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NagZ-Dependent and NagZ-Independent Mechanisms for β-Lactamase Expression in *Stenotrophomonas maltophilia*

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β-*N*-Acetylglucosaminidase (NagZ), encoded by the *nagZ* gene, is a critical enzyme for basal-level *ampC* derepression (*ampC* expression in the absence of β-lactam challenge) in *ampD* and *dacB* mutants of *Pseudomonas aeruginosa*. Three mutants with a phenotype of basal-level L1 and L2 β-lactamase derepression in *Stenotrophomonas maltophilia* have been reported, including KJΔDI (*$ampD_I$* mutant), KJΔmrcA (*mrcA* mutant), and KJΔDIΔmrcA (*$ampD_I$* and *mrcA* double mutant). In this study, *nagZ* of *S. maltophilia* was characterized, and its roles in basal-level β-lactamase derepression, induced β-lactamase activities, and β-lactam resistance of KJΔDI, KJΔmrcA, and KJΔDIΔmrcA were evaluated. Expression of the *nagZ* gene was constitutive and not regulated by AmpR, $AmpD_I$, AmpN, AmpG, PBP1a, and NagZ. Introduction of Δ*nagZ* into KJΔDI nearly abolished basal-level derepressed β-lactamase activity; conversely, introduction of Δ*nagZ* into KJΔmrcA did not affect it. At least two activator ligands (ALs) are thus considered responsible for β-lactamase expression in the *S. maltophilia* system, specifically, the NagZ-dependent (AL1) and NagZ-independent (AL2) ligands responsible for the basal-level derepressed β-lactamase activities of KJΔDI and KJΔmrcA, respectively. The contributions of AL1 and AL2 to the induced β-lactamase activities may vary with the types of β-lactams. *nagZ* inactivation did not affect aztreonam-, cefoxitin-, and carbenicillin-induced β-lactamase activities, but it attenuated cefuroxime- and piperacillin-induced β-lactamase activities. Introduction of Δ*nagZ* into KJ, KJΔDI, KJΔmrcA, and KJΔDIΔmrcA did not significantly change the MICs of the β-lactams tested except that the MICs of cefuroxime and piperacillin moderately decreased in strains KJΔZ and KJΔDIΔZ (*nagZ* mutants).

β-*N*-Acetylglucosaminidase (NagZ), a member of the glycosyl hydrolase 3 (GH 3) family, was first known as a cytoplasmic enzyme active toward *p*-nitrophenyl-β-*N*-acetyl-D-glucosaminide and was later found to be active toward anhydromuropeptides (11). Roles of NagZ in chromosomal *ampC* β-lactamase induction and β-lactam resistance were also noticed (2, 5, 25). For normal bacterial cell growth, a considerable amount of periplasmic peptidoglycan is cleaved by autolysin enzymes, with released GlcNAc-1,6-anhydromuropeptide fragments transported into the cytoplasm by AmpG permease (15, 19). The transported GlcNAc-1,6-anhydromuropeptides are competent substrates for cytosolic NagZ (encoded by *nagZ*) and *N*-acetylmuramyl-L-alanine amidase (encoded by *ampD*). The *ampD*-processed products, GlcNAc-1,6-anhydro-MurNAc moieties or 1,6-anhydro-MurNAc moieties, are further recycled into UDP-MurNAc pentapeptides acting as a repressor ligand (RL) to repress *ampC* expression (12). The *nagZ*-processed products, 1,6-anhydromuropeptides, act as the activator ligands (ALs) for induction of chromosomal *ampC* β-lactamase (6). Regulatory ligands, UDP-MurNAcpentapeptide (RL) and 1,6-anhydromuropeptides (AL), competitively regulate *ampC* induction by directly binding to the LysR-type transcriptional regulator AmpR (8, 20). Recently, Moya et al. reported that the inactivation of *dacB*, encoding penicillin binding protein 4 (PBP4), triggered the CreBC two-component regulatory system and conferred high β-lactam resistance (21). Moreover, the involvement of Δ*dacB* in *ampC* overexpression and elevated β-lactam resistance has proven to be *ampR*, *ampG*, and *nagZ* dependent (21). Therefore, the networks of *ampG-ampD-nagZ-ampR* and *dacB-creBC-ampG-nagZ-ampR* regulons exquisitely control expression of the *ampC* gene and β-lactam resistance in *Pseudomonas aeruginosa*.

