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Introduction

*Helicobacter pylori* is a Gram-negative spiral-shaped bacterium, belonging to ε-Proteobacteria specifically colonizing the gastric epithelium of humans. It causes one of the most common infections worldwide, affecting about half of the world’s population. However, it should be noted that the prevalence of *H. pylori*, particularly in the Western world, has significantly decreased coinciding with an increase of some autoimmune and allergic diseases, such as asthma. Various epidemiological studies have also documented a negative association between *H. pylori* colonization and the presence of GERD (gastroesophageal reflux disease) and risk of esophageal cancer. Additionally, an upward trend of obesity recently observed in inhabitants of developed countries raised a question about the relationship between *H. pylori* infection and the human body mass index. The first part of this review describes common, recommended anti-*H. pylori* treatments. The second part, presents the results of recent experiments aimed at evaluating the association between *H. pylori* infections and gastro-esophageal diseases, the level of stomach hormones, the human body mass index and allergic diseases. Although some studies suggest an inverse association of *H. pylori* infection with some health problems of the modern world such as asthma, obesity or GERD, *H. pylori* should be considered as a harmful human pathogen responsible for serious and sometimes lethal diseases. Thus, many scientists advocate the eradication of *H. pylori*.

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**Key words:** Helicobacter pylori, allergy, GERD, NAP protein, obesity, therapy
promoters in gastric mucosa, which are known to be methylated in cancer patients (Cover and Blanke, 2005; Ding et al., 2010; Douraghi et al., 2008; Tegtmeier et al., 2011; Yamaoka, 2008).

Sequencing of several *H. pylori* genomes and comparative genomics experiments revealed a high level of genome diversity. At first, by comparing the genomes of 15 *H. pylori* strains Salama et al. determined that *H. pylori* genes constituting the core set count 1281 genes (Salama et al., 2000). The growing number of genomes included in microarray analyses resulted in redefining the number of core genes in *H. pylori* genomes. At present, it is widely assumed that *H. pylori* core genes consist of about 1100 genes. Auxiliary genes amount to 22–27% of the genome, encoding mainly proteins of unknown function, the Cag protein, outer membrane proteins (OMP) and proteins involved in DNA metabolism (Dong et al., 2009; Gressmann et al., 2005). Some strain-specific genes are disease-specific. The diversity of *H. pylori* genomes is not only noticeable when evaluating the number of common genes, but also when examining gene nucleotide sequences. Genetic variety among *H. pylori* strains arises from intra-genomic diversification (for example – point mutations, recombination and slipped-strand mispairing) as well as inter-genomic recombination – the ability of *H. pylori* to take up exogenous DNA and incorporate it into its genome (Dorer et al., 2009). The process allows the pathogen to adapt to various niches within the same host or to the changing environments during long-lasting infections.

**Recommended anti-*Helicobacter* therapies**

According to several international guidelines, different drug regimens are recommended for treatment of *H. pylori* infections. These include triple, quadruple, (sequential or concomitant) therapy regimens. Treatment regimen should be selected according to areas of low or high clarithromycin resistance. Standard triple therapy which involves administration of a proton pump inhibitor combined with clarithromycin and amoxicillin or metronidazol for 7 to 14 days was the most commonly recommended first-line treatment by guidelines published in Europe and North America since the mid 1990s (Chey and Wong, 2007; Malfertheiner et al., 2012). However, the recent data showed that this drug combination has lost some efficacy and according to recently published worldwide guidelines should be abandoned when the clarithromycin resistance rate in the region is more than 15–20% (Malfertheiner et al., 2012). Increased doses of proton pump inhibitors had small effects on eradication rates (Fuccio et al., 2007; Vakil and Connor, 2005). Bismuth-based quadruple and levofloxacin-based triple regimens are also frequently recommended, but as second-line therapies. Third-line options (empiric regimes tailored to individual antibiotic sensitivities) include treatments based on rifabutin (an antituberculosis agent) and furazolidone. However, susceptibility testing is not common, but, when it is employed, it is only carried out in specialist research-oriented centers. Sequential therapy is an alternative to standard triple therapy for eradication of *H. pylori* (Gisbert, 2010; Gisbert et al., 2010). It aims to overcome clarithromycin resistance. During the first stage of therapy, amoxicillin is administered to weaken the bacterial cell wall, which otherwise prevents the formation of channels that block clarithromycin from entering the bacterial cell and, in effect, cause resistance to the antibiotic. Subsequently, clarithromycin and nitroimidazole are administered for a further 5 days in the second phase of the therapy. Administration of the proton-pump inhibitor is continuously employed throughout the treatment. Generally, the sequential therapy has a better rate of curing *H. pylori* infection than classical triple therapy. However, clinically the sequential administration of the two drug combinations is relatively complex. As an alternative the concomitant quadruple therapy including the same four drugs as sequential therapy given concomitantly has been tested. It appears to be equally effective as sequential therapy but less complex (Essa et al., 2009; Wu et al., 2010).

Treatment against *H. pylori* still fails in more than 20% of patients and a more acceptable eradication level is greatly anticipated. Two main obstacles against effective therapy are the resistance of *H. pylori* to different antibiotics (i.e. clarithromycin, metronidazole, amoxicillin, levofloxacin), correlated with the consumption of antibiotics in the general population, and lack of strict abidance to the rules of drug administration, which involves the efforts of both doctor and patient. The prescribed defined therapy is long and complicated. It is uncomfortable for patients and impedes complying with the recommended procedures. In view of these facts, there is an urgent need to intensify the fight against *H. pylori* by developing alternative methods of treating *Helicobacter pylori* infections (De Francesco et al., 2010; O’Connor et al., 2010). It was documented that the addition of probiotics to a standard antibiotic treatment improved slightly *H. pylori* eradication rate and considerably reduced therapy-associated adverse effects (Lionetti et al., 2010; Vitor and Vale, 2011; Zou et al., 2009).

The alarming rise of antibiotic resistant pathogenic microorganisms renewed interest in antibacterial, including anti-*Helicobacter*, research and forced scientists to search for new drugs with novel modes of action. Some new anti-*Helicobacter* drugs are currently under
development. New, potentially effective agents should fulfill several requirements, at least display strong, specific antibacterial activity against *Helicobacter*, when used in mono-therapy, and exhibit activity in low pH. Examples of such agents are: the TG44 molecule synthesized by the Nagase Chemetex Corporation and acetyl-lysyl oligomers (OAKs). The former compound, tested so far only in *in vitro* experiments, is a highly specific anti-*Helicobacter* molecule, which activity is based on the disruption of the cell outer membrane (Kamoda *et al.*, 2006). Latter compounds are synthetic antimicrobial peptides (AMPs) of broad specificity, which demonstrate high efficacy against *Helicobacter* in *in vitro* and *in vivo* tests (Makobongo *et al.*, 2009; Makobongo *et al.*, 2012). They are a unique and diverse group of molecules produced by many tissues and cell types of various organisms. As AMPs have recently elicited interest as new antibacterial drugs, one can expect that new AMPs will be tested as anti-*Helicobacter* agents soon (Brogden, 2005). However, it should be kept in mind that AMP therapy might potentially enable pathogens to overcome the innate immune response of an immunocompetent host (Brodskey and Gunn, 2005).

**Unexpected consequences of Helicobacter pylori infection/eradication**

**Impact of Helicobacter pylori infection on esophageal diseases.** Symptoms of the gastro-esophageal reflux disease (GERD) appear when the impairment of motility of the gastric system allows for the contact of the gastric content with the esophageal epithelium. This may lead to development of Barrett esophagus (BE), which is recognized as a risk factor for the subsequent development of esophageal adenocarcinoma (EAC). The pathogenesis of these diseases is complex and multifactorial, but acidity of the refluxate is a crucial factor inducing GERD development. The decreasing prevalence of *H. pylori* infections and related diseases, especially in developed countries, and, at the same time, an increase in the recognition of gastro-esophageal reflux symptoms and its complications, raise a question whether *H. pylori* is a likely etiological factor for this changing epidemiology. This phenomenon has been observed in many countries, at different geographic locations, such as Japan or USA (Blaser, 2008; Kim *et al.*, 2011; Rajendra, 2011; Yang *et al.*, 2009). The causal relationship between *H. pylori* infections and the gastro-esophageal disease has been examined in a large number of epidemiological studies. The first observations were made by Labenz *et al.*, who reported that *H. pylori* eradication in patients with duodenal peptic ulcer stimulates the development of reflux esophagitis (Labenz *et al.*, 1997). Comparative analyses concentrated on assessing the rate of *H. pylori* infection in patients with reflux esophagitis compared to those with a normal esophagus as well as on the evaluation of the effect of *H. pylori* eradication on the development of reflux esophagitis. Although some studies confirmed the inverse associations between these two analyzed phenomena, contradictory results also have been reported. For more details we recommended review papers, such as by Graham *et al.*, 2007; Hung and Wong, 2009; Sharma and Vakil, 2003; Souza and Lima, 2009. Lack of consensus between various studies may be due to many factors, such as difficulties in evaluating the clinical aspects of *H. pylori* infection or the differences in the genotype of the infecting strain. It was documented that *H. pylori* can colonize various parts of the stomach and that the site of infection is an important factor influencing the consequences of colonization. Antrum gastritis is related to high inflammation and high acid secretion, whereas corpus gastritis correlates with low acid secretion. Another important factor is the genetic and immunological status of the host. Three recent meta-analysis studies conducted by Islami and Kamangar, Qian *et al.* and Yaghoobi *et al.* summarized the data published during last twenty years (Islami and Kamangar, 2008; Qian *et al.*, 2011; Yaghoobi *et al.*, 2010) Islami and Kamangar used 19 carefully selected studies to examine the association between *H. pylori* infection and esophageal adenocarcinoma (EAC). Presented data suggested inverse association of the CagA-positive *H. pylori* colonization with risk of EAC. Meta-analysis of Yaghoobi *et al.* evaluated the risk of GERD development due to *H. pylori* eradication and showed that the frequency of GERD does not increase after *H. pylori* eradication among dyspeptic patients, whereas a two-fold higher risk of GERD development in patients with peptic ulcers was observed. Meta-analysis conducted by Qian *et al.* did not show any association between *H. pylori* eradication and the occurrence of symptomatic GERD. Potential mechanism of the protective effect of *H. pylori* gastric colonization against esophageal diseases still remains unexplainable. Apart from changes in gastric acidity, stomach colonization by *H. pylori* influences the level of at least two hormones: leptin and ghrelin (see below), which can influence the esophageus epithelium as esophagus cells contain lepin receptors. The recent progress in sequencing technology in combination with the development of new bioinformatic tools allows us to study the microbiome (a set of bacterial genes present in a specific ecological niche) of the stomach and esophagus. It is expected that this strategy will permit tracing changes in the microbiome that are correlated with disease development or are due to *H. pylori* eradication. Analysis of the stomach microbiota revealed that it is much more complex than it was assessed before. *H. pylori* was found to be
the most abundant phylotype in the stomach of individuals tested as *H. pylori*-positive by standard methods (Bik *et al.*, 2006). Structure of the human gastric bacterial community was determined to be dependent on the *H. pylori*-induced disease. For instance, the stomach microbiota of gastric cancer patients differs significantly compared to microbiota of dyspeptic individuals (Dickson *et al.*, 2009; Maldonado-Contreras *et al.*, 2011). Metagenomic study of the esophageal microbiome also revealed its astonishing complexity and significant changes in the microbiome structure connected to pathological alterations of the esophageal epithelium (Yang *et al.*, 2009). As even short term antibiotic treatment of *H. pylori* infections has tremendous repercussions for the gut microbiome structure, it may also be a factor of GERD development (Jakobsson *et al.*, 2010). Further metagenomic studies are required to shed more light on the contribution of stomach or esophageal dysbiosis on disease development.

**Correlation between Helicobacter pylori infection and asthma and allergic diseases.** In recent years, a rise in the prevalence of bronchial asthma in developed countries has been observed. Many environmental factors, such as tobacco smoke, air pollution or allergen exposure, are without doubt responsible for this documented upward tendency. However, the influence of human microbiota on allergic diseases should be also taken into account. As over the past years the prevalence of *H. pylori* infection has been decreasing, the causal relationship between *H. pylori* infection and asthma was carefully evaluated in many epidemiological studies. The relationship between these two diseases was noted for the first time in 1997 by Kosunen *et al.* (Kosunen *et al.*, 1997). Although the conducted studies provided controversial results, it is rather accepted that human colonization with *H. pylori* CagA-positive strains may have an inverse effect on development of bronchial asthma. However more studies are required to prove a real association between *H. pylori* eradication in childhood and subsequent development of asthma (Hung and Wong, 2009). At this point it should be also pointed out that *H. pylori* CagA positive strains which potentially may be protective against GERD or asthma are strongly associated with gastric cancer, which is the second leading cause of cancer-related deaths worldwide (Huang and Hunt, 2003).

For more data from epidemiological studies see papers cited by Roussos *et al.*, D’Elios *et al.* and Malfertheiner *et al.* (D’Elios *et al.*, 2009; Malfertheiner *et al.*, 2011; Roussos *et al.*, 2005).

Despite the lack of a clear hypothesis explaining the link between these two diseases, some data suggest that this inverse association might be due to the differences in the type of immune response induced.

According to the WHO definition, asthma is a chronic inflammatory disease of the airways associated with a predominant activation of CD4+ Th2 lymphocytes, which produce several Th2 cytokines, including IL-4 and IL-5 (Del Prete *et al.*, 1993; Robinson *et al.*, 1992).

In contrast, *H. pylori* gastric colonization preferentially elicits a Th1 mucosal immune response with the production of IFN-γ, IL-12, IL-18, IL-23 and TNF-α (D’Elios *et al.*, 2009). *H. pylori* neutrophil-activating protein (HP-NAP) is a main *H. pylori* virulence factor responsible for this effect. HP-NAP is a 200 kDa ball-shaped dodecamer formed by four-helix bundled subunits (17 kDa) with a hollow central part (Tsurtu *et al.*, 2012). Structurally it belongs to the Dps (DNA protecting protein under starved condition) protein family. The role of this protein in bacterial cells is still controversial. Analysis of its ability to bind to DNA resulted in incoherent data. Additionally, although HP-NAP is a bacterioferritin able to bind up to 500 atoms of iron per dodecamer, the role of this process in bacterial physiology remains unclear. Furthermore, HP-NAP was described as a cytoplasmic protein, released after cell lysis. Once released in the gastroduodenal mucosa, NAP is transported via transcytosis across endothelial cells, (de Bernard and D’Elios, 2010) stimulating subsequently human neutrophils, monocytes and dendritic cells via activation of the Toll-like receptor 2 (TLR2). In consequence, high upregulation of both the production of IL-12 and IL-23 occurs (D’Elios and de Bernard, 2010). HP-NAP activity also causes the decrease of IL-4-secreting cells. As a result, HP-NAP supported by other *H. pylori* factors induces the production of IL-12 and IL-23 that both promote the preferential development of Th-1 cells and repress the Th-2 allergic response (Amedei *et al.*, 2010; Amedei *et al.*, 2006; Cappon *et al.*, 2010). Administration of HP-NAP has a beneficial effect in case of asthma. Arnold *et al.* proved that *H. pylori* infections induced T regulatory cells (Tregs) and protected mice from asthma, especially when mice were infected neonatally. After *H. pylori* eradication due to antibiotic treatment, the protection effect was abolished (Arnold *et al.*, 2011). The efficacy of HP-NAP against asthma was also confirmed by D’Elios *et al.*, who documented that systemic and mucosal administrations of HP-NAP result in reduction of the amount of eosinophil cells, immunoglobulin E and Th2 cytokines in the mice bronchitis model. This suggests that *H. pylori* infection is able to induce long lasting Th1 type of immune response. Taking the above into account, HP-NAP seems to be an effective factor for prevention and treatment of asthma and allergic diseases (D’Elios *et al.*, 2009).

Apart from playing a role in induction of Th1 inflammation and inhibition of the Th2 response, HP-NAP may potentially be used in cancer therapy. As a very
powerful inducer of IL-12 and IL-23, HP-NAP represent the most effective cytokine in regard to tumor eradication, anti-metastatic activity and long-term anti-tumor immunity (Colombo and Trinchieri, 2002). IL-12 was recently ranked third in a comprehensive list of immunotherapeutic agents with high potential in treating cancer. Codolo and colleagues showed that local administration of HP-NAP decreases tumor growth by triggering tumor necrosis in a mouse model of bladder cancer. HP-NAP-treated tumors show also a reduced vascularization due to the anti-angiogenic activity of IFN-γ induced by treatment of cancer implants with HP-NAP (Codolo et al., 2012a; Codolo et al., 2012b). In sum it should be pointed out that even persistent infection with *H. pylori* may be linked to protection from some autoimmune diseases, it is not recommended to leave *H. pylori* infection untreated in asthmatic patients. (D’Elios and de Bernard, 2010). However based on the performed studies it is tempting to speculate that administration of HNP-NAP might be beneficial not only against allergic diseases, but also to fight cancer.

**Impact of *H. pylori* infection and anti-*Helicobacter* therapy on obesity.** The decreasing prevalence of *H. pylori* infections during the second half of the 20th century and the beginning of the 21st is noticeable across the whole developed world. For example, in some countries currently less than 10% of school children are carriers of this microorganism (Chen and Blaser, 2008; Rothenbacher et al., 1998; Segal et al., 2008). At the same time, the incidence of obesity among the same population group has been observed. It prompts many research groups to evaluate the impact of *H. pylori* infection on body weight.

Two hormones, leptin and ghrelin, play a crucial role in body weight balance by regulating food intake and energy disbursement. Leptin is a 16 kDa protein, the product of the ob gene, which is synthesized and secreted mainly by adipocytes (Zhang et al., 1994). However, it has recently been shown that this hormone is also present in rat and human gastric mucosa (Bado et al., 1998; Sobhani et al., 2000). As leptin deficiency causes obesity in humans and mice and since *H. pylori* induced gastritis may influence the leptin level, many comparative epidemiological studies have recently been conducted to examine the effect of *H. pylori* infections on gastric leptin expression and on the body mass index (BMI). Most of these studies compared infected and non-infected individuals by CagA-positive and CagA-negative strains. Infection by CagA-positive *H. pylori* strains was shown to result in more severe gastritis and more often led to gastric cancer than infection by CagA-negative *H. pylori*. Majority of obtained data has not implied any associations between the plasma leptin level and *H. pylori* infection (Azuma et al., 2001; Chuang et al., 2009; Ioannou et al., 2005). However, a study examining prepubertal children conducted by Pacifico et al. indicated that the serum leptin level was significantly lower in *H. pylori* positive patients than in *H. pylori* negative individuals (Pacifico et al., 2008). In contrast to data concerning the plasma leptin level, many investigators provided convincing results showing that *H. pylori* infection results in a significant increase of gastric leptin expression (Azuma et al., 2001; Jun et al., 2007) what, in turn, might lead to weight loss.

Conflicting results have also been published regarding the effect of *H. pylori* infection on ghrelin level. Ghrelin is a 28 amino acid peptide predominantly produced by the stomach, which is thought to be the most potent growth hormone releaser (Kojima et al., 1999). Ghrelin has also been implicated in the control of food intake (Nakazato et al., 2001). It was determined to affect metabolic functions and evoke weight gain (Wren et al., 2001). In the study performed by Gokcel et al., no differences in the plasma ghrelin concentration between adults positive and negative for *H. pylori* have been observed (Gokcel et al., 2003). In contrast, many studies performed on Japanese patients revealed a strong negative impact of *H. pylori* infection on the plasma ghrelin level, which was positively correlated with gastric ghrelin mRNA expression (Isomoto et al., 2005a; Isomoto et al., 2005b; Shiotani et al., 2005). Additionally, some analyses indicated that this effect is dependent on the severity of the disease and influenced by the patient’s gender (Chuang et al., 2009).

Although *H. pylori* infection modulates the plasma ghrelin and stomach leptin level, epidemiological studies do not document any associations between *H. pylori* infection and the body mass index of the analyzed patients. A large US-based population study showed that there is no correlation between the presence of *H. pylori* and the genetic status of the colonizing strain (CagA-positive vs. CagA-negative strains) as well as the weight of the analyzed individual (Cho et al., 2005; Ioannou et al., 2005).

The second set of experiments was directed towards analyzing the effect of *H. pylori* eradication, plasma leptin or ghrelin level and the increase in BMI. They provided rather consistent results. In many studies, it has been reported that *H. pylori* eradication is associated with the increase of the circulating leptin level, the decrease of the ghrelin level resulting in an increase in BMI. This effect was independent of the examined population and was observed both among adult Japanese as well as American individuals who underwent anti-*Helicobacter* therapy. Additionally, similar long-term effect of *H. pylori* eradication was observed among prepubertal children (Francois et al., 2011; Fujiwara et al., 2002; Osawa et al., 2006; Pacifico et al., 2008). However,
Suto et al. noticed that the effect of *H. pylori* eradication on BMI is dependent on the serum pepsinogen I/II ratio (Suto et al., 2009). Interestingly, experiments conducted by Nwokolo et al., who for the first time examined the effect of *H. pylori* eradication in *H. pylori* asymptomatic patients, showed a significant increase of the ghrelin level after therapy. Authors have thus concluded that lowering the prevalence of *H. pylori* infections in developed countries might lead to the observed increase in obesity (Nwokolo et al., 2003).

**Summary**

Collectively, it is obvious that the decreased *H. pylori* prevalence among populations of Western countries could lead to significant changes in human health. However, the plausible mechanism is extremely complex as it is influenced not only by the genotype of the pathogen, but also by the immunological and genetic status of the host. As *H. pylori* infection is almost always acquired during childhood, the microorganism is able to alter the resident stomach microbiota over a years, what leads to other pathologies such as EAC. Thus eradication of *H. pylori* can also stop transmission to healthy persons and prevent other pathologies connected with stomach microbiota changes.

*Helicobacter pylori* has accompanied humans since the dawn of time and coevolved together with its host. Thus, it is not surprising that the interrelation between the microorganism and its host is complex – the bacterium behaves as a pathogen causing some severe diseases, such as the peptic ulcer disease or gastric cancer, while, at the same time, as a commensal protecting and EAC. As such, *H. pylori* can also stop transmission to healthy persons and prevent other pathologies connected with stomach microbiota changes.

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**In vitro Study of Secreted Aspartyl Proteinases Sap1 to Sap3 and Sap4 to Sap6 Expression in Candida albicans Pleomorphic Forms**

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Received 13 January 2012, revised 20 August 2012, accepted 1 September 2012

**Abstract**

Transition from round budding cells to long hyphal forms and production of secreted aspartic proteases (Saps) are considered virulence-associated factors of Candida albicans. Although plenty of data dealing with Saps involvement in the infection process have been published, Sap expression by the different pleomorphic forms as well as the capacity of C. albicans filaments to express Sap1-6 under serum influence are poorly investigated. In this study, we used immunofluorescence and immunoelectron microscopy for the detection of Sap1-6 isoenzymes in C. albicans pleomorphic cells (blastoconidia, germ tubes, pseudohyphae, true hyphae) grown in Sap-inductive human serum and Sap non-inductive medium – yeast extract-peptone-glucose (YEPD). Isoenzymes were below the detection level in all blastoconidial cells grown in YEPD for 18 h. Sap1-6 expression was hardly detected in C. albicans cells cultivated in serum for 20 min. Increasing level of Sap1-6 expression was observed when C. albicans was incubated for 2, 6 and 18 h in serum corresponding to the development of germ tubes, pseudohyphae and true hyphae. The expression of Sap1-3 in pseudohyphae and true hyphae was more intensive compared to Sap4-6. Thus, we could show that human serum induced hyphae formation and the expression of Sap1-6 were co-regulated.

**Key words:** Candida albicans, aspartic protease expression, isoenzymes 1-3, isoenzymes 4-6, morphotypes

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**Introduction**

The opportunistic fungal pathogen Candida albicans possesses a repertoire of virulence attributes including adhesion to host tissue, the ability to undergo reversible morphogenetic transition, the secretion of extracellular hydrolytic enzymes, aspartic proteases (Saps) are considered to be key virulence determinants of C. albicans which contribute to the adhesive and invasion capabilities of strains from this species (Raška et al., 2007; Tongchusak et al., 2008; Dalle et al., 2010; Hayek et al., 2010). Among the hydrolytic enzymes, aspartic proteases (Saps) are considered to be key virulence determinants of C. albicans which contribute to the adhesive and invasion capabilities of strains from this species (Raška et al., 2007; Tongchusak et al., 2008; Dalle et al., 2010). Saps are the products of a family of 10 SAP genes divided into subfamilies based on amino acid sequence homology alignment (SAP1 to SAP3, SAP4 to SAP6, SAP9, and SAP10). Furthermore, SAP7 and SAP8 are divergent and are not represented as subfamily members (Hube et al., 1994; Monod et al., 1994 and 1998; Chen et al., 2002; Correia et al., 2010). Expression of the SAP genes varies according to the type and stage of the disease (Schaller et al., 2001; Fradin et al., 2003; Taylor et al., 2005; Jackson et al., 2007; Correia et al., 2010; Naglik et al., 2008; Abegg et al., 2011). Moreover, SAP genes expression is also regulated during the morphological transition (Argimón et al., 2007; Décanis et al., 2011). Candida albicans is able to grow in different forms (blastoconidia, germ tubes, pseudohyphae and true hyphae), a phenomenon defined as pleomorphism (Whiteway and Bachewich, 2007). Pleomorphic forms enable C. albicans to colonize and invade human tissues (Morrison et al., 2003; Kumamoto and Vinces, 2005a; 2005b; Raška et al., 2007; Barnett, 2008). It was found (Gow et al., 2002) that the
morphogenetic response (transition from budding to hyphal cells) of *C. albicans* as well as expression of SAP genes are triggered by factors existing in the environment of the host (pH, temperature, serum).

Previous studies (Schaller et al., 2000; Felk et al., 2002; Naglik et al., 2003; 2008; Lermann and Morschhäuser, 2008; Gropp et al., 2009; Dalle et al., 2010), indicate that the expression of Sap isoenzymes, for the morphogenesis of *C. albicans* varies strongly, depending on the experimental setup having a significant impact on the dependence on protease activity. Felk et al. (2002) showed that in vivo (in tissue from infected mice) expression of Sap1-3 was detected on the surface of both yeast and hyphal of wild-type cells. In contrast, the Sap4-6 antigens were identified mostly on penetrating hyphal cells (Schaller et al., 2000; 2001; Copping et al., 2005; Hornbach et al., 2009). Those authors suggested that Sap4-6 are the hyphal-associated proteins which is in striking contrast to the results obtained by Lermann and Morschhäuser (2008), who suggested that none of the SAP1-6 genes is required for invasion of vaginal RHE by hyphal morphologies. Moreover, according to Correia et al. (2010) Sap1-6 do not play a significant role in *C. albicans* virulence in a murine model of hematogenously disseminated candidiasis and that, in this model, Sap1-3 are not necessary for successful *C. albicans* infection.

In contrast, previous reports (Naglik et al., 1999; Schaller et al., 2000; Hube, 2004; Hornbach et al., 2009) showed that the Sap4-6 subfamily produced in high level by hyphal cells plays a role in immune evasion and protection from phagocytic killing by murine macrophages. Gropp et al. (2009) showed that Sap1-3 degrade and inactivate the central human complement components C3b, C4b as well as C5 and block the damaging effects of the activated complement system.

Although plenty of data dealing with the Saps involved in the infection process have been published the expression of Saps in particular morphotype is not sufficiently described. Given the role of serum in hyphae morphogenesis (Lermann and Morschhäuser, 2008; Gropp et al., 2009) and as SAP1-3 play essential role in the growth of *C. albicans* in medium consisting proteins (Felk et al., 2002; Lermann and Morschhäuser, 2008; Naglik et al., 2008; Gropp et al., 2009) as well as SAP4-6 are hypha-related genes (Lermann and Morschhäuser, 2008; Naglik et al. 2008; Gropp et al., 2009), we asked whether Sap1-3 or Sap4-6 are expressed in each morphotype under human serum influence in vitro. In addition, we analyzed whether any differences in expression between these two subfamilies exist. To investigate the expression of the Sap1-3 and Sap4-6 proteins, we studied the media, pH, and temperature shifts. Following to previous conclusion (Hube et al., 1994; Naglik et al., 1999; Wise et al., 2007; Gropp et al., 2009), that different expression profiles of Saps are regulated by pH of the maintenance medium, we studied pH, and temperature shifts during expression of the Sap1-3 and Sap4-6. In contrast to all other members of the Sap family, the proteases Sap9-10 monitored under conditions in vitro and in vivo are independent of pH and morphotype (Hornbach et al., 2009; Schild et al., 2011). Moreover, the expressions of Sap7 and Sap8 do not correlate with virulence (Hornbach et al., 2009). That is why we did not include Sap7, Sap8, Sap9 and Sap10 respectively in our study.

