JOURNAL OF BACTERIOLOGY, Oct. 1992, p. 6394–6403
0021-9193/92/206394-10$02.00/0

Vol. 174, No. 20

Murein-Metabolizing Enzymes from *Escherichia coli*: Existence of a Second Lytic Transglycosylase

HENK ENGEL, AIZE J. SMINK, LEEN VAN WIJNGAARDEN, AND WOLFGANG KECK*

Department of Biochemistry, BIOSON Research Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Received 27 February 1992/Accepted 30 July 1992

In addition to the soluble lytic transglycosylase, a murein-metabolizing enzyme with a molecular mass of 70 kDa (Slt70), *Escherichia coli* possesses a second lytic transglycosylase, which has been described as a membrane-bound lytic transglycosylase (Mlt; 35 kDa; EC 3.2.1.—). The *mlt* gene, which supposedly encodes Mlt, was cloned, and the complete nucleotide sequence was determined. The open reading frame, identified on a 1.7-kb *SalI-PstI* fragment, codes for a protein of 323 amino acids (M_r = 37,410). Two transmembrane helices and one membrane-associated helix were predicted in the N-terminal half of the protein. Lysine and arginine residues represent up to 15% of the amino acids, resulting in a calculated isoelectric point of 10.0. The deduced primary structure did not show significant sequence similarity to Slt70 from *E. coli*. High-level expression of the presumed *mlt* gene was not paralleled by an increase in murein hydrolase activity. To clarify the identity of the second transglycosylase, we purified an enzyme with the specificity of a transglycosylase from an *E. coli slt* deletion strain. The completely soluble transglycosylase, with a molecular mass of approximately 35 kDa, was designated Slt35. Its determined 26 N-terminal amino acids showed similarity to a segment in the middle of the Slt70 primary structure. Polyclonal anti-Mlt antibodies, which had been used for the isolation of the *mlt* gene, were found to cross-react with Mlt as well as with Slt35, suggesting that the previously described Mlt preparation was contaminated with Slt35. We conclude that the second transglycosylase of *E. coli* is not a membrane-bound protein but rather is a soluble protein.

When bacteria are growing or dividing, bonds must be cleaved in the murein layer to create sites for the insertion of new material into this macromolecule surrounding the cell. Among the various murein-metabolizing enzymes identified in *Escherichia coli*, only two glycan strand-degrading enzymes and two endopeptidases degrade the intact murein polymer in vitro (21). The two glycan strand-degrading enzymes are also known as the lytic transglycosylases, the soluble lytic transglycosylase (Slt), with a molecular mass of 70 kDa, being compartmentalized in the periplasm (10, 49), and the membrane-bound lytic transglycosylase (Mlt), with a molecular mass of approximately 35 kDa, described to be present in the cytoplasmic membrane as well as in the outer membrane of *E. coli* (19, 24, 37). Both transglycosylases catalyze the cleavage of the β-1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine residues, an activity comparable to that of various lysozymes. However, the bacterial transglycosylases catalyze, in addition, an intramolecular transglycosylation reaction at the *N*-acetylmuramic acid residue, resulting in the formation of a 1,6-anhydro bond. This fact has led to the proposal that the transglycosylases may play an important role in the recycling of muropeptides in the periplasm during cell elongation and division (16).

Studies on the interesting enzymatic activities of the lytic transglycosylases will contribute to a better understanding of the metabolism of murein. Furthermore, the lytic transglycosylases can be used for the production of pharmacologically active compounds via the specific degradation of murein (12). 1,6-Anhydro-muropeptides have been shown to exhibit sleep-promoting activities (26, 33) and are released by some human pathogens, eliciting cytotoxic effects on ciliated epithelial cells in higher organisms (7, 36). The *slt* gene, encoding the soluble lytic transglycosylase, was cloned (2), and the nucleotide sequence was recently determined (10). The enzyme was purified from an overproducing strain, and crystals of X-ray quality were obtained (10, 41).

This study concerns the membrane-bound lytic transglycosylase, which was described to be associated with the cytoplasmic and outer membranes but to be active only when present in the outer membrane (19).

In the present paper, we describe the cloning, nucleotide sequence, and controlled overexpression of the presumed *mlt* gene and propose, on the basis of the obtained data, that the deduced open reading frame does not encode the membrane-bound lytic transglycosylase but encodes a 37-kDa protein with an unknown function.

Nevertheless, in *E. coli* 122-1 (40), which is chromosomally deficient in the *slt* gene, encoding the soluble lytic transglycosylase, each *N*-acetylmuramic acid end of the glycan strands is still of the 1,6-anhydro type (18a), indicating the presence of a second transglycosylase in *E. coli*.

In *E. coli*, only the soluble lytic transglycosylase has been described as a glycan strand-degrading soluble murein hydrolase, being enzymatically active, in vitro, on the purified murein polymer, releasing low-molecular-weight 1,6-anhydro-muropeptides. Murein hydrolase activity was still detected in the soluble protein fraction of an *E. coli* strain that carries a chromosomal deletion of the *slt* gene. We describe the purification from this *E. coli* strain of a soluble murein hydrolase that has a molecular mass of approximately 35 kDa and that has in vitro enzymatic activity on the isolated murein polymer, releasing 1,6-anhydro-muropeptides. To avoid confusion, we designate the soluble lytic transglycosylases Slt70 and Slt35, in accordance with their respective molecular masses. Data suggesting that this second soluble lytic transglycosylase (Slt35) is identical to the previously

* Corresponding author.

reported membrane-bound lytic transglycosylase (Mlt) are presented.

MATERIALS AND METHODS

Plasmids, bacterial strains, and media. The *E. coli* gene library, established in the runaway replication vector pWK12, has been described (2). Vectors M13mp18 and M13mp19 were those used by Vieira and Messing (46). Phasmids pMa5-8 and pMc5-8 and plasmid pJRD187 have been described (8, 44). Helper phage M13KO7 was obtained from Pharmacia LKB Biotechnology Inc., Uppsala, Sweden (47). Strain CJ236 was purchased from Bio-Rad Laboratories, Richmond, Calif. *E. coli* 122-1, chromosomally deficient in the *slt* gene, encoding the soluble lytic transglycosylase, was obtained from Roeder and Sommerville (40). Wild-type *E. coli* DSM1576 was purchased from DSM, Braunschweig, Germany. *E. coli* strains were grown in Luria-Bertani medium supplemented with the appropriate antibiotics (32).

Materials. [α-^{35}S]thio-dATP was purchased from Amersham International, Amersham, United Kingdom. Unlabeled deoxynucleotide triphosphates were purchased from Pharmacia Laboratories, Uppsala, Sweden, and dideoxynucleotides were obtained from Boehringer, Mannheim, Germany. All restriction endonucleases, exonuclease *Bal*31, T4 DNA ligase, Klenow DNA polymerase, and T4 polynucleotide kinase were purchased from Boehringer. Sequenase was obtained from United States Biochemical Corp., Cleveland, Ohio. All enzymes were used under the conditions recommended by the suppliers. *N*-Succinimidyl[2,3-^{3}H]propionate was purchased from Amersham. The high-pressure liquid chromatography (HPLC) standards GlcNAc (β-1,4)1,6-anhydro-MurNAc-tripeptide and GlcNAc(β-1,4)1,6-anhydro-MurNAc-tetrapeptide were kind gifts of J.-V. Höltje (Max-Planck-Institut, Tübingen, Germany).

