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Outer Membrane Localization of Murein Hydrolases: MltA, a Third Lipoprotein Lytic Transglycosylase in *Escherichia coli*

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Lytic transglycosylases are a unique lysozyme-like class of murein hydrolases believed to be important for growth of *Escherichia coli*. A membrane-bound lytic transglycosylase with an apparent molecular mass of 38 kDa, which was designated Mlt38, has previously been purified and characterized (A. Ursinus and J.-V. Höltje, J. Bacteriol. 176:338–343, 1994). On the basis of four tryptic peptides, the gene *mltA* was mapped at 63 min on the chromosomal map of *E. coli* K-12 and cloned by reverse genetics. The open reading frame was found to contain a typical lipoprotein consensus sequence, and the lipoprotein nature of the gene product was demonstrated by [^{3}H]palmitate labeling. On the basis of the distribution of MltA in membrane fractions obtained by sucrose gradient centrifugation, a localization in the outer membrane is indicated. Overexpression of MltA at 30°C, the optimal temperature for enzyme activity, but not at 37°C results in the formation of spheroplasts. Not only a deletion mutant in *mltA*, but also double mutants in *mltA* and one of the two other well-characterized lytic transglycosylases (either *sltY* or *mltB*), as well as a triple mutant in all three enzymes, showed no obvious phenotype. However, dramatic changes in the structure of the murein sacculus indicate that lytic transglycosylases are involved in maturation of the murein sacculus.

Murein (peptidoglycan) hydrolases are believed to be essential for growth of bacteria, which have their cell walls reinforced by the cross-linked polymer murein (50, 51, 58). Because of the covalently closed, bag-shaped structure of the murein sacculus that completely surrounds the bacterium (26, 43), participation of enzymes that hydrolyze bonds in the murein is needed for at least two processes during growth: firstly, for the general enlargement of the murein net and, secondly, for the splitting of the septum to allow separation of the daughter cells.

Numerous kinds of murein hydrolases in *Escherichia coli* have been identified (23, 51). There is evidence for at least two specificities to be directly involved in the growth and division of the murein sacculus. The continuous release predominately of monomeric 1,6-anhydrodisaccharide tetra- and tripeptides during growth indicates the combined action of endopeptidases and lytic transglycosylases (LTs) (20, 29). LTs are lysozyme-like enzymes, which not only cleave the β-1,4-glycosidic bond between *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) but in addition transfer the glycosyl bond onto the C-6 hydroxyl group of the terminal muramyl moiety, thus forming 1,6-anhydromuramic acid residues (25).

Four different LTs have been previously identified (11, 45): a soluble lytic transglycosylase (Slt70 [25]) and three membrane-bound lytic transglycosylases (MltA [56], MltB [14, 15], and MltC [11]). Except for MltA, their genes have been cloned and their products have been characterized (4, 10, 11, 14). Interestingly, both MltB and MltC contain a typical lipoprotein consensus sequence. The lipoprotein character of MltB has been proven by palmitate labeling, and a localization of the enzyme in the outer membrane has been demonstrated (14). Proteolytic cleavage of MltB gives rise to the formation of an enzymatically active soluble form, called Slt35 (10, 15). MltA differs from Slt70 and MltB by accepting the peptide-free poly(GlcNAc-β-1,4-MurNAc) glycan strands as a substrate in addition to murein sacculi (56). Deletion mutants in *sltY* and *mltB*, including a double mutant, displayed no obvious phenotype (14, 53). To gain some insight into the function of this class of enzymes, we cloned and sequenced the third Mlt gene, *mltA*, which turned out to be another lipoprotein LT, and characterized a triple mutant lacking Slt70, MltA, and MltB.

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MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The *E. coli* strains and the plasmids used are listed in Table 1. Bacteriophage P1 was used for generalized transduction (41). From the Kohara phage library (34), λ458 (9A12) and λ459 (10B6) were used for cloning and deletion mutagenesis of the *mltA* gene.

Media and growth conditions. Luria-Bertani (LB) medium (41) was used for cultivation of bacteria, and LB medium with 10 mM $MgSO_4$ was used for preparation of λ phages (48). Solid medium contained 1.5% agar (Gibco BRL, Paisley, Scotland). When necessary, ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), tetracycline (12.5 μg ml^{-1}), or chloramphenicol (20 μg ml^{-1}) was added. All strains were cultivated aerobically at 37°C unless otherwise indicated.

DNA manipulations and PCR. Standard techniques were used for manipulating plasmid and genomic DNAs (48). Transformations of *E. coli* were performed as described by Hanahan (21). Restriction endonucleases and other DNA-modifying enzymes were purchased from MBI Fermentas (Vilnius, Lithuania) and Boehringer (Mannheim, Germany). Labeling of DNA with a digoxigenin labeling kit (Boehringer) and hybridization with labeled DNA probes were performed according to the manufacturer's instructions. Primers were synthesized on a GeneAssembler (Pharmacia, Uppsala, Sweden) or were purchased from Pharmacia.

