Chapter 4

Identification of New Members of the Lytic Transglycosylase Family in Escherichia coli and Haemophilus influenzae

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**Summary**

Although the bacterial peptidoglycan metabolism and numerous enzymes involved therein have been studied extensively over the years, information on the precise number and specific function of these enzymes is still lacking. This observation holds true even for the well-studied bacterium *Escherichia coli*. The determination of the complete sequences of bacterial genomes, for which that of *Haemophilus influenzae* is the archetype, provides an invaluable opportunity to obtain a complete overview of the different families of peptidoglycan metabolizing enzymes. It allows the identification of genes, for which the activity of the encoded enzymes may not be determined under normal laboratory conditions. Following this rationale, *H. influenzae* and *E. coli* genomic sequence information was searched for new members of the family of lytic transglycosylases, using structure-derived sequence information. A new putative lytic transglycosylase gene could be identified that proved to be present in both bacterial species. The *E. coli* gene coding for this third membrane bound lytic transglycosylase, called MltC, was cloned and peptidoglycan hydrolyzing activity was demonstrated for the gene product. Targeted disruption of the gene showed that it is not essential for *E. coli* grown under laboratory conditions. The *mltC* gene seems to be part of an operon, the structure of which is conserved between *E. coli* and *H. influenzae*.

**Introduction**

Bacterial peptidoglycan metabolism involves several enzyme families, the members of which have the same substrate and/or product specificity. Whether this redundancy merely serves as a backup mechanism or rather reflects a specific physiological role for each individual enzyme, remains a matter of speculation in most cases. The most extensively studied family is that of the penicillin-binding proteins (PBPs), of which numerous members have been characterized in several bacterial species. However, even for the well-studied bacterium *Escherichia coli* the exact number of proteins in this family and their specific function remains elusive. The concept of enzyme families is not limited to the synthetic part of the peptidoglycan metabolism but it is also apparent in the hydrolytic pathway. Although one might expect a redundancy in peptidoglycan hydrolases to be quite hazardous for a bacterium, this class of enzymes comprises several enzyme families with multiple members. In *E. coli*, for example, families of amidases, carboxypeptidases, endopeptidases and lytic transglycosylases are present (19, 21, 28).

In order to get a clear picture of the variety of enzymes in the different families, biochemical data and genetic data have to be combined. Using the classical approach, i.e. starting of with a measurable activity and then trying to identify the gene of which the product is responsible for this activity, certain members of a family may be overlooked because they are not expressed or are not active under the experimental conditions. On the other hand, the enzymatic activity of members that
have been identified solely on the basis of sequence homology, has to be shown biochemically.

The determination of the complete genome sequence of a bacterium supplies all the genetic information needed to get a overview of all the genes involved in the peptidoglycan metabolism, provided they can be identified by their homology to known proteins. The recent publication of the sequence of the entire genome of Haemophilus influenzae (11) provides the first opportunity for such an approach.

We have set out to search the H. influenzae genome for members of the lytic transglycosylase family. This family of peptidoglycan hydrolases was originally identified in E. coli (13), but based on the presence of homologous genes to the E. coli slt gene in Enterobacter cloacae and Salmonella typhimurium (1), and the release of the specific products of these enzymes by Bordetella pertussis (4) and Neisseria gonorrhoeae (22), it is expected to be present in a broad range of gram-negative bacteria. In E. coli, enzymatic activity has been demonstrated for three members of this family: Slt70 (13), MltA (29) and MltB (5). Moreover, two open reading frames have been identified that show homology to Slt70 but for which no activity has been shown so far: yafG and yfhD (16).