The aim of this study was to: (i) examine the expression of aspartic proteases (Sap1 to Sap3 and Sap4 to Sap6) in vitro in neutral pH during morphogenesis under human serum influence by immunofluorescence and immunoelectron microscopy; (II) establish the relationship between isoenzymes expression and pleomorphism; (iii) determine the localization of Sap1-3 and Sap4-6 in pleomorphic cells of *C. albicans* by immunoelectron microscopy.

**Experimental**

**Materials and Methods**

**Strains and growth conditions.** The *Candida* strains used in this study are listed in Table I. The clinical isolate of *C. albicans* (strain 82) was recovered from the blood of 3-year-old patient being treated for an anaplastic ependymoma. In the study, we used ATCC SC5314 reference strain to analyse the conceivable differences in Sap1-3 and Sap4-6 expression profile appearing between pleomorphic forms and various *C. albicans* strains. The stock culture of examined strains was stored on ceramic beads (Microbank<sup>TM</sup>, Pro-Lab Diagnostics, Canada) at –70°C. Prior to the respective examinations,

<table>
<thead>
<tr>
<th>Designation</th>
<th>Clone or strain</th>
<th>Relevant characteristics or genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Wild type, clinical isolate</td>
<td>82</td>
<td>URA3/URA3</td>
<td>Staniszewska et al. (2011a)</td>
</tr>
<tr>
<td>Wild type, reference strain</td>
<td>ATCC 5314</td>
<td>URA3/URA3</td>
<td>Gillum et al. (1984)</td>
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</table>
routine culturing of strains for growth was conducted at 30°C for 18 h in extract-peptone-glucose broth medium YEPD [10 g yeast extract, 20 g peptone (BBL Trypticase Peptone, Becton Dickinson) and 20 g glucose, pH 5.7] (Ness et al., 2010).

Phenotypic and biochemical characterization. The presumptive identification of the clinical strain 82 was conducted using CHROMagar Candida medium (Becton Dickenson, Sparks, MD, USA), as described previously Staniszewska et al. (2011b). Colors of the colonies were compared in reference to the C. albicans SC5314 strain. The assimilation pattern of the isolate and the reference strains SC5314 was determined using the API 20C AUX identification system (BioMérieux, France). The API 20C AUX test was used according to the manufacturer’s instructions. Results of the test were obtained based on the numerical profile read-out (Analytical Profile Index; BioMérieux) (Stantiszewa et al., 2011a).

Molecular examination. DNA was extracted from blastoconidial cells of the clinical isolate as well as C. dubliniensis ATCC MYA 581 and C. albicans SC 5314 according to Yeast DNA Miniprep Protocol as described by Amberg and Burke (2005), and DNA quantification was performed using the NanoDrop ND 1000 spectrophotometer at an absorbance of 260 nm. Ribosomal DNA region including a fragment of the 5.8S rDNA gene (GenBank) was amplified by standard PCR. The primers (CALB1, CALB2) were used for species-specific PCR (Luo and Mitchell, 2002). The species-specific PCR products were electrophoresed as previously described Luo and Mitchell (2002). DNA bands were visualized using a transilluminator SYNGENE (Division of Synoptics LTD) under UV 260 nm. Results were documented by using the GenSnap program.

In vitro study of aspartic protease enzyme expression. The expression of Sap isoenzymes in C. albicans strain cultivated in YEPD medium and, subsequently, in filtered undiluted human serum was studied. Expression of Sap1-3 and Sap4-6 was monitored in blastoconidial cells, germ tubes, pseudohyphal and true hyphal forms. The isolation of Sap1-3 and Sap4-6 was monitored in blastoconidial cells of the clinical isolate as well as C. albicans SC 5314 by using the NanoDrop ND 1000 spec-trophotometer at an absorbance of 260 nm. Ribosomal DNA region including a fragment of the 5.8S rDNA gene (GenBank) was amplified by standard PCR. The primers (CALB1, CALB2) were used for species-specific PCR (Luo and Mitchell, 2002). The species-specific PCR products were electrophoresed as previously described Luo and Mitchell (2002). DNA bands were visualized using a transilluminator SYNGENE (Division of Synoptics LTD) under UV 260 nm. Results were documented by using the GenSnap program.

Induction of pleomorphic cells. Blastocandidal cells were grown as described Staniszewska et al. (2011a). Cells were observed under a phase-contrast microscope (Docuval, Carl Zeiss, Germany). Then, blastocandidia were harvested, washed three times with distilled water, pelleted by centrifugation (300 g for 10 min), and stored at –70°C for 96 h.

To induce remaining pleomorphic forms the blastoconidial cells suspensions in YEPD (50 µl) were transferred to 500 µl of filtrated undiluted human serum (pH 7.2–7.4) and incubated separately for 20 min (pre-incubation), 2 h (to induce germ tubes), 6 h (to induce pseudohyphae), 18 h (to induce true hyphae) at 37°C. Then, pleomorphic forms were harvested, washed, pelleted and stored as described above. Particular morphotypes were observed under a phase-contrast microscope (Docuval, Carl Zeiss, Germany). To examine cell morphology, the pleomorphic cells were fixed in 2.5% glutaraldehyde (Serva, Heidelberg, Germany), dehydrated in graded ethanol, critical point dried in CO₂, coated with gold and viewed in FEI Quanta 200 Scanning Electron microscope (Czech Republic) (Stantiszewa et al., 2011a).

Immunofluorescence microscopy (IFM) Leica TCS SP (Leica, Wetzlar, Germany). The specific anti-Sap2, anti-Sap3 and anti-Sap6 rabbit polyclonal primary antibodies generated by Chen et al. (2002) were used in the study. Then, each anti-Sap rabbit antibody was separately mixed with Candida cell wall suspension to prevent unspecific labelling. Subsequently, each antibody mixture was centrifuged at 10,000 g for 5 min.

For immunofluorescence staining of Saps the cryosection (Frigocut, model 2700, Reichert-Jung) of pleomorphic forms were blocked with donkey serum (1:20; Merck & Kollegen, Ochsenhausen, Germany) and incubated with anti-Sap polyclonal rabbit antibodies (1:100), followed by positive human Candida serum (1:60; Merck & Kollegen, Ochsenhausen, Germany). Antibodies were directed against Sap1-3 and Sap4-6. Samples were then incubated with donkey-anti-rabbit Cy5 (1:500; Merck & Kollegen, Ochsenhausen, Germany) and donkey-anti-human serum Cy3 (1:500; Merck & Kollegen, Ochsenhausen, Germany), respectively. This was followed by subsequent nucleus staining with Yopro (1:2000; Invitrogen, Karlsruhe, Germany).

Immunoelectron microscopy (IEM). Electron microscopy and postembedding immunogold labelling of sections of pleomorphic forms were performed as described by Schaller et al. (1998; 1999). In brief, each pellet was fixed in periodate-lysine-paraformaldehyde (PLP) and after embedding in Lowicryl K4M, the blocks were cut using an ultramicrotome (Ultracut; Reichert, Vienna Austria). Ultrathin sections (30 nm) were mounted on formvar-coated (Serva, Heidelberg, Germany) nickel or cooper grids (Stork Veco, Eerbeek, Netherlands) and incubated with anti-Sap polyclonal rabbit antibodies, directed against Sap1 to Sap3 or against Sap4 to Sap6 followed by 10 nm or 5 nm-gold-conjugated goat-anti-rabbit IgG (Dianova, Hamburg, Germany). In control samples, the primary antibody was omitted. For examination of Sap immunogold labelling, the transmission electron microscope Zeiss Libra 120 (Zeiss, Oberkochen, Germany) operating at 80 kV was used. Evaluation of data obtained from 250 randomly chosen cells was done by determining the intensity of staining with gold particles using a plus scale ranging from 1 (+) to 4 (++++), lack of staining was mark as minus (−).
Results

Identification of isolated strain. Colony color of the clinical strain 82 grown on CHROMagar<sup>®</sup> Candida medium was determined to be light green, which is indicative of <i>C. albicans</i> species. Results of this assay indicated that the strain was beta-N-acetylhexosaminidase positive. It was capable of assimilating trehalose (TRE) after 48 h and 72 h of incubation. D-Xylose (XYL), DL-lactate (LAC) and alpha-methyl-D-glucoside (MDG) assimilation was observed after 72 h of incubation. It utilized sucrose as a carbon source for growth, which is another typical feature of <i>C. albicans</i>. The clinical strain 82 and SC5314 reference strain were phenotypically similar in all experimental system described in this study.

Application of <i>C. albicans</i> specific primers (CALB1, CALB2) allowed detecting the expected product size (273 bp) of <i>C. albicans</i> isolate. Subsequently, DNA of strain 82, ATCC SC5314 strain, excluding <i>C. dubliniensis</i> MYA 581, gave readily a PCR product with CALB primers. Primers used in this study were determined to discriminate between <i>C. albicans</i> and <i>C. dubliniensis</i> (Fig. 1).

Detection of Sap1-3 and Sap4-6 antigens by immunofluorescence and immunoelectron staining techniques. Fractions of pleomorphic forms that were examined for their Saps expression are shown in Fig. 2. For strain 82, 100% of cells were found to form blastoconidia after 18 h of incubation in YEPD medium. In undiluted human serum, germ tube formation was observed at 2 h induction and approached 100%. Hyphae were formed as pseudohyphae (chains of elongated blastoconidial cells) at 6 h induction, with addition of budding blastoconidial cells and uniformly elongated the hyphal cells. True hyphal forms appeared as homogeneous fraction after incubation for 18 h in serum. No differences were observed between the clinical strain 82 and SC 5314 reference strain (data not shown) in the ability to form pleomorphic cells.

IFM and IEM were carried out for intracellular detection of Sap1-3 and Sap4-6 in <i>C. albicans</i> pleomorphic cells grown in Sap-inductive human serum and Sap non-inductive media – YEPD. Yeast cells cultivated in YEPD medium for 18 h as well as cells transferred to undiluted human serum for 20 min showed a lack of blue fluorescence protein of Sap1-3 and Sap4-6 expressing signal. After 2 h of incubation in Sap-inductive undiluted human serum, Sap labelling became more distinct. Analysis of the different pleomorphic forms cultivated in vitro demonstrated almost similar results for all tested Sap antigens (Table II).

<table>
<thead>
<tr>
<th>Pleomorphic cells</th>
<th>Immunogold labelling intensity (immunoelectron microscopy)</th>
<th>Immunofluorescence labelling intensity (fluorescence microscopy)</th>
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<tbody>
<tr>
<td></td>
<td>Sap1-3</td>
<td>Sap4-6</td>
</tr>
<tr>
<td>Blastoconidia&lt;sup&gt;1&lt;/sup&gt;</td>
<td>–</td>
<td>22&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blastoconidia&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
<td>12</td>
</tr>
<tr>
<td>Germ tube&lt;sup&gt;3&lt;/sup&gt;</td>
<td>++</td>
<td>29</td>
</tr>
<tr>
<td>Pseudohyphae&lt;sup&gt;4&lt;/sup&gt;</td>
<td>+++</td>
<td>30</td>
</tr>
<tr>
<td>True hyphae&lt;sup&gt;5&lt;/sup&gt;</td>
<td>+++</td>
<td>24</td>
</tr>
</tbody>
</table>

<sup>1</sup> blastoconidia cultivated in YEPD medium (pH 5.7) for 18 h at 30°C  
<sup>2</sup> blastoconidia transferred to undiluted human serum (pH 7.2–7.4) for 20 min at 37°C  
<sup>3</sup> germ tubes grown in human serum for 2 h at 37°C  
<sup>4</sup> pseudohyphae grown in human serum for 6 h at 37°C  
<sup>5</sup> true hyphae grown in human serum for 18 h at 37°C  
<sup>c</sup> number of analyzed cells  
<sup>u</sup> number of analyzed images  
– lack of Sap immunogold or immunofluorescence staining; from + to ++++ the intensity of Sap staining with gold particles or immunofluorescence. Cell and images were observed by three persons independently. The experiment repeated three times gave similar results.

Fig. 1. <i>Candida albicans</i> identification based on PCR amplification of the 5.8S rDNA gene fragment using the CALB1 and CALB2 primers.

(MM) Molecular marker mass; (lane 1) analysis of <i>C. dubliniensis</i> ATCC MYA 581 showed absence of PCR product size (273 bp); (lane 2) PCR product size (273 bp) of <i>C. albicans</i> SC5314; (lane 3) PCR product size (273 bp) of <i>C. albicans</i> clinical strain 82. PCR products were separated on agarose gel (0.8%) and stained with ethidium bromide.
The results obtained for Sap1-3 (Fig. 3) express nearly similar data obtained for remaining proteins i.e., Sap4-6. The increasing immunoreactivity of Sap1-3 and Sap4-6 was seen in germ tubes, pseudohyphae and true hyphae. On the other hand, there was more intensive Sap1-3 labelling than Sap4-6 in germ tubes, pseudohyphae and true hyphae. Lack of blue fluorescence protein expressing signal was seen in the control without anti-Sap rabbit polyclonal antibodies.

Sap1-3 and Sap4-6 immunoreactivity results obtained for IEM were similar as described for IFM studies (Table II). Sap immunogold labelling was positive for all pleomorphic cells incubated in human serum for 2, 6 and 18 h at 30°C. In contrast, isoenzymes were below the detection level (estimated as minus –) in all blastoconidial cells grown in YEPD medium for 18 h. Sap1-3 and Sap4-6 were found to be active at a neutral pH in Sap-inductive medium.

Immunogold labelling showed that in pleomorphic forms Sap localizes mainly in the cell wall and in the cytoplasm. Labelling with Sap antibodies directed against Sap1-3 or Sap4-6 demonstrated lack of reactivity in blastoconidial cells cultivated in YEPD medium and intensive immunoreactivity in cells grown in Sap-inductive medium (human serum). In pleomorphic forms grown in Sap-inductive medium, intensive labelling was observed, often seen as vesicles-associated gold particles. A correlation between germination and Sap1-3 and Sap4-6 expression was demonstrated. Control experiments without polyclonal antibodies showed a complete absence of immunogold labelling with Sap1-3 or Sap4-6. The intensity of Sap1-3 gold particles immunoreactivity (Fig. 4.) bears higher immunogold labelling in germ tubes, pseudohyphae and true hyphae compared with Sap4-6 (Table II). No evidence of gold particles in control cells (without polyclonal rabbit antibodies) was observed. Strain 82 and SC5314 reference strain (data not shown) showed similar Sap1-6 expression in experimental system described in this study.
In this study, the expression of Sap1-6 during *C. albicans* morphogenesis in undiluted human serum was evaluated. Our data indicate that (1) Sap1-3 and Sap4-6 are the isoenzymes whose expression was observed in germ tubes, pseudohyphae and true hyphae of *C. albicans* (2) Sap1-3 antigens expression was significantly raised during hyphae formation compared with Sap4-6.

Recent studies (Taylor *et al.*, 2000; Leinberger *et al.*, 2005; Okawa *et al*., 2007) demonstrated that conventional biochemical tests can misidentify clinical isolates. That is why, in this study, sequence analysis of the 5.8 rDNA region amplified by using the species-specific primer pair (CALB1 CALB2) (Luo and Mitchell, 2002) confirmed that the examined isolate belongs to the *C. albicans*. In our study, identification of the clinical isolate (strain 82) based on genotypic differences confirmed results obtained through phenotypic studies.

Many authors (Lermann and Morschhäuser, 2008; Naglik *et al*., 2008; Gropp *et al*., 2009; Dalle *et al*., 2010), included the reference strain SC5314 and its mutants in studying the roles of secreted Sap hydrolases in the pathogenesis process in humans. We selected the clinical isolate in purpose in view of inconciliable results referring to strain SC5314, which are presented below.

In this study, we compared Saps expression profile of pleomorphic forms of the *C. albicans* clinical isolate recovered from blood samples as well as SC5314, which was similar (data not shown).

Taylor *et al.* (2000) showed that *C. albicans* strain SC5314 well known from animal experiments is a poor colonizer and invader of mammalian epithelia. On the contrary, it was established (Schaller *et al*., 2000; Felk *et al*., 2002; Dalle *et al*., 2010), that this strain was able to invade the host tissues, which was followed by systemic dissemination, as well as it caused damage in an *in vitro* model. It may be said that the virulence (in view of the place of recovering) of the clinical isolate and the reference strain is comparable; both strains are virulent by intravenous challenge. The clinical strain was chosen because it caused candidaemia in the patient, proving that it develops virulence factors.

Previously, we demonstrated (Staniszewska *et al*., 2011a) that the clinical isolate showed virulence determinants, it produced germ tubes, pseudohyphae, and true hyphae in undiluted human serum. Furthermore, strain 82 showed distinct differences in activity profiles of hydrolytic enzymes between hyphae and blastoconidia by using the api*ZYM* test (Staniszewska *et al*., 2011b).

In the current work, the antibodies generated by Chen *et al.* (2002) were used. The authors highlighted...
Saps expression in *C. albicans* pleomorphic forms

Detection of Sap1-3 in pleomorphic cells of *Candida albicans* using polyclonal rabbit anti-Sap2 serum and goat-anti-rabbit IgG conjugated to 5 nm gold particles. (A) Cells cultivated in Sap non-inductive medium YEPD and (B-E) in Sap-inductive undiluted human serum for (B) 20 min, (C) 2 h, (D) 6 h and (E) 18 h. (A) For blastoconidial cells, the gold particle labelling intensity is evaluated as (−). The gold labelling was not visible in the cytoplasm neither cell wall. (B) Blastocconidial cells cultivated for 20 min in human serum. The gold particle labelling density is estimated as (+). In comparison to the cell wall (cw), labelling is seen mainly in the cytoplasm (arrows). (C) Germ tube forms. The gold particle labelling density is estimated as (++). Labelling is seen mainly in the cell wall (cw) (arrow). (D) Pseudohyphae. Note the cytoplasm-located clusters of the enzyme marker surrounded by a membrane-like structure (arrows). (E) True hyphae. In comparison to the cytoplasm, labelling is seen mainly in the cell wall (arrow). Enhanced clusters of gold particles in the cell wall are seen. The gold particle labelling density is estimated as (++++). (F) The immunoreactivity of Sap4-6 was less intensive compared with that of Sap1-3 (Table II).

The difficulty with generating specific and sensitive antibodies against each Sap. It was mentioned (Chen et al., 2002) that antibodies against Sap3 showed cross reactivity with Sap2 or Sap4. The cross reactivity of anti-Sap3 might have affected results obtained in the present study. That is why in our study, additionally anti-Sap2 and anti-Sap6 were used which reacted specifically with Sap1-3 or Sap4-6, respectively (Chen et al., 2002).

The immunofluorescence microscopy studies revealed that the pattern of enzyme expression in blastoconidia grown in YEPD medium differs significantly from other *C. albicans* forms cultivated in the human serum, possibly due to the fact that YEPD medium contains no suitable substrate for Sap. Higher level of Sap1-6 expression was correlated with the course of germination process and germ tubes, pseudohyphae and true hyphae appearance during incubation blastoconidial cells respectively for 2, 6 and 18 hours in human serum. Analysis using microscopy techniques determine that Sap expression profiles demonstrate significant differences between particular pleomorphic forms grown in human serum. Sap1-3 expression gradually increased in cells during germination and was much more intensive than Sap4-6. A previous report showed (Hube et al., 1994) that deletion of *SAP4-6* did not result in differences in hyphae formation both in vitro and in vivo.

Moreover, Felk et al. (2002) showed that *SAP4-6* expression is associated with, but not required for hyphal morphology. Our findings are in line with previous data (Felk et al., 2002), showing that Sap4-6 expression is related with germination process. Additionally, we showed that Sap4-6 are expressed in each morphotype (including blastoconidial cells) under human serum influence.

These results were consistent with the *in vivo* expression pattern (Staib et al., 2001) and suggested that expression of Sap1-6 proteins is regulated by factors that also regulate *C. albicans* morphology in human serum. Yet, information on secretion of particular Sap
isoenzymes by *C. albicans* germ tubes and pseudohyphae is still missing in the literature. In our study Sap1-6 expression was observed in both morphotypes (germ tubes; pseudohyphae).

Our data allow postulating that there is a correlation between human serum induced hyphae growth *in vitro* and expression of Sap1-6. Here, we showed that the presence of high level of Sap1-3 expression throughout 18-h-incubation in human serum supports the view that Sap1-3 are probably key proteases that promote cell growth and may be dependent on morphology. Although Sap1-3 expression level was higher than Sap4-6, the latter were consistently detected in germ tubes, pseudohyphae and true hyphae at steady levels, which may also support a contributory role of Sap4-6 in *C. albicans* cell growth and fitness. Our observation is not in line with results published previously (Correia *et al.*, 2010), which suggested that yeast growth may be protease independent when *C. albicans* cells were delivered directly into the bloodstream. On the contrary, Gropp *et al.* (2009) and Staib *et al.* (2001) showed that Sap2 is essential for growth when the protein is the only nitrogen source. Another support for our findings may be the previous report (Gropp *et al.*, 2009) showing the strong complement inhibitory activity of Sap1-3 in human plasma. In the present study, it was shown that the Sap1-3 expression was highly induced in *C. albicans* hyphal cells upon host body fluid (serum) influence suggesting that also *in vivo* each of the three Sap proteins may contribute to blood infection, complement inactivation and immune evasion (Gropp *et al.*, 2009). Here, we have provided evidence that the human serum plays a important role in hyphae formation and Sap1-3 expression. However, more investigations about the expression of SAP1-3 as well as SAP4-6 under human serum influence by using RT-PCR are going to be done.

In the current study, increasing Sap1-3 and Sap4-6 expression was detected during hyphae formation induced by shift of temperature (from 30°C to 37°C) and pH (from 5.7 to 7.2) in human serum. The same results were obtained for another clinical isolate (Taylor *et al.*, 2000) as well as SC5314 reference strain (data not shown). We did not observe differences between *C. albicans* strains due to pH changes. There was no Sap1-3 or Sap4-6 activity in yeast cells grown in medium at pH 5.7, while germ tubes, pseudohyphae and true hyphae expressed these isoenzymes at a neutral pH (7.2–7.4). These characteristics may indicate the ability of *C. albicans* to survive and cause infection in a variety of host tissues.

The results obtained for the expression pattern of the immunofluorescence study were confirmed by immunoelectron microscopy studies (IEM). The use of antibodies raised against Sap1-3 or Sap4-6 made it possible to determine the precise location of these proteins in the pleomorphic forms that have been pre-cultured in human serum. Sap proteins were detected inside the cytoplasm and within the cell wall of blastoconidia, germ tubes, pseudo- and true hyphae forms, but not in the surrounding of the cell wall, which was confirmed by the negative control (non-antigens in the surrounding of the cell wall). The Sap1-3 and Sap4-6 were observed to be organized in groups and packed with the vesicles localized in the cytoplasm. The results of our study are in line with those of Stringaro *et al.* (1997) demonstrating that during murine vaginitis Sap antigen is located within the cell wall of hyphal cells. Similar results were observed in the *in vitro* model of experimental oral candidiasis (Stringaro *et al.*, 1997) and reconstituted human epidermis (Schaller *et al.*, 2000).

Our studies may support the data of Brown (2002) that both blastoconidia and filamentous forms of *C. albicans* are pathogenic and both contribute to different stages in the establishment and progress of the infection. Sap1-6 proteins tested in the above-mentioned study were determined to be expressed in pleomorphic forms during incubation in human serum which mimics *in vivo* conditions encountered during systemic blood infections. These data may indicate an important role of Sap1-6 proteins during blood infections and immune evasion (Hornbach *et al.*, 2009). We therefore conclude that there is a correlation between the increased expression of Sap1-6 proteins and germ tubes, pseudohyphae and true hyphae formation.

Acknowledgement

Martin Schaller was supported by the Deutsche Forschungsgemeinschaft (Sch 897/3, SFR773 Z2, graduate college 685), the BMBF (MedSys 0315409B) and by a NIDCR grant R01DE017514-01. Monika Staniszewska and Wiesław Kurzątkowski were supported by grant N N404 113639 from the Ministry of Science and Higher Education.

We thank Birgit Fehrenbacher, Renate Nordin, Helga Möller and Hannelore Bischof, Universität Tübingen, for excellent technical assistance.

We thank Prof. D.D. Dzierżanowska-Madalina for providing *C. albicans* clinical isolate.

Literature


**Introduction**

Organochlorine pesticides are one of the major groups of chemicals being extensively used in agriculture and responsible for environmental contamination and ecological imbalance (Tiemann, 2008). Endosulfan is a mixture of two stereoisomers (α- and β-endosulfan) which differ dramatically in their physicochemical and environmental properties, in a ratio of 7:3 and registered with several trademarks, such as Thimol, Cyclodan, Thiodan, Malix, and etc. It is extensively used throughout the world to control insect pests and mites of different crops, such as cereals, cotton, tea, fruits and vegetables (Weber et al., 2009). Due to its hydrophobic nature, abundant application and environmental transportation, endosulfan contamination has been frequently detected in soils, sediments, waters, air, food products and even the environment at considerable distance from the application point such as the Arctic (Kaushik et al., 2010). Furthermore, it is extremely toxic to aquatic fauna, and its acute and chronic toxicity is well known in a variety of mammals including human beings (Weber et al., 2009). These health and environment concerns have led to an interest in degradation and detoxification of endosulfan.

Natural degradation of endosulfan under alkaline conditions or photooxidation by UV light is not sufficient for removal of endosulfan and its derivatives from the environment (Kwon et al., 2005). The environmental degrading pathways include hydrolysis of the sulfur moiety to nontoxic endosulfan diol and oxidation to endosulfan sulfate. Endosulfan diol can be further transformed to less or non-toxic metabolites, such as endosulfan ether, endosulfan hydroxyether and endosulfan lactone. Theoretically, formation of endosulfan diol via hydrolysis might be an important detoxification way (Shivaramaiah et al., 2005). In contrast, endosulfan sulfate is produced only through biological transformation, and it is more toxic and persists longer than the parent isomers. Consequently, production of endosulfan sulfate becomes the major concern of endosulfan degradation.

Biodegradation is recognized as a promising and attractive operational means to strengthen remediation performance and has been demonstrated to successfully enhance the degradation and removal of endosulfan.
Materials and Methods

Chemicals and medium. The organic pesticide endosulfan (99.5%) and its metabolites were purchased from Sigma Aldrich (Shanghai, China). Dichloromethane and acetone used in extraction and gas chromatography analysis were purchased from Zhuoyue Chemical Co. (Jiangsu, China). The compositions of the medium used were as follows – minimal salts medium (MSM, g/l): MgCl$_2$ 0.2, NH$_4$NO$_3$ 1, KH$_2$PO$_4$ 2, K$_2$HPO$_4$ 7.5, NaCl 1, pH 6.8. For solid medium, 1.5% (w/v) agar was added to the liquid MSM. Endosulfan was dissolved in acetone at a concentration of 10$^4$ mg/l and added to the medium at appropriate concentration after sterilization.

Enrichment and isolation of endosulfan-degrading bacteria. Sludge sample was collected from a sewage outfall in Tonglu Pesticides Company, Zhejiang Province. 5 g sample was taken in a 250 ml Erlenmeyer flask containing 50 ml of the MSM and 50 mg/l of endosulfan and was incubated at 28°C with continuous shaking (160 rpm). After 7 d, 5 ml of culture was re-inoculated into 50 ml of fresh medium with 100 mg/l endosulfan and was cultured under same conditions. This process was repeated three more times. Four weeks later, dilution of the consortia was spread onto the selective MSM agar plates containing 100 mg/l of endosulfan and incubated at 28°C for 72 h. Well separated colonies were picked and repeatedly transferred onto the same solid media until obtaining pure culture.

Characterization of isolates. Phenotypic and biochemical characterization was performed according to Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994). The 16S rRNA genes of bacteria were amplified and analyzed as described by Yu et al. (2008). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Tamura et al., 2007), and the dataset was bootstrapped 1000 times.

