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$AmpD_I$ Is Involved in Expression of the Chromosomal L1 and L2 β-Lactamases of *Stenotrophomonas maltophilia*[▽]

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Two *ampD* homologues, $ampD_I$ and $ampD_{II}$, of *Stenotrophomonas maltophilia* have been cloned and analyzed. Comparative genomic analysis revealed that the genomic context of the $ampD_{II}$ genes is quite different, whereas that of the $ampD_I$ genes is more conserved in *S. maltophilia* strains. The *ampD* system of *S. maltophilia* is distinct from that of the *Enterobacteriaceae* and *Pseudomonas aeruginosa* in three respects. (i) $AmpD_I$ of *S. maltophilia* is not encoded in an *ampDE* operon, in contrast to what happens in the *Enterobacteriaceae* and *P. aeruginosa*. (ii) The AmpD systems of the *Enterobacteriaceae* and *P. aeruginosa* are generally involved in the regulation of *ampR*-linked *ampC* gene expression, while $AmpD_I$ of *S. maltophilia* is responsible for the regulation of two intrinsic β-lactamase genes, of which the L2 gene, but not the L1 gene, is linked to *ampR*. (iii) *S. maltophilia* exhibits a one-step L1 and L2 gene derepression model involving $ampD_I$, distinct from the two- or three-step derepression of the *Enterobacteriaceae* and *P. aeruginosa*. Moreover, the $ampD_I$ and $ampD_{II}$ genes are constitutively expressed and not regulated by the inducer and AmpR protein, and the expression of $ampD_{II}$ is weaker than that of $ampD_I$. Finally, $AmpD_{II}$ is not associated with the derepression of β-lactamases, and its role in *S. maltophilia* remains unclear.

Stenotrophomonas maltophilia, a gram-negative rod, shows increasing prevalence in clinical infections (2, 4). *S. maltophilia* produces two inducible β-lactamases (the L1 and L2 enzymes) which together confer resistance to all β-lactams (4). L1, a molecular class B and functional group 3 β-lactamase, is a Zn^{2+}-dependent metalloenzyme with a broad substrate profile including penicillins, cephalosporins, and carbapenems (25). L2 displays a hydrolytic ability toward penicillins, cephalosporins, and monobactams (26). A LysR-type transcriptional regulator gene, *ampR*, is contiguous to the L2 gene but divergently transcribed (10). No putative transcriptional regulator gene has been discovered in the proximity of the L1 gene. AmpR has been shown to be a key regulator for L1 and L2 β-lactamase induction in response to β-lactam challenge (24). Recently, the regulatory characteristics of AmpR have been further elucidated (17).

ampR-ampC systems, homologous to the *ampR*-L2 gene module, are widely distributed among several species of *Enterobacteriaceae* and in *Pseudomonas aeruginosa* (18, 21). A similar induction mechanism involving AmpR has been proposed for the *ampR-ampC* systems and the *ampR*-L2 gene module (17). The induction of chromosomal *ampC* is linked with bacterial cell wall recycling, and several regulatory genes, such as *ampR*, *ampG*, and *ampD*, are involved in the induction course (6). The degraded cell wall product GlcNAc-anhMurNAc-peptide (*N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-peptide), including GlcNAc-anhMurNAc-tripeptide, GlcNAc-anhMurNAc-tetrapeptide, and GlcNAc-anhMurNAc-pentapeptide, is transported into the cytosol by the AmpG protein and further cleaved into 1,6-anhydromuramic acid and peptide by AmpD. The peptide is reused by the enzymes of the cell wall-recycling pathway, resulting in the formation of UDP-*N*-acetylmuramic acid-pentapeptide. AmpR, a transcriptional regulator of *ampC* expression, is regulated allosterically by two cell wall components, anhMurNAc-peptide and UDP-*N*-acetylmuramic acid-pentapeptide. The binding between AmpR and anhMurNAc-peptide activates AmpC gene expression. In contrast, AmpR binding with UDP-*N*-acetylmuramic acid-pentapeptide represses the expression of AmpC.