Stenotrophomonas maltophilia is a Gram-negative, nonfermentative bacillus that is an important cause of nosocomial infection. *S. maltophilia* is resistant to a wide range of antimicrobials because of its intrinsic resistance determinants, including β-lactamases, aminoglycoside-modified enzymes, and a *qnrB*-like quinolone-resistant determinant (4). Among them, two inducibly expressed L1 and L2 β-lactamases inactivate β-lactam, allowing *S. maltophilia* to resist virtually all β-lactams (9, 22). Like the *ampR-ampC* module of *Enterobacteriaceae* and *P. aeruginosa*, *S. maltophilia* harbors an *ampR-L2* module (9, 22). To elucidate the L1 and L2 induction mechanisms of *S. maltophilia*, the *ampN-ampG* operon and the *$ampD_I$*, *ampR*, and *mrcA* genes, but not *nagZ*, were sequentially characterized (10, 17, 18, 26). Briefly, AmpN/AmpG permease transports degraded murein sacculus from the periplasm into the cytoplasm (10). Cytoplasmic *N*-acetyl-muramyl-L-alanine amidase ($AmpD_I$) encoded by *$ampD_I$*, plays a negative role in the expression of β-lactamase, like AmpD in *P. aeruginosa*. Inactivation of *$ampD_I$* causes a fully derepressed phenotype, resulting in L1 and L2 overexpression (26). The LysR-type transcriptional regulator AmpR regulates the expression of its contiguous L2 and unlinked L1 genes (18). Like *dacB* in *P. aeruginosa*, *mrcA*, encoding PBP1a, is also involved in basal-level β-lac-

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

Strain, plasmid, or primer	Genotype or properties[a]	Reference
S. maltophilia		
KJ	Wild type; a clinical isolate from Taiwan	9
KJΔZ	*S. maltophilia* KJ *nagZ* deletion mutant; Δ*nagZ*	This study
KJΔL1	*S. maltophilia* KJ *L1* isogenic mutant; *L1*::*xylE*	9
KJΔL1ΔZ	*S. maltophilia* KJ *L1* and *nagZ* deletion mutant; *L1*::*xylE* Δ*nagZ*	This study
KJΔL2	*S. maltophilia* KJ *L2* isogenic mutant; *L2*::*xylE*	9
KJΔL2ΔZ	*S. maltophilia* KJ *L2* and *nagZ* deletion mutant; *L2*::*xylE* Δ*nagZ*	This study
KJΔDI	*S. maltophilia* KJ $ampD_I$ deletion mutant; $\Delta ampD_I$	26
KJΔDIΔZ	*S. maltophilia* KJ $ampD_I$ and *nagZ* deletion mutant; $\Delta AmpD_I$ Δ*nagZ*	This study
KJΔR	*S. maltophilia* KJ *ampR* deletion mutant; Δ*ampR*	17
KJΔNG	*S. maltophilia* KJ *ampN* and *ampG* deletion mutant; Δ*ampN* Δ*ampG*	17
KJΔmrcA	*S. maltophilia* KJ *mrcA* deletion mutant; Δ*mrcA*	17
KJΔmrcAΔZ	*S. maltophilia* KJ *mrcA* and *nagZ* deletion mutant; Δ*mrcA* Δ*nagZ*	This study
KJΔDIΔmrcA	*S. maltophilia* KJ $ampD_I$ and *mrcA* deletion mutant; $\Delta ampD_I$ Δ*mrcA*	17
KJΔDIΔmrcAΔZ	*S. maltophilia* KJ $ampD_I$, *mrcA*, and *nagZ* deletion mutant; $\Delta AmpD_I$ Δ*mrcA* Δ*nagZ*	This study
Escherichia coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen
S17-1	λ pir^+ mating strain	26
Plasmids		
pEX18Tc	*sacB oriT* Tc^r	7
pRK415	Mobilizable broad-host-range plasmid cloning vector, RK2 origin; Tc^r	14
pX1918GT	Plasmid containing the *xylE*-gentamicin resistance gene cassette; Amp^r Gm^r	23
pΔZ	pEX18Tc with an internal-deletion *nagZ* gene; Tc^r	This study
pRK-Z	pRK415 with a complete *nagZ* gene; Tc^r	This study
$pRK188Z_{xylE}$	pRK415 with a 188-bp DNA fragment upstream from the *nagZ* start codon and a *nagZ*::*xylE* transcriptional fusion	This study
pRKDI	pRK415 with a complete $ampD_I$ gene; Tc^r	26
Primers		
NagZ5-F	5′-CTGCGCAAGCTTGCCGCCGAAC-3′	This study
NagZ5-R	5′-TTGCATGCCACCACCCCGGCCAC-3′	This study
NagZ3-F	5′-CACCGCATGCTGGTCGATGAAG-3′	This study
NagZ3-R	5′-CATGATCTAGAGGAACAGCGGCAC-3′	This study
NagZP-F	5′-CTGCGCAAGCTTGCCGCCGAAC-3′	This study
NagZP-R	5′-TTGCATGCCACCACCCCGGCCAC-3′	This study

[a] Added restriction enzyme recognition sites in primers are underlined.

tamase derepression. However, Δ*mrcA*-derived basal-level β-lactamase derepression is *creBC* independent (17).