Researchers at the Institute for Genomic Research (TIGR), the institute where the full H. influenzae genome sequence was determined, had identified two members of the lytic transglycosylase family in H. influenzae and these were assigned as such in their database: a homologue (accession number HI0872) of Slt70 and a homologue (accession number HI0232) of yfhD (Fig. 3). We searched for other members of the family using the fingerprints that were derived from the structure of Slt70 and that are most prominent in the architecture of the active site: E(478)S, containing the catalytic glutamate, G(493)LMQ and A(551)YNAG (7). Searching for the presence of these fingerprints in the correct order, allowing a high degree of freedom in the spacing of them and some mismatching, differs from the approach taken by Koonin and Rudd who defined a bacterial transglycosylase motif (16), and has previously led to the identification of a number of putative transglycosylases that could not be identified by using this motif (2, 6).

Materials and methods

Molecular cloning

DNA manipulations and cloning procedures were performed according to described protocols (20). Plasmid DNA was prepared using Qiagen (Hilden, Germany) purification systems. PCR reactions were performed with AmpliTaq DNA-polymerase and reagents from Perkin Elmer (Norwalk, CT) with the following cycling protocol: melting at 94°C for 1 minute, annealing at 56°C for 2 minutes, and extension at 72°C for 1 minute for a total of 30 cycles. Three different PCR products were generated: a full-length product, coding for ORF_o179 plus ORF_o180 (MltC), a product coding for ORF_o179 plus ORF_o180, without the putative N-terminal signal sequence and a product coding solely for ORF_o180 (C-terminus of MltC). All primer pairs used, introduced an NcoI site at the startcodon and a SalI site downstream of the ORF_o180 stopcodon. The PCR products were cloned into the pGEM-T vector (Promega, Madison, WI). Cycle sequencing was performed using the Cyclist system (Stratagene, La Jolla, CA).
Overproduction

The NcoI-SalI fragments of the PCR products were subcloned into vector pET21d+ (Novagen, Madison, WI, USA), thereby bringing the expression under the control of the T7 promoter (23). The expression constructs were transformed into host BL21(DE3) (F* dcm ompT hsdS (rB- mB-) gal (DE3)) (Stratagene, La Jolla, CA). Expression was induced by the addition of 1 mM IPTG (Boehringer Mannheim, Germany) to cultures grown in LB medium supplemented with 100 μg/ml Ampicillin, at an OD600 of 0.6. Three individual clones were evaluated for the overproduction of protein and for peptidoglycan hydrolase activity, using zymogram analysis. For larger scale preparations, 500 ml cultures were induced for expression at an OD600 of 0.6 and allowed to grow for one hour. Cells were harvested by centrifugation and resuspended in 10 mM Tris-maleate-NaOH buffer pH 7.3, containing 10 % glycerol and were disrupted by passage through an Aminco French pressure cell at 15,000 psi. Solubility of the overproduced protein and the presence of inclusion bodies was assessed by low speed centrifugation of the cell extract at 8000 x g for 30 minutes, followed by a high speed centrifugation of the supernatant at 100,000 x g for one hour.

Zymography analysis, inhibitor studies and hydrolase assay

Zymogram analysis of crude extracts was carried out essentially as described (3), using 12% PAA gels, loaded with 0.025 % purified E.coli peptidoglycan. After electrophoresis, renaturation was carried out overnight at 37°C in 20 mM phosphate buffer pH 6.8, containing 1 mM MgCl2 and 0.1% Triton X-100. After renaturation, the zymograms were washed with water and stained with 0.1% methylene blue in 0.01% potassium hydroxide solution. The zymograms were destained with water and analyzed using a model GS-700 imaging densitometer (BioRad Laboratories, Hercules, CA). For determination of the sensitivity of the protein towards bulgecin, dilutions of an extract containing the overproduced C-terminal domain to which purified Slt35 was added as an internal control, were separated on a zymogram gel. The samples were applied in duplo and after electrophoresis the zymogram was split in two halves. The washing and renaturation procedure for one half of the zymogram was carried out in the presence of 100 μg/ml bulgecin A (Takeda Chemical Industries, Osaka, Japan), whereas the other half that served as a control was processed in the absence of bulgecin.

Peptidoglycan hydrolase activity was determined by measuring solubilization of radiolabeled peptidoglycan polymer following a published procedure (9).