Degradation of endosulfan by S. sp. LD-6. Cells were grown in 50 ml of Luria-Bertani (LB) broth at 30°C, harvested by centrifugation at 10,000 g for 8 min, and washed twice with 10 mM phosphate buffer (pH 6.8). The washed cells were inoculated into the MSM containing 100 mg/l of endosulfan, which resulted in an optical density of 0.3–0.5 at 600 nm. Cultivation was conducted at 30°C in a rotary shaker (160 rpm) for 10 days, and endosulfan and its metabolites were determined at 2-day intervals. pH was measured according to the standard method (APHA, 2005). To evaluate the effect of adsorption, the experiments were repeated with heat-killed cells as described by Kwon et al. (2002). Effects of incubation conditions (shaking versus static) and temperature on biodegradation were examined according to the methods described by Hussain et al. (2007). Above experiments were carried out in triplicate.

Gas chromatographic (GC) and GC-MS analysis. Endosulfan and metabolites were analyzed by gas chromatography (GC-14B, Shimadzu) equipped with electron capture detector. The supernatant was saturated with NaCl and extracted three times with an equal volume of dichloromethane. The organic phases were combined and dehydrated by addition of anhydrous Na$_2$SO$_4$. After drying under a stream of N$_2$, the sample was redissolved in acetone and stored at 4°C before analysis. The experiment was carried out in triplicate. Samples (1 µl) were injected in splitless mode into a capillary column (DB-5, length 30 m, ID 0.2 mm, film thickness 0.25 µm). The carrier gas was N$_2$. Injector and detector temperatures were 300°C and 320°C, respectively. The temperature program used was 150°C for 1 min, then 300°C at 10°C min$^{-1}$ and a 1-min hold at 300°C. All GC-MS analyses were conducted with a gas chromatograph (Shimadzu GC-2010) equipped with mass spectrometer (GCMSQP2010), auto injector (AOC-20i) and a DB-5MS capillary column (ID 0.25 mm, film thickness 0.25 µm), coupled to MS via direct interface. Helium was used as carrier gas with a flow rate of 1.0 ml/min. GC injector temperature was held at 300°C, and the column temperature was programmed as of GC. The electron impact mass spectra were obtained at 70 eV and monitored in the range of 50 to 400 m/z.

Enzymatic degradation. Bacterial cells grown in LB media were harvested at log phase by centrifugation at 12,000 g for 15 min at 4°C. The bacterial pellets were washed twice with distilled water and then suspended in 3 ml of phosphate buffer (0.05 M, pH 6.8). Localization of degrading enzymes was conducted by the method of osmotic shock (Huang et al., 2007). To determine whether enzymes responsible for endosulfan biodegradation were inducible or constitutive, the method described by Yu et al. (2008) was used. The reaction mixture (1.0 ml) contained phosphate buffer (0.05 M, pH 6.8), endosulfan (50 mg/l) and cell crude extract. Reactions were performed at 30°C for 45 min.
without shaking, and the residual endosulfan was quantified by the GC method. All experiments were performed in triplicate.

Biodegradation of endosulfan in soil. Biodegradation of endosulfan in soil by S. sp. LD-6 was examined as described by Huang et al. (2007). Soil sample (5–15 cm soil layer) was obtained from a local farm, Linan. The soil was a sandy loam (sand 47%, silt 35%, clay 18%) with organic matter 4.74%, and a pH of 5.4. The soil has never been treated with endosulfan. The solution of endosulfan was added into the samples to give a concentration of 50 mg/kg. One set of fresh soil and sterile soil were inoculated with the strain (1 × 10^8 cells/g). Another uninoculated set was kept as a control. The inocula were thoroughly mixed with the soils under sterile conditions, and soil moisture was adjusted to 60% (w/w). Each sample was incubated under aerobic conditions at 25°C in the dark, and 5 g soil was collected at 7-day intervals and further analyzed as reported by Li et al. (2009). The experiment was carried out in triplicate.

Nucleotide sequence accession number. The 16S partial sequence of the isolated strain LD-6 was deposited in the GenBank database under accession number JQ670922.

Results and Discussion

Identification of endosulfan-degrading isolate. After selective enrichment, a total of 17 isolates were selected. They all exhibited similar characteristics: smooth and yellowish color on nutrient agar; short rod-shaped morphology; negative for Gram stain, gelatin hydrolysis, starch hydrolysis, H₂S, oxidase and dehydrogenases; while positive for catalase. One representative strain, designated as LD-6, from among those with the best endosulfan metabolism (data not shown) was chosen for further characterization as follows: growth on glucose without acidification, using xylose as a carbon source, reduction of nitrate to nitrite, positive for growth at 4°C, methyl red and citrate but negative for growth at 40°C, indole, and the Voges-Proskauer test. The 16S rDNA sequence alignment and phylogenetic analysis (Fig. 1) revealed that strain LD-6 was homologous with Stenotrophomonas spp. The organism that exhibited the highest level of homology (99%) was S. rhizophila. From these results, LD-6 was identified as Stenotrophomonas sp.

Degradation of endosulfan by S. sp. LD-6. The degradation assay revealed the bacterium could utilize endosulfan as the sole source of carbon and sulfur. Endosulfan was rapidly degraded from 100 to 28.2 mg/l (decrease of 71.8%) during the initial 4 days with increase of biomass (data not shown). By day 10, the substrate was completely degraded with an overall degradation rate of 10.0 mg/(l·day) (Fig. 2). Furthermore, degradation rates for α- and β-endosulfan were 6.8 and 3.2 mg/(l·day), respectively. This is in accordance with previous studies which revealed the degradation of α-endosulfan is faster than that of β-endosulfan (Kwon et al., 2002). The reason for above observations is still not well understood. Our finding that strain LD-6...
is capable of degrading endosulfan is another instance of the genus *Stenotrophomonas* with regard to endosulfan biodegradation. Moreover, heat killed LD-6 did not have any effect on the degradation of endosulfan (data not shown), suggesting that the decrease was not due to adsorption.

Accumulation of endosulfan sulfate was not detected in this work, but endosulfan diol and endosulfan ether appeared as detected by the GC analysis using authentic standards and matching the retention times. During the degradation, the culture pH decreased to 6.5. These results are in accordance with previous studies which revealed that some bacteria, such as *K. pneumoniae*, *Pseudomonas fluorescens*, *Arthrobacter* sp., and other Gram negative rods, form endosulfan diol, not endosulfan sulfate, while the pH value decreased (Kwon et al., 2002). Furthermore, these results suggested that the metabolites of endosulfan and the degrading mechanism might be independent of culture pH.

A significant difference between biodegradation of both α- and β-endosulfan by the strain was observed under static versus shaking incubation (Table I). Maximum biodegradation of α- and β-endosulfan (up to 98.7%) was recorded under shaking conditions, however, the counterpart was only 66.9% as found under static conditions. Better bioavailability of endosulfan to the microbes coupled with physiochemical degradation might explain the phenomenon to some extent. Furthermore, non-biological degradation could not be neglected and was more obvious under shaking conditions, which implied that aerobic conditions are relatively more conducive for abiotic degradation.

Biodegradation of α- and β-endosulfan by the bacterium was also investigated at different incubation temperatures. Biodegradation of both isomers of endosulfan was relatively greater at an incubation temperature range of 25–35°C, with a maximum at 30°C (data not shown). This was logical, because the optimum growth temperature of the strain is around 30°C. The minimum degradation of spiked endosulfan was recorded at 40°C. Besides, abiotic degradation was more pronounced at higher incubation temperatures.

**Enzymatic degradation.** Results of GC analysis indicated that no objective substance could be detected in the reaction mixture containing the intracellular fraction solution taken at 10 h. Furthermore, there was no downtrend of endosulfan content in the mixture containing the extracellular and membrane fraction solutions. These results suggested that the enzymes involved in the initial degradation of endosulfan in LD-6 were intracellular. As shown in Fig. 3, 94.1% of the α-endosulfan and 68.1% β-endosulfan were degraded by cell crude extract with induction, respectively. Similarly, 95.4% of the α-endosulfan and 69.0% β-endosulfan were degraded without induction. In controls, biodegradation was negligible. These results showed that there was no significant discrepancy in endosulfan degradation between induced and

<table>
<thead>
<tr>
<th>Treatment</th>
<th>α-endosulfan (%)</th>
<th>β-endosulfan (%)</th>
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<tbody>
<tr>
<td></td>
<td>Static</td>
<td>Shaking</td>
</tr>
<tr>
<td>LD-6</td>
<td>98.7 (1.4)</td>
<td>66.9 (2.6)</td>
</tr>
<tr>
<td>Uninoculated control</td>
<td>9.8 (2.7)</td>
<td>6.3 (1.1)</td>
</tr>
</tbody>
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The values are the average of three independent experiments, and the numbers in parentheses indicate the standard deviation (the same below).

*Shaking at 160 rpm.*
Endosulfan-degrading *Stenotrophomonas* sp. strain 4261 non-induced cells, indicating that enzymes responsible for endosulfan biodegradation might be constitutively expressed. Moreover, the optimum degrading temperature and pH value were 30°C and 6.6, respectively (data not shown).

Biodegradation of endosulfan in soil. The *in situ* endosulfan-degrading ability of LD-6 was tested, and degradation patterns of α- and β-endosulfan in soil are presented in Fig. 4. Only 7.1 mg/kg α-endosulfan and 3.6 mg/kg β-endosulfan were removed in uninoculated fresh soil after 28 days of incubation. In contrast, 24.2 mg/kg α-endosulfan and 10.1 mg/kg β-endosulfan were removed after 28 days for fresh soil inoculated with strain LD-6 (Fig. 4). Obviously, endosulfan removal was enhanced while the bacterium was inoculated. Moreover, 20.4 mg/kg α-endosulfan and 8.8 mg/kg β-endosulfan were removed from sterilized soils. Similarly, the removal in fresh uninoculated soil was superior to that in the uninoculated sterile soil.

Successful biodegradation of endosulfan from soil by isolated bacteria has been reported previously. For example, a strain, *Achromobacter xylosoxidans* CS5, originally isolated from activated sludge was examined for biodegradation. Inoculation of the strain was found to promote the removal of endosulfan in soil as described by Li *et al.* (2009). In the case of the experiment done by Arshad *et al.* (2008), *P. aeruginosa* degraded more than 85% of spiked α-endosulfan and β-endosulfan (100 mg/l) after 16 days in loam soil. Kumar and Philip reported that endosulfan was effectively degraded both in miniature and bench scale soil reactors (Kumar and Philip, 2006). In this study, addition of strain LD-6 to soil supplemented with endosulfan resulted in a higher removal rate than that observed in uninoculated soils. Meanwhile, endosulfan removal was slightly better in fresh soil inoculated with the bacteria than in inoculated sterilized soil suggesting a contribution of the indigenous flora to endosulfan removal. The data generated from this study could improve current understanding on endosulfan biodegradation.

Conclusions. In general, 17 isolates were obtained from the enrichment culture. One highly efficient aerobic degrader, strain LD-6, was selected for further examination and identified as *Stenotrophomonas* sp. The strain could utilize endosulfan as the sole source of carbon and sulfur, and 100 mg/l endosulfan could be fully

![Fig. 4. Biodegradation of endosulfan by S. sp. LD-6 in soil.](image)
degraded within 10 days. With GC-MS analysis, endosulfan diol and endosulfan ether were detected as major metabolites. The data indicates that the bacterium might degrade endosulfan by a non-oxidative pathway. Biodegradation of both isomers was relatively better at a temperature range of 25–35°C, with a maximum at 30°C. Cell crude extract of LD-6 could metabolize endosulfan rapidly. Enzyme distribution experiment showed that degradative enzymes in the strain were endoenzymes and constitutively expressed. Furthermore, inoculation of strain LD-6 was found to accelerate the removal of endosulfan in situ. The data generated from this study could improve current understandings on endosulfan biodegradation. However, further researches are still needed before practical application, such as influence of multiple parameters on bioremediation and toxicological assessment of the bacterium.

Acknowledgments
This work was supported by China National Natural Science Foundation (Grant No. 31100087), four grants (No. Y3100018, No. 2010C12001, No. 201100305 and No. 2011G23065) from Zhejiang Provincial Government, Science and Technology Support from Ocean Fishery Science and Technology in the Most Important Subjects of Zhejiang (Grant No. 20110217). We are grateful for their financial supports.

Literature


**Edwardsiella ictaluri** is a Gram-negative bacterium and the causative agent of enteric septicemia of catfish. In this study, we examined the expression and function of the LuxS from a pathogenic *E. ictaluri* strain, J901. J901 was found to produce autoinducer 2 (AI-2) activity that maximized at mid-logarithmic phase and was enhanced by glucose and repressed by high temperature. Consistently, a luxS gene (*luxS*) was identified in J901, whose expression was regulated by cell density, glucose, and temperature in a manner similar to that observed with AI-2 activity. Further analysis showed that LuxS_Ei is a biologically active AI-2 synthase that was able to complement the luxS-defective phenotype of *Escherichia coli* DH5α. To examine the functional importance of LuxS_Ei, a genetically modified variant of J901, J901Ri, was constructed, in which *luxS* expression was blocked by RNA interference. Compared to the wild type, J901Ri was (i) reduced in AI-2 activity to a level of 59% of that of the wild type; (ii) impaired in both planktonic and biofilm growth; (iii) significantly attenuated in the ability to infect cultured fish cells and to cause mortality in infected fish; (iv) unable to induce the expression of certain virulence-associated genes. Addition of exogenous AI-2 failed to rescue the growth defect of J901Ri as free-living cells but restored biofilm production and the expression of virulence genes to levels comparable to those of the wild type. Taken together, these results indicate that LuxS_Ei is a functional AI-2 synthase that is required for optimal cellular growth and host infection.

### Key words: Edwardsiella ictaluri, AI-2 activity, RNA interference, quorum sensing, virulence
AI-2 and LuxS are widespread in diverse bacteria, and the AI-2 molecule produced by one bacterial species can be sensed and responded to by different bacterial species, probably because the basic chemical structure of AI-2 is highly conserved (Surette and Bassler 1999; Waters and Bonnie 2005). For this reason, AI-2 has been called an interspecies communication signal. Accumulating evidences have indicated that LuxS/AI-2-mediated quorum sensing regulates many aspects of bacterial growth and infection, notably biofilm formation, motility, antibiotic susceptibility, and virulence development (Ahmed et al., 2007; Coulthurst et al., 2004; Coulthurst et al., 2007; Gonzalez Barrios et al., 2006; Herzberg et al., 2006; Kong et al., 2006; Parsek and Greenberg 2005; Rickard et al., 2006). In addition to participating in quorum sensing, LuxS is also involved in the activated methyl cycle by recycling SAH to homocysteine and thus contributes directly to metabolism (De Keersmaecker et al., 2006; McNab et al., 2003; Vendeville et al., 2005).

Recently, the genome sequence of E. ictaluri 93–146 has been completed, which reveals the existence of a luxS homolog (Williams et al., 2012). However, the function of E. ictaluri LuxS is not known. In this study, we cloned and analyzed the luxS gene, luxS<sub>Ei</sub>, from a pathogenic E. ictaluri strain J901. We found that LuxS<sub>Ei</sub> likely plays a role in both cellular metabolism and pathogenicity, the latter via AI-2-mediated quorum sensing system.

**Experimental**

**Materials and Methods**

**Bacterial strains and growth conditions.** Edwardsiella ictaluri J901, a pathogenic strain isolated from catfish, was purchased from Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). *Escherichia coli* DH5α was purchase from Takara (Dalian, China). *Vibrio harveyi* BB120 and BB170 were purchased from American Type Culture Collection (ATCC, USA). All strains were cultured in Luria-Bertani (LB) medium at 37°C (for E. coli) or 28°C (for all others). Where indicated, glucose was added at a final concentration of 0.5%, and ampicillin and polymyxin B were added at 100 μg/ml and 100 μg/ml respectively. The mean generation time (g) of bacterial growth was calculated as described by Eagon (1962).

**AI-2 assay.** AI-2 assay was performed according to Surette and Bassler (1999). To prepare cell-free culture supernatant, bacterial cells were grown in LB medium at 28°C for overnight, and the culture was diluted 1:100 in fresh LB medium. Two milliliters of cell culture were taken every 30 minutes, and the cell-free supernatant was obtained by centrifugation and then filtering through a 0.22-μm filter (Millipore, Billerica, MA, USA). For measurement of bioluminescence induction, overnight culture of the *V. harveyi* strain BB170 grown in AB medium at 28°C was diluted 1:5000 in fresh AB medium supplemented with cell-free culture fluids (10%) of the tested strains or with the growth medium (as the control). The growth was continued and light production was measured using a Glomax luminometer (Promega, Madison, WI, USA).

**Cloning of luxS<sub>Ei</sub>** The primers used in this study are listed in Table I. luxS<sub>Ei</sub> was cloned by PCR with primers F5/R6 designed according to the luxS sequence of *E. ictaluri* 93–146 (GenBank accession no. CP001600). The sequence of luxS<sub>Ei</sub> has been deposited in GenBank database under the accession number JQ272177.

**Plasmid and strain constructions.** pBTEiS, which expresses luxS<sub>Ei</sub>, was constructed as follows. luxS<sub>Ei</sub> was amplified by PCR with primers F5/R6, and the PCR products were ligated into the TA cloning vector pEASY-T1 (Transgen, China) resulting in pESEiS, which was digested with EcoRV, and the luxS<sub>Ei</sub>-containing fragment was inserted into pBT (Zhang et al., 2008) at the SmaI site, resulting in pBTEiS. To construct pIRSi, which expresses antisense luxS<sub>Ei</sub> under the trc promoter, the antisense strand of luxS<sub>Ei</sub> was amplified with primers F5/R5, and the PCR products were ligated into pBT as above, resulting in pBTSi, which was digested with SwaI, and the fragment containing the P<sub>trc</sub>-luxS<sub>Ei</sub> antisense RNA was inserted into the EcoRV site of pJR (Zhang et al., 2008), resulting in pIRSi. pIRSi and the control vector pJR were introduced separately into J901 via conjugation as described previously (Sun et al., 2009), and the transformants were named J901Ri and J901C respectively. J901C was used as control of J901Ri.

**Quantitative real time reverse transcriptase-PCR (qRT-PCR).** qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously (Zheng et al., 2010). PCR efficiency (99.9%) was determined as described previously (Zheng and Sun 2011). Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The expression level of the target gene was analyzed using comparative threshold cycle method (2<sup>ΔΔCT</sup>) with 16S rRNA as the control. The PCR primers for 16S rRNA gene (16SF/16SR), luxS<sub>Ei</sub> (LuxSF/ LuxSR4), and the virulence-associated genes are listed in Table I.

**Complementation by exogenous AI-2.** J901 was cultured in LB medium to OD<sub>600</sub> of 0.85, and cell-free culture supernatant (prepared as above) was added at a final concentration of 10% to the growth medium of bacterial cells under various examination.
Biofilm production analysis. Biofilm formation on a polystyrene surface was determined exactly as described by Xu et al., (2006). Briefly, cells were cultured in LB medium to exponential phase and diluted 1:100 into fresh LB. The diluted cultures were transferred into a 96-well polystyrene plate. After incubating at 28°C for 24 h, the plate was washed with PBS and the attached cells were treated with Bouin fixative and stained with 1% crystal violet. The unbound dye was removed by rinsing the plate several times with running water. The bound dye was eluted in ethanol, and the eluates were measured for absorbance at A570.

Infection of zebrafish. J901Ri and J901C were cultured to an OD600 of 0.8 in LB medium, washed with PBS, and resuspended in PBS to 1 × 10⁸ CFU/ml. Zebrafish (0.3 ± 0.05 g) were purchased from Nanshan Market (Qingdao, China) and maintained at 25°C in aerated freshwater that was changed daily. Fish were divided randomly into two groups (N = 30) and anaesthetised by immersion in a 100 µg ml⁻¹ solution of tricaine methane sulfonate (MS-222, Sigma, St Louis, MO, USA) before injection. The fish were injected intraperitoneally (i.p.) with 10 μl J901Ri or J901C and monitored for mortality for 15 days post-infection. The animal experiments were conducted in accordance with the “Regulations for the Administration of Affairs Concerning Experimental Animals” promulgated by the State Science and Technology Commission of Shandong Province.

Infection of ZF4 cells. Infection of ZF4 cells was performed as follows. J901Ri and J901C were cultured in LB medium to an OD600 of 0.80, washed with PBS, and resuspended to 1×10⁶ CFU/ml in DMEM-F12 medium (Thermo Scientific HyClone, Beijing, China) supplemented with 10% fetal calf serum. ZF4 cells were purchased from ATCC and maintained at 28°C in 96-well culture plates containing DMEM-F12 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin according to ATCC recommendations. The cells were grown to monolayer and added with J901Ri or J901C (100 μl/well). The plates were incubated at 28°C for 0.5 h, followed by washing 3× with PBS to remove unattached bacteria. To determine the number of bacterial cells associated with ZF4 cells, the washed ZF4 cells were lysed with 1% Triton X-100, and the lysates were diluted in LB medium and plated in triplicate on LB agar plates supplemented with ampicillin and polymyxin B. After
incubation at 28°C for 32 h, the colonies that appeared on the plates were counted. The genetic nature of the colonies was verified by PCR and subsequent sequence analysis of selected PCR products. Each assay was performed in triplicate.

**Statistical analysis.** All statistical analyses were performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data were analyzed with analysis of variance (ANOVA) and expressed as means ± SE. In all cases, statistical significance was defined as $P < 0.05$.

**Results**

**Regulated production of AI-2 in *E. ictaluri* J901.** To examine whether *E. ictaluri* J901 produced active AI-2, the strain was cultured in LB medium to different growth phases and examined for AI-2 activity by measuring the ability of the cell-free culture supernatant to stimulate bioluminescence in BB170, a reporter strain that is defective in AI-1 response but able to respond to AI-2 (Surette and Bassler 1999). The results showed that the AI-2 activity, as a reflection of stimulated bioluminescence production in the reporter strain, of J901 increased with cell density until OD$_{600}$ of 1.0, where peak activity was reached, and then declined at OD$_{600}$ of 1.2 (Fig. 1). However, at each of the examined point, the amount of light production induced by the culture supernatant of J901 was much lower than that induced by the supernatant of BB120, which is the parental strain of BB170. Compared to growth in standard LB medium at 28°C, growth in LB medium at 37°C decreased the AI-2 activity of J901 by 139 fold, whereas growth in LB medium supplemented with glucose increased AI-2 activity by 2.4 fold (Fig. 2).

**J901 possesses a luxS gene that encodes an active AI-2 Synthase.** The above results suggested the existence in J901 of a luxS gene, which was subsequently cloned and named lux$S_{Ei}$. Sequence analysis showed that lux$S_{Ei}$ encodes a protein of 171 amino acid residues that differs in two residues from the reported LuxS of *E. ictaluri* 93–146 (GenBank accession no. ACR70372.1) and shared 94% identity to the LuxS of *Edwardsiella tarda*. Since AI-2, the product of LuxS, is known to be an interspecies signaling molecule and able to function in a cross-species manner in many bacteria, we examined the activity of Lux$S_{Ei}$ by determining its ability to complement DH5α, which lacks a functional luxS gene and consequently exhibits an AI-2-defective phenotype (Surette and Bassler 1999). For this purpose, the plasmid pBTEiS, which expresses lux$S_{Ei}$, was constructed. *E. coli* DH5α was transformed with...

![Fig. 1. AI-2 activity detection.](image)

Cell-free culture supernatants of *Edward ictaluri* J901, DH5α/pBTEiS, DH5α/pBT, and *Vibrio harveyi* BB120 were taken at various growth points and assayed for AI-2 activity by determining the ability of the supernatant to stimulate light production in BB170. Data are presented as means ± SE (N = 3). **, $P < 0.01$.

![Fig. 2. AI-2 activity of *Edward ictaluri* J901 under different growth conditions.](image)

J901 was assayed for AI-2 activity after culturing in LB medium to OD$_{600}$ = 0.55 under various conditions, i.e., with or without 0.5% glucose, at 37°C, or at 28°C (control). AI-2 activity is presented as fold induction of light production over the control. Data are presented as the means ± SE (N = 3). **, $P < 0.01$. 

Fig. 1. AI-2 activity detection.

Fig. 2. AI-2 activity of *Edward ictaluri* J901 under different growth conditions.
Expression of luxS<sub>Ei</sub> is regulated by growth phase and growth condition. Since, as shown above, AI-2 activity in J901 was regulated by growth phase and growth condition, we examined whether luxS<sub>Ei</sub> expression was similarly regulated. For this purpose, J901 was cultured to OD<sub>600</sub> of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 respectively, and luxS<sub>Ei</sub> expression was determined by qRT-PCR. The results showed that luxS<sub>Ei</sub> expression increased with cell density and reached maximum at OD<sub>600</sub> of 0.8, after which point luxS<sub>Ei</sub> expression dropped to levels comparable to that at OD<sub>600</sub> of 0.2 (Fig. 3A). Compared to J901 cultured in standard LB medium at 28<sup>°</sup>C, J901 cultured in LB medium containing glucose exhibited significantly increased luxS<sub>Ei</sub> expression, whereas J901 cultured in LB medium at 37<sup>°</sup>C exhibited significantly decreased luxS<sub>Ei</sub> expression (Fig. 3B). These results, together with those that showed regulated AI-2 production as observed above, indicate a correlation between luxS<sub>Ei</sub> expression and AI-2 activity.

Effect of interference with luxS<sub>Ei</sub> expression. (i) Effect on AI-2 activity. To examine the functional importance of LuxS<sub>Ei</sub>, we constructed a J901 variant, J901Ri, in which luxS<sub>Ei</sub> expression was interfered by antisense RNA. We also tried but failed to create a luxS<sub>Ei</sub>-defective mutant via targeted mutation of the luxS<sub>Ei</sub> gene, which suggests a possibility of LuxS<sub>Ei</sub> being essential to the fundamental physiology of J901. qRT-PCR analysis showed that when the cells were cultured in LB medium at OD<sub>600</sub> of 0.8, which, as shown above, was the point where peak induction of luxS<sub>Ei</sub> was observed, luxS<sub>Ei</sub> expression in J901Ri was significantly (P < 0.01) decreased to a level that was approximately 13% of that in the control strain J901C or in the wild type J901, the latter two exhibited comparable levels of luxS<sub>Ei</sub> expression. These results indicate that, as designed, luxS<sub>Ei</sub> expression in J901Ri was markedly reduced. Subsequent AI-2 assay showed that the AI-2 activity of J901Ri was approximately 41% lower than that of J901C. Hence, interfering with luxS<sub>Ei</sub> expression resulted in reduced AI-2 production.

(ii) Effect on growth. Growth analysis showed that when cultured in standard LB medium and in iron-depleted LB medium caused by the presence of 50 µM iron-chelator 2,2′-dipyridyl, the mean generation time (g) values of J901Ri were 150 min and 222 min respectively, while the g values of J901C were 132 min and 169 min respectively under these conditions (Fig. 4A). Likewise, biofilm growth analysis showed that compared to J901C, J901Ri exhibited significantly reduced ability to form biofilm on polystyrene surface (Fig. 4B). To examine whether the growth defect was caused by interfered quorum sensing, AI-2-containing culture supernatant was added to J901Ri. The results showed that the presence of exogenous AI-2 failed to restore the growth of J901Ri in LB medium but rescued the defect of J901Ri in biofilm production (Fig. 4B and data not shown), suggesting that AI-2-mediated signaling is required for biofilm growth but not for planktonic growth.

Effect on infection of zebrafish and ZF4 cells. To examine whether reduced luxS<sub>Ei</sub> expression had any effect on virulence, the infectivity of J901Ri was determined using both live zebrafish and cultured zebrafish cells. For live fish infection, zebrafish were infected i.p. injection with the same dose of J901Ri or J901C and monitored for mortality. The results showed that mortality began to occur at 3 days post-infection in...
both J901Ri- and J901C-infected fish, however, J901C-infected fish reached 100% accumulative mortality by 5 days post-infection, while J901Ri-infected fish exhibited an accumulative mortality rate of 40% (Fig. 5). To examine the infectivity of J901Ri at cellular level, ZF4 cells (a cultured zebrafish cell line) were treated with J901Ri or J901C for 0.5 h, and cellular infection was subsequently examined by bacterial recovery analysis, which determined the number of J901Ri or J901C that had succeeded in adherence to or/and penetration into ZF4 cells. The results showed that the bacterial recovery from J901Ri-infected cells was 2.3-fold less than that from J901C-infected cells.

