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Front cover: Colonies of bacteria with nitrifying activity isolated from landfill leachate
(courtesy of Renata Matlakowska Ph.D.)
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IN MEMORIAM

Anna Jagoda Podhajska (1938 – 2006)

The fields of Molecular Biology and Biotechnology have lost the World-famous Polish microbiologist, Professor Anna Jagoda Podhajska. She was the Founder and former Associate Dean of the Intercollegiate Faculty of Biotechnology at the University of Gdańsk and Medical University of Gdańsk and also the Chair of the Department of Biotechnology within this Faculty. She was 68 years old and fell victim to a brain cancer.

Anna Podhajska was born on 17 April 1938 in Gdynia and her childhood coincided with the horrors of Nazi occupation of Poland (including a Concentration Camp). She graduated from the Medical University of Gdańsk in 1964 and received there her Ph.D. in microbiology in 1968. This was followed by a habilitation in 1987, based on her work on class IIS restriction endonucleases.

From 1969 Anna Podhajska was working with Professor Karol Taylor (Gene, 223 (1998) 393–394) at Department of Biochemistry at the University of Gdańsk, and then organized and chaired Department of Microbiology at the same School. For 3 years starting in 1981 and then for several months each year she spend about 10 years at Wacław Szybalski laboratory at the MaArthur Laboratory for Cancer Research, University of Wisconsin-Madison.

Among her major discoveries, Anna described the universal restriction endonucleases able to cleave DNA at any predetermined site. These were constructed of an oligo-adapters and class-IIS restriction enzymes. This work was done in the Wacław Szybalski laboratory at the University Wisconsin-Madison. Her work was published in Science (1988, 240:504–506) and Methods in Enzymology (1992, 216:303–309) and then was selected to be cited or reprinted in several books and scientific journals.

After her return to Gdańsk, in 1992 and inspired by the Madison experience, Anna began to plan and organize the Intercollegiate Faculty of Biotechnology University of Gdańsk and Medical University of Gdańsk, and became its first Associate Dean from 1993 until 1997.

Her more recent research was concentrated on several topics: (1) Development and implementation of molecular diagnostics of human viral diseases (hepatitis B virus, hepatitis C virus, human immunodeficiency virus (HIV), cytomegalovirus human papillomavirus), (2) Emerging variants of hepatitis B virus: new tools epidemiological survey, diagnosis of infection, and monitoring of drug resistance, (3) Molecular diagnostics of cancer diseases (breast cancer and cervical cancer), (4) Molecular studies on new photosensitizers as applied to photodynamic method of cancer diagnostics and treatment, (5) Application of new molecular markers to study the diversity of cyanobacteria and identification of toxic strains of cyanobacteria, (6) Human and bacterial gene cloning and protein purification for scientific and commercial application.

Anna was elected as a member of The Committee of Biotechnology and The Committee of Microbiology, Polish Academy of Sciences (PAN). She was also a member of several Scientific Boards, including those of The Institute of Marine and Tropical Medicine in Gdynia, The Institute of Sera and Vaccines in Warsaw, The Centre for Microbiology and Virology PAN in Łódź, PAN Library in Gdańsk and of The Editorial
Boards of several scientific journals (*Gene, Polish Journal of Microbiology, Polish Journal of Cosmetology*). She was elected by the assembly of the Scandinavian and Baltic Universities, Clinics and Companies as a Vice-President of ScanBalt association in 2002 (*Borderless Biotech: Europe’s First Meta-Region Taking Shape, Euro Biotech News 2003, 3:22–25*).

Anna was a very kind woman, always ready to help, true academic teacher with an outstanding sense of humor. She was an excellent experimenter, teacher and also proficient organizer who built modern Intercollegiate Faculty of Biotechnology. Moreover, she has initiated The Biotechnology Summer School, organized by Faculty every year since 1994.

Anna was a worldwide-known Polish scientist, in the fields of restriction endonucleases and later in molecular diagnostics and epidemiology of hepatitis virus B and C, and in Poland, in establishing biotechnology at both University of Gdańsk and Medical University of Gdańsk and training a new generation of Polish molecular biologists. She was also an excellent organizer of technology transfer from the university laboratory to the biotechnological companies. She had planned and established Center of Technology Transfer in Gdańsk and initiated the Pomeranian Science and Technology Park in Gdynia.

Anna was also one of Initiators and Chair of the Jury (from 2001 to 2006) of the Polish edition of the L’Oreal and UNESCO award “For Women in Science” known as “L’OREAL Polska dla Kobiet i Nauki”.

Anna Podhajska and her genius are irreplaceable. She was an exceptional person and her educational skills, organizational energy, and recently, her participation in establishment of the Scandinavian and Baltic country network (ScanBalt) will be always missed very much.

*Ewa Łojkowska*
Genetic Characterisation of the \textit{cjA}AB Operon of \textit{Campylobacter coli}

AGNIESZKA WYSZYŃSKA\textsuperscript{a}, MARCIN PAWŁOWSKI\textsuperscript{b}, JANUSZ BUJNICKI\textsuperscript{b}, DARIUSZ PAWELC\textsuperscript{c,e}, JOS P. M. VAN PUTTEN, ELŻBIETA BRZUSZKIEWICZ\textsuperscript{d} and ELŻBIETA K. JAGUSZTYN-KRYNICKA\textsuperscript{a,*}

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\textsuperscript{b}Laboratory of Bioinformatics, International Institute of Molecular and Cell Biology in Warsaw, Trojdena 4, 02-109 Warsaw, Poland;
\textsuperscript{c}Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;
\textsuperscript{d}Current address: University of Göttingen, Institute of Microbiology and Genetics, Germany;
\textsuperscript{e}Current address: Adamed Sp z o.o., Pienków 149, 05-152 Czosnów, Poland

Received 6 January 2006, revised 30 January 2006, accepted 1 February 2006

\textbf{Abstract}

We investigated the regulation of the \textit{cjA} and \textit{cjB} genes of \textit{Campylobacter coli}. These genes are seemingly arranged into one operon but appear to encode functionally different proteins \textit{i.e.} an extracytoplasmic solute receptor and a MHS – metabolite: H\textsuperscript{+} symporter transport protein. Analysis of various transcriptional \textit{cjA} and/or \textit{cjB} \textit{lacZ} fusion constructs revealed that both genes are arranged in an operon. RACE analysis located the transcription start site of the \textit{cjA}B operon 46 bp upstream of the translation start point. \textit{β}-galactosidase reporter assays yielded much higher activity for the \textit{cjA} than the \textit{cjB} gene fusion products. RT-PCR showed unequal amounts of mRNA, indicating differential post-transcriptional processing of \textit{cjA} and \textit{cjB} mRNA possibly related to the presence of inverted repeats in the intergenic region. Phylogenetic analysis grouped CjaB into a new MHS sub-family together with potential transporters with uncharacterised functions of \textit{Campylobacter} and \textit{Helicobacter}. Notably, no CjaB family members were identified in \textit{g-Proteobacteria} from different ecological niches, such as \textit{H. hepaticus} and \textit{Wolinella succinogenes}.

\textbf{Key words:} \textit{Campylobacter}, \textit{cjA}B operon, gene expression, phylogenetic analysis

\textbf{Introduction}

The Gram-negative bacteria \textit{Campylobacter coli} and \textit{Campylobacter jejuni} are commensal bacteria of warm-blooded animals and a major cause of human enteritis all over the world (Coker \textit{et al.}, 2002). Among the sequenced bacterial genomes, that of \textit{C. jejuni} is one of the most compact one; 94.3\% of the genome is occupied by protein-coding regions. Most of \textit{C. jejuni} genes are possibly expressed as polycistronic operons as judged from the genome organisation (Parkhill \textit{et al.}, 2000). In contrast to many prokaryotic genomes, the majority of \textit{C. jejuni} genes are not functionally grouped. This fact might suggest separate transcription of the majority of \textit{Campylobacter} genes. On the other hand, short intergenic DNA fragments present in the genome and the high number of overlapping genes (more than 26\% of all of the genes) rather contradicts this hypothesis (Meinersmann and Wassenaar, 2003). Such an untypical genome organisation is also an attribute of the genome of \textit{Aquifex aeolicus}, a thermophilic, chemolithoautotrophic bacterium (Deckert \textit{et al.}, 1998) and methanogenic archaeon \textit{Methanococcus jannaschii} (Bult \textit{et al.}, 1996). This unusual organisation of the genetic material raises an important question concerning the regulation of gene expression at the transcriptional level.

\textit{Campylobacter}, with a medium genome size of 1.64 Mb, like the closely related \textit{Helicobacter}, contains three sigma factors, $\sigma^{28}$, $\sigma^{54}$ and $\sigma^{70}$ encoded by \textit{fliA}, \textit{rpoN} and \textit{rpoD} genes, respectively (Parkhill \textit{et al.}, 2000; Tomb \textit{et al.}, 1997). The hitherto characterised \textit{Campylobacter} promoters are unusual compared to...
other bacteria. They have two consensus sequences TATAATT and TTTTTTG located in –10 and –16 regions but appear to lack a conserved –35 motif. The absence of a –35 region seems to be compensated by a specific periodic signal located upstream of the –16 region (Petersen et al., 2003; Wosten et al., 1998).

The present study was designed to learn more about the regulation of Campylobacter cjaA and the downstream cjaB gene expression. These genes are separated by a 41 nucleotides long DNA fragment and encode proteins that belong to different transporter superfamilies. CjaA encodes a putative amino acid binding protein of the ABC (ATP-binding cassette) transport system, while CjaB encodes a putative integral membrane protein of the MFS (major facilitator superfamily) family six (MHS – metabolite: H+ symporter). Preliminary sequence analysis suggested that the genes may be located in an operon. In the present work, we further investigated the regulation of the cjaA and cjaB genes. Since CjaA is highly immunogenic protective Campylobacter antigen the better understanding of the cjaA gene expression is crucial for elucidation its role in pathogenesis and may facilitate its use as a vaccine component.

### Experimental

#### Materials and Methods

**Bacterial strains, plasmids, media and growth conditions.** Bacterial strains and plasmids used in this work are listed in Table I. C. coli 72Dz/92 (Lior 71) was cultured as described earlier (Pawelec et al., 1997). Minimal essential medium (MEM) from GibcoBRL was used as a defined minimal medium. E. coli strains were grown in LB medium at 37°C. Antibiotics: ampicillin (50 µg ml–1), kanamycin (40 µg ml–1) chloramphenicol (20 mg µl–1), were added to the media when appropriate.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Relevant genotype or phenotype</th>
<th>Source of reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi1 hsdR17 supE4 4 relA1lac [F' proAB lacIqZ ∆M15 Tn10 (tetR)]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 (rK− mK+) supE44 ∆lacU169 F' (Φ80lacZM15)</td>
<td>Institute of Microbiology, Warsaw University, Poland</td>
</tr>
<tr>
<td>E. coli WG350</td>
<td>F− trp rpsL thi ∆(putP4)101 ∆(proP mel)121</td>
<td>Culham et al., 1993</td>
</tr>
<tr>
<td>C. coli 72Dz/92</td>
<td>serotype Lior 71, biotype 1</td>
<td>Child Health Centre, Warsaw, Poland</td>
</tr>
<tr>
<td>C. jejuni 81176</td>
<td>Lior 5; isolated in Canada from a child with a bloody diarrhoea</td>
<td>M. Blaser UV, Nashville, USA</td>
</tr>
<tr>
<td>C. coli AW4</td>
<td>C. coli 72Dz/92 cjaB::KanR</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Plasmids**

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant genotype or phenotype</th>
<th>Source of reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript II KS</td>
<td>Ap’, LacZa</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBluescript II SK</td>
<td>Ap’, LacZa</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBG518</td>
<td>Km’, LacZa</td>
<td>(Spratt et al., 1986)</td>
</tr>
<tr>
<td>pGEM-T</td>
<td>TA cloning vector, Ap’</td>
<td>Promega</td>
</tr>
<tr>
<td>pBF14</td>
<td>Km’</td>
<td>University of Utrecht, The Netherlands</td>
</tr>
<tr>
<td>pMW10</td>
<td>Km’</td>
<td>(Wosten et al., 1998)</td>
</tr>
<tr>
<td>pUWM76</td>
<td>pBG518 containing Sall-SspI 1.8 kb DNA fragment carrying cjaA gene transcribed from own promoter</td>
<td>(Pawelec et al., 1997)</td>
</tr>
<tr>
<td>pUWM201</td>
<td>pBluescript II SK containing 5.8 kb EcoRV-EcoRV DNA fragment carrying cjaAB genes transcribed from the opposite DNA strand to lacZ</td>
<td>(Pawelec et al., 1997)</td>
</tr>
<tr>
<td>pUWM272</td>
<td>pMW10 containing cjaA gene and 5’ fragment of cjaB gene (545 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM273</td>
<td>pMW10 containing 3’ fragment of cjaA gene (80 bp) and 5’ fragment of cjaB gene (545 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM472</td>
<td>pMW10 containing cjaA upstream region −591 to +34</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM471</td>
<td>pMW10 containing 5’ fragment of cjaA gene (679 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM477</td>
<td>pMW10 containing 5’ fragment of cjaA gene (361 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM421</td>
<td>pGEM-T containing cjaB gene without 418 bp central region</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM422</td>
<td>Km2 cassette of pBF14 cloned in the unique BamHI site in pUWM421 in cjaB</td>
<td>This study</td>
</tr>
</tbody>
</table>
**Table II**

Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>sequence (5’ to 3’)</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>cjaA gene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>ACCCGGATCGTATTGCTGCTGCTTT</td>
<td>Reverse</td>
</tr>
<tr>
<td>H1</td>
<td>GCTTATGATGAAACTTTTAAAAAGTC</td>
<td>Forward</td>
</tr>
<tr>
<td>327–3</td>
<td>TTACCAAGCGAGGCAATTAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>AL</td>
<td>ACTGAATCTACTTTGAAGGCAAGCCA</td>
<td>Reverse</td>
</tr>
<tr>
<td><strong>cjaB gene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5B</td>
<td>ACCCGGATCTACTTCTTACATGCTTTGCC</td>
<td>Reverse</td>
</tr>
<tr>
<td>CB1B</td>
<td>CGAATTCGTTGGTGTAGATGGA</td>
<td>Forward</td>
</tr>
<tr>
<td>CB2</td>
<td>GAGCAGCATCCTACCACCAT</td>
<td>Reverse</td>
</tr>
<tr>
<td><strong>lacZ gene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lacZ</td>
<td>AGGTTACGTTGGTGTAGATGGGAATTCACTGCGTGTTT</td>
<td>Reverse</td>
</tr>
<tr>
<td>lacZ1</td>
<td>GGAACACTGGGCGGCTGTTTT</td>
<td>Forward</td>
</tr>
<tr>
<td><strong>aph gene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KmL1</td>
<td>GAGAATATCACCAGGAATTGAACTGCTTGTTT</td>
<td>Forward</td>
</tr>
<tr>
<td>KmR1</td>
<td>CTTCTAATCCTTCCGAGCAA</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

*Capital bold letters indicate C. jejuni sequence, small letters – restriction recognition sequences introduced for cloning purposes.

**DNA sequencing and analysis.** The sequence of the *cjaB* gene was determined in the DNA Sequencing and Oligonucleotides Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. The complete nucleotide sequence of *cjaB* was submitted to the EMBL Nucleotide Sequence Database (accession number Y17971). The sequence of the *cjaA* gene is available in the EMBL under accession number Y10872.

**DNA manipulation.** DNA techniques including plasmid purification, ligation and transformation into *E. coli* were done according to standard procedures (Sambrook and Russell, 2001). Restriction endonucleases and DNA – modifying enzymes were obtained from Promega and used according to the manufacturer’s instruction. Polymerase chain reactions (PCR) were performed with Taq polymerase (Qiagen) in a Mastercycler Personal (Eppendorf). Oligonucleotide primers for PCR were from Sigma ARK (Table II).

**Mutagenesis of *cjaB* gene.** A *cjaB* mutant of *C. coli 72Dz/92* was constructed by a two-step PCR and insertion of kanamycin resistance cassette (Km) derived from pBF14. First the 5’ and 3’ ends of *cjaB* were PCR amplified using the primer combinations H1 and H5B, and CB1B and CB2. CB1B and H5B were designed with complementary protruding 5' tails with a BamHI restriction site. The obtained fragments of 432 and 447 bp were gel-purified, mixed and used as a template in a second PCR with the “outward” oriented primers H1 and CB2. The obtained PCR product that lacked the 418 bp central region of *cjaB* was cloned into the pGEM-T cloning vector. The resulting plasmid pUM421 (*cjaB*) was digested with BamH I and the BamH I-BamH I kanamycin cassette (*aph*) was inserted to obtain the final suicide plasmid pUM422. Plasmid was introduced into *Campylobacter* by electroporation (with a Bio-Rad Gene Pulser set at 0.7 kV/cm, 25 µF and 600 Ω) and transformants were selected for kanamycin resistance. *C. coli* 72Dz/92 with disrupted *cjaB* was designated AW4. The disruption of the *cjaB* gene was verified by PCR amplification.

**Transcription and translation in vitro assay.** The *in vitro* transcription and translation reaction was done with the *E. coli* Extraction System for Circular DNA kit (Promega).

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Total RNA from *C. coli* containing pMW10 derivatives was extracted using TRIZol Reagent (Molecular Research Center). RNA concentration was determined spectrophotometrically in 10 mM of Tris-HCl, pH 7.2 at 260 nm as described (Sambrook and Russell, 2001). RNA preparations (1 µg of each sample) were rendered DNA free by incubation with RNase-free DNase (Roche). RT-PCR analysis was carried out by incubating equal amounts of total RNA first with reverse transcriptase (48°C, 45 min, followed by 2 min at 95°C) and then with Taq DNA polymerase. The RNA used as a template in RT-PCR was reverse transcribed with the primer lacZ1 and the obtained cDNA was subsequently amplified with primers lacZ1 and lacZ. PCR was performed with reagents purchased from Qiagen at the concentration recommended by the supplier. The conditions for amplification were 20 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The amplified DNA fragments were analysed by 1.5% agarose gel electrophoresis.

**β-galactosidase assay.** β-galactosidase activity in *C. coli* and *E. coli* cell extracts was measured by the conversion of o-nitrophenyl-β-D-galactopyranoside (ONPG) to nitrophenol as described by Miller (1972), with the modification that *C. coli* transformants were grown for 20 h on BA plates before being harvested in LB medium and diluted until the absorbance at 600 nm was between 0.3–0.7. β-galactosidase activity assays were carried out in triplicate.

**Rapid amplification of cDNA ends (RACE) analysis.** To determine the start site of transcription the commercial “5’ RACE system for rapid amplification of cDNA ends, version 2.0” (Gibco BRL, Life Technologies) was used according to the manufacturer’s directions. RACE analysis was carried out using the primers provided by the manufacturer and *cjaA* – specific primers (327–3, AL, H3) indicated in Table II.
Database searches and sequence alignment. The amino-acid sequence of C. coli 72Dz/92 CjaB was used as a query in PSI-BLAST (Altschul et al., 1997) searches of the non-redundant database (NCBI, Bethesda, USA) to identify homologous proteins. Sequences reported with the expectation (e)-value above the threshold of 10^{-8} were downloaded and used to build the multiple sequence alignments using ClustalX (Thompson et al., 1997).

Phylogenetic analysis. Phylogenetic inference was carried out using the conserved regions of the sequence alignment of CjaB homologues based on the minimum-evolution method implemented in the Mega2 software package (Kumar et al., 2001). The stability of all branches in the tree was validated using the interior branch test and the bootstrap method. The majority-rule consensus tree was visualised using Mega2.

Results

Transcription and translation in vitro assay. Examination of the nucleotide sequence downstream of the C. coli 72Dz/92 cjaA gene (orthologue of C. jejuni NCTC11168 cj0982c) revealed the presence of an open reading frame designated cjaB. To study cjaB gene expression the plasmid pUWM201 was applied. It carries a 5.8 kb DNA fragment of C. coli 72Dz/92 including a 2.9 kb region located downstream of the cjaA gene (Pawelec et al., 1997). CjaA gene present in pUWM201 is transcribed in opposite orientation relative to lacZ gene. The size of the proteins specified by pUWM201 was determined in an in vitro coupled transcription-translation system. As shown in Fig. 1, pUWM201 encoded several proteins with molecular masses of 18, 20, 30, 50 and 55 kDa that were not expressed from the parental vector pBluescript II SK. Based on the gene sizes, the 30 kDa protein likely represented CjaA and β-lactamase. The 18 kDa protein could be a truncated product of a gene located upstream of cjaA. The predicted amino acid sequence of this protein showed 46.9% identity to the C. jejuni JlpA (Cj0983) (Jin et al., 2001). This gene together with hipO is part of 15 kb DNA fragment that has been by now recognised as C. jejuni specific (Chan et al., 2000). Our results showed that there are Campylobacter strains containing, at least, part of this DNA region and classified by PCR assays as C. coli. The remaining proteins were likely to be encoded by the DNA fragment downstream of cjaA. The sizes of the proteins were comparable to those predicted for the products of C. jejuni NCTC11168 genes surrounding cj0982c, suggesting a similarity of the genetic organisation of the analysed region in the genomes of two clinical isolates of different species, C. coli and C. jejuni. The results obtained suggest that the majority, if not all, of the promoters located within this DNA region were active in E. coli.

Sequence analysis of cjaA downstream region. Sequencing of the DNA region downstream of cjaA yielded 1347 bp starting at the last position in the sequence previously obtained from pUWM76 carrying truncated cjaB (Pawelec et al., 1997). The cjaB gene consists of 1248 nucleotides and carries an ATG (Met) start codon and TAA stop codon. Six nucleotides upstream of the proposed translation initiation codon a putative ribosome binding site (AGGA) was observed. Downstream of cjaB an orthologue of cj0980 was found as deduced from the amino acid sequence of the C – terminus of the coding region. CjaB and the
downstream gene are convergently transcribed and overlap by 18 bp. Their orthologues from *C. jejuni NCTC11168* (*cj0980* and *cj0981c*) also overlap by 18 bp.

Comparison of the nucleotide sequence of the *cjaB* gene from *C. coli 72Dz/92* with its orthologue from *C. jejuni NCTC11168* revealed 84% identity. Part of the *C. jejuni 81176* *cj0981c* orthologue was sequenced (330 bp of the 5’ end of the gene) and showed 100% identity with the corresponding fragment of the *C. jejuni NCTC11168* *cj0981c*. The start codon ATG of *cjaB* is located 41 bp downstream of the *cjaA* stop codon (TAA). Further inspection of the intergenic region revealed a lack of a putative promoter sequence for the *cjaB* gene. On the other hand, an inverted repeat (AAAGGGCTTTTGCCTTTT) which potentially might regulate mRNA stability, was found in that region. The features described above are also characteristic for the intergenic region between *cj0981c* and *cj0982c* of *C. jejuni NCTC11168* and their orthologues in *C. jejuni 81176* (Fig. 2).

**Identification of the transcription start site and the promoter region.** The transcription start site for the *cjaA*B was determined by rapid amplification of cDNA ends (RACE). Sequencing of the resulting amplicon showed that the first base of the transcript was located 46 bp upstream from the ATG start codon. Examination of the sequence located upstream of this point revealed putative –10 (TATAAT), –16 (TAAAAA) and –35 (TTGAaA) promoter sequences. It also contains periodically repeated T-stretches located upstream of the TATA box (thymes are predominant in regions –18 to –24 and –29 to –35). The transcription is initiated at a pyrimidine (cytidine) located 7 nucleotides from the first nucleotide of the TATA box. The similarity between the nucleotide sequence of the –35 region and that recognised by σ70 *E. coli* RNA polymerase probably accounted for the expression of this operon both in *Campylobacter* and *E. coli*.

**Characteristics of the putative CjaB protein.** The deduced amino acid sequence of the 49.8 kDa (415 aa) CjaB exhibits a significant overall similarity to several prokaryotic transmembrane polypeptides that belong to the MFS group, family 6 (MHS – metabolite: H+ symporter). Members of this family include transporters of citrate, β-ketoglutarate and osmoprotectants (proline and betaine) as well as some permeases of unknown transport function (Pao et al., 1998). The CjaB protein contains three signature sequences specific for the family. The MFS superfamily groups inner membrane proteins with 12 to 14 transmembrane helices (TMs). *In silico* analysis of the CjaB amino acid sequence revealed the presence of 12 hypothetical TMs, each about 20 amino acids long. The N-terminus of the protein is predicted to be cytoplasmic.