Recently, *nagZ* inactivation has been shown to attenuate β-lactam resistance and *ampC* expression in *ampD* mutant, *dacB* mutant, and *ampD-dacB* double-mutant strains of *P. aeruginosa* (27). Based on the known roles of $ampD_I$, *ampN-ampG*, *ampR*, and *mrcA* in chromosomal β-lactamase gene expression (10, 17, 18, 26), the *ampR-ampC* system of *P. aeruginosa* and the *ampR-L2* system of *S. maltophilia* share several characteristics. To date, the relationships among *nagZ*, the *ampN-ampG-*$ampD_I$*-ampR* regulon, and *mrcA* of *S. maltophilia* have not been elucidated. Here, we further characterize the *nagZ* gene of *S. maltophilia*, including its significance for β-lactamase expression and β-lactam resistance in wild-type, $ampD_I$ mutant, *mrcA* mutant, and $ampD_I$*-mrcA* double-mutant strains. Furthermore, a novel *nagZ*-independent but *ampNG-*$ampD_I$*-ampR* regulon-involved mechanism for β-lactamase expression of *S. maltophilia* is proposed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. Table 1 lists the strains, plasmids, and primers used in this study. *S. maltophilia* KJ, harboring active β-lactamases L1 and L2, has been described previously (9). Tetracycline (50 μg/ml) was added to maintain selection of cells carrying pRK415 derivatives. Primers were designed based on the *S. maltophilia* K279a genome sequence (4).

Construction of the *nagZ* knockout mutant KJΔZ. *nagZ* deletion mutants of strains KJ, KJΔL1, KJΔL2, KJΔDI, KJΔmrcA, and KJΔDIΔmrcA were constructed following a well-established procedure (26). Upstream 339-bp and downstream 247-bp DNA fragments of the *nagZ* gene were obtained by PCR using the NagZ5-F/NagZ5-R and NagZ3-F/NagZ3-R primer sets, respectively. PCR amplicons were digested with HindIII/SphI and SphI/XbaI and subsequently cloned into pEX18Tc (7). The resultant plasmid, pΔZ, has an internal 394-bp deletion in the *nagZ* gene. Plasmid pΔZ was mobilized into *S. maltophilia* KJ, KJΔL1 (9), KJΔL2 (9), KJΔDI (26), KJΔmrcA (17), and KJΔDIΔmrcA (17) via conjugation, and mutants were selected in two steps, as described previously (26). Mutant correctness was checked by colony PCR amplification (16) and sequencing.

Complementation assay. The *nagZ* gene, along with the upstream 225-bp DNA fragment, was obtained by PCR using primers NagZP-F and NagZP-R. The PCR amplicon was ligated into the complementation vector pRK415 (14), resulting in the construct pRK-Z. The sequence of the cloned PCR fragment was verified by sequencing. The orientation of the

complete *nagZ* matched that of the resident *lac* promoter of pRK415. The *nagZ* gene expressed from the recombinant plasmid pRK-Z was confirmed by quantitative reverse transcription (qRT)-PCR (data not shown).

Promoter-*xylE* transcriptional fusion. We constructed pRK188Z_{xylE} to investigate the regulation of *nagZ* expression. A 339-bp DNA fragment containing the partial 5′ terminus of the *nagZ* gene and 188 bp upstream of the *nagZ* gene was obtained by PCR using NagZ5-F and NagZ5-R as primers. The PCR amplicons were cloned into pRK415. A *xylE* gene retrieved from pX1918GT (23) was inserted following the 339-bp amplicon to create a *nagZp-xylE* fusion construct, pRK188Z_{xylE}. The orientation of the *xylE* gene in pRK188Z_{xylE} was opposite to that of *lacZp* from the pRK415 vector.

Antimicrobial susceptibility. The susceptibilities of *S. maltophilia* strains to different β-lactams were determined by a 2-fold serial agar dilution method according to CLSI guidelines (3). All chemicals were purchased from Sigma. The MIC was defined as the lowest concentration of antibiotic that prevented growth of bacteria after incubation at 37°C for 18 h.

Determination of β-lactamase activity. The procedure for β-lactamase induction is described elsewhere (18), except that different inducers were added as indicated. Cefuroxime was generally used as the inducer for determination of total β-lactamase activity; it has been proven to be a potent inducer for L1 and L2 β-lactamase (9). Cefoxitin and aztreonam are not hydrolyzed by L2 and L1 β-lactamase (9), respectively. Therefore, the inducer concentration can be kept constant during the assay process when cefoxitin and aztreonam are used as inducers in L1 and L2 deletion biological backgrounds, respectively. The β-lactamase activities were spectrophotometrically determined with 100 μM nitrocefin (Oxoid) as a substrate (18). Enzyme activity was calculated by using a molar absorption coefficient for nitrocefin of 20,500 M^{-1} cm^{-1} at 486 nm. The specific activity of β-lactamase (U/mg) was expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein. The protein concentration was determined using the Bio-Rad protein assay reagent, with bovine serum albumin as a standard. In all cases, mean β-lactamase activity values were obtained in three independent experiments.

