents who had IBD (mean age 15.08 years, 60% boys). The prevaccination GMCs are presented in Table I.

In this report, we found high prevaccination GMCs for all 13 serotypes (range from 0.55 μg/ml for serotype 4 to 4.26 for serotype 19A). This is in accordance with previous studies conducted on healthy older children (Frenck Jr *et al*., 2014; Wysocki *et al*., 2015) and adolescents (Frenck Jr *et al*., 2014). These findings support the suggestion of natural exposure to vaccine serotypes. Notably, we observed high GMCs for both the most common (1, 14 and 19F) and the most fatal (3, 4, 19F, 14 and 19A) serotypes in Polish 5- to 25-year-old subjects with IPD (Skoczyńska *et al*., 2015). For children with chronic, potentially immunosuppressing conditions such as IBD, protection against a significant proportion of the serotypes that can cause IPD is extremely important. However, mass vaccination against pneumococcal disease has not yet been introduced in Poland.

Table I
Prevaccination geometric mean concentrations (GMCs) for 13 serotypes

PCV 13 serotypes	Prevaccination GMC (95% CI)
1	0.83 (0.71–0.98)
3	0.99 (0.81–1.21)
4	0.55 (0.46–0.65)
5	1.18 (1.03–1.36)
6A	2.33 (2–2.71)
6B	2.05 (1.73–2.42)
7F	1.3 (1.03–1.64)
9V	1.31 (1.09–1.57)
14	3.86 (3.08–4.84)
18C	1.47 (1.17–1.84)
19A	4.26 (3.59–5.05)
19F	2.48 (1.98–3.1)
23F	1.52 (1.22–1.9)

We hope that the relatively high prevaccination GMCs that were observed will also offer protection against non-IPD; however, the concentration that would be required to confer protection against pneumococcal non-bacteremic pneumonia and pneumococcal otitis media remains unknown.

Our report has several advantages. Our work is only the second study to assess the use of PCV13 in older children and adolescents who are naïve to pneumococcal vaccines. Our study also provides data on the serological status of patients who were suffering from a chronic disease prior to the administration of PCV13 and adds evidence to the hypothesis that natural exposure to pneumococcal vaccine serotypes occurs in Poland: a country with very low PCV coverage and a lack of herd immunity. PCVs are still not part of the national vaccine program, and patients who elect to have vaccines are not reimbursed; thus, they are only available to a very small proportion of Polish children. Although our study group was small, it was still large enough to perform statistical analysis. A shortcoming of the present study is the lack of a functional response measurement for the serotypes.

High prevaccination pneumococcal GMCs of all PCV 13 serotypes were found in children and adolescents with IBD, suggesting that they previously acquired natural protection against IPD but not necessarily against non-IPD.

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SHORT COMMUNICATION

Prevalence of Urogenital Mycoplasmas Among Men with NGU in Upper Silesia, Poland. Preliminary Study

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Abstract

The prevalence of urogenital mycoplasmas in men with NGU in Upper Silesia (Poland) was studied. Mycoplasmas were detected in 36.7% men (*Ureaplasma parvum* and *Mycoplasma genitalium* were found in 30% and 16.7% respectively). *Urealyticum urealyticum* was not detected. We suggest including *M. genitalium* in the diagnostic scheme for nongonococcal urethritis (NGU).

Key words: *Mycoplasma genitalium*, nongonococcal urethritis, urogenital mycoplasmas

The importance of ureaplasmas as a cause of nongonococcal urethritis (NGU) is discussed. The topic of this discussion has taken on a new meaning since 1999, when Kong *et al.* (1999) divided the genera *Ureaplasma* into two new species *Ureaplasma parvum* and *Ureaplasma urealyticum*. *Mycoplasma genitalium* was being isolated from urethra of men with NGU until 1980, but only recently was confirmed as an important etiology of NGU in men (Workowski and Berman, 2010). In Poland detection of urogenital mycoplasmas is not a part of routine diagnostic procedure. Few published papers cover mainly pregnant women and newborns.

The aim of this study was to evaluate the prevalence of *M. genitalium* and *Ureaplasmas* in men with NGU, taking into account the diversity of the species (*U. parvum* and *U. urealyticum*). According to our knowledge, this is the first such study in Poland.

Thirty male patients aged 30–55 years (mean age 38 ± 5.68 years) diagnosed with NGU (clinical symptoms, microscopic preparation, after excluding *Chlamydia trachomatis)* were directed for detection of urogenital mycoplasmas. All men gave informed consent for this study. Patients were advised of the need to maintain sexual abstinence for 3–4 days and were obliged to come to testing at minimum 4 h after the last voiding. This study was approved by the Bioethical Committee of Medical University of Silesia.

Isolation of mycoplasmal DNA was performed from a pellet obtained from culture in Mycoplasma IST 2 (bioMérieux, Marcy I'Etoile, France). Identification of *U. parvum*, *U. urealyticum* and intraspecific diversity of *U. parvum* was done using species-specific primers according to Kong *et al.* (2000). Detection of *M. genitalium* was conducted using primers for adhesin genes: MgPa-1 – MgPa-3 and for 16S rRNA gene MG16-45F – MG16-447R; MG16-1204F – MG16-1301R primers were designed according to Jensen *et al.* (1991; 2003).

In 9/30 (30%) samples urogenital mycoplasmas were detected with Mycoplasma IST 2 test. PCR amplification confirmed the presence of mycoplasmas in all 9 cases and did not increase the number of positive results for ureaplasmas. However, detection of *M. genitalium* DNA by PCR increased the number of positive results for urogenital mycoplasmas from 9 cases to 11 (36.7%). Co-occurrence of two different species of urogenital mycoplasmas was shown in the majority of samples (Table I). Interestingly, species identification

Table I
Occurrence of urogenital mycoplasmas in the study group (n = 30).

	No (%)
M. genitalium	**2** (6.7)
U. parvum + *M. genitalium*	**3** (10)
U. parvum + *M. hominis*	**2** (6.7)
U. parvum	**4** (13.3)
Total	**11** (36.7)

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Table II
Results of *M. genitalium* DNA detection by PCR with selected primers in men with NGU (n = 30).

Number of studied cases	Primers			Final interpretation
	Mg16-45F + Mg16-447R	Mg16-1204F + MG16-1301R	MgPa-1 + MgPa-3	
1	Positive	Positive	Positive	Positive
4	Negative	Positive	Posititve	Positive
4	Negative	Negative	Positive	Negative
21	Negative	Negative	Negative	Negative

revealed the presence of *U. parvum* in all cases, in contrast to *U. urealyticum* strains, which were not detected. Domination of *U. parvum* in our study group was concordant with others (Tang *et al*., 2011; Vancutsem *et al*., 2011). Several studies have shown that *U. urealyticum* was significantly more common in men with NGU (Maeda *et al*., 2004; Manhart *et al*., 2013). Among isolated mycoplasmas *U. parvum* was detected significantly more often than *M. hominis* (p = 0.0453). However domination of *U. parvum* in comparison to *M. genitalium* was not significant (p = 0.3598). Although the presence of *M. genitalium* DNA by PCR using primers for adhesin genes MgPa was shown in specimens from 9 (30%) men with NGU, primers for 16S rRNA gene confirmed the presence of *M. genitalium* in only 5 samples (16.7%, Table II). The rule that double-positive amplicon for adhesin gene with primers MgPa-1/MgPa-3 and double-positive for one of the primers for 16S rRNA gene could be considered as positive was used in the interpretation of the obtained results (Tabele II). The decrease in the number of positive cases from 9/30 to 5/30 in the study group of men was not significant. According to other authors, confirmation of positive results through repetition or the use of different primers are required (Manhart *et al*., 2003). Gaydos *et al.* (2009) recognized a patient's infection only when positive results with primers for both MgPa and 16S rRNA genes were achieved. Among the analysed specimens from 719 women (Manhart *et al*., 2003) 51 were double-positive using primer pairs for MgPa genes. Furthermore, only 45 (88.2%) of 51 double-positive results were confirmed using PCR for 16S rRNA gene. At the same time, for none of 49 selected MgPa PCR-negative specimens, positive results with primers for 16S rRNA gene were demonstrated. Edberg *et al.* (2008) achieved higher sensitivity by real-time PCR for MgPa gene, compared to conventional PCR for 16S rRNA gene. Moreover, the authors demonstrated much higher sensitivity in contrary to real-time PCR for 16S rRNA gene.

The urogenital mycoplasmas are recognized in a large percentage of men with NGU. However, in order to properly interpret the presence and role of urogenital mycoplasmas in the etiology of infection, the result of the test should be complete. The outcome has to contain both: detection of *M. genitalium* DNA and identification of ureaplasmas to *U. parvum* and *U. urealyticum*. Japanese authors identified *M. genitalium*, *U. urealyticum* and *U. parvum* in men with NGU with a frequency of 17%, 16.3% and 7.8% respectively; among the patients with non-chlamydial NGU – 23.8%, 18.8% and 8.8% respectively (Maeda *et al*., 2004). In a study from Denmark, the prevalence of *M. genitalium* infection was 2.3% and 1.1% respectively in 731 men and 921 women aged 21–23 years, not seeking medical assistance (Andersen *et al*., 2007). Detection rate of *M. genitalium* DNA in urine from asymptomatic healthy young Japanese men was only 1% (Takahashi *et al*., 2006).

The absence of *U. urealyticum* in our specimens was probably due first of all to small size of the study group and secondly, the low frequency of isolation of *U. urealyticum* in the Polish population, demonstrated previously in a group of women (Ekiel *et al*., 2009). Similar results of low percentage of *U. urealyticum* were indicated in other countries (Tang *et al*., 2011; Vancutsem *et al*., 2011). A limitation of our study was the absence of a control group due to the fact that taking urethral swabs is invasive and poorly accepted by men.

The usefulness of the first – void urine (FVU) in molecular biology studies was shown by other authors. Thanks to this, availability of research on urogenital mycoplasmas certainly will increase (Takahashi *et al*., 2006; Wroblewski *et al*., 2006).

M. genitalium is now an important and established cause of approximately 9–25% cases of NGU (Workowski and Berman, 2010; Manhart *et al*., 2013). In spite of that fact that our study group was limited, 16.7% positive *M. genitalium* cases confirmed the role of this microorganism as an important causative agent of NGU. Furthermore, our study points to the requirement of including *M. genitalium* in the diagnostic scheme for patients with non-chlamydial NGUs.

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SHORT COMMUNICATION

Assessment of the Microbiological Status of Probiotic Products

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Abstract

The aim of this study was to perform the microbiological analysis of quality of 25 probiotic products, available on the Polish market. Analysis of bacterial viability in probiotic products showed that not all of these preparations possess a suitable number of bacteria. Moreover, some of the tested probiotic products contained bacterial strains other than those declared by the manufacturer. All tested strains recovered from probiotic products were found to be resistant to metronidazole and susceptible to nitrofurantoin. The susceptibility to other antibiotics was strain specific. Probiotic products should be subject to regular and thorough inspection by appropriate institutions.

Key words: antimicrobial resistance, dietary supplement, FSMP, medicinal product, probiotics

Probiotics can be defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host", according to the Food and Agriculture Organization of the United Nations and World Health Organization definition (FAO/WHO, 2001). A probiotic preparation must contain a specified minimal number of bacterial cells-colony forming units (cfu) per dose. A daily intake of minimum $10^8 – 10^{10}$ cfu per day is required to show the beneficial health effects (Czinn and Blanchard, 2009; Sanders and Huis in't Veld, 1999). These effects seem to be strain specific and dose dependent. A number of probiotic strains, particularly those belonging to species of lactic acid bacteria (LAB) group, are used to induce health benefits for a variety of conditions and diseases throughout the world (Czinn and Blanchard, 2009). Many of these effects have been scientifically supported (FAO/WHO, 2001). These include improving the condition of the intestinal tract (traveller's diarrhoea, antibiotic-associated diarrhoea) (FAO/WHO, 2001; Holzapfel *et al.*, 2001; Chapman *et al.*, 2011) decreasing the prevalence of vaginal infections (FAO/WHO, 2001), increasing immune function (FAO/WHO, 2001), and decreasing cholesterol and lipid levels (Pereira *et al.*, 2003).

There are many probiotic preparations in the Polish market that are distributed as medicinal products, dietary supplements or food for special medical purposes (FSMP). These products contain live bacteria – mostly *Lactobacillus* and *Bifidobacterium* – that supposedly produce a beneficial effect on human health. Probiotics on the market are sold in multiple forms such as capsules containing single or multiple strains, liquids or powders. A description on the label of a probiotic should include: genus, species and strain designation, minimum viable number of each probiotic cells at the end of the shelf-life, suggested serving that must deliver the effective dose related to the health claims, proper storage conditions, and corporate contact details for consumer information (FAO/WHO, 2002). In Poland, there is no national governmental agency responsible for the control of dietary supplements and FSMP products including probiotics, so the quality of these products may not comply with the information accompanying the probiotic product. Particularly important is the actual number of viable organisms present in the commercial product, which may be lower than the declared value that guarantees its beneficial properties (Czinn and Blanchard, 2009).

Although the use of lactic acid bacteria has a long history and has acquired Generally Recognised as Safe (GRAS) status, the safety of selected strains should be evaluated before use, not only for virulence factors and other potential disease-causing traits, but also for their capability of acquiring and disseminating resistance determinants. The transfer of antibiotic-resistance genes from LAB reservoir strains to bacteria in the resident

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microflora of human gastrointestinal tract, and hence to pathogenic bacteria, has not been fully documented. Lactobacilli display a wide range of types of antibiotic resistance naturally, but in most cases antibiotic resistance is not of the transmissible type. Although plasmid-linked antibiotic resistance is not very common among lactobacilli, it does occur and its influence on safety should be taken into consideration (Ashraf and Shah, 2011; Liu *et al.*, 2009; Wiatrzyk *et al.*, 2007).

The purpose of this study was to evaluate the quality of selected probiotic preparations, primarily the count of bacteria present in the different batches of several preparations, stored before distribution at temperatures recommended by the manufacturers. Additional aims included identification of the bacterial strains and designation of antimicrobial susceptibility.

A total of 16 dietary supplements, seven FSMP and two medicinal products, from two or three different batches, available on the market were tested for the viability of probiotic bacteria (Table I). Fifteen of the products were tested 3–4 times to monitor viability of bacterial strains during validity time. In the case of the 10 other products, one or two series were tested once or twice. Samples were stored at room temperature or in the refrigerator, according to the manufacturers' recommendations.

Each tested preparation was suspended in peptone water (Buffered NaCl-Peptone Solutions, Heipha), diluted and plated onto De Man Rogosa and Sharpe Agar (MRS-Agar, Merck) for *Lactobacillus* and Transgalactio-Oligosaccharides (TOS) Propionate Agar (Merck) with MUP Selective Supplement (Merck) for *Bifidobacterium*. The plates were incubated for 48–72 h at 37°C with 5% CO_2 for *Lactobacillus* and in anaerobic conditions (GENbag anaer, bioMérieux) for *Bifidobacterium*. The microbial count was expressed as cfu per one dose (Fig. 1).

The strains isolated from probiotic products were identified by two methods – (i) API 50 CHL (bioMérieux) and API 20 A (bioMérieux) biochemical tests, (ii) Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS), analysis was performed by ALAB Laboratories, Warsaw.

