Interplay among Membrane-Bound Lytic Transglycosylase D1, the CreBC Two-Component Regulatory System, the AmpNG-AmpD_I-NagZ-AmpR Regulatory Circuit, and L1/L2 β -Lactamase Expression in *Stenotrophomonas maltophilia*

Yi-Wei Huang,^a Chao-Jung Wu,^a Rouh-Mei Hu,^{b,c} Yi-Tsung Lin,^{d,e} Tsuey-Ching Yang^a

Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei, Taiwan^a; Department of Biomedical Informatics, Asia University, Taichung, Taiwan^b; Department of Biotechnology, Asia University, Taichung, Taiwan^c; Division of Infectious Diseases, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan^d; School of Medicine, National Yang-Ming University, Taipei, Taiwan^e

Lytic transglycosylases (LTs) are an important class of enzymes involved in peptidoglycan (PG) cleavage, with the concomitant formation of an intramolecular 1,6-anhydromuramoyl reaction product. There are six annotated LT genes in the *Stenotrophomonas maltophilia* genome, including genes for five membrane-bound LTs (*mltA*, *mltB1*, *mltB2*, *mltD1*, and *mltD2*) and a gene for soluble LT (*slt*). Six LTs of *S. maltophilia* KJ were systematically mutated, yielding the Δ *mltA*, Δ *mltB1*, Δ *mltB2*, Δ *mltD1*, Δ *mltD2*, and Δ *slt* mutants. Inactivation of *mltD1* conferred a phenotype of elevated uninduced β -lactamase activity. The underlying mechanism responsible for this phenotype was elucidated by the construction of several mutants and determination of β -lactamase activity. The expression of the genes assayed was assessed by quantitative reverse transcriptase PCR and a promoter transcription fusion assay. The results demonstrate that Δ *mltD1* mutant-mediated L1/L2 β -lactamase expression involved the *creBC* two-component regulatory system (TCS) and the *ampNG-ampD_I-nagZ-ampR* regulatory circuit. The inactivation of *mltD1* resulted in *mltB1* and *mltD2* upexpression in a *creBC*- and *ampNG*-dependent manner. The overexpressed MltB1 and MltD2 activity contributed to the expression of the L1/L2 β -lactamase genes via the *ampNG-ampD_I-nagZ-ampR* regulatory circuit. These findings reveal, for the first time, a linkage between LTs, the CreBC TCS, the *ampNG-ampD_I-nagZ-ampR* regulatory circuit, and L1/L2 β -lactamase expression in *S. maltophilia*.

Peptidoglycan (PG) is an important component used to maintain the shape and structural integrity of bacteria. PG consists of repeated disaccharide subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid with pentapeptides. These polysaccharide strands are cross-linked by peptide bridges into a rigid three-dimensional network (1). Enlargement and growth of the PG sacculus are achieved by a concerted action of both synthetic and lytic enzymes. Penicillin binding proteins (PBPs) and lytic transglycosylases (LTs) are such kinds of important enzymes involved in the coordinated balance. PBPs, which catalyze the transglycosylation and/or transpeptidation reaction, are responsible for peptidoglycan synthesis (2). LTs act on peptidoglycan by cleaving the glycosidic bond between *N*-acetylmuramoyl and *N*-acetylglucosaminyl residues, with the concomitant formation of a GlcNAc-1,6-anhydro-MurNAc peptide (3). In view of the vital role of PG for bacterial survival, bacteria possess an array of stress response systems to sense the defects of PG integrity, for example, the BlrAB system in *Aeromonas hydrophila* and the Rcs phosphorelay system in *Escherichia coli* (4, 5).

PG recycling has been shown to link to chromosomal β -lactamase induction in some *ampR-ampC* module-bearing Gram-negative bacteria, such as *Enterobacter cloacae*, *Citrobacter freundii*, and *Pseudomonas aeruginosa* (6). During the normal growth of members of the family *Enterobacteriaceae*, the PG fragments released are marked by a 1,6-anhydro ring structure of the terminal muramic acid due to the action of LTs (3). The GlcNAc-1,6-anhydro-MurNAc peptide is transported into the cytoplasm by AmpG permease (7). Within the cytoplasm, the GlcNAc-1,6-anhydro-MurNAc peptide is hydrolyzed by NagZ (β -*N*-acetylgluco-

saminidase), yielding 1,6-anhydro muropeptides, which function as the AmpR activator ligand (AL) for the derepression of the chromosomal *ampC* gene (8). The 1,6-anhydro muropeptides can be processed by AmpD (*N*-acetylanhydromuramyl-L-alanine amidase) and further recycled into UDP-MurNAc pentapeptide, which functions as the precursor of peptidoglycan synthesis, or the AmpR repressor ligand (RL), which represses chromosomal *ampC* expression (6). β -Lactam-mediated cellular stress or mutations in *ampR* itself are required to convert AmpR from its repressor to its activator form. In addition, the CreBC (BlrAB) two-component system (TCS) has also been reported to be associated with β -lactam resistance in *P. aeruginosa* (9). PBP 4 (encoded by *dacB*) inactivated by particular β -lactams (e.g., cefoxitin) or mutations in *dacB* itself increases the level of resistance to β -lactams in a CreBC (BlrAB)- and AmpR-dependent manner (9, 10).

Received 31 December 2014 Returned for modification 30 January 2015

Accepted 14 August 2015

Accepted manuscript posted online 17 August 2015

Citation Huang Y-W, Wu C-J, Hu R-M, Lin Y-T, Yang T-C. 2015. Interplay among membrane-bound lytic transglycosylase D1, the CreBC two-component regulatory system, the AmpNG-AmpD_I-NagZ-AmpR regulatory circuit, and L1/L2 β -lactamase expression in *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother 59:6866–6872. doi:10.1128/AAC.05179-14.