AmpD, a cytosolic anhydro-*N*-acetylmuramyl-L-alanine amidase, acts as a key enzyme to balance the concentration of GlcNAc-anhMurNAc-peptide and UDP-*N*-acetylmuramic acid-pentapeptide in the cytosol. The role of the AmpD protein in the *Enterobacteriaceae*, including *Enterobacter cloacae* and *Citrobacter freundii*, has been well studied (8, 22). In addition, three *ampD* genes (*ampD*, *ampDh2*, and *ampDh3*) are present in *P. aeruginosa*, and a new mechanism of stepwise upregulation has been proposed (13). Moreover, a recent study has demonstrated that other regulatory pathways are involved in the derepressed phenotype of *P. aeruginosa*, in addition to the three known *ampD* genes (27). Recently, an AmpD-like lipoprotein, AmiD, was identified and characterized in *Escherichia coli* (30). Apparently, multiple anhydro-*N*-acetylmuramyl-L-alanine amidase-like genes can be commonly seen in a microorganism. Multiple AmpD homologues may coordinately regulate the expression of the *ampC* gene in the *Enterobacteriaceae* and *P. aeruginosa*, resulting in a two-step or three-step derepression phenotype (13, 19).

The genome sequences of two *S. maltophilia* strains, K279a (2; http://www.sanger.ac.uk/Projects/S_maltophilia) and R553-1 (http://www.genome.gov/page.cfm?pageID = 10506376), have been determined. A genome-wide search of the sequence databases showed that two AmpD homologues are found in *S. maltophilia* K279a (Smlt0154 and Smlt1562) and R553-1 (Smal0115 and Smal1320). The exact functions of these AmpD-like proteins in

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype, sequence, or properties	Reference
S. maltophilia		
KJ	Wild type; a clinical isolate from Taiwan	9
KJΔDI	*S. maltophilia* KJ $ampD_I$ isogenic mutant; deletion of 103-bp internal DNA fragment of $ampD_I$ gene	This study
KJΔDII	*S. maltophilia* KJ $ampD_{II}$ isogenic mutant; deletion of 149-bp internal DNA fragment of $ampD_{II}$ gene	This study
KJΔDIΔDII	*S. maltophilia* KJ double mutant of $ampD_I$ and $ampD_{II}$ genes; deletion of 103-bp and 149-bp internal DNA fragments of $ampD_I$ and $ampD_{II}$ genes, respectively	This study
KJΔR	*S. maltophilia* KJ *ampR* isogenic mutant; deletion of 468-bp internal DNA fragment of *ampR* gene	This study
E. coli		
DH5α	λ^- ϕ80d*lacZ*&Dgr;M15 &Dgr;(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen
S17-1	λ pir^+ mating strain	
Plasmids		
pEX18Tc	*sacB oriT* Tc^r	6
pAmpDI	pEX18Tc vector with an 820-bp DNA fragment of *S. maltophilia* KJ containing the intact $ampD_I$ gene and the 50-bp upstream region; Tc^r	This study
pAmpDII	pEX18Tc vector with a 2,172-bp DNA fragment of *S. maltophilia* KJ containing the intact $ampD_{II}$ gene and the 858-bp upstream region; Tc^r	This study
pAmpDIIDI	pEX18Tc vector with a 2,502-bp DNA fragment containing the intact $ampD_I$ and $ampD_{II}$ genes; Tc^r	This study
pΔDI	pEX18Tc vector with a 1,918-bp DNA fragment of *S. maltophilia* KJ containing the $ampD_I$ gene with an internal 103-bp deletion, the 841-bp upstream region, and 349-bp downstream region of $ampD_I$; Tc^r	This study
pΔDII	pEX18Tc vector with a 2,089-bp DNA fragment of *S. maltophilia* KJ containing the $ampD_{II}$ gene with an internal 149-bp deletion, the 889-bp upstream region, and 523-bp downstream region of $ampD_I$; Tc^r	This study
Primers[a]		
AmpDI-F	5′-CGAAGCTTCGACAAGGAAAGGGAAGGCAG-3′	This study
AmpDI-R	5′-CAAGATCTGCACCCACCAACAGCGGCAG-3′	This study
AmpDII-F	5′-CACTTCCACTGTCCTCGTTC-3′	This study
AmpDII-R	5′-CCCTTGCCCTTCAGTTCC-3	This study
DIN-F	5′-TGAAGCTTCCAATGGTGGCAGTGG-3′	This study
DIN-R	5′-GGTCTAGAAGTGGCAGGCGGTCTTC-3′	This study
DIC-F	5′-GGTCTAGACAACAGCGGGCATTTCTAC-3′	This study
DIC-R	5′-CTGAATTCCGCACGCATCTACGCCGAC-3′	This study
16rDNAQ-F	5′-GACCTTGCGCGATTGAATG-3′	This study
16rDNAQ-R	5′-CGGATCGTCGCCTTGGT-3′	This study
L1Q-F	5′-ACCCCTGGCAGATCGGCAC-3′	This study
L1Q-R	5′-CAGCAGCACCGCCGTTTC-3′	This study
L2Q-F	5′-AACGCACCCACCGATGCC-3′	This study
L2Q-R	5′-CGCCTGTCCAGCAATGCC-3′	This study
AmpDIQ-F	5′-CTACGAAGACCGCCTGCC-3′	This study
AmpDIQ-R	5′-GAAATGCCCGCTGTTGCC-3′	This study
AmpDIIQ-F	5′-CCACCACACCGAGCAGAAG-3′	This study
AmpDIIQ-R	5′-ATCTGCGCCGCACTGAAC-3′	This study