**Effect on the expression of virulence-associated genes.** Since the above results indicated an involvement of LuxS$_Ei$ in host infection, we examined whether interfering with luxS$_Ei$ expression had any effect on the expression of virulence genes. For this purpose, qRT-PCR was carried out to determine the expression of the type three secretion system genes orf26, esrA, eseB, and eseD, the type six secretion system genes evpA and evpB, the haemolysin gene eihA, and the wbiT gene involved in O polysaccharide biosynthesis. The results showed that except for evpA and evpB, which exhibited comparable levels of expression in J901Ri and J901C, the expressions of all other genes were signifi-
cantly reduced in J901Ri (Fig. 6). To examine whether the above observed effect on gene expression was due to reduced AI-2 production in J901Ri, exogenous AI-2 was added to the cell culture of J901Ri. Subsequent qRT-PCR analysis showed that addition of AI-2 restored the expression of all the genes in J901Ri to levels similar to those in J901C (Fig. 6).

Discussion

In this study, we examined the expression, activity, and biological function of the luxS gene from a pathogenic E. ictaluri isolate, J901. J901 was found to exhibit AI-2 activity that increased with cell density and reached maximum at OD$_{600}$ of 1.0. These results suggest that J901 produced active AI-2 in a manner that was regulated by cell density, which is consistent with the role of AI-2 as a quorum sensing signal. The observation that the AI-2 activity of J901 was much lower than that of BB120 suggests that J901 may either produce less amount of AI-2 than BB120 under the culture condition or that the AI-2 of J901 is less effective in triggering the signaling process of quorum sensing that leads to light production in BB170 due to, for example, certain structural difference between the AI-2 molecules of J901 and BB120. Previous studies have shown that in bacteria such as Salmonella typhimurium and Edwardsiella tarda, AI-2 production is affected by various environmental stimuli including pH, osmolarity, and temperature (Surette et al., 1999; Zhang et al., 2008). It is known that in E. coli, AI-2 synthesis and uptake are regulated by catabolite repression through the cyclic AMP-CRP complex that indirectly represses luxS expression (Taga et al., 2003; Wang et al., 2005; Xavier and Bassler 2005). In our study, we found that compared to growth in standard LB medium at 28°C, growth in LB medium at 37°C decreased AI-2 activity, whereas growth in the presence of glucose increased AI-2 activity. These results indicate that, as observed in other bacterial species, the AI-2 activity of E. ictaluri is regulated by carbon source and temperature.

In agreement with the presence of AI-2 activity in J901, a luxS gene, luxS$_{Ei}$, was identified in J901. To examine whether luxS$_{Ei}$ encodes a functional enzyme, the gene was sub-cloned via the plasmid pBTEiS into the luxS-defective E. coli DH5α. Subsequent analysis showed that the recombinant DH5α/pBTEiS acquired the ability to produce AI-2 activity, suggesting that LuxS$_{Ei}$ is an active enzyme that is able to catalyze the production of functional AI-2 in DH5α. qRT-PCR analysis showed that luxS$_{Ei}$ expression was regulated by cell density, glucose, and temperature in a manner similar to that observed with AI-2 activity, suggesting that the AI-2 activity of J901 was controlled at the expression level of luxS$_{Ei}$. Similar observations have been made in other bacterial species such as E. coli and E. tarda (Zhang et al., 2008; Wang et al., 2005; Taga et al., 2001). In our study, the above hypothesis was supported further by the observation that J901Ri, in which luxS$_{Ei}$ expression was interfered, exhibited reduced AI-2 activity.

Growth study showed that compared to the control strain J901C, J901Ri was retarded in growth rate and impaired in the ability to form biofilm, which indicate a requirement of LuxS$_{Ei}$ for optimal growth of J901 both as free-living cells and as surface-attached cells. The observation that addition of exogenous AI-2 did not restore the growth of J901Ri in LB medium but rescued the biofilm growth of J901Ri suggests an involvement of AI-2-mediated quorum sensing in biofilm growth but not in planktonic growth. It is likely that the restored biofilm growth in J901Ri was due to the ability of the
added AI-2 to activate the quorum sensing pathway, which in turn activated the regulatory system that con-
trols biofilm production. Given that LuxS is known to play an important metabolic function in the activated methyl cycle and that luxS mutation affects metabolic genes required for normal growth (Mc Nab et al., 2003; 
Doherty et al., 2006; Kendall et al., 2007; Winzer et al., 
2002), our results suggest a likely participation of LuxS in 
the central metabolic process of E. ictaluri.

Association between LuxS and pathogenicity has been 
reported for many bacterial species (Coulthurst et al., 
2004; Coulthurst et al., 2007; Joyce et al., 2004; 
Novak et al., 2010; Plummer et al., 2011). In our study, we 
found that when zebrafish were infected with J901Ri 
or J901C, 40% accumulative mortality was observed in 
J901Ri-infected fish, whereas 100% accumulative mor-
tality was observed in J901C-infected fish. These results 
indicate that interference with luxS expression attenu-
ated the overall virulence of J901. Consistently, cellular 
mortality infection study showed that after 0.5 h of incubation 
with ZF4 cells, the number of cell-associated J901Ri was 
2.3-fold less than that of cell-associated J901C. Since 
the infection time was short, it is likely that the reduced 
Bacterial recovery of J901Ri was due to reduced infec-
tivity rather than reduced growth capacity.

In some pathogenic bacteria such as E. coli, V. har-
srevyi, Streptococcus pyogenes, and E. tarda, LuxS was 
found to regulate the expression and production of various 
virulence factors including T3SS components, proteases, and exotoxins (Henke and Bassler, 2004; 
Lyon et al., 2001; Marouni and Sela 2003; Sircili et al., 
2004). In our study, we found that interference with luxS expression significantly reduced the expression of the genes involved in T3SS, haemolysin production, and O polysaccharide biosynthesis. Since T3SS, O polysaccharide, and hemolysin are known to be associated with pathogenicity (Lawrence et al., 2003; Lawrence et al., 2001; Thune et al., 2007; Williams and Lawrence 2005), these results suggest that reduced expression of these genes may at least in part account for the reduced infectivity of J901Ri. The observation that the presence of exogenous AI-2 restored the expression of all these genes in J901Ri suggests that LuxS regulates the expression of these virulence genes through AI-2-me-
diated quorum sensing.

In conclusion, the results of this study showed that E. ictaluri J901 exhibits regulated production of AI-2 that is controlled at the level of luxS expression, and that interference with the regulated expression of luxS impairs growth and pathogenicity via different mechanisms which are, respectively, independent and dependent on quorum sensing. These results suggest a participation of LuxS in both central metabolism and AI-2-mediated signaling process, the latter may consequently affect bacterial virulence.

Acknowledgements

This work was supported by the Knowledge Innovation Pro-
gram of the Chinese Academy of Sciences grants Y02420101Q and 
KSCX2-EW-G-12B.

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Pathogenicity and Ultrastructural Studies of the Mode of Penetration by *Phoma strasseri* in Peppermint Stems and Rhizomes

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Received 15 February 2012, revised, accepted 19 September 2012

**Abstract**

Pathogenicity and ultrastructural investigation of the inoculation of peppermint stems and rhizomes with *Phoma strasseri* conidia was undertaken using scanning and transmission electron microscopy to examine the host-parasite relationship. Pathogenicity experiments demonstrated that all tested *P. strasseri* isolates had infected the stems and rhizomes of peppermint. Of all inoculation methods, direct placement of colonized agar plugs on damaged epidermis and soaking stems and rhizomes in conidial suspension were the most effective. The behavior of the conidia deposited on the stems and rhizomes was investigated at different time intervals after inoculation: 6, 16, 24, 36 and 48 h. Conidia produced an appressorium directly at the end of a short germ tube. Appressoria were formed over the cuticle, but never over stomata. Direct penetration to host tissue through the cuticle was observed. The spore and hyphae were covered with a mucilaginous sheath.

**Keywords:** *Mentha piperita*, black stem and rhizomes rot, infection process, SEM, TEM

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**Introduction**

Black stem and rhizomes rot of peppermint (*Mentha piperita* L.), also called phomosis of mint, is caused by *Phoma strasseri* Moesz (Boerema *et al.*, 2004). The occurrence of this disease has been so far found in the United States (Horner, 1971; Farr *et al.*, 1995), in Japan (De Gruyter *et al.*, 2002), in India (Kalra *et al.*, 2004), in Hungary (Paizs and Naggy, 1975) and in Poland (Zimowska and Machowicz-Stefaniak, 2005; Zimowska, 2007). The disease symptoms on the plants of peppermint cultivated in the field are visible on the stems, first in the form of necrotic, slightly hollow spots enfolding the stem around. With time, the tissue in the place of the spots gets rotten. Such symptoms are most often formed just under the ground surface to the height up to 10 cm from the base. The secondary symptom is the reddening or reduction of the leaf blades. Very young stems usually die out without any secondary symptoms of the disease (Horner, 1971; Zimowska, 2007). The rot proceeds very fast on the rhizomes. Young rhizomes rot away wholly, while the bark layer often comes off on older ones (Horner, 1971; Zimowska, 2007). The symptoms on peppermint cultivated in a glasshouse are similar to those that are observed in the field, with no secondary symptoms in the form of the reddening and reduction of the leaves, however (Horner, 1971). The yield losses as a result of plant infection can even reach 90% (Horner, 1971). Etiological signs in the form of pycnidia, including the conidia of *P. strasseri*, occur on the stems and rhizomes with the symptoms of black rot (Zimowska and Machowicz-Stefaniak, 2005; Zimowska, 2007).

The accessible literature provides information on disease symptoms caused by *P. strasseri* (Horner, 1971; Paizs and Naggy, 1975; Zimowska, 2007), biotic interactions of *P. strasseri* with the fungi colonizing the phyllosphere of peppermint stems and rhizomes as well as the effect of the thermal conditions on the formation of the infection material by the fungus (Zimowska, 2011a). The histopathological and ultrastructural aspects of the infection of peppermint by *P. strasseri* has not been so far documented. Hence, the present research undertakes studies on the ultrastructure of the inoculated stems and rhizomes of peppermint with the aim of explaining the relation between *P. strasseri* and the host plant.

**Experimental**

**Material and Methods**

**Fungal isolates.** The studies used one-spore cultures of three isolates of *P. strasseri* obtained from the naturally infected plants of peppermint with the signs of black stem and rhizomes rot from the production plantations situated in the south-eastern part of Poland (Zimowska, 2007) and isolate CBS. 126.93.
obtained from Centraalbureau voor Schimmelcultures, Utrecht Netherlands.

**Inoculation techniques.** Isolates of *P. strasseri* were incubated on a maltose agar MA throughout the first week at the temperature of 22°C without any light access, and then for 13 hours in ultraviolet light (UV). After that, the isolates were incubated in the same conditions as in the first week of the culture (De Gruyter and Noordelos, 1992). Three methods of inoculation were used to prove the pathogenicity. The first method used plugs of the colonized agar (5 mm diameter) cut out from 2-week-old cultures of each isolate. Those plugs were placed at the stem and rhizome fragments which were disinfected on the surface by being immersed for 60 seconds in 10% sodium hypochlorite. In the second method, colonized plugs were placed at disinfected fragments of stems and rhizomes, together with the epidermis injured with a needle puncture (Zimowska, 2004). The third method used a suspension of conidia with the density of 10⁶ conidia per 1 ml. It was obtained by rinsing the surface of the cultures of particular isolates with sterile distilled water. The disinfected fragments of stems and rhizomes were soaked in the suspension for 5 minutes (Horner, 1971). Each method was tested in humidity chambers. Those were 9 cm diameter Petri dishes, laid with three layers of cellulose tissue and one layer of filter paper moistened with 4 ml of distilled sterile water (Zimowska, 2004). For each method 120 fragments of stems and rhizomes were used. Control fragments of stems and rhizomes were inoculated with sterile agar plugs (methods I and II) or sterile water (method III). The experiment was conducted twice. Humidity chambers were kept in a thermostat at the temperature of 22°C for 12 days. During that time, observations were made every 3 days on the development of disease symptoms. After 12 days, the infection index was calculated on the basis of the disease scale. Next, all fragments of stems and rhizomes were analyzed for the presence of fungus according to Koch’s postulates. The results obtained from the experiment were statistically analyzed using a two-factor variance analysis (Anova) according to SAS program (Snedecor and Cochran, 1982).

**Sample preparation for scanning electron microscope (SEM).** Fragments of stems and rhizomes inoculated by conidial suspension were cut into 2–3 mm sections. Next, the specimens were fixed with 4% glutaraldehyde for 3 hours at room temperature and then, for 24 hours, at 5°C. After that time, the specimens were placed in 1% cacodylate buffer for 2 hours at room temperature (Kulik, 1988). Next, the specimens were dehydrated in an alcohol series (30%, 50%, 70%, 95% and 100%, for 15 minutes at each concentration. The specimens were then dried in liquid CO₂ by using a BAL-TEC CPD 030 Critical Point Dryer, and finally gold sputter-coated. Observations of six samples were carried out at different time intervals after inoculations: 6, 16, 24, 36 and 48 hours. Micrographs were obtained using a Vega 2, Tescan scanning electron microscope.

**Sample preparation for transmission electron microscope (TEM).** The specimens were fixed with 4% glutaraldehyde for 2 hours at room temperature and post-fixed in 2% osmium tetroxide in phosphate buffer for 2 hours at 20°C. The following fixation was conducted in 0.1 M cacodylate buffer at pH 7.4 for 2 hours at 4°C. Afterwards, the specimens were double-rinsed for 5 minutes in the same buffer and then for another 5 minutes in distilled water. After rinsing, the specimens were post-fixed in 0.5% uranyl orthosilicate dihydrate solution for 2 hours at room temperature (Maurin et al., 1993). Next, they were dehydrated in an alcohol series (30%, 50%, 70%, 95% and 100%, for 15 minutes at each concentration at 4°C, followed by 90%, 95% and 100% for 15 minutes at each concentration at room temperature). Then, ethyl alcohol was replaced with propylene oxide. After absolute alcohol, the specimens were placed in increasing concentrations of propylene oxide in alcohol: 33% for 10 min., 50% for 10 min., 67% for 10 min, and 100% twice for 10 min. (Maurin et al., 1993). Next, the specimens were hardened with increasing concentrations of Spurr Low Viscosity resin in propylene oxide: 33% for 1 hour, 50% for 1 hour, 67% for 1 hour, and 100% for 1 hour. Next, the specimens were placed in polyethylene capsules (filled with resin) and left for 12 hours at 70°C in order to polymerize. After the polymerization, the specimens were cut into ultra-thin 85-nm sections by using a Reichert Ultracut S microtome. Next, they were dyed with 8% uranyl acetate solution in 0.5% acetic acid for 45 min. Finally, they were compounded with lead citrate for 10 min. (Maurin et al., 1993).

The materials were examined by means of a FEI Tecnai Spirit G² microscope, operating at an acceleration voltage of 100 kV.

**Results**

**Pathogenicity of isolates to stems and rhizomes of peppermint and inoculation techniques.** All studied isolates of *P. strasseri* caused signs in the form of necrosis and then rot on the inoculated fragments of stems and rhizomes. As early as already 3 days after the inoculation, symptoms were observed on peppermint parts inoculated according to method II. Those were necrotic spots, 3 to 10 mm long, around the infection site. After 6 days, the necrosis grew and covered from 25% to 30% of the area of the inoculated parts. After that time, the softening of the tissues in the place of the necrosis could be seen. After 9 days, the rot covered from 40% to 70% of the surface of the stems and rhizomes, and after 12 days – from 90% to 100% of the surface of the
Phoma strasseri penetration in peppermint

The development of disease symptoms on peppermint stems and rhizomes inoculated according to method III was similar. In the combination with inoculation through the undamaged epidermis (method I), a trace of necrosis was seen after 6 days around the inoculation site. After 6 days, necrosis accompanied by the softening of the tissues covered from 15 to 20% of the surface of the inoculated parts. After 12 days, from 70% to 90% of the stem and rhizome surface of the inoculated parts was covered with the rot. The symptoms were similar to those observed on the plants of peppermint in the conditions of field cultivation. The most effective methods of inoculation proved to be methods II and III. Values of the infection index were, respectively, 96.67% and 94.79% for the stems and 96.67% and 95.00% for the rhizomes and they were significantly different from values of the index obtained for method I (Table I).

The highest values of the infection index in all inoculation methods among the tested isolates were for isolate M 743 (Tables II, III, IV). Generally, those values did not significantly differ from the values of the index obtained for the other isolates, except isolates M 289 and M 126 tested according to method II (Table III), the same isolates and isolate CBS. 126 93, which were

### Table I
Pathogenicity of *Phoma strasseri* to stems and rhizomes of peppermint using various inoculation methods (means for 4 isolates)

<table>
<thead>
<tr>
<th>Inoculation method</th>
<th>Infection index (%) after 12 days*</th>
<th>Reisolation (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stems</td>
<td>rhizomes</td>
</tr>
<tr>
<td>Colonized plugs placed at non-injured epidermis</td>
<td>87.92 a</td>
<td>84.38 a</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colonized plugs placed at injured epidermis</td>
<td>96.67 b</td>
<td>96.67 b</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stems and rhizomes soaked in conidial suspension (1×10⁶ conidia/ml)</td>
<td>94.79 b</td>
<td>95.00 b</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

HSD = 3.0537 HSD = 3.1825

1* Infection index evaluated on the basis of 5 – degree disease scale: 0° – lack of disease symptoms; 1° – sign of necrosis visible only around the inoculation point; 2° – 25% to 50% surface of inoculated organs showed disease symptoms; 3° – 51% to 75% surface of inoculated organs showed disease symptoms; 4° – 76% to 100% surface of inoculated organs showed disease symptoms

Values marked with the same letter do not differ significantly

HSD – Honest Significant Difference

### Table II
Effect of inoculation of *Phoma strasseri* isolates on occurrence of black stem and rhizomes rot – method I (mean of 6 replications)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Infection index (%) after 12 days¶</th>
<th>Reisolation (%)¶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stems</td>
<td>rhizomes</td>
</tr>
<tr>
<td>M 126</td>
<td>88.33 ab</td>
<td>84.17 a</td>
</tr>
<tr>
<td>M 289</td>
<td>82.50 a</td>
<td>77.50 a</td>
</tr>
<tr>
<td>CBS.126.93</td>
<td>88.33 ab</td>
<td>81.67 a</td>
</tr>
<tr>
<td>M 743</td>
<td>92.50 b</td>
<td>94.17 b</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

HSD = 8.7309 HSD = 9.1322

Note: see table I

¶ For stems and rhizomes showing black rot symptoms, percent isolations that resulted in *P. strasseri* colonies
used to inoculate the rhizomes tested according to method I (Table II) and isolate M 289, used to inoculate the stems in method III (Table IV). *P. strasseri* cultures were reisolated from all inoculated organs, for all methods (Tables II, III, IV). Morphological features of reisolated cultures corresponded to the features of cultures considered in the research. Uninoculated controls remained symptomless, and results from the two experiments were similar.

**Scanning electron microscope.** Six hours after inoculation, conidia of *P. strasseri* were visible on the surface of peppermint stems and rhizomes (Fig. 1 a, b). After 16 hours, single conidia formed germ tubes of length not exceeding 5 µm (Fig. 1c). Twenty four hours
after inoculation, an adhesive structure in the form of an appressorium was seen at the end of the germ tube (Fig. 1d). Conidia germination and appressorium formation always took place at a certain distance from the stomata (Fig. 1e). After 36 hours, unbranched hyphae were observed on the surface of the cuticle of peppermint stems and rhizomes (Fig. 1f).

Transmission electron microscope. The conidia of *P. strasseri* germinating on the surface of the cuticle had big vacuoles (Fig. 2a). A layer of a mucilaginous sheath not greater than 0.3 µm thick was visible on the surface of the cell wall of the conidia (Fig. 2a). Direct penetration of the pathogen by the cuticle of the host plant was observed between 36 and 48 hours after inoculation. A sheath of a mucilaginous substance µm was present on the surface of the wall of the hyphae (Fig. 2b). After 48 hours, septate hyphae was visible in epidermis cells (Fig. 2c).

### Discussion

Pathogenicity studies showed that all tested isolates of *P. strasseri* caused infection of the inoculated stems and rhizomes of peppermint. This is testified to by high values of infection indexes and the fulfillment of Koch’s postulates. Of all methods of inoculation, the most effective proved to be the one consisting in placing plugs of colonized agar on the injured tissue of the stems and rhizomes, and the method considering soaking of the fragments in a conidial suspension. These results are consistent with information from literature, according to which the enumerated inoculation methods also proved the most effective for other facultative pathogens such as *Phoma linguam* (Sock and Hoppe, 1999), *P. exigua* (Koike et al., 2006), *P. exigua var. foveata* (Giebel and Dopierała, 2004) and *P. multirostrata* (Garibaldi et al., 2010). The fact that the disease symptoms on the inoculated peppermint parts are similar to those that are observed in the conditions of field cultivation is certainly related to the production of pectolytic enzymes, especially polygalacturonase and maceration enzymes, by *P. strasseri* (Melouk and Horner, 1972a). It follows from studies conducted by Melouk and Horner (1972b) that enzymes of *P. strasseri* show the greatest activity 5 days after infection. Studies confirm the thesis posed by American researchers because already after 6 days, the symptoms of tissue softening were visible on inoculated fragments of stems and rhizomes.
Studies of the ultrastructure of the inoculated stems and rhizomes of peppermint with an conidial suspension pointed to the formation of an adhesive structure at the end of the germ tube in the form of an appressorium and to the direct infection of the pathogen by the cuticle. The ability of fungi for active infection is connected with the fact that they form special structures by means of which they first get attached to the host plant, after which they penetrate its tissues. This is an important condition of successful infection and next the development of a disease (Kulik, 1988; Maurin, 1993). It follows from the present studies that the germinating conidia of \textit{P. strasserii} were fixed to the surface of peppermint stems and rhizomes by means of the appressorium. It has been known for long that in the majority of fungi the formation of the appressorium at the end of the germ tube takes place as a result of a mechanical contact of the fungus with the substrate (Büsgen, 1893; Kerchung and Hoch, 1995). The formation of the appressorium was found in the species closely related to genus \textit{Phoma}, i.e. \textit{P. exigua} var. \textit{linicola} (Roustaee \textit{et al.}, 2000), \textit{Ascochyta pisi} (Heath and Wood, 1969), \textit{A. fabae} (Maurin \textit{et al.}, 1993) and \textit{A. rabiei} (Pandey \textit{et al.}, 1987). In \textit{P. strasserii}, the germinating conidia were also attached to the surface of stems and rhizomes by means of a mucilaginous sheath that covered the wall of the conidia and the hyphae. The presence of the mucilaginous sheath was observed during the studies on conidogenesis of \textit{P. strasserii} (Zimowska, 2011b). It is formed at the last stage of differentiation of the conidia wall to its final structure (Boerema and Bollen, 1975). Many fungi species that perform the infection directly through the cuticle form this type of mucilaginous exudates. It occurs, for example in \textit{Phyllosticta ampiclida} (Kerchung and Hoch, 1995), \textit{Phomopsis phaseoli} (Kulik, 1988) and \textit{Phoma macdonaldi} (Roustaee \textit{et al.}, 2000). Its role is to strengthen the contact with the host plant. Besides, it seals up the site where the infection hyphae penetrates and it protects the appressorium from drying out and from unfavourable atmospheric conditions (Roustaee \textit{et al.}, 2000). The studies pointed to a direct penetration of \textit{P. strasserii} by the cuticle of peppermint stems and rhizomes omitting the stomata. The majority of fungi penetrate into the tissues of their hosts directly through the cuticle. This model of infection is usually accompanied by the formation of the appressorium (Kulik, 1988). Direct penetration was observed for example in \textit{Phomopsis scabra} (Ammon and Vann, 1994), \textit{Colletotrichum lagenarium} (Bonnen and Hammerschmidt, 1989) and in \textit{C. gloeosporioides} (Dickman \textit{et al.}, 1982). It is known that direct penetration of pathogenic fungi occurs as a result of the joint action of two factors, namely the mechanical pressure of a fast growing infection hyphae and the enzymes decomposing cutin and then pectin compounds and cellulose making the composition of the cell wall (Isaac, 1992). In the case of \textit{P. strasserii}, pectolytic and maceration enzymes produced by the pathogen certainly take part in the process of active penetration. The involvement of pectolytic and hemicellulolysis enzymes has been reported for \textit{P. lingua}m (Hammond \textit{et al.}, 1985). The presence of \textit{P. strasserii} hyphae in epidermis cells as early as after 48 hours can point to a short period of the pathogen incubation.

**Literature**


INTRODUCTION

Flocculants are special natural organic macromolecule substances that can flocculate suspended solids, cells, colloidal solids, etc (Zhang, 2005). They are widely used in material separation processes, such as drinking water purification, waste water treatment, dehydration of activated sludge, dredging, downstream processing, food and fermentation process (Salehizadeh and Shojaosadati, 2001).

Flocculating agents are generally classified into three groups: (1) inorganic flocculants, such as aluminum sulfate and polyaluminum chloride (2) organic synthetic flocculants, such as polyacrylamide derivatives and polyethylene imine (3) naturally occurring flocculants, such as chitosan, sodium alginate and bioflocculant (Zhang et al., 2007).

Despite the effective flocculation performance and low cost of the synthetic chemical flocculants, their use has resulted in some health and environmental problems. For example, aluminum has been found to induce Alzheimer's disease (Arezoo, 2002). Furthermore, the acrylamide monomer is not only toxic and carcinogenic, but also non-biodegradable in nature (Ruden, 2004). On the contrary, bioflocculants have attracted considerable attention as a promising substitute for chemical flocculants because of their biodegradability and safety for ecosystems (He et al., 2004). Further, bioflocculants can be produced economically on a large scale and easily be recovered from fermentation broth. Therefore, they now have wide applications in many industrial sectors associated with textiles, detergents, adhesives, microbial enhanced oil recovery, and wastewater treatment (Kumar et al., 2004).

Heavy metals are introduced into the aquatic systems significantly as a result of various industrial operations that include agriculture, battery production, fossil fuel burning, mining and metallurgical processes (Boening, 2000). Heavy metals are a critical concern to human health and environmental issues due to their...
high occurrence as a contaminant, present in soluble form that are extremely toxic to biological systems, and the classification of several heavy metals as carcinogenic and mutagenic (Diels et al., 2002). Moreover, the metals cannot be degraded to harmless products and hence persist in the environment indefinitely. As a result, several methods have been devised for the treatment and removal of heavy metals in contaminated sites. Conventional techniques for the removal of heavy metals from wastewater, such as chemical precipitation, ion exchange, activated carbon adsorption and separation processes have limitations and become inefficient and expensive especially when the heavy metal concentration is less than 100 ppm (Yan and Viraraghavan, 2001). Finding an effective method of removal of toxic heavy metals from industrial waste water is essential from the stand point of environmental pollution control and it has directed attention to biosorption, based on the metal binding capacities of various biological materials (Al-Garni et al., 2009).

To utilize bioflocculants widely in industrial fields, it is desirable to find various microorganisms with high bioflocculant-producing ability and improve the flocculating efficiency of the produced bioflocculant. Consequently, this study aims to investigate the ability of Pseudomonas aeruginosa to produce bioflocculant. The study also includes optimization, purification and characterization of the produced bioflocculant. Moreover, the flocculating activities of the bioflocculant produced in the removal of various heavy metals which are normally present in wastewater treatment are reported in this paper.

**Experimental**

**Materials and Methods**

**Bacterial strain.** A bioflocculant-producing strain, *Pseudomonas aeruginosa* ATCC-10145 was kindly obtained from Fermentation Biotechnology and Applied Microbiology (FERM-BAM) Center, Al-Azhar University, Cairo, Egypt. The bacterium was preserved on agar slants and glycerol (20%) stocks maintained at −80°C.

**Media and cultivation conditions.** The strain was pre-cultured in 50 ml medium in 250 ml flasks on a rotary shaker (200 rpm) at 30°C for inoculation preparation. After 24 h of cultivation, the culture broth was used as a seed culture and 1% of it was inoculated into 100 ml of fermentation medium in 500 ml flask for 48 h. The seed medium contained (per liter) glucose, 10 g; yeast extract, 0.5 g; urea, 0.5 g; KH₂PO₄, 0.1 g; NaCl, 0.1 g; and MgSO₄·7H₂O, 0.2 g, pH 7 (Xiong et al., 2010). After incubation, the culture broth was centrifuged at 10000 xg for 30 min. The cell-free culture supernatant was the liquid bioflocculant, which was used for the analysis of flocculating activity.