Product analysis

Muropeptides that were liberated upon peptidoglycan hydrolysis were analyzed by HPLC, using a described method (10). Purified E. coli peptidoglycan was incubated with crude extracts of cultures that were induced for the expression of the different proteins. In a typical experiment 50 μg peptidoglycan was digested with 10 μl of a cell extract, corresponding to 100 μl of culture with an OD600 of 1. The reaction was allowed to proceed at 37°C for 2 hours, after which the sample was boiled for 5 minutes, followed by centrifugation. The released muropeptides were separated by HPLC, on a Merck Hitachi HPLC system using a Lichrospher RP18 column (Merck AG, Darmstadt, Germany). A linear gradient from 0 to 20 % acetonitrile in 0.05% trifluoroacetic acid was applied over 80 minutes with a flow of 0.5 ml/min. The
resulting HPLC pattern was compared to that of a sample of Slt70-digested peptidoglycan. Purified monomeric 1,6-anhydromuropeptides, of which the structure had been confirmed by tandem electron-spray mass spectroscopy, served as a control.

**Targeted disruption of the mltC gene**

A copy of the *mltC* gene that had been cloned into pET21d+, was disrupted by the exchange of an internal *Bsp*MI fragment by a kanamycin cassette. This was achieved by *Bsp*MI digestion of plasmid pETMltC, followed by treatment with Klenow enzyme to create blunt ends and ligation with the *Sma*I fragment from the pUC4-KIXX plasmid (Pharmacia, Uppsala, Sweden), that carries the kanamycin resistance gene. The disrupted *mltC* copy was then removed from the plasmid by digestion with *Not*I followed by Klenow treatment and digestion with *Xba*I. The resulting fragment was ligated into the pMAK705 vector, that had been treated with *Cla*I, Klenow and *Xba*I. The pMAK705 plasmid carries a temperature sensitive origin of replication, allowing targeted disruption of essential genes, following the method described by Hamilton et al. (12). *

E. coli* strain MC1061 (araD139 Δ (ara-leu)7696 Δ(lac)174 rpsL thi-1) was used as host for recombination. After transformation of plasmid pMAKmltCKan into MC1061 and growth at 30°C for several generations, dilutions were plated out on LB plates containing chloramphenicol and kanamycin and the plates were incubated at 44°C to select for integration. Cointegrates were grown at 30°C to allow a second recombination event, leading to resolution of the integrated plasmid. After plating out at 30°C, single colonies were evaluated for the ability to grow at 44°C on LB plates containing kanamycin.

**Primer extension analysis**

In order to enhance the levels of mRNA coding for MltC, the chromosomal region containing the *mltC* gene was cloned into a high copy number vector. This region was derived from phage number 467 of the Kohara library, an ordered library of overlapping phage clones that represents the whole genome of *E. coli* (15). Isolated phage DNA was digested with *Bam*HI and *Eco*RI and the 3.6 kb fragment that contained the *mltC* gene was cloned into pUC18 (Pharmacia, Uppsala, Sweden) and the identity of the cloned fragment was confirmed by sequencing. *E. coli* Xl1-blue that was transformed with the resulting plasmid, served as host for the isolation of RNA. RNA was isolated using the RNA-purification system from Qiagen (Hilden, Germany). Antisense primers that hybridized approximately 50 bp downstream of the start codons of *orf91* and *mltC* were labeled with γ-32P-ATP (Amersham, Buckinghamshire, UK), using polynucleotide kinase (Promega, Madison, WI). Primer extension reactions were performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase and reagents from Promega, following the protocol as supplied by the provider. The reactions were loaded on a 6% denaturing sequencing gel and cycle sequencing products that had been generated using the same primers and the Cyclist system from Stratagene, were run in parallel.

**Northern blot analysis**

Total RNA was analyzed by Northern blotting for the levels and length of the mRNAs that encode MltC and Orf91. RNA was purified with the aid of Qiagen purification systems, and 10 μg quantities were separated on denaturing agarose
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gels, following described protocols (20). After electrophoresis, the RNA was transferred onto a positively charged nylon membrane (Boehringer Mannheim), using a vacuum blotting device (BioRad Laboratories, Hercules, CA). The full length \textit{mltC} and \textit{orf91} genes that were used as probes, were amplified by PCR and concomitantly labeled with digoxigenin (DIG) (Boehringer Mannheim). After prehybridization at 50°C for 1 hour in EasyHyb solution (Boehringer Mannheim), the membranes were hybridized with the probes for 16 hours at 50°C. Washing of the membranes was performed with 0.5xSSC, 0.1% SDS at 68°C, after which they were incubated with anti-DIG-alkaline phosphate conjugate (Boehringer Mannheim), following the suppliers directions. Chemiluminescent detection of alkaline phosphatase activity was achieved by incubation with Disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2’-(5’-chloro)tricyclo[3.3.1.13,7]decan]-4-yl)phenyl-phosphate (CSPD) (Tropix, Bedford, MA). After incubation for 15 to 20 minutes the membranes were exposed to Biomax film (Eastman Kodak, Rochester, NY).

Results

Identification of the \textit{MltC} gene

An open reading frame for which no function was assigned in the TIGR database of the \textit{H. influenzae} genome, was found to contain the Slt70-derived fingerprints. Apart from the fingerprints this sequence shows additional homology to the catalytic domain of the \textit{E. coli} Slt70 (Fig. 1). Surprisingly, the C-terminal half of this \textit{H. influenzae} sequence also showed strong homology to an open reading frame, ORF\_o180, encoded within the 65-68 minutes region of the \textit{E. coli} genome, as determined by the \textit{E. coli} genome project (GenBank: U28377). In addition, the N-terminal half of the \textit{H. influenzae} sequence showed strong homology to an open reading frame, ORF\_o179, just upstream of ORF\_o180. Therefore two possibilities could be envisaged: either the \textit{E. coli} sequence contained a sequencing error and the two open reading frames are in fact one, or the \textit{H. influenzae} sequence codes for two proteins. We favored the first option, as the \textit{H. influenzae} sequence encoded a consensus lipoprotein signal sequence, whereas \textit{E. coli} ORF\_o179 did not. The presence of a (lipoprotein) signal sequence, resulting in a localization in the periplasm would be a prerequisite for a protein that functions as a lytic transglycosylase. This would also be in accordance with the findings of West-Hansen et al., who determined the sequence of the region comprising both ORF\_o179 and ORF\_o180 during their effort to sequence the \textit{nupG} gene that is located directly downstream of ORF\_o180 (30). Although they only determined the sequence for one strand and this sequence was never deposited in GenBank, they postulate one single rather than two open reading frames. The \textit{E. coli} DNA sequence comprising both ORF\_o179 and ORF\_o180 was amplified and the region were the suspected frame shift mutation was located was resequenced. Indeed, the presence of a one base pair deletion in the described sequence could be shown (the corrected has been submitted to GenBank, accession number U59902).
Identification of MltC

Expression of MltC

The gene coding for the potential new transglycosylase from *E. coli*, now called Membrane bound Lytic Transglycosylase C (MltC), was cloned and expressed in three different forms: the full length sequence, the sequence without the putative lipoprotein signal sequence and the C-terminal half of the sequence, corresponding to ORF_o180. All three constructs could be overproduced at high levels, using the T7 promoter (Fig. 2A).
None of the proteins provoked lysis upon overproduction, in contrast to the lytic effect of the expression of MltB (8, 24). Induction of the full length construct led to the accumulation of a 40 kDa protein, confirming that both open reading frames constitute a single gene. This finding is in accordance with the appearance of a 40 kDa protein upon expression of the nupG region in minicells (30). Overproduction of all three forms of the protein led to the formation of inclusion bodies. Whereas for the full length protein and the C-terminal domain, the bulk of the protein seemed to aggregate (data not shown), a large fraction of the protein that was overproduced in the cytoplasm proved to be soluble (Fig. 2 C).