Determining C23O activity. The activity of catechol-2,3-dioxygenase (C23O), encoded by the *xylE* gene, was measured in intact cells, as described previously (10). The rate of hydrolysis was calculated by using 44,000 M^{-1} cm^{-1} as the extinction coefficient. One unit of enzyme activity (Uc) was defined as the amount of enzyme that converts 1 nmol substrate per minute. The specific activity of the enzyme was defined in terms of Uc per optical density at 450 nm (Uc/OD_{450}).

Determining NAG activity. β-1,4-*N*-Acetylglucosaminidase (NAG) activity was assessed by using whole-cell lysates, with *p*-nitrophenyl-β-*N*-acetyl-D-glucosaminide (Sigma) used as a substrate. An overnight culture of the assayed strain was diluted in fresh LB medium to an OD_{450} of 0.15. After 0.5 h of incubation, 50 μg/ml cefuroxime was added, and growth continued for 2 h. Meanwhile, a cefuroxime-free control was simultaneously prepared. Harvested cells were washed, centrifuged, and resuspended in 50 mM Tris-HCl (pH 7.4). The cells were disrupted by sonication, and a crude extract (supernatant) was obtained after centrifugation at 150,000 × *g* for 1 h. The 500-μl sonicated cell lysate was incubated with 400 μl 1 mM *p*-nitrophenyl-β-*N*-acetyl-D-glucosaminide at room temperature. Reactions were allowed to proceed for 1, 2, 3, or 4 h and then were quenched by adding 100 μl of 2.5 M K_2CO_3. The reaction mixture was centrifuged, and *p*-nitrophenol present in the supernatant was measured at 405 nm. Specific activity was calculated by using a molar absorption coefficient for *p*-nitrophenol of 20,500 M^{-1} cm^{-1} at 405 nm. One unit of enzyme activity (U_{nagZ}) was defined as the amount of enzyme that produced 1 nmol *p*-nitrophenol per minute. The specific activity (U_{nagZ}/mg) of Nag enzyme was expressed as U_{nagZ} per milligram of protein.

Nucleotide sequence accession number. The nucleotide sequence of the *nagZ* gene of *S. maltophilia* KJ has been deposited in GenBank under accession no. JN613812.

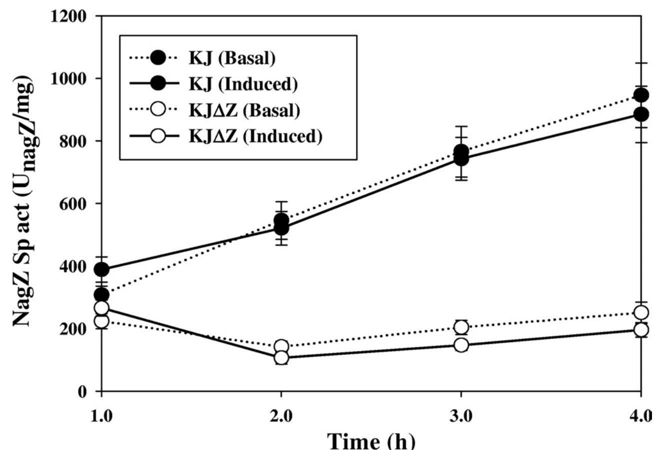

FIG 1 NagZ activity assay of wild-type *S. maltophilia* KJ and its derived *nagZ* deletion mutant, KJΔZ, under basal and induced conditions. Cefuroxime (50 μg/ml) was used as the inducer. Results shown are mean values of three experiments ± standard deviations (SD).

RESULTS

Cloning and sequence analysis of *nagZ*. PCR of *S. maltophilia* KJ chromosomal DNA with primers NagZP-F and NagZP-R (Table 1) yielded a 1,396-bp amplicon. Sequence analysis of the PCR amplicon revealed a complete *nagZ* gene and its upstream 247-bp DNA fragment. A BLAST search of protein databases revealed 97 to 100%, 81 to 84%, 50 to 53%, and 47% identity to the *nagZ* homolog-encoded (NagZ) proteins of different *S. maltophilia* strains, *Xanthomonas* spp., *Pseudomonas* spp., and *Escherichia coli*, respectively.