**Determination of flocculating activity.** The flocculating activity was measured using a kaolin clay suspension. First, 0.5 g kaolin clay was suspended in 100 ml distilled water, and 0.5 ml of the liquid bioflocculant was mixed thoroughly with 45 ml of the kaolin suspension. Then, 4.5 ml of 1% CaCl₂ solution was added to the mixture. The mixture was stirred with a vortex mixer and left standing for 5 min at room temperature. The optical density (O.D) of the supernatant and the blank control where distilled water was used instead of the supernatant was measured at 550 nm. The flocculating activity was defined and calculated as follows (Flocculating activity = (A – B)/A × 100, where A and B are the optical densities at 550 nm of the control and the sample, respectively (Zhang et al., 2007).

**Optimization of bioflocculant production.** In order to optimize the nutritional and environmental factors affecting bioflocculant production by *Pseudomonas aeruginosa*, the following variables were assayed: incubation period (1–5 days), carbon source (glucose, fructose, sucrose, lactose, galactose, mannose, maltose, starch, sodium acetate, citric acid, glycerol and ethanol), nitrogen source (yeast extract, beef extract, peptone, urea, glutamic acid, ammonium sulphate, ammonium nitrate, ammonium chloride and sodium nitrate), initial pH (pH5–12) and incubation temperature (20, 25, 30, 35 and 40°C). All experiments were performed in triplicate for calculation of the mean. Medium samples were withdrawn and monitored for final pH, cell growth (cell dry weight) and flocculating activity as described above.

**Characteristics of the bioflocculant.** The effect of different cations on flocculating activity was studied by addition of CaCl₂, KCl, MgCl₂, NaCl, ZnSO₄, CuSO₄, FeCl₃ and AlCl₃ at a concentration of (1 mM).

The effect of different bioflocculant dosages was investigated by adding different amounts of liquid biofloculant (1, 2, 3, 4 and 5%) to a constant concentration of kaolin suspension (0.5%) at pH7 containing CaCl₂ (1%). A control was prepared with distilled water in place of biofloculant. To estimate the influence of pH value on the flocculating activity, the reaction mixture was adjusted to pH value ranged from (3–11) using HCl or NaOH. The effect of temperature was studied at a temperature range of 40–90°C for 30°C. Boiling of the reaction mixture at 100°C for 1–60 min was also studied. In each case, the remaining flocculating activity of each sample was measured and calculated using the procedure described above.

**Bioflocculant purification.** Two volumes of cold ethanol were added to supernatant of the culture broth
to precipitate the bioflocculant and the mixture was left overnight at 4°C. The precipitate collected by centrifugation at 10000 x g for 30 min was dialyzed against de-ionized water overnight and then lyophilized and weighed.

**Chemical analysis.** Total carbohydrate content of the bioflocculant was measured by phenol-sulfuric acid method (Chaplin and Kennedy, 1994) using glucose as the standard solution. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Amino acid was estimated by ninhydrin method (Zhang, 2003). After hydrolysis of the bioflocculant with 2 M trifluoroacetic acid at 120°C for 2 h, neutral sugars, uronic acids and amino sugars were determined with anthrone method, carbazole sulfate acid method and the Elson-Morgan method, respectively using the procedure of Chaplin and Kennedy (1994). The percentage of carbon, hydrogen, nitrogen and sulfur of the purified bioflocculant were determined using Atomic Absorption Spectrophotometer (Perkin Elemer USA, Model 2400).

**Fourier transform infrared spectroscopy.** The purified bioflocculant (2 mg) was ground with 100 mg KBr and compressed at 7.500 Kg for 3 min to obtain translucent pellets. KBr pellet was used as the background reference. Infrared absorption spectra were recorded with a model (Jasco FTIR-6100, Japan). The spectral resolution and wave number accuracy were 4 and 0.01 cm⁻¹, respectively.

**Heavy metal adsorption.** The potential of the produced bioflocculant for removing heavy metals was assessed as described by Lin and Harichund (2011). The metal salts used were copper sulphate, lead acetate, sodium arsenate, zinc sulphate, cadmium chloride and mercury iodide (Sigma Co). 5 ml bioflocculant solution was put into dialysis tubing in flasks containing 200 ml of each appropriate metal-salt solution and shaken at 100 x g for 24 h at 30°C. The quantity of metal removed from the solution, i.e. bound to the polymer, was calculated by measuring the ions in solution at 0 h and those remaining after 24 h by Atomic Absorbance Spectrometer (Perkin Elemer USA, Model 2400) and the percentage of each metal removal was calculated (Gourdon et al., 1990). Controls were made by placing 5ml distilled water in dialysis tubing with the various metal-salt solutions. The effect of heavy metal concentrations (20, 40, 60, 80, and 100 ppm) bioflocculant concentrations(100, 1000, 5000 and 100 00 ppm) and pH value (3, 5, 7 and 9) on the metal adsorption by the biopolymer was investigated. The adsorption test procedure as well as the calculation of percentage of each metal removing were the same as described above.

**Statistical analysis.** All experiments were performed in triplicate and the results were expressed as means ± SD.

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**Results and Discussion**

**Time course assay of bioflocculant production.** The growth curve of *Pseudomonas aeruginosa* strain, the flocculating activity and pH variation of the culture broth are shown in Fig. 1. During growth, *Pseudomonas aeruginosa* showed acidification activity and the flocculating activity increased as the cultivation period increased, attaining peak flocculating activity of about 62.25% after 72 h of cultivation, beyond which flocculating activity began to decline. The flocculating activity increased in parallel with cell growth, indicating extracellular accumulation of bioflocculants in the medium during the active growth phase. This suggests that the bioflocculant was produced by biosynthesis during growth of the bacterium and not by cell autolysis (Lu et al., 2005). The observed decrease in flocculating activity might be due to partial enzymatic degradation of the polymer flocculant in the late phases of cell growth (Choi et al., 1998).

**Factors affecting bioflocculant production.** It is well known that optimization of the cultivation process is a rather powerful approach to improve the production of bioproducts. The bioflocculant production is affected by many factors, such as the constituents of the culture medium and environmental conditions (He et al., 2004). The effects of the key factors, like carbon, nitrogen sources, initial pH and culture temperature on bioflocculant production by *Pseudomonas aeruginosa* were investigated with an aim to identify the cost-optimal culture conditions.

It has been well documented that changing the carbon and nitrogen sources highly influences bacterial growth and bioflocculant production (Sheng et al., 2006). From Table I, one noteworthy result was that the bacteria grew and produced bioflocculant with all the carbon sources assayed. Ethanol seems to be the
preferred carbon source for bioflocculant production by *Pseudomonas aeruginosa*. The flocculating rate of the culture reached 70.14%, therefore, it was chosen to be the carbon source for bioflocculant production in the subsequent studies. Similarly, ethanol was the favored carbon sources for bioflocculant production by *Rhodococcus erythropolis* (Kurane *et al.*, 1991) and *Klebsiella pneumoniae* (Nakata and Kurane, 1999). Ethanol was also a good carbon source for bioflocculant production in the industrial scale. Wastes from canning factories’ and stillage from distilleries are alternatives for expensive carbon sources (Tong *et al.*, 1999).

With respect to the effect of nitrogen source on bioflocculant production, it can be observed from Table II that multiple nitrogen sources were better than a single nitrogen source. The medium containing yeast extract and sodium nitrate was the most favorable for production of bioflocculant as they caused the highest bioflocculant activity (78.24%).

It well known that the initial pH of the fermentation medium affected bioflocculant synthesis as it determines the electric charge of the cells and the oxidation-reduction potential which can affect nutrient absorption and enzymatic reaction (Xia *et al.*, 2008). The flocculating activity of the culture broth reached a maximum at pH 7.0 (78.24%) and then gradually decreased with increase of initial pH (data not shown). However, production of bioflocculant in acidic conditions was distinctly much lower than in either neutral or alkaline ones. Comparison between final pH and initial pH values clearly presented that the cultural medium had the buffer capability especially in alkaline conditions. This buffer ability may come from the organic acid contained in bioflocculant, by-production like acetic acid, or the unspent K$_2$HPO$_4$ and KH$_2$PO$_4$.

Concerning incubation temperature, various culture temperatures were tested in order to investigate their effect on bioflocculant production. The maximum flocculating activity was 80.50%, which was recorded at 35°C. The activity dropped drastically when the cultivation temperature fell below 30°C or increased above 40°C (data not shown). The metabolism of microorganisms has a direct relationship with cultivating temperature; maximum enzymatic activation can only be obtained at optimal temperature (Zhang *et al.*, 2007). A lower culture temperature might make the strain hibernate partially, and its enzyme system for bioflocculant production could not be activated completely. On the other hand, a higher culture temperature may have an adverse effect on the nucleic acid and enzyme system of the strain, further on the bioflocculant production.

**Bioflocculant characterization.** Bioflocculant characterization was determined using kaolin clay suspension as a flocculation test material because kaolin is a well-known and wide spread thickening agent. Also the surface characteristics of kaolin are well-understood to allow analysis, and in consequence kaolin has received great interest in recent years (Nasser and James, 2007).

The results of the present study indicate that environmental parameters like cationic compounds, bioflocculant dosage, pH and temperature play an important role in the flocculating activity of the produced bioflocculant.
The influence of cations on flocculating activities was studied and compared. As presented in Table III, the flocculating activity of bioflocculant from *Pseudomonas aeruginosa* was a cation-dependent whose flocculating capability was strongly increased by Ca\(^{2+}\), K\(^+\), Na\(^+\), Zn\(^{2+}\), Mg\(^{2+}\) and Cu\(^{2+}\), and dropped by the addition of Fe\(^{3+}\) and Al\(^{3+}\) compared with that of the control. Cations stimulate flocculating by accelerating bridge formation between suspended particles and bioflocculant. Moreover, the bivalent cations increase the initial adsorption of biopolymers on suspended particles by neutralizing negatively charged functional groups of both the bioflocculant molecules and the suspended particles and consequently weaken the static repulsive force thus enhancing the flocculation effect (Li et al., 2000). However, the presence of metal is not absolutely essential for bacterial bioflocculating activities. For example, bioflocculants produced by *Citrobacter* sp. TKF04 (Fujita et al., 2000) and *Bacillus* sp. F19 w (Zheng et al., 2008) were capable of flocculating kaolin clay without metals.

Concentration of the bioflocculant played an important role in bioflocculating activity, the maximum flocculation of 80.50% was recorded at 1% bioflocculant (Table III). Flocculation mainly ceased once the bioflocculant concentration exceeded as the adsorption of excess bioflocculant re-stabilized the kaolin particles; thus the attractive forces of other particles were reduced and flocculating activity decreased (Suh et al., 1997).

The effect of pH on flocculating activity was examined at pH values ranging from 3 to 11 (Table III). The activity was found to be the highest (80.50%) at pH 7. Gao et al. (2006) and He et al., 2010 reported similar optimal pH value (7.0) for the activity of the bioflocculants produced by *Vagococcus* sp. and a mutant *Halomonas* sp., respectively. At low and high pH values, the absorption of H\(^+\) ions tends to weaken the bioflocculant-kaolin complex formation process.

The flocculants with protein or peptide backbone in the structure are generally thermally labile, but those made of sugars are heat-stable. If the major component of a bioflocculant is a glycoprotein, its stability will depend on the relative contents of protein and polysaccharide (Takagi and Kadowaki, 1985). In this study, the major component of bioflocculant is a polysaccharide, and it shows heat stability (Table III). The bioflocculant maintained its stability under heating and flocculating activity was decreased only to 60.16% after heating at 100°C for 60 min, suggesting that the bioflocculant produced by *Pseudomonas aeruginosa* is thermo-stable.

The above mentioned characteristics demonstrate that the bioflocculant has strong flocculating activity and high stable quality, which affords high possibility of its practical use in industries and environmental applications. About 2.4 g bioflocculant was recovered from 1.0 l of fermentation broth, which was markedly higher than reported in the literature (Lu et al., 2005 and Xia et al., 2008). The high yield of bioflocculant can meet the need for wide application.

**Chemical analysis.** Wu and Ye (2007) propose that the composition of bacterial bioflocculants plays a major role in their flocculating activities. Several types of bioflocculants have been reported including proteins, glycoproteins, polysaccharides, lipids and glycolipids (Salehizadeh and Shojaosadati, 2003).

### Table III

Factors affecting flocculating activity of the bioflocculant produced by *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Cations (1 mM)</th>
<th>Flocculating activity (%)</th>
<th>Temperature (°C)</th>
<th>Flocculating activity (%)</th>
<th>pH</th>
<th>Flocculating activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>80.50 ± 1.89</td>
<td>40</td>
<td>80.10 ± 2.42</td>
<td>3</td>
<td>12.45 ± 0.00</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>95.42 ± 2.43</td>
<td>50</td>
<td>78.89 ± 4.66</td>
<td>4</td>
<td>21.12 ± 5.22</td>
</tr>
<tr>
<td>K(^+)</td>
<td>90.70 ± 5.20</td>
<td>60</td>
<td>78.50 ± 1.45</td>
<td>5</td>
<td>64.50 ± 1.14</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>92.39 ± 1.72</td>
<td>70</td>
<td>77.90 ± 2.13</td>
<td>6</td>
<td>72.95 ± 1.17</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>96.16 ± 4.20</td>
<td>80</td>
<td>77.45 ± 0.00</td>
<td>7</td>
<td>80.50 ± 3.70</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>84.53 ± 2.04</td>
<td>90</td>
<td>76.53 ± 1.22</td>
<td>8</td>
<td>63.80 ± 0.80</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>80.80 ± 1.22</td>
<td>100 (1 min)</td>
<td>74.00 ± 3.84</td>
<td>9</td>
<td>60.42 ± 1.12</td>
</tr>
<tr>
<td>Fe(^{3+})</td>
<td>62.49 ± 1.04</td>
<td>100 (5 min)</td>
<td>72.35 ± 1.88</td>
<td>10</td>
<td>55.45 ± 1.18</td>
</tr>
<tr>
<td>Al(^{3+})</td>
<td>65.88 ± 1.20</td>
<td>100 (1 min)</td>
<td>70.76 ± 1.43</td>
<td>11</td>
<td>50.45 ± 1.20</td>
</tr>
</tbody>
</table>

Flocculant concentration (%)

<table>
<thead>
<tr>
<th>Flocculating activity (%)</th>
<th>100 (10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65.35 ± 2.22</td>
</tr>
<tr>
<td>2</td>
<td>64.96 ± 1.34</td>
</tr>
<tr>
<td>3</td>
<td>64.12 ± 2.44</td>
</tr>
<tr>
<td>4</td>
<td>62.15 ± 1.02</td>
</tr>
<tr>
<td>5</td>
<td>60.40 ± 1.22</td>
</tr>
</tbody>
</table>
In this study, chemical analysis of the purified bioflocculant revealed that it was a sugar protein derivative composed of protein (27%, w/w) and carbohydrate (89%, w/w) including neutral sugar, uronic acid and amino sugar as the principal constituents in the relative weight proportions of 30.6%, 2.35% and 0.78%, respectively. Besides that, a ninhydrin-positive reaction illuminated that the bioflocculant contains amino acids. The elemental analysis of the bioflocculant revealed the mass proportion of C, H and N was 19.06, 3.88 and 4.32 (%), correspondently.

Sufficient content of uronic acid in a bioflocculant molecule can provide carboxyl groups to the molecular chain. The carboxyl groups presented on the molecular chain provided more effective sites for particles attachment, so many particles can be adsorbed to the long molecular chain (Aguilera et al., 2008).

**Spectroscopic characterization.** The functional groups in the polymer molecule are important determinants for the flocculating activity. FT-IR spectroscopy was performed on the purified bioflocculant between frequency ranges 4000–400 cm\(^{-1}\) to analyze the functional groups (Fig. 2). The spectrum showed a broad stretching intense absorption peak at 3425.92 cm\(^{-1}\) characteristic for hydroxyl and amine groups. A weak C–H stretching vibration band was observed at 2927.41 cm\(^{-1}\). Furthermore, an asymmetrical stretching peak was noticed at 1633.41 cm\(^{-1}\) and a week symmetrical stretching peak at 1445.39 cm\(^{-1}\), indicating the presence of carboxyl groups in the bioflocculant which may serve as binding sites for divalent cations. The carboxyl group may also work as functional moieties to generate new or modified polymer variants using different approaches like novel formulation, designing by linking such polymer with other synthetic polymers. The absorption peak at 1248.68 cm\(^{-1}\) was S = O stretching indicated the presence of sulfate. Other bands observed in the range from 1000 to 1200 cm\(^{-1}\) are generally known to be typical characteristics of all sugar derivatives such as guluronic acid, manuronic acid and uronic acid (Suh et al., 1997). The small absorption band at about 870.703 cm\(^{-1}\) could be associated with \(\beta\)-glycosidic linkages between the sugar monomers, suggested by the study of Gupta et al. (1987). The OH, COOH, COO\(^{-}\) groups in the bioflocculant and H\(^{+}\), OH\(^{-}\) group on the surface of the particles may form hydrogen bonds when the bioflocculant chains approach the surface of particles (Deng et al., 2003). In conclusion, the infrared spectrum of this partially purified exopolymers thus showed the presence of carboxyl, hydroxyl (which are the preferred groups for flocculation process), amino and sugar derivative groups.

**Heavy metal adsorption.** In the present study, the bioflocculant produced by *Pseudomonas aeruginosa* exhibited different levels of heavy metal adsorption. Differences in affinity of metals for bioflocculants are due to charge density, attractive interaction and types of conformation of polymer with adsorbed ions (Morillo et al., 2006). The mechanism and kinet-
Bioflocculant produced by *P. aeruginosa*

Chemical of metal biosorption depends on the experimental conditions particularly, medium pH, initial metal ion concentration and bioflocculant concentrations (Converti *et al.*, 2006).

Results illustrated in Fig. 3 show that the heavy metals adsorption did not increase with the increase of initial concentrations. The bioflocculant showed the highest copper and mercury removal of 87.39% and 89.09%, respectively at 20 ppm. The optimum adsorption of lead (79.70%) and cadmium (79.93%) by the bioflocculant were recorded at 40 ppm, whereas, the highest arsenate and zinc removal of 72.96% and 80.59%, respectively was recorded at 60 ppm. The enhancement in metal adsorption could be due to an increase in electrostatic interactions, involving sites of progressively lower affinity for metal ions (Puranik and Pakniker, 1999). Therefore, there was no increase in metal uptake where the binding sites were saturated by the metals.

It is clear from the results presented in Fig. 4 that the bioflocculant exhibited a better efficiency for removal of heavy metals at lower bioflocculant concentration as described by others (Das and Santra, 2007). Higher efficiencies in removing heavy metals at the low bioflocculant concentrations make them very attractive in the treatment of industrial effluents/wastewaters.

The effect of pH on the adsorption of heavy metals was examined at pH 3.0, 5.0, 7.0 and 9.0. The results presented in Fig. 5 show that the highest adsorption of Cu$^{2+}$, Pb$^{2+}$, Cd$^{2+}$ and Hg$^{2+}$ were reported at pH 7 whereas, the highest adsorption of As$^{3+}$ and Zn$^{2+}$ were reported at pH 9. Heavy metals adsorption was low at low and high pH values; it was reported that at low pH

**Fig. 4.** Effect of bacterial bioflocculant concentration on the percentage of heavy metals removal.

Cu$^{2+}$ (20 ppm), Pb$^{2+}$ (40 ppm), As$^{3+}$ (60 ppm), Zn$^{2+}$ (60 ppm), Cd$^{2+}$ (40 ppm), Hg$^{2+}$ (20 ppm).

**Fig. 5.** Effect of pH on the percentage of heavy metals removal using 100 ppm bacterial bioflocculant.
values, a high concentration of protons competes for the same anionic sites on the polymer as the divalent cations. The mass of protons leads to their preferential binding and thus divalent cation binding is low (Sahoo et al., 1992). As the pH increases to its optimum value, which differ from one metal ion to another, the adsorbing surface saturated with negative charges, resulted in increased efficiency to bind and adsorb metal ions of positive charges (Bayramoglu et al., 2003). While at pH higher than its optimum value, hydroxoo species of the metals can be formed and do not bind to the adsorption sites on the surface of the adsorbent (Kacar et al., 2000).

Therefore, this study details important implications in providing a safer alternative flocculation method for wastewater treatment.

**Literature**


Bioflocculant produced by *P. aeruginosa*. 289


**Introduction**

L-arabitol is five-carbon polyalcohol which, together with its enantiomer xylitol, has been identified as one of the top 12 biomass-derivable building block chemicals. Due to its health-promoting effects (low caloric – only 0.2 kcal/g, low-glycemic, low-insulinemic, anticariogenic, and prebiotic), arabitol can be used in many of the known applications of xylitol, as a natural sweetener, a dental caries reducer, and a sugar substitute for diabetic patients (Koganti et al., 2011). Polyols are used in the food and pharmaceutical industries due to their technological properties; for instance, they can act as texturing agents, humectants, softeners, and color stabilizers. Industrial production of most sugar alcohols is performed by catalytic reduction of sugars with hydrogen gas and nickel at a high temperature and pressure, which is expensive and requires the use of chromatographic purification steps (Monedero et al., 2010). Polyols are also produced by microorganisms from appropriate sugars, e.g., those obtained from hydrolyzates of the hemicellulosic fraction of plant biomass (Saha and Bothast, 1996). Biotechnological production may represent an efficient and cost-effective alternative to chemical production. Arabitol is known to be produced from L-arabinose by yeast such as *Debaryomyces*, *Candida*, *Pichia*, *Wickerhamomyces*, and *Saccharomycopsis* (Koganti et al., 2011). In a first screening of yeasts and fungi able to produce arabitol from L-arabinose under oxygen-limiting conditions Mc Millan and Boynton (1994) observed that the xylose-fermenting yeasts converted arabinose to arabitol, and not to ethanol, because of the relatively inefficient multistep redox assimilation pathways of the pentose sugar. Saha and Bothast (1996) observed that, among 49 yeast strains capable of growing on L-arabinose, *C. entomaea* NRRL Y-7785 and *P. guilliermondii* CCY 39501 were superior secretors of L-arabitol (yield of about 0.7 g/g). Kordowska-Wiater et al. (2008) reported that *C. parapsilosis* DSM 70125 was an efficient producer of arabitol with a yield of 0.78 g/g.
The yeast *Saccharomyces cerevisiae* is unable to assimilate and ferment pentose sugars such as D-xylose and L-arabinose. Some attempts have been made to obtain a modified *S. cerevisiae* capable of assimilating pentoses and secreting pentitols but only as byproducts of ethanol fermentation of ligninocellulosic hydrolysates. The yeast has been modified by introducing genes of araBAD operon from *E. coli* (Sedlak and Ho, 2001), genes from fungal L-arabinose pathway (Bera et al., 2010), or genes from both bacteria and fungi (Karhumaa et al., 2006). The metabolism of the yeast has also been changed by fusion of its protoplast with that of another yeast strain *Torulaspora delbrueckii* (Lucca et al., 2002). In a previous study by Kordowska-Wiater and colleagues, a special kind of fusion between protoplasts of *S. cerevisiae* V$_{34}$ and nuclei of *P. stipitis* CCY 39501 was used to obtain karyoductants able to assimilate arabinose and secrete arabitol (Kordowska-Wiater and Targoński, 2001). One of them, named SP-K7, capable of producing large quantities of this polyol, is the subject of the present study.

The process of biotransformation of L-arabinose to arabitol is highly dependent on culture conditions, especially oxygen availability, temperature, pH, and kind and concentration of medium compounds. There are some reports concerning the influence of different conditions on the process studied (Fonseca et al., 2007; Kordowska-Wiater et al., 2008; Saha and Bothast, 1996), but there is no information about the application of statistical optimization methods to biotransformation of L-arabinose to arabitol. Response surface methodology (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analyzing situations in which a response of interest is influenced by several variables. The purpose of this method is to optimize a response by choosing the level of independent variables (factors) on the basis of a quadratic function describing the relationships between the response and the studied factors (Montgomery, 2001). This method is used in different biotechnological processes, e.g., production of xylitol by yeasts (Sampaio et al., 2006; Sarrouth and da Silva, 2010; Vasquez et al., 2006), but there are no reports on the application of RSM in the process of arabitol production. The aim of this investigation was to use statistical methods of RSM to optimize biotransformation of L-arabinose to arabitol by karyoductant SP-K7 of *S. cerevisiae* V$_{34}$ and *P. stipitis* CCY 39501.

### Experimental

**Materials and Methods.** Growth and cultivation media were composed of L-arabinose as the carbon source, yeast extract, malt extract, (NH$_4$)$_2$SO$_4$ and KH$_2$PO$_4$ in different concentrations according to a Plackett-Burman design (Table I). In a next stage, the medium was composed of yeast extract, malt extract, (NH$_4$)$_2$SO$_4$ and KH$_2$PO$_4$ at a concentration of 5 g/l and L-arabinose at concentrations from 3.1 to 61.9 g/l depending on the run number in CCD (Table II). The pH was adjusted to 5.5. Media for inoculum preparation were dispensed into tubes (5 ml per tube), and media for cultivation were dispensed into 100-ml Erlenmeyer flasks (20 ml per flask) and sterilized by autoclaving at 121°C for 15 min.

**Inoculum and culture conditions.** Inocula were prepared by transferring a loopful of cells from a slant into the tubes with media and incubation at 28°C for 24 hours. Then, cultivation media were inoculated with 2% (v/v) prepared culture and incubated in a rotary shaker (Infors HT Minitron, Infors AG, Switzerland).

### Table I

<table>
<thead>
<tr>
<th>Run</th>
<th>Arabinose (g/l)</th>
<th>Yeast extract (g/l)</th>
<th>Malt extract (g/l)</th>
<th>(NH$_4$)$_2$SO$_4$ (g/l)</th>
<th>KH$_2$PO$_4$ (g/l)</th>
<th>Temp. (°C)</th>
<th>Rotation speed (rpm)</th>
<th>Max. arabitol conc. (g/l)</th>
<th>Arabitol yield (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>32</td>
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<td>0.00</td>
<td>0.00</td>
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<tr>
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<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>32</td>
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<td>2</td>
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<td>24</td>
<td>200</td>
<td>0.64</td>
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</tr>
<tr>
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<td>2</td>
<td>2</td>
<td>2</td>
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<td>200</td>
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<td>10</td>
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<td>50</td>
<td>0.24</td>
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</tr>
<tr>
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<td>5</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>32</td>
<td>200</td>
<td>16.71</td>
<td>0.355</td>
</tr>
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</table>
at 50 or 200 rpm and at 24°C or 32°C according to the Plackett-Burman design (Table I). In the second stage of the experiment, cultures were incubated at temperatures and rotation speeds according to CCD (Table II). After 48 and 72 h of incubation, the cells were collected by centrifugation at 6000 ×g for 15 min, and supernatants were used for further analysis.

**Verification of optimal model.** Inoculum of the investigated yeast strain was prepared as above. The medium was composed of yeast extract, malt extract, (NH₄)₂SO₄ and KH₂PO₄ at a concentration of 5 g/l and L-arabinose concentration of 32.5 g/l as estimated by RSM. The temperature of incubation and rotation speed were 28°C and 150 rpm, respectively. Cultures, in a volume of 100 ml, were incubated in 500-ml Erlenmeyer flasks on a rotary shaker (Infors HT Minitron, Infors AG, Switzerland). Samples for analysis were collected after 48 and 72 h and centrifuged at 6000 ×g for 15 min. Supernatants were used for L-arabinose and arabitol detection.

**Analytical methods.** L-arabinose and arabitol concentrations in supernatants were determined by HPLC (Gilson Inc., USA) equipped with a refractive index detector (Knauer GmbH, Germany) and Bio-Rad Aminex Carbohydrate HPX 42C (300 × 7.8 mm) column (Bio-Rad Laboratories Inc., USA). Deionized water was used as eluent at a flow rate of 0.5 ml/min, and the temperature of separation was 85°C. Integration and analysis of chromatograms were done using Chromax 2007 software version 1.0a.

