Cloning and expression analysis of genes of lytic endopeptidases L1 and L5 from Lysobacter sp. XL1

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ABSTRACT

Lytic enzymes are the group of hydrolases that break down structural polymers of the cell walls of various microorganisms. In this work, we determined the nucleotide sequence of a Lysobacter sp. XL1 of alpA and alpB genes, which code for, respectively, secreted lytic endopeptidases L1 (AlpA) and L5 (AlpB). In silico analysis of amino acid sequences showed these endopeptidases to be homologous proteins synthesized as precursors of similar structural organization: the mature enzyme sequence is preceded by an N-terminal signal peptide and a pro region. Based on the phylogenetic analysis, endopeptidases AlpA and AlpB were assigned to the S1E family (clan PA(S)) of serine peptidases. Expression of alpA and alpB ORFs in E. coli confirmed them to code for functionally active lytic enzymes. Each ORF was predicted to have the Shine–Dalgarno sequence located at a canonical distance from the start codon and a potential Rho-independent transcription terminator immediately after the stop codon. alpA and alpB mRNAs were experimentally found to be monocistronic; transcription start points were determined for both mRNAs. Synthesis of alpA and alpB mRNAs was shown to occur predominantly in the late logarithmic growth phase. The amount of alpA mRNA in cells of Lysobacter sp. XL1 was much higher, which correlates with higher production of endopeptidase L1 as compared with L5.

INTRODUCTION

To suppress competing microorganisms, bacteria produce and secrete into the ambient medium a broad arsenal of antimicrobial factors such as porins, nucleases, bacteriocins similar to phage tails, peptide antibiotics etc. (1, 10, 28, 35, 39). Some of the factors of microbial antagonism are lytic enzymes secreted by bacteria. A target of bacteriolytic enzymes is peptidoglycan, the main structural component of the bacterial cell wall. Depending on which bonds in peptidoglycan they hydrolyze, bacteriolytic enzymes are classified into four groups (17, 21). Glucosaminidases and muramidases cleave different bonds in peptidoglycan glucan chains, amidases hydrolyze the amide bond between muramic acid and the peptide subunit, and peptidases cleave the peptide bonds in peptide subunits or interpeptide bridges. Unlike
bacteriophage-coded lysins whose action is directed on one species or even a limited group of bacterial strains (16, 31), bacteriolytic enzymes of bacterial origin have a broad spectrum of antimicrobial activity (3, 54). Given that peptidoglycan has a conserved structure, the probability for the emergence of bacteria resistant to the action of lytic enzymes, especially with broad substrate specificity, is extremely low. Owing to these properties, the use of bacteriolytic enzymes in medicine as antimicrobial agents, especially against pathogenic microorganisms with multiple drug resistance, is promising.

The bacterium *Lysobacter* sp. XL1 secretes into the ambient medium a variety of lytic enzymes. The antimicrobial preparation lysoamidase produced based on the culture liquid of this bacterium is active against a broad range of microorganisms, in particular against bacteria of the genera *Staphylococcus*, *Bacillus*, *Streptococcus*, *Micrococcus*, *Kocuria*, *Peptostreptococcus*, *Corynebacterium*, *Streptomyces*, *Alcaligenes* and some other bacteria; yeasts of the genera *Saccharomyces*, *Candida* and *Pseudozyma* (24). Besides, the preparation prevents germination of bacterial and fungal spores (6, 37). To date, five bacteriolytic enzymes, a metalloprotease active against yeasts and a phosphatase have been isolated from the culture liquid of *Lysobacter* sp. XL1 and characterized to varying degrees (7, 43-45, 47, 48, 52).