[a] Underlining indicates the restriction sites introduced for cloning.

the induction of the L2 β-lactamase (and perhaps of L1) in *S. maltophilia* are still only partially understood. This study aimed to gain a better understanding of the functions of the AmpD homologues in *S. maltophilia*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *S. maltophilia* KJ is a clinical isolate producing the L1 and L2 β-lactamases, as identified by isoelectric focusing electrophoresis in our previous study (10). *S. maltophilia* KJ displays a low basal L2 lactamase activity, and expression of L1 and L2 is highly inducible. Moreover, the inducibility of L1 and L2 is AmpR dependent (17).

Cloning and sequencing of the $ampD_I$ and $ampD_{II}$ genes. In order to PCR amplify the two *ampD* genes of *S. maltophilia* KJ, two sets of primers derived from the K279a genome sequence (2), AmpDI-F/AmpDI-R for $ampD_I$ and AmpDII-F/AmpDII-R for $ampD_{II}$, were used (Table 1). The AmpD homologues of Smlt1562 and Smlt0154 from *S. maltophilia* KJ were designated $AmpD_I$ and $AmpD_{II}$, respectively. All the PCR amplifications were performed using the following program: 5 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by a final extension step of 10 min at 72°C. The amplified products contained the putative promoter region and structural *ampD* genes, with amplicons of 820 bp for the $ampD_I$ gene and 2,172 bp for the $ampD_{II}$ gene. The PCR amplicons were treated with the restriction enzymes HindIII and XbaI and then cloned into the plasmid pEX18Tc (7) treated with the same enzymes. Both strands of the cloned DNA were sequenced. The resultant plasmids were named pAmpDI and pAmpDII. A 1.4-kb $ampD_{II}$ gene fragment obtained from pAmpDII was inserted into the HindIII-treated pAmpDI, resulting in the recombinant plasmid pAmpDIIDI.

Construction of $ampD_I$, $ampD_{II}$, and $ampD_ID_{II}$ mutants. A gene replacement strategy was used for the construction of the $ampD_I$, $ampD_{II}$, and $ampD_ID_{II}$ mutants. A 944-bp PCR amplicon containing the upstream and N-terminal regions of the $ampD_I$ gene was obtained by PCR using the primer sets DIN-F/DIN-R (Table 1) and cloned into the yT&A vector (Yeastern Biotech Co.) (pTDIN). Similarly, the recombinant plasmid pTDIC, which contained the 793-bp C-terminal and downstream regions of the $ampD_I$ gene, was obtained

