The C-terminal Domain of *Escherichia coli* YfhD Functions as a Lytic Transglycosylase*[S]

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The hypothetical *Escherichia coli* protein YfhD has been identified as the archetype for the family 1B lytic transglycosylases despite a complete lack of experimental characterization. The *yfhD* gene was amplified from the genomic DNA of *E. coli* W3110 and cloned to encode a fusion protein with a C-terminal His$_6$ sequence. The enzyme was found to be localized to the outer membrane of *E. coli*, as would be expected for a lytic transglycosylase. Its gene was engineered for the production of a truncated soluble enzyme derivative lacking an N-terminal signal sequence and membrane anchor. The soluble YfhD derivative was purified to apparent homogeneity, and three separate *in vitro* assays involving high pressure liquid chromatography and matrix-assisted laser desorption ionization time-of-flight mass spectrometry were used to demonstrate the YfhD-catalyzed release of 1,6-anhydromuro-peptides from insoluble peptidoglycan. In addition, an *in vivo* bioassay developed using the bacteriophage λ lysis system confirmed that the enzyme functions as an autolysin. Based on these data, the enzyme was renamed membrane-bound lytic transglycosylase F. The modular structure of MltF was investigated through genetic engineering for the separate production of identified N-terminal and C-terminal domains. The ability to bind peptidoglycan and lytic activity were only associated with the isolated C-terminal domain. The enzymatic properties of this lytic transglycosylase domain were found to be very similar to those of the wild-type enzyme. The one notable exception was that the N-terminal domain appears to modulate the lytic behavior of the C-terminal domain to permit continued lysis of insoluble peptidoglycan, a unique feature of MltF compared with other characterized lytic transglycosylases.

Bacteria are enclosed by a sacculus of peptidoglycan (PG)[2] that imparts cell shape as well as retains the integrity of the cytoplasmic membrane. The PG layer consists of glycan chains of two alternating aminosugars, GlcNAc and *N*-acetylmuramic acid (MurNAc), which are cross-linked by short stem peptides attached to the lactyl moiety of the latter. This creates a covalent mesh-like structure that is vital to cell viability; significant damage to this heteropolymer precludes it from withstanding internal turgor pressures leading to cell lysis. Nonetheless, bacteria must produce a number of different autolytic PG-cleaving enzymes to allow for the regular growth and maintenance of the sacculus.

Lytic transglycosylases (LTs) are a class of autolysins that play an integral role in the metabolism of the PG sacculus (reviewed in Ref. 1). These enzymes degrade PG by cleaving the β-(1→4) glycosidic bond between MurNAc and GlcNAc residues, the same bond that is the target of lysozymes (muramidases). However, rather than functioning as hydrolases, LTs cleave PG with the concomitant formation of a 1,6-anhydromuramoyl reaction product (Fig. 1). This lytic action creates space within the PG sacculus to allow for its expansion (reviewed in Ref. 2), as well as for the insertion of various cell envelope-spanning structures such as secretion systems and flagella (reviewed in Ref. 3). These enzymes also contribute to pathogenesis as released 1,6-anhydromuro-peptides have been implicated in contributing to the infection state caused by a number of pathogens, such as *Bordetella pertussis*, *Neisseria gonorrhoeae*, *Neisseria meningitidis,* and *Haemophilus influenzae* (1).

LTs are ubiquitous among all eubacteria that produce PG (4), but the complement of enzymes produced by *E. coli* has been the most extensively examined. *E. coli* is known to produce five membrane-bound lytic transglycosylases (MltA, MltB, MltC, MltD, and EmtA) and one soluble lytic transglycosylase (Slt70). All appear to act as exoenzymes releasing GlcNAc-anhydromuro-peptides from the ends of glycan strands, except for MltE which has been shown to be endo-acting. Collectively, these enzymes form the archetypes for three of the four families of LTs identified by Blackburn and Clarke (4).

The hypothetical *E. coli* protein YfhD has been identified as the archetype for the family 1B LTs based on sequence alignments and the presence of consensus motifs (4). However, despite the fact that YfhD was first recognized as a potential LT over 10 years ago (5, 6), experimental proof confirming its activity is still lacking. That YfhD may function as an LT is supported by the finding that *E. coli* MHD79, a multiple deletion mutant strain lacking the known six LTs exhibits normal growth rates

[2] The abbreviations used are: PG, peptidoglycan; MurNAc, *N*-acetylmuramic acid; LT, lytic transglycosylase; Mlt, membrane-bound lytic transglycosylase; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; HPLC, high pressure liquid chromatography; IPTG, isopropyl 1-thio-β-D-galactopyranoside; ORF, open reading frame; NTA, nitrilotriacetic acid; KDO, 2-keto-3-deoxyoctonate; LAO, lysine, arginine, ornithine.
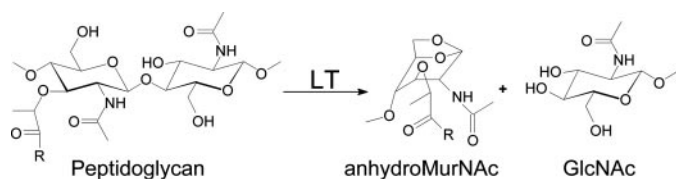
FIGURE 1. Functional activity of LT. The LTs cleave PG between MurNAc and GlcNAc residues with the formation of 1,6-anhydroMurNAc residues. *R* denotes stem peptide.

despite morphological alterations, and 1,6-anhydromuropetides are detectable within its PG sacculus (7). In this study, we present data that confirms YfhD does indeed function as an LT, and based on this evidence, we propose to rename it membrane-bound lytic transglycosylase F (MltF).

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

DNase I, RNase A, Pronase, IPTG, and EDTA-free protease inhibitor tablets were purchased from Roche Applied Science. Molecular biology kits and Ni^{2+}-NTA-agarose were obtained from Qiagen (Valencia, Ca), whereas T_4 DNA ligase and restriction enzymes were from New England Biolabs (Mississauga, Ontario, Canada). Fisher provided acrylamide, glycerol, and Luria Bertani (LB) growth medium. Unless otherwise stated, all other chemicals and reagents were from Sigma, and growth media were from Difco.

Bacterial Strains and Growth

The sources of plasmids and bacterial strains used in this study, together with their genotypic description, are listed in Table 1. *E. coli* strains DH5α, BL21[λDE3], and BL21[λDE3]pLysS were maintained on LB broth or agar at 37 °C which was supplemented with 50 μg/ml Km sulfate and 34 μg/ml Cm in the case of strains harboring plasmids pET28a(+) and BL21[λDE3]pLysS, respectively. For overexpression studies and the production of high levels of proteins, *E. coli* BL21[λDE3] and BL21[λDE3]pLysS were grown in Super Broth (5 g of sodium chloride, 20 g of yeast extract, and 32 g of tryptone) at 37 °C with agitation. All strains were maintained for long periods by storage at −70 °C in 25% glycerol.

Isolation and Purification of PG

Samples of insoluble PG were isolated from *E. coli* DH5α and *Micrococcus luteus* using the boiling SDS protocol and purified by enzyme treatment (amylase, DNase, RNase, Pronase) as described by Clarke (8).

Cloning of E. coli yfhD

Chromosomal DNA template for PCR was isolated from *E. coli* W3110. Based on the start codon indicated by the K12 genome project, the *yfhD* ORF was amplified by PCR using the primers YfhD-up and YfhD-down (supplemental Table 1), which contained NcoI and HindIII sites, respectively, to facilitate cloning. Examination and comparison of various *E. coli* genome sequences revealed the possibility of a start codon further upstream encoding a protein with an N-terminal signal sequence. This extended *yfhD* ORF and its associated truncated

form (lacking the N-terminal signal sequence) were amplified with the same downstream primer EsR28 but using different upstream primers, EsF28full and EsF28trunc, respectively. Amplified ORFs were cleaned using a PCR clean up kit (Roche Applied Science), digested with HindIII and NcoI, ligated with appropriately digested pET28a(+) plasmid DNA, and transformed into *E. coli* DH5α. Individual constructs were isolated from transformants, screened for the correct size insert, and completely sequenced to confirm nucleotide identity.