**Experimental designs.** The Plackett-Burman design, which enables screening of \( n \) variables using only \( n+1 \) experiments, was applied to limit the number of factors, selected from among media components and culture conditions, that were important for the process (Myers and Montgomery, 2002). In this experiment, a Plackett-Burman design matrix was constructed (Table I) in order to investigate the influence of the main effect of 7 selected variables on arabitol production. After the factors had been limited to three most strongly affecting the response (rotation speed, L-arabinose concentration, and temperature), a central composite design (CCD) based on three independent variables (Table II) was used to estimate response surfaces, following the general model equation:

\[
Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j ,
\]

where \( Y \) is the response variable, \( \beta_0 \) is the interception, \( \beta_i \) is the linear effect, \( \beta_{ii} \) is the quadratic effect, and \( \beta_{ij} \) are interaction effect coefficients. \( X_i \) and \( X_j \) are coded values of the factors selected as a result of the initial screening using the Plackett-Burman design. The significance of the obtained model was checked by an F-test, and

<table>
<thead>
<tr>
<th>Run</th>
<th>Rotation speed ((X_1)) (rpm)</th>
<th>Arabinose ((X_2)) (g/l)</th>
<th>Temperature ((X_3)) (°C)</th>
<th>Arabitol (g/l)</th>
<th>Arabitol yield (g/g)</th>
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</thead>
<tbody>
<tr>
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<td>15</td>
<td>24</td>
<td>1.028</td>
<td>0.163</td>
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<td>0.159</td>
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<td>100</td>
<td>50</td>
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<tr>
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<tr>
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<td>1.75</td>
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<tr>
<td>13</td>
<td>150</td>
<td>32.5</td>
<td>21.28</td>
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<td>0.941</td>
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<tr>
<td>14</td>
<td>150</td>
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<td>34.72</td>
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<td>0.516</td>
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<tr>
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<td>18.285</td>
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<tr>
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<td>150</td>
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<td>18.875</td>
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<tr>
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<td>150</td>
<td>32.5</td>
<td>28</td>
<td>16.275</td>
<td>0.5</td>
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</tbody>
</table>
goodness of fit was tested by determining coefficient $R^2$. The relationships between experimental and predicted values were shown on the response surface plots. All design matrices were generated and analyzed using a Statistica software version 7 (2007).

Results

**Plackett-Burman design.** Results of the experiments performed on the basis of the Plackett-Burman design are presented in Table I. The highest concentration of arabitol (16.71 g/l) and the highest arabitol yield (0.355 g/g) were obtained in experiment 8, in which all the variables were at high levels. Estimated values of the effect of independent factors are shown in Table III. The level of polyol concentration was mostly influenced by rotation speed (rpm) followed by concentration of L-arabinose and incubation temperature. The components of the medium such as extracts and mineral salts had less effect on biotransformation, but on the whole all the studied variables had a positive effect on arabitol production. Three factors with the highest effects were chosen for further investigation (Table III).

**CCD.** Full-factorial CCD consisted of five levels: the low and high levels, central points and star points with $\alpha = \pm 1.68$. Twenty combinations were run, as shown in Table II, and the concentration of arabitol in culture media was analyzed. Different concentrations and different yields of arabitol were obtained from 1 g of consumed sugar depending on the combination of variables. The regression coefficients obtained as a result of the CCD analysis are shown in Table IV. Since none of the factor interactions were significant, the model was simplified and the final quadratic model took the following form:

$$Y = -272 + 0.726X_1 - 0.002X_1^2 + 1.36X_2 - 0.021X_2^2 + 14.66X_3 - 0.252X_3^2$$

Table V presents the results of ANOVA which indicate that the model was statistically significant. The value of $R^2=0.93195$ suggests that this model was well fitted to the experimental data and only 6.8% of the total variations in the response were not explained by it. The response surface plots are shown in Figures 1–3.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Effect estimate</th>
<th>Coefficient</th>
<th>Ranking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>4.220</td>
<td>2.110</td>
<td>2</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>3.915</td>
<td>1.957</td>
<td>6</td>
</tr>
<tr>
<td>Malt extract</td>
<td>3.935</td>
<td>1.967</td>
<td>5</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>3.815</td>
<td>1.907</td>
<td>7</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>4.035</td>
<td>2.017</td>
<td>4</td>
</tr>
<tr>
<td>Temperature</td>
<td>4.100</td>
<td>2.050</td>
<td>3</td>
</tr>
<tr>
<td>Rotation speed</td>
<td>4.640</td>
<td>2.320</td>
<td>1</td>
</tr>
<tr>
<td>Mean/Interc.</td>
<td>2.380</td>
<td>2.380</td>
<td>–</td>
</tr>
</tbody>
</table>

Table III

Results of the Plackett-Burman design analysis

Table IV

Regression coefficients obtained on the basis of CCD

<table>
<thead>
<tr>
<th>Regression</th>
<th>Std. Err.</th>
<th>t(10)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean/Interc.</td>
<td>-269.079</td>
<td>48.03999</td>
<td>-5.60114</td>
</tr>
<tr>
<td>(1)X1-rotation speed (L)</td>
<td>0.672</td>
<td>0.18003</td>
<td>3.73497</td>
</tr>
<tr>
<td>X1- rotation speed (Q)</td>
<td>-0.002</td>
<td>0.00031</td>
<td>-7.47363</td>
</tr>
<tr>
<td>(2)X2-arabinose conc. (L)</td>
<td>1.643</td>
<td>0.48981</td>
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<tr>
<td>X2- arabinose conc. (Q)</td>
<td>-0.021</td>
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<td>-8.26249</td>
</tr>
<tr>
<td>(3)X3-temp.(L)</td>
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<td>2.90978</td>
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<td>X3-temp.(Q)</td>
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<td>0.00120</td>
<td>-0.71810</td>
</tr>
<tr>
<td>1L by 3L</td>
<td>0.003</td>
<td>0.00526</td>
<td>0.55237</td>
</tr>
<tr>
<td>2L by 3L</td>
<td>-0.005</td>
<td>0.01504</td>
<td>-0.36528</td>
</tr>
</tbody>
</table>
figures clearly demonstrate that arabitol concentration was affected by all of the investigated factors and that the selected ranges of them were appropriate. On the basis RSM, the optimal levels of factors for arabitol production from L-arabinose by karyoductant SP-K7 were defined as follows: rotation speed 150 rpm, concentration of L-arabinose 32.5 g/l, and temperature 28°C. In such conditions, the predicted concentration of arabitol after two days of incubation should be 18.367 g/l.

**Verification of the model.** The medium containing arabinose at the optimal concentration and the remaining components at a concentration of 5 g/l was inoculated with the karyoductant strain and incubated at the optimal rotation speed and temperature in order to verify the usefulness of the statistical model. After 2 days of incubation 16.80 g/l of arabitol was obtained. The yield of this process was 0.52 g/g of consumed arabinose. The result of the experiment was about 9% lower than predicted, so it may be accepted as a confirmation of the usefulness of the model.

**Discussion**

*Saccharomyces cerevisiae* has to be modified to obtain the ability to metabolize pentose sugars from hemicellulosic hydrolyzates and produce ethanol or pentitols as byproducts during ethanol fermentation. While a lot of investigations have focused on xylitol production from D-xylose (Hahn-Hagerdal et al., 2007; Jeffries and Jin, 2004), there are few reports about the application of an engineered *S. cerevisiae* for L-arabinose utilization (Bera et al., 2010; Bettiga et al., 2009; Karhumaa et al., 2006; Sanchez et al., 2010; Sedlak and Ho, 2001). Karhumaa et al. (2006) constructed different recombinants of *S. cerevisiae* containing bacterial genes of the L-arabinose pathway and/or yeast genes of xylose metabolism, which were able to utilize xylose and/or arabinose. Strain BWY02.XA slowly consumed pentose sugars in anaerobic conditions and secreted arabitol with a yield of about 1 g/g of arabinose. Sanchez et al. (2010) continued the investigation of the engineered industrial strains of *S. cerevisiae* carrying genes responsible for xylose and arabinose metabolism, obtained by Karhumaa et al. (2006). Those authors used evolutionary engineering to improve strain TMB 3061 in continuous culture. Under anaerobic conditions, all of the modified strains almost stoichiometrically converted L-arabinose to arabitol (0.94–1.03 g/g), which suggested their inability to ferment arabinose to ethanol despite the presence of appropriate genes (Sanchez et al., 2010). Bera et al. (2010) also obtained a recombinant *S. cerevisiae* 424A (LNH-ST) containing fungal genes of arabinose metabolism, which was able to ferment hemicellulosic sugars. While a control strain only produced arabitol from arabinose as sole carbon source with a metabolic yield of 82.5 ± 4.7%, the recombinant, bearing plasmid pLXR<sub>NAD</sub>-LAD, produced both arabinol and ethanol from this pentose with the metabolic yields of 33.7 ± 0.3% and 42.6 ± 2.3%, respectively (Bera et al., 2010). A recombinant obtained by Bettiga et al. (2009), TMB3664 carrying genes of the fungal

**Table V**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of square</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F-value</th>
<th>p-value</th>
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<td>Error</td>
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<td>7.474</td>
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</table>

**Fig. 2.** Effects of rotation speed ($X_1$) and temperature ($X_3$) on arabitol concentration ($Y$) with arabinose concentration ($X_2$) at its center point level.

**Fig. 3.** Effects of arabinose concentration ($X_2$) and temperature ($X_3$) on arabitol concentration ($Y$) with rotation speed ($X_1$) at its center point level.
pathway, also produced arabitol from consumed arabinose with a yield of 0.48 g/g. Karyoductant SP-K7 of S. cerevisiae V30 and P. stipitis CCY 39501 was shown to be able to assimilate arabinose and secrete arabitol with the yield of 0.45–0.95 g/g depending on culture conditions, but it could not produce ethanol from this pentose. On the other hand, the strain was demonstrated to be quite efficient producer of xylitol from D-xylose (Kordowska-Wiater and Targoński, 2001; unpublished data).

The results obtained by different scientists suggest that there are factors that might limit the L-arabinose metabolism. One such factor is an imbalance of redox cofactors which determine the kind of product that is secreted into the medium. Observations made by researchers indicate that it is necessary to check the influence of different environmental factors on L-arabinose assimilation and product secretion. On the basis of the literature and screening studies (unpublished data), 7 factors were chosen for the Plackett-Burman design (Table 1) to find out which were the most important for the process. As it could be expected, the factors that influenced arabitol production most were rotation speed, which is responsible for oxygen availability in the medium, the initial concentration of L-arabinose, which is the substrate for the process, and temperature, which affects cell growth and enzymatic activity. These variables were selected for the second part of experiment – optimization on the basis of a CCD design. This statistical method is very useful in the optimization of biotechnological processes. There is no information about the use of the statistical methods of RSM for the optimization of arabitol production from L-arabinose. To the best of our knowledge, this is the first publication on this subject. The present results can, however, be compared with results for xylitol production from xylose because the substrates and the products are chemically and functionally similar and both sugars, being components of hemicelluloses, can be metabolized by similar yeast strains.

There are several publications reporting the application of RSM for xylitol production from D-xylose and other sugars of plant biomass origin by yeast D. hansenii (Sampaio et al., 2006; Sarrouh and da Silva, 2010) and C. guilliermondii (Vasquez et al., 2006; Sarrouh and da Silva, 2010). Sampaio et al. (2006) ran a 3 full factorial design on initial concentration of xylose, rotation speed and starting biomass concentration as independent variables, and maximum xylitol concentration, yield, productivity, and specific productivity as response variables. Four equations were obtained and R2 of the model for maximum xylitol concentration was 0.9895, which meant that 98.95% of total variations in the response were explained by the model. The three remaining coefficients of determination were also above 0.9, which suggested a good fit of the model. It was shown that the increase in xylose concentration in the range 55–165 g/l resulted in a rise in xylitol concentration. A rotational speed was also shown to have positive influence on the response especially in the range 100–200 rpm (Sampaio et al., 2006). In another study, Vasquez et al. (2006) used RSM in order to optimize xylitol production from D-xylose by C. guilliermondii. They investigated the influence of the oxygen transfer coefficient and initial cell mass on xylitol yield and productivity. The optimal point corresponded to a cell mass of 9.86 g and an oxygen transfer coefficient of 32.85/h, for which predicted productivity was 1.4 ± 0.09 g/h and predicted product yield was 0.7 ± 0.02 g/g with confidence level of 95% (Vasquez et al., 2006). Sarrouh and da Silva (2010) used RSM (2 factorial design) to study the production of xylitol by C. guilliermondii from hemicellulosic hydrolysates rich in xylose in a fluidized bed reactor. They chose three independent variables, namely air flow, concentration of hydrolysate, and fluidization flux, and xylitol yield and productivity as response variables. The best values of the xylose to xylitol bioconversion parameters were observed in the central point of the factorial design (air flow, 600 ml/min; concentration of hydrolysate, 5x; and fluidization flux, 38) (Sarrouh and da Silva, 2010). Our results also showed the important role of culture aeration, which is determined by different factors (oxygen transfer, rotation speed, air flow) and can limit oxygenation processes in the cells and biomass growth. A second factor which is usually taken into consideration is the concentration of sugar as a sole carbon source and also mean values of it are the optimal for the process. It is interesting that different models are developed for different response variables of the same process, e.g., product concentration (as in this report), product yield, productivity and so on. The variety of response variables, however, impedes comparison of results obtained by different scientists.

In summary, karyoductant SP-K7 of S. cerevisiae V30 and P. stipitis CCY 39501 was able to produce arabitol from L-arabinose in batch cultures in different concentrations (0.13–18.92 g/l) and with different yields (0.005–0.94 g/g) depending on culture conditions and the amount of consumed pentose. RSM used in the optimization of this process showed that the most influential factors were rotation speed, concentration of L-arabinose and the temperature of incubation. The optimum values of these factors were 150 rpm, 32.5 g/l, and 28°C, respectively. The model predicted that the concentration of arabitol after two days of yeast incubation should be 18.367 g/l, which indicates that it is possible to achieve a yield of about 0.56 g/g during complete consumption of L-arabinose. The verification experiment confirmed the usefulness of the statistical model.
Literature


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The Capacity of Mycobacterium tuberculosis Complex Species and M. bovis BCG Substrains Specific Identification – Implications for Optimized PCR-Based Diagnostics in Adverse Events Following Vaccination Suspected Cases

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Received 26 April 2012, revised 18 September 2012, accepted 21 September 2012

Abstract

The capacities of differentiation of Mycobacterium bovis BCG from other members of M. tuberculosis complex species using PCR-RFLP, multiplex PCR, and PCR-based genomic deletion analysis approaches were compared. In the study, mycobacteria isolated from patients suspected of adverse events following vaccination with BCG, primarily classified according presence of RD1 marker as virulent and avirulent mycobacteria, were used. The PCR-based genomic deletion analysis was found the best option for mycobacteria diagnostics improvement, as it was capable precisely differentiate virulent and avirulent mycobacteria or virulent species of M. tuberculosis complex. The routine confirmation of mycobacteria species in the cases of adverse events following BCG vaccination is highly expected, especially in clinical practice of patients with primary immunodeficiency.

Key words: Mycobacterium bovis BCG, mycobacteria specific identification, PCR-based diagnostics

Introduction

Although extensive studies have been performed to develop advanced vaccines against tuberculosis (TB), vaccination with attenuated Mycobacterium bovis Bacille Calmette-Guérin (BCG) is the only commercially available vaccine against TB (WHO, 2011). The BCG vaccine is used worldwide (Brewer, 2000) and generally is regarded as being the safest in use, but the real incidence of disease evoked by BCG strains, e.g. BCG-itis or osteitis, is not known (Behr, 2002). Localized abscesses, regional lymphadenopathy, and disseminated disease in immunocompromised hosts are generally regarded as rare but well recognized complications following BCG vaccination (Bernatowska et al., 2007). Abscesses at BCG injection sites and in places other than BCG injection sites have also been described in healthy hosts (Pankowska and Roźniecki, 1997; Gołębiowska et al., 2008). In Poland, BCG vaccination has been a part of the National Immunization Program since 1951, and currently involves a single BCG dose given to neonates only. BCG AEFI (Adverse Events Following Immunization) cases are registered through passive surveillance and most of them are not routinely confirmed in the laboratory. The rate of AEFI in Poland is regarded as low (Szczuka, 2002), however, lack of simple and routine diagnostics does not allow to estimate its real incidence. Lack of routinely used species-specific diagnostics also complicates fast and reliable diagnosis of mycobacteria grown from BCG vaccinated individuals suffering from immunodeficiencies. The M. tuberculosis complex consists of mycobacteria characterized by high DNA-DNA homology (from hybridization studies) and identical 16S rRNA sequences but differ in phenotypes and pathogenicity. This group has consisted of seven closely related species: M. tuberculosis – infecting human and primates; M. africanum, a heterogeneous group causing human tuberculosis in Africa; M. microti, found as a source of infection in voles and very rarely in immunocompromised humans; M. bovis, causing tuberculosis in cattle and a wide variety of other animals, including man; M. bovis Bacille Calmette-Guérin (BCG), an attenuated vaccine strain; M. canetti, a very rare, smooth variant of M. tuberculosis, usually isolated in Africa; and M. caprae, primarily causal agent of caprine TB with transmission to human and wild animals reported (Brosch et al., 2002; Cousins et al., 2003; Rodríguez

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More recently, novel species has been described: *M. mungi*, pathogen inducing high mortality rates among banded mongooses living in close association with humans in Botswana (Alexander *et al*., 2010); *M. pinnipedii*, previously known as seal bacillus primarily infecting pinnipeds but is also pathogenic to humans and, possibly, cattle (Cousins *et al*., 2003) and *M. oryctes*, causative agent of tuberculosis in oryxes, gazelles, and waterbucks in Africa; cows and rhesus monkeys in South Asian; and humans (van Ingen *et al*., 2012).

It has been estimated that as many as 49 BCG substrains might be used in the world (Corbel *et al*., 2004) since the original Bacille Calmette-Guérin attenuated strain has been distributed worldwide. BCG daughter strains were found heterogenic as the result of the microevolution due to specific production processes, which can be currently specifically identified with molecular tools (Behr, 2002).

The use of reliable molecular tools applicable for confirmation of species identity in AEFI suspected cases are expected to precisely identify all *M. tuberculosis* complex representatives together with a possibility to identify *M. bovis* BCG substrains. In order to evaluate the capacities of molecular diagnostic tools described previously as potentially applicable for *M. tuberculosis* complex identification, in this pilot study we have used reference mycobacteria species and randomly chosen mycobacteria isolates grown from AEFI suspected cases and collected at the National Tuberculosis and Lung Diseases Research Institute (NTLDRI). The mycobacteria isolated from patients and chosen for the purpose of the study have been primarily classified as virulent and avirulent mycobacteria, as they were found harbour and not harbour RD1 marker, respectively. Three potentially applicable variants of PCR-based methods involving different species-specific or substrain-specific sequencess published previously were introduced for testing and compared.

**Experimental**

**Material and Methods**

**Strains.** A total of 10 mycobacterial strains isolated from BCG AEFI suspected cases within 2004–2010, have been included in the study. At NTLDRI they were diagnosed as virulent (7575, 3221, 1339, 5995, 5379) and avirulent (60, 4138, 868, 1078, 2714) mycobacteria according PCR performed with primers specific for RD1 region (Talbot *et al*., 1997). All strains were grown on solid Löwenstein-Jensen medium for about 20 days at 37°C, harvested, heat inactivated. The following reference strains were used: *M. bovis* BCG Moreau originating from BCG vaccine lot no. 00108, *M. bovis* BCG Danish 1331 (NIBSC, 07/270), *M. bovis* BCG Tokyo 172 (NIBSC, 07/272), *M. tuberculosis* H37Rv (ATCC 25618), *M. bovis* (ATCC 19211), *M. microti* (ATCC 19422), *M. africanum* (ATCC 25420), *M. pinnipedii* (ATCC BAA-688), and *M. canetti* (kindly obtained from Prof. Dr. Dick van Soolingen from Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment, The Netherlands).

**DNA extraction.** Chromosomal DNA was isolated from mycobacterial strains as described by van Soolingen *et al.* (1991).

**PCR.** In the study we have tested PCR-RFLP developed by Kasai *et al.* (2000), PCR-based genomic deletion analysis described by Warren *et al.* (2006) and multiplex PCR developed by Bedwell *et al.* (2001). System developed by Kasai *et al.* (2000) involved restriction of amplified product of the partial gyrB gene with *RsaI* or *TaqI* enzymes. PCR-based genomic deletion analysis involved PCR method with use of primers specific for RD1, RD4, RD9, and RD12 regions (Warren *et al*., 2006). In a multiplex PCR described by Bedwell *et al.* (2001) primers specific for RD1, RD2, RD8, RD16 and the *senX3-regX3* regions were primarily developed for purposes of identification of different substrains of *M. bovis* BCG.

**Results**

Among 10 mycobacteria isolates, 5 primarily classified at the NTLDRI as avirulent mycobacteria (60, 4138, 868, 1078, 2714), were easily identified as *M. bovis* BCG Moreau isolates (Fig. 1A) as they showed a multiplex-PCR profiles presence of 196 bp (ARD1), 252 bp (RD14), 276 bp (*senX3-regX3*), 315 bp (RD2) and the 472 bp (RD8) amplified fragments. Multiplex-PCR profiles of reference *M. bovis* BCG Danish and *M. bovis* BCG Tokyo substrains according of size of amplified fragments were easily differentiated from *M. bovis* BCG Moreau substrain. Other 5 mycobacteria isolates, classified at the NTLDRI as virulent (7575, 3221, 1339, 5995, 5379), presented profiles different from those of *M. bovis* BCG reference strains and samples of avirulent mycobacteria. In the profiles of mycobacteria samples, pre-classified as virulent, there were found products seen in the profiles of all *M. tuberculosis* complex substrains. The amplification profiles of virulent mycobacteria samples were composed of fragments RD1 (146 bp), RD14 (252 bp), RD2 (315 bp), RD16 (401 bp), RD8 (472 bp) regions but did not contained the PCR product expected for the *senX3-regX3* region. As multiplex PCR profiles did not contained 352 bp and/or 276 bp products, single PCR reactions were performed with *senX3-regX3* region primers revealing their presence and suggesting the inhibition
of the PCR reaction in cases of the virulent mycobacteria isolates (Fig. 1B).

During the PCR-based genomic deletion analysis, 5 isolates classified at the NTLDRI as avirulent mycobacteria (60, 4138, 868, 1078, 2714), were easily identified as *M. bovis* BCG (Fig. 1C) as they showed a profile of deletion regions RD9, RD1, RD4 and RD12 (with amplicon sizes: 108 bp, 196 bp, 268 bp and 306 bp, respectively). Their profiles were identical with profiles obtained for *M. bovis* BCG Moreau, *M. bovis* BCG Danish and *M. bovis* BCG Tokyo substrains. Other 5 mycobacteria isolates, classified at the NTLDRI as virulent ones (7575, 3221, 1339, 5995, 5379), presented profiles specific for *M. tuberculosis* with presence of amplified fragments...
from RD1, RD4, RD9, RD12 regions (146 bp, 172 bp, 235 bp, 369 bp products, respectively).

In the first step of PCR-RFLP gyrB analysis unique sequence for M. tuberculosis complex was amplified. Fragment of 1 020 bp was present in all mycobacteria strains used in the study (data not shown) and confirmed affiliation to the M. tuberculosis complex. At the second step, the amplified DNA fragments were digested by Rsal or TaqI and enabled four species of M. tuberculosis, M. africanum, M. bovis and M. microti to be differentiated. As shown in the Fig. 1D, M. bovis and M. microti could be differentiated from the other species by the presence of Rsal-digested fragments of 500 bp and 700 bp, respectively. TaqI digestion of the partial gyrB gene generated a 300 bp-fragment specific for M. tuberculosis as described Kasai et al. (2000) but also for M. canetti.

Discussion

The adverse events following vaccination with BCG, although registered by national surveillance after clinical recognition, are confirmed in the laboratory rarely. Moreover, the differentiation of M. bovis BCG from other members of the M. tuberculosis complex has previously been regarded difficult (Augustynowicz-Kopeć et al., 2006). Some variants of NAT (Nucleic Acid Amplification Techniques) have been described in order to improve M. tuberculosis complex or M. bovis BCG substrains diagnostics possibilities (Jagielski et al., 2010a, 2010b). In our study we have compared diagnostics capacity of gyrB-based PCR-RFLP, multiplex PCR, and PCR-based genomic deletion analysis using a set of reference mycobacteria strains and ten mycobacteria isolates originating from BCG AEFI suspected cases collected at the NTLDRI.

The first one – the gyrB-based PCR-RFLP method, previously shown to be useful for differentiation of closely related strains of bacteria such as Vibrio (Venkatesswaran et al., 1998) and Bacillus spp. (Yamada et al., 1999), was adopted by Kasai et al. (2000) to identify four M. tuberculosis complex members (M. tuberculosis, M. bovis, M. africanum and M. microti). In our study, although proper restriction profiles for all above mentioned four species were observed, the system failed to differentiate M. bovis BCG substrains, M. bovis BCG from virulent M. bovis, and M. tuberculosis from M. canetti species.

The multiplex PCR described by Bedwell et al. (2001) easily distinguished M. bovis BCG substrains, however failed to differentiate M. tuberculosis, M. africanum, M. canetti, and M. pinnipedii species. Thus, M. bovis BCG Moreau substrain was easily identified in all isolates, determined at the NTLDRI as avirulent ones, however without the value for species identification in virulent isolates.

PCR-based genomic deletion analysis (Warren et al., 2006), described originally as accurate tool for identification of M. canetti, M. tuberculosis, M. africanum, M. microti, M. pinnipedii, M. caprae or M. bovis BCG species, was found the most potent diagnostics option. Regions of differences distinguished M. bovis, M. bovis BCG, M. tuberculosis, M. canetti, M. microti and M. africanum species were described previously (Brosch et al., 2002). Differentiation of M. microti/africanum/pinnipedii species was possible with a second PCR round with additionally designed primers. Although, PCR-RFLP of the partial gyrB gene (Kansai et al., 2000) and multiplex PCR (Bedwell et al., 2001) methods declared improvement of diagnostics potential, only PCR-based genomic deletion analysis developed by Warren et al. (2006), was found capable to precisely and simultaneously differentiate virulent and avirulent mycobacteria or virulent species of M. tuberculosis complex. Multiplex PCR developed by Bedwell et al. (2001) was found a valuable diagnostic option in situations when information on the M. bovis BCG substrain might be needed, eg. in foreigners infected with M. bovis BCG.

The criteria for the diagnosis of disseminated BCG infection in people with primary immunodeficiency, including definitive cases confirmed as M. bovis BCG substrain infection by culture and/or standard PCR (Bernatowska et al., 2007) were previously proposed. However, the diagnostic details were not evaluated and discussed. As many different molecular tools were described, their evaluation is expected to improve AEFI surveillance measures. PCR-based genomic deletion analysis might be thus chosen for purposes of rapid identification of mycobacteria carriers, appropriate treatment of patients, epidemiological studies purposes, transmission studies of M. bovis between animals and humans, reliable confirmation of M. bovis BCG recovered from immunocompromised patients and healthy individuals suspected of AEFI after BCG vaccination.

Acknowledgments

This study was supported by a grant for scientific research (NN 404 207 239) from the Ministry of Science and Higher Education in Poland.

Authors express theirs gratitude to Prof. Dr. Dick van Soolingen from Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment (The Netherlands) who provided M. canetti reference strain.

Literature


Protein Profiles from Intact Cells as a Tool in Bifidobacterium Characteristics

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Received 1 June 2012, revised 18 September 2012, accepted 19 September 2012

Abstract

In this study sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles were analysed and differences were confirmed by a unweighted pair group method with arithmetic average (UPGMA) analysis between bifidobacterial species, such as B. infantis ATCC1567, B. bifidum Bb-12, B. longum KN29, B. catenulatum KD14, and B. animalis BI30. Two dimensional electrophoresis separation profiles were compared, and the most characteristic spots were characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We propose proteins extracted from intact cells as an additional trait for bifidobacteria characterization, together with molecular techniques, which can be used to analyze bacterial protein polymorphism and to distinguish among species.

Key words: bifidobacteria, liquid chromatography-mass spectrometry (LC-MS/MS), 2D-electrophoresis, proteins

Bifidobacterium is a genus of Gram-positive, microaerophiles or strict anaerobes, some of which are commonly detected in the human gut. Bifidobacteria are phylogenetically grouped in the Actinomycete branch with a high G+C content, and currently 47 species are recognized within this genus (Matsuki et al., 2003; Eztevy, 2007). Bifidobacteria are part of the resident microflora of the human large intestine and are beneficial to their host’s health (Orrhage and Nord, 2000). Some bifidobacterial strains are widely used as health-promoting or probiotic components in functional food products (Lin, 2003). For these reasons, bifidobacteria are a subject of growing interest in the pharmaceutical and food industries.