using the PCR primer sets DIC-F/DIC-R (Table 1). pTDIN was digested with HindIII and XbaI and cloned into pEX18Tc, and then the 793-bp XbaI-EcoRI fragment cut from pTDIC was further ligated. The resultant plasmid, pΔDI, with an internal 103-bp deletion of the *ampD*$_{\mathrm{I}}$ gene, was used for the construction of mutant KJΔDI. A similar strategy was employed for constructing the recombinant plasmid pΔDII, which had an internal 149-bp deletion of the *ampD*$_{\mathrm{II}}$ gene. The resultant plasmids, pΔDI and pΔDII, were transformed into *E. coli* S17-1 and further introduced into *S. maltophilia* KJ by conjugation (10). Transconjugants were selected on LB plates containing norfloxacin (2.5 μg/ml) and tetracycline (30 μg/ml). For mutant selection, the transconjugants were further transferred onto 10% sucrose-containing plates. The authenticity of the mutants was checked by colony PCR amplification (16) and sequencing. The double mutant was constructed sequentially from the single mutant by the same procedure.

Complementation studies. The plasmids pAmpDI, pAmpDII, and pAmpDIIDI were introduced into strain KJΔDIΔDII by conjugation. The transconjugants were selected on LB plates containing 2.5 μg/ml norfloxacin and 30 μg/ml tetracycline and further confirmed by colony PCR (16) using AmpDIQ-F/AmpDIQ-R and AmpDIIQ-F/AmpDIIQ-R as the primers.

Preparation of β-lactamase extracts. Cells were grown to mid-log phase, and an inducer at a specific concentration was added for the production of β-lactamase. After further incubation for 2 h, the cells were harvested by centrifugation and washed with 10 mM sodium phosphate buffer at pH 7.0. Following resuspension of the pellet in 2 ml of the same buffer, the cells were disrupted by sonication to yield the cell extract, which was ready for isoelectric focusing and β-lactamase activity assays.

Determination of β-lactamase activity. Specific β-lactamase activity was determined spectrophotometrically on crude sonic cell extracts (17). The differential L1 and L2 β-lactamase activities were determined by the modified nitrocefin-EDTA method (9). The specific activity (U/mg) was expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein, using an extinction coefficient (Δε) of 20,500 M^{-1} cm^{-1} for nitrocefin at 486 nm, as suggested by the manufacturer (Oxoid, United Kingdom). The protein concentration was determined using the Bio-Rad protein assay reagent, with bovine serum albumin as a standard.

QRT-PCR. Total RNA from cultures grown to log phase with and without inducers was isolated by using the PureLink Total RNA Purification System (Invitrogen, Carlsbad, CA) and treated with 1 unit of RNase-free DNase I (Invitrogen, Carlsbad, CA) for 15 min to eliminate DNA contamination. cDNA was generated from equal amounts of RNAs of the assayed strains by using an MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (Epicentre Biotechnologies, Taiwan). Quantitative real-time PCR (QRT-PCR) was performed with Smart Quant Green Master Mix (Protech Technology Enterprise Co., Ltd.) using the programmed ABI Prism 7000 Sequence Detection System (Applied Biosystems) (11). The primers used for QRT-PCR are listed in Table 1. The level of gene expression was calculated using the comparative $\Delta\Delta C_T$ method (20). The expression of assayed genes was normalized to the endogenous 16S rRNA gene for variation in RNA quantity and quality. Each experiment was performed three times.

Antimicrobial susceptibility test. MICs were determined in triplicate by a standard twofold serial agar dilution method according to the guidelines of the Clinical Laboratory Standards Institute (1). All antibiotics were purchased from Sigma. The MICs of cefepime, imipenem, and meropenem were quantified using Etest strips (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions.

Nucleotide sequence accession numbers. The nucleotide sequences of *S. maltophilia* KJ *ampD*$_{\mathrm{I}}$ and *ampD*$_{\mathrm{II}}$ have been deposited in GenBank under accession no. EJ447465 and EJ447466.