A homology study showed NagZ to be a putative β-*N*-acetylglucosaminidase in *S. maltophilia*. To assess the possibility, the β-*N*-acetylglucosaminidase activities of strains KJ and KJΔZ were determined under basal and cefuroxime-induced conditions, respectively. After 4 h of incubation, the β-*N*-acetylglucosaminidase activity of strain KJ increased from 308 to 946 U_{nagZ}/mg and from 389 to 685 U_{nagZ}/mg under basal and induced conditions, respectively. Still, inactivation of the *nagZ* gene nearly abolished the increase in β-*N*-acetylglucosaminidase activity regardless of the presence of the inducer (Fig. 1), which agrees with studies of *E. coli nagZ* (2) and *P. aeruginosa nagZ* (1). Hence, NagZ (a homolog of *S. maltophilia* K279a Smlt3538) is an active enzyme with *N*-acetyl-β-glucosaminidase activity in *S. maltophilia* KJ.

Role of *nagZ* in induction of L1 and L2 β-lactamase genes. NagZ is essential to produce the actual inducer molecule of 1,6-anhydroMur*N*Ac peptides in the *ampR-ampC* system (2, 25). Inactivation of *nagZ* in some *Enterobacteriaceae* (24) or *P. aeruginosa* (1) can thus affect *ampC* expression. To pinpoint the extent to which *nagZ* is involved in the expression of L1 and L2 β-lactamase, the Δ*nagZ* allele was introduced into wild-type KJ, mutant KJΔL1 (L1 isogenic), and mutant KJΔL2 (L2 isogenic) (9). The resultant mutants, KJΔZ, KJΔL1ΔZ, and KJΔL2ΔZ, were analyzed in comparison with their parental strains with regard to β-lactamase activity. Inactivation of *nagZ* did not completely abolish β-lactamase induction (Table 2). Some 22 to 25% of induced L1 and L2 β-lactamase activities were still detectable, indicating that cefuroxime-induced β-lactamase activity is partially *nagZ* dependent.

Complementation assay. A complementation assay was per-

TABLE 2 β-Lactamase activities of *S. maltophilia* KJ and its derived strains

S. maltophilia strain	β-Lactamase sp act (Un[a]/mg)	
	Basal	Induced[b]
(A) KJ	10 ± 1.8	1,628 ± 197
KJΔZ	7 ± 1.0	390 ± 51
KJΔL1	5 ± 0.9	585 ± 65[c]
KJΔL1ΔZ	15 ± 2.1	152 ± 20[c]
KJΔL2	9 ± 1.3	114 ± 18[d]
KJΔL2ΔZ	5 ± 1.0	26 ± 3.2[d]
KJΔDI	4,448 ± 752	4,592 ± 560
KJΔDIΔZ	88 ± 10	551 ± 62
KJΔmrcA	1,012 ± 190	2,496 ± 275
KJΔmrcAΔZ	981 ± 102	1,509 ± 182
KJΔDIΔmrcA	4,751 ± 876	5,940 ± 823
KJΔDIΔmrcAΔZ	955 ± 101	1,824 ± 204
KJ(pRK415)	9 ± 2.1	1,329 ± 150
KJ(pRK-Z)	8 ± 1.1	1,365 ± 143
KJΔZ(pRK415)	12 ± 1.3	402 ± 52
KJΔZ(pRK-Z)	10 ± 1.5	1,254 ± 142
KJΔmrcA(pRK415)	924 ± 112	2,135 ± 272
KJΔmrcA(pRKDI)	52 ± 10	789 ± 98

[a] One unit of β-lactamase activity is defined as 1 nanomole of nitrocefin hydrolyzed per minute. The results are expressed as the mean ± standard deviation of three independent determinations.
[b] Twenty micrograms per milliliter cefuroxime as the inducer, except for strains KJΔL1, KJΔL1ΔZ, KJΔL2, and KJΔL2ΔZ.
[c] Twenty micrograms per milliliter cefoxitin as the inducer.
[d] Twenty micrograms per milliliter aztreonam as the inducer.

TABLE 3 Induced β-lactamase activities of strains KJ and KJΔZ treated with various β-lactams

Strain	Induced-β-lactamase sp act[a] (Un/mg)				
	Aztreonam	Cefoxitin	Carbenicillin	Cefuroxime	Piperacillin
KJ	304 ± 42	793 ± 95	388 ± 40	1,868 ± 215	702 ± 84
KJΔZ	344 ± 51	818 ± 101	428 ± 52	448 ± 51	336 ± 46

[a] One unit of β-lactamase activity is defined as 1 nanomole of nitrocefin hydrolyzed per minute. The results are expressed as the mean ± standard deviation of three independent determinations.

formed by introducing plasmid pRK-Z into the wild-type strain KJ and the mutant KJΔZ. Introduction of plasmid pRK-Z into the mutant KJΔZ can restore the induced β-lactamase activity to the levels of the wild-type strain (Table 2), which further confirms that NagZ is indeed a significant enzyme responsible for β-lactamase induction. However, Table 2 shows that strains KJ(pRK415) and KJ(pRK-Z) exhibited equivalent induced β-lactamase activities. The results indicate that the activity of the chromosomally encoded NagZ enzyme of *S. maltophilia* is potent enough to accommodate the processing of all ligand precursors and murein recycling, even under induced conditions. Therefore, *nagZ* did not display a dose effect on the induced β-lactamase activity.