PCRs (47) were performed in a volume of 50 μl of 10 mM Tris-HCl (pH 8.8) containing 50 mM KCl, 1.5 to 2.5 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 100 μg of bovine bovine serum albumin ml^{-1} and 1 U of *Taq* polymerase (Biomaster, Köln, Germany) with 50 ng of genomic MC1061 DNA as a template in a Techne PHC-3 thermocycler (Thermodux, Wertheim, Germany). Generally, 600 ng of primers was used per reaction. Prior to cycling, the samples were incubated for 5 min at 94°C, followed by 30 cycles at 94°C for 1 min, 58°C for 2 min and 72°C for 1 min. The final polymerization step was an incubation at 72°C for 2 min. Alternatively, touchdown PCR conditions (12) were used. In this case, the initial denaturation step (3 min at 94°C) was followed by 5 cycles with decreasing annealing temperature (from 63 to 58°C in 1°C steps) for 1.5 min each. Denaturation was done at 94°C for 1 min. Finally, 25 cycles with an annealing temperature of 58°C under the same conditions were performed.

TABLE 1. Strains and plasmids

Strain or plasmid	Characteristics	Source or reference
Strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tc^r)]^c	Stratagene
MC1061	F′ *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2* (r_K^- m_K^+) *mcrA mcrB1*	6
MUF16	MC1061 Δ*sltY*::*kan*	53
MUF33	MC1061 Δ*mltB*::Tn*10*	This work
LT12	MC1061 Δ*mltA*::*cat*	This work
MUF45	MC1061 Δ*mltA*::*cat* Δ*mltB*::Tn*10*	This work
LT28	MC1061 Δ*mltA*::*cat* Δ*sltY*::*kan*	This work
MUF49	MC1061 Δ*mltB*::Tn*10* Δ*sltY*::*kan*	This work
MUF61	MC1061 Δ*mltA*::*cat* Δ*mltB*::Tn*10* Δ*sltY*::*kan*	This work
JC10288	*crg*(*srlR-recA*)::Tn*10*(Tc^r) *thr-1 leuB6 ara-14 proA2 lacY tsx-33 galK2 his-4 rpsL31 xyl-5 mtl-1 argE3 thi-1*	9
Plasmids		
pBluescript II SK$^+$	Ap^r *lacZ′*	Stratagene
pBC SK$^+$	Cm^r *lacZ′*	Stratagene
pJFK118EH	Km^r *tac* promoter	5
pWK12	Ap^r	4
pLC21-33	*recA mltA* Ap^r	8
pAK1	*recA* from pLC21-33 in pWK12	This work
pJL2124	pBluescript II SK$^+$ derivative (see Fig. 1)	This work
pJL873B	pBluescript II SK$^+$ derivative (see Fig. 1)	This work
pJL45832	pBluescript II SK$^+$ derivative (see Fig. 1)	This work
pJL17V5	pBluescript II SK$^+$ derivative (see Fig. 1)	This work
pJL38C	pBluescript II SK$^+$ derivative (see Fig. 1)	This work
pMAT	*mltA* in pJFK118EH (see Fig. 1)	This work

Sequencing of the *mltA* gene and DNA analysis. All plasmid clones for sequencing were deduced from plasmid pJL873B (Fig. 1). This construct contains the complete MltA coding sequence from Kohara phage λ458. By restriction digestion and by BAL 31 truncation, fragments of the sequence were generated, subcloned into pBluescript II SK$^+$, and used for sequencing. Sequencing (49) was done bidirectionally for the sense and antisense strands or unidirectionally with at least three independent reactions to verify the accuracy of the sequence. The analysis was performed on an A.L.F. DNA Sequencer (Pharmacia) by using the Autoread Sequencing kit (Pharmacia) according to the manufacturer's instructions.

The LASERGENE software package (DNASTAR, Inc., Madison, Wis.), the Genetics Computer Group (GCG) software package (GCG, Madison, Wis.), and the BLAST algorithm (1) available as e-mail server (blast@ncbi.nlm.nih.gov) were used for computer-assisted sequence analysis and similarity searches of sequence databases.

Mapping of the *mltA* gene. The *mltA* gene was mapped by using a PCR product which was obtained with degenerated oligonucleotides designed on the basis of the known peptide sequences of the MltA protein (56).

Eight different oligonucleotides, deduced from three of the peptides, with lengths of between 26 and 31 bases and a degeneration rate of 128 to 4,096 were used for PCR, and the products were cloned into pBluescript II SK$^+$ by use of restriction sites included in the primers. The cloned fragments were analyzed by DNA sequencing, and one 189-bp product obtained with primer JL21 (5′-GCTA AGCTTTNACNGGNGCRAANGAYTGNGG-3′) and JL24 (5′-CATCTCGA GAARAAYGGNCAYGCNTA-3′) was considered to be specific due to its sequence matching to the amino acid sequence of the peptides not included in the primers. This fragment, present on plasmid pJL2124 (Fig. 1), was labeled with digoxigenin and used for hybridization with the Gene Mapping Membrane (Takara Shuzo Co. Ltd., Kyoto, Japan).