Identification of lactic acid bacteria showed different results depending on the method applied (Table I). Biochemical test API 50 CHL did not properly identify *Lactobacillus rhamnosus* strains and often recognised this species as *Lactobacillus paracasei.* Moreover, API 20 A test recognised *Bifidobacterium* only to genera. Therefore MALDI – TOF MS was used to confirm the biochemical identification. Not all results of identification confirmed the strain species declared by the manufacturers. For

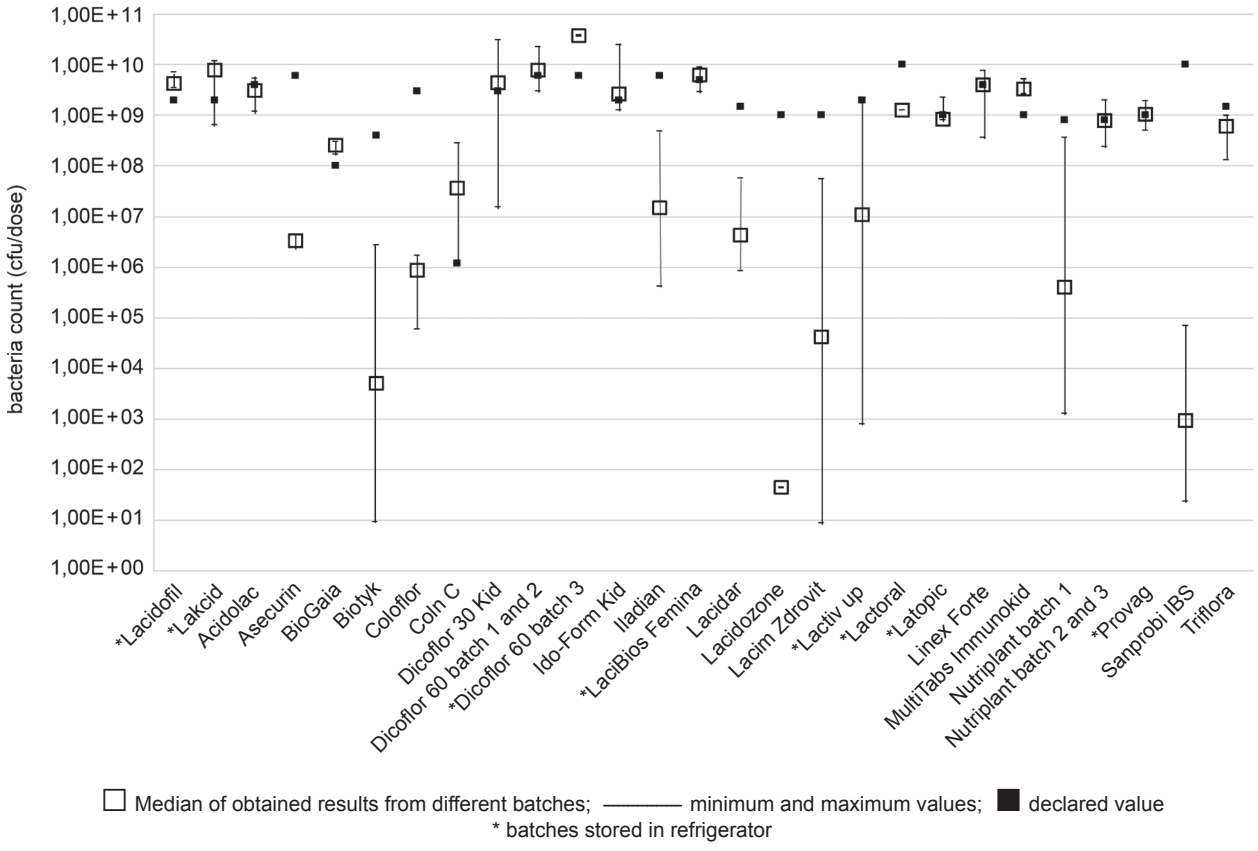

Fig. 1. Median of bacteria counts from different batches of the probiotic products (Table I) with marked minimum and maximum value of obtained result

Table I
Detailed information and results of bacterial strains` identification in analysed probiotic product.

Product	Manufacturer	Preparation form	Medicinal product/ Dietary supplement/ Food for Special Medical Purpose (FSMP)	Number of analysed batches	Time to expire when observed significant decrease in the number of bacteria (months)	Declared microorganism presented	Biochemical identification (API 50 CHL/ API 20A)	Identification MALDI-TOF MS
Lacidofil	Merck	Capsules	Medicinal product	3	Not observed	*Lactobacillus rhamnosus*, *Lactobacillus helveticus* (formerly *L. acidophilus*)	*L. rhamnosus*	*L. rhamnosus*
Lakcid	Biomed	Powder	Medicinal product	3	Not observed	*Lactobacillus rhamnosus*	*L. rhamnosus*	*L. rhamnosus*
Acidolac	Polpharma	Powder	FSMP	3	Not observed	*Lactobacillus acidophilus*, *Bifidobacterium BB-12*	*L. acidophilus* *Bifidobacterium* ssp. 1	*L. acidophilus* *B. animalis* ssp. *lactis*
Asecurin	Aflofarm	Capsules	Dietary supplement	1	11	*Lactobacillus gasseri*, *Lactobacillus reuteri* *Lactobacillus rhamnosus*	*L. rhamnosus* *L. paracasei* ssp. *paracasei* *L. debrueckii*	n.t.
BioGaia	Ewopharma	Tablets	FSMP	3	Not observed	*Lactobacillus reuteri Protectis*	*L. fermentum*	*L. reuteri*
Biotyk	Lekam	Capsules	Dietary supplement	3	21	*Lactobacillus casei*	*L. lactis* ssp. *lactis*/*L. brevis*	*L. rhamnosus**
Coloflor	Oleofarm	Capsules	FSMP	1	10	*Lactobacillus acidophilus DDS-1* *Bifidobacterium lactis VABLA-12*	*L. acidophilus* *Bifidobacterium*	n.t.
Colon C	A-Z Medica	Granulat	Dietary supplement	3	Not observed	*Lactobacillus acidophilus*, *Lactobacillus brevis*	*L. rhamnosus* *L. plantarum* *L. lactis* ssp. *lactis*	*L. rhamnosus*, *L. plantarum*
Dicoflor 30 Kid	Vitis Pharma	Capsules	FSMP	3	Depends on batch – 6 (batch 1)	*Lactobacillus rhamnosus GG*	*L. paracasei* ssp. *paracasei*	*L. rhamnosus*
Dicoflor 60	Vitis Pharma	Capsules	FSMP	3	Not observed	*Lactobacillus rhamnosus GG*	*L. paracasei* ssp. *paracasei*	*L. rhamnosus*
Ido-Form Kid	Ferrosan	Tablets	Dietary supplement	2	Not observed	*Lactobacillus rhamnosus GG*, *Bifidobacterium animalis* ssp. *lactis*	*L. paracasei* ssp. *paracasei* *Bifidobacterium* ssp.1	*L. rhamnosus*
Iladian	Aflofarm	Capsules	Dietary supplement	2	15	*Lactobacillus rhamnosus* *Lactobacillus reuteri* *Lactobacillus gasseri*	*L. rhamnosus*	n.t.
LaciBios Femina	Asa	Capsules	Dietary supplement	3	Not observed	*Lactobacillus rhamnosus GR-1*, *Lactobacillus reuteri RC-14*	*L. rhamnosus* *L. fermentum*	*L. rhamnosus*** *L. reuteri**
Lacidar	Tantus	Capsules	Dietary supplement	2	20	*Lactobacillus bulgaricus* *Lactobacillus rhamnosus* *Lactobacillus acidophilus*	*L. paracasei* ssp. *paracasei* *L. rhamnosus*	n.t.

Table I
Continued.

Product	Manufacturer	Preparation form	Medicinal product/ Dietary supplement/ Food for Special Medical Purpose (FSMP)	Number of analysed batches	Time to expire when observed significant decrease in the number of bacteria (months)	Declared microorganism presented	Biochemical identification (API 50 CHL/API 20A)	Identification MALDI-TOF MS
Lacidozone	Ozone Laboratories	Capsules	Dietary supplement	1	7	*Lactobacillus acidophilus* *Bifidobacterium breve*	*L. acidophilus*	n.t.
Lacium Zdrovit	Zdrovit	Capsules	Dietary supplement	3	17	*Lactobacillus plantarum*	*L. plantarum*	*L. plantarum*
Lactiv up	Farma-Projekt	Capsules	Dietary supplement	3	9	*Lactobacillus acidophilus*	*L. acidophilus*	*L. acidophilus*
Lactoral	Biomed	Capsules	Dietary supplement	1	8	*Lactobacillus plantarum PL 02* *Lactobacillus rhamnosus KL 53A* *Bifidobacterium longum PL 03*	*L. rhamnosus* *Bifidobacterium*	n.t.
Latopic	IBSS Biomed	Powder	FSMP	3	Not observed	*Lactobacillus paracasei ŁOCK 0919* *Lactobacillus casei ŁOCK 0900* *Lactobacillus casei ŁOCK 0908*	*L. rhamnosus* *L. paracasei* ssp. *paracasei*	n.t.
Linex Forte	Lek Pharmaceuticals	Capsules	Dietary supplement	3	Not observed	*Lactobacillus acidophilus*, *Bifidobacterium animalis* ssp. *lactis*	*L. acidophilus* *Bifidobacterium* ssp. 1	*L. acidophilus* *B. animalis* ssp. *lactis*
MultiTabs ImmunoKid	Ferrosan	Tablets	Dietary supplement	3	Not observed	*Lactobacillus rhamnosus GG*	*L. paracasei* ssp. *paracasei*	*L. rhamnosus*
Nutriplant	Agropharm	Capsules	Dietary supplement	3	3 (room temp.)	*Lactobacillus plantarum*	*L. plantarum*	*L. plantarum*
Provag	Biomed	Capsules	FSMP	2	Not observed	*Lactobacillus gasseri 57C* *Lactobacillus fermentum 57A* *Lactobacillus plantarum 57B*	*L. fermentum* *L. plantarum*	n.t.
Sanprobi IBS	Sanum Poland	Capsules	Dietary supplement	3	13	*Lactobacillus plantarum 299v*	*L. plantarum*	n.t.
Triflora	Farmapia	Capsules	Dietary supplement	2	Depends on batch – 19 (batch 2)	*Lactobacillus acidophilus* *Lactobacillus paracasei* *Bifidobacterium bifidum*	*L. acidophilus* *L. paracasei* *Bifidobacterium*	n.t.

* Good identification to genus, possible identification to species;
** Possible identification to genus
n.t. – not tested

example dietary supplement (Biotyk) was declared to contain *Lactobacillus casei* but API 50 CHL identified this strain as *Lactococcus lactis* ssp. *lactis* and MALDI-TOF as *L. rhamnosus* with good identification to genus and possible identification to species. Other case was identification of *Lactobacillus reuteri* with API 50 CHL from BioGaia and LaciBios Femina, while manufacturer declared *Lactobacillus fermentum*. However, this might be caused by a change in the nomenclature, *L. reuteri*, *L. fermentum* biotype II and *L. fermentum* subsp. *reuteri* are used sometimes as synonyms.

Several strains described on the label by manufacturers were not identified, *e.g. Lactobacillus helveticus* from Lacidofil or *Lactobacillus gasseri* and *L. reuteri* from dietary supplements (Asecurin, Iladian, Provag). In some products, the identified strains were different from those specified on the label. Dietary supplement Colon C contained *L. rhamnosus*, *Lactobacillus plantarum* and *L. lactis* ssp. *lactis*, while the manufacturer declared that preparation contains *Lactobacillus acidophilus* and *Lactobacillus brevis.* In Asecurin, we identified the *Lactobacillus delbrueckii* strain, not declared by the manufacturer. Analysis of the viability of probiotic strains (Fig. 1) showed that not all of the tested preparations contained a suitable number of lactic acid bacteria, as declared on the label. Analysis of three different batches of medicinal products – Lakcid and Lacidofil, showed a decrease in the bacteria number during the specified time interval. In the case of Lacidofil, an adequate number of living cells was maintained until the end of the validity period, while in the case of the Lakcid, cfu decrease was too far below the declared value, at the end of the validity time. A decrease in cfu values at the end of validity period was observed among all tested batches, reaching 32–78% of the declared value. Only four of the tested probiotic products (MultiTabs Immunokid, BioGaia, Colon C, Latopic), which were stored at room temperature, showed proper bacterial survival during the investigated time period. In all tested batches of these preparations, the numbers of living bacterial cells were above the minimum level declared by the manufacturers. Seven other probiotic products (Ido Form Kid, Dicoflor 30 Kid, LaciBios Femina, Acidolac, Linex Forte, Provag, Triflora) were characterised as having different levels of bacterial viability depending on the batch. In some product batches, at the date of expiry, the numbers of bacteria were above the level declared by the manufacturer – however, other batches were characterised by a large decrease in the amount of probiotic bacteria. All of these batches were kept at room temperature, except for LaciBios Femina and Provag, which were stored in a refrigerator, according to the manufacturers' recommendations.

Ten of the tested products showed a decrease in the bacteria count in all of the tested batches. In these products, which were stored in different conditions (room temperature or refrigerator), the number of live microbial cells was very low (< 10% of the declared value) at all expiry dates, which would probably result in low or no therapeutic properties. In the case of two preparations (Nutriplant and Dicoflor 60), the tested batches were stored at different temperatures (room temperature or refrigerator), depending on the storage temperature used at the time of purchase (manufacturers' recommendation below 20°C). Generally, batches of products stored in the refrigerator were characterised by better bacterial survival than preparations kept at room temperature.

Antibiotic susceptibility for 16 antibiotics was assayed using disc-diffusion method according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (www.eucast.org). Plates, with Mueller-Hinton Agar, were incubated for 48 h at 37°C in anaerobic conditions (GENbag anaer, bioMérieux). The diameter of the bacterial growth inhibition zone was measured and interpreted according to Clinical and Laboratory Standards Institute (CLSI) Table II. Experiments were performed in duplicate.

All bacterial strains were resistant to metronidazole (lack on any growth inhibition zone) and sensitive to nitrofurantoin. Tested probiotic preparations were resistant to colistin and fusidic acid (except Biotyk). Most of the tested bacterial strains were also resistant to vancomycin (except strains from Linex Forte and Biotyk) and trimethoprim/sulfametoxazole (except strains from Linex Forte, Biotyk, Nutriplant, Lacium Zdrovit and Colon C). Bacterial strains from medicinal product Lacidofil – *L. rhamnosus* and *L. helveticus*, the only of tested strains, showed resistance to cefuroxime – II generation cephalosporin. Cefotaxime, a third-generation cephalosporin, inhibited the growth of most probiotic strains – only bacteria from Lacidofil and Dicoflor 60 preparations (*L. rhamnosus* GG) were resistant. CLSI guidelines for *Staphylococcus* and *Enterococcus*, in the case of ampicilin, are different, so it cannot be clearly defined which tested strains from the probiotic products were resistant or sensitive to this antibiotic. Basing on the CLSI guidelines for reference strains, it can be concluded that strains from probiotic products Lakcid (*L. rhamnosus)*, Lactiv up (*L. acidophilus*) and Acidolac (*L. acidophilus, Bifidobacterium* BB-12) were resistant to ampicillin. In the case of clindamycin and erythromycin, only strains from preparations Lakcid, Colon C and Lacium Zdrovit (only to clindamycin) were resistant. Trimethoprim/sulfamethoxazole inhibited the growth of all *L. plantarum* strains contained in the probiotic products: Nutriplant, Lacium Zdrovit, Colon C and also the strains from Linex Forte and Biotyk.