The use of the NcoI for cloning required replacement of the second amino acid encoded in all three constructs. To correct for these replacements, QuikChange site-directed mutagenesis protocol (Stratagene) was employed using the site-directed primers listed in supplemental Table 1. Following plasmid amplification by PCR, products were digested overnight with DpnI and transformed into *E. coli* DH5α, and selected colonies were screened for loss of the NcoI site through analytical restriction enzyme digests and nucleotide sequencing. The resulting constructs were named pACES-6 (original sequence), pACES-7 (extended sequence), and pACES-8 (extended sequence truncated). Constructs were likewise generated that encoded the LT (pACES-11) and nLT (pACES-13) domains of yfhD.

Overproduction and Purification of YfhD

E. coli BL21(λDE3), freshly transformed with plasmid DNA, was inoculated into SuperBroth supplemented with 50 μg/ml Km and incubated at 37 °C until early exponential phase ($A_{600} \sim$ 0.5). Freshly prepared IPTG was added to a final concentration of 1 mM, and expression was induced for 3 h at 37 °C. Cells were collected by centrifugation (5,000 × g, 12 min, 4 °C) and frozen at −20 °C. Thawed cell pellets were washed and resuspended in IMAC buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, and 0.5% Triton X-100) containing Complete EDTA-free protease inhibitor mixture tablets, 10 μg/ml RNase A, and 5 μg/ml DNase I and incubated on ice for 30 min prior to disruption with a Sonicator Ultrasonic Liquid Processor (Heat Systems Inc., Toronto, Ontario, Canada) fitted with a macroprobe. The resulting cell lysate was clarified by centrifugation (5,000 × g, 15 min, 4 °C), and the collected soluble cell fractions were mixed with Ni^{2+}-NTA-agarose (1 ml resin/ml original culture volume) on a Nutator for 1 h at 4 °C. The resin slurry was poured into a disposable column, and the flow-through fractions were collected. Contaminating proteins were removed from the resin by washing with 1 column volume of IMAC buffer followed by 8 column volumes of IMAC buffer at pH 7, and then 1 column volume of IMAC buffer at pH 6. Purified YfhD was eluted in 3–5 ml of IMAC buffer at pH 4.5.

SDS-PAGE and Western Immunoblotting

SDS-PAGE analysis of protein samples was performed following the method of Laemmli (9) using 12% polyacrylamide gels and staining with Coomassie Brilliant Blue. Western immunoblotting for the detection of His$_6$-tagged proteins was performed as described previously (10) using a 1:1000 dilution of a commercially available mouse anti-His$_6$ antibody (Santa Cruz Biotechnology) and a 1:2000 dilution of the secondary

TABLE 1

Bacterial strains and plasmids used in this study

SOEing indicates single overlapping extension.

Strain or plasmid	Genotype or relevant characteristic	Ref. or source
Strains		
E. coli BL21[λDE3] codonPlus	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *dcm met gal*(λDE3)*endA* Hte[*argU, ileY,leuW*] Tet^R	Novagen
BL21[λDE3] pLysS	F^-*ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm met* (λDE3) pLysS (Cm^R)	Novagen
DH5α	K-12 φ80d *lacZ*ΔM15 *endA1 hsdR17* (r_K^- m_K^-) *supE44* thi-1 gyrA96 relA1 Δ(*lacZYA-argF*) U169	Invitrogen
W3110	F^- *trpA23 xyl$^-$ gly*S$_L$	38
W3110 Δ*yfhD*	W3110 derivative; Δ*yfhD*::Km^R	This study
λCI857	*E. coli* K12 lysogenic strain with a mutation at position 847 in the CI repressor gene	39
MHD79	Multiple lytic transglycosylase mutant. Δ*sltY* Δ*mltA*::Cm^R Δ*mltB*::Tet^R Δ*mltC* Δ*mltD* Δ*slt70*::Km^R	7
M. luteus ATCC 4698	Type strain	Sigma
Plasmids		
pET28a(+)	IPTG-inducible expression vector; Kan^R	Novagen
pWQ173	*aka* pAR132, pK03(pMAK) derivative *gor* gene knockouts, unstable at 45 °C, unable to grow on DL*p*-chlorophenylalanine due to presence of *pheS*; Cm^R	40
pACES-6	pET28a(+) derivative containing *yfhD* from K12 on an NcoI/HindIII fragment; Km^R	This study
pACES-7	pET28 a(+) derivative containing *yfhD* from W3110 on an NcoI/HindIII fragment; Km^R	This study
pACES-8	pET28a(+) derivative containing a *yfhD* derivative from W3110 on an NcoI/HindIII fragment encoding YfhD lacking the N-terminal segment Lys-2 to Leu-22; Km^R	This study
pACES-10	pWQ173 derivative containing *yfhD* on an SmaI/XhoI fragment encoding a Gm^R cassette (from pPS856) in place of residues in place of residues Lys-182 to Lys 421;Km^R, Cm^R	This study
pACES-11	pACES-8 derivative encoding the C-terminal (LT) domain of YfhD from Ala-263 to Asn-498; Km^R	This study
pACES-13	pACES-8 derivative encoding the N-terminal (nLT) domain of YfhD from Met-1 to Asp-250; Km^R	This study
pACES-17	pET28a(+) derivative containing *S-R-Rz* on an NcoI/HindIII fragment; Km^R	This study
pACES-20	pET28a(+) derivative containing *S-yfhD-Rz* on an NcoI/SalI fragment derived through "gene SOEing" using pACES-17 and pACES-8 as template; Km^R	This study
pACES-22	pET28a(+) derivative containing *S* of the λ lysis cassette on an NcoI/HindIII fragment; Km^R	This study
pACES-23	pET28a(+) derivative containing R of the λ lysis cassette on an NcoI/HindIII fragment; Km^R	This study
pACES-24	pET28a(+) derivative containing Rz of the λ lysis cassette on an NcoI/HindIII fragment; Km^R	This study
pACES-25	pET28a(+) derivative containing *S-yfhD* on an NcoI/HindIII fragment derived through "gene SOEing" using pACES-17 and pACES-8 as template; Km^R	This study
pACES-26	pET28a(+) derivative containing *S-LTm* on an NcoI/HindIII fragment derived through "gene SOEing" using pACES-17 and pACES-11 as template; Km^R	This study
pACES-27	pET28a(+) derivative containing *S-nLTm* on an NcoI/HindIII fragment derived through "gene SOEing" using pACES-17 and pACES-13 as template; Km^R	This study
pACES-32	pET28a(+) derivative containing *S-R* on an NcoI/HindIII fragment derived through "gene SOEing"; Km^R	This study
pACES-33	pWQ173 derivative containing *yfhD* on a SmaI/XhoI fragment encoding a Gm^R cassette (from pPS856) in place of residues Lys-182 to Lys-421;Gm^R, Cm^R	This study
pPS856	*aacC1*gene (Gm^R) from pUCGm ligated into the EcoRV site of pPS584; Gm^R cassette is flanked by identical inverted MCS	41
pNBAC 54-1	pET30a(+) derivative containing *mltB* on an NdeI/XhoI fragment encoding an N-terminal truncation of Arg-2 to Cys-17; Km^R	10

antibody, goat anti-mouse IgG + IgM alkaline phosphatase-conjugated antibody (Bio-Rad).

Cellular Localization of YfhD

The cellular localization of YfhD-His$_6$ was investigated by Western immunoblot analysis of isolated fractions of cells from 1 liter of culture. Cell fractionation using the sucrose density gradient ultracentrifugation technique was conducted as described previously (11). As indicators of purity, the isolated fractions were analyzed in triplicate for the presence of 2-keto-3-deoxyoctonate (KDO) (outer membrane) and NADH oxidase (cytoplasmic membrane) using the methods of Karkhanis *et al.* (12) and Osborn *et al.* (13), respectively.

Enzyme Assays

Zymography—Zymograms were performed using 0.1% (final concentration) purified insoluble PG as substrate in 12% polyacrylamide gels as described previously (14, 15). Cleavage of PG was visualized following staining with methylene blue/KOH. The zymoblot technique (16) was used to assess the substrate specificity of YfhD. Purified PG from different bacteria was incorporated into 2% agarose, and purified enzyme was spotted on the solid agarose. Following incubation overnight at 37 °C, PG degradation was detected in the zymodots with methylene blue/KOH staining.

