

Induction of L1 and L2 β -Lactamases of *Stenotrophomonas maltophilia*[∇]

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Isogenic L1 and L2 gene knockout mutants of *Stenotrophomonas maltophilia* KJ (KJ Δ L1 and KJ Δ L2, respectively) were constructed by *xylE* gene replacement. Induction kinetics of the L1 and L2 genes were evaluated by testing catechol 2,3-dioxygenase activity in the mutants. The results suggested that the induction of the L1 and L2 genes was differentially regulated.

Stenotrophomonas maltophilia produces two β -lactamases, known as L1 and L2 (16, 17). The expression of L1 and L2 is inducible, and β -lactams are inducers. The induction mechanism first proposed by Mett et al. (11) implies a common regulation system for both genes. Recently a differential regulation has been proposed (2). The present study investigated the induction properties of the L1 and L2 genes.

S. maltophilia KJ is a clinical isolate. Its β -lactamase extract was prepared from the periplasmic contents (9) using ampicillin (100 μ g/ml) as an inducer and subjected to an isoelectric focusing (IEF) assay (10). Two β -lactamases were revealed by IEF, of pIs 6.0 and 8.2, which can be assigned as the pIs of L1 and L2, respectively (5, 15).

PCR primers L1P-F (5'-ACATTGCCTACTACACCTCC-3'), L1P-R (5'-GCTCTTTACAGAGTCGAGCC-3'), AmpRL2-F (5'-AAGCCGCTGGATGGAAC-3'), and AmpRL2-R (5'-ATGCCGATGATGCCGAAC-3') were designed to amplify the two β -lactamase genes based on the released genome sequences of *S. maltophilia* K279a (www.sanger.ac.uk/projects/s_maltophilia). The 1.6-kb and 2.2-kb PCR amplicons obtained by PCR using the L1P-F/L1P-R and AmpRL2-F/AmpRL2-R pairs of primers, respectively, were ligated into T-vector (Yeastern Biotech Co.) and sequenced. The 1.6-kb PCR amplicon contained the L1 gene and a partial Ton B-dependent receptor gene separated by a 95-bp intercistronic region (IG). The 2.2-kb PCR amplicon contained two genes, *bla*_{L2} and *ampR*, divergently oriented and separated by a 175-bp IG. AmpR is a typical LysR transcriptional regulator protein (19) and has been shown to be a regulator for the expression of flanking β -lactamase genes in other genera (21, 24). However, the role of *ampR* in *S. maltophilia* is still unknown. For *S. maltophilia* KJ, a conserved LysR motif (TCCTAACGCTTCA) (6) was found in the IG between *ampR* and the L2 gene, implying that AmpR could be a regulator for the expression of the L2 gene. Neither a putative transcriptional regulator gene nor any conserved LysR motif is present in the region upstream of the L1 gene. The L1 and L2 proteins encoded by the isolate KJ were 87 to 98% and 69 to 100% identical, respectively, to other L1 and L2

enzymes in the literature (1, 18, 22, 23, 25) (www.sanger.ac.uk/projects/s_maltophilia; http://www.jgi.doe.gov).

Isogenic L1 and L2 gene knockout mutants, KJ Δ L1 and KJ Δ L2, were obtained by a gene replacement strategy. The 1.6-kb and 2.2-kb PCR amplicons were subcloned into the plasmid pEX18Tc, and a catechol 2,3-dioxygenase (C23O) gene (*xylE*) (20) was inserted into either the SacI site of the L1 gene or the StuI site of the L2 gene to generate transcriptional fusions in the same orientation as that of the interrupted genes. The KJ Δ L1 and KJ Δ L2 mutants were obtained by conjugation, followed by two-step antibiotics/10% sucrose selection as described previously (25). The authenticity of mutants was verified by both PCR sequencing and IEF.

MICs of strains KJ, KJ Δ L1, and KJ Δ L2 were determined in triplicate according to a standard twofold serial agar dilution method (12) (Table 1). The MICs for cefepime, imipenem, and meropenem were quantified using Etest strips (AB Biodisk, Solna, Sweden). Results of susceptibility testing with the two mutants were overall consistent with the substrate profiles of the two enzymes reported previously (4, 22).

The inducibility of L1 and L2 enzymes was further confirmed. Strains KJ, KJ Δ L1, and KJ Δ L2 were treated with and without cefuroxime (50 μ g/ml), and then levels of the expressed β -lactamase were comparatively checked with IEF and activity assay using CENTA (3) as the substrate (data not shown).