Regulation of *nagZ* expression. The regulation of *nagZ* expression was tested using a *nagZp-xylE* fusion plasmid, pRK188Z_{xylE}. KJ(pRK188Z_{xylE}) displayed a C23O activity of 133 ± 18 Uc/OD_{450}, indicating the 188 bp inserted upstream of *nagZ* contains a functional promoter of *nagZ*. The C23O activity of KJ(pRK188Z_{xylE}) was hardly changed upon the addition of β-lactam, demonstrating that *nagZ* gene expression is constitutive and β-lactam independent. To further investigate the effects of *ampR*, $ampD_I$, *ampNG*, *mrcA*, and *nagZ* on *nagZ* expression, plasmid pRK188Z_{xylE} was mobilized into KJΔR (17), KJΔDI (26), KJΔNG (17), KJΔmrcA (17), and KJΔZ, and the C23O activity in each pRK188Z_{xylE}-containing strain was determined. Plasmid pRK188Z_{xylE} displayed equivalent C23O activities in the wild-type, Δ*ampR*, Δ$ampD_I$, Δ*ampNG*, Δ*mrcA*, and Δ*nagZ* backgrounds under either basal or induced conditions. Accordingly, *nagZ* is constitutively expressed and not regulated by AmpR, $AmpD_I$, AmpN, AmpG, and PBP1a or by the addition of β-lactam. Likewise, there was no autoregulation phenomenon in the expression of *nagZ*.

Role of *nagZ* in basal-level derepressed β-lactamase activity of mutants KJΔDI, KJΔmrcA, and KJΔDIΔmrcA. Inactivating *nagZ* of *P. aeruginosa* notably reduces basal-level derepressed *ampC* expression (*ampC* expression in the absence of β-lactam challenge) in an *ampD* mutant (PAΔD) (1), a *dacB* mutant (PAΔdacB), and an *ampD-dacB* double mutant (PAΔDΔdacB) (27). The contribution of *nagZ* to basal-level derepressed β-lactamase activity in *S. maltophilia* is thus worthy of investigation. Three mutants with basal-level derepressed β-lactamase activity in *S. maltophilia* have been reported: KJΔDI ($ampD_I$ mutant) (26), KJΔmrcA (*mrcA* mutant) (17), and KJΔD_IΔmrcA (17). A Δ*nagZ* allele was further introduced into KJΔDI, KJΔmrcA, and KJΔDIΔmrcA, yielding mutants KJΔDIΔZ, KJΔmrcAΔZ, and KJΔDIΔmrcAΔZ. Basal and cefuroxime-induced β-lactamase activities were analyzed comparatively in the paired strains KJΔDI-KJΔDIΔZ, KJΔmrcA-KJΔmrcAΔZ, and KJΔDIΔmrcA-KJΔDIΔmrcAΔZ.

In a Δ$ampD_I$ background, *nagZ* inactivation nearly abolished basal-level derepressed β-lactamase activity (Table 2), indicating Δ$ampD_I$-derived basal-level derepressed β-lactamase activity is *nagZ* dependent. Still, there remained detectable β-lactamase activity in the cefuroxime-induced KJΔDIΔZ, i.e., approximately 12% of the induced β-lactamase activity of strain KJΔDI (Table 2). Surprisingly, the basal-level derepressed β-lactamase activity of KJΔmrcA was not affected by *nagZ* inactivation, whereas the cefuroxime-induced β-lactamase activity of KJΔmrcA decreased by about 40% owing to the introduction of Δ*nagZ* (Table 2). Δ*nagZ* had less effect on the KJΔDIΔmrcA double mutant than on the KJΔDI mutant but still reduced the basal (80%) and induced (70%) β-lactamase activity of KJΔDIΔmrcA (Table 2).

Role of *nagZ* in β-lactamase activities induced by different β-lactams. It is generally recognized that different β-lactams display different binding affinities to different PBPs, which is linked to the inducibility of chromosomal β-lactamase genes. As shown above, 24% of the cefuroxime-induced β-lactamase activity of strain KJ is *nagZ* independent (Table 2). Whether the same is true for different β-lactams used as inducers is of interest. Carbenicillin (CAR), piperacillin (PIP), cefoxitin (FOX), and aztreonam (ATM) were the β-lactams selected as representatives of penicillin, cephalosporin, and monobactam, respectively. Table 3 demonstrates that inactivation *nagZ* did not significantly affect aztreonam-, cefoxitin-, and carbenicillin-induced β-lactamase activities. However, 76% and 52% of cefuroxime- and piperacillin-induced β-lactamase activities were abolished by *nagZ* inactivation (Table 3).