The bacteriolytic enzymes of lysoamidase preparation are represented by three endopeptidases L1, L4 and L5, as well as *N*-acetylmuramoyl-L-alanine amidase L2 and muramidase L3 (42, 46, 48, 52). It is obvious that the occurrence of lytic enzymes specific to different bonds in peptidoglycan determines a broad range of lysoamidase antibacterial activity. From inhibition data, endopeptidases L1, L4 and L5 have been assigned to the serine protease catalytic type (42, 52). Endopeptidase L1, a small protein of a molecular weight about 22 kDa, has a broad substrate specificity and hydrolyzes the amide bonds in proteins, peptides and microbial cell walls. With respect to bacterial peptidoglycans, this enzyme exhibits both amidase and endopeptidase activities, with the latter being more potent. It hydrolyzes the amide bond between muramic acid and alanine, the peptide bonds between diaminopimelic acid and alanine, as well as between glycines of the interpeptide bridge. Endopeptidase L5 has a molecular weight of about 26 kDa. It occurs in culture medium in much smaller amounts than L1, and its enzymatic properties have been little
studied. L1 and L5 are thermostable enzymes with the reaction optima of 70 and 80 °C, respectively. Endopeptidases L1 and L5 have different spectrum of antimicrobial activity (18, 19). For instance, cells of *B. subtilis* and *M. luteus* are lyzed by both enzymes. At the same time, only L1 is capable of destroying *S. aureus* and *B. cereus* cells, whereas L5 (unlike L1) lyzes *K. rosea* and *A. faecalis*.

It has been shown that endopeptidases L1 and L5 are secreted via different pathways. Endopeptidase L5 is secreted via outer membrane vesicles, whereas L1 is not found in them. Secretion via vesicles promotes delivery of L5 to peptidoglycans of Gram-negative bacteria through outer membrane, which significantly expands the antimicrobial action spectrum of L5 (52).

Combining lytic enzymes with different substrate specificities would enable the development of novel antimicrobial preparations of a broad action range. Cloning of genes of *Lysobacter* sp. XL1 lytic enzymes and their characterization are required both for further research and for applied R&D, in particular, to create producer strains.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

The strain *Lysobacter* sp. XL1 was from the Collection of the Laboratory of Microbial Cell Surface Biochemistry. Cells of the strain were grown at 28 °C on Luria-Bertani (LB) medium for isolation of genomic DNA and on CY medium (30) for isolation of RNA.

For cloning and plasmid isolation, we used the strain *E. coli* DH5α (53).

**Oligonucleotide primers**

Oligonucleotide primers used in this study are listed in Table S1 (SUPPLEMENTAL MATERIALS).

**Isolation of nucleic acids**
Genomic DNA was isolated by phenol extraction as described (38). Plasmid DNA was isolated using a QIAquick Plasmid Purification Kit (Qiagen, USA). Total RNA was isolated from cells in the mid or late logarithmic growth phase using an RNeasy Protect Mini Kit (Qiagen, USA).

**Construction of recombinant plasmids**

Plasmids pALPI-29a and pALPII-29a bear *alpA* and *alpB* ORFs, respectively. To construct the plasmids *Lysobacter* sp. XL1 DNA fragments, which contained the *alpA* or *alpB* ORF, were amplified by PCR with primer pairs #14 and #15, #16 and #17, respectively, and inserted into the *NdeI* and *XhoI* sites of pET-29a vector (Novagen, USA). All constructs were confirmed by sequencing.

Vector pUC18-lic is intended for ligation independent cloning of PCR fragments by forming 15-nt 5′-protruding ends (Lapteva Yu. S., unpublished data). For this, the vector was digested by restriction endonuclease *SmaI* and treated with T4 DNA polymerase in the presence of dATP. The vector was used for determination of the 5′-ends of *alpA* and *alpB* mRNAs.

**Nucleic acid hybridization**

Southern and Northern blot hybridization was performed as described (38). Radioactive labelling of DNA probes was carried out using a DECAprime II DNA Labelling Kit (Ambion Inc., USA).

*DNA–DNA hybridization*. 5 µg of *Lysobacter* sp. XL1 genomic DNA was digested by restriction endonucleases, separated in 1% agarose gel and transferred to Hybond-N membrane (GE Healthcare, USA). As the probe, we used a 32P-labelled 426-bp-long DNA fragment of *Lysobacter* sp. XL1, coding for the mature part of endopeptidase L1. The fragment was amplified by PCR using primers #1 and #2.