**Peptidoglycan hydrolase activity**

Using zymogram analysis, low but significant peptidoglycan hydrolase activity could be shown for the full length MltC, suggesting that at least a small fraction of the protein is able to refold from an SDS-denatured form to an active hydrolase (Fig. 2B). No activity higher than the background activity from endogenous peptidoglycan hydrolases, could be demonstrated in cell extracts though. This may be explained by the absence of high enough amounts of correctly folded protein. However, also the cytoplasmically overproduced protein, a large fraction of which seems to be correctly folded as witnessed by the amount of soluble protein, did not show high activity in cellular extracts, although activity could be observed on zymograms. Surprisingly, the truncated C-terminal half of MltC displayed the highest activity, both on zymograms and in cellular extracts. The activity of this
domain allowed the characterization of the muramylpeptides produced by this new transglycosylase. Therefore, peptidoglycan was digested with the overproduced domain and the products were analyzed by HPLC. Indeed, they proved to be of the 1,6-anhydro type (Fig. 6). The absence of dimeric muropeptides in the displayed chromatogram, is most likely due to the presence of endogenous endopeptidases.

**FIG. 6.** HPLC analysis of muramylpeptides as produced by degradation of peptidoglycan by the C-terminal domain of the new lytic transglycosylase (upper panel) and Slt70 (lower panel). The positions of the main monomeric 1,6-anhydro-muramylpeptides are indicated. The small shift in retention times as observed for the 1,6-anhydro-muramylpeptides produced by the C-terminal domain of MltC, is often seen when the reaction mixture contains cellular extracts instead of purified proteins.

**Inhibition by bulgecin**

The homology of the C-terminal domain of MltC to the catalytic domain of Slt70, combined with the production of 1,6-anhydro-muramylpeptides by this domain, suggests that the structures of the active sites of both domains may be quite similar. To provide further evidence for this hypothesis, the effect of bulgecin on the activity
of the C-terminal domain of MltC was studied. Bulgecin is the only known inhibitor of Slt70 and has been shown to bind specifically to the active site (24, 26). It could be demonstrated that the presence of bulgecin-A during the refolding procedure, inhibits the degradation of peptidoglycan by the C-terminal domain (Fig. 5). The specificity of this inhibition was validated by the failure of bulgecin to interfere with the activity of Slt35(MltB), a lytic transglycosylase that is not sensitive to this inhibitor.

![Figure 5](image)

**FIG. 5.** Bulgecin sensitivity of the C-terminal domain of MltC. Panel A displays a zymographic analysis of dilutions of a cellular extract containing overproduced C-terminal domain, supplemented with 10 μg Slt35(MltB). Dilutions were as indicated. Panel B shows the same samples, analyzed in the presence of 100 μg/ml bulgecin.