Role of *nagZ* in β-lactam susceptibility of mutants KJΔDI, KJΔmrcA, and KJΔDIΔmrcA. To assess the effect of Δ*nagZ* on β-lactam resistance, a β-lactam susceptibility test was performed on all *nagZ* mutants and their parent strains (Table 4). Inactiva-

TABLE 4 MICs of β-lactam antibiotics for *S. maltophilia* KJ and its derived mutants

Strain	MIC (μg/ml)				
	PIP	CAR	FOX	CXM	ATM
KJ	1,024	1,024	1,024	2,048	>2,048
KJΔZ	64	512	512	512	2,048
KJΔDI	1,024	1,024	1,024	2,048	>2,048
KJΔDIΔZ	256	512	512	512	2,048
KJΔmrcA	2,048	2,048	1,024	2,048	>2,048
KJΔmrcAΔZ	1,024	1,024	1,024	1,024	2,048
KJΔDIΔmrcA	2,048	2,048	1,024	2,048	>2,048
KJΔDIΔmrcAΔZ	1,024	2,048	1,024	2,048	2,048

tion of *nagZ* of KJ and KJΔDI lowered the MICs of PIP and CXM but marginally decreased the MICs of CAR and FOX. The MICs of β-lactams tested for KJΔmrcAΔZ and KJΔDIΔmrcAΔZ were unchanged or marginally (2-fold) decreased compared to those of their parent strains, KJΔmrcA and KJΔDIΔmrcA, respectively.

Role of $ampD_I$ in Δ*mrcA*-derived basal-level β-lactamase derepression. With NagZ and AmpD critical to ligand processing for *ampC* expression in the *P. aeruginosa* system, their roles in Δ*mrcA*- and $\Delta ampD_I$-derived basal-level β-lactamase derepression are worthy of further study. To gain insight into the role of $ampD_I$ in Δ*mrcA*-derived basal-level β-lactamase derepression, an $ampD_I$-containing plasmid, pRKDI (26), was mobilized into *S. maltophilia* KJΔmrcA via conjugation. Table 2 shows that introduction of pRKDI into KJΔmrcA attenuated the basal-level derepressed β-lactamase activity of KJΔmrcA. Therefore, Δ*mrcA*-derived basal-level β-lactamase derepression is *nagZ* independent and can be attenuated by $ampD_I$ overexpression.

DISCUSSION

A significant distinction between mutants KJΔmrcA and KJΔDI was observed in this study for the role of *nagZ* in basal-level derepressed β-lactamase activities (Table 2). Notably, *nagZ* inactivation significantly reduces the basal-level derepressed β-lactamase activities of KJΔDI, which agrees well with a study of *P. aeruginosa* (1). However, introduction of Δ*nagZ* into the KJΔmrcA mutant hardly influences the basal-level derepressed β-lactamase activities of KJΔmrcA (Table 2). Distinct outcomes indicate at least two different activator ligands responsible for L1/L2 expression in the *S. maltophilia* system. One is generated by the process of NagZ (as with KJΔDI), but the other is produced independently of NagZ (as with KJΔmrcA). For concise descriptions, we designated the former activator ligand 1 (AL1) and the latter AL2. Involvement of different activator ligands in chromosomal β-lactamase expression is not an exception. It has been proposed in the *P. aeruginosa* model that GlcNAc-1,6-anhydromuropeptide, generated by processing of NagZ, acts as an activator ligand for AmpC basal-level derepression in *ampD* and/or *dacB* mutants while an unidentified *nagZ*-independent activator ligand may participate in AmpC overexpression triggered by a classical AmpC inducer, i.e., cefoxitin (27).

Our previous studies have demonstrated that Δ*mrcA*- and $\Delta ampD_I$-derived basal-level derepressed β-lactamase activities are *ampNG* permease dependent (17). Therefore, the precursors of AL1 and AL2 should be generated in the periplasm and then transported into the cytosol via the *ampNG* permease system. Based on similarity between $AmpD_I$ of *S. maltophilia* (26) and AmpD of *P. aeruginosa* (12, 13), The AL1 precursor generated in KJΔDI is likely the GlcNAc-1,6-anhydromuropeptides proposed in the *P. aeruginosa* model. In the case of *mrcA* inactivation, Δ*mrcA*-derived basal-level depressed β-lactamase activity is not affected by *nagZ* inactivation (Table 2, KJΔmrcA and KJΔmrcAΔZ). It can be reasonably speculated that a type of specific degraded murein fragment other than GlcNAc-1,6-anhydromuropeptide appears in KJΔmrcA. The specific degraded murein fragments generated in the periplasm can be AL2 precursors or AL2 itself. Although its structure is not known, AL2 should contain an amide bond that links anhydro-*N*-acetylmuramic acid and an oligopeptide of the murein subunit, since overproduced $AmpD_I$ can attenuate basal-level derepressed β-lactamase activities of KJΔmrcA (Table 2). Also, an intact amide bond in AL2 can be a critical structure for β-lactamase induction. It is worth mentioning that the GlcNAc-1,6-anhydromuropeptide proposed in *Enterobacteriaceae* and *P. aeruginosa* models is not a single entity, since it may be GlcNAc-1,6-anhydromuro-tripepetide, GlcNAc-1,6-anhydromuro-tetrapepetide, and GlcNAc-1,6-anhydromuro-pentapepetide. Therefore, it cannot be ruled out that the AL1 and AL2 proposed in this study might have a structure of anhydro-*N*-acetylmuramic acid with oligopeptides of different lengths (tri-, tetra-, or pentapeptides, for example).