The organisation into one operon of two genes, *cjaA* and *cjaB*, with seemingly unrelated functions, led us to assess the function of the *C. coli cjaAB* gene product. For this purpose, the *cjaB* gene was disrupted by insertion of an antibiotic cassette (see Materials and Methods). The mutant showed growth characteristics, at least on rich media, similar to those of their parental strains. Because the deduced amino acid sequence of the CjaB exhibits a significant overall similarity to prokaryotic transmembrane transporters of citrate and osmoprotectants (proline and betaine), its function was further investigated by complementation of the *E. coli* strain WG350. This mutant is deficient in the transport of osmoprotectants (Culham et al., 1993). Introduction of pUWM201 carrying the *cjaAB* operon into *E. coli* WG350 did not restore its ability to grow in minimal medium (MOPS) supplemented with 0.6 M NaCl and 1 mM proline. PUWM 201 was also introduced into *E. coli DH5α* and the growth of transformants was monitored on minimal citrate medium. In neither case the CjaB protein produced by pUWM201 complements the defect of *E. coli* indicating that it is involved neither in osmoprotection nor citrate transport.

To learn more about the CjaB protein, its evolutionary relationship to other transporters was investigated. According to the analysis reported by Saier et al. (1999), the MHS family comprises four subfamilies, represented by CitA Eco (Citrate transporter), PcaT Eco (dicarboxylic transporter), ProP Eco (proline/betaine transporter) and MopB Bce (4-methyl-O-phthalate transporter), respectively. Fig. 3 shows the phylogenetic tree based on the multiple sequence alignment of the MHS proteins (family metabolite: H+ symporter), comprising the new members identified in the course of our analysis, including CjaB, as well as the SHS and SP families, used as an outgroup of the MHS family. The topology of the tree with respect to the location of the MHS, SHS, and SP families as well as the distribution of the CitA, PcaT, MopB and ProP subfamilies, are in accordance with the results of the earlier analysis of the transporter family reported by
Saier et al. (1999). Strikingly, our analysis reveals that CjaB and its close homologues do not group together with any of the previously defined subfamilies, but instead form a completely new subfamily. In addition to the CjaB subfamily, our analysis has led to the identification of additional two MHS subfamilies (indicated as A and B in Fig. 3), which form clearly distinct branches in the MHS family tree, with strong bootstrap support. All three new subfamilies delineated by our analysis comprise functionally uncharacterised proteins.

Detailed analysis of the phylogeny of the CjaB subfamily reveals several duplications. The CjaB protein from C. coli has an orthologue in the completely sequenced genome of the C. jejuni strain NCTC11168, but no orthologues in the completely sequenced genomes of Helicobacter pylori strains 399 and 26695. Inter-
Characterisation of cjaAB operon of C. coli

Interestingly, CjaB is an out-paralogue of a lineage grouping orthologous proteins from both strains of *H. pylori* (present in their genomes as single copies) and two in-paralogous copies in the genome of *C. jejuni* NCTC11168. The proliferation of CjaB paralogues in *C. jejuni* suggests that these proteins may be functionally diversified and for instance exhibit different preference for the transported substrates.

Transcriptional analysis of cjaAB–lacZ reporter fusions. The arrangement of the *C. coli* DNA region containing the *cjaA* and *cjaB* genes suggested that the two genes are co-transcribed. *CjaAB*:lacZ reporter gene fusions were used to study the regulation of the putative *cjaAB* operon expression. A set of *cjaAB*-lacZ operon fusion were constructed in which progressively longer fragments of the *cjaAB* coding sequence were fused to the promotorless lacZ gene in the shuttle vector pMW10 (Wosten et al., 1998). It is equipped with translational stop codons in the three reading frames present between polylinker and lacZ gene.

Recombinant plasmids (pUWM472, pUWM477, pUWM471, pUWM272 and pUWM273) were obtained by insertion of appropriate PCR amplified *Campylobacter* DNA fragments into pMW10 previously cut with BamHI and XbaI, or by subcloning. All of them but one (pUWM273) contained the –591 to +1 *cjaA* upstream region (+1 is the experimentally determined transcription start point, see above). Details of the vector structures are depicted in Fig. 4A. β-galactosidase activity indicated that the fusion present in pUWM273 was not expressed in *Campylobacter*. The level of β-galactosidase from the fusion with lacZ downstream of the 5′ end of *cjaB* (pUWM272) was approximately six times lower than that from the fusion with lacZ within *cjaA*. Unexpectedly, the fusion carried by pUWM472, containing only the *cjaA* upstream region, was not expressed in *Campylobacter*.

Generally, the activities observed in *E. coli* were significantly lower than in *Campylobacter*. This was most pronounced for the fusion with the reporter gene inserted into *cjaA*. Interestingly, the operon fusion present on pUWM472 was expressed in *E. coli*, in contrast to the results determined for *C. coli*.
Transcriptional analysis of the putative cjaAB operon by RT-PCR. The differences in β-galactosidase activity observed for *C. coli* carrying different operon fusions could be due to different mRNA stability or its secondary structure influencing the level of translation. To distinguish between these two possibilities we measured the amount of lacZ gene transcript using RT-PCR. All the products obtained were of expected size and the absence of products in the reactions without RT (data not shown) clearly demonstrated that the bands observed with reverse transcriptase were derived from mRNA and not from contaminating DNA (Fig. 5). Two primers complementary to the aph vector gene were employed to standardise the assay (data not shown). No RT-PCR product was observed for bacteria carrying pUWM273. This confirmed that the lack of β-galactosidase activity in these cells was due to the lack of a promoter region, and thus proved that cjaA and cjaB are co-transcribed. The differences in the amount of the amplified product from bacteria carrying constructs with the reporter gene in different regions of the operon (pUWM477 vs. pUWM272) clearly correlated with the level of β-galactosidase activity. Contrary to expectations, RT-PCR showed the presence of specific mRNA transcribed from pUWM472. The nucleotide sequence of the insert carried by pUWM472 was checked by sequencing and was shown to be correct. The nucleotide sequence of the cjaA upstream region present in *Campylobacter* genome differ from that of pUWM472 by the length of the 5' untranslated region. Apparently, the secondary structure of this mRNA region completely abolished translation in *C. coli*, while it did not influence the interaction of the mRNA with *E. coli* ribosomes.

Discussion

CjaA encoded by the first gene of the cjaAB operon is an extracytoplasmic solute receptor (Wyszyńska et al., 2003), component of the ABC transport system. In most bacterial genomes the genes for proteins of the ABC-type uptake system are organised in operons containing three, four or five genes (Boos and Lucht, 1994). Thus, when compared to other Gram-negative bacteria, the *C. coli* cjaA gene is unique. The second gene of the operon, cjaB, encodes a protein belonging to the MFS group (family 6). Genes coding for MFS permeases generally are separate transcription units. However, in the light of known facts concerning the organisation of the *Campylobacter* genetic material the cjaAB operon reflects unusual and still mysterious *Campylobacter* gene arrangement. To date only a few *Campylobacter* operons have been investigated experimentally. One of them contains the fur gene involved in the ferric uptake system. It has been shown that fur is co-transcribed with two housekeeping genes, lysS and gya (van Vliet et al., 2000).

Expression of the reporter gene lacZ placed in cjaA leads to a very high level of β-galactosidase (about 2000 Miller units) ranking the cjaA promoter among the strongest so far described *Campylobacter* promoters (Wosten et al., 1998). Given that *Campylobacter* spp. are unable to use carbohydrates as a primary carbon and energy source and that amino acids can serve not only as a nitrogen but also as a carbon/energy source, the high expression of cjaAB could be expected. The protein encoded by the first gene, CjaA, is potentially present in a much higher amount compared to the product of the second gene of the operon, CjaB, as was indicated by β-galactosidase activity. High level of CjaB, an integral membrane transporter of an unknown substrate, might be toxic for cells. The RT-PCR analysis showed that the unequal amounts of the products of the two genes resulted from a modulation process that influences mRNA stability. Two inverted repeats (IR) located downstream of cjaA are possibly responsible for the protection of mRNA from 3′→5′ exonucleolitic degradation, which causes accumulation of cjaA mRNA (Grunberg-Manago, 1999).
Similar IR sequences were found downstream of the first genes of several ABC-type transporter operons (Boos and Lucht, 1994).

The C. jejuni genome is AT rich. Due to the specific nucleotide sequence of the region located upstream of the TATA box, we postulate that the cjaAB promoter is recognised by C. coli RpoD-RNAP. Transcription is initiated at a cytidine, which is uncommon for RNAP with the main sigma factor, at least in E. coli (Barrios et al., 1999). Although the –35 region of the promoter is almost identical to that recognised by E. coli σ70 RNAP the level of the reporter gene product was much lower in E. coli than in C. coli. This suggests that the cjaAB promoter specific for Campylobacter RpoD RNAP shows a rather low affinity for E. coli σ70 RNAP.

Taken together, the reporter gene experiments and RT-PCR studies showed that the cjaA and cjaB genes, belonging to different transporter superfamilies, are co-transcribed and that the stability of mRNA is responsible for varying amounts of the products of the two co-transcribed genes. In addition, unlike in other Gram-negative bacteria, but similar to some Gram-positive bacteria, the stability of mRNA is re- 

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Natural Mannose-Binding Lectin (MBL) Down-regulates Phagocytosis of Helicobacter pylori

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Abstract

Considering the role of lectin-carbohydrate interactions between Helicobacter pylori bacteria and the host cells we addressed the question on how mannose binding lectin – MBL, present in human plasma, may influence the phagocytosis of H. pylori by peripheral blood granulocytes. For phagocytosis assay the granulocytes separated from peripheral blood of healthy H. pylori-seronegative donors were used. Phagocytosis was estimated by fluorescence assay using FITC-labelled H. pylori cells. The MBL level in the serum samples as well as MBL-binding to H. pylori bacteria were estimated by ELISA. In this study all H. pylori isolates bound recombinant mannose binding lectin-MBL as shown by ELISA. The ingestion of H. pylori bacteria in the medium with human serum depleted in natural MBL (nMBL) was more intensive than in the medium with complete serum containing nMBL. Moreover, the ingestion of H. pylori bacteria in the medium with complete serum was increased by an addition of anti-rMBL IgG. The results indicate that interaction of bacterial and host lectins may regulate the phagocytosis of H. pylori bacteria and in this way influence an outcome of the infection caused by these microbes.

Key words: Helicobacter pylori, mannose binding lectin (MBL), phagocytosis

Introduction

Helicobacter pylori related gastroduodenal infections are associated with strong infiltration of the gastric mucosa by neutrophils, macrophages, lymphocytes and plasma cells (Rudnicka and Andersen, 1999). Despite mobilization of phagocytes to inflammatory foci, the bacteria are not eliminated. It has been suggested that they may evade destruction by phagocytes due to a temporary persistence in the cytosol of epithelial cells (Petersen and Krogfeld, 2003). Many H. pylori strains express adhesin proteins that bind to specific host cell macromolecule receptors. The best defined H. pylori adhesin-receptor interaction, described by Ilver et al. (1998), is that between the Lewis b (Le b) blood group antigen binding adhesin, BabA, a member of a family of H. pylori outer membrane proteins.

Mahdavi et al. (2002), identified sialyl-dimeric Lewis X glycosphingolipid as a receptor for H. pylori. The corresponding sialic-acid-binding adhesin (SabA) was isolated and the sabA gene was identified (Mahdavi et al., 2002). It has also been established that H. pylori strains express heparan sulphate binding proteins (Hirmo et al., 1995).

Two molecular mechanisms of microbial recognition by phagocytes are distinguished: direct – opsonin independent, and indirect – opsonin dependent (Ofek et al., 1995) In our previous study we found that antibodies specific to various H. pylori antigens may have opposite effects on the course of phagocytosis of these bacteria. We showed that opsonization of H. pylori with anti-Lewis X monoclonal antibody (IgM)
made Lewis X-positive but not Lewis X-negative *H. pylori* bacteria more susceptible to phagocytosis (Chmiela et al., 1997, 1998, Rudnicka et al., 2001). However, sera from dyspeptic patients with IgG against *H. pylori* surface antigens reduced the susceptibility of these bacteria to phagocytosis (Rudnicka et al., 1998). The importance of opsonic activity of the complement in the ingestion of *H. pylori* bacteria by neutrophils was also shown (Mc Kinlay et al., 1993). On the other hand, Rautelin et al. (1994), showed that about one third of *H. pylori* strains isolated from human gastric biopsy specimens, induced strong chemiluminescence in neutrophils, even without serum opsonins.

Lectinophagocytosis is a known example of opsonin independent phagocytosis. It includes the reaction between surface lectins and carbohydrates on microbial or phagocytic cells (Ofek et al., 1995). Previously we showed that interaction between bacterial surface structures such as sialic acid specific haemagglutinins, heparin binding proteins and corresponding phagocyte receptors was necessary for the ingestion of *H. pylori* (Chmiela et al., 1998). On the other hand, our results suggested that *H. pylori* can use widely distributed host compounds: sialic acid or heparin/heparan sulfate glycosaminoglycans, hyaluronic acid or vitronectin (in the presence of complement) to avoid phagocytosis (Chmiela et al., 1998).

Mannose binding lectin – MBL, a C-type lectin, interacts with various microbial carbohydrates (mannose, N-acetyloglucosamine, fucose and N-acetylmannosamine) (Sastry and Ezekowitz, 1993; Turner, 1996). The bacterial capsule and especially LPS could be a major determinant for MBL binding (Devyatyarnova-Johanson et al., 2000). MBL activates complement on lectin pathway, independent of C1q and antibodies, in the presence of MBL-associated serine proteases (MASP1 and MASP2, homologues of C1q and C1s) (Gal and Ambrus, 2001; Kase et al., 1999; Matsushita and Fujita, 1992). Garred et al. (2003), proposed a dual role of MBL dependent on the lectin’s concentration. Low concentrations have been associated with recurrent or severe infections in children and adults caused by extracellular pathogens and also with autoimmune diseases. High concentrations may enhance targeting of intracellular organisms to host phagocytes. MBL also modulates disease severity, at least in part through a complex, dose dependent influence of cytokine production (Matsushita and Fujita, 1992).

In this study we addressed the question on how MBL may influence the phagocytosis of *H. pylori* by human granulocytes. In order to answer this question we estimated: 1) interaction of MBL with *H. pylori* clinical isolates and reference strains, 2) MBL concentration in the sera from *H. pylori* infected and uninfected children/adolescents and adults, 3) the intensity of *H. pylori* ingestion by human granulocytes in the presence or absence of natural (nMBL) and recombinant (rMBL) mannose binding lectin as well as anti-rMBL IgG antibodies.

**Experimental**

**Material and Methods**

**Serum samples.** A total of 224 sera were examined for MBL concentration. Sera from *H. pylori* positive (69) and negative (49) children/adolescents (average age 13 years) diagnosed for *H. pylori* infection in Mother Health Center Institute in Łódź, Poland, were used for the study. The serum samples from *H. pylori* positive (66) or negative (40) adult dyspeptic patients (average age 53 years) were obtained from K. Jonscher Hospital in Łódź, Poland. *H. pylori* status was determined by endoscopy, rapid urease test and histology. Moreover, in all subjects the titers of anti-C1q and C1s) (Gal and Ambrus, 2001; Kase et al., 1999; Matsushita and Fujita, 1992). Garred et al. (2003), proposed a dual role of MBL dependent on the lectin’s concentration. Low concentrations have been associated with recurrent or severe infections in children and adults caused by extracellular pathogens and also with autoimmune diseases. High concentrations may enhance targeting of intracellular organisms to host phagocytes. MBL also modulates disease severity, at least in part through a complex, dose dependent influence of cytokine production (Matsushita and Fujita, 1992).

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**ELISA for serum MBL concentration.** The microtitre plates (Nunc Immunoplate Maxisorp, Nunc, Kastrup, Denmark) were coated with *S. cerevisiae* mannan (Sigma, St. Louis, Michigan, USA) at a concentration of 250 µg/ml in carbonate buffer pH 9.6 (Aittoniemi et al., 1996). The plates were washed with phosphate buffered saline (PBS), pH 7.4, containing 0.05% Tween 80 (PBS/T), and the remaining binding sites were blocked with 1% bovine serum albumin, BSA, Sigma, in PBS (PBS/BSA). Next the serum samples diluted 1:40 and 1:80 in Tris-HCl pH 8.0 with 50 mM CaCl₂ (Tris-HCl/CaCl₂) supplemented with 1% BSA were added to the wells and the plates were incubated for 2 h, at 37°C. After washing, the plates were incubated for 2 h, at 37°C with rabbit antibodies against recombinant human MBL (rMBL), and then for 1 h with swine antibodies against rabbit immunoglobulins labelled with horseradish peroxidase-HRP (Dako, Glostrup, Denmark). The colour reaction was developed in the presence of substrate solution: 1 mg o-phenylenediamine dihydrochloride-OPD (Sigma) in 1 ml citric-acid phosphate buffer, pH 5.0 supplemented with 0.5 µl/ml of 30% H₂O₂. The reaction was stopped with citric acid and the absorbance was measured at 450 nm using Victor2 reader (Wallak, Oy, Turku, Finland). The standard curve was prepared by incubation of mannan-coated wells with a known amount of rMBL (0.073–1.2 µg/ml), and then with rabbit anti-rMBL antibodies and swine-HRP antibodies to rabbit immunoglobulins. In every ELISA the control wells were used for excluding the unspecific reactions.

**Sera depleted in MBL.** For phagocytosis assay two types of serum samples were used: 1) containing nMBL; 2) depleted in nMBL by absorption with *S. cerevisiae* mannan coated sepharose (Sigma, St. Louis, Michigan, USA) (Kase et al., 1991). In brief, 500 µl of mannan bound sepharose was sedimented by centrifugation for 2 min, 300 × g, and stabilized for 18 h, at 4°C with
Tris-HCl/CaCl₂. Equilibrated sepharose was centrifuged, then resuspended in 1 ml of serum containing 2 mM CaCl₂ and incubated for 30 min, at 4°C with agitation. After sedimentation of sepharose, the supernatant was collected for estimation of MBL concentration by ELISA, as described above.

**Bacterial strains and culture conditions.** The *H. pylori* reference strains 17874 and 17875 were obtained from the Culture Collection, University of Gothenburg (CCUG), Gothenburg, Sweden. Clinical isolates (31) were from dyspeptic children/adolescents and adults being under the care of Mother Health Center Institute and K. Jonscher Hospital in Łódź, Poland. The bacteria were stored at −70°C in tryptic soy broth containing 15% glycerol. Before being used in experiments the bacteria were cultured for 48 h, at 37°C in microaerophilic conditions on blood agar containing 10% heat inactivated fetal calf serum. The *Mycobacterium bovis* reference strain from Polish Bacterial Culture Collection was grown for 2–3 weeks at 37°C; 5% CO₂ on Middelbrook 7H9 medium (Difco, Detroit, Michigan, USA) containing 0.05% Tween 80 and ADC supplement (Becton Dickinson, Sparks, USA).

**Interaction of MBL with *H. pylori* cells.** The interaction of MBL with *H. pylori* cells (33 strains) was estimated by ELISA assay on microplates coated for 18 h, at 4°C, with *H. pylori* and *M. bovis* (low binding control) bacterial suspensions, 1 × 10⁷ cells/ml in PBS, pH 7.4 (100 µl/well). Positive ELISA control with rMBL coated wells (0.5 µg/ml) was also included. Unbound plastic was blocked with PBS/BSA. After washing the plates (once with PBS and three times with Tris-HCl/CaCl₂/BSA) the rMBL (5 µg/ml in Tris-HCl/CaCl₂/BSA) was added to the wells (100 µl/well), and the plates were incubated for 2 h at 37°C. Next, the assay was continued as described above.

**FITC labelling of *H. pylori* bacteria.** The bacteria collected from the plates were washed once with PBS and resuspended in such buffer containing 100 µg/ml fluorescein isothiocyanate (FITC). The mixture was agitated for 30 min at room temperature. The bacteria, after extensive washing with PBS, were resuspended with PBS containing 4% bovine serum albumin (BSA), to bind unconjugated FITC to BSA. After 15 min incubation at room temperature, the bacteria were washed and resuspended at 1 × 10⁶ cell/ml in RPMI-1640 medium.

**Phagocytosis.** Polymorphonuclear leukocytes (PMNs) were separated from human fresh blood collected from healthy individuals by veinpuncture with heparin as an anticoagulant, by Polymorphoprep gradient centrifugation (Nycomed, Oslo, Norway). For phagocytosis, PMN suspensions (1 × 10⁵ cells/ml) were prepared in 1 ml volume of RPMI-1640 with gentamycin (5 µg/ml), containing: 1) 20% complete non-inactivated human serum containing 30 µg/ml of nMBL or 20% such serum with or without 10% rabbit anti-human rMBL antibodies; 2) 20% nMBL depleted serum; 3) 20% nMBL depleted serum and rMBL at a concentration of nMBL (30 µg/ml). The cells suspended in an appropriate medium were added in triplicate to the wells of microplate (100 µl/well) and supplemented with fluoresceine isothiocyanate (FITC)-labelled bacteria in RPMI-1640, at the ratio 1:10 or 1:100, and then incubated for 1 h, at 37°C; 5% CO₂. Phagocytosis was stopped on ice. The unbound bacteria were removed by washing the cells with ice-cold PBS with gentamycin (PBS/G). The fluorescence was measured using Victor2 reader with 480/530 nm excitation/emission filters. Afterwards extracellular fluorescence was quenched with crystalline violet (500 µg/ml in PBS). The dye was exchanged with PBS/G. The intensity of fluorescence was measured as above, and expressed in relative fluorescence units (RFU) – fluorescence counts. The wells containing FITC labelled bacteria alone were used as control of quenching effectiveness. In each experiment a standard curve for quantitation of FITC labelled *H. pylori* bacteria was prepared. Serially diluted bacterial cell suspensions in RPMI-1640 medium, were distributed into the wells, and the reference fluorescence of bacteria was measured. The values of fluorescence were plotted as a function of the number of bacteria in each well (Chmiela et al., 1997).

**Detection of C5b-C9 complement complexes.** Activation of complement during phagocytosis was estimated immunoenzymatically by dot blot method using monoclonal antibodies against C5b-C9 complexes (Dako). Two microliters of post phagocytosis supernatants were dropped three times on the BA 85 membrane (Schleicher and Schuel, Dassel, Germany). After blocking with 2% BSA in Tris-HCl/200mM NaCl, pH 7.4 (Tris-HCl/NaCl/BSA) the membranes were incubated for 18 h, at room temperature with mouse monoclonal antibodies to C5b-C9 complex, diluted 1:40 in Tris-HCl/NaCl/BSA. After washing the membranes with Tris-HCl/NaCl containing 0.5% Tween 80, rabbit antibodies to mouse immunoglobulins labelled with HRP (diluted 1:1000 with mouse monoclonal antibodies to C5b-C9 complexes, diluted 1:40 in Tris-HCl/NaCl/BSA) were added and the membranes were stored for 2 h at room temperature. The color reaction was developed microaerophilically on blood agar containing 10% heat inactivated fetal calf serum. The *Mycobacterium bovis* reference strain from Polish Bacterial Culture Collection was grown for 2–3 weeks at 37°C; 5% CO₂ on Middelbrook 7H9 medium (Difco, Detroit, Michigan, USA) containing 0.05% Tween 80 and ADC supplement (Becton Dickinson, Sparks, USA).

**Results**

The level of MBL in *H. pylori* infected and uninfected individuals. There was a high variation in MBL amount in the serum samples in each group under the study (Table I). The MBL concentration was in the range 2–50 µg/ml. There was no significant difference in the frequency of the MBL concentration: 0–2 mg/ml; 2–4 µg/ml; 4–10 µg/ml and >10 µg/ml, between the groups of *H. pylori* infected and uninfected children/adolescents and adults or between males and females.

The interaction of *H. pylori* with MBL. The *H. pylori*-MBL interaction was evaluated for 31 clinical isolates and two reference strains. The results showed that all *H. pylori* strains interacted with rMBL when investigated by ELISA. The specific ELISA OD450 counts for *H. pylori* strains were in the range 1.0–2.0 (mean 1.5 ± 0.25) and for *M. bovis* 0.2–0.6 (mean 0.4 ± 0.05) (Table II). Positive ELISA counts for rMBL coated wells were in the range 0.8–1.0.

The intensity of phagocytosis of FITC-*H. pylori* bacteria by granulocytes in the presence or absence of MBL. The phagocytosis of MBL-binding *H. pylori* strain CCUG 17874 by human granulocytes,

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**Role of MBL in *H. pylori* phagocytosis**

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The concentration of MBL in the sera from children/adolescents and adults infected or uninfected with *H. pylori*

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<tr>
<th>Group investigated</th>
<th>Serum MBL concentration (µg/ml)</th>
<th>Prevalence (%)</th>
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<td></td>
<td>Range (r)</td>
<td>Below 2.0</td>
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<td>Children/adolescents</td>
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<td><em>H. pylori</em> (+) mean:</td>
<td>r = 2.0–38.0 10.1 ± 9.0</td>
<td>12/69</td>
</tr>
<tr>
<td>n = 69</td>
<td></td>
<td>17%</td>
</tr>
<tr>
<td><em>H. pylori</em> (-) mean:</td>
<td>r = 2.0–49.6 12.2 ± 10.9</td>
<td>5/49</td>
</tr>
<tr>
<td>n = 49</td>
<td></td>
<td>10%</td>
</tr>
<tr>
<td>Adults</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. pylori</em> (+) mean:</td>
<td>r = 2.0–30.5 9.6 ± 7.6</td>
<td>10/66</td>
</tr>
<tr>
<td>n = 66</td>
<td></td>
<td>15%</td>
</tr>
<tr>
<td><em>H. pylori</em> (-) mean:</td>
<td>r = 2.0 – 43.2 10.4 ± 10.7</td>
<td>7/40</td>
</tr>
<tr>
<td>n = 40</td>
<td></td>
<td>18%</td>
</tr>
</tbody>
</table>

n – number of subjects

The interaction of recombinant mannose binding lectin (rMBL) with *H. pylori* and *M. bovis* estimated by ELISA

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>ELISA</th>
<th>Range of unspecific OD450 counts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific OD450 counts</td>
<td>mean</td>
</tr>
<tr>
<td><em>H. pylori</em></td>
<td>1.0 – 2.0</td>
<td>1.5 ± 0.25</td>
</tr>
<tr>
<td><em>M. bovis</em> (low binding control)</td>
<td>0.2 – 0.6</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td>rMBL (positive ELISA control)</td>
<td>Range 0.8–1.0</td>
<td></td>
</tr>
</tbody>
</table>

The interaction of rMBL with *H. pylori* and *M. bovis* was estimated by ELISA using *S. cerevisiae* mannan as coating antigen. Polyclonal rabbit anti-rMBL IgG were used for recognition of MBL bound with bacterial cells and swine antibodies against rabbit immunoglobulins labeled with horse-radish peroxidase for detection of such complex.

The intensity of phagocytosis of FITC-labelled *H. pylori* bacteria by human granulocytes in the presence or absence of natural (nMBL) or recombinant mannose binding lectin (rMBL) and anti-rMBL antibodies

<table>
<thead>
<tr>
<th>Phagocytosis milieu</th>
<th>Fluorescence counts</th>
<th>Phagocytosis index</th>
<th>Fluorescence counts</th>
<th>Phagocytosis index</th>
<th>Fluorescence counts</th>
<th>Phagocytosis index</th>
<th>Fluorescence counts</th>
<th>Phagocytosis index</th>
</tr>
</thead>
<tbody>
<tr>
<td>supplemented with: 20% serum containing 30 µg/ml nMBL (control culture)</td>
<td>1284</td>
<td>1.0</td>
<td>1940</td>
<td>1.5</td>
<td>3372</td>
<td>2.6</td>
<td>2848</td>
<td>2.2</td>
</tr>
<tr>
<td>supplemented with: 20% nMBL-depleted serum</td>
<td>1048</td>
<td>5.4</td>
<td>5707</td>
<td>5.4</td>
<td>4578</td>
<td>4.3</td>
<td>1125</td>
<td>1.0</td>
</tr>
<tr>
<td>supplemented with: 20% serum containing 30 µg/ml nMBL and anti-rMBL rabbit IgG</td>
<td>1486</td>
<td>2.8</td>
<td>4169</td>
<td>2.8</td>
<td>6180</td>
<td>4.1</td>
<td>1161</td>
<td>0.8</td>
</tr>
<tr>
<td>supplemented with: 20% nMBL depleted serum and 30 µg/ml rMBL</td>
<td>2560</td>
<td>2.3</td>
<td>6074</td>
<td>2.3</td>
<td>5759</td>
<td>2.2</td>
<td>2926</td>
<td>1.1</td>
</tr>
<tr>
<td>Mean: 1595 ± 668</td>
<td>Mean: 4473 ± 1879</td>
<td>Mean: 4972 ± 1264</td>
<td>Mean: 5752 ± 1264</td>
<td>Mean: 6180 ± 1264</td>
<td>Mean: 3.3</td>
<td>Mean: 3.3</td>
<td>Mean: 2015 ± 1008</td>
<td>Mean: 1.3</td>
</tr>
</tbody>
</table>

The ingestion of *H. pylori*-FITC labeled bacteria by granulocytes was estimated fluorimetrically. The intensity of phagocytosis was expressed as mean of the fluorescence counts from four experiments, evaluated in the fluorescence reader Victor2. The phagocytosis index was calculated with relation to the intensity of phagocytosis in the medium supplemented with 20% of serum containing 30µg/ml nMBL (control culture). Difference statistically significant (p<0.05).
in the medium with 20% of complete human serum containing natural MBL-nMBL (30 µg/ml), was expressed as relative fluorescence units – RFU (1595 ± 668) and as Phagocytosis Index 1.0 (Table III). The replacement of the complete serum by the same serum depleted in nMBL (nMBL-depleted serum) on mannan coated sepharose caused a significant (p<0.05) increase of fluorescence counts from 1595 ± 668 to 4473 ± 1879 and Phagocytosis Index up to 3.0. The preservation of complement activity in MBL-depleted serum was proved by using monoclonal anti-C5-C9 complex antibodies. Data in Table III also show that addition of rabbit IgG against recombinant MBL (rMBL) to the phagocytosis samples with human complete serum containing natural MBL increased the fluorescence counts from 1595 ± 668 to 4972 ± 1264 and Phagocytosis Index from 1.0 to 3.3. The replenishment of removed human serum by the addition of rMBL did not cause the increase of phagocytosis intensity (RFU 2015 ± 1008, Phagocytosis Index 1.3) as compared with the intensity of ingestion in the medium with nMBL (RFU 1595 ± 668, Phagocytosis index 1.0).

Discussion

Previously we showed that interaction between H. pylori surface structures, namely sialic acid-specific haemagglutinin or heparin/heparan sulfate binding proteins, and corresponding macrophage receptors is required for engulfment of H. pylori bacteria. On the other hand, these microbes can use host's sialylated compounds, heparin/heparan sulfate glycosaminoglycans, hyaluronic acid or vitronectin in the presence of complement to escape phagocytosis (Chmiela et al., 1998; Drogari-Apiranthitou et al., 1997; Rudnicka et al., 1998, 2001).

In this study we found that all H. pylori strains bound recombinant MBL as estimated by ELISA. These bacteria bound MBL more intensively than the cells of M. bovis. Fungi of Candida species and Aspergillus fumigatus as well as bacteria Staphylococcus aureus, exhibited strong binding of MBL, whereas Escherichia coli, Klebsiella spp., and Haemophilus influenzae type b were characterized by heterogeneous binding patterns (Neth et al., 2000). In contrast, beta-haemolytic group B streptococci, S. pneumoniae and S. epidermidis showed low levels of binding. The MBL binding by H. pylori could be mediated by mannose residues in various bacterial cell surface structures but also by fucose moieties of Lewis X or Lewis Y determinants present in the LPS of the majority of H. pylori strains (Moran et al., 1996). Jack et al. (2001) and Devyatarnova-Johanson et al. (2000), showed that bacterial LPS was of major importance in determining the binding of MBL to Gram-negative organisms Salmonella spp. and Neisseria spp.

In general, it is thought that MBL mediates protection against infections due to its opsonic activity, by activating the complement system in the presence of MASP (Garred et al., 2003; Matsushita and Fujita, 1992). However, in this study the H. pylori bacteria were ingested more intensively by human granulocytes in the medium with MBL-depleted or anti-MBL sera as compared with the intensity of phagocytosis in the medium with complete fresh sera containing natural MBL (nMBL). The complement was possibly involved in this process. During phagocytosis, in the presence of complete serum, the lytic complex could be generated on lectin pathway due to the interaction of nMBL with H. pylori bacteria. In the post-phagocytosis supernatants the C5b-C9 terminal complement complex was detected. The lysis due to complement could diminish the number of ingested bacteria in the milieu of nMBL. The complement could be activated on lectin pathway both in the medium with or without nMBL, by serum ficolins which may bind mannose or GlcNAc present on the surface structures of H. pylori (Holmskov et al., 2003; Matsushita et al., 2001). During the depletion of the sera in nMBL the activity of C5b-C9 complex was preserved. The mechanism of antibody blocking of the MBL inhibition of phagocytosis could be through blocking of nMBL binding to H. pylori or blocking of its inhibiting qualities. The interaction of anti-MBL IgG with nMBL bound to mannose residues on the surface of granulocytes or binding of nMBL-anti-nMBL IgG complexes to phagocyte Fc receptors could not be excluded. Another explanation for diminished phagocytosis of H. pylori in the medium containing nMBL as compared to the medium without nMBL is that bacteria avoid phagocytosis by intensive nMBL binding, a phenomenon which was earlier observed by us for vitronectin and sialic acid (Chmiela et al., 1998). MBL may mask the H. pylori surface adhesins important for recognition and engulfment of these bacteria by phagocytes. A weak H. pylori phagocytosis in nMBL-depleted serum with rMBL confirms this suggestion. The more extreme environment for MBL binding in the gastric mucosa, where phagocytic cells infiltrate during infections, can be neutralized by H. pylori urease. Similarly, to the results of our study, Swanson et al. (1998), showed the 50% inhibition of the interaction of Chlamydia trachomatis, C. pneumoniae and C. psittaci with the leukocytes by rMBL.
Considering the known role of phagocyte receptors for Fc fragment of IgG (FcyR) in the ingestion of bacteria it was interesting to compare the outcome of H. pylori phagocytosis in the medium with the sera from H. pylori infected individuals, seropositive for anti-H. pylori IgG and from uninfected,seronegative donors. In this study, we could see no difference in the ingestion of MBL-binding H. pylori bacteria in the medium with sera containing or free of anti-H. pylori IgG. Similarly, we observed no significant difference in the MBL levels in the sera from H. pylori infected or uninfected children and adults. Also Klabunde et al. (2000), showed no differences in serum MBL concentration in the patients infected with Schistosoma sp. and in healthy controls though Schistosoma cercariae and adult worms, like H. pylori, bind MBL. In contrast, MBL deficiencies were detected with a high frequency in the patients infected with HIV, hepatitis B virus or Neisseria meningitides (Devyatynova-Johanson et al., 2000; Saifuddin et al., 2000; White et al., 2000). The lack of significant correlation between MBL concentration and H. pylori infection in this cohort study implies that MBL is not an essential factor in the disease process. However, in some H. pylori infected patients, the elevated MBL concentration by blocking H. pylori phagocytosis may allow these bacteria permanent colonization of gastric mucosa. In the summary, our results indicate that H. pylori bacteria may use MBL to avoid engulfment by phagocytes. The interactions of bacterial compounds and host lectins may regulate H. pylori phagocytosis and on this way influence an outcome of H. pylori related infections.

Acknowledgments. The authors wish to thank Prof. R.A.B. Ezekowitz for providing rMBL and anti-rMBL antibodies. This study was supported by a grant of the State Committee for Scientific Research (KBN), Poland 3P05 E045 24.

Literature


Role of MBL in *H. pylori* phagocytosis


Phenotypic and Genotypic Characteristics of Pseudomonas aeruginosa Strains Isolated from Hospitals in the North-West Region of Poland

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Received 22 June 2005, revised 2 March 2006, accepted 8 March 2006

Abstract
A total of 90 Pseudomonas aeruginosa strains isolated from 4 hospitals in the west-north region of Poland were studied by arbitrarily primed polymerase chain reaction (AP-PCR). AP-PCR results revealed the presence of 11 main groups of patterns (A-K) and 5 unique patterns among isolates. Generally, they were characterized by high resistance to antibiotics tested and significant differences in serogroups and types of growth on Cetrimide Agar medium. It was observed that clonally related strains were isolated from patients within the same ward, among different wards as well as in distant hospitals.

Key words: Pseudomonas aeruginosa, arbitrarily primed PCR (AP-PCR), fingerprinting, antibiotic resistance, nosocomial infections

Introduction

Pseudomonas aeruginosa is a Gram-negative, non-fermenting rod widely distributed in nature and human environment. It is responsible for one of the most serious opportunistic infections in humans. In recent years nosocomial infections caused by P. aeruginosa has been recognized as an acute problem in hospitals due to its antibiotic multi-resistance. P. aeruginosa is one of the main causes of nosocomial respiratory tract, urinary tract and surgical site of infection. It is the primary cause of ventilated-associated pneumonia in intensive tract unit (ICU), where individuals are highly susceptible to infection than patients from the other wards of the same hospital. Severity of illness, underlying disease, immunosupression and invasive devices especially mechanical ventilation are risk factors for P. aeruginosa infection (Ayats et al., 1997; Bertrand et al., 2000; Boddie et al., 2003; Cheol-In Kang et al., 2003; Garcia-Garmendia et al., 1999; Garrouste-Orgeas et al., 1996). Patients hospitalized in ICUs have three-fold greater risk of contracting nosocomial infection and mortality may increase from 13% to 47% (Nikodemski, 1997).

According to the studies conducted by the European Prevalence of Infection in Intensive Care (EPIC) which covered 10 038 patients in 17 western European countries, P. aeruginosa was the third most frequently isolated microorganism (28.7% of the total number of isolates) (Spencer, 1993). As reported by Peacock and Garrad (1997) 16–31% of pneumonia cases diagnosed among artificially ventilated patients were connected with P. aeruginosa infections (Peacock et al., 1997). Pneumonia occurrences caused by P. aeruginosa result in 30–80% of death cases among ICU patients in Polish hospitals (Piotrowska, 1998).

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Quite recently epidemiological investigations could rely only on classical methods based on analyses of phenotypic features, including biotype, serotype and phagotype identification as well as bacteriocin and antibiotic sensitivity of tested strains. To identify a strain precisely several method had to be applied. Phenotypic tests are expensive, time and labor consuming and very often their results are ambiguous to interpret, e.g. in case of endemic strains (Gospodarek and Waszak, 1995; Gillespie et al., 2000; Hancock et al., 1983; van Belkum, 1994; Versalovic et al., 1993).

Presently, genetic techniques supported by phenotypic tests enable to conduct a detailed characteristic of strains isolated from particular time and environment. The aim of such analyses is to precisely evaluate if strains isolated from infected patients are clonally related and transmitted horizontally within the ward or infections are caused by representatives of various clonal groups. It provides data about sources of infection and route of microorganism transmission and is of crucial importance for epidemiological investigations, especially those focused on tracing nosocomial infections.

Reliability of performed analyses relies both on a typing method selected as well as on interpretation of results. Rightly performed analysis should cover three basic criteria of typing bacterial strains, i.e. typeability, reproducibility and discrimination (Power, 1996; Versalovic et al., 1993). To fulfill the above criteria, a preliminary optimization of applied typing method is usually essential. Lack of standardization leads to non-reproducibility of the results and their wrong interpretation and may also limit wider application of genetic methods in routine clinical diagnostics. Macrorestriction analysis of genomic DNA followed by pulsed field gel electrophoresis (PFGE) has become the gold standard for molecular typing, however, interpretation of PFGE results for *P. aeruginosa* is very complicated. Efficiency of the method may be limited by species features, e.g. interpretation of pulsed-field gel electrophoresis (PFGE) typing patterns is complicated (Fiett et al., 1998; Kersulyte et al., 1995). Promising results were obtained by application of arbitrarily chosen primers in random amplified polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF) and arbitrary primed PCR assay (AP-PCR).

The aim of the work was to estimate intra-species differentiation of *P. aeruginosa* strains isolated from 4 hospitals in the west-north region of Poland and to evaluate effectiveness of phenotypic methods such as biotyping, serotyping, susceptibility to chemotherapeutic agents and type of growth on Cetrimide Agar medium in the epidemiological investigation.

### Experimental

#### Materials and Methods

**Bacterial strains.** A total of 90 *P. aeruginosa* strains isolated from various clinical specimens in hospitals nos. 1–4 in the west-north region of Poland during 1996–2000 were examined. Strains were isolated from 56 patients admitted to different wards of these hospitals. Some patients had *P. aeruginosa* isolated from one, two sites or from the same specimens collected at different days of hospitalization.

**Phenotypic study.** *P. aeruginosa* isolates were identified by the biochemical profile index procedure ID 32 GN (bioMérieux, France). Pyocin production was tested on selective Cetrimide Agar (Merck, Germany). O-type lipopolysaccharide was determined according to Habs (1957) protocol based on agglutination test with anti-O sera (Sanofi Pasteur, France). Susceptibility to antibacterial drugs was studied by the disk diffusion method according to CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS-National Committee for Clinical Laboratory Standards) for following agents: carbenicillin (Cb), piperacillin-tazobactam (Tzp), ceftazidime (Caz), imipenem (Imp), meropenem (Mem), gentamicin (Gn), tobramycin (Tb), netilmicin (Net), amikacin (Ak), pefloxacin (Per), ciprofloxacin (Cip), colistin (Ks) (Performance standards for antimicrobial susceptibility testing. 1999).

**Genetic analysis.** DNA was extracted using DNA ZOL reagent (Gibco, USA) according to the manufacturer’s instructions. DNA concentration was quantified spectrophotometrically at 260 nm/280 nm in GeneQuant RNA/DNA Calculator (Pharmacia Biotech, UK) and diluted if necessary to obtain concentration of 20 ng/µL. To obtain complex and stable amplicon profiles, the orthogonal array assay designed by Taguchi and Wu (1980) and modified by Cobb and Clarkson (1992) was applied.

The PCR was performed in a volume of 25 µL containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 1 U of *Taq* DNA polymerase (Roche, USA), 3.0 mM MgCl₂, 2.0 mM of each nucleotide, 30 pM of each primer: CagA2 (5’ ATT TAG AAG CAG GCT TTA GC 3’) and CMVin2 (5’ GGT AGC ACC GCG GGT TTC GAC 3’) and 20 ng/µL of template DNA in thermocycler Gene Amp PCR System 9600 (Perkin Elmer, USA). The thermal profile consisted of an initial denaturation step at 94°C for 2 min followed by 36 cycles of a 49°C denaturation for 30 sec, a 30°C annealing for 5 min, a 72°C elongation for 1 min, proceeded with 37 cycles of a 94°C denaturation for 30 s, a 30°C annealing for 1 min and a 72°C elongation for 2 min. At the end of amplification mixture was subjected to the final extension at 72°C for 7 min. Amplified products including a negative control and a molecular weight marker (Mass Ruller™ DNA Ladder, MBI Fermentas, Lithuania) were analyzed by electrophoresis in 6% polyacrylamide gel stained with 0.5 mg/L ethidium bromide and visualized, photographed and analyzed by GelDoc 2000 (BioRad Laboratories, USA). Dice coefficient was calculated and compared to evaluate similarity among strains by BIOGENE software (Vilber Lourmat, France).
Results

The isolation sites of 90 strains \textit{P. aeruginosa} for the 56 patients are shown in Table I. Most of strains (63–70\%) were isolated from lower respiratory tract of patients admitted to ICU, 16 (17.8\%) from postoperative wounds and single from catheters (4), throat (4), bile (2) and anus (1). \textit{P. aeruginosa} was isolated from one site or from sequential samples of various respiratory tract exudates in 52 patients, and from two different sites in 4 patients (in all from respiratory tract and wound-2, or catheter-1, or anus-1).

<table>
<thead>
<tr>
<th>Hospital/ward</th>
<th>1996 number of strains</th>
<th>1997 number of strains</th>
<th>1998 number of strains</th>
<th>1999 number of strains</th>
<th>2000 number of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>N°1 ICU</td>
<td>1a 1</td>
<td>1c 1</td>
<td>5b 1</td>
<td>4b 4</td>
<td>16(15b,1f) 1</td>
</tr>
<tr>
<td>Sur1</td>
<td>1b 1</td>
<td>1c 1</td>
<td>3(2b,1c) 1</td>
<td>1d 1</td>
<td>2d 2</td>
</tr>
<tr>
<td>Sur2</td>
<td>1e 1</td>
<td>3d 3</td>
<td>1d 1</td>
<td>2g 2</td>
<td>2d 2</td>
</tr>
<tr>
<td>DSur</td>
<td>1c 1</td>
<td>2d 2</td>
<td>1b 1</td>
<td>1d 1</td>
<td>1d 1</td>
</tr>
<tr>
<td>N°2 ICU</td>
<td>2b 2</td>
<td>1b 1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N°3 ICU</td>
<td>2(1a,1b) 1b 1</td>
<td>1b 1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N°4 ICU Sur</td>
<td>1b 1</td>
<td>1e 1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2 2 3 3 39 24 10 10 36 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Genetic types were determined based on cluster analysis comparing values of Dice coefficient for AP-PCR patterns. Two main groups (I–II) at the level of 30–40\% of Dice coefficient were observed. AP-PCR typing revealed 11 groups of genotypes (A–K) containing from 2 to 20 isolates of a high similarity according to Dice coefficient values (70–100\%) and 5 unique isolates (L, M, N, O, P) (Fig. 1 and 2). Among them, genotypes persistently present in a particular ward for some years were detected, e.g. AP-PCR types A and D isolated from a ICU in the hospital no 1 from 1996 to 1998, as well as strains typical of different wards of the same hospital, e.g. AP-PCR type C (ICU, Sur1 and DSur) and type F (ICU, Sur1, Sur2, DSur). It was
Fig. 2. Dendrogram demonstrating the genetic relationship among 90 isolates *Pseudomonas aeruginosa*. The scale corresponds to the percentage of similarity. A – K AP-PCR types, P – O-unique types.
observed that some AP-PCR types occurred simultaneously for a year or longer in different hospitals, e.g. AP-PCR types H and K detected in 1997 (hospital no 2) and 1998 (hospitals nos 2–4). Details are presented in Table II. Most often the same clones were isolated from two to ten patients, but the different clones in sequential samples from respiratory tract or from two sites of the same patient were found also.

Strains classified to the same genotypic type were not phenotypically similar, i.e. they did not display the same susceptibility to antimicrobial agents and the same type of growth on selective medium as well as they did not belong to the same serotype. Within the same type up to several resistance profiles, serotypes and types of growth on selective medium were presented. Detailed data on comparison of phenotypic and genotypic strain features are presented in Table III.

Irrespectively of the hospital and/or ward, most of \textit{Pseudomonas aeruginosa} isolates showed very differentiated resistance to antimicrobial agents tested. Different resistance patterns in various arrangements were observed, from sensitivity to all tested antibiotics, through single resistance to carbenicillin or/and pefloxacin to multidrug resistance for almost all tested drugs. All strains were susceptible to colistin. Strains isolated in 1996 and 1997 and 4 from 5 unique types (L, M, N, P) were generally less resistant to chemotherapeutic agents than isolated since the end of 1998. Nevertheless, no correlation was found between susceptibility to antibiotics and serotype or the type of growth on selective medium.

The total of 90 \textit{Pseudomonas aeruginosa} strains were tested on selective cetrimide agar. A green-yellow type of growth appeared most frequently (59 strains – 65.5%) whereas a yellow type was the most rarely found (11.1%). Details are presented in Table III.

From all tested strains 81 isolates (89.9% of) reacted with applied sera. 11 serotypes were distinguished. Most strains (70–77.7%) typed by monovalent sera. Two serotypes: FO:11 (46–51.1%) and EO:16 (17–18.9%) were observed most frequently. Individual strains reacted with following sera EO:15 (2), AO:1 (1), AO:4 (2), AO:6 (1), CO:10 (1). Some strains were typed only by polyvalent sera: PMA (6), PME (3) and PMF (2) whereas 9 (10.0%) of strains was non-typed at all. In general, 21 of strains (23.3%) gave ambiguous typing results. A great variety of serotypes were observed among isolates from hospitals no 1 and 2. Only all strains isolated from the hospital no 3 belonged exclusively to EO:16 serotype whereas isolates from the hospital no 4 were identified as FO:11.

**Table II**

<table>
<thead>
<tr>
<th>Hospital/ward</th>
<th>AP-PCR type (number of strains)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N°1 ICU</td>
<td>A (1), D (1)</td>
</tr>
<tr>
<td>Sur1</td>
<td>H (1), K (1)</td>
</tr>
<tr>
<td>Sur2</td>
<td>H (3)</td>
</tr>
<tr>
<td>DSur</td>
<td>H (1)</td>
</tr>
</tbody>
</table>


**Discussion**

The hospital environment remarkably promotes selection and quick distribution of resistant strains (Dzierżanowska, 1997; Giedrys-Kalemba, 2000; Hanberger et al., 2004; Jaworski et al., 1993; Łopaciuk, 1996; Sader et al., 2004). One of the essential steps leading to a reduction of nosocomial infections is a constant monitoring of etiological agents and resistance of intrahospital strains. It is of crucial importance to carry out epidemiological surveys including a detailed characteristic and relationship among strains isolated in particular environment and time, as well as to become aware of risk factors, sources and ways of infection distribution (Czekajło-Kołodziej, 2001; Łopaciuk, 1996). To obtain reliable results,
### Table III

AP-PCR types *Pseudomonas aeruginosa* isolated in the north – west region of Poland and their phenotypic differentiation

<table>
<thead>
<tr>
<th>AP-PCR type</th>
<th>Resistance pattern</th>
<th>Date of isolation</th>
<th>Serotype</th>
<th>Type of growth on Cetrimide Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td>CbTzpPef</td>
<td>1996</td>
<td>1</td>
<td>green-yellow (4)</td>
</tr>
<tr>
<td></td>
<td>CbGnTbPef</td>
<td>1997</td>
<td>1</td>
<td>green (1)</td>
</tr>
<tr>
<td><strong>B</strong></td>
<td>CbTzpGnTbAkPefCip</td>
<td>1999</td>
<td>2</td>
<td>green-yellow (2)</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>Cb</td>
<td>1999</td>
<td>1</td>
<td>celadan (3)</td>
</tr>
<tr>
<td></td>
<td>CbGnTbNetAkPefCip</td>
<td>1999</td>
<td>1</td>
<td>green-yellow (2)</td>
</tr>
<tr>
<td></td>
<td>TzpGnTbNetPefCip</td>
<td>2000</td>
<td>1</td>
<td>green (1)</td>
</tr>
<tr>
<td></td>
<td>CbTzpIpmMemGnTbNetAkPefCip</td>
<td>2000</td>
<td>1</td>
<td>blue-green (1)</td>
</tr>
<tr>
<td></td>
<td>CbTzpGnTbNetAkPefCip</td>
<td>1999</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>D</strong></td>
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especially in case of isolates without characteristic phenotypic markers, application of molecular methods seems to be inevitable.

To differentiate precisely among particular \textit{P. aeruginosa} strains isolated from 4 hospitals in the west-north region of Poland, AP-PCR typing was carried out. However, due to the large number of strains and their different origin, the classification of strains/genetic patterns was conducted at the level of 70\% and more of Dice coefficient (Struelens \textit{et al.}, 1993). Dendrogram analysis enabled to divide strains into main groups (40\%–50\% of similarity), then subgroups (55\%–65\%), genotypes (70\%–75\%) and subtypes (76\%–100\%). AP-PCR typing revealed presence of 16 AP-PCR types of \textit{P. aeruginosa}.

A high number of AP-PCR types pointed to marked intrahospital differentiation of \textit{P. aeruginosa} strains that are widely distributed in nature, especially in humid environments. It indicated various sources of strains and their constant exchange, also with the same patients. Such strains were generally highly resistant to antibiotics what confirmed the development of secondary resistance and their intrahospital selection. At the same time strains of unique fingerprints, frequently expressing higher susceptibility to chemotherapeutic agents, were isolated. It gave evidence of the temporary incidence of new endogenous strains entering the hospital environment.

Some of the genetic types expressing the same/similar of AP-PCR pattern were numerically dominant within the ward(s) for some months/years. It might prove horizontal transmission of clones or clonally-related groups and epidemic/endemic character of registered infections (Fiett \textit{et al.}, 1998). The incidence of the same genetic types of \textit{P. aeruginosa} in different hospitals drew attention to a possibility of a long-distance strain transmission. It might be linked to the movement of patients, visitors, medical and paramedical staff.

Based on dates of strains isolation and their resistance to antibiotics, it is highly probable that selection of highly resistant isolates takes place in ICUs, where \textit{P. aeruginosa} is one of the most frequent and severe cause of infections, especially in patients with mechanical ventilator. In the absence of epidemic clones, secondary resistance development during combined antibacterial therapy appeared to be the main factor contributing to the prevalence of resistance in ICU, what was observed in sequential samples from the same patient. On the other side, the significance of patients relocated from different wards to ICUs and colonized with ward-specific microflora should not be underestimated.

Phenotypic methods are based on a presence or absence of expressed and strain characteristic features. Instability of such features in various environmental conditions is the main disadvantage of phenotypic assays that frequently precludes a precise strain characteristic. Types of \textit{P. aeruginosa} growth on Cetrimide Agar medium differed significantly within strains belonging to one genotype as well as among strains of different genotypes. The green-yellow type was observed most frequently (65.6\%) whereas the yellow type was the rarest (1.1\%). Also serotyping based on the somatic antigen evaluation according to Habs protocol did not reveal correlation between genotypes and serotypes. Both FO:11 (51.1\%) and EO:16 (18.9\%) were the most widespread serotypes. The FO:11 serotype was isolated from almost all wards in hospitals covered by our studies. Intriguingly, a FO:12 serotype (the dominant type among multi-resistant \textit{P. aeruginosa} strains in the hospitals all over the world) was not detected (Bingen, \textit{et al.}, 1996; Cavallo \textit{et al.}, 2000). Serotyping is not considered as the method of high discriminatory power. The occurrence of antigenic variations within strains causes that some strains do not respond to commercial sera (10.0\% of strains analyzed in our studies). A susceptibility to antibiotics is not also the most practical tool for unambiguous epidemiological evaluation of strains. However, it may serve as the preliminary criterion indicating the incidence of a potential intrahospital strain and signaling the necessity of conducting further investigations.

Results of AP-PCR typing did not reveal consistence between strain fingerprints and their phenotypic features. A majority of \textit{P. aeruginosa} strains presented a high differentiation of phenotypic patterns within a genotype. It confirmed lack of correlation between molecular and conventional typing, e.g.: types of growth on Cetrimide Agar, serotype or susceptibility/resistance pattern to antimicrobial agents. Similar results also with the other genera of microorganisms were proved (Bouza \textit{et al.}, 1999; Fierobe \textit{et al.}, 2001; Dinesh \textit{et al.} 2003; Giedrys-Kalemba \textit{et al.}, 2001). The lack of correlation between \textit{P. aeruginosa} AP-PCR types and their phenotypic features indicates that phenotypic analysis should not be exclusive method of evaluating of strain relationship and conducting epidemiological investigations of nosocomial infections. It is necessary to carry out analysis at the molecular level (Cavallo \textit{et al.}, 2000; Gomez \textit{et al.}, 2000). However, phenotypic studies are a valuable tool supplementing genotyping as they enable tracing of phenotypic feature expression influenced by different environmental conditions (Biendo \textit{et al.}, 1999).


Detection of Enterotoxic *Bacillus cereus* Producing Hemolytic and Non Hemolytic Enterotoxins by PCR Test

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

**Abstract**

Nine strains belonging to *Bacillus cereus* group has been isolated from food and environmental samples. Their taxonomic position was confirmed by RFLP analysis of 16S rRNA gene digested with TaqI. The detection of DNA sequences encoding the hemolysin BL complex and enterotoxin NHE, was studied in *Bacillus* sp. isolates. Set of primers was used to amplify fragment of *hblD* gene by PCR. For the detection of *nheB* gene a new primer set was developed which allowed to amplify 273 bp fragment from wide number of strains belonging to *B. cereus* group. The *hblD* gene was present in 7 out of 9 isolates whereas *nheB* gene occurred in all of them. Reference strains of *B. cereus* LOCK 0807, and *B. thuringiensis* NCAIM 01262 contained both genes. Strains of *B. subtilis* ATCC 6633 and *B. pumilus* LOCK 0814 do not contain both genes. Obtained results showed that *B. thuringiensis* NCAIM 01262 contains both genes and therefore may be harmful for human beings.

**Key words:** enterotoxin BL, enterotoxin NHE, *Bacillus cereus* group, PCR

**Introduction**

*Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* are members of the *Bacillus cereus* group of bacteria sharing common properties and strong genetic similarity especially in the sequence of 16S rRNA, the number of rRNA operons, their organization, localization and ability to produce numerous toxins responsible for pathogenicity and food poisonings. *B. anthracis* causes the acute fatal disease, anthrax, and is widely recognised as powerful biological weapon due to its high toxicity for human beings. *B. thuringiensis* is a well known producer of crystalline intracellular proteins called δ-endotoxins, toxic to wide number of insect larvae belonging to *Diptera* and *Coeloptera*. For this reason cells of *B. thuringiensis* are widely used as biological pesticide and its genes coding for δ-endotoxins were used for the construction of pest resistant transgenic crop plants. *B. cereus* is a very common soil bacterium which may contaminate starchy food products and raw materials causing diarrheal and emetic food poisonings. Formation of thermoresistant spores by *Bacillus cereus* may also cause sterility problems in ready to eat dishes consisting of boiled rice, noodles, potato and other starch-containing food products.

Advent of modern genetics brought a lot of information on chromosome organization of the members of *B. cereus* group. So far two *B. anthracis* strains Ames and Sterne (Read et al., 2003; Brettin et al., 2004c), three *B. cereus* strains ATCC 14579 (Ivanova et al., 2003), ATCC 10987 (Rasko et al., 2004) and ZK (Brettin et al., 2004b) as well as one *B. thuringiensis* serovar konkukian strain 97–27 (Brettin et al., 2004a) were sequenced and the data obtained were disseminated to the public through computer databases. This

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makes ideal situation for comparative genomics in silico to study distribution of disease related genes in all members of that group. From the viewpoint of food poisonings caused by *B. cereus*, presence of two gene clusters responsible for the production of hemolytic HBL and nonhemolytic NHE enterotoxins are very important. Search of computer databases revealed presence of non hemolytic enterotoxin gene cluster *nheABC* in all sequenced members of *B. cereus* group showing that even recognized as “non toxic” *B. thuringiensis* serovar *konukkian* str 97–27 can cause food poisonings. Gene cluster responsible for the production of hemolysin BL *hblCDA* was found only in one strain of *B. cereus* ATCC 14579 and in *B. thuringiensis* serovar *konukkian* str 97–27. This findings also confirmed possible toxicity of strain known as “harmless”. Therefore detection of enterotoxin production potential of strains belonging to the *B. cereus* group is very important for the epidemiology of food poisonings. Available methods of detection of enterotoxic *B. cereus* group were as follows: 1 – blood cell hemolysis on Columbia Agar medium, 2 – immunological tests, *Bacillus* diarrheal enterotoxin visual immunoassay (BDE kit Tecra), (BCET-RPLA kit Oxoid), 3 – PCR test. The *B. cereus* enterotoxin test kit RPLA, Oxoid is specific to the L3 component of the hemolysin BL complex whereas BDE kit Tecra detects two non toxic proteins of 40 and 41 kDa characteristic to enterotoxic strains (Beecher and Wong, 1994). Detection of both enterotoxins with PCR based technique has been developed by Hansen and Hendriksen (2001) who proposed sets of primers for individual genes of *hblCDA* and *nheABC* operons. Mantynen and Lindstrom (1998) and Neil et al. (2003) used PCR technique for detection of *hblCDA* operons. Comparison of immunological methods for detection of enterotoxin producing strains with PCR-based technique showed good correlation of obtained results and therefore both techniques can be equally reliable and replaceable (Mantynen and Lindstrom, 1998). These authors have also shown that presence of enterotoxin coding genes are not characteristic for the genus *B. cereus* but also can be found in *Bacillus mycoides*, *Bacillus pasteurii*, *Bacillus smithii* and *Bacillus thuringiensis*. However among 50 strains of *B. cereus*, only 26 contained enterotoxin coding genes suggesting that this feature is strain specific. Presence of enterotoxin genes in non cereus group, *Bacillus pasteurii* and *Bacillus smithii*, may indicate that ability to produce the same enterotoxins can be found in other species belonging to the genera *Bacillus*.

The aim of the present study was to develop rapid and accurate diagnostic tests for detecting *Bacillus cereus* group genes responsible for the production of enterotoxins. In order to determine phylogenetic position of studied strains we performed 16S rRNA gene RFLP analysis.

### Experimental

#### Materials and Methods

**Bacterial strains used in this study.** Bacterial strains applied in this study (*Bacillus* sp. 1 – sp. 9) were isolated from different food products according to the method described in the PN-EN ISO 7132 using MYP selective medium (Oxoid). *B. cereus* £OCK 0807, *B. thuringiensis* NCAIM 01262, *B. pumilus* £OCK 0814 and *B. subtilis* ATCC 6633 were used as reference strains. They originated from Culture Collection of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź.

**DNA preparation.** Chromosomal DNA was isolated according to the modified method of Marmur (1961). Additional lysozyme treatment of digested cell suspension with proteinase K was the major modification of original method.

**PCR amplification of 16S rRNA, hblD and nheB fragments.** Primer sequences for amplification of *hblD*, *nheB* and 16S rRNA fragments were derived from data records published in NCBI Database (Table I). Amplification of *hblD* fragment (430 bp) was performed in the following manner. About 20 ng of DNA template, 20 pmol of primer *hblDF*, 20 pmol of primer *hblDR*, 12.5 µl Red-Taq ReadyMix DNA polymerase (Sigma-Aldrich) were mixed together and supplemented with PCR grade water to a total volume of 25 ml. The amplification procedure consisted of one cycle of 2 min at 94°C, followed by 35 cycles for 30 sec at 94°C,

<table>
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<td></td>
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<td>5’-ATTCCCAAAGTTAAGTATCACTTG-3’</td>
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Differentiation of enterotoxic *B. cereus* by PCR test

30 sec at 48°C and 90 sec at 72°C with final extension cycle for 2 min at 72°C was performed using Uno II thermocycler, Biometra, with tube lid heating block set for 105°C. No overlay oil was added to the tubes. The reaction mix for amplification of *nheB* fragment (273 bp) was the same except of primers replaced by nheBF and nheBR in the concentration of 20 pmol each. The amplification procedure for *nheB* fragment consisted of one cycle of 2 min at 94°C, followed by 35 cycles for 30 sec at 94°C, 30 sec at 50°C and 90 sec at 72°C with final extension cycle for 2 min at 72°C. In case of 16S rRNA gene amplification the reaction mix was the same but primers were replaced by FRNA and RRNA in the concentration of 20 pmol each. The amplification procedure for 16S rRNA fragment consisted of one cycle of 2 min at 94°C, followed by 35 cycles for 1 min at 94°C, 1 min at 55°C and 3 min at 72°C with final extension cycle for 2 min at 72°C.

**RFLP analysis.** The 16S rRNA amplification fragments were digested with *TaqI* (MBI Fermentas) for 1 h at 65°C according to the product instruction. The digested PCR products were run at 60V for 4 h in 2% (w/v) agarose gel (0.5 TBE buffer containing 0.5 µg/ml ethidium bromide) by using gel electrophoresis apparatus (Biotec Fisher). Gels placed on UV transiluminator were photographed with digital camera through yellow filter.

**Agarose gel analysis of PCR products.** PCR products of *hblD* and *nheA* fragments after amplification were analysed on 1% (w/v) agarose gel in 0.5 TBE buffer containing 0.5 µg/ml ethidium bromide. Gels were run at 60 V for 3 h and photographed as described above.

**Results and Discussion**

RFLP analysis of 16S rRNA amplicons of bacterial isolates and reference strains (Fig. 1) confirmed previous findings from classical diagnostic procedure (PN-EN ISO 7132) that all *Bacillus* sp. 1–9 isolates belongs to the cereus group. Profile comparison of lane 1 and 2 in Fig. 1 showed that RFLP patterns for the

![Fig. 1. RFLP profiles of 16S rRNA gene amplicons digested with *TaqI*.](image)

W – DNA size marker; lanes 1 – *B. thuringiensis* NCAIM 01262; 2 – *B. cereus* LOCK 0807; 3 – *B. subtilis* ATCC 6633; 4 – *B. pumilus* LOCK 0814; 5 – *Bacillus* sp. 1; 6 – *Bacillus* sp. 2; 7 – *Bacillus* sp. 3; 8 – *Bacillus* sp. 4; 9 – *Bacillus* sp. 5; 10 – *Bacillus* sp. 6; 11 – *Bacillus* sp. 7; 12 – *Bacillus* sp. 8; 13 – *Bacillus* sp. 9.

![Fig. 2. Electrophoregram of *hblD* gene fragment amplicons.](image)

W – DNA size marker; lanes 1 – *B. thuringiensis* NCAIM 01262; 2 – *B. cereus* LOCK 0807; 3 – *B. subtilis* ATCC 6633; 4 – *B. pumilus* LOCK 0814; 5 – *Bacillus* sp. 1; 6 – *Bacillus* sp. 2; 7 – *Bacillus* sp. 3; 8 – *Bacillus* sp. 4; 9 – *Bacillus* sp. 5; 10 – *Bacillus* sp. 6; 11 – *Bacillus* sp. 7; 12 – *Bacillus* sp. 8; 13 – *Bacillus* sp. 9.
reference strains *B. thuringiensis* NCAIM 01262 and *B. cereus* LOC8 0807 are the same and therefore, these species can not be distinguished with this method. This finding has been confirmed by computer analysis of published sequences of 16S rRNA genes from all four members of cereus group (*B. anthracis*, *B. cereus*, *B. mycoides*, *B. thuringiensis*), showing that TaqI recognition sites within the region of that gene resides at 63, 201 and 972 bp positions. Taking into account that amplified fragment is 7 bp shorter from the front and
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4 bp from the end of complete 16S rRNA gene, resulting \textit{TaqI} digestion products should have the following size: 56, 138, 579, 771 bp. Obtained results confirmed usefulness of applied RFLP analysis for genetic confirmation of taxonomic position of isolated strains.

Application of primers developed by Hansen and Hendriksen (2001) allowed to detect \textit{hblD} gene (Fig. 2). The obtained results showed that in seven isolates of \textit{Bacillus} sp. 1–3 and sp. 4–8 as well as in both reference strains \textit{B. thuringiensis} NCAIM 01262 and \textit{B. cereus} LOCK 0807 this gene was present. Strains of \textit{Bacillus} sp. 4 and 9 showed presence of \textit{hblD} related bands (430 bp) with very low intensity what may be interpreted as lack of this gene. However this result can be obtained because the incompatibility of primers with the polymorphic DNA of \textit{hblD} gene in that strains. The other reference strains of \textit{B. subtilis} ATCC 6633 and \textit{B. pumilus} LOCK 0814 did not have that gene. Presence of \textit{hblD} gene in the reference strain of \textit{B. thuringiensis} NCAIM 01262 shows that this organism can be potentially harmful for human beings what is in disagreement with the common opinion of microbiologists.

Polymorphism of \textit{nheB} genes found in \textit{B. anthracis} (strains Ames and Sterne), \textit{B. cereus} (strains ATCC 10987, ATCC 14579, ZK) and \textit{B. thuringiensis} serovar konkukian str. 94–27 analysed \textit{in silico} showed that primers designed by Hansen and Hendriksen (2001) are not 100% identical to the corresponding regions of that genes. Similarity of forward primer varied from 94.7 to 100% and reverse primer from 88.2 to 100%. This observation lead us to the conclusion that such primers can not be used for reliable PCR-based detection of \textit{nheB} gene in the wide number of bacterial species. Therefore, an attempt has been made to design our own set of universal primers with 100% similarity to the corresponding regions of \textit{nheB} genes of all already sequenced \textit{Bacillus} strains. Figure 3 shows alignment of 273 bp long fragment of \textit{nheB} DNA sequence from genomes of \textit{Bacillus} strains belonging to the \textit{B. cereus} group. PCR reaction made with this primers for 9 \textit{B. cereus} isolates and 4 reference strains from culture collection revealed presence of characteristic 273 bp long amplicons for all isolates of \textit{Bacillus} sp. 1–9, \textit{B. cereus} LOCK 0807 and \textit{B. thuringiensis} NCAIM 01262. Strains of \textit{B. pumilus} LOCK 0814 and \textit{B. subtilis} ATCC 6633 were deprived of \textit{nheB} gene (Fig. 4). Obtained results confirmed observations from \textit{in silico} analysis of \textit{B. cereus} group genomes that \textit{nheABC} operon is characteristic for that group of organisms. This finding also confirmed previous statement that \textit{B. thuringiensis} can be harmful for human being and its use for the production of biological pesticides may create biological hazard.

\textbf{Literature}


\textit{Brettin} T.S., D. \textit{Bruce}, J.F. \textit{Challacombe}, \textit{et al}. 2004b. Complete genome sequence of \textit{Bacillus cereus} ZK. \textit{ACCESSION CP000001}. 


Optimization and Purification of Alkaline Proteases Produced by Marine Bacillus sp. MIG Newly Isolated from Eastern Harbour of Alexandria

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Abstract

A marine Bacillus strain was isolated from the eastern harbour of Alexandria and identified as Bacillus sp. MIG. Maximum activity of studied proteases was obtained when the bacterium was grown in medium with 1% wheat bran and 0.5% yeast extract in addition to the mineral salts and incubated for 48 h at 30°C and 120 rpm. Two alkaline proteases (Pro 1 and Pro 2) were purified to homogeneity using cation exchange chromatography on CM-Sepharose CL-6B followed by Sephadex G-75 superfine. The optimum activities were at pH 11 or 12, and temperatures of 50 and 55°C for Pro 1 and Pro 2 respectively. These two enzymes were relatively stable over pH range from 7.0–11. Pro 2 was found to be more stable at 50°C in absence of Ca²⁺ and retained about 47% of its activity after 3 h at this temperature, while Pro 1 lost its activity completely at the same conditions. The two enzymes were active against haemoglobin and casein; in addition, Pro 2 exhibited moderate activity against keratin. Both enzymes were partially inhibited by Ag⁺ and Hg²⁺. PMSF completely inhibited the enzymes, while dithiothreitol and 2-mercaptoethanol stimulated their activities, suggesting to be thiol-dependent serine proteases. The enzymes were stable in the presence of the surfactants and bleaching agent (H₂O₂) and relatively stable in presence of some commercial detergents.

Key words: marine Bacillus sp. MIG; alkaline serine protease; thiol-dependent serine protease

Introduction

Alkaline proteases are a physiologically and commercially important group of enzymes used primarily as detergent additives. This group of enzymes has other industrial applications such as in food, pharmaceutical, leather industry and recovery of sliver from used X-ray films (Anisworth, 1994; Inhs et al., 1999; Outtrup et al., 1995). In 1994, the total market for industrial enzymes accounted for approximately 400 million $, of which enzymes worth 112 million $ used for detergent purposes (Hodgson, 1994). Alkaline protease added to laundry detergents plays a specific catalytic role in the hydrolysis of protein stains such as blood, milk, human sweat, etc. The increased usage of the protease as a detergent additive is mainly due to its cleaning capabilities in environmentally acceptable, nonphosphate detergents (Mei and Jiang, 2005).

Although microbes from terrestrial sources are employed for industrial production of enzymes, the potential for synthesis of several novel enzymes by marine microorganisms has been recognized (Chandrasekaran, 1997). Diverse techniques have been used for the screening of novel enzymes with new biocatalytic capabilities and great potential for several industrial processes and other applications (Manachini and Fortina, 1998). Large number of microorganisms produces proteases, but Bacillus strains are recognized as important sources of commercial alkaline proteases because of their ability to secrete large amounts of enzymes with high activity (Beg and Gupta, 2003; Joo et al. 2004).

Joo and Chang (2005) reported that around 30–40% of the production cost of the industrial enzymes accounted for the cost of the growth medium. Research efforts have been directed mainly towards evaluating the effect of various carbon and nitrogen nutrient cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimization of environmental and fermentation parameters such as pH, temperature, aeration and agitation. In addition, no defined medium
has been established for the optimum production of alkaline proteases from different microbial sources. Each organism has its own special conditions for maximum enzyme production (Kaur et al., 2001; Kumar and Takagi, 1999; Razak et al., 1994). In order to reduce medium costs, different low-cost substrates were screened and in course of this wheat bran, molasses, soybean meal and feather were used for cost-effective production of multiple forms of alkaline proteases by marine Bacillus sp. MIG. The present work also describes purification of two alkaline proteases from this strain to homogeneity. Some properties of the pure enzymes were also tested.

Experimental

Materials and Methods

Microorganisms. Thirty-five marine bacteria were isolated from the eastern harbour of Alexandria (Egypt). All isolates were cultured on skim milk agar plates. The isolates, which show clear zones on the plates, were cultivated in liquid production medium to test their ability to produce alkaline proteases.

Enzyme production. The production of alkaline protease from Bacillus sp. MIG was carried out in marine medium which have the following composition, (g l\(^{-1}\)): peptone, 3; yeast extract, 1; casein, 10; ferric citrate, 0.1; NaCl, 19.45; MgCl\(_2\), 8.8; Na\(_2\)SO\(_4\), 3.24; CaCl\(_2\), 1.8, the pH of the medium was adjusted to 7.5. The medium was inoculated with 1% of 24 h seed culture and incubated under shaked condition (120 rpm) and 30°C for different periods. Different carbon and nitrogen sources were used in the present work for optimization of nutritional factors. The physical parameters including (pH of the medium and temperature of incubation) were also tested. At the end of the incubation period, the cell-free enzyme supernatant was obtained by centrifugation at 10,000 g for 15 min at 6°C.

Enzyme purification. The cold cell-free supernatant obtained from the optimized medium after 48-h incubation was precipitated with two volumes of cold acetone and then collected by centrifugation. The precipitate was resuspended in a minimal volume of Tris-HCl buffer (0.02 M, pH 7.5) containing 5 mM CaCl\(_2\). After removing the insoluble materials by centrifugation for 15 min at 6°C and 10,000 g, the clear supernatant was applied to CM-Sepharose CL-6B ion exchange column (Pharmacia) per-equilibrated with the same buffer. The column was washed with the same buffer and eluted with stepwise gradient (0–0.5 M) of NaCl in the same buffer. Fractions of 5 ml were collected at a flow rate of 1 ml/min, and then the protein content and enzyme activity of each fraction were determined. The active fractions were collected and dialyzed against the same buffer without NaCl. The fractions, which are required further purification, were applied to Sephadex G-75 superfine gel filtration column. The column was equilibrated with the last mentioned buffer and eluted with the same buffer. Fractions of 2 ml were collected at a flow rate of 0.5 ml/min, and the protein content and enzyme activity were assayed. All active fractions were stored at –20°C for further study.

Enzyme assay. Protease activity was determined using casein as substrate. Casein was dissolved at 1% in glycine-NaOH buffer (0.1 M, pH 10). The assay mixture consisted of 650 µl buffer, 250 µl substrate and 100 µl of diluted enzyme and incubated at 37°C for 10 min. The reaction was terminated by the addition of 500 µl of trichloroacetic acid reagent (TCA), and then centrifuged in eppendorf centrifuge for 10 min to remove the undigested protein. Protease activity was determined as released tyrosine in the supernatant (Lowry et al., 1951). One unit of the enzyme was defined as the amount of the enzyme resulting in the release of 1 µg of tyrosine ml\(^{-1}\) min\(^{-1}\) under the assay conditions. The protein content was also determined by Lowry et al. (1951) using bovine serum albumin as a standard.

SDS-Polyacrylamide gel electrophoresis and detection of enzymatic activities in the gel. The purity and relative molecular weights of the purified enzymes were estimated by SDS-PAGE using a 12% polyacrylamide gel according to the method of Laemmli (1970). The enzyme activities were tested in the gel following the method described by Heussen and Dowdle (1980).

Substrate specificity. Protease activities of purified enzymes against various protein substrates including BSA, gelatin, azocasein, azoalbumin, haemoglobin and keratin were assayed using 1% from each substrate dissolved in glycine-NaOH buffer, pH 11. The reaction was terminated with TCA and released products were measured at the suitable wavelength for each substrate. The activity toward casein was taken as control.

Effect of metal ions, inhibitors, surfactants and detergents. The effect of different metal ions, protease inhibitors, surfactants and commercial detergents on the activity of purified proteases was determined by incubating them with the enzymes for 30 min at 40°C and the residual activities were measured by the standard assay procedure.

Results and Discussion

Selection of the best alkaline protease-producing organism. From the tested isolates, four have the ability to produce alkaline proteases. The best alkaline protease producer was identified using 16S rRNA methodology. Part of 16S rRNA gene was amplified, sequenced and deposited in the GeneBank database with accession number DQ076248 as Bacillus sp. MIG. Comparing the obtained sequence with the sequences available in the NCBI revealed 97% similarity with Bacillus pumilus strain: M1-9-1. This organism was selected for production and purification of alkaline proteases.

Correlation between growth and protease production. The protease production by the test organism started in the exponential growth phase and enzyme activity showed linearity with growth. The maximum activity (1070.86 Uml\(^{-1}\)) was obtained in the stationary phase after 48 h and remains more or less stable.
Alkaline proteases in marine Bacillus sp.

until 72 h, and then decreased with increasing the incubation time (data not shown). A similar trend was reported for extracellular protease production by Bacillus sp. (Oberoi et al., 2001), Bacillus subtilis PE-11 (Adinarayana et al., 2003) and by Bacillus clausii (Kumar et al., 2004). The cell density increased with time and reached its maximum after 24 h.

Effect of temperature and pH on the enzyme production. The effect of incubation temperature and pH of the production medium are critical factors and need to be optimized. The optimum temperature for enzyme production was found to be 30°C, increasing the temperature led to decrease the enzyme production (Fig. 1). Higher temperatures at 40 and 45°C caused more than 37 and 77% loss in the enzyme compared to 30°C. The protein content increased with the increase in enzyme activity except at 45°C, while the enzyme activity decreased, the protein content was increased. Ray et al. (1992) reported that temperature could regulate the synthesis and secretion of extracellular proteases by microorganisms. The effect of the initial pH of the culture medium on the enzyme production was studied in pH range of 4–10, the initial pH was adjusted with NaOH or HCl. No growth was found at pH 4. The enzyme was produced over pH range from 6–10 with a maximum value at pH 7, in which no pH adjustment was made. In order to prove that the enzyme was produced in neutral to weak alkaline pH, the initial pH was adjusted using sodium carbonate. In this experiment, maximum enzyme activity was obtained in pH range from 7–8. Drastic decrease in bacterial growth and enzyme production was observed, when the initial pH was in the alkaline range (data not shown). These results indicate that the Bacillus sp MIG is a neutralophilic organism and produces alkaline proteases.

Effect of carbon sources on enzyme production. The effect of carbon sources was investigated in medium containing (3 g l⁻¹ peptone and 1 g l⁻¹ yeast extract). The tested carbon sources were supplemented to the medium at 1%. The results presented in Figure 2 showed that the addition of wheat bran, molasses,
starch or maltose enhanced the enzyme production with the maximum value in the presence of wheat bran (1449.15 U ml\(^{-1}\)). The use of wheat bran in the production medium is very important, because it is one of the cheap and readily available carbon sources. The estimated cost of wheat bran was found to be 0.002 $ for one liter production medium. More recently, alkaline protease production from \textit{Bacillus} sp. was investigated in solid-state fermentation using wheat bran and lentil husk, where wheat bran was found to be a better source (Uyar and Baysal, 2004). Studies on alkaline proteases reported that the addition of starch to the culture medium induced enzymes synthesis (Chauhan and Gupta, 2004; Fang \textit{et al.}, 2001). On the other hand, addition of other carbon sources like sucrose, glucose or fructose reduced the activity by about 60, 42 and 40% (respectively) compared to the control. Removal of casein from the medium with peptone and yeast extract had no effect on the enzyme production, indicating the constitutive nature of \textit{Bacillus} sp. MIG enzyme. Production of microbial proteases has been found to vary from being constitutive to partially inducible in nature (Gupta \textit{et al.}, 2002; Puri \textit{et al.}, 2002). Increasing the concentration of wheat bran up to 2.5% had no affect on enzyme activity.

\textbf{Effect of nitrogenous compounds.} Effect of different nitrogen containing compounds was evaluated in medium with wheat bran as the sole carbon source (Fig. 3). The nitrogen source in the basal medium (3 g l\(^{-1}\) peptone and 1 g l\(^{-1}\) yeast extract) was replaced on their nitrogen equivalent by different organic and inorganic nitrogen sources. It was found that yeast extract was the best organic nitrogen source and gave the highest activity (1601.34 U ml\(^{-1}\)), while sodium and potassium nitrate were the best inorganic nitrogen source. Ammonium nitrate and chloride totally repressed the enzyme production and ammonium sulphate gave only 8.6% compared to the control medium. Johnvesly and Naik, (2001) reported that protease production by \textit{Bacillus} sp. JB-99 was supported by nitrate nitrogen viz. sodium and potassium nitrate, while ammonium nitrogen completely inhibited the production. Soybean meal, casein hydrolysate and casein also supported high enzyme activity. Peptone and urea reduced the activity by about 20 and 47% (respectively) compared to the control. The addition of 1% feather to the medium with 1% wheat bran supported the enzyme production and gave high activity, but the incubation time was 4-days. \textit{Bacillus} sp. P-001A was found to degrade feather after at least 5-days (Atalo and Gashe, 1993). It was also found that the use of medium with 1% feather as the sole carbon and nitrogen source gave about 60% of the activity obtained in presence of wheat bran and feather and the addition of 1% glucose to the feather reduced the activity by about 30% (data not shown). On the other hand, the protein content in the culture filtrate was not greatly affected by the nitrogen sources.

\textbf{Enzyme purification.} The purification scheme of the extracellular proteases produced by \textit{Bacillus} sp. MIG is summarized in Table I. Two proteases were purified to homogeneity using cation exchange chromatography on CM-Sepharose CL-6B, followed by gel filtration on Sephadex G-75 superfine. The steps were very effective and combined to give overall purification of 19.3 and 16.1-fold for the protease 1(Pro 1) and protease 2 (Pro 2) respectively. Figure 4 shows that two fractions were obtained after the cation exchange. The first one was eluted using 0.1 M NaCl and contained three main protein bands, in addition to minor proteins and the second was eluted with 0.5 M NaCl and contained one major band and some minor bands.
Alkaline proteases in marine Bacillus sp.

The rechromatography of the first fraction from the cation exchange on Sephadex G-75 gave two pure bands. The specific activities for both proteases are 5023.1 and 4185.5 U mg\(^{-1}\) respectively. The purified enzymes were also confirmed to be homogeneous by SDS-PAGE with molecular weights of about 36 and 23 kDa respectively (Fig. 4). The molecular masses of microbial alkaline proteases ranged between 15 and 36 kDa, with few exceptions of high molecular mass, such as 42 kDa from Bacillus sp. PS719 (Towatana et al., 1999) and very high (90 kDa) from Bacillus subtilis (Kato et al., 1992). Zymogram activity staining revealed three major clear zones of proteolytic activities that are active over broad range of pH and fourth one that only active in the alkaline range (Fig. 5).

### Effect of pH on activity and stability of both enzymes.

The effect of pH on the activity of both purified proteases was determined at 40°C in the pH range from 6.0–12.0. The two proteases were more active in the alkaline range with maximum activity at pH 11 and 12 for Pro 1 and Pro 2 respectively (Fig. 6a). The two enzymes were relatively stable over a pH range from 7–10 for 20 h at 30°C and retained about 88 and 92% at pH 10 after the same time (Fig. 6b). Two alkaline proteases AP-1 and AP-2 from alkalophilic Bacillus spp. were also optimally active at pH 11 and 12, respectively, and were stable for 4 h in pH range of 6–12 for AP-1 and 6–9 for AP-2 (Kumar et al., 1999). The optimum pH obtained for enzymes reported in this study, are higher than the values pH 9 (Rahman et al., 1994) and pH 10.5 (Beg and Gupta, 2003) for other alkaline proteases.

### Effect of temperature on activity and stability of both enzymes.

The optimum temperatures for Pro 1 and Pro 2 were found to be 50 and 55°C respectively (Fig. 7a). It was also found that Pro 2 was relatively

### Table I

**Summary of purification steps**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg(^{-1}))</th>
<th>Yield (%)</th>
<th>Fold purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>400250</td>
<td>1542</td>
<td>259.6</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td>300650</td>
<td>589</td>
<td>510.4</td>
<td>75.1</td>
<td>1.9</td>
</tr>
<tr>
<td>CM-Sepharose CL-6B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eluted with 0.1M NaCl</td>
<td>121300</td>
<td>90</td>
<td>1347.78</td>
<td>30.3</td>
<td>5.2</td>
</tr>
<tr>
<td>Eluted with 0.5M NaCl</td>
<td>30213</td>
<td>15</td>
<td>2014.2</td>
<td>7.5</td>
<td>7.8</td>
</tr>
<tr>
<td>Sephadex G-75 superfine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro 1</td>
<td>50231</td>
<td>10</td>
<td>5023.1</td>
<td>12.5</td>
<td>19.3</td>
</tr>
<tr>
<td>Pro 2</td>
<td>54412</td>
<td>13</td>
<td>4185.5</td>
<td>13.6</td>
<td>16.1</td>
</tr>
</tbody>
</table>

The rechromatography of the first fraction from the cation exchange on Sephadex G-75 gave two pure bands. The specific activities for both proteases are 5023.1 and 4185.5 U mg\(^{-1}\) respectively. The purified enzymes were also confirmed to be homogeneous by SDS-PAGE with molecular weights of about 36 and 23 kDa respectively (Fig. 4). The molecular masses of microbial alkaline proteases ranged between 15 and 36 kDa, with few exceptions of high molecular mass, such as 42 kDa from Bacillus sp. PS719 (Towatana et al., 1999) and very high (90 kDa) from Bacillus subtilis (Kato et al., 1992). Zymogram activity staining revealed three major clear zones of proteolytic activities that are active over broad range of pH and fourth one that only active in the alkaline range (Fig. 5).

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**Effect of temperature on activity and stability of both enzymes.** The optimum temperatures for Pro 1 and Pro 2 were found to be 50 and 55°C respectively (Fig. 7a). It was also found that Pro 2 was relatively
active at high temperatures than Pro 1. The thermal stability of the two enzymes was examined by preincubation of the enzymes at pH 11 for 3 h at temperatures 40 and 50°C in presence and absence of 5 mM CaCl$_2$, then the residual activities were determined under the standard assay conditions. Both enzymes are stable in presence of CaCl$_2$ at 40°C and relatively stable in its absence at the same temperature (Fig. 7b). At 50°C, in the absence of calcium chloride, Pro 1 lost about 46 and 100% of its activity after 90 and 180 min, while Pro 2 lost only 24 and 53% after the same time. On the other hand, the two enzymes retained more than 80% after 2 h at 50°C in presence of 5 mM CaCl$_2$. These results revealed that calcium chloride increased the thermal stability of both enzymes. Similar optimum temperatures were reported for AP-1 and AP-2 from Bacillus spp. (Kumar et al., 1999). Alkaline protease from B. mojavensis was optimally active at temperature of 60°C, with rapid loss of activity above 65°C (Beg and Gupta, 2003), while lower optimum temperature (45°C) was reported for other protease (Rattary et al., 1994). Alkaline proteases from Nocardiopsis sp. retained only 60% after 2 h at 50°C (Moreira et al., 2003). Generally, most of the commercial available Subtilisin-type proteases are active in the pH and temperature range between 9.0–12.0 and 50–60°C, respectively (Beg and Gupta, 2003).
Substrate specificity. The ability of the Pro 1 and Pro 2 to hydrolyze different proteins was tested. It was found that Pro 1 exhibited the highest activity towards casein and bovine serum albumin and good activity with haemoglobin, while low level of activity was detected with modified substrates (azocasein and azoalbumin). On the other hand, Pro 2 showed the highest activity toward casein but no activity with bovine serum albumin and azoalbumin. Pro 2 also showed a moderate activity against keratin, while Pro 1 had no activity on the same substrate. Both enzymes hardly hydrolyze gelatin. Many of the previous studies also revealed that alkaline proteases showed highest activity towards casein relative to other proteins including BSA, haemoglobin, keratin and azocasein (Freeman et al., 1993; Rahman et al., 1994). The two alkaline proteases isolated from some Bacillus spp. exhibited very low keratinolytic activity (Kumar et al., 1999). The ability of the Pro 1 and Pro 2 produced by the test organism to hydrolyze a wide range of proteins substrate may be advantageous for its use in detergents against a wide variety of stains.

Effect of metal ions. The effect of different metal ions on the activity of both purified enzymes was also tested. Of all the tested ions Hg$^{2+}$ and Ag$^+$ inhibited the activity of both enzymes by about 50–70%. Many alkaline proteases were reported to be inhibited by mercury and sliver (Banerjee et al., 1999; Beg and Gupta, 2003; Kumar et al., 1999). However, metal ions such as Mn$^{2+}$, Mg$^{2+}$, Co$^{2+}$ and Ca$^{2+}$ increased or stabilized the activity of the two enzymes, confirming that these cations take part in the stabilization of the protease structure and are required for protection against thermal denaturation (Paliwal et al., 1994). Another important feature of the enzymes used in leather industry was the salt tolerant capacities. In the presence of 1 M NaCl, the two enzymes retained 100% of their activities after 60 min preincubation at 40°C (data not shown). It was reported that alkaline protease produced from haloalkaliphilic Bacillus sp. lost about 20% when perincubated with 0.17 M NaCl for 30 min at 37°C (Gupta et al., 2005), while Bacillus sp. JB-99 protease lost only 16% in the presence of 1 M NaCl after 2 h at 45°C (Johnvesly et al., 2002).

Effect of inhibitors, surfactants and detergents. The results obtained in this study revealed that both enzymes were completely inhibited by 2 mM PMSF, a serine protease inhibitor, while 2-mercaptoethanol and dithiotheretiol stimulate the activity suggesting that both are thiol-dependent serine proteases. Similar results were observed for B. mojavensis protease (Beg and Gupta, 2003). The two proteases were found to be very stable against non-ionic surfactants (such as Tween 80 and Triton X-100) and stable in the presence of anionic surfactants, SDS. Matta and Punj (1998) reported that protease from B. polymyxa B-17 lost approximately 10% of its activity on treatment with 1 mM SDS. The enzymes also exhibited strong stability against bleaching agent, hydrogen peroxide and relatively stable in presence of commercial detergents. Singh et al. (2001) reported that SSR1 protease retained 40–90% of its activity in the presence of local detergents. The stability of both enzymes in the presence of EDTA suggesting that metal cofactors are not required for enzyme activity. This property of the enzyme was very useful for application as detergent additive since chelating agents, which function as water softeners and are involved in the removal of stains are components of the detergent and then specifically bind divalent cations (Beg and Gupta, 2003).

From these results, it is envisage that Bacillus sp. MIG can be a potential source of alkaline proteases for use in different industrial application, because it produced the enzymes at a prominent level using cost-effective medium. The broad substrate specificity of the proteases from Bacillus sp. MIG may be also advantageous for its use in detergents against wide variety of stains and the stabilities in the presence of high sodium chloride concentration permit their application in leather industry as an unhairing agent, removing the need to use toxic reagents.

Literature


Novel Yeast Cell Dehydrogenase Activity Assay in situ

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

Abstract

The aim of this research was to develop a suitable method of succinate dehydrogenase activity assay in situ for different industrial yeast strains. For this purpose different compounds: EDTA, Triton X-100, sodium deoxycholate, digitonin, nystatin and β-mercaptoethanol were used. The permeabilization process was controlled microscopically by primuline staining. Enzyme assay was conducted in whole yeast cells with Na-succinate as substrate, phenazine methosulfate (PMS) as electron carrier and in the presence one of two different tetrazolium salts: tetrazolium blue chloride (BT) or cyanoditolyl tetrazolium chloride (CTC) reduced during the assay. In comparable studies of yeast vitality the amount of intracellular ATP was determined according to luciferin/luciferase method. During the succinate dehydrogenase assay in intact yeast cells without permeabilization, BT formazans were partially visualized in the cells, but CTC formazans appeared to be totally extracellular or associated with the plasma membrane. Under these conditions there was no linear relationship between formazan color intensity signal and yeast cell density. From all chemical compounds tested, only digitonin was effective in membrane permeabilization without negative influence on cell morphology. Furthermore, with digitonin-treated cells a linear relationship between formazan color intensity signal and yeast cell number was noticed. Significant decreasing of succinate dehydrogenase activity and ATP content were observed during aging of the tested yeast strains.

Key words: yeasts cells permeabilization, succinate dehydrogenase, formazans

Introduction

Succinate dehydrogenase (SDH) plays a crucial role in the energy supply for physiological activity of every living cell, included microorganisms (Samokhvalov et al., 2004). Therefore SDH activity assay is an important method for measurement of the yeast vitality in scope to control of different fermentation processes.

Reduction of various tetrazolium salts by dehydrogenases of metabolically active cells leads to production of highly colored end products – formazans. Tetrazolium salts commonly used in microbiological applications include: 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Saliola et al., 2004), 2,3,5-triphenyl tetrazolium chloride (TTC) (Rosa and Tsou, 1963), and more recently, new generation tetrazolium salts: 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Bernaś and Dobrucki, 1999), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Berridge et al., 1996; Stowe et al., 1995; Freimoser et al., 1999), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) (Kuhn et al., 2003) or 4-[3-(4-idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST1) (Berridge et al., 1996; Tsukatani et al., 2003).

Enzymatic reactions in yeasts are usually studied in cell-free extracts which requires disruption of cells and as consequence, inactivation of particular enzymes often can be observed. In the case of enzymatic reaction conducted in situ the plasma membrane forms a barrier with low degree of penetration. Therefore, cell permeabilization is recommended as an alternative method for the study of intracellular enzyme activities.
The aim of this research was to develop a suitable method of yeast cells permeabilization in succinate dehydrogenase activity assay in situ which can be used for different industrial Saccharomyces cerevisiae strains.

**Experimental**

**Materials and Methods**

**Yeast strains used in this study.** Five yeast strains Saccharomyces cerevisiae (races: Bc16a, Jaa, Ila, Ja 64 and baker’s industrial strain) used in this study originated from the International Collection LOCK 105 of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź.

**Media and cultivation.** Yeast strains were cultivated in 50 ml of worth broth (Merck) at 30°C for 20 hours or 7 days with shaking at 220 rpm/min.

**Yeast cells permeabilization.** Different compounds: 1% Triton X-100 (Sigma-Aldrich), 0.1% sodium deoxycholate (Sigma-Aldrich), 0.05% digitonin (Fluka), 185 mg/100 ml EDTA (Sigma-Aldrich), 0.39 g/10 ml β-merkaptoetanol (POCh) were used to treated tested yeast strains before the assay. The permeabilization process was controlled microscopically by staining with 0.01% primuline (Sigma-Aldrich) (Duffus et al., 1984).

**Formazans formation.** Two different tetrazolium salts blue chloride BT (Sigma-Aldrich) or cyanoditolyl tetrazolium chloride CTC (Polysciences) were used in the enzyme assay at concentration 0.68 mM and 4 mM respectively and were reduced during assay to form crystals of formazan, which were observed microscopically. BT formazans in the bright-field microscopy were an opaque blue intracellular deposits and the CTC formazans formed red, highly fluorescent crystals with fluorescence emission primarily in the red region, when excitation wavelength was in the region 360–370 nm.

**Succinate dehydrogenase SDH assay.** This enzyme activity was assayed in whole cells. The cells were collected, washed and resuspended in Ringer solution. Standardized cell suspensions with 9×10^7 to 5×10^8 cell/ml were transferred to the tubes and centrifuged (10 min, 2100×g). Supernatants were discarded and 0.3 ml 0.05 M Na-succinate solution (Merck), one small crystal of PMS (Sigma-Aldrich) and 3 ml of solution contained 50 mg BT, 185 mg EDTA, 300 mg sodium azide (Sigma-Aldrich) in 100 ml were added to the biomass. The mixture was incubated for 60 min at 37°C and then the reaction was stopped with 0.4 ml of 37% formaldehyde (POCh) and the samples were centrifuged as before (10 min, 2100×g). Supernatants were discarded and the pellets were resuspended in DMSO (Sigma-Aldrich) for extraction of formazan crystals formed in yeast cells during the assay. Extraction was conducted with three sequenced volumes of 3, 2 and 2 ml DMSO, then extracts were pulled and the final absorbance was measured at 540 nm by spectrophotometer Specol 210 (Carl Zeiss, Jena, Germany). Each experiment was performed in triplicate and each data was the mean of three measurements. One SDH activity unit was equal to 1 µmol of formazan formed by 1×10^8 yeast cells at 37°C in 60 minutes.

**ATP content.** Intracellular ATP content was determined by luciferin/luciferase method using Hy-Lite2 luminometer (Merck) as relative light units (RLU). (De Luca et al., 1979).

**Results**

In microscopic studies for SDH activity assay of yeast cells without permeabilization, BT formazans (BTf) can be seen partially in the cells, but CTC formazans (CTCf) appeared to be totally extracellular or associated with the plasma membrane (Fig. 1 Ia, b; Fig. 2 Ia, b). Under these reaction conditions no simple linear correlation was observed between formazan absorbance and cell density (Fig. 3). Microscopic observations after primuline staining showed that the yeast plasma membrane was the barrier for tetrazolium salt in SDH activity assay in situ. In the case of cell permeabilization, when different active agents were added, such treatment changed the morphology of cells. Only digitonin was effective in membrane permeabilization without negative influence on cell morphology (Fig. 4). After digitonin treatment visible

![Fig. 1. BTf crystals.](Image)

I) outside yeast cells; II) inside yeast cells; a) bright-field microscopy; b) electron microscopy
Yeast dehydrogenase assay in situ

Fig. 2. CTCf crystals.
I) outside yeast cells; II) inside yeast cells; a) bright-field microscopy; b) electron microscopy

Formazan crystals were observed inside the yeast cells but not outside them (Fig. 1 IIa,b; Fig. 2 IIa,b). Good correlation ($R^2 = 0.97$) between BTf absorbance intensity after DMSO extraction and number of cells was seen. Linear correlation was observed in the concentration range of yeast cells from $9 \times 10^7$ to $5 \times 10^8$ per sample. For yeast cell concentrations below $1 \times 10^7$ per sample the formazan color intensity signals were too low to detect with good precision (Fig. 3).

The results obtained for SDH activity were in good agreement with ATP content in yeast cells. Significant decreasing of succinate dehydrogenase activity and ATP content were observed during aging of tested strains (Table I).

![Graph showing yeast cells density and BTf signal](image)

**Table I**

SDH activity and ATP content in different populations of *Saccharomyces cerevisiae* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>20 h culture</th>
<th>7 day culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP content</td>
<td>SDH activity</td>
</tr>
<tr>
<td></td>
<td>[fM/cell]</td>
<td>[µM/10^8 cells]</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Be16a</td>
<td>95.9787</td>
<td>0.028</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Jaa</td>
<td>115.9787</td>
<td>0.0973</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> IIa</td>
<td>1.6037</td>
<td>0.0146</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Ja64</td>
<td>201.7287</td>
<td>0.1069</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> industrial strain</td>
<td>86.1037</td>
<td>0.0631</td>
</tr>
</tbody>
</table>

Correlation factor $R^2 = 0.72$  
Correlation factor $R^2 = 0.97$
The earlier research described in the literature and conducted with different tetrazolium salt and yeast genera showed that colorimetric signal of extracted formazans was not proportional to the cell number (Kuhn et al., 2003; Tsukatani et al., 2003). In our study the plasma membrane was the barrier to study SDH activity in situ. This fact was confirmed by microscopic studies and primuline staining. This may explain the lack of linear relationship between cell number and formazan absorbance in our previous experiments. Gentle permeabilization of yeast cell membrane, increasing its penetrability for tetrazolium salt, appeared to be an important step for BT formazan signal and SDH assay in yeast cells (Caldwell, 1987). In earlier studies on permeabilization, this process was conducted with digitonin at concentration from 0.01% to 0.1% during 10–30 min (Alamäe and Järviste, 1995; Freire et al., 1998; Samokhvalov et al., 2004). For digitonin-treated yeast cells linear relationship between cell number and BTf absorbance was observed.

The reaction with tetrazolium salt can be used in a wide range of biological assays including tests of cell viability. The developed method of SDH assay can be used in the study not only of yeast cell activity but to detect respiring cells in different ecosystems: water, food matrices, activated sludge or biofilms.

Acknowledgements. We gratefully acknowledge financial support of UE 6PR Grant NMP3-CT-2003- 504937 PERCERAMICS

Literature


Yeast dehydrogenase assay in situ


In vitro Activity of Caspofungin against Planktonic and Sessile Candida sp. Cells

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

Abstract

Candida sp. may be regarded as one of the leading etiologic agents of hospital-acquired infections, including those related with the indwelling medical devices, which become colonized by the yeasts, accompanied by biofilm formation. In this paper we assayed in vitro susceptibility to caspofungin of planktonic and sessile cells of nasopharyngeal isolates of Candida sp. Two types of biomaterials were used – silicone elastomer-coated latex urinary Foley catheter and PCV Thorax catheter. The minimal inhibitory concentrations (MIC) of caspofungin for planktonic Candida sp. cells ranged from 0.008 to 0.031 mg/l, while the minimal fungicidal concentrations (MFC) from 0.008 to 0.062 mg/l, with MFC/MIC ratios ≤2. The minimal concentration of caspofungin preventing adhesion process of Candida sp. on both biomaterials ranged from 0.004 to 0.031 mg/l, while preventing biofilm formation from 0.004 to 0.062 mg/l. In contrast, much higher minimal concentrations of caspofungin were needed to eradicate the mature biofilm (0.25 to >8 mg/l). In all cases, drug concentrations depended on the strain and the biomaterial used. Our preliminary data suggest that caspofungin, showing good anti-adherent activity in vitro against Candida sp., appears to be a potential agent rather for prophylaxis of the yeast infections associated with biomaterials but not for their treatment.

Key words: caspofungin, Candida sp., biomaterials, adhesion, biofilm

Introduction

Recently, the yeasts belonging to the genus Candida may be regarded as one of the leading etiologic agents of hospital-acquired infections, including those related with the indwelling medical devices such as catheters, prosthetic joints and heart valves, dentures etc. These devices become colonized by the yeasts, accompanied by biofilm formation (Hawser and Douglas, 1994; Douglas, 2002; Jabra-Rizk et al., 2004). Treatment of candidiasis often presents a challenge for clinicians, since the number of effective antifungal agents at their disposal is limited, due to increasing resistance of the pathogens. In addition, sessile cells of Candida sp. within a biofilm are even more insensitive to current antimicrobial therapy in comparison to their planktonic (free-floating) counterparts. Therefore, the pharmaceutical industry is still working on the discovery of novel drugs which might reinforce conventional antifungal armamentarium (Chandra et al., 2001; Bachmann et al., 2002; Kuhn et al., 2002; Jabra-Rizk et al., 2004).

Caspofungin is the first antifungal compound of a new echinocandin class with a unique mode of action. It inhibits the synthesis of 1,3-β-D-glucan, a key component of the fungal cell wall, that is essential for osmotic stability, cell growth and cell division (Letscher-Bru et al., 2003). This antibiotic should be effective in combating both planktonic and biofilm-associated Candida sp. populations. However, the literature data regarding the effect of caspofungin in vitro on Candida sp. biofilm formation and its eradication are controversial (Bachmann et al., 2002; Kuhn et al., 2002; Ramage et al., 2002; Soustre et al., 2004).

Here we presented data on the in vitro susceptibility to caspofungin of planktonic and sessile cells of nasopharyngeal isolates of Candida sp.
Experimental

Materials and Methods

Microorganisms. A total of 7 clinical isolates of Candida sp. possessing hydrophilic or hydrophobic cell structure were studied. The hydrophobicity of cell surface was assessed using salt aggregation test (Lindahl et al., 1981). The collection included the following isolates: C. albicans (1 hydrophilic and 2 hydrophobic isolates), C. famata (1 hydrophilic and 1 hydrophobic isolates), C. glabrata (1 hydrophobic isolate), C. krusei (1 hydrophilic isolate). The isolates were obtained from nasopharynx of patients with lung cancer undergoing pulmonary resection and were stored on Sabouraud dextrose agar and before each experiment they were subcultured on Sabouraud glucose broth.

Caspofungin. Standard antifungal powder of caspofungin (caspofungin acetate) was examined (Merck & Co., Inc., USA). Stock solution containing 16 mg/ml was prepared in distilled water and was stored frozen at –20°C.

Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of caspofungin. Determination of MIC of caspofungin for planktonic Candida sp. cells was performed by a broth microdilution method in accordance with the guidelines recommended by CLSI (Clinical Laboratory Standards Institute), using serial two-fold dilutions of caspofungin in Sabouraud glucose broth. Final concentrations of caspofungin ranged from 0.0002 to 16 mg/l. Stock inoculum suspensions of yeasts were prepared in Sabouraud medium and adjusted to optical density corresponding with 0.5 Mc Farland standard i.e. 150×10^6 CFU (colony forming units). After 48 h of incubation at 35°C, the MICs were assessed visually as the lowest drug concentration showing complete growth inhibition. In order to determine the MFC of caspofungin for the planktonic cells of the yeasts, 10 µl from each tube that showed thorough growth inhibition, from the last positive one and from the growth control was streaked onto Sabouraud dextrose agar plates. After 48 h of incubation at 35°C, the MFCs were assessed visually as the lowest drug concentration at which there was no growth. All experiments were done in triplicates. The representative data are presented.

Biomaterials. All assays were carried out on two types of catheters that differed in unevenness of surface from each other – silicone elastomer-coated latex urinary Foley catheter and PCV Thorax catheter.

The effect of caspofungin on adhesion of Candida sp. and biofilm formation on the biomaterials. The adhesion process and biofilm formation were determined by using the MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay (Levitz et al., 1985). The catheters used were cut aseptically into 0.5 cm² fragments and placed into Petri dishes. The standardized yeast suspensions (optical density of 0.5 Mc Farland standard) were prepared. Various concentrations of caspofungin (0.004–8 mg/l) were used. (i) In order to assay the effect of caspofungin on adhesion process, the yeast suspensions in sterile PBS (phosphate-buffered saline) containing various concentrations of caspofungin were incubated with biomaterials for 1 h at 35°C. Nonadherent cells were removed by careful rinsing catheter discs with sterile PBS and then resuspended in Sabouraud glucose broth, followed by overnight incubation at 35°C. (ii) In order to assay the effect of caspofungin on biofilm formation, the yeast suspensions in Sabouraud glucose broth containing various concentrations of caspofungin were incubated with biomaterials for 24 h at 35°C. Nonadherent cells were removed by careful rinsing catheter discs with sterile PBS and then resuspended in fresh Sabouraud glucose broth. Medium changing and catheters washing procedures after overnight incubation at 35°C were repeated thrice (total incubation period lasted 72 h). (iii) In order to assay the effect of caspofungin on biofilm eradication, the mature biofilms were incubated in the presence of various concentrations of caspofungin for 24 h. In all assays a drop of 1% MTT solution was added to each dish. After incubation for 24 h at 35°C, in the presence of Candida sp. viable cells tetrazolium salt was reduced to the violet tetrazolium formazan product, accompanied by violet colour of the medium. In each experiment control free-drug assays were carried out. All experiments were done in triplicates. The representative data are presented.

Results

The MICs of caspofungin for planktonic Candida sp. cells ranged from 0.008 to 0.031 mg/l (Table I). Paradoxical turbidity at the highest drug concentrations, imitating fungal growth was observed for 3 isolates. However, this so-called “eagle” effect was ignored for the MIC determinations according to the literature data (Bartizal and Odds, 2003; Stevens et al., 2004). The MFCs of caspofungin for planktonic Candida sp. cells was 0.008–0.062 mg/l (Table I). For 6 strains tested MFC/MIC ratios were = 2, while for one strain MFC/MIC = 1. It is worth notifying that the turbidity observed at the highest drug concentrations during the MIC readings was accompanied by the lack of fungicidal effect of caspofungin, which was revealed during MFC determinations.

All assayed Candida sp. isolates were able to adhere both to Thorax and Foley catheters, following by biofilm formation on both biomaterials. This was monitored by formation of violet tetrazolium formazan product inside and outside of the catheters and violet coloured medium after addition of MTT solution. The structure of the biofilm of hydrophobic strain of C. albicans on Thorax catheter was presented in Fig. 1.
Capsofungin activity against *Candida* sp.

The minimal concentration of caspofungin preventing adhesion process on both biomaterials ranged from 0.004 to 0.031 mg/l (Table II). The above data indicate that caspofungin significantly inhibited *in vitro* the adherence capacity of the yeasts to the biomaterials assayed at concentrations ranged from 0.5×MIC or 1×MIC.

The minimal concentration of caspofungin preventing biofilm formation ranged from 0.004 to 0.062 mg/l. In contrast, much higher minimal concentrations of caspofungin (0.25 to >8 mg/l) were needed to eradicate the mature biofilm of *Candida* sp., depending also on the strain and the biomaterial used (Table III). The total eradication of *Candida* sp. biofilms with the drug concentrations below 8 mg/l was obtained for 2 strains only (Table III). The above data indicate that caspofungin significantly inhibited *in vitro* the biofilm formation capacity of the yeasts to the biomaterials assayed at concentrations ranged from 0.5×MIC to 2×MIC, while eradication of the mature biofilm required concentrations from 4×MIC to >1032×MIC.

Generally, using the Thorax catheter higher concentrations of caspofungin were needed in order to achieve the same effects on adhesion process of *Candida* sp. and the yeast biofilm formation as were obtained with the Foley catheter (Table II and III).

**Discussion**

*Candida* sp. infections can be regarded as an important medical problem, especially in the immunocompromised patients, e.g. in lung cancer patients. Additionally, according to our unpublished data, *Candida* sp. isolates have shown to possess a potential ability to colonize pleural drains in patients undergoing pulmonary resection. Although *C. albicans* remains a predominant etiologic agent of candidiasis, other species that tend to be less susceptible to the commonly used antifungal agents such as *C. krusei*, *C. glabrata* or *C. famata* have emerged as opportunistic pathogens (Chandra *et al*., 2001; Jabra-Rizk *et al*., 2004).

The results presented in this paper confirmed the good inhibitory activity of caspofungin at low concentrations against planktonic cells of *Candida* sp., observed also by other authors (Bachmann *et al*., 2002;

<table>
<thead>
<tr>
<th>Strain</th>
<th>Minimal concentration preventing adhesion process (mg/l)</th>
<th>Minimal concentration eradicating biofilm (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thorax catheter</td>
<td>Foley catheter</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrophilic*</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>hydrophobic</td>
<td>0.031</td>
<td>0.031</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrophilic*</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td><em>C. famata</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrophilic*</td>
<td>0.008</td>
<td>0.015</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrophilic</td>
<td>0.015</td>
<td>0.015</td>
</tr>
<tr>
<td><em>C. krusei</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hydrophilic</td>
<td>0.015</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* In these cases the “eagle” effect was observed.
Ramage et al., 2002; Bartizal and Odds, 2003; Stevens et al., 2004). The MICs and MFCs determined for the nasopharyngeal Candida sp. isolates were lower than those reported in the literature (Bartizal et al., 2003; Stevens et al., 2004). However, such discrepancies are possible since the type of medium used in antifungal susceptibility testing is of the utmost importance. The standard medium for testing drug susceptibility of Candida sp. was RPMI 1640 medium, whereas Sabouraud glucose broth was used for studies on Candida sp. biofilm formation (Chryssanthou and Cuenca-Estrella, 2002; Bartizal et al., 2003). The MFC/MIC ratio, not higher than 2 for the tested strains, confirmed the excellent in vitro fungicidal activity of caspofungin against planktonic cells of Candida sp.

Our data demonstrating in vitro anti-adherent activity of caspofungin are in accordance with those from literature (Soustre et al., 2004). The drug concentrations as low as the 0.5×MIC and 1×MIC were sufficient to deprive biofilm-precursor planktonic cells of Candida sp. of adherence capacity and ability to form a mature biofilm. It’s worth mentioning that these values are within the range of mean through levels of caspofungin in serum in humans after standard dosing – 1–2 mg/l (Letscher-Bru and Herbrecht, 2003). As shown by other authors (Bachmann et al., 2002; Kuhn et al., 2002; Ramage et al., 2002; Soustre et al., 2004), exposure of planktonic Candida sp. cells even to subinhibitory concentrations of caspofungin inhibited adhesion process and subsequent biofilm formation. On the other hand, all examined strains of Candida sp. became much more resistant to caspofungin when adhered to the biomaterials and embedded in the mature biofilm. Eradication of sessile Candida sp. populations required much higher concentrations of the antibiotic in comparison with their free-floating counterparts. Analogous investigations also have shown that the complete sterility of the mature Candida sp. biofilms as well as the thorough eradication of adherent cells was hard to attain (Bachmann et al., 2002; Ramage et al., 2002). Nevertheless, a significant reduction in metabolic activity in caspofungin-treated sessile yeast cells was detected by other authors (Bachmann et al., 2002; Ramage et al., 2002; Soustre et al., 2004). In addition, caspofungin-treated yeast biofilms appeared to be less hyphal and showed minor defects in the overall biofilm architecture (Bachmann et al., 2002; Ramage et al., 2002; Soustre et al., 2004). However, Ramage et al. (2002) found that caspofungin killed >99% of sessile cells of some strains of C. albicans within the mature biofilm at therapeutically attainable concentrations (0.125 mg/l and 1 mg/l). The highest concentration of caspofungin (8 mg/l) was less efficacious due to paradoxical lack of fungicidal effect of caspofungin at this concentration. Therefore, in studies on the effect of caspofungin on removal of Candida sp. cells adhered to the biomaterials or embedded in the biofilm, the “eagle” effect observed in case of some yeast strains should be taken into account (Bartizal and Odds, 2003; Stevens et al., 2004).

The observed in this paper differences in antifungal activity of caspofungin against catheter-associated cells of Candida sp. depended on the type of biomaterial used. Using the Thorax catheter higher concentrations of the drug were needed in order to achieve the same outcomes as were obtained with the Foley catheter.

To conclude, the controversial literature data (Bachmann et al., 2002; Kuhn et al., 2002; Ramage et al., 2002; Soustre et al., 2004) and our studies concerning in vitro effect of caspofungin against sessile Candida sp. cells suggest that this antibiotic, showing good anti-adherent activity in vitro, appears to be a potentially effective agent rather for prophylaxis of the yeast infections associated with biomaterials but not for their treatment.

Acknowledgments. The part of this work was supported by a grant from European Social Found (Agreement No Z/2.06/II/2.6/09/04/U/06/04).

Literature


Capsofungin activity against Candida sp.


Enhancement of Oil Degradation by Co-culture of Hydrocarbon Degrading and Biosurfactant Producing Bacteria

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Abstract

In this study the biodegradation of oil by hydrocarbon degrading Pseudomonas putida in the presence of a biosurfactant-producing bacterium was investigated. The co-culture of test organisms exhibited improved degradation capacities, in a reproducible fashion, in aqueous and soil matrix in comparison to the individual bacterium culture. Results indicate that the in situ biosurfactant production not only resulted in increased emulsification of the oil but also change the adhesion of the hydrocarbon to cell surface of other bacterium. The understanding of interactions between microbes may provide opportunities to further enhancement of contaminants biodegradation by making a suitable blend for bioaugmentation.

Keywords: biodegradation, biosurfactant, co-culture, BATH assay, bioaugmentation

Introduction

Mass transfer from sorbed or insoluble phases is considered to be the rate-limiting step in biodegradation of organic contaminants because the compounds must be released to the aqueous phase prior to entering the microbial cell and subsequent intracellular transformation by the necessary catabolic enzymes (Dean et al., 2001). The availability of slightly soluble organic compounds can be enhanced by biosurfactants. Biosurfactants can enhance biodegradation by (i) increasing available substrate surface area (dispersion) and (ii) improving affinity of microbial cells for the substrate (Ito and Inoue, 1982; Zhang and Miller, 1994; Rahman et al., 2003; Chang et al., 2004; Bonilla et al., 2005). However, effect of biosurfactant on degradation is less straightforward. There are also evidences that biosurfactants may interfere with the interaction between biosurfactant-dispersed substrates and microbial cells resulting in decreased biodegradation (Falatko and Novak, 1992).

There are several reports on improved hydrocarbon degradation by addition of biosurfactant or chemical surfactant (Boonchan et al., 1998; Stelmack et al., 1999; Noordman et al., 2002; Rahman et al., 2003; Kuyukina et al., 2005). However, the effect of in situ biosurfactant production by co-culture of the biosurfactant-producing and hydrocarbon degrading bacteria on hydrocarbon degradation is relatively less studied (Dean et al., 2001; Van Hamme and Ward, 2001). For field bioremediation application based on bioaugmentation, addition of the biosurfactant-producing bacteria may be beneficial and more practical than exogenously adding purified biosurfactant. Bacterial strain designated as DHT-GL isolated in our laboratory from hydrocarbon contaminated soil, forms excellent emulsions between 24 and 48 h of incubation with wide the range of hydrocarbon while other strain, 5a1, efficiently degrades pure hydrocarbons in aqueous system. We hypothesized that a co-culture of the two organisms will enhance hydrocarbon degradation through the combination of superior emulsification and degradation capabilities. In this study, we used simple pure cultures and co-culture to study metabolic and physiological interactions that may occur in...
a mixed-culture fermentation system and in soil matrix. An understanding of these interactions is necessary when developing treatment techniques for hydrocarbon biodegradation.

The goal of this study was to determine if biodegradation of the oil could be accelerated through bioaugmentation with hydrocarbon degrading and biosurfactant-producing bacteria. Batch aqueous and soil degradation experiments using diesel oil as representative complex hydrocarbon, were conducted involving inoculation with hydrocarbon degrading and biosurfactant producing bacterial strains separately or in combinations.

Experimental

Materials and Methods

Enrichment and isolation of bacteria. The bacterial strains were isolated by the enrichment culture technique from the soil obtained from Guanaco Asphalt Belt, Venezuela. 5 g of soil was inoculated into 100 ml of minimal salt medium (MSM) containing (g/l) Na₂HPO₄ – 6.0; KH₂PO₄ – 3.0; NH₄Cl – 1.0; NaCl – 0.5; MgSO₄ – 0.1 and 2.5 ml of trace element solution (pH 7.0). Trace elements solution contained (mg/l) MnCl₂ × 2H₂O – 23; MgCl₂ × 6H₂O – 30; H₂BO₃ – 31; CoCl₂ × 6H₂O – 36; CuCl₂ × 2H₂O – 10; NiCl₂ × 6H₂O – 20; Na₂MoO₄ × 2H₂O – 30 and ZnCl₂ – 50. Crude oil (5% w/v) was used as carbon source and incubated at 30°C or 60°C on a rotary shaker (200 rpm) for 4 days. After five cycles of such enrichment, 1 ml of the culture was diluted and plated on MSM agar (2% w/v) plates containing crude oil (5% w/v) as sole carbon source and incubated at 30°C or 60°C. The bacterial colonies obtained were further purified on Luria-Bertani (LB) agar plates. The isolated strains were stored as frozen stock cultures at –70°C in 25% glycerol.

Plasmid curing and mutagenesis. Plasmid DNA was isolated by the method of Hansen and Olsen (1978). Ethidium bromide was used as plasmid curing agent (Guerry and Colwell,1977). Random mutagenesis using N-methyl-N'-nitro-N-nitrosoguanidine was carried out according to Tahzibi et al. (2004) to isolate bacteria with lost ability to produce biosurfactant.

Growth on hydrocarbons: Growth in the presence of hydrocarbons was studied using liquid minimal salt medium containing 0.1 g of substrate/liter. Erlenmeyer flasks (250 ml) containing 50 ml of MSM and the hydrocarbon were inoculated with 100 µl Luria-Bertani (LB) broth grown pre-culture and incubated at 30°C and 200 rpm. Growth was followed by visually observing the increase in cell density in comparison to un-inoculated control with respective carbon source. When no difference was noticed in the turbidity of the flask and that of the control, it was taken as no growth (–), where slight increase in turbidity was noticed it was taken as poor growth (+). Significant increase in turbidity was taken as good growth (++) while luxuriant growth and increases in turbidity was stated as very good growth (+++).

The solid polycyclic aromatic hydrocarbons (PAHs) (except naphthalene) were dissolved in 5% (w/v) diethyl ether and sprayed on surface of MSM agar. Naphthalene was provided as crystals directly placed on the plate lid. Growth on PAHs in solid media was considered positive by the formation of clear zone around the growing colonies or appearance of pigments.