Turbidometry—The turbidometric assay of Hash (17) was used to monitor the time course of PG solubilization by YfhD. Purified PG from *E. coli* or *M. luteus,* or whole cells of *M. luteus,* were suspended in 50 mM sodium acetate buffer, pH 6.5, and subjected to brief sonication to provide homogeneous suspensions. Purified YfhD (0–100 μg) was added to 1-ml aliquots of substrate suspension, and the decrease in turbidity of the reaction mixtures was monitored continuously at OD_{660} for a period of 15 min to 2 h.

HPLC-based Assays—The HPLC-based assay developed by Blackburn and Clarke (18) was adapted to assay and identify the soluble muropeptides released from insoluble substrate after enzyme treatment. A typical assay reaction mixture consisted of freeze-dried PG suspended in 25 mM sodium acetate buffer, pH 5.8, containing 0.1% Triton with a total volume of 300 μl. As described above, these suspensions were sonicated, and final substrate concentrations ranged between 0.25 and 4.0 mg/ml. Reactions were initiated by the addition of enzyme followed by incubation at 37 °C with gentle shaking. Reaction mixtures lacking enzyme addition served as negative controls. At appropriate intervals of time, individual reactions were quenched by plunging the sample into a dry ice/ethanol bath. Samples were thawed, and undigested PG was immediately removed by ultracentrifugation at 100,000 × *g* for 20 min at 22 °C (Beckman

Airfuge, Beckman Instruments, Mississauga, Ontario, Canada). Each sample was divided into 2 aliquots and treated separately as follows. (i) One-half of the soluble muropeptides recovered in the supernatants were reduced with sodium borohydride and then hydrolyzed to their constituent aminosugars by incubation in 6 M HCl for 1 h at 95 °C. The hydrolyzed samples were dried *in vacuo* and resuspended in sodium diluent (Beckman Instruments) for muramic acid and muramitol analysis. Muramic acid/muramitol content was determined using a Beckman System Gold amino acid analyzer with ninhydrin detection as described previously (18). Initial activities were determined from plots of muramic acid content as a function of time and expressed as nanomoles of PG monomeric units (*viz.* GlcNAc-AnhMurNAc-peptide) released per min. (ii) The remaining half of the soluble reaction products were subjected to reverse phase HPLC on a 4.6 × 250 mm Gemini C18 (5 μ) analytical column (Phenomenex Inc., Torrance, CA). Detection of eluting muropeptides was achieved by monitoring A_{205}. Fractions collected by hand were concentrated *in vacuo* and desalted with a C18 Ziptip[TM] (Millipore). These separated PG components were then analyzed by MALDI-TOF MS as described previously (10) in a Bruker Reflex III MALDI-TOF in reflectron mode using a 337 nm nitrogen laser set to 109–121 mJ output (Biological Mass Spectrometry Facility, Department of Molecular and Cellular Biology, University of Guelph).

The optimal conditions for enzymatic activity were determined using the turbidometric assay described above. For the determination of optimal pH, suspensions of PG were prepared in 25 mM sodium acetate, pH 3.0–5.5, and 25 mM sodium phosphate, pH 5.0–8.0. In other reactions, buffers included 0.05–0.2% glycerol or Triton X-100.

Construction of Strains for Phage Lysis Assay

The bacteriophage λ lysis cassette encoding the genes *S, R,* and *Rz* (19) was amplified from *E. coli* λCl$_{857}$ using the primer pair SRRz-for and SRRz-rev. The cleaned PCR product was digested with enzymes and cloned into pET28a(+). This construct, pACES-17, was then used with pACES-8 as templates in a gene single overlap extension experiment to create constructs containing *yfhD* in combination with the λ lysis genes *S* and *Rz*. For this, each gene was individually amplified with primers containing extensions that overlapped with the planned adjacent gene. Next, amplified products were diluted 1:10, and PCRs were prepared containing *yfhD* and either *S* or *Rz* to amplify *S-yfhD* and *yfhD-Rz*. Finally, these PCR products were gel-extracted, mixed, and used as template to amplify *S-yfhD-Rz* on a single amplicon. This final PCR product was gel-extracted, digested with NcoI and HindIII or NcoI and XhoI, as appropriate, and ligated with appropriately digested pET28 to generate constructs pACES-25 (*S-yfhD*) or pACES-20 (*S-yfhD-Rz*). Construct pACES-32 (S-R in pET28a(+)) was prepared in a similar fashion.

Lysis Bioassay

A 1:20 dilution of an overnight culture of *E. coli* BL21 pLyS-competent cells, freshly transformed with plasmids of interest, was used to inoculate 100 ml of LB containing 50 μg/ml Km and 34 μg/ml Cam and incubated at 37 °C with aeration. At mid-exponential phase of growth ($A_{600} \sim 0.6$), expression of the plasmid constructs was induced by the addition of 1 mM IPTG, and growth was maintained at 37 °C with continued monitoring of the culture absorbance. At appropriate intervals of time, samples of the cultures were withdrawn, diluted with sterile saline, plated on LB containing 50 μg/ml Km and 34 μg/ml Cam, and incubated at 37 °C overnight for enumeration.

PG Binding Assay

A PG "pulldown" assay (20) was used to assess the ability of sYfhD derivatives to bind insoluble substrate. Purified samples (15 μg) of sYfhD derivatives were incubated with 0.15 mg of purified, insoluble *E. coli* PG in 300 μl (total volume) of 50 mM sodium acetate buffer, pH 6.0, for 1 h at 4 °C. The insoluble PG was collected by ultracentrifugation (100,000 × *g*, 20 min, room temperature), using a Beckman Airfuge, and washed free of unbound material. Bound protein was recovered by extraction of the PG pellet with 4% SDS. Each of the collected fractions was analyzed by SDS-PAGE and Western immunoblotting using the anti-His$_6$ antibody.

Chromosomal Mutation of yfhD

To construct a chromosomal mutation of *yfhD* in *E. coli*, the first 546 and final 291 nucleotides of the gene were amplified using primers KO1 and -2, and KO3 and -4. These fragments were then digested with MluI and XhoI, respectively, and ligated with either a Km^R cassette that had been amplified from pET28a(+) with the primers KO5 kanforward and KO7 kanreverse, or with a Gm^R cassette that had been amplified from pPS856 with the primers GmMluF and GmXhoR. These DNA ligation fragments of an antibiotic resistance cassette flanked by the most N-terminal and C-terminal regions of *yfhD* were digested with SmaI and XbaI and ligated into the suicide vector pWQ173 (also digested with SmaI and XbaI). Constructs were isolated from *E. coli* DH5α transformants, screened for the correct size insert, and sequenced to confirm nucleotide identity. The resulting constructs, named pACES-10 (Km^R) and pACES-33 (Gm^R), were then transformed into competent *E. coli* MC1061 and/or *E. coli* MHD79, plated on the appropriate antibiotic, and incubated overnight at 30 °C. From overnight broth cultures of isolated colonies, transformants were subcultured 1:20 in 5 ml of LB + Km or LB + Gm and incubated at 30 °C until an absorbance at 660 nm of 0.7 was reached. Cells were collected by centrifugation (5,000 × *g*, 4 °C), washed, and then resuspended in PBS prior to plating on YEG-Cl (0.5% yeast extract, 1% NaCl, 0.2% DL-*p*-chlorophenylalanine, 0.4% glucose) with Km or Gm. Plates were incubated overnight at 42 °C to facilitate loss of the plasmid, and cells from isolated colonies were re-streaked onto LB plates containing the appropriate antibiotic to identify those that contained the gene interruption but not the plasmid. PCR using the primers KO20/KO21 and KO22/KO23 was used to confirm the absence of pACES-10 and pACES-30, respectively, and to confirm the chromosomal interruption of *yfhD* with the antibiotic resistance cassette.