Radioactive labeling of the isolated murein polymer. Murein was isolated from wild-type *E. coli* DSM1576 as described previously (12). The isolated murein was labeled with tritium in a manner similar to the procedure by which the Bolton-Hunter reagent is used to iodinate proteins (5). The isolated murein (24 mg) was treated with *N*-succinimidyl[2,3-^{3}H]propionate (37 MBq; specific activity, 3.40 TBq/mmol), which reacts with free amino groups in murein, which are present only on diaminopimelic acid, the third amino acid of the stem peptides of *E. coli* murein, leaving diaminopimelic acid ^{3}H propionated. The reaction was performed with 0.1 M sodium borate buffer (pH 8.5) with stirring for 15 min. Unincorporated label was subsequently removed by several washes with water until the 15,000 $\times$ *g* supernatant was free of label. The 15,000 $\times$ *g* murein pellet was resuspended in water containing 0.02% NaN_3 at a concentration of 1.3 μg/μl. The specific activity of ^{3}H-propionated murein was 7,500 cpm/μg of murein.

DNA isolation and transformation. Plasmid DNA was purified by cesium chloride-ethidium bromide density gradient centrifugation (32). The alkaline extraction procedure was used to prepare small-scale plasmid DNA (3). *E. coli* host strains were transformed with plasmids as described previously (31).

DNA sequencing. Restriction endonuclease fragments or fragments created by exonuclease *Bal*31 treatment of the 1.7-kb *Sal*I-*Pst*I chromosomal insert from pWK71 were subcloned into M13mp18 or M13mp19 and sequenced by the dideoxyribonucleotide chain termination reaction with Sequenase (42). Both strands were completely sequenced. DNA sequences were analyzed with the PCGENE program (Genofit, Geneva, Switzerland). Amino acid sequences were compared with the Swiss-Prot protein data base (EMBL, Heidelberg, Germany) by use of the FASTA program (30).

Construction of an overproducing plasmid and controlled overexpression. The 1.1-kb *Sal*I-*Nru*I fragment of pWK71, carrying the presumed *mlt* gene, was cloned into pHEMa101 linearized with *Sal*I and *Xba*I, the *Xba*I site having been blunted with Klenow DNA polymerase. Plasmid pHEMa 101, in which the thermosensitive phage lambda repressor (*c*I857), the p_R promoter, the Shine-Dalgarno sequence, and the ATG start codon of the phage lambda *cro* gene, together with a multiple cloning site, are cloned in the pMa5-8 mutagenesis vector, has been described (11). In addition, the *Bam*HI site was converted to an *Asp*718 restriction site. Cloning of the presumed *mlt* gene in pHEMa101 resulted in the 5.8-kb plasmid designated pHEMa300.

Site-directed mutagenesis was performed exactly as described by Kunkel et al. (27). For the incorporation of uridine, strain CJ236 was used as a host for plasmid pHEMa300; helper phage M13KO7 was used for the production of single-stranded DNA (47). The synthetic oligonucleotide 5′-TTTTTTTCGGTACCATGCTTT-3′, carrying an *Asp*718 restriction site (underlined), was synthesized on a model 380B DNA synthesizer (Applied Biosystems, Warrington, United Kingdom) by Eurosequence (University of Groningen, Groningen, The Netherlands). The oligonucleotide was used to clone the gene of interest directly in-frame and downstream of the translation initiation signals of the phage lambda *cro* gene under the control of the phage lambda p_R promoter being regulated by the thermosensitive *c*I857 repressor. The 0.2-kb *Asp*718 fragment was subsequently deleted, resulting in the 5.6-kb plasmid designated pHEMa301.

E. coli HB101 harboring pHEMa101 or pHEMa301 was grown at 28°C in 500 ml of Luria-Bertani medium containing 100 μg of ampicillin per ml. When the cultures had reached the mid-exponential growth phase (optical density at 600 nm = 0.4), the temperature was shifted to 42°C. Growth was continued for 1 h, and cells were collected by centrifugation (15 min, 4,000 $\times$ *g*, 4°C), resuspended in 10 ml of 10 mM Tris-maleate-NaOH buffer (pH 6.8), disrupted in a French press at 10,000 lb/in^2 (homogenate), and centrifuged at 100,000 $\times$ *g* for 1 h. The soluble fraction and the pellet were subjected to further analysis, the latter being washed several times with the above-described buffer and ultimately resuspended in the same buffer containing 2% Triton X-100. A small volume of this suspension was centrifuged at 15,000 $\times$ *g*, resulting in a solubilized membrane fraction and an insoluble particulate fraction. Both fractions were assayed for murein hydrolase activity by use of the isolated murein labeled with [^{3}H]propionate as a substrate. Host strain HB101 carrying vector pHEMa101 served as a control.

Murein hydrolase assay. Murein degradation was assayed by measuring the release of low-molecular-weight muropeptides from the radiolabeled murein polymer as described for the soluble lytic transglycosylase in the absence of Triton X-100 (1).

Isolation of soluble murein hydrolase. Soluble murein hydrolase was isolated from *E. coli* 122-1 (40), deficient in the soluble lytic transglycosylase (Slt70). The culture was grown at 37°C in a 65-liter mobile-plant fermentor (New Brunswick, Edison, N.J.) containing Luria-Bertani medium supplemented with 0.5% glucose. The bacteria were stirred (200 rpm) and aerated (90 liters/min), and the pH of the culture was kept at 7.0. After reaching the early stationary phase

(optical density at 600 nm = 6.0), the cells were harvested by centrifugation in a continuous centrifuge. The yield from the fermentation was 870 g of cells (wet weight). A 450-g quantity of cells was resuspended in 10 mM Tris-maleate-NaOH buffer (pH 6.8) at 1 g (wet weight) per ml, disrupted in a French press at 10,000 lb/in^2, and centrifuged at 100,000 $\times$ *g* for 1 h. The soluble murein hydrolase was isolated from the soluble fraction. The soluble fraction (760 ml; 22.5 mg of protein per ml) was dialyzed against 10 mM Tris-maleate-NaOH buffer (pH 6.8) and loaded onto a carboxymethyl-Sepharose CL-6B column (volume, 500 ml) equilibrated with the same buffer. The murein hydrolase was eluted at a concentration of 0.3 M NaCl when a linear gradient of 0.01 to 0.6 M NaCl in the same buffer (total volume, 1 liter) was applied. After dialysis of enzymatically active fractions (95 ml; 0.65 mg of protein per ml) against 10 mM potassium phosphate (pH 6.8) (dialysis buffer), the second purification step was carried out on a hydroxylapatite column (volume, 20 ml) equilibrated with dialysis buffer. Fractions containing the murein hydrolase were eluted at a concentration of 0.1 M buffer when a gradient of 0.01 to 0.4 M potassium phosphate buffer (total volume, 100 ml) was applied. The enzymatically active fractions were pooled (27 ml; 0.46 mg of protein per ml) and dialyzed against 20 mM Tris-HCl buffer (pH 7.5). The final purification step was performed on a fast protein liquid chromatography system (Pharmacia Laboratories) by use of a Mono Q HR 5/5 column (5 mm by 5 cm) with portions of 2 ml. The murein hydrolase was eluted at a concentration of 0.18 M NaCl when a linear gradient of 0.0 to 0.3 M NaCl (21 min; flow rate, 1 ml/min) in dialysis buffer was applied, yielding 340 μg of purified murein hydrolase.