Biosurfactant production: To study biosurfactant production and activity, bacteria were either grown in YPG medium (g/l): Yeast extract – 5; Peptone – 5; Glucose – 15) or in MSM containing 3% (w/v) glucose and/or 2% (w/v) hexadecane. The cultures were incubated at 30°C and 150 rpm. After 24 h the culture broth was centrifuged at 8000 rpm for 10 min and the supernatant was used for measurement of surface-active properties. The surface tension of the biosurfactant was measured by the Ring method using a CSC-DuNouy Tensiometer at room temperature. Drop test and oil spread test was carried out according to Youssef et al. (2004). The emulsification activity (E₂₄) was determined by the addition of the respective hydrocarbon (kerosene, gasoline, diesel oil, gas oil, hexadecane, and alpha methyl naphthalene) to the same volume of cell free culture broth, mixed with a vortex for 2 minutes and left to stand for 24 h. The emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm). To study the stability of emulsion the emulsified solutions were allowed to stand at 60°C and emulsification index was analyzed at different time intervals. Surface active compounds were extracted by liquid – liquid extraction from cell free culture broth acidified with 1N HCl to pH 2.0 (Rahman et al., 2003). Supernatant fluid was mixed with an equal volume of a chloroform: methanol (2:1) mixture. The solvent was evaporated and the material was used as crude biosurfactant and weighted to evaluate the yield. Determination of the carbohydrate content of the partially purified biosurfactant was done by anthrone reagent method at 620 nm (Spiro, 1966). Protein was assayed by the Bradford (1976) method using bovine serum albumin as standard. Lipid was analysed as described by Ilori and Amund (2001).

16S rDNA partial gene sequencing and bacteria identification. Bacterium total DNA was isolated according to Chen and Kuo (1993). Partial gene sequence coding for 16S rRNA was amplified by PCR using universal primers U1 (5’-CCA GCA GCC GGC GTA ATA CG-3’) corresponding to nucleotide positions 518 to 537 (forward primer) and U2 (5’-ATC GG(T/C) TAC CTT GTT ACG ACT TC-3’) corresponding to nucleotide positions 1513 to 1491 (reverse primer) according to the Escherichia coli numbering system (Weisburg et al., 1991). The PCR were performed as described by Lu et al. (2000) using A PCT-100TM thermal cycler unit (MJ Research, Inc. USA). The PCR product was separated by agarose gel electrophoresis and visualized by SYBR Green I staining (Sigma, St. Louis, USA) and finally purified by using a Wizard PCR Preps Purification System (Promega Corp., USA) according to manufacturer’s instructions. DNA sequencing the reaction was performed with an ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and the sequencing products were separated by capillary electrophoresis by using a 310 Sequencer (Perkin-Elmer Corp., Applied Biosystems, USA) according to standard procedures. Sequence data were analyzed with DNAMAN version 5.2.9 (Lynnon BioSoft, USA) to obtain a consensus sequence. To identify the isolated bacterial strain, the 16S rDNA consensus sequence was compared with 16S rRNA gene sequences from the public GenBank, EMBL, and DDBJ databases using the advanced gapped n-BLAST program, version 2.1. The program was run via Internet through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast/). Sequences with more than 98% identity with a GenBank sequence were considered to be of the same species as the highest score-matching sequence on the public sequence databases.
Degradation of oil in aqueous medium. Standard diesel oil, obtained directly from Petróleos de Venezuela (PDVSA) gas station in Caracas, Venezuela, was used as a representative complex hydrocarbon mixture for biodegradation studies. The selected bacterial isolates of *Pseudomonas putida* strain 5a1 and *Pseudomonas aeruginosa* strain DHT-GL were inoculated into 100 ml sterile nutrient broth. The flasks were incubated and shaken at 200 rpm, for 12 h at 30°C. One ml volumes from each broth culture were mixed to prepare mixed bacterial consortium. Individual bacterial cultures and bacterial consortium (1%) were transferred to 250 ml conical flasks, each containing 100 ml of sterile defined mineral salts medium with 5% diesel (v/v). An uninoculated control was also studied. The influence of crude biosurfactant(s) produced by strain DHT-GL was evaluated by adding 500 mg/l crude biosurfactant in culture medium inoculated with strain 5a1. The flasks were incubated at 30°C and shaken at 200 rpm for three weeks. At 2-day intervals, a set of flasks was used for the enumeration of microbial population and estimation of diesel content. To extract the diesel at the timed interval, the culture broth was acidified to pH 3.0 using the 6 N HCl and was amended with 20% NaCl. The resulting solution was extracted with equal volume of ethyl acetate and extracted hydrocarbon concentration was determined according to Rahman et al. (2002). A standard curve plotted using known concentration of diesel was used to estimate the amount of hydrocarbons. Degradation was estimated as difference between the initial and final concentration of the oil content in the medium. Growth of the bacterial strains was verified by demonstrating an increase in bacterial protein concentration or increase in number of colony forming units (CFU) concomitant with a decrease in the diesel concentration.

Degradation of oil in soil medium. For diesel oil degradation tests, soil was obtained from the Campus of IDEA, Caracas, Venezuela. To equalize soil particles, the soil was air-dried, passed through sieves, oven dried at 80°C for 24 hours and steam autoclaved (3 times at 121°C, 15 lbs, 15 min). Fifty grams of soil, 10 ml of the culture (separately and in combinations), 0.25% (w/w) yeast extract and 5% (v/v) standard diesel were mixed, sealed and cultivated in incubator at 30°C. An uninoculated control was also incubated under same conditions. To extract the total petroleum hydrocarbons (TPH), 10 g of soil was twice extracted with ethyl acetate (2×20 ml) and analyzed for hydrocarbon according to Rahman et al. (2002) at different time intervals. Extract was dried at room temperature by evaporation of solvents in a fume hood. After evaporation, the amount of residual TPH recovered was also determined gravimetrically.

Analysis of cell surface hydrophobicity. The bacterial adhesion to hydrocarbons (BATH) assay was used in order to test the hydrophobicity of the two strains (Rosenberg and Rosenberg, 1985). Bacterial cells were harvested from growth culture by centrifugation at 8000×g for 10 min at 4°C and washed twice. The cells were then resuspended in a buffer salts solution (pH 7.0) containing (g/l) 16.9 of K,HPO₄, 7.3 of KH₂PO₄, 1.8 of urea, and 0.2 g MgSO₄ × 7H₂O to give an optical density (OD) at 600 nm = 1.0. Cells (4.0 ml) and hexadecane (1.0 ml) were mixed in a screw-top test tube and the test tube was vortexed for 1 min. After vortexing, the hexadecane and aqueous phases were allowed to separate for 45 min. The aqueous phase was then carefully removed with a Pasteur pipette, and the turbidity of the aqueous phase at 600 nm was measured. Hydrophobicity is expressed as the percentage of adherence to hexadecane, which is calculated as follows: 100 x (1 – OD of the aqueous phase/OD of the cell suspension).

Results

Isolation and identification of bacteria. From the enrichment culture established at 30°C several bacterial strains were obtained. Essentially on the basis of its ability to grow on various hydrocarbons, strain 5a1 was selected for further studies. Like wise from the enrichment at 60°C, one strain DHT-GL, was selected for further study because of its ability to produce efficient biosurfactant and growth at wide temperature range (manuscript under preparation). Taxonomical identification of these organism, designated as strain 5a1 and strain DHT-GL, was performed by amplification and sequencing the 16S rRNA genes and comparing them to the database of known 16S RNA sequences. Alignment of the 16S rRNA gene sequences of strain 5a1 with sequences obtained by doing a Blast searching revealed maximum similarity to *Pseudomonas putida*, while the strain DHT-GL resembles *Pseudomonas aeruginosa*.

Growth on hydrocarbons. Bacterial strains were tested for its ability to grow on variety of carbon source including various low and high molecular weight PAHs as well as other simple aromatic hydrocarbons and n-alkanes (Table I). These chemicals represent the most common organic pollutants and are the main components of crude oils. Strain 5a1 grows very efficiently on diverse hydrocarbons and within 24 hours maximal growth was observed. In comparison strain DHT-GL grows slow and it takes around 24 hours to show the visible growth in liquid and solid media. When colonies were grown on PAHs coated agar plates, zone of the clearing appeared, indicating PAH degradation. In the presence of dibenzothiophene on plate as well as in liquid media bacterium 5a1 produced orange or reddish brown product(s) (Kodama et al., 1973), while no pigmentation was observed in the case of strain DHT-GL. Beside that 5a1 can also grow in the model crude oil containing 200 ppm of each: pyrene, dibenzothiophene, phenanthrene, hydroxyquinolone and 50 ppm of hexadecane dissolved in alpha-methyl-naphthalene (data not shown). Results shows that strain 5a1 can effectively grow in wide range of the pure hydrocarbons but grew poorly in diesel. In comparison the strain DHT-GL grew in hydrocarbon less effectively. The cured strain of 5a1 lost its ability to grow on PAHs and alkanes used in this study. This indicates that the hydrocarbon degrading ability of the 5a1 was plasmid mediated. There are several reports describing plasmid mediated hydrocarbon degradation (van Hamme et al., 2003; Plaza et al., 2005)

Biosurfactant production. In contrary to 5a1 the strains DHT-GL give positive result in drop collapse and oil spreading test. These qualitative test are indicative of the surface and wetting activities (Youssef et al.,
The strain DHT-GL was capable of the surface reduction on YPG media from 54.9 to 30.4 dN/cm in comparison to 49.4 dN/cm by strain 5a1 after 96 hours of growth. Interestingly, most of the surface activity of the strain DHT-GL was confined to the culture supernatant, while almost no significant potential of surface activity was observed for the cells. Moreover, no significant effect was observed on the activity of the extracellular biosurfactant when it was autoclaved. Emulsification activities of the culture supernatant were measured with several water immiscible substrates. The results show that culture supernatant has high emulsification activities against diesel oil (78 ± 4%), kerosene (74 ± 3%), gas oil (76 ± 5%) and gasoline (62 ± 5%). The emulsification activity for the mixture of n-hexadecane and 2-methylnaphthalene (100%) was better than that for hexadecane (82 ± 4%) alone and almost the same as of the 2-methylnaphthalene (80 ± 5%) alone. These findings are in agreement with the results of Kalpan and Rosenberg (1982) who reported that a mixture of aliphatic and aromatic hydrocarbons was required for maximum emulsion formation. Similar finding were obtained when the culture supernatant obtained from growth of strain DHT-GL in MSM containing glucose and/or hexadecane as carbon source, was analyzed for various tests related to surface activity (data not shown). No significant change in emulsification index was noticed up to 48 h when the emulsion was in 60°C (data not shown). Highest emulsification activity and biosurfactant production (10.05 g/l) was obtained in YPG after 96 hours of growth. No protein was detected in partially purified biosurfactant, which however contained lipid and carbohydrate, therefore putatively was classified as a glycolipid. Pseudomonas aeruginosa is well known to produce biosurfactant of glycolipid type. Several types of the rhamnolipids biosurfactant produced by *P. aeruginosa* are well characterized and studied (Banat et al., 2000).

The function of biosurfactant for microbial cell is not fully understood. However, there has been speculation about their involvement in emulsification of water-insoluble substrates. Direct contact of cells with hydrocarbon droplets and their interaction with emulsified droplets has been described. The biosurfactant-mediated solubilization of the hydrocarbon can be helpful for bacteria to increase bioavailability by enhancing the solubility of the water insoluble compounds (Koch et al., 1991; Carrillo et al., 1996; Wei et al., 2005).

### Synergistic effects of co-culture on oil emulsification

We observed the physical state of oil in shake flasks containing strain 5a1, DHT-GL, or a co-culture of the two. The strain DHT-GL culture passed through a stage (24 to 48 h) where emulsification occurred and the co-culture produced a similar but more stable emulsion. The lack of emulsification in case of strain 5a1 was noticed. Emulsification activity is an indicator used extensively to quantify biosurfactant produced by bacteria (Rahman et al., 2003). We detected bioemulsifiers with a kerosene-water emulsification assay during the growth of the individual bacteria and their co-culture in degradation assay (Fig. 1). Detection of biosurfactant in strain DHL-GH occurred for the first time in 2nd day showing E$_{24}$ 26 ± 2% for culture supernatant. The peak of biosurfactant production was obtained on 7th day with E$_{24}$ value 74 ± 3%. After one-week cultures on diesel of strain 5a1 had an emulsification value of 14 ± 1%. In case of co-culture detection of biosurfactant occurred for the first time in 2nd day showing E$_{24}$ 30 ± 2% and the peak of biosurfactant production was obtained on 8th day with E$_{24}$ value 79 ± 4%. This indicates that strain DHT-GL produced biosurfactants in co-culture condition. The addition of crude biosurfactant caused stable emulsion formation after few hours of shaking (data not shown).

### Oil degradation by co-culture and biosurfactant addition

The effect of biosurfactant producing bacteria on biodegradation of diesel oil by strain 5a1 was investigated in aqueous and soil system. Figure 2A depicts the time dependent increase in protein content and concomitant decrease in the hydrocarbon content
Oil degradation by co-cultures of bacteria

in aqueous system while Figure 2B shows the diesel degradation in the soil. It showed that co-culture enhanced the degradation of diesel than compared with the individual strain. The maximum extent of degradation by strain 5a1, DHT-GL and co-culture is 44 ± 2%, 24 ± 1% and 92 ± 3%, respectively, after 20 days in liquid media while this was 38 ± 2%, 20 ± 1% and 80 ± 4% in case of soil matrix. Co-inoculation with the biosurfactant-producing bacterium (P. aeruginosa strain DHT-GL) in soil matrix enhanced both the rate and the extent of diesel degradation by P. putida strain 5a1. After 20 days of incubation the total degradation by the co-culture was higher than the arithmetic sum of the degree of degradation by individual microbe. When the crude biosurfactant was added with strain 5a1 in aqueous medium, after 20 days around 85 ± 2% degradation in diesel oil content was observed. However, no significant impact of biosurfactant addition was observed in case of strain DHT-GL. A primary goal for conducting parallel experiments with exogenously
added biosurfactant was to check the hypothesis where *P. aeruginosa* strain DHT-GL would be releasing biosurfactant in co-inoculated experiments. The degradation patterns in aqueous co-inoculated experiments was reproduced by adding biosurfactant suggesting that biosurfactant produced by strain DHT-GL was responsible for the enhanced diesel degradation.

When the strain DHT-GL was co-inoculated with the cured strain of the strain 5a1, we could not find any significant enhancement in the degradation (27 ± 2%) but we could get E24 69 ± 2%. This indicates that possible interaction of two microbes is responsible for the enhancement of the degradation and strain 5a1 plays important metabolic role in the degradation of the diesel. When the mutant strain of the DHT-GL unable to produce biosurfactant was co-inoculated with strains 5a1, no enhancement of degradation as well as poor emulsification was observed, indicating possible role of the biosurfactant production in enhancement of degradation. This was further supported by enhancement of diesel degradation by the addition of crude biosurfactant. Abalos *et al.* (2004) also reported that addition of rhamnolipids produced by *Pseudomonas aeruginosa* AT10 accelerated the biodegradation of total petroleum hydrocarbons from 32% to 61% at 10 days of incubation.

**Analysis of cell surface hydrophobicity.** The strain DHT-GL has higher cell hydrophobicity (55%) than the 5a1 (29%). When both grown in co-culture the hydrophobicity of the mix culture increased to 68%, what is more than the hydrophobicity of the individual bacterium. This indicates that biosurfactant produced by the DHT-GL cause alteration in the cell hydrophobicity and increased cell affinity for hydrophobic substrate. Strain 5a1 cells growing in presence of hexadecane (2%) and glucose (2%) was collected by centrifugation, incubated (1% w/v) with the supernatant of strain DHT-GL (hexadecane and glucose as carbon source) for 4 hours in shaking condition and then BATH assay was carried out. We found enhanced hydrophobicities (around 29% increased) in the treated cells in comparison to the untreated cells. But when DHT-GL was incubated with 5a1 supernatant only little (7%) improvement was noticed. Similar finding was observed when the cells were incubated with crude biosurfactant (data not shown) further indicating that the biosurfactant not only increased bioavailability of hydrocarbon by emulsifying the hydrocarbon but also increased cell affinity for hydrophobic substrate by altering the cell surface. Wouter and Dick (2002) found increased biodegradation of long-chains alkanes by addition of rhamnolipids, probably due to increased cell surface hydrophobicity of cellular envelope by rhamnolipids. Plaza *et al.*, 2005 isolated two bacteria from oil contaminated soil showing adhesion to hydrocarbon and bioemulsifiers production.

**Discussion**

The co-culture of test organisms, strain DHT-GL and 5a1 and addition of crude biosurfactant exhibited improved degradation capacities in both soil and aqueous systems in a reproducible fashion. The emulsification studies shows that the *Pseudomonas aeruginosa* produces the biosurfactant under experimental condition. It is likely that the surfactant in some way enhanced cell-cell interactions or change the surface properties of each other or increased bioavailability which resulted in an increased rate and extent of diesel degradation. Lower diesel oil degradation in microcosms inoculated with cured strain of 5a1, suggested that the biosurfactant-producing strain contributed to diesel oil degradation through an indirect mechanism rather than degrading diesel directly and the strain 5a1 play important role in the degradation. The results of this study, although preliminary, illustrate species-specific commensal interactions between contaminant-degrading and surfactant-producing bacteria resulting in the overall enhancement of diesel degradation. Enhancement of diesel oil degradation by addition of crude biosurfactant ruled out the fact that the improved diesel degradation was due to synergistic catabolic routes in the two test strains rather than due to enhanced cell-cell interactions or increased diesel bioavailability. The overall impact of addition of a surfactant on biodegradation depends on how the basic diffusion pathways are altered and whether the surfactant itself affects the cells. If the surfactant is neither toxic nor a growth substrate, it can either increase the rate of biodegradation by carrying hydrocarbons in relatively accessible micelles or it can decrease the rate by inhibiting the adhesion of cells to the hydrocarbon-water interface. The overall impact depends on the importance of each pathway. The presence of surfactants at concentrations above the critical micelle concentration does inhibit adhesion of bacteria to the surfaces of droplets of liquid hydrocarbons and thus inhibits biodegradation (Ortega-Clavo and Alexender, 1994). Surfactant can dissolve hydrocarbon by forming the micelles into aqueous solution (Rosenberg and Rosenberg, 1981). Direct interactions between cells and micelles can occur. A number of species of bacteria are able to degrade liquid hydrocarbons after adhering to the surfaces of droplets (Dahlback *et al.*, 1981; Rosenberg and Rosenberg, 1985; Malachowsky *et al.*, 1994). This direct
contact between a bacterial cell and a target hydrocarbon can significantly increase the rate of diffusion into the cell, thereby enhancing growth and increasing the apparent rate of dissolution of the hydrocarbon. Surfactants are known to change the surface property of the hydrocarbon degrading bacteria and resulting in altered degradation of the contaminant (Neu, 1996; Stelmack et al., 1999; Zinjarde and Pant, 2002). The sorption of surfactants to bacteria and to interfaces can either enhance or inhibit adhesion, depending on the nature of the surfaces and the surfactant itself. Recently, Calfee et al., 2005 demonstrated importance of a bacterial surfactant in the solubilization and bioactivity of a cell-to-cell signal. The alterations of surfaces depend only on the concentration of free surfactant. Results from the present study indicates that the biosurfactant produced by one bacteria not only emulsify the oil but also change the adhesion of the hydrocarbon to cell surface and result in the increased degradation of the diesel oil.

**Literature**


Abstract

The paper presents the activity of anaerobic bacterial communities isolated from soil polluted by aircraft fuel on distillery decoctions with phosphogypsum. The microorganisms were selected using the microcosms method, and then enriched on Postgate medium with ethanol. The isolated communities became the inoculum to establish a culture on potato and rye distillery decoctions. The obtained results show that a simultaneous removal of two industrial wastes such as phosphogypsum and distillery decoctions is possible. The introduction of an inoculation comprising a selected anaerobic bacterial community into the culture does not influence the increase of the biotransformation process efficiency.

Key words: biotransformation, sulphate reducing bacteria, distillery decoctions

Introduction

Phosphogypsum represents industrial waste originated during the production of phosphoric acid from apatites or phosphorites. Phosphogypsum is composed mainly of gypsum (CaSO$_4$·2H$_2$O), accompanied by bassanite (CaSO$_4$·0.5H$_2$O). The mineralogical characteristic of phosphogypsum was presented by Kowalski et al. (1990). Phosphogypsum, containing about 50% sulphates, undergoes biotransformation in cultures of sulphate reducing bacteria (Przytocka-Jusiak et al., 1995). Sulphate reducing bacteria (SRB) are heterotrophic and absolute anaerobes, using sulphates as well as other oxidised sulphur compounds (sulphites, tiosulphites, tritronians, tertrationians, elementary sulphur) as the final electron acceptors in respiration processes (Postgate, 1984; Gibson, 1990).

Equally hazardous wastes as phosphogypsum are fluid organic wastes, e.g. distillery decoctions. They originate during the production of ethyl alcohol from rye and potatoes. In order to be applied, as the fluid phase to dissolve phosphogypsum, fluid organic wastes should contain organic compounds available for the SRB; nitrogen and a low content of sulphates. The main organic components of decoctions are proteins, pectines, cellulose and fibres (Mayer and Hillebrandt, 1997). They represent a group of organic compounds rather poorly available for the SRB due to the fact that the sulphate reducing bacteria do not produce hydrolytic enzymes. At present the only one species of SRB, Archeoglobus fulgidus is known to take part in the hydrolysis process, as it produces amylase (Labes and Schonheit, 2002). The organic components of distillery decoctions after initial fermentation by the accompanying microflora undergo transformations into alcohols or organic acids, being a good source of carbon for SRB (Szewczyk and Pfennig, 1990). The simultaneous biodegradation of two industrial wastes such as phosphogypsum and distillery decoctions would be an interesting solution from the economical point of view. Costs linked with simultaneous biodegradation of two industrial wastes, hazardous to the environment, are always lower than in the case of two separate biodegradation processes.

The main focus of the presented research was obtaining a sulphidogenic microorganisms community containing SRB and testing the possibility of phosphogypsum biotransformation in stationary cultures with simultaneous treatment of distillery decoctions.
**Experimental**

**Materials and Methods**

**Phosphogypsum.** The studied sample of phosphogypsum was collected from a waste dump from Wizów near Bolesławiec in Lower Silesia (Table I A).

**Microorganisms.** Community of anaerobic bacteria isolated from soil polluted by aircraft fuel and autochthonic microflora from distillery decoctions.

**Media.** (a) Modified Postgate medium (Postgate, 1984), in which Na$_2$SO$_4$ (4.5 g/dm$^3$) was replaced by phosphogypsum (5.0 g/dm$^3$). The source of carbon in the medium was ethanol (3 cm$^3$/dm$^3$). (b) Potato and rye distillery decoctions. The standard composition of distillery decoctions is presented in Table I B. Resasurine in the concentration 0.001 g/dm$^3$ was added to all cultures.

<table>
<thead>
<tr>
<th>Component (% weight)</th>
<th>Kowalski et al. (1990) – Wizów</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO$_4^{2-}$</td>
<td>42.18</td>
</tr>
<tr>
<td>CaO</td>
<td>29.61</td>
</tr>
<tr>
<td>P$_2$O$_5$</td>
<td>2.24</td>
</tr>
<tr>
<td>SrO</td>
<td>1.62</td>
</tr>
<tr>
<td>SiO$_2$</td>
<td>0.65</td>
</tr>
<tr>
<td>F</td>
<td>0.5</td>
</tr>
<tr>
<td>Al$_2$O$_3$</td>
<td>0.24</td>
</tr>
<tr>
<td>Na$_2$O</td>
<td>0.37</td>
</tr>
<tr>
<td>Fe$_2$O$_3$</td>
<td>0.14</td>
</tr>
<tr>
<td>K$_2$O</td>
<td>0.10</td>
</tr>
<tr>
<td>BaO</td>
<td>0.03</td>
</tr>
<tr>
<td>MgO</td>
<td>0.05</td>
</tr>
<tr>
<td>H$_2$O cryst.</td>
<td>20.18</td>
</tr>
</tbody>
</table>

**Culture conditions.** The cultures were enriched in 100 or 300 cm$^3$ volume bottles, tightly sealed with rubber plugs, with permanently attached needles with syringes. The relation of the inoculum to the medium was 1:10. The cultures were incubated in temperature of 30°C or/and 55°C.

**Enrichment of microorganisms.** The microorganisms were enriched from soil polluted by aircraft fuel using the microcosm method. The soil supplemented with phosphogypsum (5 g/dm$^3$) was placed in 100 cm$^3$ boxes, and next the Postgate medium with ethanol was added as the only source of carbon. The boxes were tightly sealed and incubated for 6 weeks in temperature of 30°C and 55°C in order to select anaerobic sulphidogenic consortions able to biotransform phosphogypsum.

**Measurements.** The following parameters were determined in the cultures: sulphides using the iodometric method, sulphates using the bar method and emission spectrometry method (induction excitation in an ICP medium), COD using the bi-chromate method. The reaction (pH) of the culture was corrected using 0.1N HCl or 0.1N NaOH.

The post-culture deposits were analysed with the radiography method using a DRON-2 diffractometer.

