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 [3H]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 nonmeric 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-acetylgalactosamine (GlcnAc) but in addition transfer the 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). Peptolytic 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.

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), λ58 (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 MgSO₄ 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.

PCR products 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 MgCl₂, 250 μM deoxynucleoside triphosphates, 100 μM of 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.
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'-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'-AGGGAATTTCATGAAAGAGCGTTGGTAAAG TACC-3') and MLTA2 (5'-AAAGATCTCTGGGAGCGTAAAG TACC-3') and the downstream region, a 1.1-kb EcoRI/HindIII fragment from pBCSK+; and the downstream region, a 1.1-kb EcoRI/HindIII fragment from pBCSK+, and the downstream region, a 1.1-kb EcoRI/HindIII fragment from pBCSK+. The amplified fragments were purified, digested with EcoRI and BamHI, and cloned into the corresponding sites of pJFK118EH (5), a vector carrying the tac promoter and the lacI3 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 XhoI/Smal fragment from pJL8582 (Fig. 1), was ligated with a chloramphenicol acetyltransferase gene, a 1.6-kb Hael fragment from pBCSK+, and the downstream region, a 1.1-kb EcoRI/HindIII 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 phase-mediated transduction. Accordingly, E. coli MC1061 harboring pJL38C was infected with the Kohara phage λ458, 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).
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 slyV deletion was MU616 (53); a deletion of the mltB gene was found in JC10288 (9). To verify the absence of the different Lts mutants were grown aerobically in L broth at a density of about 108 (OD600) 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 BamHI fragment from pl2C1-33 (8, 55) was cloned into pJK12 (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 \( \mu \)g of [\( ^{3}H \) ]palmitate (54 Ci mmol \(^{-1} \); Amersham Buchler) was inoculated with XL-1-Blue(pFK118EH) and XL-1-Blue(pMAT) to an OD578 of 0.01. The cells were grown aerobically at 37°C to an OD578 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 \( \mu \)g of gentamicin (Sankyo Co., Ltd., Tokyo, Japan) ml \(^{-1} \); Amersham Buchler). LB medium (10 ml) containing 50 \( \mu \)g of [\( ^{3}H \) ]palmitate (54 Ci mmol \(^{-1} \); Amersham Buchler) was inoculated with XL-1-Blue(pFK118EH) and XL-1-Blue(pMAT) to an OD578 of 0.01. The cells were grown aerobically at 37°C to an OD578 of 0.4, and expression of MRA was induced for 30 min by the addition of 1 mM IPTG (30) in the presence or absence of 100 \( \mu \)g of gentamicin (Sankyo Co., Ltd., Tokyo, Japan) ml \(^{-1} \); Amersham Buchler). LB medium (10 ml) containing 50 \( \mu \)g of [\( ^{3}H \) ]palmitate (54 Ci mmol \(^{-1} \); Amersham Buchler) was inoculated with XL-1-Blue(pFK118EH) and XL-1-Blue(pMAT) to an OD578 of 0.01. The cells were grown aerobically at 37°C to an OD578 of 0.4, and expression of MRA was induced for 30 min by the addition of 1 mM IPTG (30) in the presence or absence of 100 \( \mu \)g of gentamicin (Sankyo Co., Ltd., Tokyo, Japan) ml \(^{-1} \); Amersham Buchler).

Membrane fractionation. Inner and outer membranes were separated by sonic transduction as described previously (14).

Murein hydrolyase activity assay. Murein sacculi were prepared and hydrolyzed with cellolysin (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 sequencing project (accession number U32251). 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).

RESULTS

Mapping and cloning of the mltC gene. The previously determined peptide sequences from the mltC 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 mltC 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 \( \lambda 458 \) (9A12) and \( \lambda 459 \) (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 \( \lambda 459 \) 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 lacZ 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).

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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 anhydromuropeptides by 35.5%. In agreement with the earlier demonstration in earlier reports (32, 35).

**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 and outer 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.

**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 [3H]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).

**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
muramic acid; Lys Arg muropeptides, stem peptides substituted at the A2pm
two different stem peptides; anhydros, all muropeptides carrying a 1,6-anhydro-
muropeptides 24.20
Cross-linked muropeptides 24.20
transpeptidase-transglycosylase penicillin-binding proteins
reactions catalyzed by the bifunctional synthetic murein
murein precursors (19, 29, 42). Hence, a concerted action of
would enable the cell to take up the murein turnover products
released glycan strands into their monomeric subunits by LTs
recycling of murein turnover products. Degradation of the
role of pacemaker enzymes, whereas LTs may be involved in
pulling into the layer under stress. Removal of the docking
hydrolases, the triple pack of new murein is automatically
neath one strand (the docking strand) in the murein layer.
coli, three new cross-linked glycan strands are attached under-
other murein hydrolases such as the endopeptidases or that the
mutant were normal. We have to conclude either that LTs in
six experiments have shown that normally the average length of
strands compared to that of wild-type murein. Pulse-chase
experiments have shown that normally the average length of
the glyc an 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 anhydromu-
rop eptides compared to uncross-linked anhydromuropeptides
in the mutant can be explained by assuming that in the wild
type, the LTs preferentially shorten the glyc an 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-A2pm-m-A2pm cross-links by
about 60%, which is contrasted by a decrease in DD-Ala-m-
A2pm 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-A2pm-m-A2pm 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 exclu-
sively to the outer membrane (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, multi-
layered peptidoglycan of gram-positive bacteria, murein hydro-
lases are thought to be active exclusively in the outermost
layers (33), whereas the synthases, which are anchored to the
cyttoplasmic 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 expec-
tation, murein-synthesizing activity in general (28) and the
protein PPB1 in particular (3) were shown to be enriched in
membrane contact sites.