*RNA–DNA hybridization*. 30 µg of *Lysobacter* sp. XL1 total RNA was separated in 1.2% denaturing agarose gel and transferred to Hybond-N membrane. DNA probes specific to *alpA* or *alpB* genes were amplified by PCR with pairs of primers #3 and #4, #5 and #6, respectively.
Determination of mRNA 5′-ends

The 5′-ends of alpA and alpB mRNAs were determined by the 5′-RACE protocol. The first cDNA strand was obtained with 5 µg total RNA and 10 nmol primer #7 or #8 specific to the ORF of alpA or alpB, respectively, using a RevertAid First Strand cDNA Synthesis Kit (Fermentas). RNA was removed by alkaline hydrolysis. The cDNA was cleaned up from nucleotides and primers. One third of the purified DNA was ligated with 5 ng oligonucleotide #9 using T4 RNA ligase at room temperature for 48 h. Amplification of DNA was carried out using PCR with primers #12 (0.5 µM), #10 (0.025 µM) and #13 (0.5 µM) for alpA; and #12 (0.5 µM), #11 (0.025 µM) and #13 (0.5 µM) for alpB. PCR fragments were purified from 2% agarose gel using a QIAquick Gel Extraction Kit (Qiagen, USA), treated with T4 DNA polymerase in the presence of dTTP, cloned into plasmid vector pUC18-lic and sequenced.

Phylogenetic analysis of proteins

Protein sequences homologous to AlpA and AlpB were searched for in the GenBank database (http://www.ncbi.nlm.nih.gov/) using the BLAST program. Amino acid sequences of proteins were aligned using the CLUSTAL X program (50). The tree was constructed by means of the TREECON package (51) using the neighbour joining method. The statistical significance of branching was assessed by bootstrap analysis of 1000 alternative trees using the respective function of the TREECON program. The evolutionary distance was expressed as the number of substitutions per 100 amino acids.

Expression of alpA and alpB ORFs in E. coli

Cells of E. coli BL21(DE3) (49) were transformed by plasmids pALPI-29a or pALPII-29a, plated on dishes with agarized LB medium with kanamycin (25 µg/ml) and grown overnight at 37 °C. The grown colonies were replated on agarized Davis medium (12) containing disrupted cells of S. aureus 209P, 0.1 mM IPTG and 25 µg/ml kanamycin. Cells were grown at 28 °C for 48–72 h.
Nucleotide sequence accession number

The final sequence of the 9017-bp fragment of Lysobacter sp. XL1 genome determined in this study has been deposited in GenBank under accession number GU188567.

RESULTS AND DISCUSSION

Search for and determination of the nucleotide sequence of endopeptidase L1 gene

When cloning the gene of Lysobacter sp. XL1 endopeptidase L1 we based our approach on earlier data of amino acid sequences of the N-terminus of the mature enzyme and its proteolytic fragments (UniProtKB acc. #P85142). Considering the high similarity of the sequences for peptides of endopeptidase L1 and α-lytic protease of Lysobacter enzymogenes (GenBank acc. #AAA74111) (14), which are 88–92% identical (29), we designed a pair of degenerate primers (#1 and #2), amplified with them by PCR a Lysobacter sp. XL1 genome fragment 426 bp long and determined its nucleotide sequence. Analysis of the in silico translated sequence showed the fragment to contain a segment of endopeptidase L1 gene. The fragment synthesized by PCR was used as a probe to identify by hybridization the genomic-DNA restriction fragments bearing the endopeptidase L1 gene (SUPPLEMENTAL MATERIALS, Fig. S1).

Then, by means of inverse PCR, we amplified EcoRI and SalI fragments (contain the alpA and alpB ORFs, Fig. 1) and established their nucleotide sequences. As we found, in addition to endopeptidase L1 gene (alpA), this contig contained one more ORF that was named alpB. Its product AlpB exhibited high degree of homology with AlpA (see below). Analysis showed that the putative protein AlpB contained a sequence corresponding to the experimentally established sequence of the N-terminus of the mature region of Lysobacter sp. XL1 endopeptidase L5 (UniProtKB acc. #P85158). As the ORFs of endopeptidases L1 and L5 were found to be located next to each other, we assumed that the genes of lytic enzymes in the Lysobacter sp. XL1 genome could form a single cluster. For this reason, we determined the sequences of genome segments flanking the alpA and alpB ORFs. As the result, a 9017-bp contig was constructed, in which no other lytic enzyme ORFs were found at a distance of 2.4 kb upstream of alpA and 3.6 kb
downstream of alpB (Fig. 1, GenBank acc. #GU188567). Downstream of the alpB gene in the sequenced fragment, there is the ORF designated as orfA, oriented oppositely with respect to alpA and alpB. Analysis of the Pfam database (http://pfam.sanger.ac.uk, (15)) showed that 600 aa protein OrfA presumably belongs to the family of ABC transporters and contains TMD (transmembrane domain, pf00664) and ABC (ATP binding cassette, pf00005) domains characteristic of these proteins.