RESULTS AND DISCUSSION

The *ampD*$_{\mathrm{I}}$ gene and its genomic context organization are conserved in different *S. maltophilia* strains. Sequence analysis of the 820-bp PCR amplicon from strain KJ revealed a putative *ampD*$_{\mathrm{I}}$ gene. The predicted 188-amino-acid AmpD$_{\mathrm{I}}$ protein exhibited 98, 94, 67, 32, 33, 33, and 31% identity, respectively, to Smlt1562 of *S. maltophilia* K279a (accession no. NC_010943), Smal1320 of *S. maltophilia* R551-3 (accession no. NC_011071), XCC1539 of *Xanthomonas campestris* pv. campestris ATCC 33913 (accession no. NC_003902), PA4522 (AmpD) of *P. aeruginosa* PAO1 (accession no. NC_002516), AmpD of *E. cloacae* 14 (accession no. Z14003), AmpD of *C. freundii* OS60 (accession no. Z14002), and AmpD of *E. coli* K-12 (accession no. AAC73221). The intraspecies diversity in the AmpD$_{\mathrm{I}}$ protein was at least 6%, as revealed by comparison among *S. maltophilia* strains KJ, K279a, and R551-3. This diversity value is consistent with those of *E. cloacae* (22) and *C. freundii* (29). A highly conserved "common core region" of the AmpD protein and seven strictly conserved residues revealed by Jacobs et al. (12) were also identified in the AmpD$_{\mathrm{I}}$ protein of *S. maltophilia* KJ. Furthermore, the AmpD$_{\mathrm{I}}$ protein of *S. maltophilia* KJ conserved the four residues essential for *C. freundii* AmpD activity proposed by Genereux et al. (5).

The AmpD$_{\mathrm{I}}$ protein is likely a cytoplasmic protein, according to the SignalP3.0 Server prediction (http://www.cbs.dtu.dk/services/SignalP/).

Two whole genomic sequences of *S. maltophilia* strains K279a and R551-3 are available in the public database. The genomic organizations surrounding *ampD*$_{\mathrm{I}}$ in strains K279a and R551-3 were compared. Highly conserved genetic contexts were found to surround the *ampD*$_{\mathrm{I}}$ gene. A 528-bp open reading frame (ORF) encoding a hypothetical protein was located upstream of the *ampD*$_{\mathrm{I}}$ gene, while a putative sulfurtransferase-encoding gene was observed downstream. However, no *ampE* homologue was present in the neighborhood of the *ampD*$_{\mathrm{I}}$ gene. This structure is different from the genomic organization of the *ampD-ampE* operon of the *Enterobacteriaceae* and *P. aeruginosa* (8, 15).

The *ampD*$_{\mathrm{II}}$ gene and its genomic context are more divergent in different *S. maltophilia* strains. The *ampD*$_{\mathrm{II}}$ gene from strain KJ was located on a 2,172-kb PCR amplicon. Sequencing of the amplicon revealed a complete 747-bp ORF and two truncated ORFs. The complete ORF encoded AmpD$_{\mathrm{II}}$, which showed 99, 90, 67, 38, 33, and 40% homology, respectively, to Smlt0154 of *S. maltophilia* K279a (accession no. CAQ_43763), Smal0115 of *S. maltophilia* R551-3 (accession no. ACF_49820), XCC3805 of *X. campestris* pv. campestris ATCC 33913 (accession no. AAM_43051), PA5485 (AmpDh2) (accession no. NP_254172) and PA0807 (AmpDh3) (accession no. NP_249498) of *P. aeruginosa* PAO1, and AmiD of *E. coli* (accession no. NP_415388). AmpD$_{\mathrm{I}}$ and AmpD$_{\mathrm{II}}$ of *S. maltophilia* KJ displayed 29% identity. As predicted by the LipoP 1.0 server (14; http://www.cbs.dtu.dk/services/LipoP/), a putative signal peptidase II cleavage site was detected between the 18th Ala and the 19th Cys residues, indicating that AmpD$_{\mathrm{II}}$ could be a lipoprotein. According to the "+2 rule" of lipoprotein sorting (28), AmpD$_{\mathrm{II}}$ is likely to be transferred to the outer membrane.