Considering the significant increase in the annual consumption of probiotic products, it is very impor-

Table II
Susceptibility of microbial strains present in tested probiotic products to antimicrobial agents according to CLSI guidelines for *Enterococcus* spp. and *Staphylococcus* spp.

Product/antibiotic	Lakcid	Lacidofil	LaciBios Femina	Lactiv up	Acidolac	Linex Forte	IdoForm Kid	Biotyk	Nutriplant	Dicoflor 30 Kid	Dicoflor 60	Lacium Zdrovit	BioGaia	MultiTabs ImmunoKid	Colon C
Ampicillin AMP (10 µg)	R	S	S	R	R	S	S	S	S	S	S	S	S	S	S
Cefaclor CEC (30 µg)	R	R	R	R	R	S	I	S	S	R	R	I	I	R	R
Ciprofloxacin CIP (5 µg)	S	S	S	R	I	I	I	S	R	S	S	R	S	I	S
Gentamycin GEN (10 µg)	S	S	S	S	S	R	R	S	S	S	S	S	I	S	S
Colistin CST (10 µg)*	10	6	12	6	6	6	6	21	12	6	6	6	6	6	6
Cefotaxime CTX (30 µg)	S	R	I	S	S	S	I	S	S	S	R	S	I	I	I
Cefuroxime CXM (30 µg)	S	R	S	S	S	S	S	S	S	S	S	I	S	I	S
Clindamycin CLI (2 µg)	R	S	S	S	S	S	I	S	I	S	I	R	I	S	R
Doxycycline DOX (30 µg)	R	I	S	I	S	S	S	S	R	S	S	R	S	S	R
Erythromycin ERY (15 µg)	R	S	S	S	S	S	S	S	S	S	S	S	S	S	R
Nitrofurantoin NIT (300 µg)	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic acid FA (10 µg)	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R
Cefazolin CFZ (30 µg)	R	R	S	R	R	S	R	S	S	R	R	I	I	R	R
Metronidazole MTZ (50 µg)*	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Trimethoprim/sulfametoxazole SXT (1.25/23.75 µg)	R	R	R	R	R	S	R	S	S	R	R	S	R	R	S
Vancomycin VAN (30 µg)	R	R	R	R	R	S	R	S	R	R	R	R	R	R	R

* Lack in CLSI guidelines, presented diameter (mm) of growth inhibition zones. Resistant strains are shaded background.
S – susceptible; I – intermediately susceptible; R – resistant

tant that these products should be of proper quality, containing probiotic strains that are well documented regarding safety and functionality (Sanders and Huis in't Veld, 1999). However, the analysis of the obtained microbiological results clearly shows that the quality of tested probiotic products is far from ideal.

Bacteria viability of probiotic medicinal products in the Polish market were previously analysed by Szajewska *et al.* (2002). From five tested products, four possessed the bacteria count in accordance with the manufacturer's declaration. One product had a low number of probiotic bacteria. Szajewska *et al.* (2004) also investigated the quality of probiotic products licensed for medicinal purposes. Microbiological and genetic analysis showed that, in terms of quality, only three of five products contained the bacterial strains claimed on the label. Quantitative analysis demonstrated that 89% (57 of 64) of samples contained bacterial counts at the cell densities (doses) claimed on the label. Coeuret *et al.* (2004) performed some analyses of European probiotic products. In food supplements, the numbers of colonies were in accordance with data declared on the label – however, in the tested nutritional supplements, there were no viable lactobacilli found, even though the labels claimed that the product contained high numbers of various lactic acid bacteria. A recent study made by Temmerman *et al.* (2002) showed that numbers of viable bacteria were generally lower in food supplements than in dairy products, with no viable bacteria being found in 37% of food supplements. Moreover, 9 of 30 tested food supplements contained microorganism species other than those indicated on the product label. Research carried out by Hamilton-Miller *et al.* (1998) on 21 different kinds of supplements showed that only seven UK products completely fulfilled their label quantitative claims. Moreover, only 9 of 21 products contained exclusively the species stated on the label, with the other 12 products lacking one or more of the stated species. Sometimes the species have been incorrectly identified, or a contaminant strain was present. Only 7 of 21 products tested were both qualitatively and quantitatively bacteriologically satisfactory (Hamilton-Miller *et al.*, 1998).

The profiles of antimicrobial susceptibility of LAB have been documented in many countries (Liu *et al.*, 2009). *Lactobacillus* strains are usually susceptible to cell-wall-targeting penicillins, but are more resistant to cephalosporins. Many *Lactobacillus* species showed a high level of resistance to vancomycin. On the other hand, lactobacilli are generally susceptible to low concentrations of many inhibitors of protein synthesis, such as chloramphenicol, macrolides, lincosamides,

and tetracyclines, but their resistance to aminoglycosides is often high (Gueimonde *et al*., 2013), which was not confirmed by our studies (only strains from product Linex Forte were resistant to gentamicin). *Lactobacillus* strains are naturally resistant to nalidixic acid, trimethoprim/sulfametoxazole and metronidazole (Hummel *et al*., 2007). Resistance of lactobacilli to metronidazole might be caused by the absence of hydrogenase activity (Danielsen and Wind, 2003). Moreover, *L. rhamnosus* strains are resistant to vancomycin, which distinguishes them from vancomycin-sensitive *L. acidophilus* (Wiatrzyk *et al*., 2007). Research carried out by Temmerman *et al.* (2002) on 187 strains isolated from probiotic products subjected to antibiotic susceptibility testing showed that 79% and 65% of these isolates were resistant to kanamycin and vancomycin, respectively. In our study, in 2 of 15 tested probiotic products, bacteria susceptible to vancomycin were observed. Wiatrzyk *et al*. (2013) confirmed that all tested *L. rhamnosus* were resistant to vancomycin. Moreover, this study demonstrated resistance of probiotic strains present in products Lakcid and Lakcid forte to: penicillin, ampicillin, amoxicillin, piperacillin, cefuroxime, cefotaxime, ceftazidime, cefepime, cefradine, cloxacillin, imipenem, meropenem, gentamicin, neomycin, netilmicin, tobramycin, streptomycin, erythromycin, vancomycin, teicoplanin, doxycycline, trimethoprim/sulfamethoxazole, nalidixic acid, metronidazole, clindamycin and colistin. Investigations conducted in our laboratory have not confirmed resistance of bacterial strains from Lakcid to ampicillin, cefotaxime, cefuroxime and gentamicin. In the case of colistin, there are no guidelines from the CLSI for the interpretation of bacterial sensitivity. Moreover, sensitivity to nitrofurantoin was observed. Strains from products Lacidofil and EcoVag tested by Wiatrzyk *et al.* (2013) were resistant to aminoglycosides, glycopeptides and clindamycin, colistin and chemotherapeutics (trimethoprim/sulfamethoxazole, metronidazole, nalidixic acid). These results are also not entirely consistent with ours, where probiotic strains from Lacidofil were sensitive to gentamicin and clindamycin, and as above there were no CLSI guidelines for colistin. Wiatrzyk *et al.* (2013) used European Food Safety Authority (EFSA) guidelines for the interpretation of their results. Moreover, comparison of the antimicrobial susceptibility of *L. rhamnosus* PEN to gentamycin was assayed by two methods, disc diffusion and the E-test, which showed different results. In the disc-diffusion method, the *L. rhamnosus* PEN strain was susceptible to gentamycin, but when using the E-test, this strain was resistant. Similarly, contradictory results were obtained in the case of *L. delbrueckii* subsp. *bulgaricus* strain. Nawaz *et al*. (2011) also observed resistance of lactic acid bacteria to nalidixic acid, vancomycin and kanamycin, while susceptibility of all tested strains to ampicillin was noted. In our study, bacteria in 3 of 15 tested probiotic products showed resistance to ampicillin.

Many probiotic products from the Polish market (medicinal products, dietary supplements and food for special medical purposes) contain too few probiotic bacteria cells, which probably cause low or even no beneficial effect on health. In addition some of the tested probiotic products contained different bacterial strains than those declared by the manufacturer. Taking into account the fact that each strain is characterised by different properties, such situations should not occur. Each strain has a different antibiotic resistance profile, and incorrect labelling of strains will also results in no beneficial effect on health. Only one medicinal product, two dietary supplements and two FSMP of all tested 25 different products showed a good quality with respect to the number of bacterial cells. In 15 of 25 tested products, compliance of species with the label was proved. Products with inappropriate number of bacterial cells or with not confirmed properties, which may even cause serious health problems, should be withdrawn from the market. On the basis of the obtained results, it can be concluded that all probiotic products available on the market should be subjected to routine and thorough inspection by appropriate institutions.

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SHORT COMMUNICATION

Antimicrobial Activity of Penicillin G and N-acetylcystein on Planktonic and Sessile Cells of *Streptococcus suis*

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Abstract

The aim of this study was to investigate the capacity of *Streptococcus suis* strains to form biofilms and to evaluate the antimicrobial activity of Penicillin G and N-acetylcystein (NAC) on both *S. suis* sessile and planktonic forms. Only non-typeable isolates of *S. suis* were correlated with a greater biofilm formation capacity. The MCI of Penicillin G and NAC required for inhibiting biofilm growth were higher than the required concentration for inhibiting planktonic growth. The combinations of NAC and Penicillin G showed a strong synergistic activity that inhibited biofilm formation and disrupted the pre-formed biofilm of *S. suis*.

Key words: *Streptococcus suis*, biofilm, N-acetylcystein, Penicillin G

In most parts of the world, *Streptococcus suis* is the predominant agent of streptococcal infections in the swine industry and it is associated with a variety of diseases including meningitis, arthritis and pneumonia (Gottschalk *et al*., 2007; Lun *et al*., 2007). Thirty--five serotypes of *S. suis* have been characterized, the most virulent type to swine and humans, being serotype 2 (Gottschalk *et al*., 2007; Lun *et al*., 2007; Wang *et al*., 2011).

Zoonotic disease due to *S. suis* does occur sporadically in Western countries, but is encountered more commonly in countries such as China and Hong Kong. It affects predominantly individuals with occupational exposure to pigs (Huang *et al*., 2005). The antibiotics are widely used to control *S. suis* infections (in humans and in animals), but the emergence of antibiotic-resistant strains represents a problem for both pig production and public health (Hui *et al*., 2005).

Bacterial biofilm formation is a mechanism that allows them to become persistent colonizers and enhance their resistance to antibiotics (Guo *et al*., 2012). Many drugs and compounds have been described as biofilm inhibitors (Aslam *et al*., 2007), specifically Ampicillin and Penicillin G have been examined on *S. suis* biofilm (Grenier *et al*., 2009); biocides such as N-acetylcysteine (NAC) decrease biofilm formation for a variety of bacteria (Marchese *et al*., 2003; Schwandt *et al*., 2004), but their effect on *S. suis* biofilm remains unknown. Therefore, we investigated the biofilm-forming potential of *S. suis* strains collected from farms in Cuba; the effect of two antimicrobial agents, Penicillin G and N-acetylcysteine, on biofilm formation was also studied.

S. suis strains tested for biofilm formation in this study belonged to different serotypes: two serotype 1 (Ss181, ss1S), one serotype 1/2 (Ss1/2M), two serotype 2 (Ss213, Ss211), two serotype 3 (Ss36, Ss364), one serotype 8 (Ss8O), one serotype 9 (SsS9A), one serotype 16 (Ss16X) and four non-typeable isolates (SsNTF, SsNTV, SsS9Q, SsNTY). The isolates were recovered as predominant bacteria from lungs (pigs with pneumonia) in different farms in Cuba. Appropriate amounts of materials from infected lungs were aseptically transferred to the surface of a Columbia agar plate (Oxoid), supplemented with 5% bovine blood. All the isolates were biochemically typed using the API 20 STREP test kit (Bio Mérieux, France). All the strains are part of the collection of Bacteriology Laboratory at National Centre for Animal and Plant Health (CENSA). Strain confirmation was carried out by PCR assays proposed

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by (Marois *et al*., 2007). Serotyping of *S. suis* was performed by a coagglutination test as previously described (Higgins and Gottschalk, 1990).

The biofilm formation was screened using the conditions previously described (Grenier *et al*., 2009). Briefly, *S. suis* strains were cultured in Todd Hevit Broth (THB) until the late-log phase (1×10^8 CFU $\cdot$ ml^{-1}). Then, aliquots of 0.05 ml were added into wells containing the minimal medium (MM). The adherent cells in the wells were quantified measuring the optical density (OD) at $\lambda = 492$ nm using a microtiter plate reader (SUMA, PR-621, Cuba).

The antibacterial activity of Penicillin G and NAC on planktonic cells were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute, document M100-S18 (CLSI, 2008). Briefly, serial two-fold dilutions of Penicillin G (500–0.5 μg $\cdot$ ml^{-1}) and NAC (16–0.016 mg $\cdot$ ml^{-1}) were respectively prepared in THB. Then, aliquots corresponding to a cell concentration of approximately 5×10^5 CFU $\cdot$ ml^{-1} were inoculated into wells containing each antimicrobial. After incubation at 37°C for 24 h, the absorbance at 620 nm was determined.

The effect on biofilm formation was evaluated as described previously, but the dilutions of both antimicrobials (Penicillin G and NAC) were prepared in MM. Each well was inoculated with a final concentration of approximately 5×10^8 CFU $\cdot$ ml^{-1}. The antibacterial activity of Penicillin G in combination with NAC (P + NAC) was investigated as previously described (Mackay *et al*., 2000). Two concentrations of NAC (4 and 8 mg $\cdot$ ml^{-1}) and 10 fold dilutions (500–0.5 μg $\cdot$ ml^{-1}) of Penicillin G were tested. The effect of antimicrobials on preformed biofilms was also evaluated after incubation at 37°C for 4, 8 and 24 h in MM. Preformed biofilms were then exposed to 200 μl of test agent-containing MM broth.

The activity of Penicillin G and NAC was evaluated on planktonic growth of the following strains Ss213, Ss36, SsS9A, while the effect on both planktonic and sessile growth was evaluated in the strains *i.e.* SsNTF, SsS9Q and SsNTV.

The results of the antimicrobial activity were expressed as percentage of the growth inhibition compared with the untreated wells. All experiments were repeated independently three times. All microtiter plate assays were performed in duplicate wells. One-way ANOVA was used to compare groups followed by Bonferroni's multiple comparison post-test by using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). The significance level was $p < 0.05$.

Only one isolate was classified as strongly adherent (SsNTF), one isolate as moderately adherent (SsNTQ), while SsNTV and SsNTY were weakly, the rest were non-adherent. According to our results, only few biofilm-producing isolates could be detected, in agreement with the observations of other researchers who found non-typeable isolates producer of biofilms (Bonifait *et al*., 2008; 2010). The ability to form biofilms is not required for virulence, but instead contributes towards long-term colonization, transmission and difficulties to eradicate these infections (Huaijie *et al*., 2013).