Induction experiments were performed to monitor the C23O activity of KJ Δ L2 and KJ Δ L1. Overnight cultures were diluted to an optical density at 450 nm of 0.15 and subsequently grown at 37°C for 0.5 h. Unless otherwise stated, induction was carried out using 50 μ g/ml of inducer for 2.5 h. The C23O activities in intact cells were determined (8); meanwhile, the optical density of cell suspension at 450 nm was recorded. One unit of enzyme activity was defined as the amount of enzyme that converted 1 nmol substrate per minute. The specific activity of the enzyme was defined in terms of units per A_{450} units (one A_{450} unit corresponds to approximately 3.6×10^8 cells/ml). Each experiment was repeated at least three times.

An induction time course experiment was designed to monitor the C23O activities of KJ Δ L1 and KJ Δ L2 at an interval of 30 min, using cefuroxime as an inducer (Fig. 1). Without the addition of an inducer, the C23O activity was below the level of detection for both mutants. In the presence of the inducer, the C23O activity was detectable starting with the first sampling, without any appar-

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TABLE 1. MICs of β -lactam antibiotics for *S. maltophilia* KJ, KJ Δ L1, and KJ Δ L2

Antibiotic	MIC (μ g/ml) of antibiotic for strain:		
	KJ	KJ Δ L1	KJ Δ L2
Penicillins			
Ampicillin	>2,048	>2,048	>2,048
Piperacillin	1,024	256	512
Carbenicillin	1,024	1,024	64
Cephalosporins			
Cefuroxime	2,048	1,024	1,024
Cefoxitin	1,024	128	1,024
Cefoperazone	128	32	64
Cefotaxime	256	128	128
Ceftriaxone	256	128	128
Cefepime	64	64	4
Carbapenems			
Imipenem	>32	8	>32
Meropenem	>32	0.5	>32
Monobactam			
Aztreonam	>2,048	>2,048	16

ent lag period. This is consistent with studies with other *S. maltophilia* strains (14–16) and with *Enterobacter cloacae* (7) but different from that with *Pseudomonas aeruginosa*, for which a long and concentration-dependent lag phase was observed (13). Maximum C23O activities for KJ Δ L1 and KJ Δ L2 were obtained at 3 h and 2.5 h after induction, respectively. Thereafter, the C23O activity of KJ Δ L2 decreased at a rate significantly faster than that of KJ Δ L1 up to 4.5 h of induction.

Figure 2 shows the C23O activities of KJ Δ L1 and KJ Δ L2 as a function of the cefuroxime concentration. The expression of *xyIE* of KJ Δ L1 and KJ Δ L2 was readily induced by a small amount of cefuroxime (1 μ g/ml). Apparently, the level of L1 induction was less dependent on the cefuroxime concentration than that of L2 induction.

Rosta and Mett found that total induced β -lactamase activity in *S. maltophilia* decreases at a rate of 50% per generation after the maximal peak (15). In the present study, a difference between the induction of L1 and that of L2 was apparent. The difference in enzyme decline after maximum induction (Fig. 1)

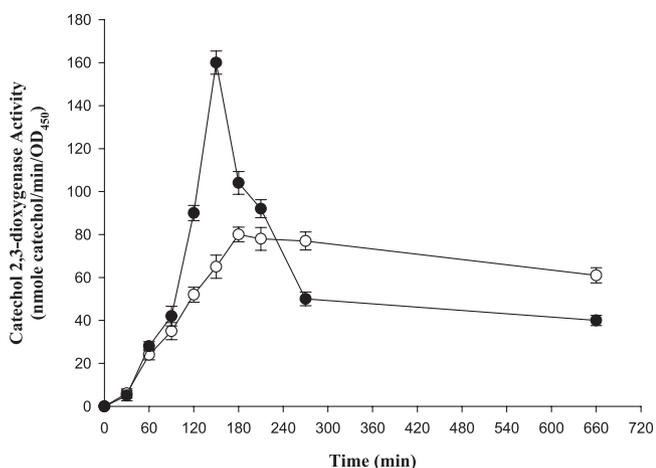


FIG. 1. Induction of C23O activity in *S. maltophilia* KJ Δ L1 and KJ Δ L2. The error bars indicate standard deviations ($n = 3$). Symbols: \circ , mutant KJ Δ L1; \bullet , mutant KJ Δ L2. OD₄₅₀, optical density at 450 nm.