Interestingly, the genetic contexts of *ampD*$_{\mathrm{II}}$ in *S. maltophilia* strains K279a and R551-3 were different. In K279a, *ampD*$_{\mathrm{II}}$ was flanked by a putative ammonia transporter gene (Smlt0153) and a putative murein-degrading transglycosylase gene (Smlt0156), while in R551-3, there was an approximately 17.5-kb DNA fragment inserted between the putative ammonia transporter (Smal 0111) and *ampD*$_{\mathrm{II}}$ (Smal0115) genes, as well as an extra 4-kb DNA fragment inserted between *ampD*$_{\mathrm{II}}$ and the putative murein-degrading transglycosylase (Smal0119) gene. The *ampD*$_{\mathrm{II}}$ genomic organization of strain KJ is more similar to that of strain K279a.

AmpD$_{\mathrm{I}}$, but not AmpD$_{\mathrm{II}}$, is involved in regulation of β-lactamase expression. To investigate the biological functions of

TABLE 2. Basal and induced β-lactamase activities of *S. maltophilia* strains

Strain	β-Lactamase activity (U[a]/mg)					
	Basal			Induced[b]		
	(L1 + L2)[c]	L1[d]	L2[e]	(L1+L2)[c]	L1[d]	L2[e]
KJ	20	0	20	1,958	605	1,437
KJΔDI	4,478	1,155	3,323	4,379	1,064	3,315
KJΔDII	16	1	15	2,105	624	1,481
KJΔDIΔDII	4,431	896	3,535	4,478	1,006	3,472
KJΔDIΔDII (pKJAmpDI)	20	1	19	1,186	282	904
KJΔDIΔDII (pKJAmpDII)	4,706	1,272	3,434	4,539	1,475	3,064
KJΔDIΔDII (pKJAmpDIIDI)	14	2	12	743	366	377

[a] One unit of β-lactamase activity is defined as 1 nanomole of nitrocefin hydrolyzed per minute. The results are means of three independent determinations. Standard derivations were within 10% of the means in all cases.
[b] With 50 μg/ml cefoxitin as the inducer.
[c] The total β-lactamase activity (L1 and L2) was determined by the nitrocefin method.
[d] The L1 β-lactamase activity was calculated by subtracting the L2 β-lactamase activity from the total β-lactamase activity.
[e] The L2 β-lactamase activity was determined by the nitrocefin-EDTA method.

TABLE 3. MICs of β-lactam antibiotics for *S. maltophilia* KJ and its derived *ampD*-associated mutants

Antibiotic	MIC (μg/ml) of antibiotic for strain:			
	KJ	KJΔDI	KJΔDII	KJΔDIΔDII
Penicillins				
Piperacillin	1,024	1,024	1,024	1,024
Carbenicillin	1,024	1,024	1,024	1,024
Cephalosporins				
Cefuroxime	2,048	2,048	2,048	2,048
Cefoxitin	1,024	1,024	1,024	1,024
Ceftriaxone	256	256	256	256
Cefepime	64	96	64	96
Carbapenems				
Imipenem	>32	>32	>32	>32
Meropenem	>32	>32	>32	>32
Monobactam				
Aztreonam	>2,048	>2,048	>2,048	>2,048

$AmpD_I$ and $AmpD_{II}$, the isogenic mutants KJΔDI, KJΔDII, and KJΔDIΔDII were constructed. It has been found that inactivation of *ampD* of the *Enterobacteriaceae* and *P. aeruginosa* generally leads to a derepression phenotype (12, 19). Therefore, induction assays and β-lactamase activity measurements were performed with the wild-type strain KJ and the isogenic mutants KJΔDI, KJΔDII, and KJΔDIDII. Cefoxitin (50 μg/ml) was used as the inducer, and the nitrocefin-EDTA method (9) was used to differentially determine the L1 and L2 β-lactamase activities. Strain KJΔDII displayed an induction pattern (low basal level and high induced activity) similar to that of the wild-type strain KJ, whereas strains KJΔDI and KJΔDIΔDII had a fully derepressed phenotype (constitutive hyperexpression) with an equivalent fully derepressed β-lactamase activity (Table 2). Furthermore, the mutants KJΔDI and KJΔDIΔDII expressed both the L1 and L2 β-lactamases in the basal condition (Table 2), indicating that $AmpD_I$ is involved in the expression of both the L1 and L2 genes.