Penicillin G is still the drug of choice for the treatment of *Streptococcus* spp. infections because it remains susceptible to the antibiotic despite its intensive use (Grenier *et al*., 2009). In this work, the minimum effective concentration of Penicillin G for reducing the 50% of planktonic growth of *S. suis* was found to be 2 μg $\cdot$ ml^{-1}; however, the needed concentration for reducing their sessile counterparts was 32 μg $\cdot$ ml^{-1}. On the other hand, MIC for reducing the 90% of planktonic cells were over 128 μg $\cdot$ ml^{-1} and 500 μg $\cdot$ ml^{-1} for sessile cells (Fig. 1A). Significant limitations to biofilm penetration have been reported for beta-lactams antibiotics, which act at the surface of the bacteria due to the impermeability of their stable architecture. Bacteria within a biofilm multiply very slowly and therefore are much less susceptible to growth-dependent antimicrobial killing (Mackay *et al*., 2000; Marchese *et al*., 2003).

Thus, the need for more effective biofilm dissolution treatments becomes imperative. One the ways is to enhance the antimicrobial activity by combining antibiotics with other compounds such as NAC. MIC of NAC for reducing the 90% of planktonic cells were over 2 mg $\cdot$ ml^{-1} (Fig. 1B). Interestingly, the MCI of Penicillin G in the presence of 8 mg $\cdot$ ml^{-1} of NAC was reduced from 500 μg $\cdot$ ml^{-1} to 128 μg $\cdot$ ml^{-1} for sessile bacteria inhibition and to 1 μg $\cdot$ ml^{-1} for the inhibition of planktonic cells (Fig. 1C). Therefore, the combinations of Penicillin G and NAC exerted a synergistic inhibition of both planktonic and sessile forms.

Biofilm formation by the *S. suis* strains was shown to occur earlier than 4 hours. The effects of the tested antibacterial agents on the development of established biofilms is shown in Fig. 2. Among the concentrations of Penicillin G tested, 250 μg $\cdot$ ml^{-1} and 500 μg $\cdot$ ml^{-1} showed the highest reduction activity against preformed biofilm, permitting only the following percentages of biofilm formation $47.16 \pm 5.54\%$; $P < 0.05$ and $48.14 \pm 8.64\%$; $P < 0.05$, respectively (Fig. 1A). N-acetylcysteine (16 mg $\cdot$ ml^{-1}) reduced biofilm development approximately to $60.16 \pm 2.33\%$ ($P < 0.05$) compared to the control group during the first 4 hours (Fig. 1B). However, the combination of Penicillin G and NAC showed a higher disruptive effect on the preformed biofilms and reduced the development of biofilm approximately to $80.84 \pm 18.73\%$ ($P < 0.05$) with respect to the control after 4 hours (Fig. 1C). None of the treatments inhibited biofilm development at more than 50% with respect to the untreated group after 8 or 24 hours.

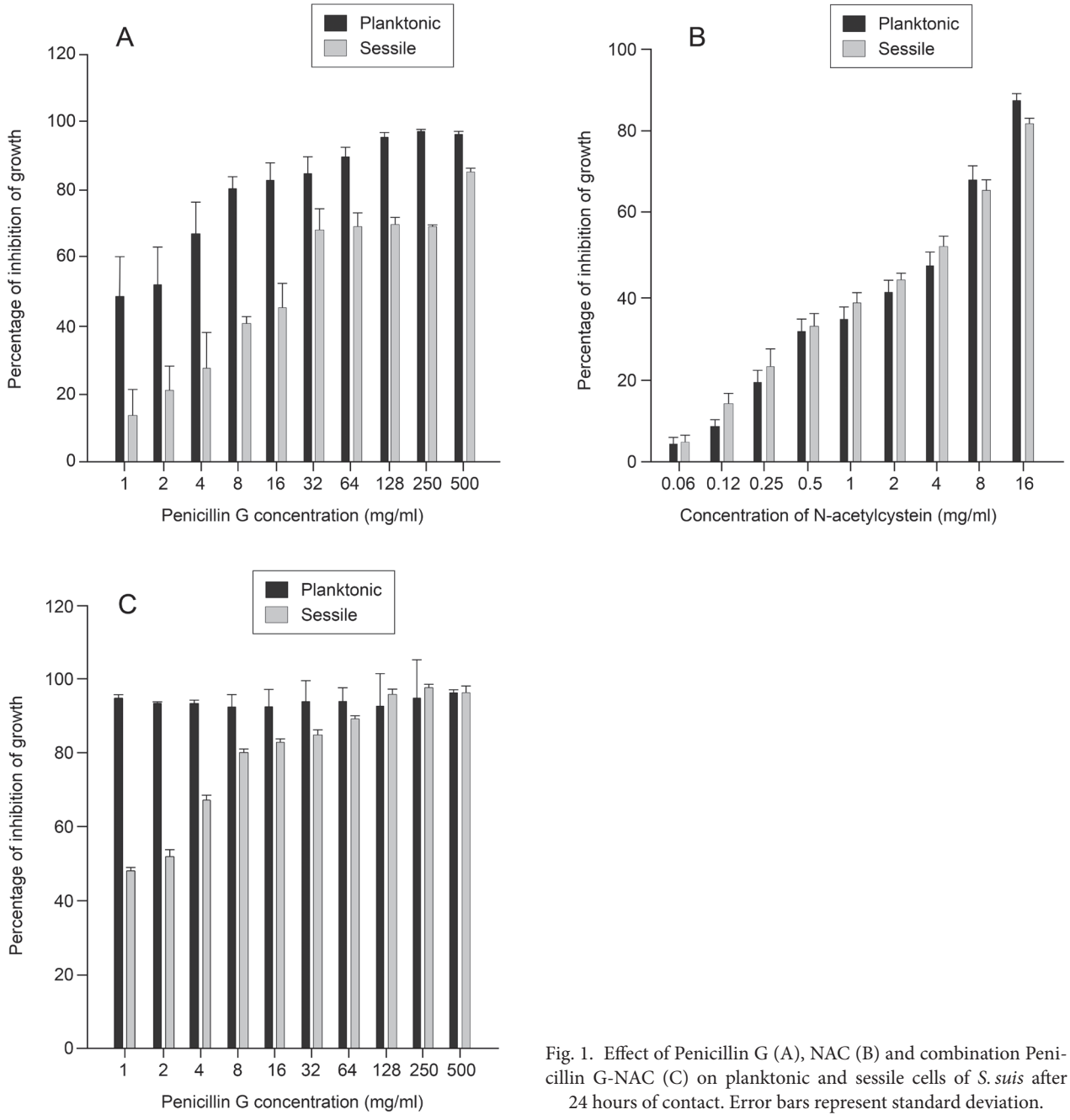

Fig. 1. Effect of Penicillin G (A), NAC (B) and combination Penicillin G-NAC (C) on planktonic and sessile cells of *S. suis* after 24 hours of contact. Error bars represent standard deviation.

Biofilm formation can be divided into three stages: early, intermediate and mature. During the first stage, the bacteria are still susceptible to antibiotics and perioperative antibiotic prophylaxis can be critical for successful treatment (Merle *et al.*, 2012). Some studies show that, when an antimicrobial agent is used for exopolysaccharide matrix disruption, the penetration of other antimicrobials into the pre-formed biofilm can be facilitated (Hajdu *et al.*, 2009). The results of this study showed the Penicillin G and NAC disrupted preformed biofilms by up to 50% and 38% from microtiter plates when were used separately at the concentrations 500 µg/ml and 16 mg/ml respectively. However the application of both mixed compounds permitted only the development of biofilm by up to 20% at 4 hours. NAC is a sulfhydryl group-containing antioxidant and a mucolytic agent that is used in therapy of bronchitis (Marchese *et al.*, 2003; Aslam *et al.*, 2007). Previous studies showed that NAC could decrease biofilm formation by a variety of bacteria and that it inhibited bacterial adherence, reduced the production of extracellular polysaccharide matrix, while promoting the disruption of mature biofilms, and reduced sessile cell viability (Aslam *et al.*, 2007).

Antibiotic treatment due *S. suis* infections in pigs is rarely successful, probably because of poor antibiotic penetration of the porcine tonsillar tissues, which act as a source of infection. Development of new therapeutic

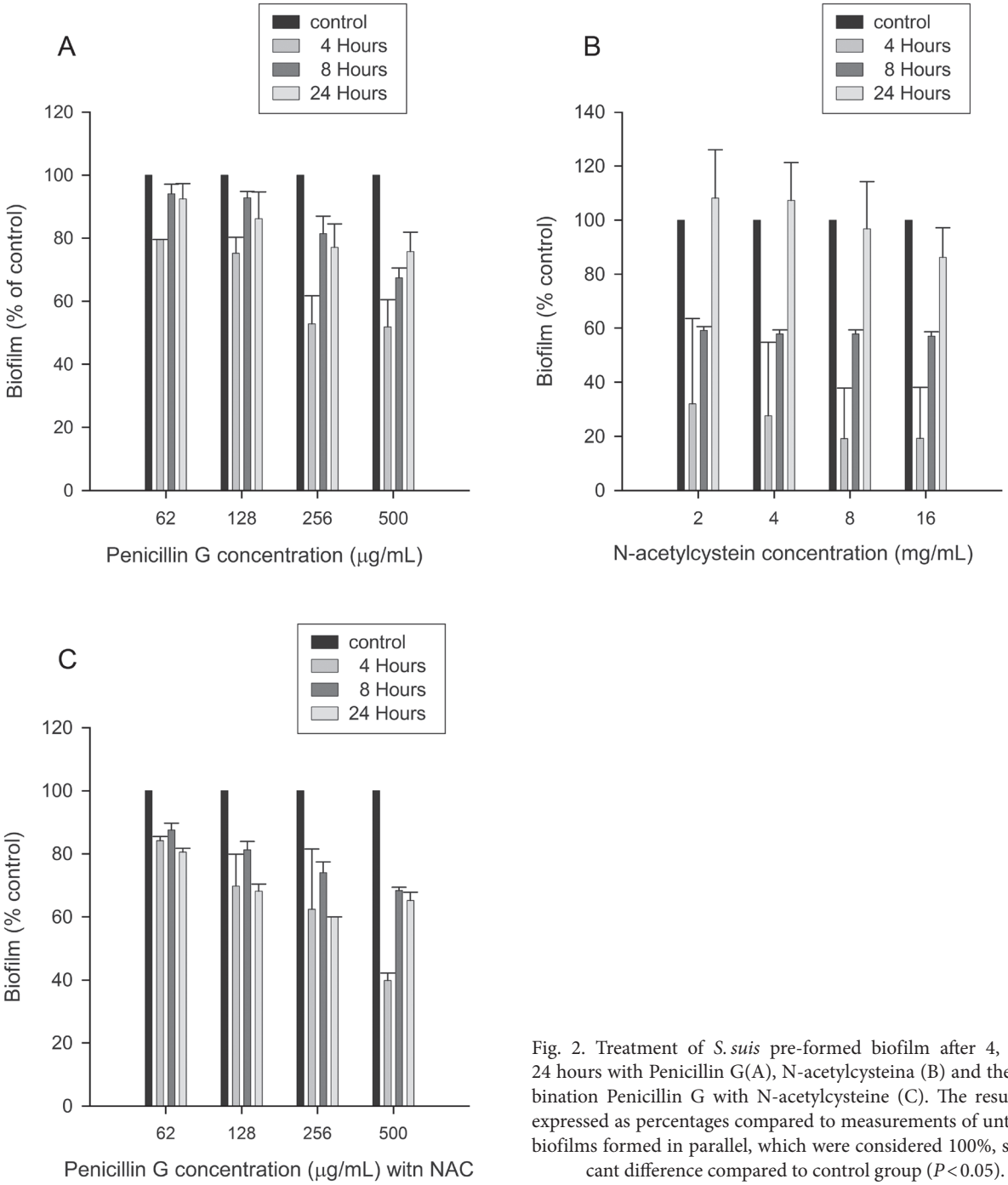

Fig. 2. Treatment of *S. suis* pre-formed biofilm after 4, 8 and 24 hours with Penicillin G(A), N-acetylcysteina (B) and the combination Penicillin G with N-acetylcysteine (C). The results are expressed as percentages compared to measurements of untreated biofilms formed in parallel, which were considered 100%, significant difference compared to control group ($P < 0.05$).

options against the most prevalent strains might be one possible way to prevent colonization of female pigs and to protect those working with pigs.

Traditionally, microbiologists have evaluated the efficacy of an antibiotic by measuring the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). These measurements are made on freely floating, planktonic, laboratory phenotypes. In conclusion, the results of this study agrees with other works in emphasizing the importance of considering the growth in biofilms when evaluating antimicrobials to control *S. suis* – associated infections, and suggests to use combinations of NAC and Penicillin G to increase their antibiotic activity against *S. suis* because of a strong synergic effect.

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SHORT COMMUNICATION

Usefulness of CHROMagar Candida Medium, Biochemical Methods – API ID32C and VITEK 2 Compact and Two MALDI-TOF MS Systems for *Candida* spp. Identification

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Abstract

This study was conducted to compare of the yeasts identification results obtained with two new systems using the MALDI-TOF MS technique with the ones obtained using the routine identification methods of *Candida* spp. in clinical microbiology laboratories. All 124 *Candida* spp. isolates were recovered from the routine examination of clinical specimens in microbiological laboratories and collected in the Centre of Quality Control in Microbiology in Warsaw (Poland). Our findings confirm the high agreement (98%) of fungal identification using the standard, biochemistry laboratory methods and mass spectrometry technique.

Key words: *Candida* spp. – identification, chromogenic medium and biochemical methods, MALDI-TOF MS

The rise of fungal infections correlates with the widespread use of broad-spectrum antibacterial agents, prolonged hospitalization of critically ill patients and the increased number of immunocompromised patients (Bassetti *et al.*, 2006; 2009). *Candida albicans* is still the most frequently isolated, but the frequency of isolation of non-albicans species such as *Candida krusei*, *Candida glabrata, Candida kefyr*, *Candida tropicalis* and *Candida parapsilosis* is steadily increasing globally (Levy *et al.*, 1998; Marklein *et al.*, 2009). It is crucial to identify the *Candida* isolates to the species level in order to make therapeutic decisions. The species such as *C. glabrata* and *C. krusei*, when compared with *C. albicans*, are generally less susceptible to commonly used antifungal drugs such as fluconazole. The majority of *Candida dubliniensis* clinical isolates examined so far are susceptible to commonly antifungal drugs (Sullivan and Coleman, 1998). However, oral *C. dubliniensis* isolates with significantly reduced susceptibility to fluconazole have been recovered from immunocompromised patients who had previously been treated with fluconazole (Ruhnke *et al.*, 2000; Martinez *et al.*, 2002). Commercially available chromogenic agar media as well as biochemical and enzymatic panels are widely used for rapid identification of yeasts (Graf *et al.*, 2000; Freydiere *et al.*, 2001; Campanha *et al.*, 2005). These chromogenic media are species-specific, enabling the microorganism identification to the species level by their colour and colonial characteristics after 48-hour incubation at 37°C. As an alternative to the conventional and biochemical methods, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) has emerged as a rapid and accurate tool for identifying pathogens, including bacteria, mycobacteria, moulds and yeasts (Prod'hom *et al.*, 2010; Pulcrano *et al.*, 2013).