Other Analytical Techniques

Protein concentrations were determined using a bicinchoninic acid assay (Pierce). Identification of open reading frames,
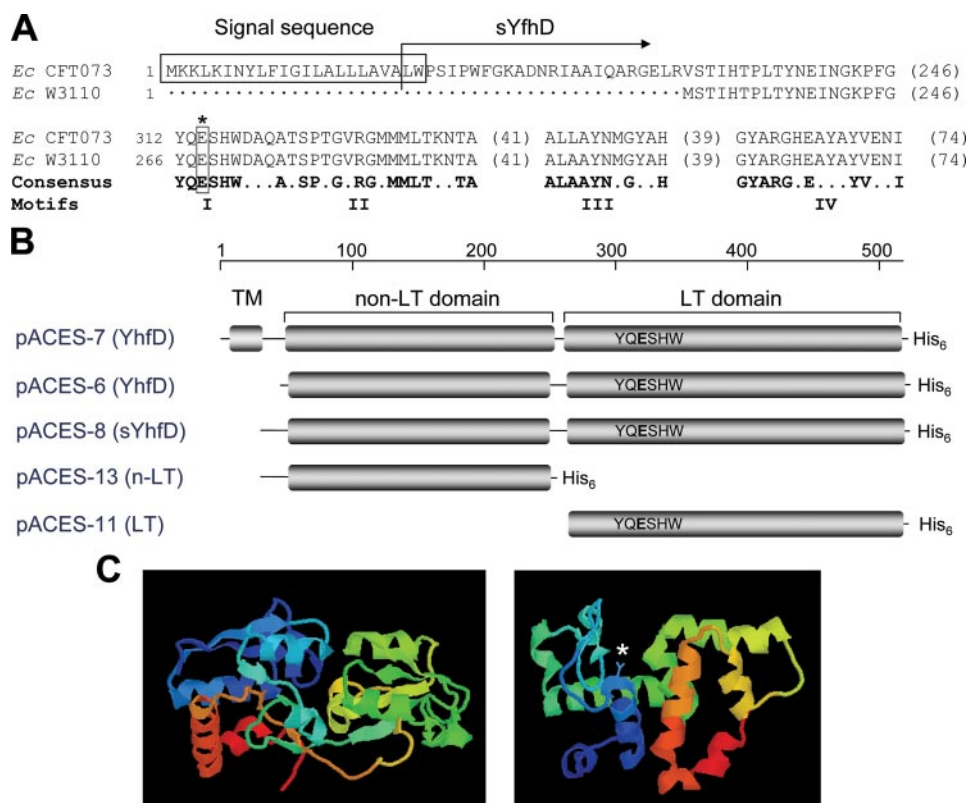
FIGURE 2. **Hypothetical amino acid sequences of YhfD and modular structure of proteins encoded in engineered constructs.** *A,* amino acid sequences of *E. coli* YhfD from strains CFT073 and W3110. Depicted are the hypothetical N-terminal sequences and consensus motifs of the family 1B LTs. The putative catalytic Glu residue within motif I is *boxed* and denoted by an *asterisk*. *B,* scaled modular structure of YfhD encoded on plasmids used in this study with the amino acid sequence of motif I is presented within the LT domain. *TM,* transmembrane domain. *C,* three-dimensional structures of the N-terminal, n-LT (*left*) and C-terminal LT domains of YfhD (*right*) as predicted using PHYRE. The N-terminal residues 1–270 were threaded onto the periplasmic LAO-binding protein from *S. enterica* serovar Typhimurium (Protein Data Bank 1LST) with 19% identity and 9.7^{-21} estimated precision, whereas the C-terminal residues 283–518 were modeled on *E. coli* Slt70 (Protein Data Bank 1QSA) with 23% identity and $2e^{-15}$ estimated precision. The *asterisk* denotes the postulated catalytic Glu-314 residue that is appropriately positioned within a groove through the middle of the enzyme.

As the genome sequences of different *E. coli* strains became available, we discovered some discrepancies in *yfhD* annotation, specifically at their 5′ ends. Comparison of the hypothetical proteins predicted by the annotated *yfhD* sequences from strains W3110 and CFT073 gave 97% identity. However, closer inspection indicated that the only difference between the two was an extended N-terminal stretch of 46 amino acids associated with the CFT073 sequence (Fig. 2). Analysis of the two gene sequences with promoter prediction software identified the larger of the two ORFs as being more probable for transcription. Moreover, the larger ORF encodes a protein with a predicted cleavable N-terminal signal sequence of 22 amino acids complete with an inside-to-outside transmembrane helix that would allow the protein to access the periplasm and PG within; a predicted signal sequence was lacking in the annotated ORF of strain W3110. It is important to note that although the original annotation of *yfhD* in W3110 was incorrect, the larger "complete" ORF is present within its genome and each of those of all of the other sequenced *E. coli* strains (including the 13 *E. coli* O157:H7 strains currently in the data base).

The CFT073 *yfhD* gene encodes a relatively large hypothetical protein of 518 amino acids with a calculated molecular mass of 58.3 kDa and an estimated pI value of 5.33. However, only the C-terminal 270 amino acids of the protein bear sequence similarity to other family 1 LTs. This similarity includes all four consensus motifs characteristic of the family and a potential catalytic Glu residue, Glu-314, located within motif 1 (Fig. 2). Consequently, this region of YfhD was labeled the LT domain. Whereas being conserved within the subfamily 1B sequences, the extended N-terminal, non-LT (n-LT) sequence is found only in a subset of the other family 1 LT enzymes. This sequence was analyzed with the Phyre algorithm using the N-terminal 250 amino acids of the hypothetical protein as the probe. The results of this analysis indicated that the n-LT domain of YfhD is similar to ABC-type periplasmic binding proteins (E value of $5.8\ e^{-13}$) (Fig. 2). No cleavage site was predicted to exist between the two protein domains. Hence, it appears that *yfhD* encodes a bi-modular protein with two potential functions, one of which may be as an LT.

Cloning and Overexpression of yfhD—Based on the analysis described above identifying two versions of the *yfhD* nucleotide

protein translations, and isoelectric points (pI) were performed using Clone Manager 5. Signal sequence predictions were performed using SignalP 3.0 TMpred. Searches for homologs of YfhD were carried out using the NCBI protein-protein BLAST data base to tentatively identify functions for each of the genes in the cluster (www.ncbi.nlm.nih.gov). Protein alignments were performed using ClustalW version 1.8 software, and shading was done using BOXSHADE version 3.21. Three-dimensional modeling of YfhD was performed using both Phyre and 3D-PSSM.

RESULTS

Identification and Characterization of the yfhD Gene—The nucleotide sequence of *yfhD* was originally obtained from the NCBI *E. coli* W3110 (K12) *g*enome data base. This sequence was used by Blackburn and Clarke (4) to identify 13 homologs from other bacteria and subsequently organize them into family 1B of their classification system. We conducted a more recent analysis of the genome data bases, which revealed at least 200 sequences that fit into this family, from both Gram-positive and Gram-negative bacteria (data not shown).

sequence, appropriate oligonucleotide primers were synthesized for PCR amplification of both. Amplified products were cloned into pET28a($+$), and the two constructs, pACES-6 (encoding 472 amino acids) and pACES-7 (encoding 518 amino acids), were transformed into *E. coli* DH5α. The nucleotide sequences of both constructs were confirmed prior to further experimentation.

Initial experiments designed to overproduce YfhD from plasmids pACES-6 or pACES-7 encoding the two versions of the full-length enzyme resulted in poor expression levels using several hosts and induction conditions. The low level production of these full-length enzymes could be followed after induction by Western immunoblot analysis using anti-His$_6$ tag antibody (data not shown). Most of the protein produced appeared to form insoluble aggregates thus compromising the efficiency of subsequent purification processes and the provision of sufficient amounts for characterization. Induction of cultures with IPTG at 18 °C (10) did improve yields of soluble protein, but the majority still remained insoluble.

Given these difficulties, a truncated derivative of the enzyme encoded on pACES-8 was engineered, which lacked its N-terminal 22 amino acid residues. These residues were selected for deletion because they include the predicted cleavable signal sequence and transmembrane helix. Consequently, the protein product of this construct would remain within the cytoplasm. Cells of *E. coli* BL21[λDE3] freshly transformed with pACES-8 were grown to late exponential phase, and enzyme production was found to increase with time as assayed by SDS-PAGE with both Coomassie Blue staining and Western immunoblotting and zymography (supplemental Fig. 1). Importantly, a greater proportion of this derivative of the enzyme remained soluble, and hence amenable for purification in large quantities. To avoid confusion, this soluble form of the enzyme was labeled sYfhD.