Address correspondence to Tsuey-Ching Yang, tcyang@ym.edu.tw.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.05179-14>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

Stenotrophomonas maltophilia is an aerobic Gram-negative bacillus and harbors two chromosomally encoded β -lactamase genes, L1 and L2 (11). Two different mechanisms of β -lactamase induction in *S. maltophilia* have been proposed. One is the *nagZ*-dependent mechanism; the other is the *nagZ*-independent mechanism (12, 13). The *nagZ*-dependent one, which has been shown to be involved in the uninduced β -lactamase overexpression of the *ampD*₁ mutant (12), is basically similar to the mechanism of the known model of β -lactamase induction involving *ampG-ampD-nagZ-ampR* in the *Enterobacteriaceae*. Briefly, the degraded PG sacculus (primarily the GlcNAc-1,6-anhydro-MurNAc peptide) is transported by AmpN/AmpG permease from the periplasm into the cytoplasm (14). In the case of *ampD*₁ inactivation, the transported degraded PG sacculus can largely be processed by β -N-acetylglucosaminidase (encoded by *nagZ*) to form the cytoplasmic activator ligand 1 (AL1; likely to be 1,6-anhydromuropeptides), which can induce β -lactamase expression in the presence of a functional AmpR protein. The *nagZ*-independent mechanism was first observed in an *mrcA* (predicted to encode PBP 1a) mutant (15). Distinct from the β -lactamase activity of the wild-type strain, the Δ *mrcA* mutant exhibits uninduced β -lactamase activity. *nagZ* inactivation of the Δ *mrcA* mutant does not compromise the uninduced β -lactamase activity, indicating that there can be a *nagZ*-independent activator ligand 2 (AL2) or another unidentified homologous NagZ enzyme responsible for uninduced β -lactamase expression in the Δ *mrcA* mutant (13). However, the involvement of a TCS in the β -lactamase expression of *S. maltophilia* has not been proposed.

S. maltophilia harbors six putative LT genes according to the sequenced *S. maltophilia* genomes (16). The impact of LTs on β -lactamase expression has not been described. In this study, we demonstrate that inactivation of membrane-bound lytic transglycosylase D1 (*mltD1*) triggers cross talk between the CreBC TCS, LTs, and the *ampNG-ampD₁-nagZ-ampR* regulatory circuit and then switches on L1/L2 β -lactamase expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. Table S1 in the supplemental material lists the strains, plasmids, and primers used in this study. *S. maltophilia* KJ harboring two active β -lactamases, L1 and L2, has been described previously (17). Tetracycline (30 μ g/ml) was added to maintain the selection of cells carrying the pRK415 derivatives.

Construction of LT-knockout mutants. The Δ *mltA*, Δ *mltB1*, Δ *mltB2*, Δ *mltD1*, Δ *mltD2*, and Δ *slt* LT deletion mutants were constructed by double-crossover homologous recombination between the wild-type KJ chromosome and plasmids p Δ *mltA*, p Δ *mltB1*, p Δ *mltB2*, p Δ *mltD1*, p Δ *mltD2*, and p Δ *slt*, respectively. The basic strategy for the construction of these plasmids was as follows: two DNA fragments targeting the 5' terminus and the 3' terminus of the mutated LT gene were obtained by PCR using the primer sets MltAN-F/MltAN-R and MltAC-F/MltAC-R for p Δ *mltA*, MltB1N-F/MltB1N-R and MltB1C-F/MltB1C-R for p Δ *mltB1*, MltB2N-F/MltB2N-R and MltB2C-F/MltB2C-R for p Δ *mltB2*, MltD1N-F/MltD1N-R and MltD1C-F/MltD1C-R for p Δ *mltD1*, MltD2N-F/MltD2N-R and MltD2C-F/MltD2C-R for p Δ *mltD2*, and Sltn-F/Sltn-R and SltnC-F/SltnC-R for p Δ *slt* (see Table S1 in the supplemental material). These PCR amplicons (as shown in Fig. S1 in the supplemental material) were digested and subsequently cloned into pEX18Tc (18). Plasmid mobilization, transconjugant selection, and mutant confirmation were performed as described previously (12). The double mutant was sequentially constructed from the single mutant by use of the same procedure.

Determination of β -lactamase activity. The induction of β -lactamase was studied using a published procedure (13) with nitrocefin (100

μ M) as the substrate. Cefuroxime was used as the inducer and was added at 30 μ g/ml. Enzyme activity was calculated by using a molar absorption coefficient for nitrocefin of 20,500 M⁻¹ cm⁻¹ at 486 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that converts 1 nmol nitrocefin per minute. Specific activity (U/mg) was expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. The protein content was determined by use of the Bio-Rad protein assay reagent, and bovine serum albumin was used as a standard. All experiments were performed in triplicate.

Construction of LT-overexpressing strains. The full-length genes of six LTs were amplified by PCR and cloning into an expression plasmid, pRK415 (19), yielding pMltA, pMltB1, pMltB2, pMltD1, pMltD2, and pSltn. The PCR primer sets used for *mltA*, *mltB1*, *mltB2*, *mltD1*, *mltD2*, and *slt* were MltAN-F/MltAC-R, MltB1N-F/MltB1C-R, MltB2N-F/MltB2C-R, MltD1N-F/MltD1C-R, MltD2N-F/MltD2C-R, and Sltn-F/SltnC-R, respectively (see Table S1 in the supplemental material). The recombinant plasmids were transferred into wild-type strain KJ, obtaining the LT *mltA*⁺, *mltB1*⁺, *mltB2*⁺, *mltD1*⁺, *mltD2*⁺, and *slt*⁺ LT-overexpressing strains. The orientation of these cloned LT genes was the same as that of the resident *lac* promoter of pRK415. To avoid the possibility that PCR-introduced errors would affect LT activity, the PCR for each LT was performed at least three times to ensure the consistency of each LT gene sequence. The expression of the cloned gene was checked by quantitative reverse transcriptase PCR (qRT-PCR).