The aim of the study was to compare of the identification results obtained with two new systems using the MALDI-TOF MS technique: MALDI Biotyper (Bruker Daltonics, Germany) and VITEK MS (bioMérieux, France) with the ones obtained using the routine identification methods of *Candida* spp. in clinical microbiology laboratories: API ID32C and VITEK 2 YST (bioMérieux, France) and CHROMagar Candida Medium (Becton Dickinson).

The same set of strains was used in all methods compared in the study. All methods were duplicated. All 124 *Candida* spp. isolates were recovered from the routine examination of clinical specimens in microbiological laboratories throughout Poland, and submitted to the

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Centre of Quality Control in Microbiology (CQCM) located in Warsaw (Poland) for further evaluation and deep frozen. They were deposited in the CQCM collection. The strains used during the study were transferred from storage at –70°C onto the non-selective Sabouraud agar medium (bioMérieux, France) and incubated for 48 h at 30°C.

The biochemical identification of the strains was carried out at the same time using the API ID32C as based method of yeasts identification in routine laboratory and YST card system, according to the manufacturer's instructions. In parallel with the biochemical identification the study strains were inoculated on CHROMagar Candida Medium which shows different colour colonies for *C. albicans* (green), *C. tropicalis* (dark blue, with a pink halo), *C. krusei* (pink and downy appearance).

All tested *Candida* strains were simultaneously identified to species level by MALDI-TOF MS Biotyper and VITEK MS in line with the manufacturer's instructions. The results of the pattern-matching process were expressed as log score values ranging from 0 to 3 using the MALDI-TOF MS Biotyper application and software. The score values ranging from 2.300 to 3.000 meant "highly probable species identification"; 2.000–2.299 – "secure genus identification, probable species identification"; 1.800–1.999 – "identification to the genus level", and a score of ≤ 1.800 was interpreted as "no reliable identification".

The resulting slides were then processed in the VITEK MS device with MYLA software offering the automated analysis of the obtained mass spectra against the built-in database. A single identification is displayed (green), with a confidence value (% probability) from 60.0 to 99.9 (good confidence level), when one significant yeast or yeast group is retained. "Low-discrimination" identifications are displayed (red) when two or four significant yeasts or yeast groups are retained. When no match is found, the yeast is considered unidentified (orange).

The results of analysis are shown in Table I. The growth characteristics of 124 *Candida* spp. strains on CHROMagar Candida Medium enabled the unambiguous identification of isolates belonging to three species: green colonies as *C. albicans* (n = 68; 55%), steel-blue colonies – *C. tropicalis* (n = 21; 17%) and pink colonies – *C. krusei* (n = 35; 28%). Green colony colour suggested *C. albicans* strains, but different intensity of colour was difficult to evaluate objectively. Thirty five of strains (28.2%), which grew on chromogenic medium, formed pink/violet to pink colour colonies: pink colonies (n = 1), pink/violet-pink colonies (n = 24), white large glossy pink colonies (n = 2), matt pink colonies (n = 3), pink colonies with white "halo" (n = 3) or ivory to lavender colonies (n = 2). According to manufacturer instructions, pink colony colour suggested *C. krusei* strains.

Biochemical analysis with the API ID 32C system identified 123 (99,2%) *Candida* isolates. The isolates, which grew on chromogenic medium forming pink/violet-pink colonies (n = 24) and white large glossy pink colonies (n = 2), were identified by API ID 32C as *C. glabrata* (n = 26). All isolates growing as matt pale pink colonies (n = 3) were identified as *C. krusei*. Only four from five isolates which grew forming pink with a white "halo" or ivory colonies were identified as *C. parapsilosis*. The green colour colonies were identified by API ID 32C as *C. albicans* (n = 67) and *C. dubliniensis* (n = 1).

The identification levels of VITEK 2 YST were: excellent – 58.1%, very good (34.7%), good (4.8%) and acceptable (only 2.4%). The results obtained with VITEK 2 YST card were correct with API identification for the majority of common clinical yeasts, *C. albicans*

Table I
Cumulative results of *Candida* strains identification

CHROMagar Candida Medium		Identification (no strains)			
Colony colour (no strains)		API ID 32C	VITEK 2 YST	MALDI Biotyper	VITEK® MS
Study strains (n = 124)					
Dark green, green or light green colonies (n = 68)		*C. albicans* (n = 67)	*C. albicans* (n = 66)	*C. albicans* (n = 66)	*C. albicans* (n = 66)
		C. dubliniensis (n = 1)	*C. dubliniensis* (n = 2)	*C. dubliniensis* (n = 2)	*C. dubliniensis* (n = 2)
Dark blue colonies (n = 21)		*C. tropicalis* (n = 21)	*C. tropicalis* (n = 21)	*C. tropicalis* (n = 21)	*C. tropicalis* (n = 21)
Pink/violet colonies (n = 35)	Pink/violet-pink colonies (n = 24)	*C. glabrata* (n = 26)	*C. glabrata* (n = 26)	*C. glabrata* (n = 26)	*C. glabrata* (n = 26)
	White large glossy pink colonies (n = 2)				
	Pink colonies (n = 1)	*C. kefyr* (n = 1)	*C. kefyr* (n = 1)	*C. kefyr* (n = 1)	*C. kefyr* (n = 1)
	Matt pale pink colonies (n = 3)	*C. krusei* (n = 3)	*C. krusei* (n = 3)	*C. krusei* (n = 3)	*C. krusei* (n = 3)
	Pink colonies with white halo (n = 3)	*C. parapsilosis* (n = 3)	*C. parapsilosis* (n = 5)	*C. parapsilosis* (n = 5)	*C. parapsilosis* (n = 5)
	Ivory to lavender colonies (n = 2)	*C. parapsilosis* (n = 1)			
		Not ID (n = 1)			

(n = 66), *C. glabrata* (n = 26) and *C. krusei* (n = 3). The problematic isolate with light-green colonies was identified with API ID 32C as *C. albicans*, whereas VITEK 2 system identified it as *C. dubliniensis* (n = 1). The only strain unidentified by API ID 32C was later identified by YST card as *C. parapsilosis*.

The MALDI Biotyper correctly identified 71.8% and 26.6% and 1.6% of isolates at the species level using scores of ≥ 2.300 and ≥ 2.000 and ≥ 1.800, respectively. The MALDI Biotyper performed better than the routine methods for identification of *C. kefyr*, *C. dubliniensis* and *C. parapsilosis*. *C. dubliniensis* (n = 2) was correctly identified using the MALDI Biotyper (score ≥ 2.000), while it was misidentified as *C. albicans* (n = 1) using the traditional methods (API ID 32C), likely due to the close phylogenetic relationship between the two species. VITEK MS offered a good identification to the species level of all *Candida* isolates (n = 124). The confidence interval for this *Candida* identification method ranged from 95.6% to 99.9%.

The identification methods used in this study were characterised by a favourable parameter profile. We analysed the number of identified strains of the same species. The degree of identification correspondence expressed as a percentage of the total number of tested strains was shown in Table II.

Quality control strains were obtained from the American Type Culture Collection (ATCC): *C. albicans* ATCC 14053, *C. glabrata* ATCC 2950, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *C. tropicalis* ATCC 13803. *C. albicans*, *C. krusei* and *C. tropicalis* reference strains grew on the chromogenic medium in line with the manufacturer's description. The colonies of *C. parapsilosis* ATCC 22019 strain looked like *C. krusei* on this medium. *C. glabrata* ATCC 2950 grew as violet-pink colonies.

Several authors have reported difficulties in differentiation of *C. albicans* from *C. dubliniensis* due to the phenotypic similarity of the two species (Sullivan and Coleman, 1998; Raut and Varaiya, 2009). Both species produce the germ tube, chlamydospores and grow on CHROMagar Candida as green colonies. Although *C. dubliniensis* colonies are dark green unlike the green or light green colonies of *C. albicans*, this difference may be inadequate for primary identification and often lost after serial subculture (Kirkpatrick *et al*., 1998). *C. dubliniensis* may be underreported in clinical samples because most currently used isolation and identification methods fail recognize this yeast (Campanha *et al*., 2005; Pravin *et al*., 2015).

Our findings confirm the high agreement of fungal identification using the standard, biochemistry laboratory methods and mass spectrometry technique. Although our high 98% agreement is based on the analysis of 124 clinical, patient-derived strains only, these results remain in accordance with previous observations on larger numbers of clinical isolates (Van Veen *et al*., 2010; Sow *et al*., 2015).

Conventional methods including macroscopic/microscopic characteristic and biochemical profile by API identification system offer efficiency and accuracy to facilitate the diagnosis. We found that CHROMagar Candida Medium offers easy identification of several species based on colony colour and morphology, as well as an accurate differentiation between the three most common *Candida* species *i.e. C. albicans*, *C. tropicalis* and *C. krusei*. As shown in this paper, different shades of green or pink colour of colonies growing on a chromogenic media may indicate the same or different yeast species. This supports the need to use in a laboratory different diagnostic methods, such as biochemistry or mass spectrometry. In our study the API ID 32C identification had to be repeated twice in a few cases, which delayed the time until the results were available after an additional 24–48 hours.

The VITEK 2 YST card was able to reliably differentiate between *C. dubliniensis* and *C. albicans*. The percentage of correct identification is similar to the ones by other commercial identification systems (Graf *et al*., 2000; Freydiere *et al*., 2001; Sow *et al*., 2015). We believe that for microbial identification tests, there should be at least 90% agreement with the existing system or reference method before the new method is considered verified (Clark *et al*., 2009).

Table II
Compliance of *Candida* strains identification results using different methods

MALDI Biotyper		VITEK® MS		VITEK 2 YST		Compatibility with MS* results (%)	
Score/ID Level	No/% strains	Confidence interval (%)	No/ % strains	ID Level	No/ % strains		
2.300–3.000 / excellent	89/71,8%	99.9	11/8.9%	Excellent	72/58.1%	API ID 32C	98,4%
2.000–2.299 / very good	33/26,6%			Very good	43/34.7%	VITEK 2 YST	100%
1.800–1.999 / good	2/1,6%	95.6–99.9	113/91.1%	Good	6/4.8%	CHROMagar Candida Medium	72,6%
≤1.8 / acceptable	–			Acceptable	3/2.4%		

*MS – mass spectrometry method (MALDI Biotyper and VITEK® MS)

In summary, conventional methods of fungal identification are very useful in clinical microbiology laboratories. Identification by conventional microbiology methods takes at least 16 hours, if a culture is positive. The introduction of MALDI-TOF MS in the microbiology laboratory could mean a radical change in the identification accuracy, reducing time (10 minutes per a single isolate) and cost (about 5 times cheaper than a conventional identification) (Sow *et al*., 2015). MALDI-TOF clearly outperformed the diagnosis capacities of phenotypic methods by reducing the delay of results and giving accurate identification at species level (Van Veen *et al*., 2010; Marinez-Lamas *et al*., 2011). Moreover, this approach appears to be cost-effective and should be implemented especially in resource-poor context (Sow *et al*., 2015).

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SHORT COMMUNICATION

Enterobacter asburiae KUNi5, a Nickel Resistant Bacterium for Possible Bioremediation of Nickel Contaminated Sites

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Abstract

Nickel resistant bacterial strain *Enterobacter asburiae* KUNi5 was isolated and showed resistance up to 15 mM and could remove Ni optimally better at 37°C and pH 7. Maximum removal was found at initial concentration of 0.5 to 2 mM, however, growth and Ni removal were affected by other heavy metals. Major amount of the metal was accumulated in the membrane fractions and certain negatively charged groups were found responsible for Ni binding. KUNi5 could also produce 1-aminocyclopropane-1-carboxylate deaminase, indole-acetic acid and siderophore. It seems that KUNi5 could be a possible candidate for Ni detoxification and plant growth promotion in Ni-contaminated field.

Key words: *Enterobacter* sp., bioremediation, nickel

Nickel (Ni) contamination of the environment from industrial sources has been reported to affect living organisms and is being considered as a global concern (Khodadoust *et al*., 2004; Cecchie and Zanchi, 2005). Irrigation of agricultural fields with Ni containing wastewater is affecting crop yield and also creating potential risk of biomagnification by entering into the food chain (Sanders *et al*., 1987; Singh *et al*., 2012).

Different mechanisms of Ni resistance have earlier been documented (Gadd, 1988; Nies, 1999; Sar *et al*., 2001; Salvador *et al*., 2007; Desale *et al*., 2014), of which certain mechanisms could be exploited for successful remediation of sites contaminated with such hazardous metal. Screening of Ni resistant microbes from contaminated sites with potential Ni immobilizing ability could provide a significant impact on environmental management. Moreover, such bacterial inoculants having metal immobilizing ability with plant growth promoting (PGP) features have opened other eco-friendly measures for sustainable agriculture (Burd *et al*., 2000; Faisal and Hasnain, 2006; Denton, 2007; Rajkumar and Freitas, 2008). Considering this background, the objectives of this study were to isolate and characterize a Ni resistant bacterial candidate from Ni contaminated soil and to examine its remediating ability with PGP features under experimental conditions.

Ni resistant bacterial strain was isolated from industrial waste contaminated soil (pH 7.4, Ni content – 2.9 mg/Kg of soil) near Durgapur, West Bengal, India. Dilution plate technique was followed to isolate the Ni resistant strain. The bacterial strain that showed maximum tolerance to Ni (as $NiCl_2$, $6H_2O$) on nutrient agar (NA) medium (HiMedia, India) was selected and designated KUNi5. The colony was further transferred to modified Tris minimal agar (TMA) medium (Sar *et al*., 1998) for culture purification and was maintained. The maximum tolerance levels of KUNi5 to other heavy metals were also assessed in modified Tris minimal broth (TMB). The isolate KUNi5 was identified on the basis of its morphological and biochemical features (Vos *et al*., 2009) and 16S rDNA sequence analysis. The base sequence was aligned using BLAST function (Altschul *et al*., 1990) at the National Center for Biotechnology Information (NCBI) database for its identification.

To determine the effect of Ni on bacterial growth under aerobic culture conditions, equal volume of young cell suspensions were inoculated (~4 log CFU/mL) into modified TMB with different concentrations of Ni (0.5, 1.0, 2.0, 3.0 and 4.0 mM) as $NiCl_2$, $6H_2O$ separately and each set including the control set was incubated at 37°C on a rotary shaker. The growth of KUNi5 was measured by colony count method at certain time intervals on modified TMA plates. Ni removal by KUNi5 at each treatment regime was also determined by quantifying the residual amount of Ni present in the medium. Supernatant was harvested by centrifugation and was acid-digested; Ni contents in the supernatants were measured by Atomic Absorption Spectrophotometer

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(iCE 3000 AAS, Thermo Scientific, Austria). An uninoculated set for each treatment regime was also maintained and processed similarly to monitor the artifacts that might arise owing to metal chelation on the surface of the container. The percentage of Ni removal was calculated following the equation: $R\ (\%) = (C_o - C_e) \times \times 100/C_o$ (C_o = Initial Ni concentration; C_e = Equilibrium Ni concentration).

To study the effect of initial pH on Ni removal under culture conditions, modified TMB supplemented with 2 mM Ni with pH values ranging from 5 to 8 was inoculated with equal volume of bacterial inoculum and incubated at 37°C. The growth patterns of KUNi5 and Ni removal at respective treatment regimes were also measured. The effect of temperature on Ni removal and growth under aerobic growth conditions was determined in TMB (pH 7) having 2 mM of Ni at different temperatures (30°C, 37°C, and 40°C).

The effects of other heavy metals separately (0.1 mM Cu as $CuSO_4$, $5H_2O$ or 0.1 mM Co as $CoCl_2$, $6H_2O$ or 0.1 mM Zn as $ZnCl_2$ or 0.1 mM Cd as $CdCl_2$, $5H_2O$) on Ni removal were studied. Equal volume of cell suspension was inoculated in modified TMB (pH 7, 2 mM Ni) to each treatment regime and incubated for 48 h at 37°C. Culture set without having any selected heavy metals other than Ni was considered as control. After 48 h the amount of Ni removal was measured as mentioned earlier.

Amount of Ni in different cell fractions were measured by growing the cells in modified TMB supplemented with 2 mM Ni for 48 h at 37°C. Cellular fractions were harvested and prepared following Sar *et al.* (2001). The amount of Ni in different fractions was measured after acid digestion as mentioned before. Fourier transform infra red (FTIR) spectroscopy was performed to visualize the shifts in the absorption spectrum of the treated cell fractions in comparison to the control one. Cells from both control (Ni free) and treated (2 mM Ni) sets were harvested after 48 h of growth and washed with HEPES-NaOH buffer (100 mM, pH 7.2), and were then lyophilized. Infra red spectra were obtained by using Spectrum One FT-IR Spectrometer (Perkin Elmer, USA).

For determining possible PGP features of KUNi5, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-acetic acid (IAA) and siderophore producing abilities were considered. ACC-deaminase activity of KUNi5 was qualitatively assayed following Dworken and Foster (1958). Siderophore and IAA production by KUNi5 were qualitatively determined following respective standard methods (Schwyn and Neilands, 1987; Sosa-Morales *et al*., 1997).

KUNi5 cells were found to be Gram-negative and rod shaped. Based on the analysis of 16S rDNA sequence (NCBI GenBank Accession No. KM277458), KUNi5 has been identified as a strain of *Enterobacter asburiae*. It could tolerate 15 mM Ni in nutrient broth and 10 mM Ni in modified TMB. The difference in tolerance limit in two different media might be due to the complexation of Ni with undefined ingredients in the nutrient broth thus lowered toxicity (Sau *et al*., 2008). KUNi5 showed varied degree of tolerance to heavy metals in the order of Ni>Zn>Cu>Co>Cd (10, 7, 5, 2 and 1 mM respectively) in TMB. Tolerance to different toxic metals might be due to the selection pressure of contaminant metals at the sampling site posed on the KUNi5 population. The strain showed ability to produce ACC deaminase, IAA and siderophore, which might be of important agronomic value for using as PGP candidate. PGP features of *E. asburiae* and its suitability of using in sustainable agriculture were also documented earlier (Ahemad and Khan, 2010; Zhao *et al*., 2011).

Growth retardation and extended lag phase of KUNi5 were found with increasing Ni concentration in the medium (Fig. 1a). Irrespective to the Ni concentration, highest cell mass was obtained after 48 h of growth. The extended lag phase might be due to the requirement of time for buffering the metal stress (Patel *et al*., 2006; Das *et al*., 2014). KUNi5 grew optimally better at pH 7 and 37°C under Ni stress (2 mM) conditions (Fig. 1b, c). Irrespective of the initial Ni concentration, pH of the medium and incubation temperature, highest Ni removal was found after 48 h and thereafter remained same (Fig. 1d, e, f). When initial Ni concentration was considered as the factor, maximum removal was found at an initial concentration ranging from 0.5 m to 2 mM of Ni in the medium. Incubation temperature of 37°C and pH 7 were found to be conducive for Ni removal. From the experimental analysis it seems that active biomass might play a crucial role on Ni removal. Other heavy metals were found to antagonize growth and Ni removal (Fig. 2). Cadmium was found to be most antagonistic than Co, Zn and Cu. Similar types of results were also observed with other bacteria (Fu and Maier, 1991; Hussein *et al*., 2004; Das *et al*., 2014). Decrease in Ni removal might be due to the growth inhibitory or competitive effect of other metals on Ni binding (Kaltwasser and Frings, 1980; Fu and Mier, 1991).

From the cellular fractionation study it was observed that most of the metals were accumulated (nmol of Ni/mg dry weight) in the membrane fractions (~53%) followed by periplasmic space (~34%) and cytosol (~13%). Such pattern of accumulation and sequestration of toxic cations was also reported earlier (Cha and Cooksey, 1991; Sar *et al*., 2001). In order to ascertain the role of different functional groups involved in Ni binding, FTIR analysis were done for both lyophilized control and treated (2 mM Ni) biomass. A large shift was found due to Ni exposure in the intensity of absorption band peak for OH stretch from 3300.61 cm^{-1} to 3344.68 cm^{-1} (Fig. 3). Small shifts occurred in the intensity of the bands 1539.03 cm^{-1} to 1546.21 cm^{-1}

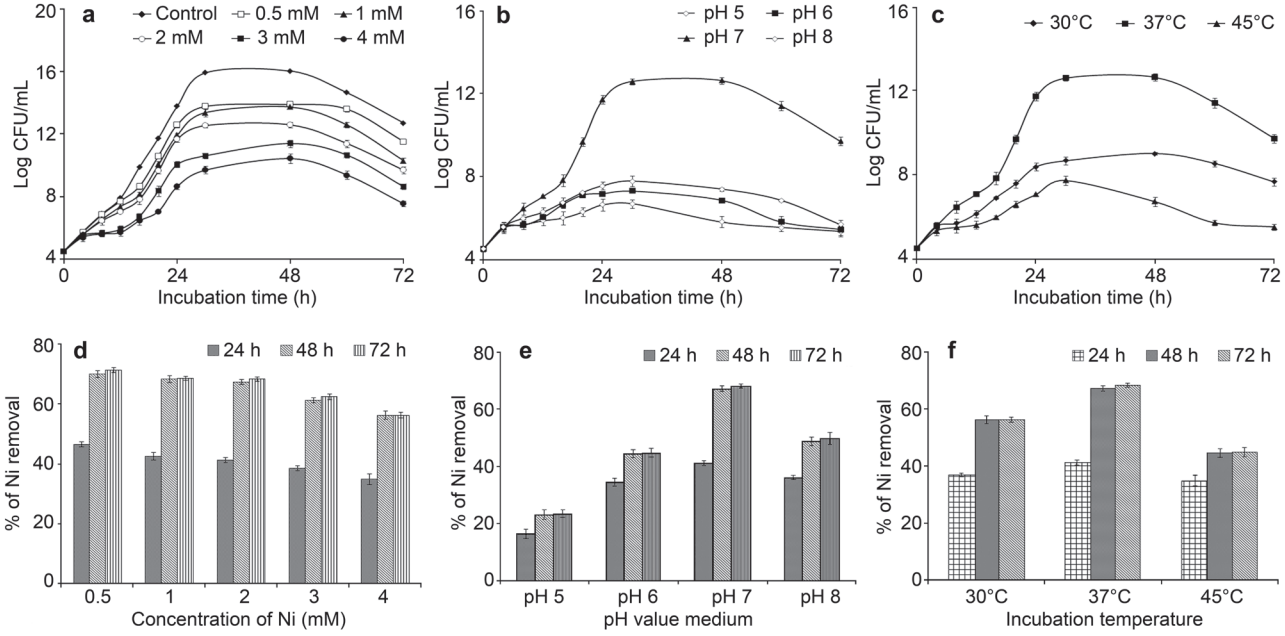

Fig. 1. Growth response of KUNi5 in TMB with different concentration of Ni and without Ni as control (a), having 2 mM Ni at different pH (b), and having 2 mM Ni at different temperatures (c). Ni removal ability of KUNi5 grown in TMB at different time intervals with increasing Ni concentrations (d), at different pH values (e), and at different temperatures (f). Data are the mean of three replications with ± SE ($p < 0.05$)

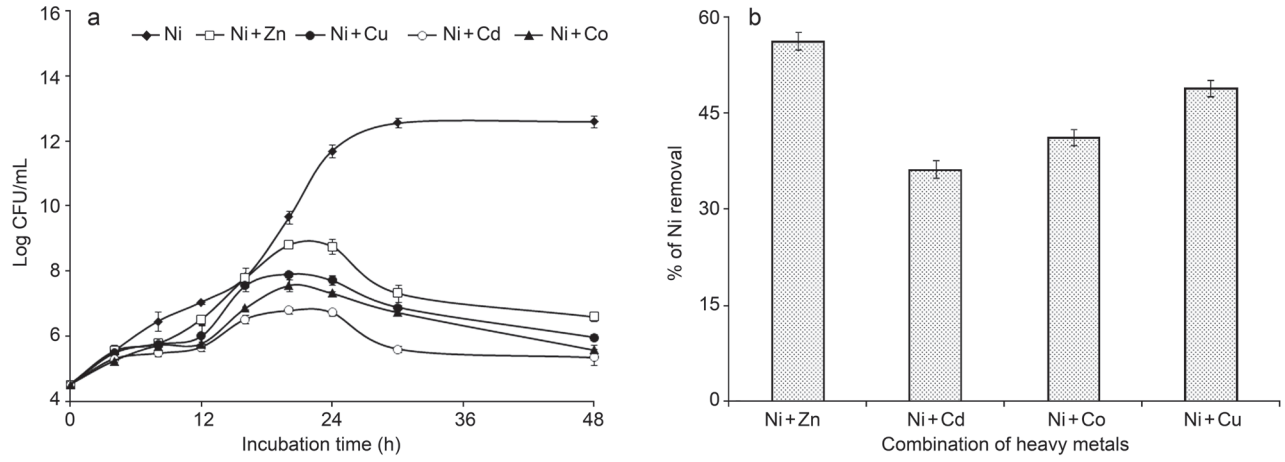

Fig. 2. Growth response (a) and Ni removal ability (b) of KUNi5 grown in TMB having 2 mM Ni after 48 h in presence of other heavy metals (0.1 mM). Data are the mean of three replications with ± SE ($p < 0.05$).

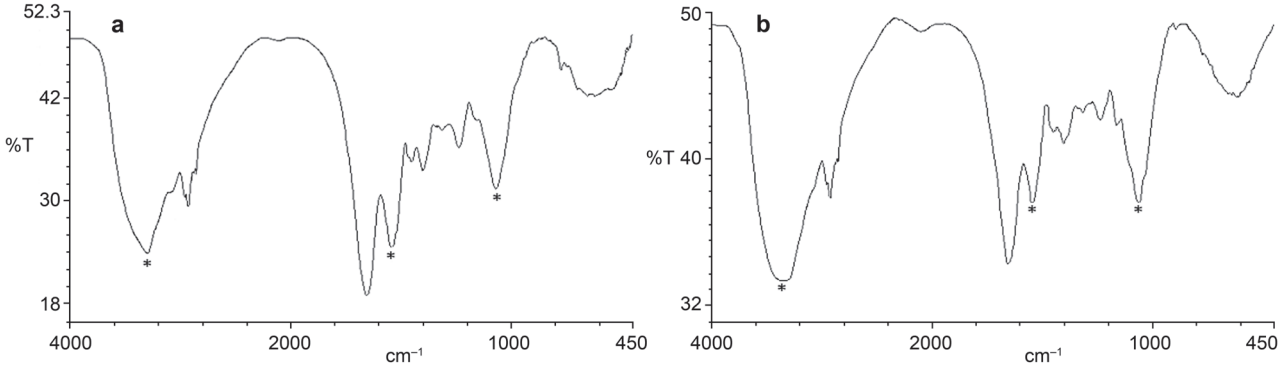

Fig. 3. FTIR spectra of untreated (a) and 2 mM Ni treated (b) cell biomass of KUNi5. Major shifting in the intensity of absorption band peaks are indicated by [*].

indicating N-H bending and C-H stretching in amide II, and 1068.86 cm^{-1} to 1063.29 cm^{-1} suggesting asymmetric and symmetric stretching of PO^{2-} and $P(OH)_2$ or C-OH bonds in alcohols or polysaccharide of bacterial biomass (Jiang *et al*., 2004). It seems that positively charged metal ions bind to such negatively charged functional groups present on the bacterial cell fractions (Anand *et al*., 2006; Desale *et al*., 2014).

Thus, the strain KUNi5 showed its promise for bioremediation of Ni-contaminated crop field and also as a plant growth promoting agent, although the extent of Ni immobilization and plant growth promotion under actual metal-polluted field conditions warrants further investigation.

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SHORT COMMUNICATION

IgG Avidity: an Important Serologic Marker for the Diagnosis of Tick-Borne Encephalitis Virus Infection

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Abstract

A total of 52 serum samples from patients with symptoms suggestive of tick-borne encephalitis virus (TBEV) infection and positive IgM and/or IgG antibodies were tested for IgG avidity. Acute/recent TBEV infection was confirmed by low/borderline avidity index (AI) in 94.8% IgM positive/IgG positive samples, while in 5.2% high AI was found indicating persisting IgM antibodies. Majority of IgM negative/IgG positive samples (78.6%) showed high AI consistent with past TBEV infection. However, in 21.3% patients without measurable IgM antibodies current/recent infection was confirmed by AI. IgG avidity represents an additional serologic marker that improves diagnosis of TBEV, especially in cases of atypical antibody response.

Key words: IgG avidity, serology, tick-borne encephalitis virus

Tick-borne encephalitis virus (TBEV) is a small, enveloped virus that belongs to the family *Flaviviridae*, genus *Flavivirus*, tick-borne encephalitis serocomplex. There are three subtypes of TBEV: the European, the Far Eastern and the Siberian subtype which differ in geographical distribution, tick vector and clinical manifestation of disease (Lindenbach *et al.*, 2007). TBEV cause a wide spectrum of symptoms, from a subclinical course to aseptic meningitis, encephalitis, myelitis and radiculitis (Bogovic *et al.*, 2010). Infections caused by European type usually take a biphasic course (~75%). The first phase presents as a nonspecific influenza-like illness. After an afebrile and relative asymptomatic period, the second phase occurs with symptoms of central nervous system (CNS) (Mansfield *et al.*, 2009). Reverse-transcriptase polymerase chain reaction (RT-PCR) can be of diagnostic value in the first viremic phase of infection (Saksida *et al.*, 2005). As the patients usually seek medical assistance when neurologic symptoms develop, diagnosis of TBEV is most commonly performed by serological methods, usually enzyme-linked immunosorbent assay (ELISA) (Holzmann, 2003; Niedrig *et al.*, 2010; Donoso-Mantke *et al.*, 2011). IgM antibodies appear within six days of illness, and usually decline within few months. An early diagnosis of TBEV confirmed by detecting IgM is sometimes questionable, since IgM antibodies can persist for more than 9 months in some vaccinees or individuals who acquired the infection naturally (Stiasny *et al.*, 2012). This may lead to a misinterpretation of the serological results in case of another CNS disease within this time period (Holzmann, 2003; Kleiter *et al.*, 2007). In addition, in some patients with TBEV infection, the IgM response may be delayed or weak. IgG avidity determination could be helpful to prove or exclude a recent TBEV infection in these patients (Gassman and Bauer, 1997). The term avidity denotes the affinity of IgG antibodies to bind the antigen. IgG antibodies are of low specificity after primary antigenic challenge which results in low avidity, but increases progressively thereafter within few months (high avidity) (Fox *et al.*, 2006).