Product analysis of murein hydrolase. The isolated murein (60 μg) was degraded with the purified murein hydrolase in 10 mM potassium phosphate buffer (pH 6.8). The products were analyzed by HPLC as described previously (15). Fractionation was performed at 55°C on a 4-μm Supersphere 100 RP-18 column (250 by 4 mm) with a gradient optimized for the separation of 1,6-anhydro-muropeptides, obtained by the degradation of murein with Slt70 (12); the gradient ranged from methanol-free 50 mM sodium phosphate (pH 4.3)

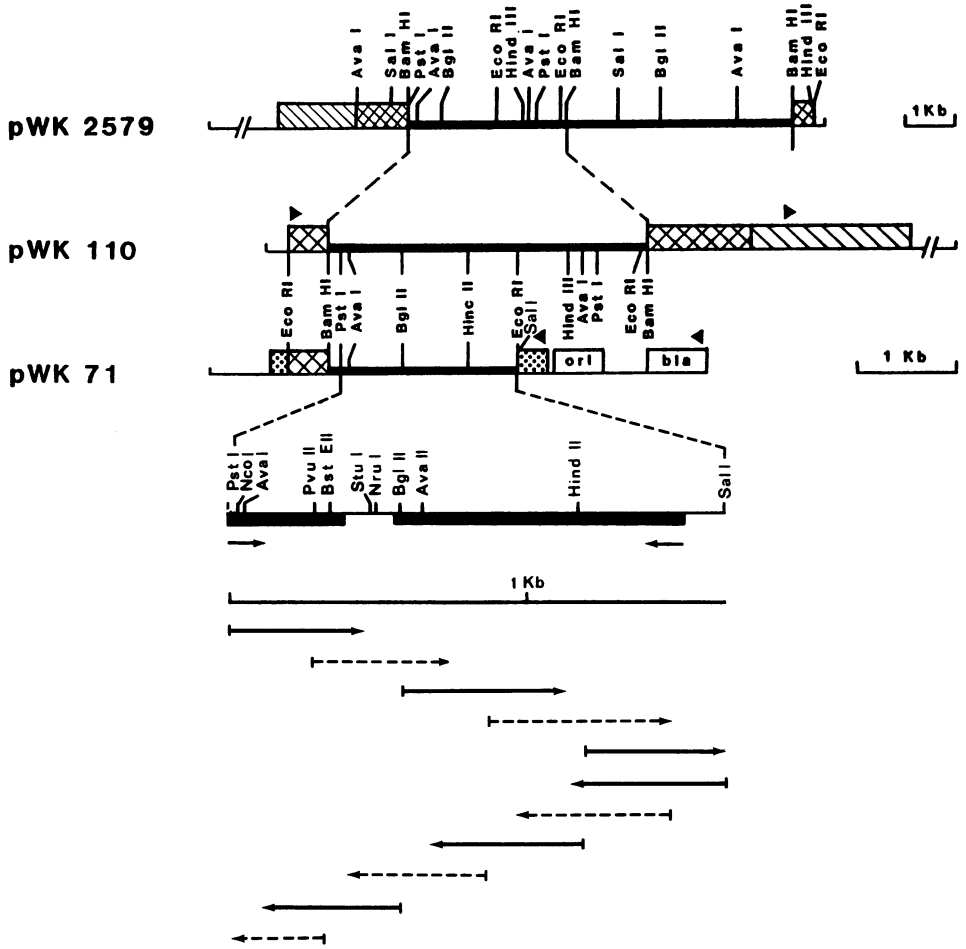

FIG. 1. Restriction map of plasmid pWK2579 and the strategy used for sequencing the presumed *mlt* gene. The subcloning is described in Results. The 1.7-kb *SalI-PstI* DNA fragment of plasmid pWK71, containing the complete *mlt* gene and a truncated C-terminal open reading frame, is shown. The two coding regions are indicated by thick bars. Initially, bases were determined from the *SalI* and *PstI* sites in one direction and from the *Hin*dII and *Bgl*II sites in both directions (long solid arrows). To read bases that were not obtained from these fragments, we performed *Bal*31 exonuclease digestions from the *SalI* and *PstI* sites to produce fragments that allowed further reading (broken arrows). The two small solid arrows and the arrowheads indicate the direction of transcription. The origin of replication is indicated by ori, and bla indicates the β-lactamase gene; the other regions are derived from lambda (▨ in pWK2579), Tn*3* (▨ in pWK110), R1 (—), pBR322 (▩), and the β-galactosidase gene (▥).

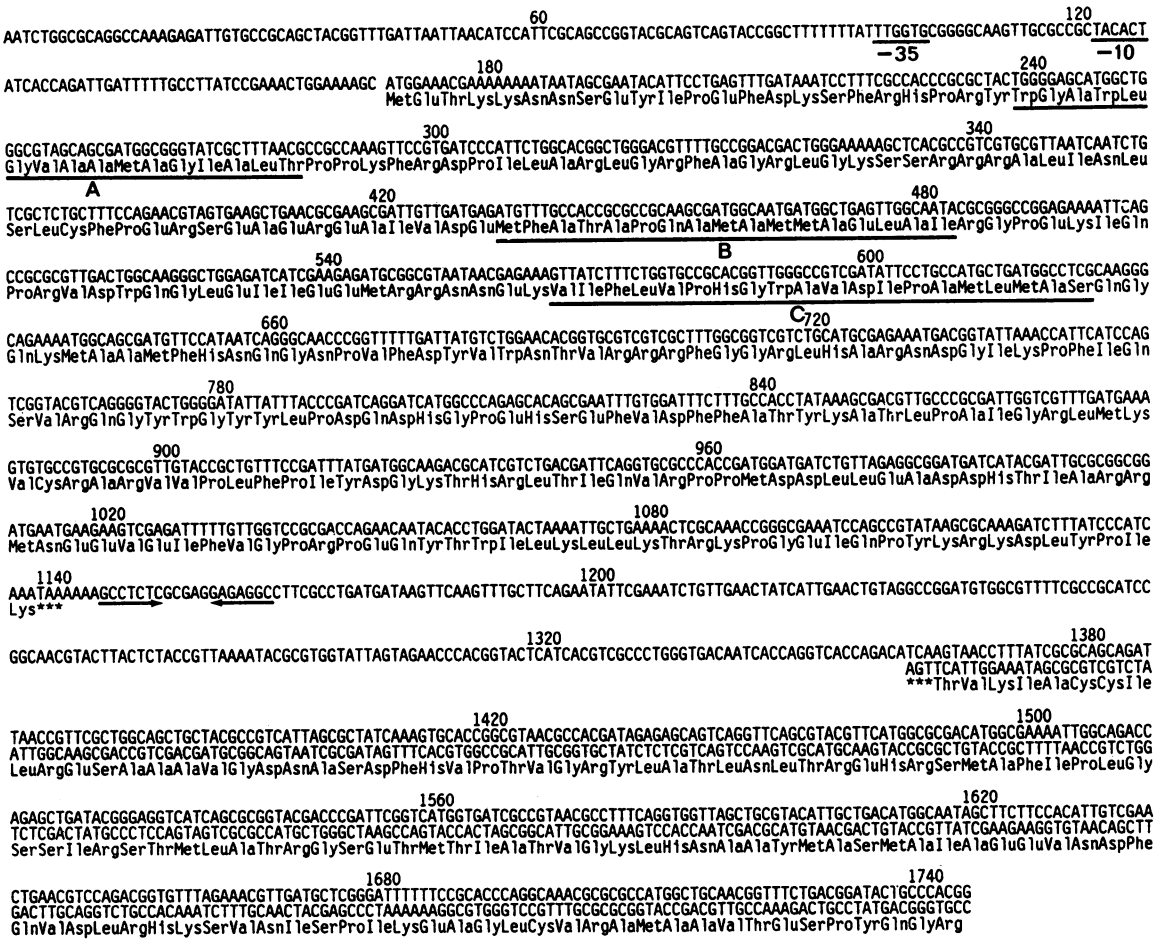

FIG. 2. Nucleotide sequence of the presumed *mlt* gene and deduced amino acid sequence. Shown is the 1,743-bp *SalI-PstI* fragment of plasmid pWK71. The coding region for the 37-kDa protein starts at nucleotide 168 (ATG) and ends at nucleotide 1137 (TAA). An additional C-terminal open reading frame, translated in the opposite direction, ends at nucleotide 1361 (TGA). The corresponding amino acids are shown directly below the triplet codons in the three-letter code. The complementary strand of the C-terminal open reading frame is also shown. The proposed medium-strength promoter of the presumed *mlt* gene is underlined and designated −35 and −10. The putative transcriptional terminator sequence is indicated with horizontal arrows. The asterisks indicate the translation termination triplets. The predicted transmembrane helices are underlined and designated A and B; the predicted membrane-associated helix is underlined and designated C.