AlpA and AlpB are secreted lytic peptidases

The secreted proteases of microorganisms are synthesized, as a rule, in the form of inactive preproenzymes and contain at the N-end a signal peptide required for translocation across the cytoplasmic membrane, a pro region and a mature enzyme. The pro region, as it has been shown for α-lytic endopeptidase of L. enzymogenes (2, 4, 40), catalyzes the folding of the enzyme and, after autocatalytic cleavage, inhibits its activity. The latter function is important for preventing the digestion of peptidoglycan of the producing cell before the enzyme leaves the periplasmic space. We analyzed the amino acid sequences of AlpA and AlpB to establish the structural organization of these proteins. Search for signal peptides was carried out using the SignalP 3.0 program (13). Using the program, the signal peptides for both proteins were predicted to exist by the algorithm of the hidden Markov model with the probability of 1.000. According to the prediction, the position of the processing site is located, with the probability of 1.000, between 33 and 34 aa for AlpA and between 28 and 29 aa for AlpB. The boundaries between the pro regions and mature enzymes were determined based on the data on the N-terminal sequences of mature enzymes L1 and L5 isolated from the culture liquid of Lysobacter sp. XL1. Based on the analysis, the proenzyme processing site was found to be between 199 and 200 aa for AlpA, and 194 and 195 aa for AlpB. Summing up the data of the amino acid sequence analysis, it can be concluded that AlpA of 398 aa in length has the following structure: signal peptide, 33 aa; pro region, 166 aa; mature enzyme, 199 aa. Close by its size, AlpB of 399 aa in length has a similar structural organization: signal peptide, 28 aa; pro region, 166 aa; mature enzyme, 205 aa.
The mature enzymes AlpA and AlpB have close values of molecular weight, 19.8 and 20.8 kDa, and pI, 9.63 and 9.44, respectively. Nevertheless, the electrophoretic mobilities of mature naturally secreted AlpA and AlpB in the denatured PAGE (25) differ significantly and correspond to the mobilities of proteins with molecular weights of 22 and 26 kDa (52). The same electrophoretic mobilities were observed for mature recombinant endopeptidases AlpA and AlpB, whose preparations were obtained by in vitro renaturation of purified recombinant proenzymes and subsequent autocatalytic processing (O. R. Latypov, unpublished data). Besides, during the expression in E. coli of the alpA and alpB ORFs fused at the 3'-end with the sequence coding for 6×His, the mature endopeptidases secreted into the culture liquid contained a His tag at the C-terminus and, herewith, preserved the difference in electrophoretic mobilities (A. E. Kalinin, unpublished data). These data make it unlikely that there is posttranslational modification of AlpB or processing of AlpA at the C-terminus. Apparently, an anomalous electrophoretic mobility of AlpB is due to features of the enzyme’s structure, which is more resistant to the action of SDS. In favor of this assumption is a significantly higher temperature stability of AlpB as compared with AlpA. It has been shown that the activity of AlpB drops down almost twofold after incubation at 75 °C for 15 min, whereas AlpA loses half of its activity for the same time already at 55 °C (42). Besides, both endopeptidases are capable of preserving their activity in the presence of SDS (A. E. Kalinin, unpublished data).

In modern classification, peptidases are divided into clans, families and subfamilies based on their evolutionary relationship, catalytic mechanism and order in the polypeptide chain of amino acid residues involved in catalysis (5). A clan may contain enzymes that use various mechanisms of catalysis, whose relationship is exhibited only in the similarity of tertiary structures. The phylogenetic analysis of the sequences of mature endopeptidases AlpA and AlpB (Fig. 2) enabled us to assign these enzymes to clan PA, subclan S of serine peptidases, family S1 and subfamily S1E, the prototype of which is chymotrypsin A. Clan PA is a mixed clan and unites serine and cysteine peptidases. The sequences of AlpA and AlpB exhibit the highest similarity to the α-lytic protease of L. enzymogenes: the precursor of this enzyme is identical by 78% and 58% to preproenzymes AlpA and AlpB, respectively. Alignment of the sequences of
L. enzymogenes α-lytic protease, AlpA and AlpB (Fig. 3) revealed in the two latter the catalytic triad His (H235 for AlpA, H234 for AlpB), Asp (D262 for AlpA, D261 for AlpB) and Ser (S343 for AlpA and AlpB), characteristic of serine proteases of subclan PA(S) (33). It should be noted that AlpA and AlpB, as all characterized representatives of the chymotrypsin family, are endopeptidases (7, 34) MEROPS Database: http://merops.sanger.ac.uk/.

Well-characterized α-lytic protease of L. enzymogenes has specificity toward peptide substrates with small hydrophobic side chains before scissile bond. For this enzyme it was shown that residues M343 and M363 (numbered according to Fig. 3), that are lining primary specificity pocket S1, as well as the most of amino acid residues of surface loop 367-385 adjacent to the S1 pocket, are essential for the enzyme specificity (26). Substitutions of these residues for alanine broadened the specificity of α-lytic protease significantly. In particular, the enzyme containing either M343A or M363A substitutions is able to cleave efficiently the peptides with large hydrophobic side chains before scissile bond (9). Substrate specificity of AlpA and AlpB has not been studied yet. Sequence alignment of AlpA, AlpB and α-lytic protease of L. enzymogenes (Fig. 3) shows that amino acid residues of AlpA and α-lytic protease at the above sequence positions are identical, whereas those of AlpB differ significantly. In AlpB, Met at positions 343 and 363 are replaced by Thr, and there are 11 substitutions in the 19 aa surface loop. Therefore, it is feasible to suggest that substrate specificity of AlpA is similar to that of α-lytic protease, while AlpB has different specificity. The different spectrum of antibacterial activity of AlpA and AlpB counts in favor of this suggestion. Nevertheless, it is not possible to definitively predict substrate specificity of AlpB because of lack of knowledge about composition of the cell wall of the bacteria lysed by L5 and cleavage sites in interpeptide bridges.

To check the functional activity, the alpA and alpB genes were individually cloned under control of T7lac promoter and expressed in E. coli. For this, E. coli BL21(DE3) cells transformed by corresponding plasmids (see MATERIALS AND METHODS) were grown in the presence of the inducer on an agarized medium containing disrupted S. aureus cells. Lytic activity appeared as zones of lysis.
around colonies. As seen from the results of the assay shown in Fig. 4, zones of lysis were observed only around colonies of *E. coli* cells that expressed *alpA* and *alpB* genes, but were absent around colonies of the host *E. coli* cells transformed with plasmid vector. It should be noted that *alpA* and *alpB* gene products proved toxic for *E. coli* cells, which was manifested in a significantly slower growth rate on Davis minimal medium in the presence of the inducer. It appears that peptidoglycan of this bacterium is sensitive to the action of AlpA and AlpB peptidases.

Based on the conducted analysis, it can be concluded that *alpA* and *alpB* genes code for the secreted peptidases L1 and L5 of *Lysobacter* sp. XL1. It is unlikely that the putative ABC transporter OrfA, which we identified in this study, may be involved in secretion of AlpA or AlpB. ABC transporters carry diverse substrates including proteins across cellular membranes. Nevertheless, sequence analysis does not allow prediction of their substrate specificity. In Gram-negative bacteria, ABC transporter is a component of the type I secretion system which carries the proteins that are synthesized without N-terminal signal peptide (8). Whereas the proteins with N-terminal signal peptide, as in the case of AlpA and AlpB, are usually secreted by the type II or V secretion systems.

**alpA** and **alpB** ORFs are transcribed from their own promoters

*Lysobacter* sp. XL1 releases ~20 times as much endopeptidase L1 as L5 into the culture liquid (52). To explain the difference, we analyzed the translation and transcription signals for their ORFs.

Analysis of the nucleotide sequences upstream of *alpA* and *alpB* ORFs established the occurrence of a Shine–Dalgarno sequence before the initiating ATG codon at a distance of eight nt for *alpA* and seven nt for *alpB*. For both ORFs, it is a polypurine sequence AGGAG, which is complementary to the sequence at the 3′-end of 16S rRNA gene for various species of *Lysobacter* deposited in the GenBank database (Fig. 5A). The occurrence of a Shine–Dalgarno sequence at a canonical distance from the start codon, as well as the absence of stem–loop structures in this region (data not shown) does not make it possible to assume a significant difference in the efficiency of *alpA* and *alpB* translation initiation.
Analysis of the nucleotide sequences located after the stop codons of the *alpA* and *alpB* ORFs made it possible to identify the occurrence of putative intrinsic transcription terminators (Fig. 5B)(36). Thus, at a distance of 26 nt from *alpA* ORF stop codon there is a 29-member palindromic sequence capable of forming a hairpin with a perfect stem, rich in G/C pairs (75%), of 12 bp and a central loop of five unpaired bases. So high a content of G/C pairs is usually characteristic of Rho-independent terminators (11). At a distance of 27 nt from *alpB* ORF stop codon, there is also a 24-member palindromic sequence that forms a stem–loop structure of 20 paired (80% G/C) and four unpaired bases. Immediately after the hairpin there is an oligo(dT) sequence.

The finding of potential transcription terminators, as well as the distance of 602 nt between these ORFs suggested that *alpA* and *alpB* genes have their own promoters. This suggestion was examined by Northern blot hybridization of the total RNA of *Lysobacter* sp. XL1 with DNA probes specific to the *alpA* or *alpB* ORF (Fig. 6). As seen from the hybridization results, cells of *Lysobacter* sp. XL1 contain transcripts of about 1.5 kb long that comprise the *alpA* or *alpB* ORF. Larger-size transcripts potentially containing both ORFs were not found. Besides, mRNA of *alpA* is found in the mid-exponential growth phase, and its level significantly increases by the late exponential phase – early stationary phase of growth, whereas the amount of *alpB* mRNA is significantly lower than that of *alpA*, and the transcript is detected only by the late exponential growth phase.

Using the 5′-RACE technique, we determined the 5′-ends of *alpA* and *alpB* mRNA (Table 1). For eight out of 11 clones in the case of *alpA* and seven out of nine clones of *alpB*, the positions of transcription start points coincided. Three clones of *alpA* cDNA and two clones of *alpB* cDNA corresponded to a shorter 5′-UTR. We assume that this could be due to the premature termination of cDNA synthesis or a partial degradation of mRNA from the 5′-end. Thus, the length of the 5′-UTR of *alpA* mRNA is 134 nt; at the same time, the length of the *alpB* mRNA 5′-UTR is 140 nt. For both mRNAs, the 5′ terminal nucleotide residue is G.
We analyzed the nucleotide sequences of \textit{alpA} and \textit{alpB} genes in the –35 and –10 regions by aligning them with the corresponding consensus sequences for \textit{E. coli} \( \sigma^{70} \) promoter (Fig. 5C). The analysis showed that the putative promoter of \textit{alpB} greater corresponds to the \textit{E. coli} sequence than the \textit{alpA} one.

In the case of \textit{alpA}, three out of six nucleotides were found to match the \textit{E. coli} consensus sequence in the –35 region and two in the –10 region. In the case of \textit{alpB}, there are five matches to the \textit{E. coli} consensus sequence in the –35 region and two in the –10 region. In spite of the evolutionary conservatism of \( \sigma^{70} \) (20, 22, 32), it is evident that the sequences of the promoters of \textit{Lysobacter} bacteria in regions –35 and –10 can differ significantly from the consensus sequences of \textit{E. coli} \( \sigma^{70} \)-promoter. In particular, for \textit{Xanthomonas campestris}, which, as \textit{Lysobacter} sp. XL1, is attributed to the Xanthomonadaceae family, analysis of the frequency of occurrence of potentially strong gene promoters, conducted based on \textit{E. coli} \( \sigma^{70} \)-consensus sequences has shown that such promoters are in fact absent in the genome (41). Besides, the activity of \textit{alpA} and \textit{alpB} gene promoters can be regulated by transcription factors or else depend on alternative \( \sigma \)-factors. In favor of this is the significant increase in the amount of \textit{alpA} and \textit{alpB} mRNAs in cells at the late exponential growth phase. Involvement of transcription factors in the regulation of expression of lytic enzymes was shown for \textit{Lysobacter enzymogenes} str. C3. In this strain, production of lytic enzymes is regulated by the product of gene \textit{clp} assigned to the CRP family of global transcription regulators (23).

Thus, based on the results of the analysis of the nucleotide sequences and cellular transcripts, it can be concluded that \textit{alpA} and \textit{alpB} genes are transcribed each from its own promoter. Nevertheless, these findings are not sufficient enough to explain the differences in the levels of \textit{alpA} and \textit{alpB} mRNAs, which can be due to differences in promoter strength, mRNA stability or both.