We analyzed the value of IgG avidity determination in diagnosis of TBEV infection.

From 2012–2014, a total of 52 serum samples from hospitalized patients with clinical symptoms suggestive of TBE (meningitis/meningoencefalitis) and

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serologically confirmed current or previous TBEV infection were tested for IgG avidity. Majority of them were from northwestern Croatia, an endemic area for TBE (Golubić and Dobler, 2012). TBEV IgM and IgG antibodies were detected using a commercial ELISA test (Euroimmun, Lübeck, Germany). IgG avidity was determined by ELISA test using urea as a denaturing agent (Euroimmun, Lübeck, Germany). The IgG avidity index (AI) was calculated and expressed as percentage by dividing the OD values with and without urea treatment and interpreted as follows: <40% low AI indicating acute primary infection; 40–60% borderline AI indicating recent infection; >60% high AI indicating past TBEV infection.

According to IgM/IgG results, samples were divided into two groups as follows: I – IgM positive/IgG positive samples indicating acute/recent TBEV infection (N=38; 73.1%), II group – IgM negative/IgG positive indicating past TBEV infection (N=14; 26.9%).

Using IgG avidity, 39 (75.0%) patients are classified as having current/recent TBEV infection while in 13 patients (25.0%) a previous TBEV infection was documented. Avidity indices for each patient are presented in the Figure 1.

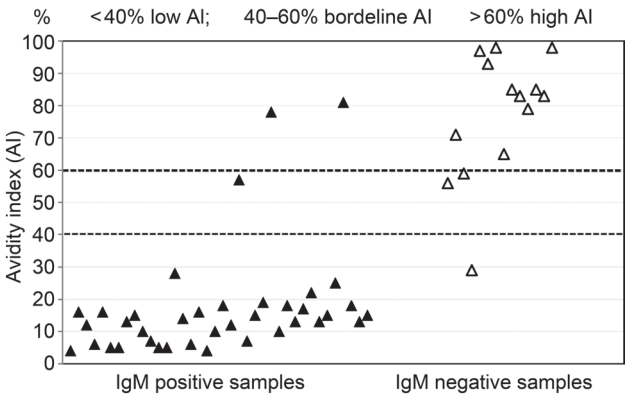

Fig. 1. Avidity indices in 52 patients with current/previous tick-borne encephalitis virus infection.

Figure 2 shows AI according to the IgM/IgG results. Current/recent TBEV infection was documented by low AI in 35/38 (92.1%) patients with positive IgM antibodies and 1/14 (7.1%) patients with negative IgM antibodies. In addition, one patient with positive IgM (2.6%) and two patients with negative IgM (14.2%) showed borderline AI (57%, 56%, and 59% respectively) consistent with recent TBEV infection. In 2/38 patients (5.2%) with positive IgM antibodies and 11/14 patients (78.6%) with negative IgM antibodies, high AI was found consistent with past TBEV infection.

Detection of IgM antibodies is usually considered as a marker of acute/recent infection. However, virus serology based on IgM detection alone may lead to misdiagnosing of acute infection since IgM antibodies may exhibit irregular patterns (Holzmann, 2003). IgM antibodies may be long-persisting for several months after primary TBEV infection which could result in a false-positive diagnosis of acute infection. Since cross-reactions are common in flavivirus infections, especially among viruses within the same serocomplex, IgM antibodies may be cross-reactive, induced by other flaviviruses (Stiasny *et al*., 2013). In addition, IgM antibodies may be detectable for a very short period or at low titers and thus lead to a false exclusion of current TBEV infection (Gasmann and Bauer, 1997; Stiasny *et al*., 2012). The monitoring of an increase of IgG titers in paired serum sample two weeks later is rarely carried out, since for many patients only one sample is available for testing. Avidity assays have been used for the diagnosis of some flaviviruses such as dengue virus (Prince *et al*., 2011) and West Nile virus (Levett *et al*., 2005; Fox *et al*., 2006). To our knowledge, there is only one published study in 1997 which analyzed the IgG avidity in diagnosis of TBEV infection (Gassmann and Bauer, 1997).

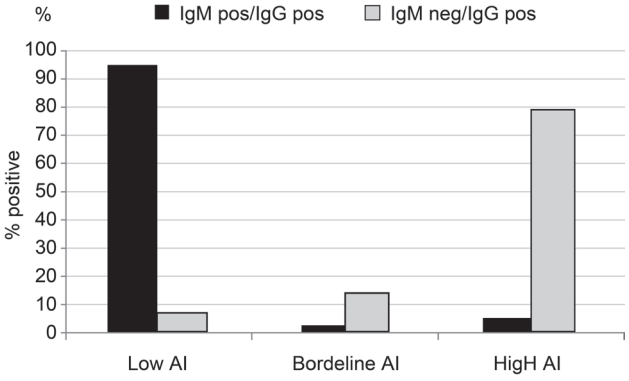

Fig. 2. IgG avidity index according to the IgM/IgG results.

In this study, a very high proportion of IgM positive samples (94.8%) showed low/borderline AI indicating current/recent infection. Small proportion IgM positive patients (5.2%) showed high AI indicating persisting TBEV IgM antibodies unrelated to a current infection. In addition, some other clinical situations can be related to the presence of IgM antibodies such as polyclonal stimulation or cross-reactive IgM due to other antigens which could be recognized by IgG avidity (Holzmann, 2003).

Majority of IgM negative patients (78.6%) showed high AI consistent with past TBEV infection. However, serological testing for IgM/IgG fails to detect current/recent infection in 21.3% patients with no measurable IgM antibodies. This percentage is even higher than that reported from Gassman and Bauer (1997) who detected acute TBEV infection in 11.1% IgM negative patients. In these cases, current or recent infection could be recognized only by demonstration of low/bor-

derline AI. These findings highlight the importance of IgG avidity testing not only in IgM positive/IgG positive but also in IgM negative/IgG positive patients.

The results of this study showed that 9.6% of patients with atypical serologic response were probably incorrectly classified based on the routine serology (TBEV IgM/IgG result). Incorrect classification was confirmed in both IgM positive and IgM negative group. Using IgG avidity, 21.3% IgM negative patients were diagnosed as having current/recent TBEV infection and 5.2% of IgM positive patients as having past TBEV infection. Since clinical symptoms of TBE resemble some other CNS diseases which may require treatment, rapid and accurate TBEV diagnosis is of a particular importance.

In conclusion, presented results confirmed IgG avidity as an additional serologic marker that improves diagnosis of TBEV, especially in cases of atypical antibody response. Combination of IgM and IgG avidity seems to be highly helpful for reliable diagnosis of TBEV infection in both IgM positive and IgM negative patients. Our results highlight the need for IgG avidity testing in the routine TBEV serology.

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SHORT COMMUNICATION

Etiologic Agents and Antifungal Susceptibility of Oral Candidosis from Romanian patients with HIV-infection or type 1 *diabetes mellitus*

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Abstract

This is the first Romanian investigation of oral candidosis in patients suffering of HIV-infection or type 1 *diabetes mellitus* (T1DM). *Candida albicans* was the dominant species in both types of isolates: n = 14 (46.7%) in T1DM, n = 60 (69.8%) in HIV. The most frequent non-*albicans Candida* spp. were *Candida kefyr* (n = 6; 20%) in T1DM and *Candida dubliniensis* (n = 8; 9.3%) in HIV. Resistance to fluconazole was detected only in the HIV non-*albicans Candida* group (n = 8; 9.3%). All isolates were susceptible to VOR. The experimental drug MXP had MIC values equal or close to the ones of VOR. Echinocandin resistance was more frequent than azole resistance.

Key words: antifungal susceptibility, MXP-4509, oral candidosis, Romanian HIV and diabetes patients

The presence of oral *Candida* yeasts is considered a biomarker indicative of immune system impairment and, in immunodeficiency disorders, can be correlated with a progressive disease (Vargas and Joly, 2002). Oral candidosis (OC) is the most frequent type of yeast infection and occurs especially in denture wearers and individuals with severe conditions, such as HIV-infected patients, those under antibiotic or chemotherapy, organ transplantation recipients and patients with systemic diseases such as diabetes (Vergani *et al.*, 2013).

HIV-infected patients are susceptible to opportunistic mycoses as cell-mediated immunity decays (Sangeorzan *et al.*, 1994). Before the era of highly active antiretroviral therapy (HAART), oropharyngeal candidosis (OPC) occurred in as many as 90% of patients, at some point during the course of HIV infection (Lortholary *et al.*, 2012). Since the initiation of HAART in 1996, there has been a decrease in the incidence of OPC (Leigh *et al.*, 2004) while oropharingeal colonization varies from 44% to 62% (Lin *et al.*, 2013a).

To the best of our knowledge, the present study is the first Romanian investigation providing data regarding the etiologic spectrum and the antifungal susceptibility profile of OC isolates from patients with either HIV-infection or diabetes.

The 116 clinical yeast isolates included in this study were collected in three tertiary hospitals from different regions of Romania (*i.e.* Iasi, Timisoara and Brasov), from patients with overt OC. Of these patients, 30 were suffering from type 1 (insulin-dependent) *diabetes mellitus* (T1DM), while the other 86 were HIV infected (CD4+ T lymphocytes count $< 500/mm^3$). The final identification was performed using ID32C strips (bioMérieux, France). Isolates identified as *Candida albicans* or *Candida dubliniensis* were further verified with duplex PCR (Romeo and Criseo, 2011). The isolates for which the ID32C strips gave inconclusive results were sent to the CBS-KNAW Fungal Biodiversity Centre, Utrecht (The Netherlands), where they were identified by MALDI-TOF MS or the sequence analyses of the ITS

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(Internal Transcribed Spacer) and the D1/D2 domains of the LSU (Large SubUnit) of the ribosomal DNA, as previously reported (Kolecka *et al.*, 2013).

In vitro susceptibility testing was performed following the EUCAST E. Def 7.1 guidelines (Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2008), for six antifungal agents: fluconazole (Sigma – St. Louis, USA), voriconazole (Pfizer Ltd. – Sandwich, UK), caspofungin (Merck & Co, Inc.), micafungin (Astellas Pharma, Japan), anidulafungin (Pfizer, Inc.) and the MXP-4509 experimental compound ("Petru Poni" Institute of Macromolecular Chemistry – Iasi, Romania), which is a triazole based nanoconjugate with β-cyclodextrin as a carrier molecule (Marangoci *et al.*, 2011). Two reference strains (*C. albicans* ATCC 90028 and *Candida krusei* ATCC 6258) were used for quality control. The interpretation of the MICs for the commercial antifungal agents was done according to the recent EUCAST document "Antifungal Agents. Breakpoint tables for interpretation of MICs", version 7.0 (Subcommittee on Antifungal Susceptibility Testing (AFST) of the European Committee for Antimicrobial Susceptibility Testing (EUCAST), 2014).

Specific statistical parameters (Mode, MIC_{50} and MFC_{50} – for $n \geq 5$, MIC_{90} and MFC_{90} – for $n \geq 10$ and Geometric Mean–for $n \geq 2$, where n = the number of isolates) were calculated for each tested drug using Microsoft® Excel® (Dannaoui *et al.*, 2008). Statistical analysis was performed using a fully functional trial version of GraphPad Prism version 6.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Two-tailed *P*-values were calculated and $P < 0.05$ was considered significant. The level of significance was signalled in the text with one superscript asterisk (*) for $P \leq 0.05$ and two superscript asterisks (**) for $P \leq 0.01$. To calculate the geometric means and run the statistical tests, right censored values (MIC > the maximum tested concentration) were treated as the next theoretical value *i.e.* "> 8 mg/l" was treated as "16 mg/l" (Dannaoui *et al.*, 2008).

The overall species distribution and some of the calculated statistical parameters of the MICs are shown in Table I. Nine species were identified. In both types of chronic condition *Candida albicans* was the dominant species. Although it was surpassed by non-*albicans Candida* in the T1DM isolates, the statistical analysis revealed no significant differences in the distribution of species (*C. albicans* vs. non-*albicans Candida*) between the two categories. Cumulative antifungal susceptibility data (MIC_{50}, MIC_{90}, MFC_{50}, MFC_{90}) along with susceptibility and resistance rates for *C. albicans* and the non-*albicans Candida* group are presented in Table II. All the T1DM isolates and all *C. albicans* HIV isolates were susceptible to FLC. Based mostly on the 2 mg/l non-specific BP for FLC, eight (30.77%) non-*albicans Candida* HIV isolates can be considered resistant, *i.e.* four *C. krusei* isolates, two of *Candida inconspicua* and two of *Candida norvegensis*. There was also two intermediately susceptible *Candida utilis* isolate. All isolates were susceptible to VOR. Two *C. albicans* and two *Candida tropicalis* T1DM isolates were resistant to all echinocandins, but they were susceptible to azoles. The *C. tropicalis* isolates also had high MFC values for CAS and ANI. There were also two *Candida lusitaniae* T1DM isolates resistant to CAS. Within the HIV isolates there were four of *C. albicans* that were resistant to MCA and ANI but were susceptible to azoles. All the non-*albicans Candida* HIV isolates were susceptible to echinocandins.

The antifungals MICs for the reference strains used for quality control were: *C. albicans* ATCC 90028 (0.125–0.25 mg/l for FLC, 0.0156 mg/l for VOR, 0.0156–0.0312 mg/l for MXP, 0.0625–0.125 mg/l for CAS, 0.0156–0.0312 for MCA, and 0.0156 mg/l for ANI); *C. krusei* ATCC 6258 (16–32 mg/l for FLC, 0.0625–0.125 mg/l for VOR, 0.0312–0.0625 mg/l for MXP, 0.0312–0.0625 mg/l for CAS, 0.0156–0.0312 for MCA, and 0.0312–0.0625 mg/l for ANI).

Articles regarding species distribution and antifungal susceptibility of oral isolates from patients with diabetes are relatively scarce and, unlike our study, they investigate isolates resulted from colonisation and not from OC. Even fewer go as far as testing antifungal susceptibility. Despite reports of increased presence of non-*albicans Candida* species, the most recent surveys from Brazil (Sanitá *et al.*, 2013; Bremenkamp *et al.*, 2011) or Western Europe (Manfredi *et al.*, 2002; 2006) document isolation rates of 70% and higher for *C. albicans*. The proportion in our study, approximately 50%, is more similar to reports from geographically closer areas such as Poland (Drozdowska and Drzewoski, 2008; Nawrot *et al.*, 2006), Slovakia (Dorko *et al.*, 2005) or Turkey (Kadir *et al.*, 2002). Regarding the non-*albicans* species, most studies report the isolation of *C. tropicalis*, but also *Candida glabrata* and *Candida parapsilosis*; the latter two did not occur in our investigation. The number of isolates and also the geographical gradient are important reasons for these differences. The Turkish survey reports *Candida kefyr*, while an older British survey reports *C. lusitaniae* (Manfredi *et al.*, 2002), species also reported by this study.

Our findings regarding FLC susceptibility are in agreement with the most recent Brazilian (Sanitá *et al.*, 2013) and British-Italian (Manfredi *et al.*, 2006) researches that found no FLC resistance. In contrast to the situation in Brazil, the Romanian isolates had a high rate of CAS resistance. Since there are no established BPs for CAS, we did use a non-specific BP of 0.25 mg/l which encompasses most of the already

Table I
Species distribution and *in vitro* antifungal susceptibility in oral candidosis isolates

Species (no. of isolates % of all isolates)	Compound	MIC (μg/ml)			MFC (μg/ml)		
		Range	Mode	GM[1]	Range	Mode	GM
T1DM isolates (n = 30)	FLC	≤ 0.125–0.25	≤ 0.125	0.1682			
C. albicans	VOR	NA[1]	≤ 0.0156	0.1566			
(14–46.67%,	MXP	≤ 0.0156–0.0625	≤ 0.0156	0.0210			
95% CI = 24.80–69.89%)	CAS	0.0312–0.5	0.0312	0.0464	0.125–2.0	0.25	0.4529
	MCA	≤ 0.0156–0.25	≤ 0.0156	0.0283	0.0625–2.0	0.125; 0.25	0.2500
	ANI	0.0312–0.25	0.0312	0.0420	0.125–1.0	0.125	0.2051
C. kefyr	FLC	0.25–0.5	0.25	0.3150			
(6–20.00%)	VOR	NA	≤ 0.0156	0.0156			
	MXP	≤ 0.0156–0.0312	≤ 0.0156	0.0197			
	CAS	NA	0.0312	0.0312	0.0625–0.25	0.25	0.1575
	MCA	0.0312–0.0625	0.0625	0.0496	0.125–0.5	NA	0.2500
	ANI	0.0625–0.25	NA	0.1250	0.125–0.5	0.5	0.3150
C. lusitaniae	FLC	≤ 0.125–0.5	≤ 0.125	0.1984			
(6–20.00%)	VOR	NA	≤ 0.0156	0.0156			
	MXP	≤ 0.0156–0.0312	≤ 0.0156	0.0197			
	CAS	0.0312–0.5	NA	0.1249	0.5–1.0	0.5	0.6300
	MCA	0.0312–0.25	NA	0.0787	0.125–0.5	0.5	0.3150
	ANI	0.0625–0.25	0.0625	0.0992	0.25–1.0	0.25	0.3969
C. tropicalis	FLC	≤ 0.125–1.0	NA	0.3536			
(4–13.33%)	VOR	≤ 0.0156–0.0312	NA	0.0221			
	MXP	≤ 0.0156–0.0625	NA	0.0884			
	CAS	0.0312–1.0	NA	0.1766	0.25–16.0	NA	2.0000
	MCA	0.0625–1.0	NA	0.2500	0.25–1.0	NA	0.5000
	ANI	0.0625–2.0	NA	0.3536	1.0–8.0	NA	2.8284
Non-albicans *Candida*	FLC	≤ 0.125–1.0	≤ 0.125	0.2726			
(16–53.33%,	VOR	≤ 0.0156–0.0312	≤ 0.0156	0.0170			
95% CI = 30.11%–75.20%)	MXP	≤ 0.0156–0.0625	≤ 0.0156	0.0286			
	CAS	0.0312–1.0	0.0312	0.0810	0.0625–16.0	0.25	0.5000
	MCA	0.0312–1.0	0.0625	0.0884	0.125–1.0	0.5	0.3242
	ANI	0.0625–2.0	0.0625	0.1487	0.125–8.0	0.25; 0.5; 1.0	0.5946
Overall	**FLC**	**≤ 0.125–1.0**	**≤ 0.125**	**0.2176**			
	VOR	**≤ 0.0156–0.0312**	**≤ 0.0156**	**0.0163**			
	MXP	**≤ 0.0156–0.0625**	**≤ 0.0156**	**0.0248**			
	CAS	**0.0312–1.0**	**0.0312**	**0.0624**	**0.0625–16.0**	**0.25**	**0.4774**
	MCA	**≤ 0.0156–1.0**	**≤ 0.0156; 0.0625**	**0.0519**	**0.0625–2.0**	**0.125; 0.25; 0.5**	**0.2872**
	ANI	**0.0312–2.0**	**0.0312**	**0.0824**	**0.0625–8.0**	**0.125**	**0.3618**
HIV isolates (n = 86)	FLC	≤ 0.125–0.5	0.5	0.2872			
C. albicans	VOR	NA	≤ 0.0156	0.0156			
(60–69.77%,	MXP	NA	≤ 0.0156	0.0156			
95% CI = 54.80%–81.49%)	CAS	≤ 0.0156–0.0312	0.0312	0.0291	0.0625–2.0	0.0625	0.1984
	MCA	≤ 0.0156–0.0312	≤ 0.0156	0.0163	0.0312–2.0	0.0625	0.1575
	ANI	≤ 0.0156–0.0625	0.0312	0.0312	0.0625–2.0	0.0625	0.1469
C. dubliniensis	FLC	0.25–0.5	0.25	0.2973			
(8–9.30%)	VOR	NA	≤ 0.0156	0.0156			
	MXP	NA	≤ 0.0156	0.0156			
	CAS	NA	0.0312	0.0312	0.5–1.0	1.0	0.8409
	MCA	0.0312–0.0625	0.0312; 0.0625	0.0442	1.0–4.0	1.0	1.4142
	ANI	0.0625–0.125	0.125	0.1051	0.125–1.0	0.5	0.4204
C. kefyr	FLC	NA	0.5	0.5000			
(4–4.65%)	VOR	NA	≤ 0.0156	0.0156			
	MXP	NA	≤ 0.0156	0.0156			
	CAS	NA	0.0312	0.0312	0.0312–0.0625	NA	0.0442
	MCA	NA	0.0625	0.0625	0.125–0.25	NA	0.1768
	ANI	NA	0.125	0.1250	0.25–0.5	NA	0.3536

Table I. Continued.

Species (no. of isolates % of all isolates)	Compound	MIC (μg/ml)			MFC (μg/ml)		
		Range	Mode	GM[1]	Range	Mode	GM
C. krusei (4–4.65%)	FLC	32.0–64.0	NA	45.2548			
	VOR	0.25–0.5	NA	0.3536			
	MXP	0.25–0.5	NA	0.3536			
	CAS	NA	0.125	0.1250	0.125–0.25	NA	0.1768
	MCA	0.0625–0.125	NA	0.0884	0.125–0.25	NA	0.1768
	ANI	NA	0.0625	0.0625	NA	0.125	0.1250
C. tropicalis (4–4.65%)	FLC	0.25–0.5	NA	0.3536			
	VOR	NA	0.0312	0.0312			
	MXP	0.0312–0.0625	NA	0.0442			
	CAS	NA	0.0312	0.0312	0.125–2.0	NA	0.5000
	MCA	NA	0.0312	0.0312	NA	1.0	1.0000
	ANI	NA	0.0625	0.0625	0.25–1.0	NA	0.5000
C. inconspicua (2–2.33%)	FLC	NA	32.0	32			
	VOR	NA	0.25	0.25			
	MXP	NA	0.125	0.125			
	CAS	NA	0.125	0.125	NA	0.125	NA
	MCA	NA	0.0312	0.0312	NA	0.0625	NA
	ANI	NA	0.0625	0.0625	NA	0.125	NA
C. norvegensis (2–2.33%)	FLC	NA	16.0	16			
	VOR	NA	0.0312	0.0312			
	MXP	NA	0.0312	0.0312			
	CAS	NA	0.0625	0.0625	NA	0.25	NA
	MCA	NA	0.0312	0.0312	NA	0.125	NA
	ANI	NA	0.0312	0.0312	NA	0.125	NA
C. utilis (2–2.33%)	FLC	NA	4.0	4			
	VOR	NA	0.125	0.125			
	MXP	NA	0.0625	0.0625			
	CAS	NA	0.0312	0.0312	NA	0.0312	NA
	MCA	NA	0.0312	0.0312	NA	0.0625	NA
	ANI	NA	0.0312	0.0312	NA	0.0312	NA
Non-*albicans Candida* (26–30.23%, 95% CI = 18.51%–45.20%)	FLC	0.25–64.0	0.25; 0.5	1.7044			
	VOR	≤ 0.0156–0.5	≤ 0.0156	0.0430			
	MXP	≤ 0.0156–0.5	≤ 0.0156	0.0408			
	CAS	0.0312–0.125	0.0312	0.0453	0.0312–2.0	0.125; 1.0	0.2370
	MCA	0.0312–0.125	0.0312	0.0453	0.0625–4.0	1.0	0.3631
	ANI	0.0312–0.125	0.0625	0.0733	0.0312–1.0	0.125	0.2370
Overall	**FLC**	**≤ 0.125–64.0**	**0.5**	**0.4920**			
	VOR	**≤ 0.0156–0.5**	**≤ 0.0156**	**0.0212**			
	MXP	**≤ 0.0156–0.5**	**≤ 0.0156**	**0.0209**			
	CAS	**≤ 0.0156–0.125**	**0.0312**	**0.0333**	**0.0312–2.0**	**0.125**	**0.2094**
	MCA	**≤ 0.0156–0.125**	**0.0156**	**0.0222**	**0.0312–4.0**	**0.0625**	**0.2027**
	ANI	**≤ 0.0156–0.125**	**0.0312**	**0.0404**	**0.0312–2.0**	**0.125**	**0.1698**

[1] GM–Geometric Mean; [2] NA–Not Applicable

established echinocandin BPs. This situation requires further research, especially considering the findings of a recent study that echinocandins would be a safer choice for diabetes patients since they do not seem to be affected by glucose, which appears to significantly lower the antifungal activity of azoles and polyenes (Mandal *et al.*, 2014).

Studies that investigate isolates from HIV patients are more abundant, but similarly to those targeting diabetes, more of them focus on the asymptomatic carriage of yeasts in the oral cavities. Nevertheless, oral colonisation of HIV-infected patients in conjunction to low counts of CD4 cells are strong premises for subsequent development of OPC (Fong *et al.*, 1997). The same increase of prevalence for the non-*albicans* species is documented for HIV patients and, equally, *C. albicans* remains the dominant species. *C. dubliniensis*, *C. glabrata*, and *C. tropicalis* are considered as

Table II
Cumulative antifungal susceptibility data and resistance (R) rates of oral candidosis isolates

Species	T1DM				HIV				
	Compound	MIC_{50} (μg/ml)	MFC_{50} (μg/ml)	R (n–%)	MIC (μg/ml)		MFC (μg/ml)		R (n–%)
					MIC_{50}	MIC_{90}	MFC_{50}	MFC_{90}	
Candida albicans	FLC	≤0.125		0–0.0%	0.25	0.5			0–0.0%
	VOR	≤0.0156		0–0.0%	≤0.0156	≤0.0156			0–0.0%
	MXP	≤0.0156		NA	≤0.0156	≤0.0156			NA
	CAS	0.0312	0.25	2–14.3%	0.0312	0.0312	0.125	1.0	0–0.0%
	MCA	≤0.0156	0.25	4–28.6%	≤0.0156	≤0.0156	0.0625	2.0	4–6.7%
	ANI	0.0312	0.125	2–14.3%	0.0312	0.0312	0.125	0.5	4–6.7%
Non-*albicans Candida*	FLC	0.25		0–0.0%	0.5	32.0			8–30.8%
	VOR	≤0.0156		0–0.0%	0.0312	0.25			0–0.0%
	MXP	≤0.0156		NA	0.0312	0.25			NA
	CAS	0.0312	0.25	4–25.0%	0.0312	0.125	0.25	1.0	0–0.0%
	MCA	0.0625	0.25	2–12.5%	0.0312	0.0625	0.25	1.0	0–0.0%
	ANI	0.0625	0.5	2–12.5%	0.0625	0.125	0.25	1.0	0–0.0%
Overall	FLC	0.25		0–0.0%	0.5	4.0			8–9.3%
	VOR	≤0.0156		0–0.0%	≤0.0156	0.0312			0–0.0%
	MXP	≤0.0156		NA	≤0.0156	0.0625			NA
	CAS	0.0312	0.25	6–20.0%	0.0312	0.0312	0.125	1.0	0.0
	MCA	0.0625	0.25	6–20.0%	≤0.0156	0.0625	0.125	2.0	4–4.7%
	ANI	0.0625	0.25	4–13.3%	0.0312	0.125	0.125	0.5	4–4.7%

emerging pathogens (Lin *et al.*, 2013; Drozdowska and Drzewoski, 2008; Binolfi *et al.*, 2005).

Regarding *C. albicans* proportion within the HIV isolates, values similar to the one in this study (70%) have been reported for Taiwan (Ho *et al.*, 2014), Cameroun (dos Santos Abrantes *et al.*, 2014), USA (Merenstein *et al.*, 2013), Spain (Ramírez *et al.*, 2006) or Turkey (Erköse and Erturan, 2007). Percentages can go as high as 90% in India (Maurya *et al.*, 2013), Italy (Giammanco *et al.*, 2002) or UK (Cartledge *et al.*, 1999), 83% in South Africa (dos Santos Abrantes *et al.*, 2014), 79% in Serbia (Mitrovic *et al.*, 1996), or can go as low as 62% in Turkey (Erköse and Erturan, 2007) or Brazil (Costa *et al.*, 2006). Again, *C. glabrata* is missing from the isolates in our non-*albicans Candida* group.

Reported levels of FLC resistance vary widely from 0.9% in Taiwan (Ho *et al.*, 2014) and 3.4% in China (Li *et al.*, 2013) to about 50% in South Africa and Cameroun (dos Santos Abrantes *et al.*, 2014) for *C. albicans*. The differences can have a few possible causes such as street availability of antifungals, without prescription (dos Santos Abrantes *et al.*, 2014), or the different susceptibility testing methods used in each investigation. In our study, FLC resistance was present only in the non-*albicans Candida* group, in agreement with the above mentioned sources which found higher resistance rates for this group by up to 13% (Li *et al.*, 2013).

All the Romanian isolates were susceptible to VOR, a situation similar to that in Taiwan (Ho *et al.*, 2014). Some resistance was found in China–3% for *C. albicans* and 14.5 % for non-*albicans Candida* (Li *et al.*, 2013) and very high values, were reported for *C. albicans* from South Africa and Cameroun (dos Santos Abrantes *et al.*, 2014). This study also signals the occurrence of resistance to echinocandins that other investigations did not report. Although these antifungals are not the first choice in treating patients with OC, they can be an effective alternative if topical or systemic azoles have definitely failed (Lortholary *et al.*, 2012).

Our study confirms a few worldwide reported tendencies such as the increasing prevalence and lower antifungal susceptibility of non-*albicans Candida* species, and *C. dubliniensis* as an emerging oral pathogen in HIV patients. It also supports the status of FLC as the first option for treatment, but not advisable for prophylaxis, and VOR as a viable second line of defence.

As a triazole based antifungal, MXP-4509 inhibits the ergosterol biosynthesis, similar to FLC and VOR. The experimental drug had a good antifungal activity with MIC values similar to those of VOR. Further, *in vivo* studies are warranted.

In conclusion, strict oral hygiene and adherence to specific treatment are the best prophylactic approaches to prevent OC in both chronic conditions, while FLC is recommended only as a first line of defense after the occurrence of the infection. As a second line of defense, in case of FLC therapeutic failure, echinocandins are a viable option for HIV patients. In the case

of diabetes patients, however, the risk of azole cross-resistance should be evaluated first; for patients without prior exposure to azoles, VOR may be a better option.

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