**Results and Discussion**

**Isolation and enrichment of SRB from soil polluted by aircraft fuel.** The most characteristic environments in which SRB occur are marine sediments (Boopathy et al., 1998; Kuever et al., 1999), where the concentration of sulphates typically reaches an average of 28 mM (de Wit, 1992), oil fields and oil reservoirs (Voordouw et al., 1996; Telang et al., 1997; Jenneman and Gevertz, 1999; Magot et al., 2000; Gieg and Suflita, 2002), as well as environments polluted by oil-derived products (Wolicka and Kowalski, 2005). The analyses were preceded by the enrichment of an active community of anaerobic microorganisms in conditions favouring the selection of SRB.

The microorganisms were isolated from soil polluted by aircraft fuel. After 6-week incubation, the enrichment of selected anaerobic communities of microorganisms began on a Postgate medium with ethanol and phosphogypsum. The obtained sulphidogenic microorganisms community (ethanol community) was passaged 5 times. Each passage lasted for 14 days. The maximal content of sulphides, 720 mg HS$^-$/dm$^3$, etc.
was observed in the 4th passage of the culture, what corresponded to the reduction of 2030 mg SO$_4$/$dm^3$, i.e. 46% of phosphogypsum introduced into the medium (Fig. 1).

**Enrichment of bacterial communities on distillery decoctions.** The isolated ethanol community was used as the inoculum to establish cultures on potato and rye distillery decoctions. Ten cultures were established: 4 on potato distillery decoctions (P) and 4 on rye distillery decoctions (R). Control cultures (C), cultures no 5 and 10, were cultured on a Postgate medium with ethanol inoculated with the ethanol community. They were managed in two variants: with and without pH regulation in the medium. All cultures were passaged 6 times in order to adapt the microorganisms to the distillery decoctions environment, and also in order to select a bacterial community able to actively biotransform phosphogypsum. The obtained results are presented in Table II.

As shown in Table II, the activity of the selected microorganisms in the enriched cultures depends on three factors: temperature, pH and type of decoctions. Higher concentrations of HS$^-$ were observed in cultures where the pH during the incubation was optimal for the SRB and reached 6.6–7 (cultures no 1 and 3). In these cultures the content of HS$^-$ reached over 380 mg HS$^-$/dm$^3$. In cultures no 2 and 4, in which the pH values were not corrected (pH 4.8–5.2) was observed lower activity of the SRB. Only some SRB can be active in media with such pH, they include bacteria growing in acidic mine waters (Elliot *et al.*, 1998).

Analysing the data from Table II it can be concluded that higher values of HS$^-$ were obtained in mesophilic cultures. In cultures no 1 and 3 a similar concentration of HS$^-$ was obtained – 612 and 610 mg/dm$^3$, what corresponds to the reduction of 1726 and 1720 mg SO$_4$/dm$^3$ and 34.5% phosphogypsum/dm$^3$ in relation to 5 g/dm$^3$ introduced into the medium. In cultures of thermophilic communities of microorganisms, regardless the type of applied decoctions and the pH value in the medium, high concentrations of HS$^-$ were not observed. The maximal content of HS$^-$ – 280 mg/dm$^3$ was observed in cultures no 6 and 8, what

![Fig. 1. Maximal concentration of sulphides obtained in the following passages of the ethanol culture](image)

<table>
<thead>
<tr>
<th>Type of medium</th>
<th>No of culture</th>
<th>pH</th>
<th>Incubation temp. (°C)</th>
<th>No of passage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>reg.</td>
<td>30</td>
<td>448 460 495 612 568 497</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>n. reg.</td>
<td>30</td>
<td>320 280 280 220 200 220</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>reg.</td>
<td>30</td>
<td>380 470 520 610 580 540</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>n. reg.</td>
<td>30</td>
<td>472 442 408 529 612 529</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>n. reg.</td>
<td>30</td>
<td>380 420 470 628 640 620</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
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C – control cultures; reg. – pH regulation of the medium; n.reg. – without pH regulation of the medium
corresponded to the reduction of 790 mg SO₄/dm³ and 15.8% phosphogypsum/dm³ in relation to 5 g/dm³ introduced into the medium.

Regardless the applied decoction (potato or rye), the activity of the isolated bacteria was comparable.

Influence of the inoculum on the activity of phosphogypsum biotransformation in distillery decoctions. The next stage of the study was focused on testing the influence of the applied inoculum on the effectiveness of the phosphogypsum biotransformation process in distillery decoctions. In this case the most active microorganism community no 1 was used as the inoculum to establish a culture on distillery decoctions. Three cultures were established: 1G – on barren potato decoctions inoculated with the inoculum; 2G – on non-barren decoctions inoculated with the ethanol inoculum. Culture 3G comprised non-barren non-inoculated potato decoctions (control culture). All cultures contained phosphogypsum as the only electron acceptor. The production of sulphides obtained in these cultures is shown in Fig. 2.

The increase of concentration of hydrogen sulphide was observed in all cultures. The highest concentration of HS⁻ – 488 mg/dm³ was noted in culture 3G, on non-barren non-inoculated decoctions. In 1G and 2G similar values of 336 and 343 mg/dm³ of HS⁻ were noted. This corresponded to the reduction of about 55%, 38% and 39%, respectively, of phosphogypsum in relation to 5 g/dm³ introduced into the medium.

The costs linked with simultaneous biodegradation of two industrial wastes, particularly hazardous to the environment, are always lower than for each of these wastes treated separately. Therefore, the removal of organic wastes from decoctions is equally important as the biotransformation of phosphogypsum. The COD of distillery decoctions was high and reached 2.2 g O₂/dm³, whereas the content of sulphates was rather low – 50 mg/dm³. Addition of phosphogypsum to distillery decoctions makes them high-sulphur wastes as in the case of wastes from the production of citric acid from sugar cane (2.9 g SO₄/dm³). The calculated ratio COD/SO₄ reached 8.8. If this ratio is higher, then more organic compounds are decomposed during the formation of methane. At COD/SO₄ > 10 sulphides are not produced (Li and Humphre, 1989; Oude Elferink et al., 1995). It is assumed that the co-existence of the two groups of microorganisms is possible when COD/SO₄ ratio is between 1.7 and 2.7; below 1.7 the SRB dominate (Clancy et al., 1992). The reduction of COD and biotransformation of phosphogypsum in the particular cultures are presented in Fig. 3.

It can be assumed that addition of a selected anaerobic inoculum comprising a community of microorganisms to the decoctions did not increase the effectiveness of phosphogypsum transformation. Community 3G comprising non-barren non-inoculated distillery decoctions most effectively took part in the biotransformation process (55% reduction of phosphogypsum) with simultaneous purification of the distillery decoctions (24% reduced COD). Calculations showed similar results for cultures 1G and 2G: 38% reduction of phosphogypsum and about 13.5% reduction of COD. The activity of SRB in COD reduction was 22.7% in culture 1G, 22.2% in 2G and 17.7% in 3G. The obtained results of a ca. 20% activity of SRB in the utilisation of organic compounds are similar to those obtained by Gupta et al. (1994) in bioreactors purifying sewage.
After incubation the post-culture deposits were separated. The observed reduction of the phosphogypsum mass was: 15% for culture 1G (0.9 g/dm³), 2G – 20% (1 g/dm³) and 3G – 55% (2.75 g/dm³). Diffractometric analyses did not show any substantial phase changes in the sediment in comparison to phosphogypsum.

In conclusion the community of bacteria isolated from soil contaminated by air fuel contained SRB capable of the biotransformation of phosphogypsum. It can be also stated that the biotransformation of phosphogypsum in cultures in distillery decoctions is possible but is not very effective and its optimization requires further research.

**Literature**


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Survival of *Proteus mirabilis* O3 (S1959), O9 and O18 Strains in Normal Human Serum (NHS) Correlates with the Diversity of their Outer Membrane Proteins (OMPs)

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

**Abstract**

Urinary tract infections are frequently caused by *Proteus mirabilis* strains. In the previous studies there were defined the complete structures of O-polysaccharide parts of lipopolysaccharides from strains: *P. mirabilis* O3 (S1959), *P. mirabilis* O9 and *P. mirabilis* O18. In the present study it was investigated bactericidal effect of normal human serum (NHS) to *P. mirabilis* strains. We also focused on the diversity of outer membrane proteins (OMPs) being separated on a gel isolated from tested strains. Serial passage of *P. mirabilis* O18 in 90% normal bovine serum (NBS) contributed to over-expressing some classes of OMPs.

**Key words**: *Proteus mirabilis*, serum, the complement system, outer membrane proteins (OMPs)

*Proteus mirabilis* strains are a common cause of urinary tract infections and also have been described as opportunistic etiological agents in infections of the respiratory tract and of wounds, burns, skin, eyes, nose and throat diseases (Różalski *et al*., 1997). Pathogenic bacteria have worked out many different ways to overcome the host defense system for example proteolytic degradation of secretory immunoglobulin or steric shielding or modification of exposed pathogen-associated molecular patterns (PAMPs) (Hornef *et al*., 2002). One of the mechanisms which protect the macroorganism from infections is the bactericidal activity of serum. The complement system is a complex of serum proteins which interact in a cascade. It recognizes and promotes clearance, by phagocytosis, of invading microorganisms and also causes lysis of Gram-negative bacteria itself. Activation of the complement system is achieved through three major pathways: the classical pathway (CP), which is activated by certain isotypes of antibodies bound to antigens (immune complexes); the alternative pathway (AP), which is activated on microbial cell surfaces in the absence of antibodies and the lectin pathway (LP), which is activated by a mannose binding lectin – MBL, that binds to mannose residues on microbes (Sim and Tsiftsoglou, 2004). The mechanism of the bacterial resistance to the bactericidal effect of the serum is still not fully understood, but it is known that some outer membrane proteins (OMPs) and lipopolysaccharides (LPSs) are the factors determining the resistance (Pilz *et al*., 1992; Prasadarao *et al*., 2002; White *et al*., 2005) or the sensitivity (Alberti *et al*., 1993; Merino *et al*., 1998; Weber *et al*., 1992) of bacteria to the bactericidal action of the serum.

Lipopolysaccharide (LPS, bacterial endotoxin), the major component of the outer membrane is one of the virulence factors of *Proteus*, preventing the *Proteus* rods against bactericidal effects of the complement proteins deposition. There are different mechanisms for LPS mediated complement activation (Vukajlovich *et al*., 1992). The antibody-independent classical pathway (CP) is mediated only by the lipid A portion of the molecule (Vukajlovich *et al*., 1987). Activation of the alternative pathway (AP) requires the polysaccharide moieties of LPS, core oligosaccharide and/or O-antigen polysaccharides (Vukajlovich *et al*., 1992; Vukajlovich *et al*., 1987). Previous studies tested complement activation by LPSs isolated from *P. mirabilis* O10, O23, O30, and O43 strains, which differ in the number of negative COO-groups on their polysaccharide...
components. Four *P. mirabilis* strains studied were resistant to complement-mediated killing, despite of the complement binding by their LPSs (Kaca et al., 2000). In the other studies of two smooth *P. mirabilis* strains O9 and O49 bacterial resistance to the bactericidal effect of the serum was found to be depended on the chemical structure and polysaccharide lenght of O9 and O49 LPSs (Fudala and Kaca, 2004). The complete structures of O-polysaccharide parts of lipopolysaccharides of studied strains were established. *P. mirabilis* O3 (S1959), O9 and O18 LPSs have the unique structures with lysine, furanosic-form of ribose and phosphocholine residues, respectively (Fudala et al., 2003; Kondakova et al., 2003; Ziolkowski et al., 1997). In the above mentioned studies the role of the outer membrane proteins in the bacterial complement mediated-resistance has not been investigated yet.

Below, we focused on electrophoretic patterns of OMPs isolated from *P. mirabilis* O3 (S1959), O9 and O18 strains. A correlation between the presence of some OMPs and the susceptibility of bacteria to the bactericidal activity of serum was observed.

Three strains: *P. mirabilis* O9, *P. mirabilis* O18 and *P. mirabilis* O3 (S1959) obtained from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague, Czech Republic) and Institute of Microbiology and Immunology, University of Łódź, Poland (strain *P. mirabilis* O3 (S1959)) were used. Normal human serum (NHS) was obtained from three healthy volunteers which had not been previously treated with any antimicrobial drug. The sample serum were collected and kept frozen (−70°C) for a period no longer than 4 months. A suitable volume of serum was thawed immediately before use. Each portion was used only once. Normal bovine serum (NBS) was obtained from five healthy animals, prepared and stored in the same way like NHS. To define bacterial sensitivity to the bactericidal serum activity the modified Doroszkiewicz method (Doroszkiewicz, 1997) was used. Briefly, the strains were grown overnight, and then bacterial cells of early exponential growth phase were transferred to a fresh nutrient broth and incubated at 37°C for 1 hour. After incubation the bacterial cells were centrifuged (4000 × g, 20 min, 4°C). The bacteria were then added to 50% and 75% NHS or 90% NBS (serum was diluted with 0.1 M NaCl) and incubated in a water bath at 37°C. After 0, 60 and 180 min samples were collected, diluted and cultured on nutrient agar plates for 18 h at 37°C. The number of colony forming units (CFU) at the time zero was taken as 100%. Strains with a survival ratio not less than 50% after 180 min of incubation in NHS or NBS were regarded as resistant. The control samples of NBS and NHS were decomplemented by heating at 56°C for 30 min (Doroszkiewicz et al., 1995; Eidinger et al., 1977; Mielnik et al., 2001). The level of C3 and C4 components in NHS was determined using monoclonal polyclonal antibodies anti-C3 and anti-C4 proteins (Roitt and Male, 2000). Outer membrane proteins (OMPs) were isolated (Murphy and Bartos, 1989) from the bacteria grown in 100 ml of Brain Heart Infusion (BHI) broth (Difco) at 37°C for 18 h. *P. mirabilis* O18 strain after serial passage in 90% NHS was also grown in BHI broth at 37°C for 18 h before the isolation procedure. After incubation the bacterial cells were centrifuged (4000 × g, 15 min, 4°C) and the pellet was suspended in 2.5 ml of buffer β (1 M sodium acetate, 0.001 M β-mercaptoethanol, pH 4.0). Then 22.5 ml of water solution containing 5% (v/v) Zwittergent 3–14 (Calbiochem-Behring) and 0.5 M CaCl₂ was added. That mixture was stirred at room temperature for 1 h. To precipitate nucleic acids a 6.25 ml volume of 96% (v/v) cold ethanol was added very slowly. The mixture was then centrifuged at 17000 × g for 10 min. The remaining proteins were precipitated by addition of 96% (v/v) cold ethanol and were centrifuged at 17000 × g for 20 min at 4°C. The pellet was then suspended in buffer Z (0.05% (v/v) Zwittergent, 0.05 M Tris and 0.01 M EDTA, pH 8.0) and stirred for room temperature for 1 h. The solution was kept at 4°C overnight and centrifuged at 12000 × g for 10 min at 4°C. OMPs were present in the buffer Z soluble fraction after centrifugation. To determine a concentration of outer membrane proteins in the samples the Bradford protein assay (Bradford, 1976) was used. Discontinuous sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was carried out on slabs with 12.5% acrylamide according to Laemmli (Laemmli, 1970). The Wide Molecualr Weight Range Sigma Marker protein standard was used for molecular mass calibration. OMPs prepared in the sample buffer were loaded in each of the wells in the same volume of 10 µl. The samples consisted of almost the same amounts of protein in the range of 0.9–1.2 mg/ml. After electrophoresis the gels were kept for 1h in a solution containing 25% (v/v) methanol, 10% (v/v) acetic acid and 0.05% (w/v) Coomassie Brilliant Blue and then destained in 10% (v/v) acetic acid for 3–5 h. For the molecular analysis of OMPs the BIO-CAPT v. 99 software as well as the BIO-1D++ v.99 (Vilber Lourmat, France) were used.

The data concerning the determination of the level of C3 and C4 in NHS showed that the level of C3 was 78 mg/dl (standard for a normal human serum is 55–120 mg/dl) and C4 was 37 mg/dl (standard for a normal human serum is 20–50 mg/dl).

To determine the killing effect of the serum on bacteria, we studied the survival of bacteria after three hours of incubation in serum. The results concerning the sensitivity of three *P. mirabilis* strains to NHS after
180 min of incubation are given in Table I. The results presented in the Table I indicate that the strains \textit{P. mirabilis} O9 and \textit{P. mirabilis} O18 were more sensitive to the bactericidal action of NHS than \textit{P. mirabilis} O3 (S1959) strain. The latter one was resistant to the lytic action of the serum and the number of cells increased in 50% and 75% human serum dilution. When NHS decomplemented by heating at 56°C for 30 min was used in the experiment, the bacteria proliferated very intensively. It confirmed that the complement was responsible for the killing action of NHS. The serial passage (23 times in 90% NBS) of \textit{P. mirabilis} O18 strain increased the level of the resistance of bacteria to the bactericidal action of 50% NHS (Table I).

Also some differences appeared in SDS-PAGE patterns of the outer membrane proteins. \textit{P. mirabilis} O18 treated by the sequential passage in 90% NBS (data not shown) over-expressed some of the outer membrane proteins compare to \textit{P. mirabilis} O18 before the passage (Fig. 1). It was observed, that OMPs with the molecular masses: 36 kDa, 35 kDa, 34 kDa, 33 kDa, 22 kDa, 21 kDa, 19 kDa, 18 kDa were present in all tested strains of \textit{P. mirabilis}. Particular analysis of bands on 12.5% polyacrylamide gel have shown, that \textit{P. mirabilis} O18 after passage in 90% NBS produced some excess of proteins which were unexpectedly related in their quantity to these isolated from the resistant strain \textit{P. mirabilis} O3 (S1959) and the main were: 22 kDa, 19 kDa, 18 kDa, 17 kDa. As a result of the passage of \textit{P. mirabilis} O18 in 90% NHS there appeared OMPs with the molecular masses 110 kDa, 67 kDa, 62 kDa and 34 kDa which were not produced by \textit{P. mirabilis} O18 before the passage in 90% NBS. Densitometric analysis of OMPs (data not shown) was helpful to the preliminary defining of the dependence of the survival of bacteria in NHS and the presence and ratio of some outer membrane proteins. The following investigations will be comprise protein elution and determining the proportional contents of particular OMPs in the samples.

In the previously published papers the participation of OMPs in the bacterial serum resistance phenomena (Cisowska \textit{et al.}, 2005; Futoma \textit{et al.}, 2004; Murphy \textit{et al.}, 2000; Prasadarao \textit{et al.}, 1992) was discussed. It has been suggested that outer membrane protein A (OmpA) of \textit{Escherichia coli} contributes to serum resistance by binding to C4b binding protein (C4bp), a complement fluid phase regulator (Prasadarao \textit{et al.}, 2002). In other bactericidal assays, the mutants of \textit{Moraxella catarrhalis} were more readily killed by normal human serum compared to the isogenic parent strains which possessed outer membrane protein E (OmpE). Those results indicated that OmpE was involved in the expression of serum resistance (Murphy \textit{et al.}, 2000). Some OMPs can inhibit complement activation on the C3, C9 and C5b-9 level (Prasadarao \textit{et al.}, 1992). It has been shown that porin (OmpK36) from \textit{Klebsiella pneumoniae} activated the classical pathway of the complement system by forming complexes with C1q, a component of the complement. Together with an activated complement in the alternative pathway it came to effective elimination of serum-sensitive \textit{K. pneumoniae} cells (Alberti \textit{et al.}, 1993).
The presented data indicate that the serum resistance of *P. mirabilis* rods may be mediated by outer membrane proteins patterns in a concert with O-polysaccharides LPSs structures.

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**Literature**


Lack of an Association between *Helicobacter* Infection and Autoimmune Hepatitis in Children

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Abstract

A total of 32 children (mean age 12.8 years; range 3–18 years; 20 females, 12 males) with autoimmune hepatitis presenting for control investigation including liver biopsy were included in the study. The autoantibody profile was consistent with type I AIH in 30 children (anti-nuclear antibodies and/or smooth muscle antibodies), whereas the remaining two patients had type II AIH (antibodies to liver-kidney microsome type 1). From each child 2 ml of blood was taken for serology and a transcutaneous liver biopsy was obtained for histology, culturing and PCR. The control group for the comparison of seroprevalence of antibodies to *H. pylori* and non-gastric *Helicobacter* species consisted of 44 healthy children (mean age 10.3 years; range 1–18 years, 20 females, 24 males) presenting either for a routine vaccination or otherwise healthy children treated in the Department of Orthopedic Surgery.

The study was undertaken with the approval of the Ethics Committee of Children’s Memorial Health Institute, and informed consent was obtained from all participants.

Antibodies to *Helicobacter pylori* and non-gastric *Helicobacter* species (i.e. *H. pullorum, H. bilis, H. hepaticus*), were detected using immunoblot assay, as described previously (Nilsson et al., 1997; Kornilovs’ka et al., 2002). Cultures of liver samples were performed on Wilkins Chalgren agar with 7% horse blood and Dent’s selective supplement SR 147 (Oxoid) under microaerophilic conditions at 37°C for

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up to 21 days. All liver samples from AIH patients were analysed by PCR to detect Helicobacter sp. DNA. The DNA was extracted by use of QIAamp DNA Mini kit (Qiagen). The purified DNA was subjected to two separated PCRs with Helicobacter genus-specific primers (Fox et al., 1998) generating 16S rRNA amplicons of 1200 or 400 bp. Amplified DNA fragments were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide and visualized under UV light. The 400 bp PCR products were purified using PCR-purification kit (Qiagen) and subjected to direct sequencing of both strands with an automated DNA sequencer (ABI PRISM 310).

The prevalence of antibodies to Helicobacter in AIH patients and controls was compared using the chi-squared test. The prevalence of antibodies to H. pylori and non-gastric Helicobacter species did not differ significantly between AIH children and control subjects. Antibodies to H. pylori were found in 7 of 32 (22%) AIH patients compared with 6 of 44 (14%) controls. Antibodies against non-gastric Helicobacter species were not detected in any patient from either group. Helicobacter DNA was found in the liver samples from two children with AIH (6%). The sequencing analysis revealed that both sequences closely resembled H. pylori, with the similarity rates exceeding 99.7%. The two patients whose liver samples contained H. pylori DNA were also anti-H. pylori positive. None of the liver cultures showed the presence of Helicobacter species.

The present study revealed that the seroprevalence of antibodies to H. pylori did not differ significantly between children with AIH and a control group. This finding is consistent with previous observations, indicating that the frequencies of antibodies to H. pylori in adult AIH patients were similar to those in controls (Durazzo et al., 2002; Nilsson et al., 2003). On the other hand, AIH and PSC adult patients were reported to have a higher prevalence of anti-H. pullorum, anti-H. bilis and anti-H. hepaticus antibodies as compared to blood donors (Nilsson et al., 2003), which is in conflict with the present results, where no such antibodies were detected in either AIH or control subjects. Since both, the former (Nilsson et al., 2003) and the present, serological studies have been performed in the same laboratory (Department of Medical Microbiology, Lund University) using the same methods, this discrepancy cannot be attributed to methodological differences. It could, however, be related to regional variability and differences in patient populations. The Polish children participating in the study were much younger than the Swedish patients, which could influence the exposure to Helicobacter species.

Helicobacter DNA was found in only two AIH liver tissues, and both sequences showed high similarity to H. pylori. This finding is in line with the serological results, since both patients were also anti-H. pylori positive and did not have antibodies to other Helicobacter species tested. However, despite prolonged incubation, we were not able to culture Helicobacter sp. from any of tested samples. Similarly, with one exception (Queiroz et al., 2001), all other attempts to cultivate Helicobacter sp. from human hepatobiliary materials have been unsuccessful, in spite of the presence of their genetic material (Fox et al., 1998; Avenaud et al., 2000; Silva et al., 2003). The inability to cultivate Helicobacter in some of the former studies was suggested to be attributed to the fact that long-stored frozen samples have been used for culturing (Fox et al., 1998; Silva et al., 2003). In our study, however, all the samples were cultured directly after collection, and therefore sample processing was unlikely to influence the results. Detection of H. pylori DNA, but not culturable cells in the liver tissues of AIH patients could therefore indicate that the bacteria were either nonviable or that they were present in very low numbers or in a non-culturable coccoid form. The fact that Helicobacter genetic material was found only in two AIH liver samples and the seroprevalence of anti-Helicobacter antibodies did not differ between the AIH and the control group suggests that these bacteria are unlikely to be associated with the pathogenesis of autoimmune hepatitis in children.

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Literature


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