containing 1.0 mg of sodium azide per ml (buffer A) to 75 mM sodium phosphate (pH 5.0) containing 15% methanol (buffer B). The flow rate was 1.2 ml/min for 75 min: from min 0 (100% A, 0% B) to min 5 (85% A, 15% B), from min 5 to min 40 (50% A, 50% B), and from min 40 to min 75 (0% A, 100% B). For comparison of the obtained muropeptides with the Slt70 degradation products, murein was also degraded with the previously isolated Slt70 (10).

Protein analysis. The method of Bradford was used to determine the protein concentrations (6). Proteins from the different fractions were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (29). The proteins were either stained with Coomassie brilliant blue R-250 or transferred to nitrocellulose filters and stained immunochemically (4) with polyclonal antibodies raised previously against either the isolated Mlt or the penicillin-insensitive murein endopeptidase (MepA) (23, 24).

N-terminal sequence analysis of soluble murein hydrolase. N-terminal sequence analysis was carried out with 1 nmol of soluble murein hydrolase (Slt35) that had been electrotransferred from a 10% SDS-polyacrylamide gel onto a polyvinylidene difluoride membrane (34). Sequencing was done with an Applied Biosystems model 477A protein sequencer (pulsed-liquid sequencer) connected on-line to a 120A phenylthiohydantoin analyzer (Eurosequence).

The N-terminal amino acid sequence was compared with the primary structure of Slt70 by use of the FASTA program (30).

Nucleotide sequence accession number. The primary nucleotide and amino acid sequence data reported in this paper have been deposited in GenBank (Los Alamos National Laboratory) and are available under accession number M87660.

RESULTS

Presumed *mlt* gene and its nucleotide sequence. The presumed *mlt* gene was isolated from an *E. coli* gene expression library as described for the *slt* gene (2) by screening immunochemically (18) a gene overexpression library established in a runaway replication vector with polyclonal antibodies (24). HB101 harboring plasmid pWK2579 overexpressed a 35-kDa protein, detectable on Western blots (immunoblots)

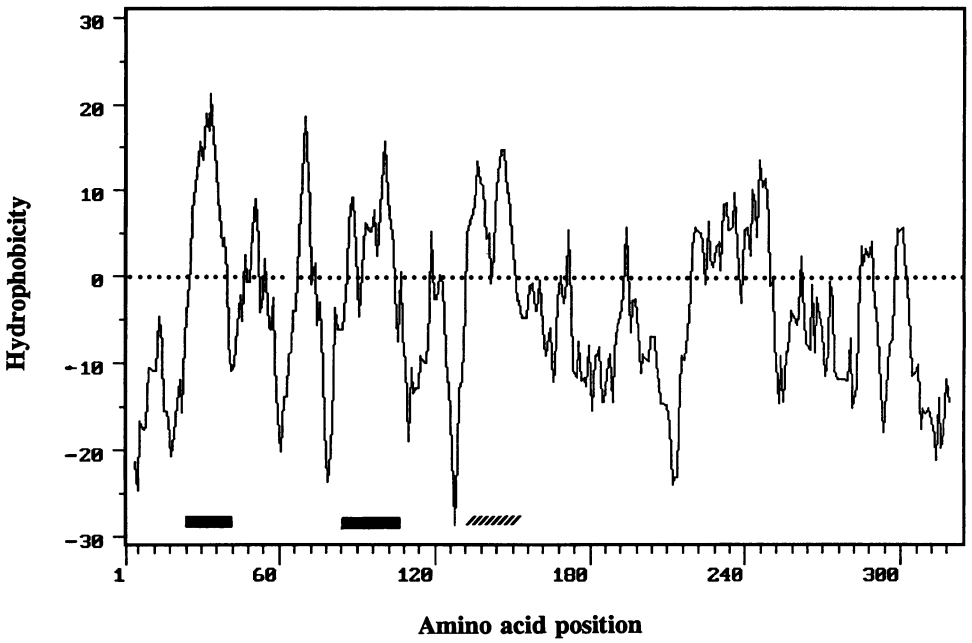

FIG. 3. Hydropathy plot of the 37-kDa protein. The relative hydrophobicities of the amino acids are depicted with an interval of 9 amino acids (28). The positions of the two putative transmembrane helices are indicated by black boxes, and that of the putative membrane-associated helix is indicated by a striped box.

with polyclonal antibodies raised against purified Mlt (19). The presumed *mlt* gene was localized by subcloning fragments of the insert in pWK2579 either into the *Bam*HI site of pWK12 (pWK110) or the multiple cloning site of pUC9 (pWK71) as shown in Fig. 1. Plasmid pWK71 overexpressed the 35-kDa protein (data not shown).

To determine the complete nucleotide sequence of the presumed *mlt* gene, we subcloned subfragments of the 1.7-kb *Sal*I-*Pst*I fragment of pWK71 into M13 vectors and then sequenced them by the method of Sanger et al. (42). The sequencing strategy is depicted in Fig. 1. The complete nucleotide sequence and the corresponding open reading frames are shown in Fig. 2. A large complete open reading frame and a truncated open reading frame were identified. The large one codes for a protein with a molecular weight of 37,410. Translation initiates at nucleotide 168 (ATG), terminates at nucleotide 1137 (TAA), and results in a polypeptide of 323 amino acid residues. A search for transmembrane helices (39) and membrane-associated helices (9) revealed that the 37-kDa protein possesses in its N-terminal half two putative transmembrane helices and one putative membrane-associated helix. The hydrophobicity plot along the amino acid sequence (28) is shown in Fig. 3.

In addition, a 3′ open reading frame was found in the opposite direction and encodes a truncated C terminus of 127 amino acid residues (Fig. 2).

Promoter sequence and translation initiation and transcription termination signals. A possible promoter sequence was identified upstream of the coding region, as shown in Fig. 2; it showed significant similarity to −35 and −10 consensus promoter sequences and was classified as a medium-strength promoter sequence (17, 38). A ribosome-binding site upstream of the translation initiation site and showing similarity to the consensus Shine-Dalgarno sequence was not found (43). A putative transcription terminator structure was identified downstream of the translation termination site and consisted of a GC-rich stem of 7 bp and a loop of 5 bases, as shown in Fig. 2.

Controlled overexpression of the presumed *mlt* gene. An overproducing plasmid was constructed as described in Materials and Methods with plasmids pHEMa101 and pWK71 (Fig. 4). Because of the creation of the *Asp*718 restriction site, the N terminus of the native 37-kDa protein (MetGluThrLys) is converted to MetValProLys. A 500-fold overproduction was achieved 1 h after temperature induction (42°C) (Fig. 5, lane 3). The degree of overproduction was estimated from Western blot analysis with various dilutions of the homogenates. The overproduced 37-kDa protein could be solubilized from the 15,000 × *g* pellet in 10 mM Tris-maleate-NaOH buffer (pH 6.8) containing 2% Triton X-100 (Fig. 5, lane 6). However, upon overproduction, no increase in murein hydrolase activity was detected in either of the fractions containing the overproduced protein.

Purification of the second soluble lytic transglycosylase (Slt35). The purification of Slt35 is summarized in Table 1. The increase in yield after the CM-Sepharose CL-6B column step in comparison with the yield from the soluble fraction might be explained by the presence of inhibitors in the soluble fraction. Therefore, the final purification (10,784-fold) and the final yield (21%) were in fact somewhat lower than calculated. The purification steps are shown in Fig. 6A and resulted ultimately in the purification of a 35-kDa protein. The 26 N-terminal amino acids of the 35-kDa protein were determined and are shown in Fig. 7A, starting with a leucine. At least the N-terminal methionine, if not a signal peptide, was cleaved off. A signal peptide should be cleaved off to transport soluble Slt35 into the periplasm, the compartment in which its substrate, the murein polymer, is localized.

Analysis of muropeptides released from the murein polymer by Slt35. The isolated murein polymer was degraded by purified Slt35. The obtained muropeptides were separated by HPLC as described in Materials and Methods. Concerning

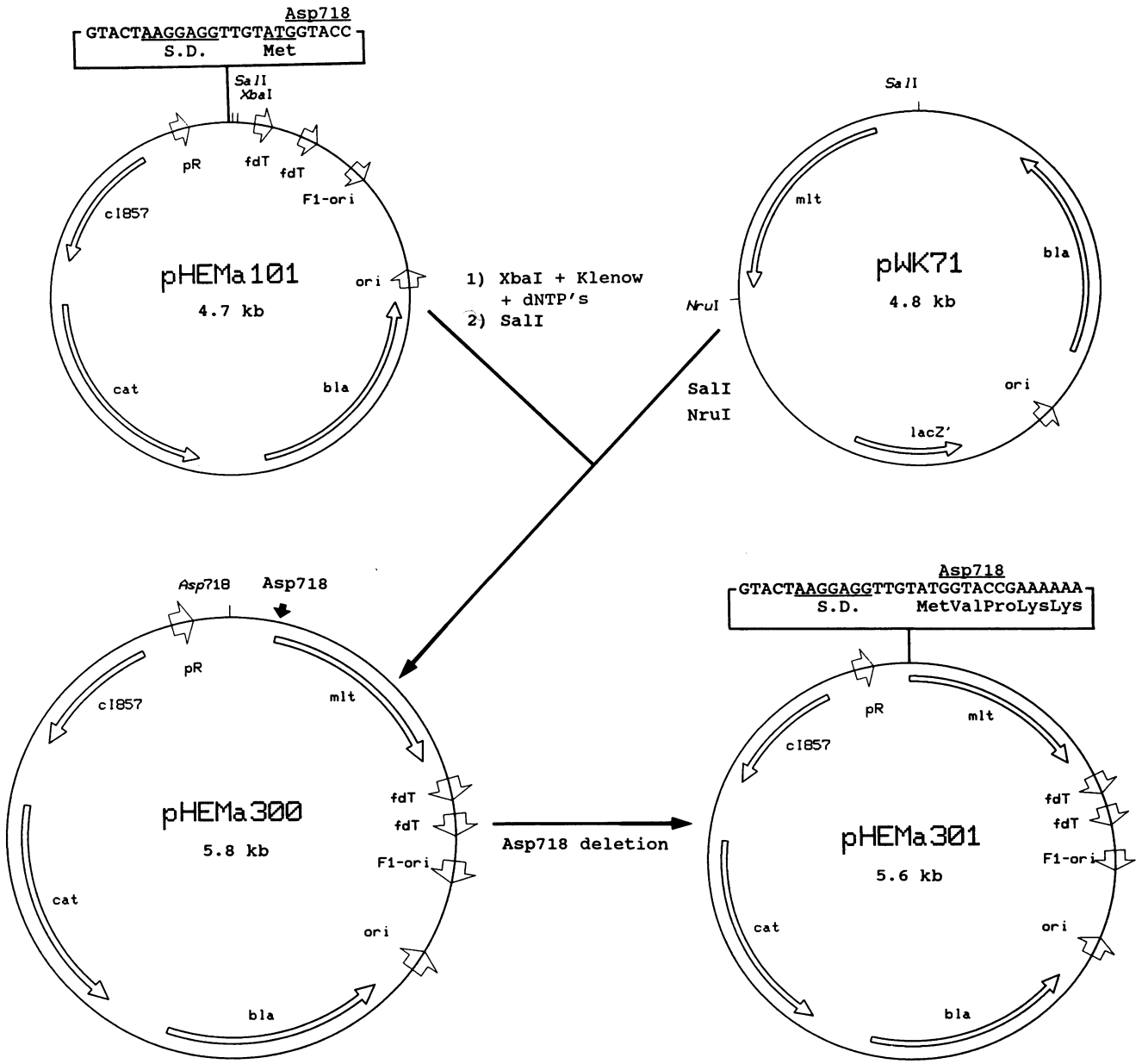

FIG. 4. Construction of plasmid pHEMa301, from which the 37-kDa protein was overproduced. Some restriction sites are indicated. Long open arrows show the relative positions of the genes coding for the 37-kDa protein (mlt), phage lambda repressor (cI857), β-lactamase (bla), chloramphenicol acetyltransferase (cat), and β-galactosidase (lacZ'). Short open arrows indicate the direction of transcription. The origin of replication of phage f1 (F1-ori), the central transcription terminator of phage fd (fdT), the ColE1 origin of replication (ori), and the phage lambda p_R promoter (pR) are also indicated. The nucleotide sequence of the Shine-Dalgarno ribosome-binding site (S.D.) and the triplet of the start codon (ATG) of the phage lambda *cro* gene as well as the flanking sequences of ATG are shown. The position of the created *Asp*718 restriction site is indicated by a closed arrow, and the nucleotide sequence and corresponding amino acid sequence obtained for pHEMa301 after deletion of the small *Asp*718 fragment from pHEMa300 are shown. dNTP's, deoxynucleoside triphosphates.

the monomeric muropeptides, the HPLC profile was identical to the profile of murein degraded by Slt70; all muropeptides were of the 1,6-anhydro type (Fig. 8). The disappearance of all dimeric 1,6-anhydro-muropeptides and the corresponding increase in the amount of monomeric 1,6-anhydro-muropeptides indicate the presence of an endopeptidase that can cleave both types of cross-bridges (D-Ala-*m*-DAP and *m*-DAP-*m*-DAP) present in murein. This enzymatic activity has been ascribed to MepA, the penicillin-insensitive murein endopeptidase (23). Western blot analysis and immunostaining of the Slt35 preparation with the MepA-specific antibodies revealed the presence of traces of MepA, an enzyme with a molecular mass of 30 kDa (Fig. 6B, lane 2).