Construction of an inducible expression system. The coding region of *mltA* was amplified from genomic MC1061 DNA by touchdown PCR with the following primers: MLTA1 (5′-AAGGAATTCATGAAAGGACGTTGGGTAAAG TACC-3′) and MLTA2.1 (5′-AATGGATCCTCAGCCGCTAAAGACGTTA CCTGCG-3′). The amplification products were purified, digested with *Eco*RI and *Bam*HI, and cloned into the corresponding sites of pJFK118EH (5), a vector carrying the *tac* promoter and the *lacI*^{q} allele of the *lac* repressor. By the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), overexpression of MltA could be induced.

Construction of an *mltA* deletion. The method of Kulakauskas et al. (38) was used to generate a chromosomal deletion mutation in MC1061. First, a plasmid-encoded insertional deletion of the *mltA* gene was constructed. The upstream region of the *mltA* gene, a 3.2-kb *Xho*I/*Sma*I fragment from pJL45832 (Fig. 1), was ligated with a chloramphenicol acetyltransferase gene, a 1.6-kb *Hha*I fragment from pBCSK$^+$, and the downstream region, a 1.1-kb *Eco*RV/*Hin*dIII fragment from pJL17V5, generating plasmid pJL38C. In this construct, almost the complete coding region of the *mltA* gene is replaced by the resistance cassette (Fig. 1). This mutant allele of the *mltA* gene was introduced in strain MC1061 by lambda phage-mediated transduction. Accordingly, *E. coli* MC1061 harboring pJL38C was infected with the Kohara phage λ459, and the lysate containing recombinant phages was used to infect strain MC1061. Chloramphenicol-resistant clones were selected and shown to contain the insertional deletion by hybridization of their digested chromosomal DNAs to corresponding probes from the *mltA* and *cat* genes (data not shown).

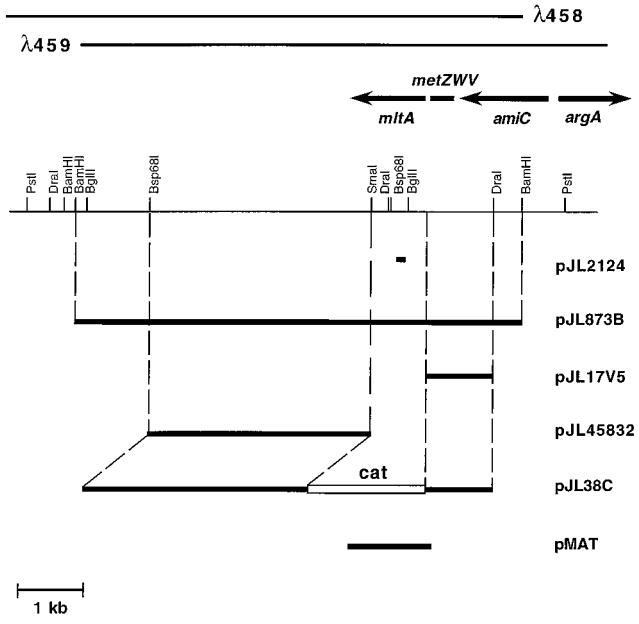

FIG. 1. Physical map of the *mltA* region (63.4 min) of the *E. coli* chromosome. The overlapping lambda phages from the Kohara miniset library (34) are shown, and characterized genes in this region are indicated. The inserts of the plasmids used in this study are shown beneath the physical map.

Construction of multiple deletions in LTs. To combine the *mltA* deletion with mutations of other known LTs, P1 transduction was employed. The source for an *sltY* deletion was MUF16 (53); a deletion of the *mltB* gene was found in JC10288 (9). To verify the absence of the different LTs, transductants were grown aerobically at 37°C in L broth to an optical density at 578 nm (OD_{578}) of 0.5. The cells were centrifuged, washed once with phosphate-buffered saline, resuspended in 1/10 volume of phosphate-buffered saline, and mixed with 1/3 volume of loading buffer. After boiling, cellular proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (40), and blotted onto nitrocellulose (54). Western blot analysis with specific polyclonal antisera against the purified transglycosylases confirmed the absence of MltA, MltB, and Slt70.

Since the deletion found in JC10288 not only includes *mltB* but also covers the closely linked *recA*, a growth defect due to the absence of this gene was found with the *mltB* mutants. To complement this defect, a low-copy-number plasmid containing *recA* was constructed. A 3-kb *Bam*HI fragment from pLC21-33 (8, 55) was cloned into pWK12 (4). The resulting construct was named pAK1. The presence of functional RecA protein was shown by determination of UV sensitivity (7).