**Targeted disruption of the mltC gene**

All three lytic transglycosylase genes that have been identified in *E. coli* so far, *slt*, *mltB* and *MltA*, proved not to be essential (8, 17, 24). Of these three, only the Slt70 encoding gene is present in *H. influenzae*. As the newly identified MltC is present in both species, the question regarding its essentiality was addressed by targeted disruption of the gene. The method used for this targeted disruption allows the inactivation of the chromosomal copy of essential genes and is summarized in figure 6. A disrupted copy of the gene was cloned into a plasmid with a temperature sensitive origin of replication that carries a chloramphenicol resistance marker (12). When this plasmid was introduced in *E. coli* strain MC1061, integration of the plasmid could be selected for by growth on chloramphenicol at 44°C, because the plasmid cannot replicate at this temperature anymore. A second recombination event at 30°C allowed resolution of the plasmid, resulting in an inactivated chromosomal copy and an intact copy under control of the *lac* promoter on the plasmid. The *mltC* mutants that were constructed this way were tested for the ability to grow at 44°C. If the gene would have been essential the bacteria should stop growing after the temperature shift, due to the loss of the intact copy of the gene on the plasmid. This turned out not to be the case for the *mltC* deletion mutant. Upon a shift to 44°C the bacteria did not lose their viability but lost the plasmid, as witnessed by a loss in chloramphenicol resistance. The resulting *mltC* deletion was confirmed by PCR. When the *mltC* was amplified, the resulting fragment differed from the wild type gene both in size and in the presence of an *NcoI* restriction site, derived from the kanamycin resistance gene.
FIG. 6. Targeted disruption of the \textit{mltC} gene. The map of plasmid pET\textit{mltC}kan, carrying a kanamycin cassette in the \textit{mltC} gene, is not depicted. Only the crossover events leading to the desired exchange are shown. A second cross-over in the 5' end of the gene would result in resolution of the original plasmid. Details on the followed procedure are described in the materials and methods section.
The deletion mutant proved not to display differences in growth characteristics and cellular morphology, and the penicillin-induced lysis profile was also comparable to that of the parent strain, showing that MltC is not directly involved in autolysis. MltC was shown to be a target for bulgecin (see above), a glycopeptide that potentiates the effects of certain beta-lactam antibiotics, in particular PBP3 inhibitors (14). The combined use of these antibiotics and bulgecin causes the formation of bulges. The observation that a strain carrying a deletion in the slt gene also showed this phenotype, when treated with PBP3 inhibitors, pointed to the Slt70 as the target for bulgecin (24). Therefore we tested the effects of these antibiotics on the mltC deletion strain. The minimal inhibitory concentrations (MIC’s) for azthreonam and cefmenoxime were not significantly altered in comparison with the parent strain, nor was the MIC for mecillinam, that was described to be lowered for the slt mutant (24). Also the lysis induction by azthreonam was not affected in the mltC mutant, which again was a prominent feature of the slt mutant.

Operon structure of MltC

From a comparison of the genomic context of the MltC encoding genes in E. coli and H. influenzae it appeared that mltC belongs to a cluster of three genes that were conserved in these two bacterial species. The two other genes, that are both located upstream of the mltC gene, are an open reading frame, orf91 of unknown function and the gene encoding MutY, an adenine glycosylase that is involved in DNA mismatch repair (18, 27). West-Hansen et al. analysed several chromosomal fragments containing the nupG gene, that is located down stream of mltC and is not conserved at that position in H. influenzae, for expression of proteins in minicells (30). A fragment encoding the whole orf91 open reading frame plus downstream region resulted in the expression, apart from that of NupG, of both a protein band corresponding to the size of orf91 and one corresponding to MltC, as judged from the PAA gels displayed in the paper by West-Hansen et al. However, when a fragment starting from an HincII site internal to the orf91 was analyzed, it appeared that both orf91 and MltC were not expressed. This may indicate that at least orf91 and mltC form an operon. To investigate this possibility, an attempt was made to identify transcriptional start sites upstream of both genes. Using and identical RNA preparation as a template, only the primer extension analysis of the region upstream of orf91 produced a clear product, whereas that of the region upstream of mltC failed to do so. This observation supports the hypothesis that both genes form an operon, the promoter of which is located upstream of orf91 (Fig. 7).

Upstream of the transcriptional start as identified by the primer-extension analysis, a reasonable -10 and -35 sequence could be identified. A very faint additional product could be identified, the length of which would correspond to a transcriptional start within the proposed -10 region. As alternative -10 regions further upstream could not be identified from the sequence, the reason for the appearance of this fragment is unclear.