qRT-PCR. Total cellular RNA and c-DNA preparation and the levels of transcription of the LT genes were determined by qRT-PCR as described previously (12). The primers used for qRT-PCR were MltAQ-F/MltAQ-R, MltB1Q-F/MltB1Q-R, MltB2Q-F/MltB2Q-R, MltD1Q-F/MltD1Q-R, MltD2Q-F/MltD2Q-R, SltnQ-F/SltnQ-R, and CreDQ-F/CreDQ-R for *mltA*, *mltB1*, *mltB2*, *mltD1*, *mltD2*, *slt*, and *creD*, respectively (see Table S1 in the supplemental material). The 16S rRNA gene was chosen for use for normalization of transcription levels. The relative quantities of mRNA from each gene of interest were determined by the comparative cycle threshold (*C_T*) method (20).

Construction of promoter-*xylE* transcriptional fusions. A 227-bp fragment and a 578-bp DNA fragment upstream of the *mltB1* and *mltD2* genes were obtained by PCR amplification from the chromosome of *S. maltophilia* KJ using primer sets MltB1N-F/MltB1N-R and MltD2N-F/MltD2N-R, and the genes were cloned upstream of the promoterless *xylE* gene in plasmid pRKXylE (21), yielding plasmids pMltB1_{*xylE*} and pMltD2_{*xylE*}, respectively.

C23O activity assay. Catechol-2,3-dioxygenase (C23O) activity was measured as described previously (22). One unit of enzyme activity (Uc) was defined as the amount of enzyme that converted 1 nmol substrate per minute. The specific activity was expressed as Uc/optical density at 450 nm (OD₄₅₀).

Antimicrobial susceptibility test. The susceptibilities of the *S. maltophilia* strains to a number of β -lactams were tested following Clinical and Laboratory Standards Institute (CLSI) guidelines (23). The MIC was defined as the lowest concentration of antimicrobial that inhibited the visible growth of the bacteria.

Statistical analyses. Data are presented as means $\pm$ standard deviations. Statistical significance was assessed by Student's *t* test.

RESULTS AND DISCUSSION

Multiple lytic transglycosylases are present in *S. maltophilia*. The annotation of the *S. maltophilia* K279a genome indicates the presence of five putative *mlt* genes and a putative soluble LT (*slt*), including *mltA* (Smlt0155), two *mltB* genes (Smlt4052 and Smlt4650), two *mltD* genes (Smlt0994 and Smlt3434), and *slt* (Smlt4007) (see Table S2 in the supplemental material) (16). The genomic organization surrounding these putative LTs is pictured in Fig. S1 in the supplemental material. The predicted LTs of *S. maltophilia* shared 27 to 41% identity with those of *E. coli* (see Table S2 in the supplemental material). For convenience, the ho-

FIG 1 Roles of lytic transglycosylases in β -lactamase expression by *S. maltophilia*. The strains assayed included the wild-type strain KJ and its derived LT deletion mutants (the $\Delta mltA$, $\Delta mltB1$, $\Delta mltB2$, $\Delta mltD1$, $\Delta mltD2$, and Δslt mutants). Data are the means from three independent experiments. Error bars indicate the standard deviations for triplicate samples. *, $P \leq 0.01$, calculated by Student's *t* test. (A) Uninduced β -lactamase activity. The overnight cultures assayed were inoculated into fresh LB with an initial OD₄₅₀ of 0.15. After 2 h of culture, the uninduced β -lactamase activity was determined. (B) Cefuroxime-mediated inducibility. The overnight cultures assayed were diluted to an OD₄₅₀ of 0.15 and subsequently grown at 37°C for 1 h. Induction was carried out using 30 μ g/ml cefuroxime for 1 h. β -Lactamase activities were determined before and after induction.

mologues of Smlt0155, Smlt4052, Smlt4650, Smlt0994, Smlt3434, and Smlt4007 in *S. maltophilia* KJ were named *mltA*, *mltB1*, *mltB2*, *mltD1*, *mltD2*, and *slt*, respectively.

LT-generated fragments are involved in β -lactamase expression in the *E. coli* in-trans recombinant *ampC* and *ampR* genes (24, 25). Therefore, reduced LT activity should mean less PG breakdown and lower levels of β -lactamase induction. However, it is possible that a loss of LT activity may have little effect on PG breakdown and β -lactamase induction because of the functional redundancy of these LTs. For this purpose, the uninduced and cefuroxime-induced β -lactamase activities between wild-type KJ and its derived LT deletion mutants (the $\Delta mltA$, $\Delta mltB1$, $\Delta mltB2$, $\Delta mltD1$, $\Delta mltD2$, and Δslt mutants) were comparatively assessed. Inactivation of *mltD1* resulted in an increase in uninduced β -lactamase activity (Fig. 1). The following study thus focused on elucidating the underlying mechanism of the $\Delta mltD1$ -mediated increase in uninduced β -lactamase activity. For the convenience of description, the term “ $\Delta mltD1$ mutant phenotype” used in this article represents the $\Delta mltD1$ -mediated increase in uninduced β -lactamase activity.

FIG 2 Expression of lytic transglycosylase and β -lactamase genes by strain KJ and its derived strains determined by qRT-PCR. cDNA was prepared from the RNA of exponentially growing cells by RT-PCR and used as the template for qRT-PCR. The levels of expression of the target gene transcripts were normalized to the level of expression of the 16S rRNA gene using the $\Delta\Delta C_T$ method. Data are the means from three independent experiments.