Comparison of the induced β-lactamase activities of the wild-type KJ and of KJΔZ can elucidate the contribution of AL1 and AL2 to the induced β-lactamase activities, since NagZ dependence is a critical determinant to distinguish AL1 and AL2. If the induced β-lactamase activities of strains KJ and KJΔZ are equivalent, AL2 nearly accounts for the activator ligand responsible for the induction of β-lactamase. Inducers of aztreonam, cefoxitin, and carbenicillin are examples of this (Table 3). It can be inferred that aztreonam, cefoxitin, and carbenicillin may have significant affinity for PBP1a. However, AL1 and AL2 appear to be involved in the induced β-lactamase activities when cefuroxime and piperacillin are used as inducers (Table 3). In conclusion, the contributions of AL1 and AL2 to the induced β-lactamase activities may vary with the types of inducers (β-lactams).

Inactivation of *nagZ* restores the wild-type β-lactam MICs for PAO1 *dacB* and *ampD* mutants and dramatically reduces the MICs for the *ampD-dacB* double mutant in the *P. aeruginosa* system (27). In *S. maltophilia*, *nagZ* inactivation moderately attenuates the PIP and CXM resistances of the wild-type KJ and mutant KJΔDI (the MICs are reduced 4- to 16-fold) while it marginally reduces resistance to CAR and FOX, i.e., 2-fold, as shown in Table 4. The difference can be explained based on the results shown in Table 3. The activator ligands produced in the CAR- and FOX-treated bacteria are mainly non-NagZ processed (Table 3), which may reflect the marginal MIC changes of CAR and FOX in the paired strains KJ-KJΔZ and KJΔDI-KJΔDIΔZ. Both the NagZ-processed and non-NagZ-processed ligands are generated in PIP- and CXM-treated *S. maltophilia*; thus, the differences in the MICs of PIP and CXM are more significant in the paired strains KJ-KJΔZ and KJΔDI-KJΔDIΔZ. Nevertheless, *nagZ* inactivation does not significantly affect the β-lactam resistance of Δ*mrcA*-associated mutants (KJΔmrcA and KJΔDIΔmrcA), since the non-NagZ-processed ligand exists because of *mrcA* inactivation.

Several conclusions emerge. (i) When PBPs are not saturated with any β-lactam (the basal level of the wild type), the concentration of repressor ligands dominates over that of activator ligands (presumably AL1) in the cytosol. Therefore, the β-lactamase genes are repressed. (ii) Two kinds of activator ligands, NagZ

processed (AL1) and non-NagZ processed (AL2), were observed with $\Delta ampD_I$ and $\Delta mrcA$, respectively. AL1, likely the known activator ligand of 1,6-anhydromuropeptides of the *Enterobacteriaceae* model, is massively formed under the condition of $ampD_I$ inactivation, and such formation is NagZ dependent. AL2 is generated when *mrcA* is inactivated; formation of AL2 does not depend on NagZ, whereas $AmpD_I$ can process AL2 into a less potent form for β-lactamase induction. (iii) After adding β-lactam, the β-lactam results in saturation of certain PBPs, which depends on its affinity for different PBPs and retardation of peptidoglycan synthesis. Surplus degraded peptidoglycan fragments are transported into the cytosol by the AmpN/AmpG permease system and further processed into activator ligands for β-lactamase induction with the assistance of AmpR. The types of β-lactam and the amounts of PBPs bound by the added β-lactam determine the contributions of AL1 and AL2 to induced β-lactamase activities. Specifically, more AL2 ligand is generated when more PBP1a is bound. (iv) *nagZ* inactivation moderately decreases PIP and CXM MICs for the wild-type and the *ampDI* mutant whereas it has an insignificant effect on the CAR and FOX MICs for the wild type and the *ampDI* mutant and the PIP, CAR, FOX, and CXM MICs for the *mrcA*-associated mutants.

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