DISCUSSION

Nucleotide sequence of the presumed *mlt* gene and deduced amino acid sequence. The largest open reading frame that

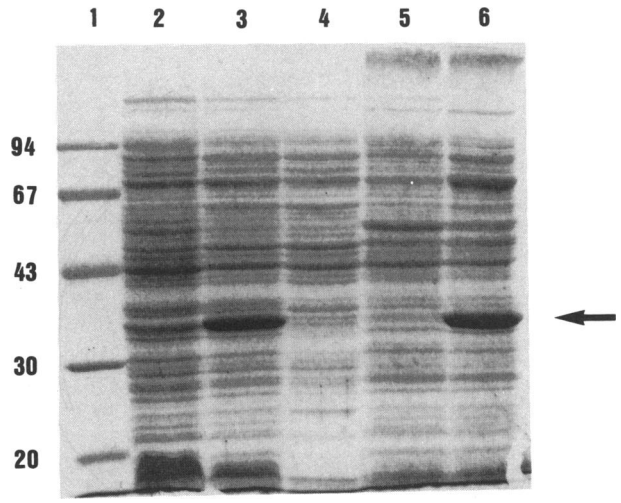

FIG. 5. Analysis of the overproduction of the 37-kDa protein by SDS-polyacrylamide gel electrophoresis. Lanes: 1, molecular mass markers, the masses being given in kilodaltons; 2 and 3, total cellular protein before and 1 h after temperature induction, respectively; 4, soluble fraction 1 h after temperature induction; 5 and 6, solubilized protein (in 2% Triton X-100) from the 15,000 × *g* pellet before and 1 h after temperature induction, respectively. Lanes 2 to 6 contained 20 μg of protein each. The arrow indicates the position of the overproduced 37-kDa protein. The 10% polyacrylamide gel was stained with Coomassie brilliant blue R-250.

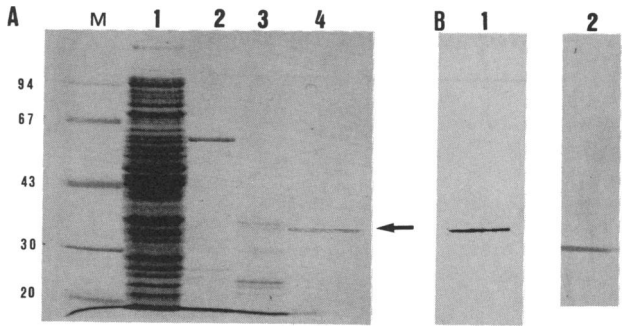

FIG. 6. Analysis of the purification of Slt35 by SDS-polyacrylamide gel electrophoresis (A) and Western blotting (B). (A) Lanes: M, molecular mass markers (the masses are given in kilodaltons); 1, total soluble protein of strain 122-1 (20 μg); 2, enzyme pool obtained after elution from CM-Sepharose CL-6B (7 μg); 3, enzyme pool obtained after elution from hydroxylapatite (5 μg); 4, enzyme pool obtained after elution from Mono Q HR 5/5 (0.5 μg). The 10% polyacrylamide gel was stained with Coomassie brilliant blue R-250. The arrow shows the position of Slt35. (B) Lanes 1 and 2, enzyme pool obtained after elution from Mono Q (0.5 μg) and detected with polyclonal antibodies previously raised against the isolated membrane-bound lytic transglycosylase (Mlt) and penicillin-insensitive murein endopeptidase (MepA), respectively.

contains the coding region for the structural gene that supposedly corresponds to the membrane-bound lytic transglycosylase codes for a protein of 323 amino acids. Analysis of the primary structure revealed that the N-terminal half of the encoded protein has a hydrophobic character (Fig. 3); the amino acid sequence possesses two putative regions long enough to span the membrane as transmembrane helices (39) and one putative region that could function as a membrane-associated helix (9).

The percentage of positively charged amino acids is relatively high; lysine and arginine residues represent up to 15% of the total amino acids in the protein, being distributed randomly over the complete primary structure. The isoelectric point, calculated on the basis of the complete primary structure, was estimated to be 10.0. This isoelectric point is in contrast to the previously experimentally determined isoelectric points of Mlt in the cytoplasmic membrane fraction and in the outer membrane fraction, 6.7 and 5.8, respectively (19).

The primary structure of the 37-kDa protein was compared with those of other proteins present in the Swiss-Prot protein sequence data base (21,795 entries) by use of the FASTA program (30). We found no significant similarity to any other protein, including the soluble lytic transglycosylase (70 kDa) from *E. coli* and the phage lambda endolysin (17 kDa), the only two proteins that possess identical enzymatic activities and whose primary structures are known (10, 22, 45).

Comparison of the primary structure of the truncated C-terminal open reading frame with those for other proteins. In addition to the large open reading frame, we identified a truncated C-terminal open reading frame. This C terminus shows significant sequence similarity to the C termini of all pyruvate kinases present in the Swiss-Prot protein sequence data base, including pyruvate kinase F from *E. coli*. Since it is known that *E. coli* possesses two pyruvate kinases, pyruvate kinase A and pyruvate kinase F (13), we most likely sequenced the 3′ end of the pyruvate kinase A (AMP-dependent pyruvate kinase) encoding region. The pyruvate kinase F encoding gene has been mapped at minute 36.5 on the *E. coli* map. Unfortunately, we were unable to localize the chromosomal insert present in pWK2579 on the *E. coli* restriction map (25, 35), suggesting that this insert might originate from one of the very few gaps in this map.

Overexpression of the presumed *mlt* gene. The 500-fold overproduction of the 37-kDa protein encoded by the presumed *mlt* gene after temperature induction did not lead to an increase in murein hydrolase activity in the cell homogenate, the soluble fraction, or even the Triton X-100 extract of the membrane fraction, in which the major portion of the

TABLE 1. Purification of the second soluble lytic transglycosylase from *E. coli slt* chromosomal deletion strain 122-1[a]

Step	Vol (ml)	Protein concn (mg/ml)	Total protein (mg)	Sp act (U/mg)	Purification (fold)	Total activity (U)	Yield (%)
Soluble fraction	760	22.50	17,100	0.03	1	513	100
CM-Sepharose CL-6B pool	95	0.65	61.75	8.72	291	538	105
Hydroxylapatite pool	27	0.46	12.42	22.04	735	274	53
Fast protein liquid chromatography (Mono Q) pool	17	0.02	0.34	323.53	10,784	110	21

[a] Enzymatic activity was defined in terms of micrograms of murein released per minute.

A

```
Leu-Leu-Glu-Pro-Gln-His-Asn-Val-Met-Gln-Met-Gly-Gly-Asp-Phe-
Ala-Asn-Asn-Pro-Asn-Ala-Gln-Gln-Phe-Ile-Asp-
```

B

```
1    LLEPQHNVMQMGGDFANNPNAQQFID   26
       :  :++::  : :    +::++ +
338  AKEILHQLMQQRG-FYPMVAAQRIGE  362
```

FIG. 7. N-terminal sequence of Slt35 and comparison with the primary structure of Slt70. (A) The 26 N-terminal amino acids were determined as described in Materials and Methods. (B) The upper and lower sequences represent the N terminus of Slt35 and a portion of Slt70, respectively. Numbers denote the amino acid positions in the respective primary structures. Amino acid identities and evolutionarily related residues are indicated by colons and plus signs, respectively, and a gap in the alignment is indicated by a dash.

overproduced protein was detected immunochemically. This same phenomenon was observed earlier when the protein was overproduced at a much lower level in experiments with runaway replication plasmid pWK2579 (19). This result was explained by the finding that apparently only a given number of protein copies are inserted into the outer membrane and bind to murein, by which they might be activated or at least stabilized. The major portion of the overproduced protein, however, did not undergo this translocation and activation and was therefore detected in an inactive form in the cytoplasmic membrane.