Inactivation of *mltD1* led to the upexpression of L1 and L2 β -lactamase genes. Given the presence of two active β -lactamases, L1 and L2, in *S. maltophilia* KJ (17), we first assessed the involvement of L1 and L2 in the $\Delta mltD1$ mutant phenotype. The L1 and L2 transcripts between the wild type and the $\Delta mltD1$ mutant were quantitatively compared by qRT-PCR. Compared with their levels of expression in wild-type strain KJ, the L1 and L2 transcripts in the $\Delta mltD1$ mutant had 13.5 ± 1.3 -fold and 14.7 ± 1.5 -fold increments, respectively (Fig. 2). As a result, *mltD1* inactivation resulted in the coordinate overexpression of the L1 and L2 genes.

Relationship between the $\Delta mltD1$ mutant phenotype, the *ampNG-ampD₁-nagZ-ampR* regulatory circuit, and the CreBC TCS. The *ampNG-ampD₁-nagZ-ampR* regulatory circuit is known to be responsible for β -lactamase expression in *S. maltophilia* (12–14, 22). In order to understand the role of the *ampNG-ampD₁-nagZ-ampR* regulatory circuit in the $\Delta mltD1$ mutant phenotype, we constructed *ampNG*, *ampR*, and *nagZ* deletion mutants of $\Delta mltD1$, yielding the $\Delta mltD1 \Delta ampNG$, $\Delta mltD1 \Delta ampR$, and $\Delta mltD1 \Delta nagZ$ mutants, respectively. The uninduced β -lactamase activities of these mutants and their parent strain, the $\Delta mltD1$ mutant, were determined. In the meantime, the β -lactamase activities of the KJ ΔNG , KJ ΔR , and KJ ΔZ mutants (consisting of wild-type strain KJ with the deletion of *ampNG*, *ampR*, and *nagZ*, respectively) reported in our previous publications (13, 14) were also included for comparison. Inactivation of *ampNG* and *ampR* decreased the uninduced β -lactamase activity of the $\Delta mltD1$ mutant to the wild-type level (Fig. 3), indicating that the $\Delta mltD1$ mutant phenotype relies on functional AmpN/AmpG permease and AmpR. *nagZ* inactivation partially reduced the uninduced β -lactamase activity of the $\Delta mltD1$ mutant (Fig. 3).

The CreBC TCS has been known to be involved in β -lactamase gene expression in *Aeromonas* spp. (5) and β -lactam resistance in *P. aeruginosa* (9). The homologues of *creBC* were identified within the *S. maltophilia* K279a genome by amino acid sequence alignment using *P. aeruginosa* CreBC as the query sequence. The homologues of CreBC in *S. maltophilia* K279a were identified to be Smlt1436 and Smlt1437, and their predicted amino acid sequences were found to have 50% and 48% identity to *P. aeruginosa*

FIG 3 Uninduced β -lactamase activities of *S. maltophilia* KJ and its derived mutants. Bacteria that had been cultured overnight were inoculated into fresh LB with an initial OD_{450} of 0.15 and cultured for 2 h, and the β -lactamase activities were determined. The data represent the means from three repetitions. Error bars indicate the standard deviations for triplicate samples. *, $P \leq 0.01$, calculated by Student's t test. $\Delta mltD1\Delta NG$, $\Delta mltD1\Delta R$, $\Delta mltD1\Delta Z$, and $\Delta mltD1\Delta ABC$, the $\Delta mltD1 \Delta ampNG$, $\Delta mltD1 \Delta ampR$, $\Delta mltD1 \Delta nagZ$, and $\Delta mltD1 \Delta creBC$ double mutants, respectively.

CreB and CreC, respectively, and 51% and 46% identity to *Aeromonas* BlrA and BlrB, respectively (see Fig. S2 in the supplemental material). To explore the relevance of the CreBC system to the $\Delta mltD1$ mutant phenotype, we constructed the *creBC* deletion mutant of the $\Delta mltD1$ strain, yielding the $\Delta mltD1 \Delta creBC$ strain. Inactivation of *creBC* in the $\Delta mltD1$ mutant decreased the β -lactamase activity to nearly the wild-type level (Fig. 3).

Inactivation of *mltD1* resulted in the upexpression of *mltB1* and *mltD2*. To verify the contribution of *mltD1* inactivation to the β -lactamase activity increment, an *mltD1* complemented strain was constructed by transferring plasmid pMltD1 harboring an intact *mltD1* gene into the $\Delta mltD1$ strain, yielding the $\Delta mltD1$ (pMltD1) mutant. The empty pRK415 plasmid was also transferred into the $\Delta mltD1$ mutant, generating the $\Delta mltD1$ (pRK415) mutant as a control. The uninduced β -lactamase activities of strain KJ(pRK415) and the $\Delta mltD1$ (pRK415) and $\Delta mltD1$ (pMltD1) mutants were assessed. To our surprise, the uninduced β -lactamase activities of $\Delta mltD1$ (pRK415) and $\Delta mltD1$ (pMltD1) mutants were comparable and ca. 38-fold higher than the uninduced β -lactamase activity of KJ(pRK415). Therefore, complementation of the $\Delta mltD1$ mutant with an intact *mltD1* gene did not restore the uninduced β -lactamase activity of the $\Delta mltD1$ mutant to the wild-type level.