Cellular Localization of YfhD—Various cell fractions of *E. coli* BL21[λDE3] overexpressing pACES-6, pACES-7, and pACES-8 were prepared using sucrose gradients. Three distinct membrane fractions were obtained following their ultracentrifugation. NADH oxidase and KDO assays confirmed the identification and purity of the low density fraction as cytoplasmic membrane material, whereas the high density fraction was purified outer membrane components. The middle density fraction included a mixture of both membranes. Each of the two pure membrane preparations, together with the separately isolated periplasmic and cytoplasmic fractions, were analyzed for the presence of YfhD by SDS-PAGE coupled with Western immunoblotting (Fig. 3). The full-length YfhD produced from pACES-7 was found to be associated with the outer membrane fraction, consistent with previous observations that LTs are localized to this site (2, 10). In contrast, however, the shorter form of YfhD produced from pACES-6 was found mostly concentrated in the cytoplasmic fraction (data not shown). These data thus suggested that pACES-7 encodes the correct form of YfhD. As expected, the engineered sYfhD derivative lacking an N-terminal leader sequence and encoded on pACES-8 was also found to be retained within the cytoplasm (data not shown).

Overproduction and Purification of sYfhD—Recombinant sYfhD overproduced in *E. coli* BL21[λDE3] cells was isolated
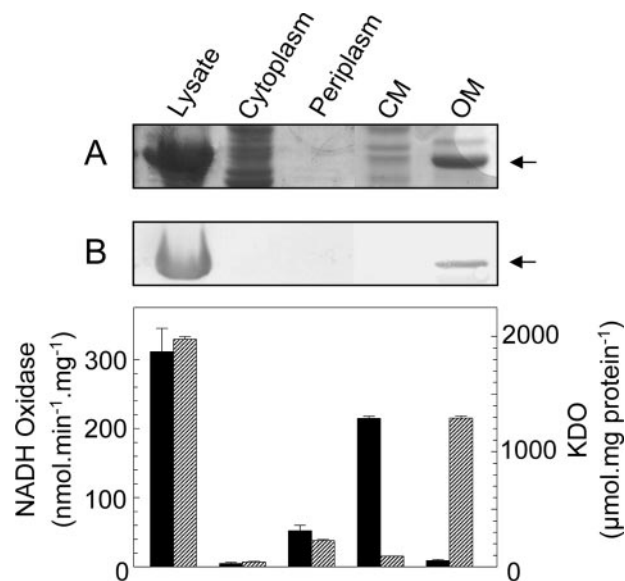

FIGURE 3. **Localization of recombinant YfhD by *E. coli* BL21.** Cells from a 1-liter culture of *E. coli* BL21[λDE3] freshly transformed with pACES-7 grown in Superbroth at 37 °C were collected, and cellular fractions were prepared as described under "Experimental Procedures." YfhD was detected by both staining with Coomassie Brilliant Blue (*A*) and Western immunoblotting using an anti-His$_6$ antibody (*B*). The *arrows* denote YfhD. The levels of NADH oxidase (*solid bars*) and KDO (*hatched bars*), as markers for cytoplasmic (*CM*) and outer membrane (*OM*) purity, respectively, are presented below the corresponding fractions ($\pm$S.D.; $n = 3$).

and purified by immobilized metal affinity chromatography using Ni^{2+}-NTA-agarose. Lowering the pH of the wash buffer to 7 served to remove most of the contaminating proteins prior to elution of YfhD at pH 4.5 (supplemental Fig. 2). The few remaining contaminants were readily removed by anion-exchange chromatography on Source Q. Yields of 1.1 mg of protein were routinely prepared from 1 liter of culture medium by this protocol.

Enzymatic Activity of sYfhD—Zymogram analysis of *E. coli* cells overproducing sYfhD showed clearings on the gel with an apparent molecular ratio of 55,000 (supplemental Fig. 1), which is consistent with that calculated for the truncated enzyme (M_r 55,960). It should be noted that the clearing bands observed in these zymograms are typical of those arising from lytic activity and not the false-positive "shadowing" that can arise from proteins that simply bind or adhere to PG (10). Indeed, boiling sYfhD for 30 min prior to zymogram analysis abolished these clearings within zymogram gels after renaturation thus confirming the lytic activity of the enzyme in its native state.

The turbidometric assay of Hash (17) was used to assess the lytic activity of purified samples of sYfhD with insoluble PG as substrate. This assay is based on monitoring the decrease in turbidity as insoluble PG is cleaved into soluble products by lytic enzymes. sYfhD was found to reduce PG turbidity over time, and the rate of this activity was dependent on enzyme concentration (Fig. 4*A*). Interestingly, however, the shapes of the progress curves of PG clearing were not similar to those observed previously with MltB and the CAZy family 23 glycoside hydrolases, which includes the goose-type lysozymes (10). With these latter enzymes, an initial rapid burst of activity is followed by a leveling to a slower rate. In contrast to this biphasic progress curve, sYfhD activity appeared to be constant as
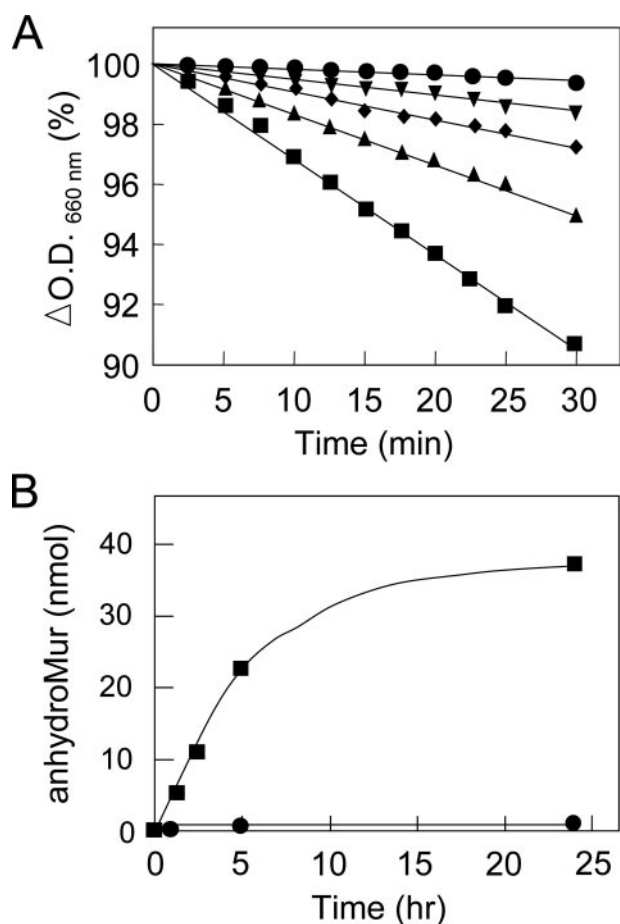
FIGURE 4. **Assays for LT activity.** *A,* turbidometric assay of PG solubilization by sYfhD-His$_6$. Cells of *M. luteus* in 50 mM sodium phosphate buffer, pH 6.5, were incubated at 25 °C with the following: (●) 0, (▼) 7.5, (◆) 15, (▲) 30, and (■) 60 μg/ml purified enzyme. The resulting decrease in OD$_{660}$ is expressed as a percentage of the initial value for respective suspensions. *B,* representative assay for the YfhD-catalyzed release of soluble 1,6-anhydromuramyl-containing muropeptides from PG. Insoluble PG suspended in 25 mM sodium acetate buffer, pH 5.8, containing 0.1% Triton was incubated in the (●) absence and (■) presence of enzyme at 37 °C with gentle shaking. At the times indicated, samples were withdrawn, and soluble reaction products were recovered by ultracentrifugation, reduced with sodium borohydride, and hydrolyzed to their constituent aminosugars by incubation in 6 M HCl for 1 h at 95 °C. The levels of 1,6-anhydromuramyl residues in the original samples were determined as muramic acid by HPLC-based aminosugar analysis.

reflected by linear progress curves. Also unlike the other characterized LTs, YfhD activity continued upon prolonged incubation to effect the complete solubilization of insoluble PG.