Within the forty seven species currently recognized as belonging to the genus Bifidobacterium, only a few have been sequenced (Lukjancenko et al., 2011). There is limited information on the polymorphism of bifidobacterial cell-wall proteins (BIFOP). Mattarelli et al. (1993) showed phenotypic differences among BIFOP on the basis of an examination of 150 strains of Bifidobacterium globosum. Proteomic analysis has been used to show differences among Bifidobacterium longum strains (Aires et al., 2010). It has been demonstrated that SDS-PAGE of whole cell proteins is a reliable and specific method for the identification of the lactic acid bacteria down to the species level (Tae-Woon et al., 2003). Hébert et al. (2000) showed that SDS-PAGE fingerprinting of cell-wall proteins allowed to distinguish L. helveticus from L. delbrueckii subsp. lactis. Two subspecies of Lactobacillus delbrueckii were characterized by different SDS-PAGE cell-wall protein profiles. Gatti et al. (2001) have also shown that this method can be an efficient taxonomic tool. It has been reported that cell-wall proteins can be used as immunoreactive markers for the identification of some pathogenic microorganisms (Betts et al., 2000; Duffs et al., 2000; Enroth et al., 2000).

The aim of the present study was to employ proteins extracted from intact cells for characterization of bifidobacterial species. Bifidobacterium infantis ATCC1567 was obtained from the American Type Culture Collection (ATCC, Manassas, Va., U.S.A., http://www.atcc.org/). B. bifidum Bb-12, B. animalis BI30, B. catenulatum KD14, and B. longum KN29 were kindly provided by Prof. M. Bielecka (Department of Food Microbiology, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences in Olsztyn, Poland). Bacteria were cultured on Garche’s medium (Rasic, 1990) (10 ml) at 37°C in anaerobic conditions. After 24 h, stock cultures were inoculated in a new medium using 5% (v/v) of inoculum. Extractions were performed in triplicate from three independent cultures.
All species (0.5 l of liquid culture) were harvested by centrifugation (15 min, 4°C, 8 000 × g) at the early stationary growth phase. Cell-wall proteins were extracted using three protocols: the Mattarelli et al. (1993) method, a method using 8 M urea, and a method using 2 M guanidine hydrochloride (Rosenberg, 2005). Protein concentrations were estimated using the Bradford Assay Kit (Bradford, 1976). Proteins from all extracts were solubilised in loading buffer and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a final polyacrylamide concentration of 10% w/w (Laemmli, 1970).

Protein pattern profiles were scored manually for the presence (1) or absence (0) of protein bands. These binary data matrices were used for calculating Jaccard similarity coefficients (Sneath and Sokal, 1973) using the SIMQUAL (similarity for qualitative data) module of NTSYS-pc software version 2.02 g (Rohlf, 1998). Subsequently, similarity coefficients were used to construct UPGMA (unweighted pair group method with arithmetic average) dendrograms using SAHN (sequential, hierarchical, agglomerative and nested cluster methods) clustering implemented in NTSYS-pc 2.02g.

The samples of extracted proteins were prepared using a 2D Clean-Up kit (LG Healthcare) and two dimensional electrophoresis was performed according to Nezhad et al. (2012). Upon completion of 2D SDS-PAGE, the gels were stained with BioSafe Coomassie Stain. Stained protein spots were scanned on a GS-800 Calibrated Densitometer (Bio-Rad, USA). The 2D gels were calibrated using a 2D SDS-PAGE standard (Bio-Rad, USA). Spot detection and analysis was performed using PDQuest software version 8.0.1 (Bio-Rad, USA).

Gel slices were subjected to a standard in-gel tryptic digestion (Shevchenko et al., 1996). The peptide mixtures obtained were then applied to a RP-18 pre-column (Waters) using a 0.1% (v/v) TFA solution as the mobile phase, and transferred to a nano-HPLC RP-18 column (Waters, length: 250 mm, bead diameter: 1.7 μm). The mass spectra obtained were preprocessed with the Mascot Distiller software (v.2.3, Matrix Science) and searched against the non-redundant protein database from the NCBI (NCBiR, 14259576 sequences; 4884494093 residues) using the 8-processor on-site licensed MASCOT search engine (Mascot Server v.2.2.03). The search parameters were set as follows: enzyme, Trypsin; fixed modifications, carbamidomethylation (C); variable modifications, oxidation (M); protein mass, unrestricted; peptide mass tolerance, ± 40 ppm; MS/MS fragment ion mass tolerance, ± 0.8 Da; max missed cleavages, 1. Only peptide hits exceeding a Mascot expectation value of 0.05 were accepted.

The acquired raw data were processed using the Mascot Distiller followed by a Mascot search (MatrixScience, London, UK, locally installed http://proteom.pl/mascot) against the NCBI nonredundant database. Search parameters for precursor and product ion mass tolerances were ± 40 ppm and ± 0.8 Da, respectively, with allowance made for one missed semi Trypsin, fixed modifications of cysteine through carbamidomethylation, and variable modification through lysine carbamidomethylation and methionine oxidation. The Mascot program reports an individual ion score for each assignment of an MS/MS spectrum to a database sequence, and groups correctly identified peptides into sets according to the corresponding database deposited proteins. The ion score is −10*Log(P), where P is the probability (P < 0.05) that the observed match is a random event.

The protein extraction methods were used to differentiate among the examined species of bifidobacteria. We showed that specific proteins isolated from intact cells could be species-specific. The different protein profiles obtained using the examined methods are shown in Fig. 1. The highest number of protein bands were identified using the extraction method by Mattarelli (1993). It can be assumed that the diversity of the collected proteins was due to ultracentrifugation, and it was only using this extraction method that low molecular weight proteins could be detected.

Dendrogram obtained by numerical comparison of the protein patterns of the investigated bifidobacterial species is shown in Fig. 2. A numerical analysis of the SDS-PAGE protein patterns grouped all the detected
patterns into two main branches. The phenotypes of the examined *Bifidobacterium* species were grouped into two clusters, 1) *B. infantis* ATCC1567 and *B. bifidum* Bb-12 and 2) *B. longum* KN29, *B. catenulatum* KD14, and *B. animalis* BI30, at similarity levels of 0.55 and 0.64, respectively. Canzi *et al.* (2005), who analyzed RAPD patterns of *B. bifidum* and *B. longum*, derived a dendrogram showing higher similarity levels (0.66 and 0.72) than those in our study. Also a phylogenetic analysis of all taxa included in the family *Bifidobacteriaceae* using 16S rRNA showed a similarity level of 0.9 (Sidarenka *et al.*, 2008). In another study, a sequence homology analysis of the 16S rRNA gene demonstrated very high similarities for some species groups: a *B. catenulatum* and *B. pseudocatenulatum* group (similarity 0.99), a *B. longum* and *B. infantis* group (similarity 0.99), and a *B. lactis* and *B. animalis* group (similarity 0.99) (Ward and Roy, 2005). Lukjancenko *et al.* (2011) have analyzed 19 genomes from 9 *Bifidobacteria* species. They reported high similarity (99.5%) between strains and the low level of similarity between species (28–55%). This statement is consistent with our results. However, in our study the level of similarities between species was higher (0.23–0.76) which can be explain by the same ecological niche sharing by different species of *Bifidobacteria* used in our study. Moreover, our study did not concern the whole proteome of bifidobacteria, but embraced the selected group of proteins extracted from intact cells. In this work we propose these proteins as additional trait for bifidobacteria characterization.

There is evidence that proteomic analysis could be a tool for differentiation between some bacterial species. Two-dimensional electrophoresis has been used by other authors to analyze bacterial protein polymorphism and to distinguish among closely related pathogenic organisms. Proteomic comparison of membrane and extracellular proteins has been performed to identify biomarkers for *Helicobacter pylori* (Carlosohn *et al.*, 2006), *Listeria innocua* (Calvo *et al.*, 2005), and *Pseudomonas aeruginosa* (Nouvens *et al.*, 2002). Hitherto, 2D-electrophoresis has not been used to compare bifidobacteria. In the present study 2D-electrophoresis was employed in the analysis of the protein content of five *Bifidobacterium* species. A total of 29 to 53 spots were isolated, out of which only 12% to 41% were common for all the examined species. The analysis revealed that there were 149 spots which distinguished all the species, examples of them are shown in Figure 3. Identification of the most characteristic spots was performed using peptide mass fingerprinting and automated MS/MS analysis (Table I). The 18 identified proteins fell mainly into the following functional categories: metabolism-related proteins, especially membrane-related proteins; proteins involved in energy production and conversion; and proteins related to transcription and translation. Spots 4 and 13 were identified as ATP binding proteins that energize transport of sugars through the ABC transport system. Such activities can be associated with the cytoplasmic membrane (Gilad *et al.*, 2010). Spot 7 was recognized as a trigger factor. A trigger factor is involved in protein export. It is a ribosome-associated molecular chaperone, which is the first to interact with nascent polypeptide chains. It acts as a chaperone by maintaining the newly synthesized protein in an open conformation (Kramer *et al.*, 2004). The trigger factor...
<table>
<thead>
<tr>
<th>Spot No. extracted from</th>
<th>Putative function</th>
<th>Bifidobacterial species</th>
<th>Accession No.</th>
<th>Theoretical molecular mass (kDa)</th>
<th>MS/MS</th>
<th>MWE</th>
<th>Signal peptides*</th>
<th>Anchoring*</th>
<th>PSORTb*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. B. catenulatum KD14</td>
<td>Not identified</td>
<td><em>Bifidobacterium</em> catenulatum DSM 16992</td>
<td>gi</td>
<td>212716076</td>
<td>92.43</td>
<td>46</td>
<td>1486</td>
<td>No –</td>
<td>Unknown</td>
</tr>
<tr>
<td>2. B. infantis ATCC</td>
<td>Hsp60</td>
<td><em>Lactobacillus</em> plantarum</td>
<td>gi</td>
<td>23979186</td>
<td>21.3</td>
<td>30</td>
<td>1567</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>3. B. animalis BD10</td>
<td>protein grpE HSP-70 cofactor</td>
<td><em>Bifidobacterium</em> animalis subsp. lactis HN019</td>
<td>gi</td>
<td>183602853</td>
<td>24.71</td>
<td>139</td>
<td>9065</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>4. B. animalis BI30</td>
<td>ATP binding protein of ABC transporter for sugars</td>
<td><em>Bifidobacterium</em> animalis subsp. lactis HN019</td>
<td>gi</td>
<td>183602647</td>
<td>41.38</td>
<td>67</td>
<td>2208</td>
<td>No –</td>
<td>Cytoplasmic/Membrane</td>
</tr>
<tr>
<td>5. B. bifidum Bb-12</td>
<td>Pyruvate kinase</td>
<td><em>Bifidobacterium</em> animalis subsp. lactis BB12</td>
<td>gi</td>
<td>289178753</td>
<td>52.51</td>
<td>79</td>
<td>3657</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>6. B. bifidum Bb-12</td>
<td>L-lactate dehydrogenase 2</td>
<td><em>Bifidobacterium</em> animalis subsp. lactis HN019</td>
<td>gi</td>
<td>183602408</td>
<td>34.27</td>
<td>67</td>
<td>1855</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>7. B. bifidum Bb-12</td>
<td>trigger factor</td>
<td><em>Bifidobacterium</em> animalis subsp. lactis HN019</td>
<td>gi</td>
<td>183601396</td>
<td>49.92</td>
<td>77</td>
<td>2588</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>8. B. bifidum Bb-12</td>
<td>hypothetical protein pLP9000_05</td>
<td><em>Lactobacillus</em> plantarum</td>
<td>gi</td>
<td>21218447</td>
<td>16.35</td>
<td>21</td>
<td>758</td>
<td>No –</td>
<td>Unknown</td>
</tr>
<tr>
<td>9. B. infantis ATCC</td>
<td>fructose-bisphosphate aldolase</td>
<td><em>Lactobacillus</em> plantarum WCFS1</td>
<td>gi</td>
<td>23977250</td>
<td>31.02</td>
<td>48</td>
<td>2078</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>10. B. infantis ATCC</td>
<td>pyruvate kinase</td>
<td><em>Lactobacillus</em> plantarum WCFS1</td>
<td>gi</td>
<td>23978548</td>
<td>62.94</td>
<td>122</td>
<td>4112</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>11. B. infantis ATCC</td>
<td>ribosome recycling factor</td>
<td><em>Bifidobacterium</em> animalis subsp. lactis HN019</td>
<td>gi</td>
<td>183601689</td>
<td>18.98</td>
<td>81</td>
<td>2164</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>12. B. animalis BI30</td>
<td>ribosome recycling factor</td>
<td><em>Bifidobacterium</em> longum NCC2705</td>
<td>gi</td>
<td>23466062</td>
<td>20.12</td>
<td>30</td>
<td>1227</td>
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<td>Cytoplasmic/Membrane</td>
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<tr>
<td>13. B. longum KN29</td>
<td>ATP binding protein of ABC transporter for sugars</td>
<td><em>Bifidobacterium</em> longum NCC2705</td>
<td>gi</td>
<td>23465255</td>
<td>40.76</td>
<td>24</td>
<td>1035</td>
<td>No –</td>
<td>Cytoplasmic/Membrane</td>
</tr>
<tr>
<td>14. B. longum KN29</td>
<td>COG0264: Translation elongation factor Ts</td>
<td><em>Bifidobacterium</em> longum DJ010A</td>
<td>gi</td>
<td>23351570</td>
<td>29.96</td>
<td>38</td>
<td>939</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>15. B. longum KN29</td>
<td>heat shock protein GrpE</td>
<td><em>Bifidobacterium</em> longum NCC2705</td>
<td>gi</td>
<td>23465108</td>
<td>23.69</td>
<td>49</td>
<td>2385</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>16. B. catenulatum KD14</td>
<td>hypothetical protein</td>
<td><em>Bifidobacterium pseudocatenulatum</em> DSM 20438</td>
<td>gi</td>
<td>225352151</td>
<td>29.82</td>
<td>133</td>
<td>5765</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>17. B. catenulatum KD14</td>
<td>hypothetical protein</td>
<td><em>Bifidobacterium</em> pseudocatenulatum DSM 20438</td>
<td>gi</td>
<td>225351598</td>
<td>40.94</td>
<td>37</td>
<td>1808</td>
<td>No –</td>
<td>Cytoplasmic/Membrane</td>
</tr>
<tr>
<td>18. B. catenulatum KD14</td>
<td>hypothetical protein</td>
<td><em>Bifidobacterium</em> catenulatum DSM 16992</td>
<td>gi</td>
<td>12715680</td>
<td>48.93</td>
<td>166</td>
<td>4755</td>
<td>No –</td>
<td>Cytoplasmic</td>
</tr>
</tbody>
</table>

* Signal peptides and final subcellular localization were predicted using the PSORTb package (Gardy et al., 2005).
is a ribosome-associated protein that interacts with the translation elongation protein and with a wide variety of polypeptides to catalyze their folding (Ventura et al., 2003). Spots 11, 12, and 14 were identified as ribosomal proteins. These proteins are often exposed on the bacterial surface. Several surface-associated ribosomal proteins have been identified in Streptococcus pyogenes (Ventura et al., 2003), Bacillus subtilis (Severin et al., 2007), and Lactobacillus rhamnosus GG (Sanchez et al., 2009). Spot 14 was also identified as a translation elongation factor. Spot 2 was identified as the cytoplasmic Hsp60 protein. It has been confirmed that the hsp60 gene can be used for detection, characterization, and species identification of bifidobacteria (Ward and Roy, 2005). The diversity of the gene sequences indicates that the encoded proteins will differ from one another in various bifidobacterial species. Spots 5, 6, 9, and 10 were identified as cytoplasmic enzyme proteins involved in the sugar catabolism pathway.

The present study shows that the method of extraction of surface-associated proteins by Mattarelli et al. (1993) is the most suitable for the isolation of proteins from intact cells of bifidobacteria. The proteomic analysis revealed that these specific proteins make it possible to distinguish among Bifidobacterium species such as B. infantis ATCC1567, B. bifidum Bb-12, B. longum KN29, B. catenulatum KD14, and B. animalis B130. This observation was borne out by SDS-PAGE and 2D electrophoresis and further confirmed by an MS/MS analysis, in which the most characteristic protein profiles were identified.

Further research and comparative analyses are needed to develop appropriate proteomic profiles for different species and to find the specific cell-wall proteins, their sequences and characteristics. If characteristic profiles of these proteins are known, scientists will have an additional tool for accurate identification of bifidobacteria. Interesting possible application could be in MALDI-TOF mass spectrometry. This technique is cost effective and allow for highly accurate identification of bifidobacteria in a faster way than traditional methods.

Literature


Nosocomial Outbreak due to *Klebsiella pneumoniae* at a Neurosurgery Unit

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Received 11 October 2012, revised 1 September 2012, accepted 2 September 2012

**Abstract**

Thirty three isolates of *K. pneumoniae* were studied. The strains were cultured from different clinical specimens received from patients hospitalised at a Neurosurgery Unit at the Dr Jurasz University Hospital in Bydgoszcz. Production of ESBL was assessed using double disk synergy test. The genomic DNA was extracted from the strains separated by PFGE after digesting with XbaI endonuclease. Production of ESBL was detected in 81.8% of *K. pneumoniae* isolates. Molecular typing results revealed a great genetic diversity among *K. pneumoniae* isolates. All repeated PFGE patterns were detected in 12 (36.3%) *K. pneumoniae* isolates.

**Key words:** *Klebsiella pneumoniae*, extended-spectrum beta-lactamases, nosocomial outbreak, pulsed-field gel electrophoresis

*Klebsiella* rods are Gram-negative bacteria which usually cause infections of respiratory tracts, urinary system or systemic infections. As opportunistic microorganisms, they mainly cause infections in patients with lower immunity and hospitalised at intensive care units, surgery units and paediatric units (Podschun and Ullmann, 1998). *Klebsiella* rods are the most common pathogen isolated from neurosurgical patients with meningitis (Tang *et al*., 1997). Gram-negative rods may produce different types of beta-lactamases. Strains which produce extended-spectrum beta-lactamases (ESBL) are a serious epidemiologic and therapeutic problem. To be more specific, therapeutic options against infections with ESBL-producing bacteria are limited because they are often coresistant to multiple non-beta-lactam antibiotics. *K. pneumoniae* rods, including ESBL-producers, may cause nosocomial outbreaks (Hernández *et al*., 2005; Peña *et al*., 2001). The commonly applied phenotypic methods (biotyping, phagetyping, antimicrobial patterns) do not always provide a proper interpretation of an epidemiological situation at a hospital unit. Molecular methods allow for a genetic differentiation of strains belonging to the same species.

The purpose of this study was to evaluate genetic relatedness of *K. pneumoniae* strains isolated from patients hospitalised at a Neurosurgery Unit at the Dr Jurasz University Hospital in Bydgoszcz.

Thirty three clinical strains of *K. pneumoniae* were included. The strains were isolated from different specimens: wound swabs – 12, urine – 11, cerebrospinal fluid – 8 and sputum – 2. Every strain came from other patient. The patients were hospitalised because of: aneurysm – 15 (45.5%), brain tumour – 8 (24.3%), meningitis – 4 (12.2%), neuroinfections – 4 (12.1%) and spinal fractures – (6.0%). Eighteen (54.5%) strains were cultured from men and 15 (45.5%) from women, aged between 22 and 81 years. Twenty eight patients were treated with antibiotics. The strains were identified on the basis the results of biochemical reactions achieved in ID32E tests (bioMérieux). Production of ESBL was assessed using double disk synergy test (Gniadkowski *et al*., 2009). *K. pneumoniae* ATCC 700603 were used as reference strain. Macrorestriction analysis of chromosomal DNA with XbaI endonuclease (Fermentas) was carried out by pulsed-field gel electrophoresis (PFGE). PFGE was performed by using a CHIEF-MAPPER™ apparatus (Bio-Rad) with pulses ranging from 2 to 35 s, at a voltage of 6V/cm, at 14°C for 20 h. As reference chromosomal DNA pattern Lambda Ladder standard (Bio-Rad) was used. The interpretation of PFGE patterns was according to the method of Tenover *et al.* (1995).

Out of all *K. pneumoniae* strains five biochemical profiles were detected. Two biotypes: 44074757331 (17 strains – 51.5%) and 45074757331 (13 strains – 39.4%) were prevailing. These two biotypes differentiated from...
each other only by one biochemical reaction – the ability to urea hydrolysis. Out of all analysed *K. pneumoniae* strains, 27 (81.8%) produced ESBLs. All *K. pneumoniae* strains isolated from cerebrospinal fluid were ESBL-positive. Ten *K. pneumoniae* strains cultured from wound swabs, eight strains from urine and 1 from sputum were also ESBL-positive.

Considering the results of PFGE testing, the studied strains were divided into four groups: identical – 12 (36.5% – group A), closely related – 2 (6.0% – group B), potentially related – 1 (3.0% – group C) (Fig. 1). Among *K. pneumoniae* strains, eighteen (54.5% – group D) unrelated were detected (Fig. 2). Among *K. pneumoniae* strains belonging to the same genetic group, ESBL-positive and ESBL-negative phenotypes were detected. All genetically identical *K. pneumoniae* strains had the same biotype (44074757331). *K. pneumoniae* strains group A were isolated for a period of seven months from 5 men and 7 women. Ten isolates that belonged to clone A carried ESBL.

Of the *Klebsiella* genus, *K. pneumoniae* is the most common one to cause human infections. *Klebsiella* is usually found in the gastrointestinal tract in humans and animals. As it is known, colonisation can last very long and lead to infection. This mainly concerns hospital patients. *K. pneumoniae* are one of the most frequent ESBL producers, including Poland (Empel et al., 2008). The percentage of the *K. pneumoniae* ESBL-positive strains is varied and ranges from 7.5% to 44% (Falagas and Karageorgopoulos, 2009). In this study almost 82% of the *K. pneumoniae* ESBL-producing strains achieved. Such a high percentage might result from a specific character of the hospital unit, where patients within this study were hospitalised, from applied antibiotic prophylaxis and treatment in this institution as well as hygienic-sanitary activities. It is known that the use of the wide range cephalosporins affects the selection of the ESBL-positive strains (Morosini et al., 2006). In our study 19 patients were treated with third-generation cephalosporins. The selective pressure exerted by cephalosporins has been reported as a significant risk factor for the emergence of ESBL-producing *K. pneumoniae* strains earlier (Peña et al., 1997).

In this study *K. pneumoniae* strains group A were isolated for a period of 7 months with a one-month break from 12 patients. These strains had the same biotype, but differed with ESBL phenotype. On this ground it can be assumed that certain *K. pneumoniae* strains survive in the unit environment, in which stayed colonised patients or patients infected with these rods. Patient-to-patient transmission of ESBL-producing *K. pneumoniae* by hands of health care workers in the neurosurgery unit seemed to be possible. In the present study, we have used antibiotic sensitivity profiles and PFGE profiles as the typing methods. Antibiogram typing is one of the simplest and widely used method of bacteriological typing, although not comparable to genetic methods in discriminatory power. Molecular methods allow for a genetic differentiation of strains belonging to the same species.

In our study, PFGE showed the circulation of some different groups of *K. pneumoniae* at a Neurosurgery Unit. The analysed genetically identical *K. pneumoniae* strains have the same biochemical profiles, but different ESBL phenotype, so molecular typing should be supported with conventional diagnostics.

**Literature**


High Prevalence of Bartonella henselae and Bartonella quintana Antibodies in Croatian Patients Presenting with Lymphadenopathy

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2School of Medicine, University of Zagreb, Zagreb, Croatia

Received 27 December 2011, accepted 3 July 2012

Abstract

Between 2007 and 2010, a total of 268 Croatian patients with lymphadenopathy were tested for IgM/IgG antibodies to Bartonella (B.) henselae and B. quintana. Samples from 44.4% patients showed positive IgG antibodies: 35.8% to B. henselae, 6.7% to B. quintana and 1.9% to both Bartonella species. There was no difference in seropositivity between males and females (47.4% vs. 41.5%). Seroprevalence was high in all age groups (40.4–60.9%). Patients from urban and rural areas showed a similar seroprevalence rate (44.1% vs. 44.8%). Positive IgM antibodies were found in 28.3% patients varying from 17.5% and 37.5% among age groups. Most cases were reported from August to March.

Key words: Bartonella, croatia, lymphadenopathy, prevalence

Bartonella (B.) henselae and B. quintana are the causative agents of cat-scratch disease, the most common zoonosis caused by Bartonella spp. (Boulouis et al., 2005). Domestic cats are the main reservoir of B. henselae, which is transmitted among cats by the cat flea (Chomel et al., 2004). Seropidemiological studies have demonstrated the worldwide distribution of B. henselae infection in domestic cats with antibody prevalence from 5–86% (Podsiadly et al., 2002; Fabbi et al., 2004; Boulouis et al., 2005; Celebi et al., 2009). Transmission to humans mainly occurs directly by a cat scratch and possibly via a cat bite (Boulouis et al., 2005; Breitschwerdt et al.; 2007; Westling et al., 2008). In immunocompetent individuals, cat-scratch disease is characterized by a benign regional lymphadenopathy. Low-grade fever, malaise and aching are often reported (Ridder et al., 2002; Chomel et al., 2004). Since the isolation of Bartonella spp. is difficult, serologic tests are commonly used for etiologic diagnosis of cat-scratch disease (Vermeulen et al.; 2007, Hoey et al., 2009).

Human Bartonella infections have been reported from several continents, including Europe (Chomel et al., 2004; Lamas et al., 2008). The prevalence of antibodies varies in different geographic regions and among the individuals living in the same geographic area (Maruyama et al., 2000; Massei et al., 2003; da Costa et al., 2005). Seropositivity is lower among blood donors (McGill et al., 2005) and up to 53.3% in certain risk groups such as veterinarians and cats owners (Chmielewski et al., 2007).

In Croatia, data on the prevalence of bartonellosis are very limited (Pandak et al., 2009). The aim of this study was to determine the prevalence of B. henselae and B. quintana in patients presented with lymphadenopathy. During a four year period (2007–2010), a total of 268 serum samples from children and adults aged 1–73 years presented with lymphadenopathy were tested for the presence of specific IgM and IgG antibodies to B. henselae and B. quintana. Serologic tests were performed using commercial indirect immunofluorescence assay (Euroimmun, Lubeck, Germany). According to manufacturer recommendation, titer ≥100 for IgM and ≥320 for IgG were considered positive. Chi-square test was used to compare differences between groups. P < 0.05 was considered to be statistically significant.

Serum samples from 119/44.4% (95% CI = 38.6–50.4) patients showed IgG antibodies to Bartonella spp. Ninety-one patients (35.8%) were seropositive to B. henselae, 18 (6.7%) to B. quintana and 5 (1.9%) to both Bartonella species.

Prevalence of B. henselae/B. quintana antibodies according to characteristic of participants is shown in Table I. The difference in IgG seropositivity between males and females was not significant (47.4% vs. 41.5%, p = 0.397). According to age, IgG seropositivity rates varied from 40.4% to 60.9% with no statistically significant differences between age groups (p = 0.669). A similar IgG seroprevalence was found in patients residing in urban areas (44.1%) and patients residing in rural areas (44.8%, p = 0.999).

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Acute \textit{B. henselae}/\textit{B. quintana} infection was found in 76/28.3\% (95\% CI = 23.3–34.0) patients. The prevalence of IgM positive patients was high in all age groups (17.5–37.5\%, \( p = 0.386 \)) (Table I). Most cases of bartonellosis were reported from August to March with a peak incidence (%) in November (Fig. 1). Cat-scratch disease is frequently reported in children and young adults (Podsiadly et al., 2002; Massei et al., 2002), but many cases may go undiagnosed in older adults (Lamas et al., 2008). In this study, acute \textit{B. henselae}/\textit{B. quintana} infection (positive IgM antibodies) was demonstrated in 28.3\% patients presented with lymphadenopathy. No significant difference in IgM seropositivity was found between age groups (17.5–37.5\%, \( p = 0.386 \)) (Table I). A Thai study showed similar results suggesting that \textit{Bartonella} infection may occur in various age groups (Maruyama et al., 2000).

Seasonality is different in the Southern and Northern hemispheres. In Peru, most cases of cat-scratch disease occur in December and January (summer school vacation and exposure to pets) (Huarcaya et al., 2002). In contrast, a study conducted in France (1999–2009) showed that the majority of cases (87.5\%) were reported during September-April and peaked in December (Sanguinetti-Morelli et al., 2011). This study demonstrated a seasonality of \textit{B. henselae} and \textit{B. quintana} infection similar to that reported for France, but shifted one month earlier (from August to March and peaked in November). This pattern may be explained by seasonality in cat reproductive behavior. In the Northern hemisphere cat reproduction increases in spring and summer, and kittens stay with their mother until 12–16 weeks of age (Chomel et al., 1995). In addition, during summer cats spend most time outside the house, whereas during autumn they stay indoors (Sanguinetti-Morelli et al., 2011).