Next, the *mltD1* transcripts in strain KJ(pRK415) and the $\Delta mltD1$ (pRK415) and $\Delta mltD1$ (pMltD1) mutants were checked by reverse transcriptase PCR (RT-PCR) and qRT-PCR. The *mltD1* transcript was indeed undetectable in the $\Delta mltD1$ (pRK415) mutant; however, the level of the *mltD1* transcript in the $\Delta mltD1$ (pMltD1) mutant was increased ca. 26-fold compared with that in wild-type strain KJ(pRK415) (Fig. 2). As a result, the situation in the $\Delta mltD1$ (pMltD1) mutant appeared to represent *mltD1* overexpression. To verify the impact of LT overexpression on the uninduced β -lactamase activity, the uninduced β -lactamase activities of the six LT-overexpressing strains (the *mltA*⁺, *mltB1*⁺, *mltB2*⁺, *mltD1*⁺, *mltD2*⁺, and *slt*⁺ strains) were assessed. All LT-overexpressing strains tested except the *mltD1*⁺ strain had increased uninduced β -lactamase activity, and it was especially

FIG 4 Impact of LT overexpression on uninduced β -lactamase activities. The overnight cultures assayed were inoculated into fresh LB with an initial OD_{450} of 0.15. After 3 h of culture, the uninduced β -lactamase activity was determined. The strains assayed included the wild-type KJ(pRK415) and its LT-overexpressing constructs derived from the wild type (the *mltA*⁺, *mltB1*⁺, *mltB2*⁺, *mltD1*⁺, *mltD2*⁺, and *slt*⁺ strains). Data are the means from three independent experiments. Error bars indicate the standard deviations for triplicate samples. *, $P \leq 0.01$, calculated by Student's t test.

high for the *mltB1*⁺ strain (Fig. 4). Therefore, overexpression of *mltD1* does not appear to be the key point for the increased uninduced β -lactamase activity observed in the $\Delta mltD1$ (pMltD1) mutant.

Since LTs have redundant functions (3) and overexpression of the genes for all LTs except *mltD1* results in increased uninduced β -lactamase activity in *S. maltophilia* (Fig. 4), we considered whether *mltD1* disruption results in altered expression of the remaining LT genes. The levels of expression of the six LT genes in strain KJ(pRK415) and the $\Delta mltD1$ (pRK415) mutant were compared by qRT-PCR. Most notably, the enhanced expression of *mltB1* and *mltD2* was observed in the $\Delta mltD1$ (pRK415) mutant (Fig. 2). To further confirm the correctness of the qRT-PCR results, the DNA segments comprising the *mltB1* and *mltD2* promoters were cloned into a modified pRKXyle reporter plasmid (21) to generate transcription fusion plasmids pMltB1_{xyle} and pMltD2_{xyle}, respectively. The effect of the $\Delta mltD1$ deletion on *mltB1* and *mltD2* expression was further investigated by determining the C23O activity following the growth of wild-type KJ and the $\Delta mltD1$ mutant carrying pMltB1_{xyle} or pMltD2_{xyle}. *mltB1* and *mltD2* were intrinsically expressed in wild-type KJ, and in response to *mltD1* inactivation, their levels of expression were further increased (Fig. 5).

Given the evidence that *mltD1* inactivation confers increased expression of the *mltB1* and *mltD2* genes (Fig. 5) and the uninduced β -lactamase activities of the *mltB1*⁺ and *mltD2*⁺ strains were higher than the β -lactamase activity of wild-type strain KJ (Fig. 4), we speculated that the *mltB1* and *mltD2* upexpression in the $\Delta mltD1$ mutant may be involved in the $\Delta mltD1$ mutant phenotype. Therefore, the *mltB1* and *mltD2* alleles were deleted from the $\Delta mltD1$ mutant, yielding the $\Delta mltD1 \Delta mltB1$ and $\Delta mltD1 \Delta mltD2$ double mutants, respectively. Moreover, the $\Delta mltD1 \Delta mltB1 \Delta mltD2$ triple mutant was also constructed. Figure 3 demonstrates that the uninduced β -lactamase activities expressed by the $\Delta mltD1 \Delta mltB1$ and $\Delta mltD1 \Delta mltD2$ mutants were lower than the β -lactamase activity expressed by the $\Delta mltD1$ mutant but still higher than the β -lactamase activity expressed by wild-type strain KJ, supporting the suggestion that *mltB1* and *mltD2* overexpression in the

FIG 5 Roles of *creBC* and *ampNG* in $\Delta mltD1$ -mediated *mltB1* and *mltD2* overexpression. The bacteria assayed were cultured overnight, inoculated into fresh LB with an initial OD₄₅₀ of 0.15, and cultured for 3 h, and the C23O activities were determined. The data represent the means from three repetitions. Error bars indicate the standard deviations for triplicate samples. *, $P \leq 0.01$, calculated by Student's *t* test.

$\Delta mltD1$ mutant indeed contributes to the $\Delta mltD1$ mutant phenotype. However, the $\Delta mltD1 \Delta mltB1 \Delta mltD2$ triple mutant still displayed detectable uninduced β -lactamase activity (Fig. 3). As a result, the simultaneous inactivation of *mltD1*, *mltB1*, and *mltD2* may actually upregulate the expression of the remaining LT genes. Thus, the levels of the *mltA*, *mltB2*, and *slt* transcripts in wild-type strain KJ and the $\Delta mltD1 \Delta mltB1 \Delta mltD2$ triple mutant were determined by qRT-PCR. Compared to the levels of *slt* expression by wild-type KJ, the *slt* transcript of the $\Delta mltD1 \Delta mltB1 \Delta mltD2$ triple mutant had a 4.85 ± 0.8 -fold increment. Therefore, the overexpression of *slt* may be responsible for the uninduced β -lactamase expression of the $\Delta mltD1 \Delta mltB1 \Delta mltD2$ triple mutant.