The optimal conditions for activity were determined using different buffer conditions. A plot of initial reaction rates as a function of pH was bell-shaped with the optimal pH for lytic activity between 6.5 and 7.0 (data not shown). At pH 6.5, a specific activity of the enzyme was calculated to be 317 ± 15.6 $\Delta\text{OD}_{660}\cdot\text{min}^{-1}\cdot\mu\text{mol protein}^{-1}$. The inclusion of up to 0.2% Triton X-100 or glycerol neither enhanced nor inhibited activity.

Functional Activity of sYfhD—A previously described assay for LTs (18) was used to determine whether YfhD produces 1,6-anhydromuro-peptides during PG cleavage. In this assay, soluble reaction products liberated from insoluble substrate by enzyme action are reduced with sodium borohydride, which converts any reducing GlcNAc or MurNAc into their corre-

sponding alditols, *N*-acetylglucosaminitol or *N*-acetylmuramitol, respectively, whereas leaving 1,6-anhydromuramyl residues unaltered. Strong mineral acid is then used to hydrolyze muropeptides into their corresponding free aminosugars and amino acids. This hydrolysis will also hydrolyze any 1,6-anhydromuramyl residues back into muramic acid. As positive and negative controls for this assay, the soluble products of PG treated with *Pseudomonas aeruginosa* sMltB and hen egg white lysozyme, respectively, were processed accordingly. Of these two latter enzymes, only sMltB yielded a muramitol product upon aminosugar analysis confirming the specificity of the assay (data not shown). Similar treatment of the soluble reaction products generated by sYfhD yielded only muramic acid and glucosamine. The lack of any alditol products thus indicated that the liberated glycan products terminated in a 1,6-anhydromuramoyl residue. Sampling reaction mixtures over time permitted a kinetic analysis of the enzyme (Fig. 4*B*). Using this assay, a specific activity of 0.0619 ± 0.0061 nmol of anhydromuro-peptide released per min$^{-1}\cdot$nmol protein^{-1} was determined. This compared with 21.2 nmol released per min$^{-1}\cdot$nmol protein^{-1} for sMltB.

Confirmation of the chemical structure of reaction products released from insoluble PG by YfhD was obtained by MALDI-TOF mass spectrometry following their separation by reverse phase HPLC. After incubating the enzyme with *E. coli* PG as substrate, residual insoluble material was removed by ultracentrifugation, and the soluble fraction was subjected to HPLC. The major fractions from these HPLC separations were collected, desalted using Ziptips$^{\text{TM}}$, and analyzed by MALDI-TOF MS. The results of these analyses are presented in Table 2. No muropeptides were detected in control reactions with no added enzyme. As a positive control for this analysis, other aliquots of the insoluble PG preparation were digested with a homogeneous preparation of *P. aeruginosa* sMltB and treated similarly. As seen in Table 2, the two enzymes were found to generate the same major reaction products, each of which involved anhydromuramoyl residues (supplemental Fig. 3) that have been observed previously (10, 22). These data thus confirm YfhD to function as an LT, and hence we propose to rename the protein membrane-bound lytic transglycosylase F (MltF).

Whereas the experiments described above provided evidence that sYfhD functions to cleave PG, its observed rate of activity was very weak. This relatively low activity could result from the *in vitro* conditions used involving a homogeneous preparation of the enzyme in the absence of other potential required factors, in particular other membrane components. Hence, we investigated the ability of sYfhD to function *in vivo* by using it to replace the LT of the bacteriophage λ lysis system. This phage encodes three proteins required for lysis of host cells during the lytic stage of their life cycle (19), R, S, and Rz, where R is the LT necessary for degradation of the cell wall during bacteriolysis (21), and S is a transmembrane protein (holin) that facilitates the localization of R into the periplasm; the function of Rz remains unclear (19). The three genes encoding S, R, and Rz were cloned and placed under the control of an IPTG-inducible promoter to create plasmid pACES-17. A control construct, pACES-22 was engineered that encoded only S. Following growth to early exponential phase, IPTG induction

TABLE 2

MALDI-TOF MS analysis of soluble muropeptide products released from insoluble PG by YfhD

Following reaction of the YfhD and MltB (positive control) with insoluble PG as described in the legend to Fig. 4*B*, the soluble muropeptide products were separated by reverse phase HPLC on a 4.6 × 250 mm Gemini C18 (5 μm) analytical column. Fractions were concentrated *in vacuo*, desalted with C18 Ziptips, and analyzed by MALDI-TOF-MS in a Bruker Reflex III MALDI-TOF.

Muropeptide	Identity	Ion	m/z Expected	Observed (Δm) MltB	Observed (Δm) YfhD
1	Anhydro-disaccharide-tripeptide-tetrapeptide-MurNAc	$[M + H]^+$	1569.72	1569.40 (0.32)	1569.45 (0.27)
2	Anhydro-disaccharide-tripeptide-tetrapeptide-(anhydro)MurNAc	$[M + H]^+$	1551.71	1551.35 (0.36)	1551.42 (0.29)
3	Anhydro-disaccharide-tripeptide-tetrapeptide	$[M + H]^+$	1294.49	1294.14 (0.35)	1294.17 (0.37)
4	Anhydro-disaccharide-tetrapeptide	$[M + Na]^+$	944.03	944.84 (0.81)	944.72 (0.69)

of *E. coli* BL21[λDE3]pLys harboring pACES-17 led to rapid lysis as detected by a decrease in A_{600} (Fig. 5). No such loss of absorbance was observed upon IPTG induction of *E. coli* transformed with control plasmid pACES-22, confirming the requirement of a lytic protein for lysis. Instead, cells continued to grow at the same rate as other control cells harboring the pET vector with no insert. A third construct was engineered that involved replacement of the gene coding for R with *yfhD*, generating plasmid pACES-20. As with the complete λ phage lysis system, IPTG induction of *E. coli* transformed with pACES-20 led to cell lysis but at a slightly slower rate. These data thus confirmed that YfhD in its appropriate environment does degrade PG and may function as an autolysin.

Substrate Specificity of MltF—The specificity of sMltF for different PG chemotypes was assessed using the dot blot technique. Zones of clearing were readily detected when samples of sMltF were spotted onto agarose containing insoluble PG from *E. coli*, *P. aeruginosa* (both chemotype A1γ), *M. luteus* (chemotype A4α), and *Bacillus subtilis* (chemotype A1α) (data not shown). These data suggested that the nature and composition of the stem peptide associated with PG does not greatly influence the activity of sMltF. However, given sMltF functions as an LT, it was not surprising to find that it could not degrade PG from *Proteus mirabilis,* a bacterium that *O*-acetylates its PG (reviewed in Ref. 23). PG *O*-acetylation only occurs at the C-6 hydroxyl position of MurNAc, and thus the presence of the ester-linked acetate at this position would preclude formation of 1,6-anhydro linkages during the catalytic pathway of MltF.

Modular Structure of MltF—As noted above, MltF appears to be bi-modular composed of an N-terminal, non-LT domain with similarity to ABC-type periplasmic binding proteins, and a C-terminal LT domain (Fig. 2). To confirm this modular structure, the *yfhD* gene was genetically engineered to encode each domain separately. Thus, two constructs were generated from pACES-8 encoding sMltF (sYfhD). pACES-13 coded for the N-terminal 250 amino acids of sMltF, whereas pACES-11 encoded the C-terminal LT domain from Ala-263 to Asn-498 (sYfhD numbering). Overexpression of these constructs in *E. coli* BL21[λDE3] led to the overproduction of the respective mutant proteins possessing C-terminal His$_6$ tags. The proteins were recovered by affinity chromatography on Ni^{2+}-NTA-agarose and then purified by anion-exchange chromatography on Source Q.