In this study, the overall IgG seropositivity to \textit{B. henselae} and/or \textit{B. quintana} was 44.4\%. A total of 35.8\% patients were seropositive only to \textit{B. henselae}, 6.7\% patients only to \textit{B. quintana} and 1.9\% patients to

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Tested N/%</th>
<th>IgM positive N/%</th>
<th>95% CI</th>
<th>( p ) value</th>
<th>IgG positive N/%</th>
<th>95% CI</th>
<th>( p ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>133/49.6</td>
<td>45/33.8</td>
<td>26.3–42.2</td>
<td>0.066</td>
<td>63/47.4</td>
<td>39.1–55.8</td>
<td>0.397</td>
</tr>
<tr>
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<td>135/50.4</td>
<td>31/22.9</td>
<td>16.6–30.8</td>
<td></td>
<td>56/41.5</td>
<td>33.5–49.9</td>
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</tr>
<tr>
<td>Age group (years)</td>
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<td></td>
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<tr>
<td>&lt;10</td>
<td>82/30.6</td>
<td>27/32.9</td>
<td>23.7–43.7</td>
<td>0.386</td>
<td>36/43.9</td>
<td>33.7–54.7</td>
<td>0.669</td>
</tr>
<tr>
<td>10–19</td>
<td>57/21.3</td>
<td>17/29.8</td>
<td>15.5–42.7</td>
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<td>23/40.4</td>
<td>28.6–53.3</td>
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</tr>
<tr>
<td>20–29</td>
<td>24/8.9</td>
<td>9/37.5</td>
<td>21.1–57.4</td>
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<td>1/11.5</td>
<td>7.9–64.9</td>
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<tr>
<td>30–39</td>
<td>42/15.7</td>
<td>9/21.4</td>
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<td>7/30.4</td>
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<td>14/60.9</td>
<td>40.7–77.9</td>
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<tr>
<td>50+</td>
<td>40/14.9</td>
<td>7/17.5</td>
<td>8.3–32.3</td>
<td></td>
<td>18/45.0</td>
<td>30.7–60.2</td>
<td></td>
</tr>
<tr>
<td>Area of residence</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Urban</td>
<td>152/56.7</td>
<td>41/26.9</td>
<td>20.5–34.5</td>
<td>0.661</td>
<td>67/44.1</td>
<td>36.4–52.0</td>
<td>0.999</td>
</tr>
<tr>
<td>Rural</td>
<td>116/43.3</td>
<td>35/50.2</td>
<td>22.5–39.1</td>
<td></td>
<td>52/44.8</td>
<td>36.1–53.9</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Seasonal distribution of \textit{Bartonella henselae}/\textit{quintana} cases
both Bartonella species. Serologic studies of bartonellosis in healthy population across the Europe showed the IgG seropositivity of 8.7% in Spain (Pons et al., 2008), 16.3% in the United Kingdom (Harrison and Doshi, 1999), 22.4% in Greece (Tea et al., 2003) and 30% in Germany (Sander et al., 1998). However, a Polish study conducted in a group of patients with lymphadenopathy showed a higher IgG seropositivity rate (57%) (Podsiadly et al., 2002). In addition, a study conducted in Italy showed very high IgG prevalence (61.6%) to B. henselae among Italian children without evidence of cat-scratch disease (Massei et al., 2003).

Similar to other published studies (Harrison et al., 1999; Maruyama et al., 2000; Tea et al., 2003; Pons et al., 2008; Pandak et al., 2009), we observed no significant difference in the IgG seropositivity between males (47.4%) and females (41.5%). In addition, no difference in IgG seropositivity was found between children and adults which is consistent with a previous Croatian study (Pandak et al., 2009). The IgG seroprevalence rate was high in all age groups varying from 40.4% to 60.9%.

According to place of residence, there was no difference in the IgG positivity among patients who live in urban areas (44.1%) and patients who live in rural areas (44.8%). The other authors reported similar results (Pons et al., 2008; Pandak et al., 2009).

In conclusion, the results of this study indicate a high prevalence of cat-scratch disease (28.3%) both in children and adults presented with lymphadenopathy. Therefore, testing to Bartonella antibodies should be included in differential diagnosis of lymphadenitis in children as well as in adults.

Literature


Lack of Association between *Helicobacter pylori* Infection and Biliary Tract Diseases

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Received 15 February 2012, revised 9 July 2012, accepted 1 September 2012

**Abstract**

There are ambiguous results about the involvement of *Helicobacter* species in production of hepatobiliary diseases. This study was aimed to investigate any possible association between the presences of *Helicobacter* spp., their genotypes and occurrence of different biliary diseases. Cultures of 102 bile samples for *Helicobacter* spp. did not show any growth, but the presence of *Helicobacter* genus specific DNA (16s rRNA gene) was detected in 3.92% of them. No significant association was found between development of the diseases and presence of the bacteria. All the *Helicobacter* genus positive samples belonged to *H. pylori* species and showed vacA*+* (s/m), cagA− genotypes.

**Key words:** *Helicobacter pylori*, biliary diseases

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*Helicobacter pylori* had been found by Marshall in 1986 in gastric biopsy specimens. The bacterium is associated with many diseases in the gastrointestinal tract. *H. pylori* DNA had been detected in human liver tissue samples of patients with primary sclerosing cholangitis and primary biliary cirrhosis (Warren and Marshall, 1983, Kawaguchi et al., 1996, Roe et al., 1999). There are also some reports confirming the presence of non *Helicobacter pylori* species, such as *H. pullorum, H. canis, H. cholecystus, H. rappini, H. hepaticus,* and *H. bilis* in the liver, bile and gallbladder tissues (Vorobjova et al., 2006, Matsukura et al., 2002).

Several species of *Helicobacter* genus are believed to play major roles in the causation of gallbladder cancer. Previously, it was found that *H. pylori* was not associated with the gallbladder diseases (Roe et al., 1994), but in some studies it was found to be associated with the biliary tree and gallbladder cancers (Cover et al., 1992, Presser et al., 2003).

Free bile acids in the human bile can kill *H. pylori,* however the inhibitory effect of bile acids on the survival of this bacterium is still unclear (Hanninem, 1991). It can guess that at numbers of certain pathological conditions such as bile duct obstruction, bile composition can be altered and thereby its inhibitory effect on the growth of *H. pylori* might decrease or disappear (Roe et al., 1994).

Two major important virulence markers of *H. pylori,* cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) had been well described (Xiang et al., 1995). It has been reported that the cagA is present in approximately 60% of *H. pylori* strains from Western populations but over 90% of the strains from Southeast Asian populations (Truong et al., 2009).

In contrast to cagA, vacA is present in nearly all the *H. pylori* strains around the world, only half of which express cagA, concurrently (Atherton, 1998). CagA is considered as the sole bacterial oncoprotein responsible for gastric carcinogenesis and VacA is a virulence marker that induces cell vacuolation. Colonization of *Helicobacter* spp. in the biliary tract has been implicated as a possible cause of hepatobiliary diseases ranging from chronic cholecystitis and primary sclerosing cholangitis to gallbladder cancer and primary hepatic carcinoma (Mishra et al., 2010). Although Helicobacter species have identified in the bile, tissue and stones of patients with benign biliary diseases, due to differing results that have been obtained from different studies in diverse geographical regions, no causative relationship could be established for their roles in the disease occurrence (Neri et al., 2005, Abayli et al., 2005, Francavilla et al., 2000).

In this study, we investigated the presence of *Helicobacter* species in the bile samples of patients with...
gallstone disease. We also evaluated any probable associations between the presence of *Helicobacter* DNA and the biliary diseases. From August 2010 through February 2011, 102 bile samples were collected with ERCP (Endoscopic Retrograde Cholangiopancreatography) from patients referred to Taleghani hospital in Tehran, Iran. Obstruction of bile duct, bile duct cancer, gallstone and related inflammatory disease were considered as reasons for the ERCP. All the bile samples were obtained from biliary drainage tubes; at least two milliliters of the bile were taken by needle aspiration from each patient during the operation and were collected in a sterile container. Bile samples were cultured on Brucella agar supplemented with 10% (v/v) sheep blood and selective supplement (vancomycin 2.0 mg, polymixin B 0.05 mg, trimethoprim 1.0 mg) (Merck). The cultured plates were incubated at 37°C for three to five days in a microaerophilic atmosphere (5% O2, 10% CO2, 85% N2) in a CO2 incubator (Innova-Co 170; New Brunswick Scientific, Edison, NJ, USA). The remainders of the bile samples were stored at −20°C for further analysis. DNAs from the bile samples were extracted by using phenol-chloroform method (Wilson *et al.*, 1995). To detect the bacterial DNA, the 16S rRNA gene of the *Helicobacter* genus was amplified by PCR assay. PCR was also used for seeking the presence of *H. pylori* DNA using glmM, cagA, and vacA gene specific primers compared to positive control *H. pylori* strain RIGLD-133 (Table I). For vacA genotyping among the positive samples, signal region s1/s2 alleles and midregion m2 allele of the gene were determined by multiplex PCR (Table I). The PCRs were performed in applied thermal cyclers (Eppendorf, Hamburg, Germany). All the PCRs in this study were performed in a volume of 25 μL containing: 1X PCR buffer, 500 nM of each primer, 2 mmol/L of MgCl2; 200 μM of each deoxyribonucleotide triphosphate (dNTP), 1.5 U of Taq DNA polymerase, and 200 ng of DNA sample under the following conditions: initial denaturation for 5 min at 94°C followed by 30 cycles of 93°C for 1 min, 58°C for 30 s and 72°C for 1 min. The amplified products were identified by electrophoresis in 1.0% agarose gel.

Among 102 patients under the study, highest clinical problems were belonged to biliary stone disease. The biliary stones were detected in 52% of the patients. All the cultured bile samples for *Helicobacter* spp. did not show any growth for the bacterium, but *Helicobacter* sp. specific PCR result for 16S rRNA gene were positive in four samples (3.92%). PCR for glmM gene demonstrated all of the identified *Helicobacter* spp. as *H. pylori* species (Fig. 1). No significant association was detected between the type of diseases and presence of the bacterium. Although vacA gene was found in all of the *H. pylori* DNA samples (100%), but they did not harbor cagA gene, interestingly (Table II).

![Fig. 1. PCR results for 16S rDNA, glmM and vacA genes. Lane 1 and 5 ladder mix; lane 2: PCR product for 16S rRNA; lane 3: PCR product for glmM and lane 4: PCR product for vacA gene.](image)

### Table I

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene specific primer sequences (5’→3’)</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vacA s1/s2</td>
<td>F: ATGGAAATAC AACAACACAC&lt;br&gt;R: CTCCTTGAAATGCCAAAAC</td>
<td>259/286</td>
<td>55</td>
</tr>
<tr>
<td>vacA m2</td>
<td>F: GGAAGCCCCAGAAAACATTG&lt;br&gt;R: CATAAATGGCGGCTACAC</td>
<td>352</td>
<td>55</td>
</tr>
<tr>
<td>glmM</td>
<td>F: GGATAAGCTTTTAGGGGTGTTAGGG&lt;br&gt;R: GCTTACTTTTCAACACTAAGCGCGC</td>
<td>296</td>
<td>58</td>
</tr>
<tr>
<td>cagA</td>
<td>F: AATACCCCAACGGCTCAAAG&lt;br&gt;R: TTGGTCGGCGTTCGCTC</td>
<td>349</td>
<td>57</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>F: GGCGTGACCGGCGCGCGCG</td>
<td>764</td>
<td>57</td>
</tr>
</tbody>
</table>
Benign diseases of the hepatobiliary system and the stone diseases are encountered as clinical problems in all parts of the world. Correlation of bacterial infections and their products with these diseases are of the major concerns. Presence of *Helicobacter* DNA has been investigated in the bile and biliary tissue of human beings with diverse biliary diseases (Warren and Marshall, 1983, Kawaguchi et al., 1996, Roe et al., 1999). Presser Silva et al., 2003 investigated the presence of *Helicobacter* species by culture of gallbladder tissue and bile samples. Result of this investigation was similar to our results, as their efforts for culture of *Helicobacter* spp. from the bile samples had not been successful (Presser et al., 2003). Some species of *Helicobacter* genus may be unculturable in common culture media. Viability of these bacteria strictly affected in the bile duct during their infections that limits their detection in these samples by conventional methods. Molecular studies can confirm the existence of *Helicobacter* spp. DNA in the culture negative samples. Some studies from Germany and Mexico failed in detecting the presence of DNA of *Helicobacter* spp. (Mendez-Sanchez et al., 2001, Rudi et al., 1999), but Matsukura et al. had detected different non *H. pylori* strains by analyzing the 16s rRNA gene in the bile samples (Matsukura et al., 2002). Frequency of this presence was 59.2%. Lowered risk of the infection by the pets, major reservoirs of non- *Helicobacter pylori* species, in Iran can explain absence of non *Helicobacter pylori* species in our study among the different bile samples. However, in comparison to results obtained by Farshad et al., (18.2%) and Abayli et al., (9.1%), frequency of *H. pylori* in our bile samples was lower (3.92%) (Abayli et al., 2005, Farshad et al., 2004).

In addition to the direct role of *H. pylori* in biliary diseases, it may also promote the risk of stone formation by acting as a foreign body to form a nidus around which the stone may develop or it may produce hydrolyzing enzymes or nucleating proteins like immunoglobulins. CagA protein of *H. pylori* has been found to have a homology with aminopeptidase and hence can increase the risk for gallstone formation (Maurer et al., 2005). In our study, 50% of the bile positive samples were belonged to patients with bile stone and 50% to patients with malignancy or other diseases. There wasn’t any statistically significant association between the presence of *H. pylori* and the bile diseases. All of the *H. pylori* isolates in our study were cagA negative which can to some extent explain the lack of this association. No other studies have yet analyzed this association.

According to several studies on gastric biopsies, s1/m1 is the most frequent vacA gene subtype in Mexico (Mendez-Sanchez et al., 2001) and Japan (Ito et al., 1997), in contrast to other countries such as Iran. In a recent study that was conducted in our research center on gastric biopsy samples, the s1m2 genotype was a frequently observed genotype in Iranian strains while s1m1 was more common in strains isolated from Afghani patients (Dabri et al., 2010). Similarity of the common vacA genotypes between the gastric and biliary tract isolates could propose their gastric source of infection. This relationship was established in studied patients, as all of the positive samples showed vacA s1m2, and cagA+ genotypes. In Asian countries, such as Japan (Maeda et al., 1998) and Korea (Miehlke et al., 1996), the proportion of cagA+ *H. pylori* strains was usually over 90% in all of the isolates that is higher than the isolates in Iran (~60%). Additional studies in this field are needed to clear more details about roles of non-pylori and *H. pylori* genotypes and diversity in their virulence factors in the production of biliary diseases.

In conclusion, according to our results the relationship between *Helicobacter* spp. infections and biliary tract diseases was not supported in our patients. Homology of the identified virulence gene markers of *H. pylori* in the positive samples proposed their initial roles for pathogenesis of the biliary tract. Low rates of *H. pylori* infection among the studied samples propose a possible role for other bacteria or other predisposing factors that need future analysis.

### Table II

<table>
<thead>
<tr>
<th></th>
<th>Gallstone disease (74)</th>
<th>Biliary– pancreatic Malignancy (15)</th>
<th>Other disease (13)</th>
<th>Total (102)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive culture1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>gln gene</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>vacA gene</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>cagA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>s1-s2</td>
<td>S1</td>
<td>S1</td>
<td>S1</td>
<td>–</td>
</tr>
<tr>
<td>m1–m2</td>
<td>m2</td>
<td>m2</td>
<td>m2</td>
<td>–</td>
</tr>
<tr>
<td><em>H. pylori</em> positive samples</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

1 Culture of samples was done on *Helicobacter* specific media.
Literature


Identification and Antibacterial Resistance of Bacteria Isolated from Poultry

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Received 11 october 2011, revised 5 July 2012, accepted 4 September 2012

Abstract

Food-borne infections are among the prominent health hazards. Antibacterial agents (ABA) are usually administered to poultry in Lebanon as antibiotic growth promoters (AGP), which might lead to the dissemination of resistant bacterial strains. The aims of this study were to isolate potential food borne pathogens from poultry and investigate an association between AGP usage and antibacterial resistance (ABR). Isolates were obtained from the culture of cloacae swabs and identified. Escherichia coli was the predominant isolate. There was a significant association between the use of tetracycline and gentamicin as AGP and the number of E. coli isolates resistant to these ABA.

Key words: antibacterial agents, antibacterial resistance, association; confidence interval, growth promoting factor

The abuse or misuse of antibacterial agents (ABA) as growth promoters (AGP) for livestock is of major concern in Lebanon and other developing countries. Antibacterial resistance (ABR) of enteric bacteria isolated from poultry attracts attention due to its direct influence on public health, through elevating the morbidity, mortality, and treatment costs of infectious diseases caused by cross-resistance to drugs used in human medicine (Warren et al., 2008). Increased exposure of intestinal bacterial flora to ABA which is administered for disease prophylaxis, treatment, and growth promotion is one of the causes for the emergence of resistant strains (Gyles, 2008).

ABA have been used intensively in poultry farming since 1950’s, with blurry figures representing the annual administered amounts for “non-therapeutic use”, namely disease prevention and growth promotion, ranging from 27.3 million to 16 million pounds of ABA in USA at 2001, according to the Union of Concerned Scientists and the Institute of Medicine respectively (Shea, 2003).

Taking into consideration the recommendations of the Swann report (Swann Report, 1969) and WHO report (WHO, 1997) to phase out the use of ABA as AGP many European countries cut down the non-therapeutic use of ABA. On January 1, 2006, the European Union (EU) abandoned the use of AGP in feed for livestock (Regulation EC No. 1831/2003). Fruitful results were reflected in decreased ABR in zoonotic bacteria without loss of food productivity (Aarestrup et al., 2001; Phillips et al., 2004).

On the other hand, many countries, including Lebanon, continue to use AGP in food production (Bywater and Casewell, 2000; Phillips et al., 2004). It is noteworthy to mention that recently in the United States, during the 111th Congress, a legislation entitled: the Preservation of Antibiotics for Medical Treatment Act of 2009 (H.R. 1549; S. 619) was introduced to restrict the non-therapeutic use in livestock and poultry of “critical antimicrobial animal drugs” such as penicillin, tetracycline, macrolide, aminoglycosides, and sulfonamides. This controversial legislation was supported by some federal agency officials and was opposed by most U.S. livestock and poultry producers, driven by concerns about animal welfare, food safety, and possible increases in production costs (Johnson, 2010).

The aims of this study were to identify potentially pathogenic Gram-negative isolates obtained from poultry farms in Lebanon, identify ABA used as AGP by farms and determine their ABR patterns, and relate the...
AGP used to the resistance patterns. The results might alert the public health authorities to take the necessary measures to control the use of AGP in animal feeds.

During a 2-month period, 111 cloacae swabs were collected from six layer farms. Four of six layer farms used AGP (farms A, B, C and D). The average age of layers was 9.2 ± 7.7 months. A questionnaire was used to record name of farm (A to F), location, breed/strain, age of birds and AGP administered (Table I).

Sterile cotton swabs were used to take specimens from live chickens. The cloacae swabs were placed in tryptic soy broth at room temperature. Prior approval by the Animal Care Committee was obtained. Only isolation of Gram-negative rods, excluding Salmonella species, was attempted. All broth cultures were sub-cultured on MacConkey agar plates (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) and the most dominant characteristic colony was selected and sub-cultured on MacConkey agar plates. The final pure culture was identified using the API 20E kit (BioMerieux, Paris, France). A number of species were identified from 111 isolates (Table II), and some of them such as E. coli, K. pneumoniae, P. aeruginosa and Enterobacter cloacae, are causes of various human infections, such as pneumonia, meningitis and urinary tract infections (Moniri and Dastehgoli, 2005, Warren et al., 2008; Harajly et al., 2010). Two of the isolates, Hafnia alvei and Pseudomonas putida are not human pathogens but it is worth noting that the latter bacterium is the only patented living organism. Its discoverer reported that it degrades oils (Connors et al., 1997; Van Beilen and Funhoff, 2007).

Antimicrobial susceptibility testing using the Kirby-Bauer disk diffusion method following the Clinical and Laboratory Standards Institute, CLSI (CLSI, 2006) guidelines was performed.

Bacterial inoculums were streaked on a Mueller-Hinton II agar plate (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) to obtain confluent growth. All isolates were tested for six antimicrobial agents (Oxoid, Basingstoke, UK): amoxicillin/clavulanic acid (30 µg ml⁻¹), ceftriaxone (30 µg ml⁻¹), gentamicin (10 µg ml⁻¹), tetracycline (30 µg ml⁻¹), ciprofloxacin (5 µg ml⁻¹), and sulfamethoxazole/trimethoprim (SXT) (25 µg ml⁻¹). Selection of ABAs tested was based primarily on antibacterial agents that were used as a food additive.

The majority of isolates were resistant to tetracycline (76 isolates; 68.5%) followed by SXT (49 isolates; 44.1%), ciprofloxacin/enrofloxacin (34 isolates; 30.6%), gentamicin (26 isolates; 23.4%) and amoxicillin/clavulanic acid (11 isolates; 9.9%). Only 1 isolate was resistant to ceftriaxone (0.9%) (Table II). E. coli isolates were used to investigate a link between ABA administration and ABR patterns because they were the predominant isolates obtained (82 of 111 isolates) and E. coli is used as an indicator of food and water contamination (Angulo et al., 2004).

Relative Risk (RR) and 95% Confidence Interval (CI) were determined to see if an ABA administered as a AGP was associated with the number of E. coli isolates resistant to the ABA in a statistically significant manner. RR and CI were determined by the method described by Hutchon, DJR (Hutchon, 1999). An RR more than one and a CI that does not include the number “1” indicated significance.

Tetracycline was used as an AGP in 3 of 6 and gentamicin in 2 of 6 layer farms. There was a significant

<table>
<thead>
<tr>
<th>Farm</th>
<th>Location</th>
<th>B</th>
<th>E</th>
<th>C</th>
<th>D</th>
<th>F</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AMC</td>
<td>CRO</td>
<td>GN</td>
<td>TE</td>
<td>CIP</td>
<td>SXT</td>
</tr>
<tr>
<td>Number of isolates</td>
<td></td>
<td>3</td>
<td>7</td>
<td>12</td>
<td>12</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td>N</td>
<td>6 days</td>
<td>2</td>
<td>4</td>
<td>11</td>
<td>14</td>
<td>9</td>
<td>17</td>
</tr>
<tr>
<td>Resistant to</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Abbreviations:** AGP = Antibiotic Growth Promoter; AMC: amoxicillin/clavulanic acid; GN: gentamicin; TE: tetracycline; CIP: ciprofloxacin, SXT: sulphamethaxazole/trimethoprin; CRO: ceftriaxone; ND: not done; N: number of E. coli isolates; –: no AMA was administered as AGP. AUB: American University of Beirut.

Total number of layers (Farms A, B, C, D, E and F) = 103,000.

The average age of layers was 9.2 ± 7.7 months.
association between tetracycline or gentamicin used as an AGP and the number of resistant *E. coli* isolates to these ABA (RR for Tetracycline = 19.1, 95% CI = 2.8 to 130.3. RR for Gentamicin = 4.2, 95% CI = 2.5 to 6.9) (Table III).

On the other end, there were no or few isolates that were resistant to an ABA that was not used as a AGP. None of the layer farms administered ceftriaxone or amoxacillin/clavulonic acid as an AGP and none of the isolates were resistant to ceftriaxone while only 3 of 82 *E. coli* isolates were resistant to amoxacillin/clavulonic acid.

Interestingly, 32 of 82 (39%) *E. coli* isolates from farms that did not use ciprofloxacin (a quinolone) as an AGP and 38 of 68 (56%) isolates from farms that did not use SXT as an AGP were resistant to these ABA. Cross resistance with other ABA that block bacterial porins and/or modify bacterial influx/efflux pumps, thus preventing the assimilation of SXT and ciprofloxacin to their intracellular targets, might be involved (Boerlin and Reid-Smith, 2008; Warren et al., 2008).

Deciphering the intricacies of bacterial resistance against SXT has been a controversial issue. In our study, no significant association existed between the use of SXT as an AGP and the number of resistant isolates to this ABA (RR = 0). Our findings indicate that in farms not using SXT as an AGP, 38 of 68 (56%) isolates were resistant. Moreover, in farm D where SXT was used as an AGP, none of the *E. coli* isolates from these birds were resistant to SXT (Table I). It therefore appears that

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Number isolated</th>
<th>Number of isolates resistant to</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>AMC</td>
<td>CRO</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>82</td>
<td>3</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>5</td>
<td>1</td>
</tr>
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<td><em>P. vulgaris</em></td>
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<td>1</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>7</td>
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<tr>
<td><em>Ps. aeruginosa</em></td>
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<td>1</td>
</tr>
<tr>
<td><em>Ps. Putida</em></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td><em>H. alvei</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>E. sakozaki</em></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>C. freundi</em></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>111</td>
<td>11</td>
</tr>
</tbody>
</table>

**Table II**
Number of isolates resistant to each antibacterial agent

**Table III**
Antibacterial agent(s) used as an AGP and number of *E. coli* isolates resistant and susceptible to the AGP(s)

<table>
<thead>
<tr>
<th>AGP as an AGP</th>
<th>Number of isolates resistant to AGP</th>
<th>Number of isolates susceptible to AGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline* as AGP</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>Tetracycline* not an AGP</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>Gentamicin† as AGP</td>
<td>20</td>
<td>13</td>
</tr>
<tr>
<td>Gentamicin† not an AGP</td>
<td>2</td>
<td>47</td>
</tr>
<tr>
<td>Sulfamethoxazole/Trimethoprim‡ as AGP</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Sulfamethoxazole/Trimethoprim‡ not as AGP</td>
<td>38</td>
<td>30</td>
</tr>
<tr>
<td>Ciprofloxacin§ not as an AGP</td>
<td>32</td>
<td>50</td>
</tr>
<tr>
<td>Ceftriaxone§ not as an AGP</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td>Amoxicillin/Clavulonic§ acid not as an AGP</td>
<td>3</td>
<td>79</td>
</tr>
</tbody>
</table>

AGP = Antibiotic Growth Promoter.
* Relative Risk for Tetracycline = 19.1, 95% Confidence Interval = 2.8 to 130.3
† Relative Risk for Gentamicin = 4.2, 95% Confidence Interval = 2.5 to 6.9
‡ Relative Risk for Sulfamethoxazole/Trimethoprim = 0
§ Ciprofloxacin, Ceftriaxone and Amoxicillin/Clavulonic acid were not used as an AGP in any of the farms.
abuse or misuse of SXT is not the only factor involved in the development of resistance, warm climates and poor hygiene may play a role (Lester et al., 1990). Moreover, Huovinen et al. (1995) reported that E. coli isolates in developing countries were more resistant to SXT than those from developed countries.

It may be argued that E. coli isolated from older birds are longer exposed to ABAs and are more likely to develop resistance to ABAs than isolates from younger birds. Referring to Table I, the number of resistant isolates from birds did not appear to correlate with the age of the birds.

The dissemination of resistant strains is of concern since the Lebanese ministry of agriculture permits the use AGP in poultry farming and estimates that Lebanon produces 144,023 tons of poultry food products, exports 894 tons of its local production of poultry food products, and the consumption per person of poultry meat in 2007 was 24.3 kg (Ministry of Agriculture, 2007).

In conclusion, ABR epidemiology is sparked by the concern of transmitting drug resistant food born pathogens to humans, either through food supply or direct contact with animals, contributing to human illnesses with elevated rates of treatment failures. Our findings suggest a relationship between ABA administration and emergence of ABR of E. coli isolates obtained from the normal flora of layers, arising fears of consequent dissemination of resistant bacteria to humans and urging the need to reevaluation and change in practice. Abolishing the use of AGP alone is not enough (Van Den Bogaard et al., 2000) if uncoupled with prudent ABA therapeutic usage, eradication programs for specific pathogens, and ABR surveillance programs coordinating their efforts on the national, continental, and international level to maintain an achievable high quality of food and health.

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ACKNOWLEDGEMENTS

The Editors of Polish Journal of Microbiology wish to express their gratitude to following scientists from various fields of microbiology, who have reviewed the manuscripts submitted to our Journal in the past year:
