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Cloning and controlled overexpression of the gene encoding the 35 kDa soluble lytic transglycosylase from *Escherichia coli*

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Abstract **The lytic transglycosylases of *Escherichia coli* are involved in peptidoglycan metabolism and resemble the lysozymes not only in activity, but in the case of the 70 kDa soluble lytic transglycosylase (Slt70), also structurally. Here we report the cloning of the gene that encodes the 35 kDa soluble lytic transglycosylase (Slt35) of *E. coli*. Based on the sequence of the full-length gene, Slt35 is very likely to be a proteolytically truncated form of a slightly larger protein. The homology between Slt35 and Slt70, albeit poor, indicates that the active site architecture of both proteins may be alike. Using the T-7 promoter system, Slt35 was overproduced in large quantities and purified to homogeneity for crystallographic purposes.**

Key words: Lytic transglycosylase; Peptidoglycan metabolism; Lysozyme; *Escherichia coli*

1. Introduction

The peptidoglycan layer of bacteria is a polymeric macromolecule that is composed of glycan strands of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid, cross-linked by short peptide bridges (for a recent review see [1]). This macromolecule encloses the bacterial cell, thereby serving as a casing that protects the cell from mechanical stress and allows it to maintain a high internal osmotic pressure. Although the protective nature of the peptidoglycan layer implies rigidity, flexibility is required for the cell to be able to grow and divide. These paradoxical characteristics are reflected in a complex metabolic machinery that involves the well-balanced action of an array of synthesizing and hydrolyzing activities. Of the different enzymes involved, most attention has been given over the years to the penicillin-binding proteins, as it is this class of enzymes that is the direct target of β-lactam antibiotics. The emergence of resistance against this extensively used class of antibiotics has, however, resulted in a quest for new antibacterial targets and the non-penicillin sensitive enzymes involved in the peptidoglycan metabolism can be considered potential candidates. Among these are the lytic transglycosylases, a class of peptidoglycan hydrolyzing enzymes that was originally identified in *Escherichia coli*. Based on the presence of genes homologous to the *E. coli slt* gene in *Enterobacter cloacae* and *Salmonella typhimurium* [2], and the release of the specific products of these enzymes by *Bordetella pertussis* [3] and *Neisseria gonorrhoeae* [4], members of this class are expected to be present in a broad range of Gram-negative bacteria. The activity of the lytic transglycosylases is the cleavage of the β-glycosidic bond between the *N*-acetylglucosamine and *N*-acetylmuramic acid residue, an activity resembling that of the lysozymes, but in addition these enzymes introduce an internal 1,6-anhydro bond in the muramic acid residue of the monomeric muropeptide product [5]. Of the *E. coli* transglycosylases, the 70 kDa soluble protein (Slt70) has been the most extensively studied, and the three-dimensional structure of this enzyme has recently become available [6]. The activity of Slt70, however, does not seem to be essential for growth of *E. coli* under laboratory conditions and the only known inhibitor of this enzyme, bulgecin, is not an antibiotic in its own right but only shows synergistic antibacterial activity in combination with β-lactam compounds [7]. The reason for this may be the presence of at least two more transglycosylases in *E. coli* that are not inhibited by bulgecin, and may substitute for the activity of Slt70 [8–10]. From an antibacterial point of view, the main object will therefore be to tackle this class of enzymes as a whole, and for this purpose more functional and structural information is needed about the two smaller enzymes.

This report describes the cloning of the gene coding for one of those, the 35 kDa soluble lytic transglycosylase (Slt35). After confirmation of the transglycosylase activity of the gene product, a stable form of the protein was purified for further crystallographic studies.

2. Materials and methods

2.1. Molecular cloning methods

General DNA manipulations and cloning procedures were performed according to described protocols [11]. Plasmid DNA was prepared using Qiagen (Hilden, Germany) purification systems. Dideoxy sequencing was performed using the Sequenase version 2.0 kit (Amersham, Buckinghamshire, UK), following the manufacturers instructions. The Chameleon system (Stratagene, La Jolla, CA, USA) was used to introduce site-directed mutations. PCR reactions were performed with AmpliTaq DNA-polymerase and reagents from Perkin Elmer (Norwalk, CT, USA) with the following cycling protocol: melting at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min for a total of 30 cycles.

2.2. Cloning and sequencing of the Slt35 encoding gene and surrounding region

Two degenerate oligonucleotides (*A-11:* 5′-CAYAAYGTNATGC-ARATGGG-3′; *A12:* 5′-RTCRATRAAYTGYTGNGC-3′), the sequences of which were based on the N-terminal amino acid sequence of Slt35 [8], were used to amplify a 63 base pair fragment. The amplified fragment was sequenced, reamplified to incorporate [α^{32}P]dATP, and was used as a probe in a Southern hybridization procedure on *KpnI* and *PstI* digested genomic DNA of *E. coli* strain 122-1, which has a deletion for the gene coding for Slt70 [12]. Two subgenomic libraries were constructed by cloning of genomic DNA fragments of the size range corresponding to that of the hybridization signal into pUC18 (Pharmacia, Upsalla, Sweden). After transformation into super competent XL1-blue (Stratagene, La Jolla, CA, USA), the transformant colonies were screened using the same 63-mer as a probe. One positive clone from each library was selected for further analysis, and the gene

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coding for Slt35 plus the surrounding region was sequenced by primer walking.

2.3. Overproduction and purification

Using site-directed mutagenesis, an *Nco*I site was constructed, changing the codon for Leu-40 into an ATG start codon (primer *A62*: 5'-GCGGCTCAACCATGGAGCCGCCAGACCGGT-3'). For selection of the mutant strand, an oligonucleotide mutating the unique *Bsa*I site in the ampicillin resistance gene of pUC18 was used (primer *A44*: 5'-GGTGAGCGTGGTTCTCGCGGTAT-3'). The 1.9 kb *Nco*I fragment of the resulting plasmid pAD115 was cloned into the *Nco*I site of the vector pET21d+ (Novagen, Madison, WI, USA), thereby bringing the expression of Slt35 under the control of the T7 promoter [13]. The expression construct pAD121 was transformed into host BL21(DE3) and expression was induced by the addition of 1 mM IPTG. For the large scale purification, 60 g of induced cells (wet weight) were resuspended in 10 mM Tris-maleate-NaOH buffer, pH 7.3, disrupted by passage through an Aminco French pressure cell at 15,000 psi, and the soluble fraction was obtained by centrifugation at 100,000 × *g* for one hour. The soluble fraction was loaded on a 80-ml SP-sepharose fast flow column (Pharmacia, Uppsala, Sweden) and bound protein was eluted by applying a linear gradient of 0–1 M NaCl in 10 mM Tris-maleate-NaOH. Fractions containing Slt35 (eluting at 0.2 M NaCl) were pooled, dialyzed against 10 mM phosphate buffer, pH 6.8, and loaded in three consecutive runs on a 20 ml Bio-scale hydroxylapatite column (Bio-Rad Laboratories, Hercules, CA, USA). Using a linear gradient from 10 mM to 500 mM phosphate, bound proteins were resolved and only the peak fractions containing Slt35 in its purest form (eluting at 150 mM phosphate) were pooled, yielding a total of 310 mg of Slt35.

2.4. Peptidoglycan hydrolase assay, product analysis and zymography

Peptidoglycan hydrolase activity was determined by measuring solubilization of radiolabeled peptidoglycan polymer following a published procedure [14].

HPLC analysis of the produced muropeptides was performed essentially as described [8]: isolated peptidoglycan was degraded with purified Slt35 and the released muropeptides were separated by HPLC, using a linear gradient from 0.05% trifluoroacetic acid to 0.035% trifluoroacetic acid containing 20% acetonitrile over 60 min. The retention times of the released muropeptides were compared with those of purified monomeric 1,6-anhydro-muropeptides, of which the structure had been confirmed by tandem electron-spray mass spectroscopy.

Zymogram analysis of crude extracts and purified protein was carried out essentially as described [15], using 10% PAA gels, loaded with 0.03% purified *E. coli* peptidoglycan.

3. Results and discussion

Based on the N-terminal sequence information, two overlapping clones carrying the full-length gene coding for the 35 kDa soluble lytic transglycosylase were isolated. Sequence comparison revealed that the first 276 base pairs of the coding region were present in the GenBank database, as being part of the upstream region of the *gut* operon, although the authors did not identify the open reading frame [16]. This information allowed mapping of the gene at 60.8 min on the *E. coli* chromosomal map, between the *gut* operon and the *recA* gene [17] (Fig. 1). During preparation of this manuscript, a sequence was submitted to GenBank (accession number U18785), which covers the same region. The gene coding for Slt35 is called *mltB* by these authors and to avoid confusion, we will use this gene name in the future.

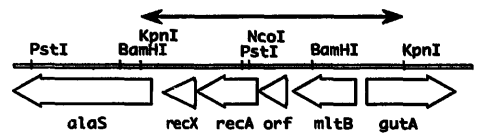

Fig. 1. Mapping position of the Slt35-encoding *mltB* gene. The 60.8 min region of the *E. coli* chromosomal map between *alaS* and *gutA* is depicted. The position and direction of transcription of the genes in this region is indicated by the open arrows, whereas the solid arrow represents the 4.8 kb *Kpn*I fragment that was cloned and from which the sequence of the *mltB* gene was determined.

Primary sequence analysis revealed that the gene product is a very positively charged protein (a calculated pI of 9.4 for the precursor protein). This positive charge may ensure an optimal interaction with the peptidoglycan substrate, which is highly negatively charged.

The spacing between the published N-terminal sequence and the first possible ATG start codon upstream is unusually large. Although this possible start codon is followed by a stretch of sequence displaying the characteristics of a consensus signal sequence, i.e. a short stretch of sequence containing positively charged residues followed by a longer hydrophobic stretch [18], no consensus signal peptidase cleavage site at the start of the published N-terminal sequence could be detected. Rather, directly following the hydrophobic stretch, a possible cleavage site for the lipoprotein signal peptidase II is present [19]. The cysteine of this cleavage site is separated from the first residue of the published N-terminal sequence by 20 amino acid residues (Fig. 2). It therefore seems likely that the protein encoded by the *mltB* gene is a lipoprotein and that the lipid anchor and the active domain are linked by a proteolysis-prone spacer. This

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MFKRRYVTLLPLFVL LAACSSKPKPTETDT TTGTASGGFLLEPQH NVMQMGGDFANNPNA QQFIDKMVNKHGFDR  75

QQLQEILSQAKRLDS VLRLMDNQAPTTSVK PPSGPNGAWLRYRKK FITPDNVQNGVVFWN QYEDALNRAWQVYGV 150

PPEIIVGIIGVETRW GRVMGKTRILDALAT LSFNYPRRAEYFSG- ELETFLLM--ARDEQ DDPLNLKGSFAGAMG 222
                                487 YTSGK EIPQSYAMAIARQES AWMPKVK-SPVGASG

YGQFMPSSYKQYAVD FSGDGHINLWDPVDA IGSVANYFKAHGWVK GDQVAVMANGQAPGL PNGFKTLYSISQLAA 297
LMQIMPGTATHTVKM FSIPG  540

AGLTPQQPLGNHQQA SLLRLDVGTGYQYWY GLPNFYTITRYNHST HYAMAVWQLGQAVAL ARVQ           361
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Fig. 2. Amino acid sequence of the *mltB* gene product and alignment with Slt70. The putative signal peptidase cleavage site is indicated by an arrow. The arrow head marks the N-terminus of the overproduced protein. Of the alignment with Slt70, only the region containing the two conserved motifs (underlined) is shown (see text for further details). Identical residues are indicated by bars.

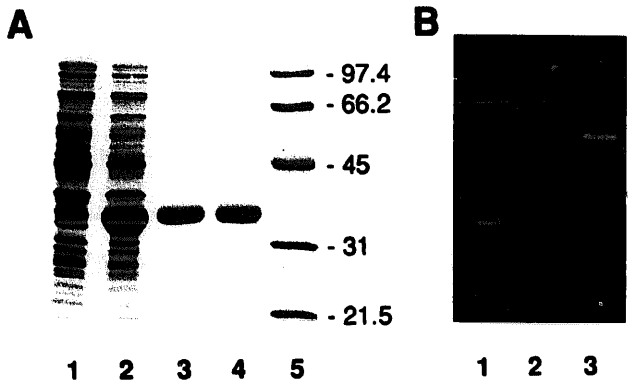

Fig. 3. Purification of overexpressed Slt35. (A) Analysis of cell homogenates and pooled fractions by SDS-polyacrylamide gel electrophoresis. Lanes 1 and 2 = total cellular protein (50 μg) of strain BL21(DE3) containing plasmid pAD121 before and after induction, respectively; lanes 3 and 4 = 10 μg of protein from pooled fractions after elution from SP-sepharose and hydroxylapatite, respectively; lane 5 = molecular mass markers (sizes given in kilodaltons). (B) Zymogram analysis of overexpressed and purified Slt35. Lane 1 = total protein (2.5 μg) after induction; lane 2 = 1.25 μg purified Slt35; lane 3 = 1.25 μg purified Slt70 serving as a control. Cleared zones indicate peptidoglycan hydrolysis activity.

hypothesis, and the issue of whether the clipping of the linker region is a purification artifact or has physiological relevance, remains to be investigated.

Downstream of the *mltB* gene a possible open reading frame of unknown function was identified and database searching using the BLAST algorithm [20] showed that this open reading frame is significantly homologous to an open reading frame, *ydeJ*, downstream of the *marB* locus [21] (data not shown). This homology sheds no light on the possible function of the protein encoded by the small open reading frame, as the function of *ydeJ* is not known either.

Although the described homology between the N-terminal sequence of Slt35 and a stretch of sequence in Slt70 suggested a high degree of sequence conservation between these proteins [8], the overall homology between the C-terminal part of Slt70 and Slt35 turns out to be less convincing, and neither does the sequence of Slt35 show significant homology to the proposed transglycosylase fingerprint [22]. Based on the fact that the catalytic mechanism of Slt70 and proteins that are structurally as well as functionally related to Slt70 involves a glutamic acid residue, we attempted to identify possible candidates for this residue in Slt35. It appeared that introduction of a large gap in the primary sequence of Slt70, thereby aligning the catalytic glutamic acid of Slt70 with Glu-206 of Slt35, results in a reasonably good alignment in the region comprising two of the three conserved boxes that were identified on basis of the structure of Slt70 and are indicative for the presence of a lysozyme fold (Fig. 2) [23]. The validity of this alignment is currently being investigated by mutagenesis of the proposed catalytically important Glu-206. The degree of conservation of the conserved boxes is, however, less in Slt35 than in a newly identified family of putative transglycosylases involved in a variety of transport processes (A. Dijkstra, unpublished) which suggest a more distant evolutionary relationship.

For crystallographic purposes, we set out to overexpress the protein in its soluble, stable form lacking the proteolysis-sensitive putative linker region. A very high degree of overproduction of soluble protein could be achieved using the T-7 promoter system [13] (Fig. 3, panel A). The protein was shown to be overproduced in an active form, as judged by its ability to break down polymeric peptidoglycan both in solution and after renaturation from SDS in a zymogram analysis (Fig. 3, panel B). The specific production of 1,6-anhydro-muropeptides by the overproduced protein could be demonstrated by means of HPLC (data not shown).

The high isoelectric point of Slt35 allowed selective binding to a strong cation exchanger, resulting in a very efficient purification of Slt35 from the soluble fraction of a lysate, as is shown in Fig. 3, lane 3. An additional purification step involving hydroxylapatite was applied to get rid of minor contaminating proteins. This two-step purification scheme was used to purify 100 mg amounts of Slt35 for future crystallographic studies.

It will be very interesting to see to what extent the structure of Slt35 resembles that of Slt70. The above-mentioned lack of high overall sequence homology does not exclude the possibility of a structural similarity, as has been shown to be the case for Slt70 and the G-type lysozymes [23]. If it indeed turns out that Slt35 also contains a lysozyme-like domain, the question remains as to the function of the N-terminal-half of the protein. Furthermore, obtaining structure–function information on Slt35 will help to elucidate the catalytic mechanism of the whole class of enzymes, as the precise mechanism of the transglycosylases and even that of the lysozymes is still a matter of debate [24], even though the latter mechanism is considered to be text-book knowledge.

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