Localization of the 37-kDa protein. Mlt has been reported to be localized in the inner membrane as well as in the outer membrane of *E. coli* (19). All *E. coli* proteins localized in the outer membrane are, to our knowledge, translated as preproteins. As a consequence of transportation across the cytoplasmic membrane, the N-terminal signal peptide is cleaved off, a process that ultimately can result in the insertion of the protein into the outer membrane. However, primary structure analysis of the 37-kDa protein revealed that this protein does not possess an N terminus with the typical features of a signal peptide (48). Furthermore, the murein hydrolase activity was associated, in the wild-type situation, predominantly with the outer membrane fraction (19); the calculated and previously experimentally determined isoelectric points of the translated 37-kDa protein and Mlt, respectively, differed significantly; and, upon overproduction, no increase in murein hydrolase activity was detected. Taking all these data into consideration, we question the earlier explanation and suggest that we are dealing with a gene that does not encode the membrane-bound lytic transglycosylase but rather a cytoplasmic membrane-bound protein with an unknown function. Since polyclonal antibodies previously raised against purified Mlt cross-reacted with isolated Slt35, we conclude that Slt35 was present in the Mlt preparation and was responsible for the detected murein hydrolase activity. Because of their very similar molecular masses, the two proteins could not be detected as separate bands on SDS-polyacrylamide gels. Unfortunately, by screening of a gene overexpression library with the polyclonal antibodies, the gene encoding the 37-kDa membrane-bound protein with an unknown function was isolated.

Is Slt35 identical to previously described Mlt? The determination of the subcellular localization of soluble murein-metabolizing enzymes expressed at low levels has been shown to be difficult (49). Since these enzymes act on murein and murein is pelleted with membranes after the disruption of cells, the soluble murein-metabolizing enzymes can be

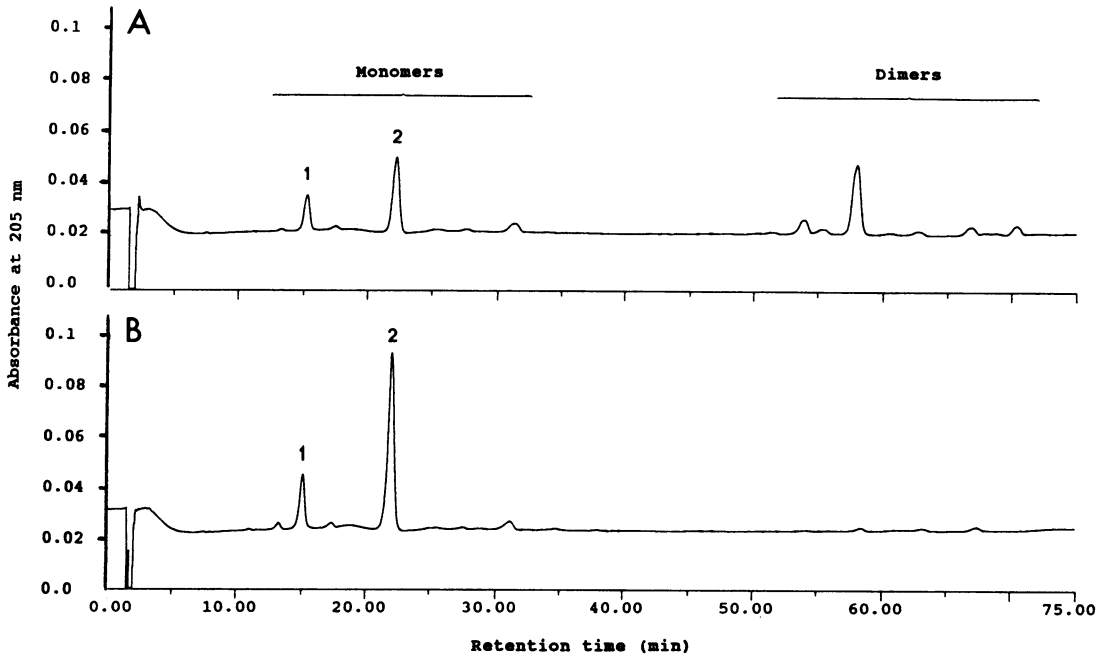

FIG. 8. HPLC profiles of muropeptides obtained after degradation of murein with Slt70 (A) or Slt35 (B). Muropeptides were separated as described in Materials and Methods. Shown is the A_{205} with respect to the retention time in minutes. The positions of the monomeric and dimeric 1,6-anhydro-muropeptides are indicated; the peaks corresponding to GlcNAc(β-1,4)1,6-anhydro-MurNAc-tripeptide and GlcNAc(β-1,4)1,6-anhydro-MurNAc-tetrapeptide are indicated by 1 and 2, respectively. The positions of the muropeptides in the HPLC profiles were determined with standards.

purified from the soluble fraction as well as from the membrane fraction. Therefore, it is possible that Slt35 was present in the previously described Mlt preparation in addition to the membrane-bound 37-kDa protein with an unknown function. Copurified Slt35, but not the membrane-bound 37-kDa protein, could therefore have been responsible for the murein hydrolase activity detected in this preparation. This conclusion is supported by the finding that the polyclonal antibodies previously raised against the Mlt preparation cross-reacted with purified Slt35 (Fig. 6B, lane 1). Therefore, we suggest that the second transglycosylase in *E. coli* is not membrane bound, as was stated earlier (19, 24, 37).

E. coli seems to possess, for all essential reactions to be catalyzed during murein metabolism, at least two enzymes with identical enzymatic activities (20). The high-molecular-weight penicillin-binding proteins (PBPs) 1A and 1B are both described as cytoplasmic membrane-bound murein-synthesizing transglycosylases and transpeptidases, PBP 4 and the penicillin-insensitive murein endopeptidase (MepA) are described as soluble DD-endopeptidases, PBP 5 and PBP 6 are described as cytoplasmic membrane-bound DD-carboxypeptidases, and now Slt70 and Slt35 are described as soluble lytic transglycosylases.

Slt70 has been described as an exo-enzyme in vitro, starting at the *N*-acetylglucosamine end of the glycan strand and proceeding towards 1,6-anhydromuramic acid (1). It will be of interest to determine whether Slt35 acts as an endo-enzyme involved in the trimming of sugar chains after the polymerization process. Within 5 min after synthesis, the sugar chains are reduced in average length from 50 to 31 disaccharide units (14). Since no corresponding release of muropeptides has been observed, the existence of an endo-transglycosylase has been proposed.

The enormous difference in the molecular masses of the two soluble lytic transglycosylases may indicate that Slt70 possesses an additional function in comparison with Slt35. Some carbohydrate-degrading enzymes have been described to possess a second substrate-binding domain in addition to a catalytic domain. Such an idea may be supported by the finding that the N-terminal amino acid sequence of Slt35 shows similarity to a stretch of amino acids in the middle of the primary structure of Slt70 (Fig. 7B). About 30% of the amino acid residues in a stretch of 26 amino acids are identical, and 54% are similar when evolutionarily related substitutions are included. Cloning of the gene encoding Slt35 and its nucleotide sequence as well as the determination of the three-dimensional structure of Slt70 (41) may prove this idea.

ACKNOWLEDGMENTS

We are grateful to B. Kazemier and P. Terpstra for help with the nucleotide sequence determination and the computer analysis, respectively.

ADDENDUM IN PROOF

Recently, we learned from M. Karow and C. Georgopoulos (University of Utah, Salt Lake City) about the identity of the *mlt* gene with a very recently sequenced gene, *msbB* (J. Bacteriol. **174**:702–710, 1992) (GenBank accession number M77039).