A more interesting observation was that overexpression of *mltD1* in wild-type strain KJ and the $\Delta mltD1$ mutant had different influences on the uninduced β -lactamase activity. KJ(pMltD1) displayed a basal level of uninduced β -lactamase activity (Fig. 4, *mltD1*⁺); however, the $\Delta mltD1$ (pMltD1) mutant had an elevated level of uninduced β -lactamase activity (471 ± 29 U/mg) comparable to that of the $\Delta mltD1$ (pRK415) mutant (360 ± 30 U/mg). For further elucidation, the L1/L2 and LT transcripts of KJ(pMltD1) and the $\Delta mltD1$ (pMltD1) mutant were evaluated by qRT-PCR. Compared with those of wild-type KJ(pRK415), a substantial increase in the L1 and L2 gene transcript levels was observed in the $\Delta mltD1$ (pMltD1) mutant but not in KJ(pMltD1) (Fig. 2), consistent with the results of β -lactamase activity determination. Furthermore, except for the *mltD1* transcript level, the levels of all LT transcripts of KJ(pMltD1) were comparable to those of KJ(pRK415), indicating that the overexpression of *mltD1* in wild-type KJ hardly affects the expression of the other LTs (Fig. 2). However, the levels of the *mltB1* and *mltD2* transcripts in the $\Delta mltD1$ (pMltD1) mutant were comparable to those of the $\Delta mltD1$ (pRK415) mutant (Fig. 2), indicating that overexpression of *mltD1* in the $\Delta mltD1$ mutant background barely blocks $\Delta mltD1$ -mediated *mltB1* and *mltD2* upexpression. This may be the reason why the complementation of *mltD1* did not return the L1 and L2 gene transcripts and the uninduced β -lactamase activity of the $\Delta mltD1$ mutant to the wild-type levels.

The $\Delta mltD1$ -mediated *mltB1* and *mltD2* upexpression is *creBC* and *ampNG* dependent. Inactivation of *creBC* or *ampNG* led to the loss of the $\Delta mltD1$ mutant phenotype (Fig. 3), empha-

sizing the importance of *creBC* and *ampNG* for the $\Delta mltD1$ mutant phenotype. The effect of *creBC* and *ampNG* deletion on the $\Delta mltD1$ -mediated *mltB1* and *mltD2* upexpression was further assessed by a promoter transcription fusion assay. Compared with the activities in the wild type, the *P_{mltB1}* and *P_{mltD2}* activities in the $\Delta mltD1$ mutant were increased and nearly reverted to the wild-type level once the $\Delta creBC$ or $\Delta ampNG$ deletion was introduced into the chromosome of the $\Delta mltD1$ mutant (Fig. 5). Accordingly, the $\Delta mltD1$ -mediated *mltB1* and *mltD2* upexpression was processed by a *creBC*- and *ampNG*-dependent pathway. Because $\Delta mltD1$ -mediated *mltB1* and *mltD2* upexpression was *creBC* dependent, whether *mltD1* inactivation activates the CreBC TCS is of great importance. *creD* expression is positively regulated by activated CreBC in *E. coli* and *P. aeruginosa* (9, 26). Therefore, the upexpression of *creD* is customarily used as an indicator for *creBC* TCS activation in *E. coli* and *P. aeruginosa* systems. To evaluate whether the *mltD1* deletion leads to *creBC* activation in *S. maltophilia*, *creD* transcript levels in wild-type strain KJ and the $\Delta creBC$ and $\Delta mltD1$ mutants were assessed. *creD* was expressed in wild-type KJ, and its expression increased 3.83 ± 0.33 - and 1.96 ± 0.24 -fold in the $\Delta creBC$ and $\Delta mltD1$ mutants, respectively. Moya et al. have shown that deletion of *creBC* from *P. aeruginosa* PAO1 has little influence on *creD* expression (9). Therefore, the relationship between *creD* expression and *creBC* activation in *S. maltophilia* may be distinct from that in *E. coli* and *P. aeruginosa* systems. In fact, our unpublished data revealed that, in addition to the CreBC TCS, the promoter activity of *P_{creD}* was also regulated by other factors in *S. maltophilia*, which negates the possibility that *creD* expression can simply be linked to *creBC* activation. In view of the lack of a linkage of expression of a definite gene to the activation of CreBC in *S. maltophilia* so far, we cannot immediately conclude whether the *mltD1* deletion directly activates CreBC TCS.

The impact of *creBC*, *ampNG*, and *ampR* on MltB1 and MltD2 overexpression-mediated uninduced β -lactamase activity was assessed. Strains KJ(pMltB1) and KJ(pMltD2) represent constructs overexpressing *mltB1* and *mltD2*, respectively. The uninduced β -lactamase activities of KJ Δ BC(pMltB1) and KJ Δ BC(pMltD2) were comparable to those of KJ(pMltB1) and KJ(pMltD2), respectively (Fig. 6). Nevertheless, the uninduced β -lactamase activities were rarely detectable in strains KJ Δ NG(pMltB1), KJ Δ R(pMltB1), KJ Δ NG(pMltD2), and KJ Δ R(pMltD2) (Fig. 6). These results allow us to conclude that the *mltB1* and *mltD2* overexpression-mediated uninduced β -lactamase activities are dependent on functional AmpNG permease and AmpR systems but the *creBC* TCS is less relevant. In addition, the uninduced β -lactamase activity expressed in the *mltB1*-overexpressing strain was partially *nagZ* dependent; however, that in the *mltD2*-overexpressing strain was *nagZ*-independent (Fig. 6).