Palmitate labeling. LB medium (10 ml) containing 50 μCi of [^{3}H]palmitate (54 Ci mmol^{-1}; Amersham Buchler) was inoculated with XL1-Blue(pJFK118EH) and XL1-Blue(pMAT) to an OD_{578} of 0.01. The cells were grown aerobically at 37°C to an OD_{578} of 0.4, and expression of MltA was induced for 30 min by the addition of 1 mM IPTG (30) in the presence or absence of 100 μg of globomycin (Sankyo Co., Ltd., Tokyo, Japan) ml^{-1}. The cells were centrifuged, washed in 10 ml of 50 mM sodium phosphate (pH 7.2), resuspended in 120 μl of 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA and 1% SDS, and lysed by heating to 100°C for 5 min. After centrifugation (10 min; 12,000 × *g*; 4°C), the proteins in the supernatant were precipitated with 10 volumes of ice-cold acetone at 4°C overnight, pelleted by centrifugation as described above, and resuspended thoroughly in 50 μl of 1% SDS. Aliquots corresponding to approximately 10^6 cpm were separated by SDS-12% PAGE (40), and the labeled proteins were visualized by fluorography.

Membrane fractionation. Inner and outer membranes were separated by sucrose gradient ultracentrifugation as described previously (14).

Murein hydrolase activity assay. Murein sacculi labeled with *meso*-2,6-diamino-[3,4,5-^{3}H]pimelic acid (10^6 cpm ml^{-1}; 0.5 mg ml^{-1}) were used as a substrate to determine murein hydrolase activity as described previously (39).

Murein structure analysis. Murein sacculi were prepared and hydrolyzed with cellosyl (kindly given to us by Hoechst AG, Frankfurt, Germany), and the resulting muropeptides were fractionated by reverse-phase high-pressure liquid chromatography as described by Glauner (16).

Nucleotide sequence accession number. The GenBank accession number for the *mltA* gene is U32224. During this work, the *mltA* gene was also sequenced and submitted to GenBank as part of the *E. coli* sequencing project (accession number U29581). This sequence differs in 1 bp from the sequence that we determined (C instead of A at position 1246, resulting in an exchange of amino acid 346 [His instead of Asn]) (Fig. 2).

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   1 CGTCCGTGGATGCGCACTATACTCCGCTCGCGTTTTCATGCAACCTTTTTTTTACCCCGA   60

  61 TCACGGATTTGGCATGATTTTGAACAAAAACAAATCACTAAAGCCTATTTTTTTGTGCAAA  120

 121 ATTTGCGTCAGGGAGAGGCTGCTCATTTCATTAAACGCGGCGGTTTGTTATCTTCGTTGC  180

 181 GCCTTATTTTTTAACCTGAAGAAGGAACAATGAAAGGACGTTGGGTAAAGTACCTTCTT  240
                     RBS       M  K  G  R  W  V  K  Y  L  L    10

 241 ATGGGCACGGTTGTGGCAATGCTTGCCGCCTGCTCTTCCAAACCAACCGATCGCGGACAG  300
  11  M  G  T  V  V  A  M  L  A  A  C  S  S  K  P  T  D  R  G  Q   30
                             *

 301 CAATATAAAGACGGGAAATTTACCCAGCCTTTCTCTCTGGTGAACCAGCCAGATGCCGTT  360
  31  Q  Y  K  D  G  K  F  T  Q  P  F  S  L  V  N  Q  P  D  A  V   50

 361 GGCGCGCCGATTAACGCCGGTGATTTTGCCGAGCAAATTAACCATATCCGTAATTCGTCA  420
  51  G  A  P  I  N  A  G  D  F  A  E  Q  I  N  H  I  R  N  S  S   70

 421 CCGCGTCTGTATGGCAACCAGAGTAATGTTTATAACGCGGTGCAAGAGTGGCTGCGCGCA  480
  71  P  R  L  Y  G  N  Q  S  N  V  Y  N  A  V  Q  E  W  L  R  A   90

 481 GGCGGTGATACCCGCAATATGCGCCAGTTCGGCATTGATGCCTGGCAGATGGAAGGTGCC  540
  91  G  G  D  T  R  N  M  R  Q  F  G  I  D  A  W  Q  M  E  G  A  110

 541 GACAACTATGGTAACGTGCAGTTTACCGGTTATTACACGCCGGTAATTCAGGCGCGCCAT  600
 111  D  N  Y  G  N  V  Q  F  T  G  Y  Y  T  P  V  I  Q  A  R  H  130

 601 ACCCGCCAGGGCGAGTTCCAGTATCCTATTTACCGTATGCCGCCAAAACGTGGTCGTCTG  660
 131  T  R  Q  G  E  F  Q  Y  P  I  Y  R  M  P  P  K  R  G  R  L  150

 661 CCGTCTCGTGCGGAGATCTACGCGGGGGCATTGAGTGATAAATATATTCTCGCTTACAGT  720
 151  P  S  R  A  E  I  Y  A  G  A  L  S  D  K  Y  I  L  A  Y  S  170

 721 AACTCCCTGATGGATAACTTCATTATGGATGTGCAGGGTAGTGGGTATATCGACTTTGGT  780
 171  N  S  L  M  D  N  F  I  M  D  V  Q  G  S  G  Y  I  D  F  G  190

 781 GATGGCAGTCCGCTTAACTTTTTCAGCTATGCAGGGAAAAACGGTCATGCCTATCGCAGC  840
 191  D  G  S  P  L  N  F  F  S  Y  A  G  K  N  G  H  A  Y  R  S  210

 841 ATTGGTAAGGTGCTGATCGACCGTGGCGAAGTGAAAAAAGAAGATATGTCGATGCAGGCG  900
 211  I  G  K  V  L  I  D  R  G  E  V  K  K  E  D  M  S  M  Q  A  230

 901 ATTCGTCACTGGGGCGAAACACACAGTGAAGCCGAGGTTCGCGAGCTGCTGGAACAGAAC  960
 231  I  R  H  W  G  E  T  H  S  E  A  E  V  R  E  L  L  E  Q  N  250

 961 CCGTCTTTCGTCTTCTTTAAACCGCAATCTTTTGCTCCGGTGAAAGGGGCAAGTGCGGTG 1020
 251  P  S  F  V  F  F  K  P  Q  S  F  A  P  V  K  G  A  S  A  V  270

1021 CCGCTGGTTGGTCGCGCGTCAGTTGCCTCTGATCGTTCCATTATTCCGCCAGGTACTACC 1080
 271  P  L  V  G  R  A  S  V  A  S  D  R  S  I  I  P  P  G  T  T  290

1081 TTGCTGGCAGAAGTGCCGTTGCTGGATAATAACGGCAAATTTAATGGTCAGTACGAACTG 1140
 291  L  L  A  E  V  P  L  L  D  N  N  G  K  F  N  G  Q  Y  E  L  310

1141 CGTCTGATGGTGGCGCTGGATGTCGGTGGTGCAATCAAAGGCCAACACTTCGATATCTAT 1200
 311  R  L  M  V  A  L  D  V  G  G  A  I  K  G  Q  H  F  D  I  Y  330

1201 CAAGGGATCGGGCCGGAAGCCGGACACCGCGCAGGTTGGTACAACAACTATGGACGTGTC 1260
 331  Q  G  I  G  P  E  A  G  H  R  A  G  W  Y  N  N  Y  G  R  V  350

1261 TGGGTGCTGAAAACCGCCCCGGGCGCAGGTAACGTCTTTAGCGGCTGATGTGATATTCTG 1320
 351  W  V  L  K  T  A  P  G  A  G  N  V  F  S  G                365

1321 TATGCCGTTTTTACGGATATGACAGGGTGAGGGGTAACCTCACATCCTGATTATTGACTA 1380

1381 AGTGCGCGTTGTTCATGCCGGATGC                                    1405
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FIG. 2. Nucleotide sequence of the *mltA* gene. The nucleotide sequence is shown with the corresponding deduced amino acid sequence of MltA. RBS, potential ribosome binding site; boldface, the consensus sequence of the lipoprotein processing site; asterisk, the modified N-terminal cystein of the mature lipoprotein; underlining, the amino acid sequences of the peptides used to design degenerated oligonucleotides. The amino acid (N) in position 346 of our sequence, which is different from the one in the GenBank database (accession no. U32224) is indicated by a boldface letter.

RESULTS

Mapping and cloning of the *mltA* gene. The previously determined peptide sequences from the MltA protein (formerly Mlt38 [56]) were used to design degenerated oligonucleotides to amplify parts of the coding region of the gene by PCR. With the primer pair JL21 and JL24 (see Materials and Methods), a DNA fragment of approximately 180 bp in length was amplified, cloned into pBluescript II SK$^+$, and sequenced. Sequence analysis revealed the presence of a possible reading frame, which contained parts of the known peptide sequences that were not provided by the primers. Therefore, the cloned DNA fragment was considered part of the *mltA* gene.

The amplified fragment was used for hybridization with the Gene Mapping Membrane (Takara Shuzo Co. Ltd.), which contains immobilized DNA from the Kohara miniset collection (34). A positive hybridization signal was obtained for Kohara phages λ458 (9A12) and λ459 (10B6), two overlapping clones, which share an identical region of 7.5 kb (Fig. 1). Therefore, the *mltA* gene maps at 63.4 min on the *E. coli* restriction map (46). This result was confirmed by Southern blot analysis of restriction digested chromosomal DNA and hybridization with the 180-bp probe (data not shown). This region from λ459 was cloned into pBluescript II SK$^+$, yielding pJL873B.

Nucleotide sequence analysis. About 1.8 kb of DNA was sequenced and analyzed for open reading frames (ORFs). One complete ORF of 1095 bp could be detected. As for the sequences of MltB (14) and MltC (11), this coding region contains a signal peptide followed by a typical lipoprotein processing site (Fig. 2). Therefore, the determined sequence was expected to code for a lipoprotein with a calculated molecular mass of 39 kDa. Sequence similarity searches revealed the presence of homolog proteins of unknown function in *Synechocystis* species and *Haemophilus influenzae*. We failed to detect significant similarity to any other known proteins. Surprisingly, the other known LTs (MltB, MltC, and Slt70) share no region of similarity with this enzyme. Consequently, we were not able to detect the LT motif defined by Koonin and Rudd (36).