Isoelectric focusing analysis of β-lactamases in the cell extracts revealed two identical bands, corresponding to L1 and L2, in the induced strain KJ and in the uninduced strain KJΔDI. This finding confirmed that both the L1 and L2 genes are coderepressed in the $AmpD_I$-null mutant.

The RNA transcripts of the L1 and L2 genes from strains KJ and KJΔDI were quantified by QRT-PCR. Under the uninduced condition, the transcript ratio of KJΔDI to KJ was 89 for the L1 gene and 184 for the L2 gene. However, this ratio was 3 for the L1 gene and 21 for the L2 gene under the induced condition. Consequently, the $AmpD_I$ protein has a stronger repressor effect on L1 and L2 β-lactamase expression regardless of the presence of β-lactams.

Complementation assays were performed by introducing pAmpDI, pAmpDII, and pAmpDIIDI into strain KJΔDIΔDII, and the basal and induced β-lactamase activities of these transconjugants were quantified. Strains KJΔDIΔDII(pAmpDI) and KJΔDIΔDII(pAmpDIIDI) exhibited the wild-type β-lactamase expression pattern, i.e., low basal level and inducibility, as observed in the wild-type strain KJ, while complementation with pAmpDII did not restore the inducible phenotype in KJΔDIΔDII (Table 2).

These results confirmed that $AmpD_I$ is a functional anhydro-*N*-acetylmuramyl-L-alanine amidase in *S. maltophilia* and is associated with the regulation of L1 and L2 gene expression. Although strains KJ, KJΔDIΔDII(pAmpDI), and KJΔDIΔDII(pAmpDIIDI) displayed β-lactamase inducibility (Table 2), the induced β-lactamase activities were notably different, since KJΔDIΔDII (pAmpDI) showed an induced β-lactamase activity lower than that of the wild-type strain, KJ (Table 2). This difference could be related to the copy number of plasmid pAmpDI. More $AmpD_I$ proteins in KJΔDIΔDII(pAmpDI) could result in a stronger repressor effect.

As for $AmpD_{II}$, results obtained with KJΔDII suggested that $AmpD_{II}$ has no significant effect on the expression of the L1 and L2 genes, and this hypothesis was confirmed by data from the complementation assays. However, it is noteworthy that the induced β-lactamase activity of strain KJΔDIΔDII(pAmpDIIDI) was lower than that of strain KJΔDIΔDII(pAmpDI) (743 versus 1,186 [Table 2]), suggesting that the $AmpD_{II}$ protein could exhibit a synergistic effect on the function of the $AmpD_I$ protein. The synergistic effect of $AmpD_{II}$ on the $AmpD_I$ function was not detectable with mutants, which could be due to lower expression of the $ampD_{II}$ gene when carried in the chromosomal context than when carried on the multicopy plasmid.

The effect of AmpD inactivation on β-lactam resistance was also determined. All tested β-lactams exhibited the same MICs for the wild-type strain, KJ, and the *ampD*-associated mutants except cefepime, which was slightly less active against the KJΔDI and KJΔDIΔDII mutants (Table 3). These results suggest that $ampD_I$ inactivation likely does not have clinical relevance.

The $ampD_I$ and $ampD_{II}$ genes are constitutively expressed. The expression of the $ampD_I$ and $ampD_{II}$ genes under different conditions, such as the basal state, induced state, and $AmpD_I$-null state, was studied using QRT-PCR (Fig. 1). The expres-