The isolated, separated domains of MltF were assayed for lytic activity using both turbidometry and the bioassay and their
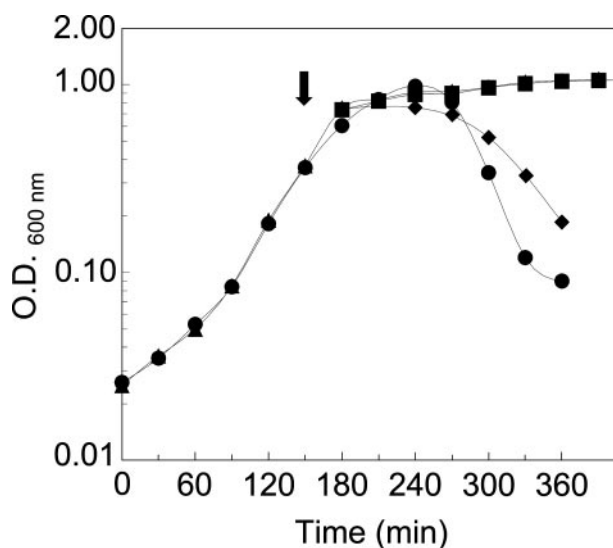

FIGURE 5. **Bioassay for LT activity.** Competent cells of *E. coli* BL21[λDE3]pLys were freshly transformed with the plasmids of interest and cultured in 100 ml of LB containing 50 μg/ml Km and 34 μg/ml Cm at 37 °C with aeration. Expression of plasmid constructs was induced by the addition of 1 mM IPTG (*arrow*), and growth was maintained at 37 °C with continued monitoring of the culture A_{600} (●) pACES-17 (S-R-Rz), (▲) pACES-22 (S), (◆) pACES-20 (S-YfhD-Rz), and (■) pACES-7 (YfhD, control).

ability to bind insoluble peptidoglycan. As predicted, the C-terminal LT domain possessed both the ability to bind insoluble PG (Fig. 6) and lytic activity with PG as substrate (Fig. 7). Using the turbidometric assay, the specific activity of this truncated form of MltF was 267 ± 12.3 $\Delta OD_{660} \cdot min^{-1} \cdot \mu mol\ protein^{-1}$, which was very similar to that of the wild-type MltF. However, this level of activity was only evident for an initial period because the lytic behavior of the isolated LT domain changed over time. Rather than catalyzing the clearing of insoluble PG in a linear fashion as before, a biphasic pattern of lysis was observed with the isolated LT domain which more resembled that seen with other LTs and family 23 glycoside hydrolases. Thus, the specific activity of the isolated LT domain decreased to 13.5 ± 8.5 $\Delta OD_{660} \cdot min^{-1} \cdot \mu mol\ protein^{-1}$ during the second phase of the lysis.

The N-terminal, non-LT domain was found to be devoid of both PG binding capacity (Fig. 6) and lytic activity by both turbidometry (Fig. 7) and the bioassay (data not shown). Also, recombination of the two separately prepared domains did not return the activity profile of the LT domain to that of the full-length wild-type enzyme; turbidometric activity curves remained biphasic.

FIGURE 6. **Affinity assay of wild-type sMltF and its derivatives for insoluble PG.** Binding reactions were performed with 10 μg of protein and 100 μg of *E. coli* PG, and after incubation for 15 min on ice, PG was isolated, washed, and then extracted with 2% SDS. Following each step, the PG was recovered by centrifugation, and supernatants were subjected to SDS-PAGE with Western immunoblot analysis using the anti-His$_6$ probe.
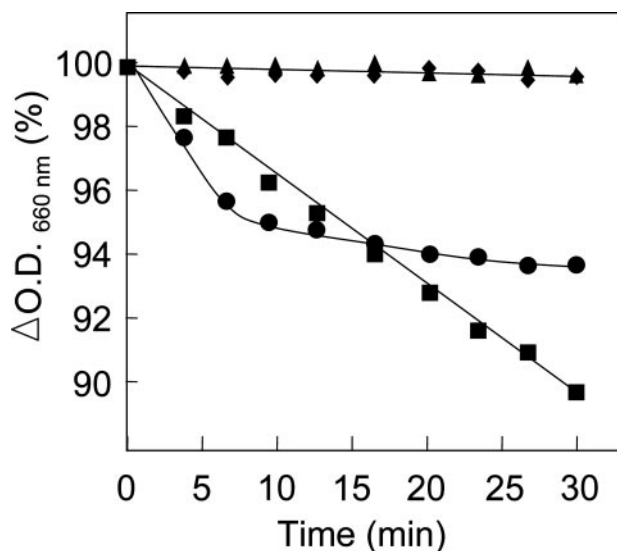
FIGURE 7. **Turbidometric assay of MltF and its isolated domains.** The decrease in turbidity of insoluble PG in 25 mM sodium acetate buffer, pH 6.5, containing 0.1% Triton was monitored with time after addition of (▲) no enzyme, (◆) the isolated non-LT domain, (●) the isolated LT domain, and (■) wild-type MltF, at final concentrations of 60 μg/ml.

Deletion of mltF in E. coli—Mutation of the chromosomal copy of yfhD in *E. coli* MC1061 was achieved by the insertion of either a Gm or Km resistance cassette within the gene using an allelic exchange strategy using the suicide vector pWQ173. The plasmid constructs pACES-10 (Km^R) and pACES-33 (Gm^R) containing the interrupted LT gene were isolated from *E. coli* DH5α transformants and were then transformed into competent *E. coli* MC1061. No noticeable change in cell phenotype was observed with these knock-out mutant strains compared with wild-type MC1061 cells. Thus, there was no discernible difference between wild-type and mutated cells with respect to cell shape by phase microscopy, doubling time in LB, or the minimal inhibitory concentration values against Km and tetracycline. In contrast, repeated attempts to transform *E. coli* MHD79, a mutant strain of *E. coli* lacking functional copies of each of the six previously known LTs, with either of these two plasmids failed.

DISCUSSION

The hypothetical *E. coli* protein YfhD (MltF) had been identified as the archetype for the family 1B LTs based on sequence alignments and the presence of consensus motifs (4). Despite the fact that YfhD was first recognized as a potential LT over 10 years ago (5, 6), there was no prior experimental proof to confirm this activity. In this study, we thus demonstrated for the first time that MltF does indeed function as an LT; it degrades peptidoglycan producing 1,6-anhydromuramoyl residues in the process. Moreover, its activity appears to provide compensation for the loss of six other LTs in *E. coli* MHD79. The enzyme was found to be localized by *E. coli* to its outer membrane. This is consistent with observations made on other characterized LTs that have been shown to be membrane-associated, specifically with the inner leaflet of the outer membrane (24–26). *E. coli* is known to also produce two soluble LTs, Slt35 which is a natural degradation product of MltB and Slt70 which contains a cleavable N-terminal signal sequence (27). The latter has been shown to be tightly associated with both the PG sacculus and the outer membrane, as we found MltF to be.

MltF degrades PG *in vitro* as demonstrated with zymography and turbidometric analysis of PG digests. More interestingly, however, MltF is capable of replacing the bacteriophage λ LT *in vivo*. This temperate phage utilizes a holin-endolysin system involving three late transcripts whose products, S, R, and Rz, are involved in host lysis and virion release (28). S encodes a holin, a small membrane-spanning protein that forms oligomers that accumulate in the inner membrane prior to developing into a lesion, or hole, through which R and Rz can gain access to the periplasm and PG within. Rz is a membrane protein that may act as an endopeptidase, but it is not absolutely required for host cell lysis. R, also known as λ lysozyme (LaL), represents the family 4 LT archetype, and its absence precludes lysis unless complemented by another PG-degrading enzyme. That lysis only occurred when a functional form of MltF, such as sMltF or its LT motif, was introduced and confirmed its autolytic activity.