Controlled overexpression of MltA. Controlled overexpression of the MltA was achieved from pMAT, an expression vector based on the pBR322 derivative pJFK118EH (5). In this construct, the *mltA* gene is cloned behind the *tac* promoter. SDS-PAGE analysis of the cellular proteins after induction of expression by the addition of IPTG revealed the enrichment of a protein in the membrane fraction with an apparent molecular mass of about 39 kDa, which nicely corresponds to the mass of the purified MltA protein (56).

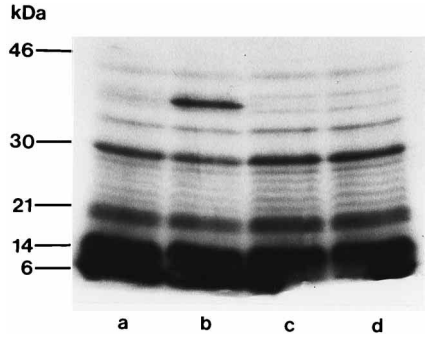

FIG. 3. Demonstration of the lipoprotein character of MltA. *E. coli* XL1-Blue harboring pMAT or, as a control, pJFK118EH growing in the presence of [^{3}H]palmitate was induced by the addition of 1 mM IPTG for 30 min at 37°C in the presence or absence of globomycin (100 μg/ml). Cells were lysed as described in Materials and Methods. Samples were separated on an SDS-12% PAGE gel, and the labeled proteins were visualized by fluorography of the gel. Lanes: a, pMAT vector in the presence of globomycin; b, pMAT vector; c, control vector pJFK118EH in the presence of globomycin; d, control vector pJFK118EH. Molecular masses of marker proteins are indicated on the left.

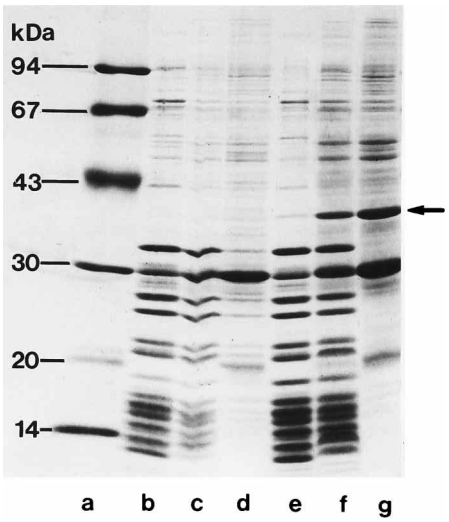

FIG. 4. Subcellular distribution of MltA. Membranes of IPTG-induced *E. coli* XL1-Blue harboring pMAT (lanes e to g) or, as a control, pBluescript II SK$^+$ (lanes b to d) were separated by sucrose gradient centrifugation into cytoplasmic membrane fraction (lanes b and e), intermediate membrane fraction (lanes c and f), and outer membrane fraction (lanes d and g) as described in Materials and Methods and were resolved by SDS-12% PAGE. Proteins were visualized by silver staining. Lane a shows molecular mass markers. The position of the mature MltA protein is indicated by an arrow.

Whereas the constitutive overproduction of MltA did not affect growth rate and yield or the morphology of the cells, spheroplast formation and lysis of the culture resulted when MltA expression was induced by IPTG from plasmid pMAT. However, these morphological alterations occurred only when cells were induced at 30°C, the optimal temperature for enzymatic activity (56), but not at 37°C. Since the level of expression was the same at both temperatures, this is strong evidence that spheroplasting and lysis are due to the enzymatic activity of the overproduced MltA rather than to an unspecific effect of the overproduction of a membrane protein.

Well in accordance with this conclusion, murein hydrolase activity in a Triton X-100 extract of the membranes was increased compared to that for control cells harboring the empty plasmid by a factor of 114 when cells were grown at 37°C and by a factor of 142 when growth was at 30°C. In addition, the expressed activity was able to hydrolyze both murein sacculi and isolated glycan strands, a specific feature demonstrated earlier for purified MltA (56).

Demonstration of the lipoprotein character of MltA. To test for the lipoprotein character of MltA, the protein was expressed in cells growing in the presence of [^{3}H]palmitate. The proteins were separated by SDS-PAGE and visualized by fluorography. As shown in Fig. 3, a labeled band with an apparent molecular mass of about 39 kDa was indeed present in the membranes of induced cells harboring pMAT but not in control cells harboring pJFK118EH. In addition, processing of the preform could be inhibited by the addition of globomycin, a specific inhibitor of signal peptidase 2 (27).