Surprisingly, the proposed promoter appeared to be located in the coding region of the mutY gene. This observation, combined with the fact that the proposed open reading frames coding for the MutY and Orf91 homologs in H. influenzae actually overlap (see Fig. 9), suggest that MutY may also be part of the same transcriptional unit. To investigate this in more detail, Northern blot analysis was performed.
Identification of \textit{MltC}

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Identification of \textit{MltC}

\textbf{FIG. 7.} Primer extension analysis of the region upstream of orf91. The extension product, marked \textquotedblleft P\textquotedblright, is indicated and the transcriptional start, marked with a box, can be derived from the sequencing pattern that was generated with the same primer. The relevant part of the sequence is shown and the proposed -10 sequence is depicted in bold in the left panel and underlined in the right panel, as is the proposed -35 sequence.

Figure 8 shows the results of analysis of total RNA for the presence of transcripts hybridizing with the \textit{mltC} gene or the \textit{orf91} gene.

\textbf{FIG. 8.} Transcriptional analysis of the \textit{mltC} operon. Panel A: Lane 1, total RNA hybridized with a \textit{orf91} probe; lane 2, DIG-labeled RNA markers of indicated sizes. Panel B: Lane 1, total RNA hybridized with a \textit{mltC} probe; lane 2, markers. Panel C: Lane 1: Markers (high range); lane 2, total RNA hybridized with a \textit{groEL} probe, serving as a control. The expected size of the \textit{gro} operon mRNA is 2.3 kb; lane 3, markers (low range).
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Hybridization with an orf91 probe revealed that the most abundant transcript had a size corresponding to the orf91 message only (ca. 400 bp). Inspection of the sequence between orf91 and mltC revealed the presence of an inverted repeat sequence which could possibly function as a rho-independent transcriptional terminator. However, two further transcripts could be detected, the sizes of which could possibly correspond to a transcript carrying both mltC and orf91 (expected size: 1.5-1.6 kb) and one carrying mutY, orf91 and mltC (expected size 2.5-2.6 kb). The latter two were also seen in an Northern analysis which involved mltC as a probe. These observations may indicate that the mutY, orf91 and mltC genes are transcribed from both the putative mutY promoter and the promoter upstream of orf91, the latter being the stronger of the two. The terminator sequence would then negatively regulate mltC transcription by allowing only a certain amount of read-through. Care must be taken though with the interpretation of these results as a control hybridization with groEL also showed a band in the size range of the putative orf91-mltC transcript, in addition to the expected signal of 2.3 kb for the groEL-ES operon. Therefore the signal that possibly corresponds to the orf91-mltC transcript may be unspecific. In an analysis of RNA isolated from stationary phase cells though, this signal had disappeared from the groEL hybridization, whereas it was still present in the mltC hybridization, suggesting that it is specific. Furthermore, a preliminary analysis of H. influenzae RNA showed a clear band of a size corresponding to an orf91-mltC transcript after hybridization with a H. influenzae mltC probe. After hybridization with an H. influenzae orf91 probe, the appearance of a band of the same size plus a band that could correspond to a separate orf91 transcript could be observed, although this hybridization appeared to be less specific (A. J. Dijkstra and D. Wildt-Perinic, unpublished observations).

The results of the analysis of the proposed mltC operon are shown schematically in figure 9.

**FIG. 9.** Features of the proposed mltC operon. Large arrows represent open reading frames. Stem-loops indicated the position of inverted repeats, the one downstream of E. coli mltC overlapping with the nupG promoter (30). Open arrowheads mark the position of promoters, the one upstream of mutY only being putative (27). Small filled arrowheads denote the position of the primers that were used for the primer-extension analysis. The position of the HincII site is indicated as are the proposed mRNA transcripts and their expected sizes.
Discussion