Concluding remarks. The impact of LTs on β -lactamase induction was studied in *E. coli*. Inactivation of LTs decreases the level of induced β -lactamase expression, and overexpression of LTs enhances β -lactamase induction (24, 25). The inhibition of LT activity has thus been proposed to be an alternative strategy for the control of β -lactam resistance caused by β -lactamase induction (24). However, in this study, the loss of *mltD1* unexpectedly increased the level of uninduced β -lactamase expression, which is contrary to the previous perception. We have provided further evidence that *mltB1* and *mltD2* upexpression, rather than the

FIG 6 Roles of *creBC*, *ampNG*, *ampR*, and *nagZ* in *mltB1* or *mltD2* overexpression-mediated uninduced β-lactamase activities. The bacteria assayed were cultured overnight, inoculated into fresh LB with an initial OD₄₅₀ of 0.15, and cultured for 3 h, and the β-lactamase activities were determined. The data represent the means from three repetitions. Error bars indicate the standard deviations for triplicate samples. *, $P \leq 0.01$, calculated by Student's *t* test.

mltD1 inactivation itself, provides the link between the Δ *mltD1* deletion and uninduced β-lactamase expression in *S. maltophilia* and that the *creBC* TCS and *ampNG-ampD1-nagZ-ampR* regulatory network are involved in this regulatory circuit. Figure 7 presents the relationship among the *creBC*, *ampNG-ampD1-nagZ-*

ampR regulatory network, *mltB1* and *mltD2* upexpression, and the uninduced L1 and L2 β-lactamase response driven by *mltD1* inactivation. We propose that the disruption of *mltD1* in some way upregulates the expression of *mltB1* and *mltD2* in a *creBC*- and *ampNG*-dependent way. It has been proposed that the murein turnover products can function as a signal to affect gene expression (27). The dependence of the Δ *mltD1*-mediated *mltB1* and *mltD2* upexpression on AmpNG strongly suggests that some degraded PG fragments which are transported from the periplasm to the cytoplasm by the AmpNG permease may be recognized by CreBC or by some members of the CreBC regulon and mediate *mltB1* and *mltD2* upexpression. The overexpressed MltB1 and MltD2 can further process peptidoglycan cleavage, and more PG fragments are transported into the cytoplasm by the AmpNG permease system and activate β-lactamase expression in an AmpR-dependent manner.

In our previous study, we have demonstrated that disruption of *ampD1* or *mrcA* causes the coordinated hyperproduction of both the L1 and L2 β-lactamases *in vitro* (12, 15). As revealed in this article, *mltD1* is the third identified gene whose inactivation confers a phenotype of β-lactamase hyperproduction in *S. maltophilia*. Talfan et al. have shown that the mutations in *ampD1* or *mrcA* are linked to the phenotype of β-lactamase hyperproduction, as evidenced by the β-lactamase hyperproduction by mutants selected in the laboratory (28). They also noticed that some β-lactamase-hyperproducing mutants selected in the laboratory and clinical isolates harbored wild-type *ampD1* and *mrcA* genes.

FIG 7 Model of coordinated regulation of L1 and L2 expression by the *creBC* TCS, the *ampNG-ampD1-nagZ-ampR* regulatory circuit, and lytic transglycosylase systems. Inactivation of *mltD1* results in the upexpression of *mltB1* and *mltD2* in a *creBC*- and *ampNG*-dependent manner. The increased periplasmic MltB1 and MltD2 activity results in the accumulation of a variety of degraded murein fragments, which are then imported into the cytoplasm by the AmpNG permease system. The imported murein fragments are processed into the activator ligands (ALs) by either a NagZ-dependent or a NagZ-independent pathway. In the presence of ALs, the AmpR acts as an activator for the expression of L1 and L2 β-lactamases. OM, outer membrane; IM, inner membrane.

Therefore, they proposed that at least one additional gene mutation in another gene, with the exception of *ampD*₁ and *mrcA*, is involved in β -lactamase hyperproduction in *S. maltophilia* (28). On the basis of the findings presented in the present article, *mltD1* is likely the candidate whose inactivation contributes to β -lactamase hyperproduction.

The conditions that directly perturb the PG balance have been noted to activate the Rcs phosphorelay-, CreBC-, and Cpx-mediated PG stress responses (4, 5, 9, 29). These conditions reported to trigger the PG stresses are generally associated with PBP inactivation, such as β -lactam treatment (4), PBP 4 inactivation of *P. aeruginosa* (9), and the simultaneous inactivation of PBP 4, PBP 5, PBP 7, and AmpH of *E. coli* (29). This study is the first to demonstrate that the inactivation of one LT triggers the upregulation of two others and that the *creBC* TCS is involved.

ACKNOWLEDGMENT

The research described here was supported by grant MOST 104-2320-B-010-023-MY3 from the Ministry of Science and Technology of Taiwan.