Subcellular distribution of the MltA. The lipoprotein murein hydrolase MltB has been shown to be localized in the outer membrane of the cell wall (14). Likewise, membrane fractionation by sucrose gradient centrifugation shows that the MltA protein is also localized in the outer membrane (Fig. 4).

Characterization of various LT deletion mutants. The deletion in the *mltA* gene was combined with mutations in two other LTs, the soluble enzyme Slt70 (53) and the lipoprotein enzyme MltB (14), to create two different double mutants and one triple mutant in order to obtain information about the function of LTs for growth of *E. coli*. Surprisingly, no change in the morphology of any of the double deletion mutants, including a combined *sltY mltB* deletion, or of the triple mutant could be detected when they were studied by phase-contrast microscopy. In addition, growth rate and growth yield were not affected. The MICs for mecillinam and aztreonam were changed in mutants lacking Slt70, which is an effect already described for the Slt70 deletion mutant *E. coli* MUF16 (53). However, the effect of β-lactamase-sensitive penicillins in the mutants harboring the pAK1 plasmid that carries ampicillin resistance could not be determined (see above). However, the involvement of LTs in bacteriolysis induced by β-lactams has been demonstrated in earlier reports (32, 35).

Structure of the murein of the LT triple mutant. Muropeptide analysis of the mutant lacking Slt70, MltA, and MltB revealed a number of significant changes (Table 2). As was expected for a mutant with decreased LT activity, the relative amounts of anhydromuropeptides that represent glycan chain ends were decreased, indicating an increase in the average lengths of glycans. A decrease of 35.5% for all anhydromuropeptides was determined. This was due to a decrease in the dimeric anhydromuropeptides by 38.5% and in the trimeric amhydromuropeptides by 45.9%. However, monomeric anhydromuropeptides were not changed at all (+1.6%). More surprisingly, the relative amount of murein peptide bridges cross-linked by the unusual LD-*m*-A_2pm-*m*-A_2pm peptide bond was dramatically increased by 63.8%. Other significant changes were the decrease in Lys-Arg-substituted muropeptides representing lipoprotein attachment sites by 21.2% and an increase in tripeptide stem peptides by 25.6%. In addition, the amount of all cross-linked muropeptides was decreased by 8.6% resulting from decreases in dimeric muropeptides by 5.4% and in trimeric muropeptides by 26.3%. In agreement with the earlier finding that the murein in a mutant lacking Slt70 was not substantially affected (53), a double mutant with deletions in *mltA* and *mltB* showed changes quite similar to those found for the triple mutant (data not shown).

DISCUSSION

There is still no experimental proof for the proposal that murein hydrolases are essential for bacterial growth (51). As shown here, even the construction of a deletion mutant that

TABLE 2. Muropeptide composition of *E. coli* MUF61 (*sltY mltA mltB*) and *E. coli* MC1061

Muropeptide[b]	% Muropeptide composition (± SE)[a]		Δ%
	MC1061	MUF61	
Monomers	53.45 ± 0.19	57.12 ± 0.28	6.9
Tri	8.25 ± 0.30	10.36 ± 0.03	25.6
Tetra	41.79 ± 0.26	42.44 ± 0.22	1.6
Dimers	41.01 ± 0.20	38.80 ± 0.20	−5.4
Ala-A_2pm	38.28 ± 0.50	34.21 ± 0.30	−10.6
A_2pm-A_2pm	2.73 ± 0.30	4.59 ± 0.10	68.1
Trimers	5.54 ± 0.01	4.08 ± 0.08	−26.3
Anhydros	3.77 ± 0.14	2.43 ± 0.01	−35.5
Monomers	0.64 ± 0.05	0.65 ± 0.01	1.6
Dimers	2.70 ± 0.12	1.66 ± 0.01	−38.5
Trimers	0.43 ± 0.03	0.12 ± 0.02	−72.1
Lys Arg muropeptides	2.36 ± 0.28	1.86 ± 0.03	−21.2
Cross-linked muropeptides	24.20 ± 0.10	22.12 ± 0.16	−8.6
Ala-A_2pm	22.71 ± 0.26	19.68 ± 0.20	−13.3
A_2pm-A_2pm	1.49 ± 0.16	2.44 ± 0.04	63.8

[a] The relative amounts of the muropeptides and the degree of cross-linkage were calculated as described by Glauner (16). The averages of two determinations each from two independent experiments are given.

[b] The abbreviations used for the muropeptides are as follows: Tri, GlcNAc-β-1,4-MurNAc-L-Ala-D-Glu-*m*-A_2pm; Tetra, GlcNAc-β-1,4-MurNAc-L-Ala-D-Glu-*m*-A_2pm-D-Ala; Ala-A_2pm, cross-linkage via D-Ala and *m*-A_2pm of two different stem peptides; A_2pm-A_2pm, cross-linkage via *m*-A_2pm and *m*-A_2pm of two different stem peptides; anhydros, all muropeptides carrying a 1,6-anhydromuramic acid; Lys Arg muropeptides, stem peptides substituted at the A_2pm residue by a Lys Arg dipeptide, a leftover from the covalently linked murein lipoprotein (26).

lacks three LTs is not lethal. Growth and division of the triple mutant were normal. We have to conclude either that LTs in general are dispensable for growth and can be substituted by other murein hydrolases such as the endopeptidases or that the simultaneous deletion of MltA, MltB, and Slt70 can be overcome by the presence of additional LTs. Indeed, further LTs have been described for *E. coli*. A membrane-bound LT, which is referred to as MltC, has recently been identified (11). Furthermore, a novel membrane-bound LT has been isolated and characterized to be an endoglycosylase-type enzyme, which is in contrast to all other LTs that are exoglycosylases (37).