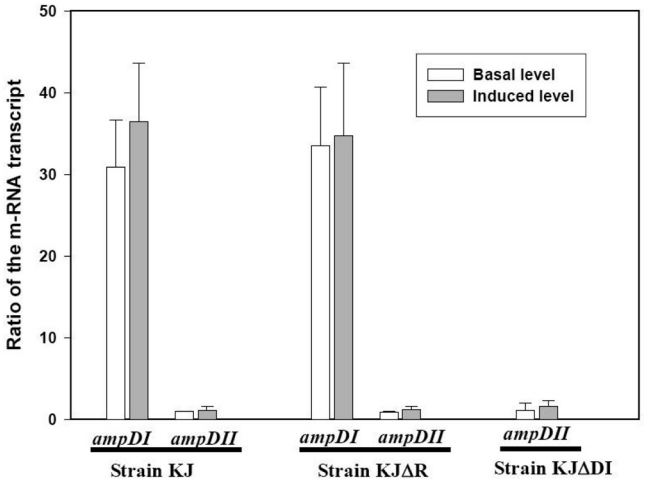

FIG. 1. The relative increase in $ampD_I$ and $ampD_{II}$ mRNA of the basal and induced *S. maltophilia* strains KJ, KJΔR, and KJΔDI. Each pair of bars indicates the ratio of the mRNA transcript of a specific gene to that of the strain KJ basal $ampD_{II}$, as measured by QRT-PCR. The induced condition is 30 μg/ml cefoxitin for 2 h. The mean value is based on three independent experiments. The error bars indicate the standard deviations.

sion of the $ampD_I$ and $ampD_{II}$ genes was constitutive and was hardly influenced by the addition of the inducer. Under basal or induced conditions, the expression of $ampD_I$ was higher than that of $ampD_{II}$, suggesting that in wild-type *S. maltophilia*, $AmpD_I$ could play a significant role in the cell wall-recycling process. Since strains KJΔDI and KJ expressed equivalent amounts of $ampD_{II}$ transcript (Fig. 1), no compensatory expression of the $ampD_{II}$ gene occurs in the absence of the $AmpD_I$ protein.

AmpR is not involved in the expression of $ampD_I$ and $ampD_{II}$. In our previous study, AmpR was shown to act as an important regulator in the induction of the L1 and L2 genes (10). To investigate the role of AmpR in the expression of the $ampD_I$ and $ampD_{II}$ genes, the $ampD_I$ and $ampD_{II}$ transcripts of strains KJ and KJΔR were evaluated under the basal and induced conditions. Figure 1 shows that the expression of $ampD_I$ and $ampD_{II}$ was unaffected by *ampR* inactivation, suggesting that AmpR is not involved in the expression of the $ampD_I$ and $ampD_{II}$ genes.

Concluding remarks. Following the release of whole genomic sequences, several bacteria have been found to have multiple *ampD*-like alleles, such as *ampD* and *amiD* in *E. coli* (30) and *ampD*, *ampDh2*, and *ampDh3 in P. aeruginosa* (13). *S. maltophilia*, like the *Enterobacteriaceae*, has genes for two AmpD homologues, i.e., $AmpD_I$ and $AmpD_{II}$, in its genome. However, in the present study, the previously mentioned semiconstitutive β-lactamase hyperproduction phenotypes in *E. cloacae* (19) and the moderate-level (or high-level) hyperinducible phenotypes in *P. aeruginosa* (13) were not observed in the *ampD*-associated mutants of *S. maltophilia*. Recently, Okazaki and Avison have isolated 12 β-lactamase hyperexpression *S. maltophilia* mutants, 2 of which exhibit the phenotype of semiconstitutive overexpression (24). The $ampD_{II}$ genes of those mutants have been sequenced, and mutations in the $ampD_{II}$ gene were not observed. However, $ampD_I$ sequence results were not reported. Nevertheless, the finding of the semiconstitutive overexpression mutants suggests that, in addition to the regulation involving *ampD*, alternative mechanisms may contribute to the L1 and L2 gene derepression, which is consistent with the results of Schmidtke and Hanson for *P. aeruginosa* (27).

For some specific enterobacterial strains, such as *E. cloacae* NOR-1 and *Proteus vulgaris*, a second set of *ampR* class A β-lactamase modules exists, in addition to the *ampR-ampC* module (3, 23). AmpD of *E. cloacae* NOR-1 has been shown to be involved in the expression of both of the *ampR*-linked β-lactamases, NmcA and AmpC (22). In this study, $AmpD_I$ in *S. maltophilia* was found to play a key role, not only in the expression of the *ampR*-linked L2 gene, but also in the expression of the non-*ampR*-linked L1 gene. This, together with our previous findings (17), indicates that the L1 and L2 genes may be under the control of the *ampD-ampR*-associated regulatory system.

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