Using fingerprints that were derived from the structure of Slt70, we were able to identify a new type of lytic transglycosylase that is present in *H. influenzae* and *E. coli*. Based on the presence of a consensus lipoprotein signal sequence and the fact that the expressed protein fractionates with the membrane fraction of minicells (30), we propose to dub this new member: membrane bound lytic transglycosylase C (mltC). The validity of this homology was proven by showing the activity of the *E. coli* protein on zymograms and demonstrating the specific production of 1,6-anhydro-muramylpeptides and the inhibition of the enzyme by bulgecin. As the homology is limited to the C-terminal domains of both proteins, it is unlikely that the structural resemblance between this new transglycosylase and Slt70 extends beyond the lysozyme-like catalytic domain. Rather, a different domain seems to be attached to the lysozyme module and this domain may be involved in the regulation of the hydrolase activity. This would be an explanation for the observation that when the C-terminal domain is uncoupled from the N-terminal domain, its activity is enhanced dramatically. The presence of such a regulatory domain makes sense, as the tight regulation of the enzyme’s potentially lethal activity is probably of vital importance to the bacterial cell. It cannot be excluded that other proteins are also involved in this regulation. The presence of these in the cellular extracts tested could be the reason for the difference between the activity seen on zymogram and in liquid assays.

The results presented here add yet another member to the lytic transglycosylase family of *E. coli*, which already consisted of three established and two putative members (Fig. 10). With the addition of this new member, the issue of the functions of the individual lytic transglycosylases becomes ever more intriguing. As was also shown for the three established members, MltC proved not to be essential for survival of *E. coli* under laboratory conditions. An often proposed explanation for this observation is the possibility that another enzyme of the family may substitute for the lost activity as the lytic transglycosylase system is highly redundant. However, the system seems to be less redundant in *H. influenzae*, with MltC being one of only three lytic transglycosylase homologs (Fig. 10). An alternative explanation for the observed dispensability could be that these enzymes are involved in processes that are not needed for normal lab growth. This latter possibility is supported by our identification of lytic transglycosylase homologues that may be involved in *in vivo* processes like toxin secretion, DNA transfer and fimbrial assembly (6).

When the *mltC* deletion mutant was compared with the parent strain by zymography, no difference in the pattern of hydrolase bands could be detected. On the other hand, when the mltC region was present on a high copy number plasmid, no induction of a band on zymogram could be observed (data not shown). As mRNA analysis indicates that the mltC gene is transcribed under normal growth conditions, these observations suggest that the activity of enzyme is regulated on a protein level rather than on a transcriptional level. This regulation may be mediated through the N-terminal domain, as is mentioned above.

The mRNA analysis further suggests that mltC is part of an operon. This is a very interesting finding as, so far, little information is available on the regulation of gram-negative peptidoglycan hydrolases on a transcriptional level and this would, in fact, be the first demonstration of a certified gram-negative peptidoglycan hydrolase
being present in an operon. Although the possibility that mltC has an own promoter cannot be completely ruled out, it is highly likely that its expression is driven by the promoter that was identified upstream of orf91 and that read-through of the putative terminator results in transcription of mltC at a level that is much lower than the transcription of orf91. The organization of the genes even suggests that mutY may be part of this transcriptional unit. This is a surprising finding as a link between peptidoglycan metabolism and DNA repair is not directly obvious. This link may not be an isolated case though as another protein that is involved in DNA repair, mutL, was shown to form an operon with a gene coding for an amidase, AmiB (28). This amidase shows high homology to gram-positive autolytic amidases and overproduction of this protein was shown to have effects that support its putative peptidoglycan hydrolase function, although this activity remains to be proven. In the case of the amiB-mutL operon an internal mutL promoter is located in the amiB coding region, a situation comparable to the internal promoter in mutY that probably drives expression of orf91 and mltC. The physiological implications of these findings are presently unclear, but most likely the explanation lies somewhere in the complex connection between DNA replication and cell division.

**FIG. 10.** The lytic transglycosylase family of *E. coli*. The length of the bars is proportional to the length of the sequences and boxes mark the positions of the fingerprints. The parts of the sequence of Slt70 that form the ring domain and the lysozyme domain are shown. (25) Shaded stretches indicate homology to Slt70 that extends beyond the fingerprints. A flag marks the presence of a consensus lipoprotein signal sequence. Mapping positions are indicated in minutes. The TIGR accession numbers of homologous *H. influenzae* open reading frames, and the percentage of similarity to the *E. coli* sequence are shown.

### References