Assays used to assess sMltF activity also involved sMltB from *P. aeruginosa* (10) as a positive control. Whereas sMltF was found to be consistently active, its specific activity was considerably lower compared with sMltB using these assays. This difference could arise from several different factors. One obvious possibility is that the *in vitro* conditions used to assay the activity do not emulate appropriately those *in vivo*. This is extremely likely given that MltF functions as a peripheral membrane protein and acts upon an insoluble substrate. Clearly, accurately reproducing such a natural situation *in vitro* is virtually impossible. A second, and related, possible reason for observing low activity could concern the lack of other factors that may naturally stimulate the LT activity of MltF. In general, LTs have been demonstrated to facilitate PG biosynthesis and the assembly of secretion systems (1). For both functions, it has been suggested that LTs interact in membrane-associated, multienzyme complexes, thereby experiencing a regulation of their activity to prevent destructive degradation of the sacculi. Indeed, that
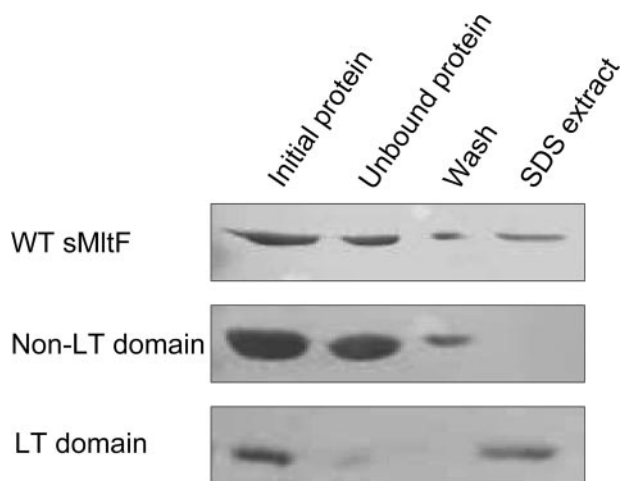
E. coli Membrane-bound Lytic Transglycosylase F

MltF appears to be bimodular with the LT activity associated with the C-terminal domain, whereas the N-terminal domain has sequence similarity to amino acid transport proteins, suggests that it may include a larger complex of proteins involved with transport (discussed below). If so, it is conceivable that the LT activity is enhanced when associated with these other proteins. However, given the technical complexity of monitoring LT activity *in situ*, no work has yet demonstrated that these associations modulate enzymatic activity and function.

A third possible explanation for the comparatively low specific activity of MltF could be related to a difference in the mode of action between the two enzymes that represent two different families. MltB, like most other LTs, is an exo-acting enzyme that cleaves anhydrodisaccharides from the nonreducing end of the glycan strand. However, examples of endo-acting LTs have been identified, MltE (EmtA) from *E. coli* (24) and MltD from *H. pylori* (29). Both of these latter enzymes were found to generate larger fragments from PG oligomers, which for *E. coli* MltE were characterized to be tetra- and hexasaccharides ((GlcNAc-MurNAc)$_n$-GlcNAc-anhMurNAc, $n = 1$ or 2). The phylogenetic analysis of the LT family 1 archetypes conducted by Blackburn and Clarke (4) placed MltF closer to MltE than the other family 1 enzymes. Hence, it is possible that MltF is also an endo-acting enzyme, and thus the lower apparent specific activity could be a consequence of our using inappropriate assays. Indeed, the assays we employed were designed to detect smaller, soluble PG muropeptides. If MltF is endo-acting, the majority of larger PG fragments would either remain insoluble or unresolved by the HPLC separations thereby resulting in an apparent low product yield. Obtaining conclusive proof that MltF, or for that matter any LT, is an endo-acting enzyme using natural PG substrates is complicated by the fact that cleaved glycan products may remain cross-linked to each other. For the unequivocal identification of the mode of action, a defined substrate is required. Unfortunately, LTs are unable to act on commercially available chitooligosaccharides (oligomers of β-1,4-linked GlcNAc) or any of their chromogenic derivatives, such as p-nitrophenyl-GlcNAc or methylumbelliferyl-GlcNAc, which have proved useful in work with lysozymes. This is because LTs seem to have an absolute requirement for the presence of the C-3 lactyl moiety of MurNAc to facilitate ligand recognition (30). Furthermore, this moiety has been proposed to participate in substrate-assisted catalysis (31). We are currently attempting to prepare PG-derived glycans of defined length and free of stem peptides in sufficient quantities for use in further enzymatic analysis of MltF.

An intriguing finding of this study was the lytic behavior of MltF on insoluble PG and its change upon removal of the non-LT domain. As an intact, full-length protein, MltF solubilized PG in a linear time-dependent fashion in a manner analogous to hen egg white lysozyme. However, this pattern of solubilization is not catalyzed by the other LTs studied to date. Previously, these enzymes, along with the CAZy family 23 glycoside hydrolases, which includes the G-type lysozymes, were found to catalyze a rapid burst of PG lysis followed by a slower gradual rate of activity (10 and references therein). Interestingly, the unique lytic behavior of MltF changed with the removal of the non-LT domain such that the isolated LT domain now followed the same pattern of PG degradation as seen with the other LTs. This would suggest that the non-LT domain serves to modulate the activity of the LT, with the implication being that in the correct biological context (association) the enzyme remains fully active. How this modulation happens is currently not understood, but our preliminary studies suggest that the association of the two domains needs to be covalent; simply recombining the separate domains *in vitro* did not appear to affect the activity of the LT domain. Clearly, this warrants further investigation. Likewise, it would be interesting to determine whether or not this modulation of lytic behavior applies to the other LTs upon their association with other proteins as they assemble into macromolecular complexes.

Other functions, if any, of the non-LT domain remain unknown. Its amino acid sequence is similar to HisJ and the lysine-arginine-ornithine (LAO)-binding protein from *E. coli* and *Salmonella enterica* serovar Typhimurium, respectively, periplasmic binding proteins involved with the ABC transport of the basic amino acids. However, further structural analysis revealed that key residues known to be important for ligand binding in HisJ and LAO (32, 33) do not align in the non-LT motif. This would suggest that if functioning to facilitate transport, the non-LT domain has a different ligand specificity. One obvious possibility could be the stem peptides of PG given that LTs of *E. coli* and other Gram-negative bacteria are required for PG turnover and the recycling of released 1,6-anhydromuropeptides, but this was discounted based on the results of the PG binding assays. A very recent study involving a microarray-based genetic technique identified *yfhD*, among other genes, as critical for the fitness of *E. coli* in the absence of aromatic amino acids (34), suggesting its role in their transport. This function, if correct, may apply to the homologs present in other Gram-negative bacteria because the genomic organization around *mltF* is largely maintained. However, some differences are noted. For example, in *P. mirabilis* it is found at the end of a fimbrial operon (35) suggesting that MltF in this instance may be associated with the assembly of this structural organelle.

In an effort to determine the functional role of MltF, an *E. coli* strain containing a chromosomal disruption of *mltF* was constructed using a kanamycin resistance cassette. The mutant had no obvious phenotype other than being slightly more sensitive to ampicillin-induced cellular lysis. This finding was not unexpected given previous studies with *E. coli* where up to six LTs (*viz.* MltA-E and Slt70) have been disrupted without impairment of viability and only subtle changes in cellular morphology (7, 24, 35, 36). Interestingly, 1,6-anhydromuramoyl residues were still detected in the MHD79 strain lacking all six LTs suggesting the existence of at least one other LT. Presumably this remaining LT would provide functional equivalency and compensate for the six LTs missing in this strain. That repeated attempts to knock-out MltF within strain MHD79 were unsuccessful supports this view. It would also suggest that LT activity is essential and the redundancy in their production ensures continued cell viability. This situation would thus be analogous to the apparent redundancy and functional equivalency observed with the penicillin-binding proteins (37), enzymes that are responsible for the final stages of PG biosynthesis and the target of the β-lactam class of antibiotics.

LTs can be considered as space-making enzymes cleaving glycosidic bonds within the PG sacculus to permit a number of different processes to occur. They appear to be important for the expansion of the sacculus and consequently cell growth by creating sites for the insertion of PG precursors (1, 2). Together with amidases, LTs function to split the septum thereby permitting the separation of dividing cells (7). They have also been implicated with facilitating the insertion of protein complexes that extend through the PG sacculus, such as secretion systems, flagella, and pili (reviewed in Ref. 3). With respect to the latter, the protein complexes responsible would be too large to pass through the natural pores within the PG sacculus thus requiring the action of an LT to remodel the layer locally and form a large enough space for insertion of the system. Given these important functions, intuitively one would expect the LTs to be essential for the viability of bacteria, a feature that has not been demonstrated previously. Our discovery of a seventh LT in *E. coli* would account for this apparent discrepancy. More importantly, our observations support the hypothesis that the LTs represent a potential target for the development of a novel class of antibacterials.

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