The obtained results enable a more profound study of the regulation of \textit{alpA} and \textit{alpB} gene expression, as well as a study of the structural features of the encoded proteins responsible for the choice of secretion pathway and substrate specificities.

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**Figure legends**

Table 1. Analysis of 5′-ends of *alpA* and *alpB* mRNAs by the 5′-RACE protocol.

*Given in accordance with GenBank acc. # GU188567.*
Fig. 1. Map of the Lysobacter sp. XL1 cluster of genes alpA, alpB and orfA (GenBank, GU188567). Gray arrows indicate the arrangement and orientation of the ORFs. P1 and P2 are, respectively, transcription start points for alpA and alpB genes. Locations of restriction endonuclease sites are shown.

Fig. 2. Phylogeny of endopeptidases AlpA and AlpB. Phylogenetic tree of bacterial serine proteases was constructed based on homology between amino acid sequences of mature enzymes. Accession numbers of sequences in the UniProt database are given. Enzymes from the following organisms are presented: P00778, Lysobacter enzymogenes; D2K8B3 and D2K8B4, Lysobacter sp. XL1; P52320 and Q07006, Streptomyces griseus; Q03424 and P41140, Streptomyces fradiae; Q05308, Rarobacter faecitabidus; P0C1U8 and Q9FD08, Staphylococcus aureus; P00784, L. enzymogenes α-lytic protease; CAA00270 (GenBank accession number), Neisseria gonorrhoea; Q9EZE7, Escherichia coli; P00784, Carica papaya. Papain was selected as an outgroup. Protease clans (PA and CA) and families (S1E, S1B, S1D and S6) are given in the right-hand side of the figure in accordance with the MEROPS peptidase database (34), http://merops.sanger.ac.uk/).

Fig. 3. Alignment of amino acid sequences of Lysobacter sp. XL1 lytic endopeptidases AlpA, AlpB and L. enzymogenes α-lytic protease (GenBank acc. #P00778). Sequences corresponding to signal peptides, pro regions and mature enzymes are shown. The amino acid residues of the catalytic His-Asp-Ser triad are boxed.

Fig. 4. Expression of genes of Lysobacter sp. XL1 lytic endopeptidases AlpA and AlpB in E. coli. A lytic activity test is shown. Cells of E. coli BL21(DE3) transformed with vector pET-29a (3) or with plasmids pALPI-29a (2) or pALPII-29 (1) bearing alpA or alpB genes, respectively, were grown on an
agarized medium containing cell walls of *S. aureus* 209-P (see Materials and methods). Lytic activity is seen as clarification zones around colonies.

Fig. 5. Analysis of translation and transcription signals for *alpA* (1) and *alpB* (2) of *Lysobacter* sp. XL1. A. Fragments of the 5′-UTR sequences of *alpA* (2391–2407 bp) and *alpB* (4190–4205 bp) mRNAs and sequence at the 3′-end of 16S rRNA of *Lysobacter* sp. Shinshu-th3 (1530–1543 bp, GenBank # AB121774) are shown. The putative ribosome-binding site in mRNA of *alpA* and *alpB* is bolded and underlined. The start codon is bolded, italicized and underlined. B. Fragments of the 3′-UTR sequences of *alpA* (3628–3656 bp) and *alpB* (5430–5453 bp) mRNAs capable of forming stem–loop structures are shown. The sequences were analyzed using the UNAFold package (27), http://mfold.rna.albany.edu/). Hairpin energy (in kcal/mol) is given on the right. Positions of the ribosome-binding sites and stem–loop structures are given in accordance with GenBank sequence # GU188567. C. Analysis of *alpA* and *alpB* regions –35 and –10 as compared with the corresponding consensus sequences for *E. coli* σ70 promoter (bolded and underlined). The transcription start point (+1) is bolded.

Fig. 6. Transcription analysis of *alpA* and *alpB* expression. Total RNA of *Lysobacter* sp. XL1 cells in the mid (1) and late (2) logarithmic growth phase was hybridized with 32P-labelled probe to *alpA* or *alpB.*
Table 1. Analysis of 5′-ends of *alpA* and *alpB* mRNAs by the 5′-RACE protocol.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Positions of transcription start points*</th>
<th>5′-UTR length, nt</th>
<th>Number of clones</th>
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<tr>
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<tr>
<td></td>
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</table>

*Given in accordance with GenBank acc. #GU188567.