REFERENCES

- Turner RD, Vollmer W, Foster SJ. 2014. Different walls for rods and balls: the diversity of peptidoglycan. *Mol Microbiol* 91:862–874. <http://dx.doi.org/10.1111/mmi.12513>.
- Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. 2008. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol Rev* 32:234–258. <http://dx.doi.org/10.1111/j.1574-6976.2008.00105.x>.
- Scheurwater E, Reid CW, Clarke AJ. 2007. Lytic transglycosylases: bacterial space-making autolysins. *Int J Biochem Cell Biol* 40:586–591.
- Laubacher ME, Ades SE. 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. *J Bacteriol* 190:2065–2074. <http://dx.doi.org/10.1128/JB.01740-07>.
- Taylor AE, Ayala JA, Niumsups P, Westphal K, Baker JA, Zhang L, Walsh TR, Wiedemann B, Bennett PM, Avison MB. 2010. Induction of β -lactamase production in *Aeromonas hydrophila* is responsive to β -lactam-mediated changes in peptidoglycan composition. *Microbiology* 156:2327–2325. <http://dx.doi.org/10.1099/mic.0.035220-0>.
- Jacobs C, Frere JM, Normark S. 1997. Cytosolic intermediates for cell wall biosynthesis and degradation control inducible beta-lactam resistance in gram-negative bacteria. *Cell* 88:823–832. [http://dx.doi.org/10.1016/S0092-8674\(00\)81928-5](http://dx.doi.org/10.1016/S0092-8674(00)81928-5).
- Lindquist S, Weston-Hafer K, Schmidt H, Pul C, Korfmann G, Erickson J, Sanders C, Martin HH, Normark S. 1993. AmpG, a signal transducer in chromosomal beta-lactamase induction. *Mol Microbiol* 9:703–715. <http://dx.doi.org/10.1111/j.1365-2958.1993.tb01731.x>.
- Votsch W, Templin MF. 2000. Characterization of a beta-N-acetylglucosaminidase of *Escherichia coli* and elucidation of its role in muropeptide recycling and beta-lactamase induction. *J Biol Chem* 275:39032–39038. <http://dx.doi.org/10.1074/jbc.M004797200>.
- Moya B, Dotsch A, Juan C, Blazquez J, Zamorano L, Haussler S, Oliver A. 2009. β -Lactam resistance response triggered by inactivation of a non-essential penicillin-binding protein. *PLoS Pathog* 5:e1000353. <http://dx.doi.org/10.1371/journal.ppat.1000353>.
- Zamorano L, Moya B, Juan C, Mulet X, Blazquez J, Oliver A. 2014. The *Pseudomonas aeruginosa* CreBC two-component system plays a major role in the response to β -lactams, fitness, biofilm growth, and global regulation. *Antimicrob Agents Chemother* 58:5084–5095. <http://dx.doi.org/10.1128/AAC.02556-14>.
- Brooke JS. 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* 25:2–41. <http://dx.doi.org/10.1128/CMR.00019-11>.
- Yang TC, Huang YW, Hu RM, Huang SC, Lin YT. 2009. AmpD₁ is involved in expression of the chromosomal L1 and L2 β -lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 53:2902–2907. <http://dx.doi.org/10.1128/AAC.01513-08>.
- Huang YW, Hu RM, Lin CW, Chung TC, Yang TC. 2012. NagZ-dependent and NagZ-independent mechanisms for β -lactamase expression in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 56:1936–1941. <http://dx.doi.org/10.1128/AAC.05645-11>.
- Huang YW, Lin CW, Hu RM, Lin YT, Chung TC, Yang TC. 2010. AmpN-AmpG operon is essential for expression of L1 and L2 beta-lactamases in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 54:2583–2589. <http://dx.doi.org/10.1128/AAC.01283-09>.
- Lin CW, Lin HC, Huang YW, Chung TC, Yang TC. 2011. Inactivation of *mrcA* gene derepresses the basal-level expression of L1 and L2 β -lactamases in *Stenotrophomonas maltophilia*. *J Antimicrob Chemother* 66:2033–2037. <http://dx.doi.org/10.1093/jac/dkr276>.
- Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebahia M, Saunderson D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream MA, Harris D, Churche C, Bentley SD, Parkhill J, Thomson NR, Avison MB. 2008. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. *Gen Biol* 9:R74. <http://dx.doi.org/10.1186/gb-2008-9-4-r74>.
- Hu RM, Huang KJ, Wu LT, Hsiao YJ, Yang TC. 2008. Induction of L1 and L2 β -lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 52:1198–1200. <http://dx.doi.org/10.1128/AAC.00682-07>.
- Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77–86. [http://dx.doi.org/10.1016/S0378-1119\(98\)00130-9](http://dx.doi.org/10.1016/S0378-1119(98)00130-9).
- Keen NT, Tamaki S, Kobayashi D, Trollinger D. 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* 70:191–197. [http://dx.doi.org/10.1016/0378-1119\(88\)90117-5](http://dx.doi.org/10.1016/0378-1119(88)90117-5).
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(delta delta C(T)) method. *Methods* 25:402–408. <http://dx.doi.org/10.1006/meth.2001.1262>.
- Huang YW, Liou RS, Lin YT, Huang HH, Yang TC. 2014. A linkage between SmeIJK efflux pump, cell envelope integrity, and σ^E -mediated envelope stress response in *Stenotrophomonas maltophilia*. *PLoS One* 9:e111784. <http://dx.doi.org/10.1371/journal.pone.0111784>.
- Lin CW, Huang YW, Hu RM, Chiang KH, Yang TC. 2009. The role of AmpR in the regulation of L1 and L2 β -lactamases in *Stenotrophomonas maltophilia*. *Res Microbiol* 160:152–158. <http://dx.doi.org/10.1016/j.resmic.2008.11.001>.
- Clinical and Laboratory Standards Institute. 2010. Performance standards for antimicrobial susceptibility testing, 20th informational supplement. M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.
- Kraft AR, Prabhu J, Ursinus A, Hölte JV. 1999. Interference with murein turnover has no effect on growth but reduces beta-lactamase induction in *Escherichia coli*. *J Bacteriol* 181:7192–7198.
- Korsak D, Liebscher S, Vollmer W. 2005. Susceptibility to antibiotics and β -lactamase induction in murein hydrolase mutants of *Escherichia coli*. *Antimicrob Agents Chemother* 49:1404–1409. <http://dx.doi.org/10.1128/AAC.49.4.1404-1409.2005>.
- Cariss SJ, Taylor AE, Avison MB. 2008. Defining the growth conditions and promoter-proximal DNA sequences required for activation of gene expression by CreBC in *Escherichia coli*. *J Bacteriol* 190:3930–3939. <http://dx.doi.org/10.1128/JB.00108-08>.
- Boudreau MA, Fisher JF, Mobashery S. 2012. Messenger functions of the bacterial cell wall-derived muropeptides. *Biochemistry* 51:2974–2990. <http://dx.doi.org/10.1021/bi300174x>.
- Talfan A, Mounsey O, Charman M, Townsend E, Avison MB. 2013. Involvement of mutation in *ampD*₁, *mrcA*, and at least one additional gene in β -lactamase hyperproduction in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 57:5486–5491. <http://dx.doi.org/10.1128/AAC.01446-13>.
- Evans KL, Kannan S, Li G, de Pedro MA, Young KD. 2013. Eliminating a set of four penicillin binding proteins triggers the Rcs phosphorelay and Cpx stress response in *Escherichia coli*. *J Bacteriol* 195:4415–4424. <http://dx.doi.org/10.1128/JB.00596-13>.