Theoretically, growth and division of the murein sacculus could be accomplished by the participation of either LTs or endopeptidases. According to a model called three-for-one (22, 24), which has been proposed to explain the safe enlargement of the stress-bearing monolayered murein sacculus of *E. coli*, three new cross-linked glycan strands are attached underneath one strand (the docking strand) in the murein layer. Upon the specific release of the docking strand by murein hydrolases, the triple pack of new murein is automatically pulled into the layer under stress. Removal of the docking strand could be done either by degrading the strand by the action of LTs or by cutting out the intact strand by endopeptidases. Therefore, it is feasible that endopeptidases play the role of pacemaker enzymes, whereas LTs may be involved in recycling of murein turnover products. Degradation of the released glycan strands into their monomeric subunits by LTs would enable the cell to take up the murein turnover products into the cytoplasm and to reuse them for the formation of new murein precursors (19, 29, 42). Hence, a concerted action of endopeptidases and LTs, which represents the reversal of the reactions catalyzed by the bifunctional synthetic murein transpeptidase-transglycosylase penicillin-binding proteins (PBPs), is likely to be important for an efficient turnover and recycling of murein during growth.

Indeed, some dramatic changes in the structure of the murein sacculus are present in the triple mutant. One major change was a relative decrease in the amount of chain ends, which in *E. coli* are marked by 1,6-anhydromuramyl residues. This indicates an increase in the average length of the glycan strands compared to that of wild-type murein. Pulse-chase experiments have shown that normally the average length of the glycan strands decreases during maturation of the murein sacculus (17). LTs known to function as exoglycosylases (45) are probably responsible for the shortening of the murein strands, a reaction that is likely to be severely hampered in the mutant strain. The larger decrease in cross-linked anhydromuropeptides compared to uncross-linked anhydromuropeptides in the mutant can be explained by assuming that in the wild type, the LTs preferentially shorten the glycan strands from one end in a processive manner until the enzyme reaches a cross-linkage where it stops. This increases the amount of cross-linked muropeptides in comparison to that of uncross-linked muropeptides at the chain ends. The absence of three of the LTs in the mutant thus prevents the accumulation of cross-linked chain ends. More difficult to explain are the findings of the dramatic increase in LD-*m*-A_2pm-*m*-A_2pm cross-links by about 60%, which is contrasted by a decrease in DD-Ala-*m*-A_2pm cross-links, in particular of the trimeric muropeptides, by about 26%. In addition, there was a significant increase (by about 25%) of the amount of disaccharide tripeptides. One explanation could be that the absence of Slt70, MltA, and MltB in the triple mutant affects the rate of murein turnover and as a consequence results in a relative increase in aged murein. Murein from the stationary-growth phase is known to be rich in disaccharide tripeptides and LD-*m*-A_2pm-*m*-A_2pm cross-links (17, 18).

It has been speculated that murein hydrolases, which are potentially suicidal enzymes (autolysins), might be controlled by a specific topological distribution in the bacterial cell wall (13, 31, 33). In this context, it is interesting to find that three of the LTs, namely, MltA, MltB, and MltC, are lipoproteins residing in the outer membrane of the cell envelope. In addition, Slt70 has been shown by electron microscopy to bind exclusively to the outer surface (facing the outer membrane) of the murein sacculus (57). Thus, quite analogously to gram-positive bacteria, even in the gram-negative bacterium *E. coli*, murein hydrolases and synthases interact with the murein sacculus from opposite sites. According to the widely accepted concept of an inside-to-outside growth mechanism of the thick, multilayered peptidoglycan of gram-positive bacteria, murein hydrolases are thought to be active exclusively in the outermost layers (33), whereas the synthases, which are anchored to the cytoplasmic membrane (2, 52), act upon the inner surface of the peptidoglycan shell. Such a topological distribution of the enzymes was also predicted on the basis of the three-for-one growth model (24) for gram-negative bacteria.

The different types of anchorage to the inner and outer membranes of the murein synthases and hydrolases do not mean that both enzyme systems cannot come in direct contact with each other or could not form multienzyme complexes, as has been proposed elsewhere (22, 24, 44). However, in this case, the formation of contact sites between both membranes would be expected to take place. In accordance with this expectation, murein-synthesizing activity in general (28) and the protein PBP1B in particular (3) were shown to be enriched in membrane contact sites.

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