REFERENCES

1. **Beachy, E. H., W. Keck, M. A. de Pedro, and U. Schwarz.** 1981. Exoenzymatic activity of transglycosylase isolated from *Escherichia coli*. Eur. J. Biochem. **116**:355–358.
2. **Betzner, A., and W. Keck.** 1989. Molecular cloning, overexpression and mapping of the *slt* gene encoding the soluble lytic transglycosylase of *Escherichia coli*. Mol. Gen. Genet. **219**:489–491.
3. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
4. **Bittner, M., P. Kupferer, and C. F. Morris.** 1980. Electrophoretic transfer of proteins and nucleic acids from slab gels to diazobenzyloxymethyl cellulose or nitrocellulose sheets. Anal. Biochem. **102**:459–471.
5. **Bolton, A. E., and W. M. Hunter.** 1973. The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Application to the radioimmunoassay. Biochem. J. **133**:529–539.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Cookson, B. T., H.-L. Cho, L. A. Herwaldt, and W. E. Goldman.** 1989. Biological activities and chemical composition of purified tracheal cytotoxin of *Bordetella pertussis*. Infect. Immun. **57**: 2223–2229.
8. **Davison, J., M. Heusterpreute, N. Chevalier, and F. Brunel.** 1987. A 'phase-shift' fusion system for the regulation of foreign gene expression by lambda repressor in gram-negative bacteria. Gene **60**:227–235.
9. **Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall.** 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. J. Mol. Biol. **179**:125–142.
10. **Engel, H., B. Kazemier, and W. Keck.** 1991. Murein-metabolizing enzymes from *Escherichia coli*: sequence analysis and controlled overexpression of the *slt* gene, which encodes the soluble lytic transglycosylase. J. Bacteriol. **173**:6773–6782.
11. **Engel, H., H. Mottl, and W. Keck.** 1992. A modified vector for the controlled high-level overproduction of staphylococcal protein A fusion proteins in the periplasm of *Escherichia coli*. Protein Expression Purification **3**:108–113.
12. **Engel, H., A. M. van Leeuwen, A. Dijkstra, and W. Keck.** Enzymatic preparation of 1,6-anhydromuropeptides by immobilized murein hydrolases from *Escherichia coli* fused to staphylococcal protein A. Appl. Microbiol. Biotechnol., in press.
13. **Garrido-Pertierra, A., and R. A. Cooper.** 1983. Evidence for two distinct pyruvate kinase genes in *Escherichia coli* K-12. FEBS Lett. **162**:420–422.
14. **Glauner, B., and J.-V. Höltje.** 1990. Growth pattern of the murein sacculus of *Escherichia coli*. J. Biol. Chem. **265**:18988–18996.
15. **Glauner, B., J.-V. Höltje, and U. Schwarz.** 1988. The composition of the murein of *Escherichia coli*. J. Biol. Chem. **263**:10088–10095.
16. **Goodell, E. W.** 1985. Recycling of murein by *Escherichia coli*. J. Bacteriol. **163**:305–310.
17. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11**:2237–2255.
18. **Helfman, D. M., J. R. Feramisco, J. C. Fiddes, G. P. Thomas, and S. H. Hughes.** 1983. Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library. Proc. Natl. Acad. Sci. USA **80**:31–35.

18a. **Höltje, J.-V.** Personal communication.

19. **Höltje, J.-V., and W. Keck.** 1988. Organization of the major autolysin in the envelope of *Escherichia coli*, p. 181–188. *In* P. Actor, L. Daneo-Moore, M. L. Higgins, M. R. J. Salton, and G. D. Shockman (ed.), Antibiotic inhibition of bacterial cell surface assembly and function. American Society for Microbiology, Washington, D.C.
20. **Höltje, J.-V., and U. Schwarz.** 1985. Biosynthesis and growth of the murein sacculus, p. 77–119. *In* N. Nanninga (ed.), Molecular cytology of *Escherichia coli*. Academic Press, London.
21. **Höltje, J.-V., and E. I. Tuomanen.** 1991. The murein hydrolases of *Escherichia coli*: properties, functions and impact on the course of infections in vivo. J. Gen. Microbiol. **137**:441–454.
22. **Imada, M., and A. Tsugita.** 1971. Amino-acid sequence of lambda phage endolysin. Nature (London) **233**:230–231.

23. **Keck, W., A. M. van Leeuwen, M. Huber, and E. W. Goodell.** 1990. Cloning and characterization of mepA, the structural gene of the penicillin-insensitive murein endopeptidase from *Escherichia coli*. Mol. Microbiol. **4**:209–219.
24. **Keck, W., F. Wientjes, and U. Schwarz.** 1985. Comparison of two murein transglycosylases of *Escherichia coli*. Eur. J. Biochem. **148**:493–497.
25. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495–508.
26. **Krueger, J. M., M. L. Karnovsky, S. A. Martin, J. R. Pappenheimer, J. Walter, and K. Biemann.** 1984. Peptidoglycans as promoters of slow-wave sleep. II. Somnogenic and pyrogenic activities of some naturally occurring muramyl peptides; correlations with mass spectrometric structure determination. J. Biol. Chem. **259**:12659–12662.
27. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
28. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
31. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Martin, S. A., M. L. Karnovsky, J. M. Krueger, J. R. Pappenheimer, and K. Biemann.** 1984. Peptidoglycans as promoters of slow-wave sleep. I. Structure of the sleep-promoting factor isolated from human urine. J. Biol. Chem. **259**:12652–12658.
34. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**:10035–10038.
35. **Médigue, C., A. Hénaut, and A. Danchin.** 1990. *Escherichia coli* molecular genetic map (1000 kbp): update I. Mol. Microbiol. **4**:1443–1454.
36. **Melly, M. A., Z. A. McGee, and R. S. Rosenthal.** 1984. Ability of monomeric peptidoglycan fragments from *Neisseria gonorrhoeae* to damage human fallopian-tube mucosa. J. Infect. Dis. **149**:378–386.
37. **Mett, H., W. Keck, A. Funk, and U. Schwarz.** 1980. Two different species of murein transglycosylases in *Escherichia coli*. J. Bacteriol. **144**:45–52.
38. **Mulligan, M. E., D. K. Hawley, R. Entriken, and W. R. McClure.** 1984. *Escherichia coli* promoter sequences predict in vitro RNA polymerase selectivity. Nucleic Acids Res. **12**:789–800.
39. **Rao, J. K. M., and P. Argos.** 1986. A conformational preference parameter to predict helices in integral membrane proteins. Biochim. Biophys. Acta **869**:197–214.
40. **Roeder, W., and R. L. Sommerville.** 1979. Cloning the *trpR* gene. Mol. Gen. Genet. **176**:361–368.
41. **Rozeboom, H. J., B. W. Dijkstra, H. Engel, and W. Keck.** 1990. Crystallization of the soluble lytic transglycosylase from *Escherichia coli*. J. Mol. Biol. **212**:557–559.
42. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1979. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
43. **Shine, J., and L. Dalgarno.** 1976. The 3'-terminal sequence of *Escherichia coli* 16 S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71**:1342–1346.
44. **Stanssens, P., C. Opsomer, Y. M. McKeown, W. Kramer, M. Zabeau, and H.-J. Fritz.** 1989. Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers. Nucleic Acids Res. **17**:4441–4454.
45. **Taylor, A., B. C. Das, and J. van Heijenoort.** 1975. Bacterial-cell-wall peptidoglycan fragments produced by phage lambda or Vi II endolysin and containing 1,6-anhydro-N-acetylmuramic acid. Eur. J. Biochem. **53**:47–54.
46. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
47. **Vieira, J., and J. Messing.** 1987. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468–500.
48. **von Heyne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
49. **Walderich, B., and J.-V. Höltje.** 1991. Subcellular distribution of the soluble lytic transglycosylase in *Escherichia coli*. J. Bacteriol. **173**:5668–5676.