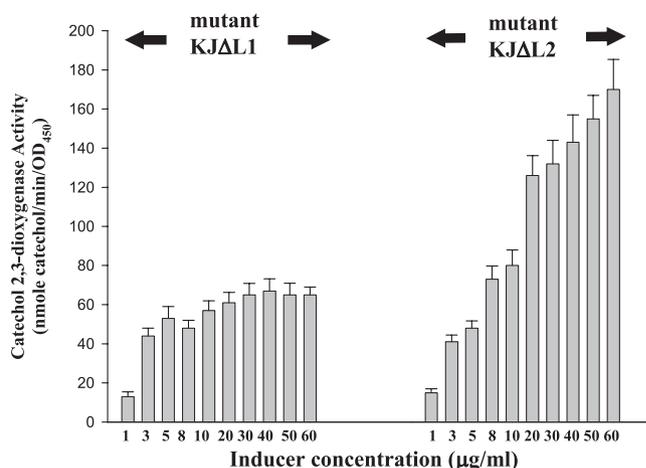


FIG. 2. Induction of C23O activity in *S. maltophilia* KJ Δ L1 and KJ Δ L2 as a function of the inducer concentration. The error bars indicate standard deviations ($n = 3$). OD₄₅₀, optical density at 450 nm.

between KJ Δ L1 and KJ Δ L2 could be explained by the decrease in the inducer concentration in the assay system owing to its hydrolysis by the induced β -lactamase, which has a greater effect on induction of L2 (Fig. 2). In addition, the different activities of L1 and L2 with the inducer cefuroxime might also contribute to this difference.

The induced β -lactamase activity has been shown to be linearly correlated to the inducer concentration for *Pseudomonas aeruginosa* and *S. maltophilia* (13, 15). In the present study, a roughly linear correlation was observed with KJ Δ L2 but not with KJ Δ L1. This lower dependence of the induction of L1 on the inducer concentration is a phenomenon that has not yet been reported in β -lactamase induction of gram-negative bacteria (13, 15).

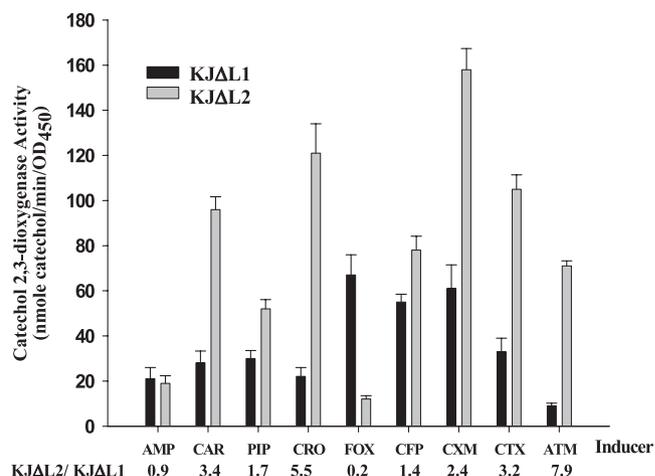


FIG. 3. Induction of C23O activity by various β -lactam antibiotics in *S. maltophilia* KJ Δ L1 and KJ Δ L2. Inducers (shown on the x axis): AMP, ampicillin; CAR, carbenicillin; PIP, piperacillin; CRO, ceftriaxone; FOX, cefoxitin; CFP, cefoperazone; CXM, cefuroxime; CTX, cefotaxime; ATM, aztreonam. The induction ratio of KJ Δ L2 to KJ Δ L1 for each inducer is also included. The concentration of each inducer is 50 μ g/ml, except that cefoperazone's is 16 μ g/ml and aztreonam's is 8 μ g/ml. The error bars indicate standard deviations ($n = 3$). OD₄₅₀, optical density at 450 nm.

Figure 3 shows the induction of the L1 and L2 genes by various β -lactams. At a concentration of 50 $\mu\text{g/ml}$, most of the inducers hardly affected the growth of KJ Δ L1 or KJ Δ L2, with the exception of cefoperazone against KJ Δ L1 and aztreonam against KJ Δ L2. Consequently, induction experiments using cefoperazone and aztreonam as the inducers were also performed using lower concentrations, i.e., 16 $\mu\text{g/ml}$ cefoperazone and 8 $\mu\text{g/ml}$ aztreonam. In general, the induced C23O activity of KJ Δ L2 was higher than that of KJ Δ L1 against a specific inducer, except ampicillin and ceftiofexim. The ratio of induction for KJ Δ L2 to that for KJ Δ L1 ranged from 0.2 to 7.9. Consequently, the induction potencies toward the L1 and L2 genes differed significantly for different inducers.

In conclusion, based on the results of induction experiments with KJ Δ L1 and KJ Δ L2 for the aspects of the induction course, the inducer type, and its concentration, this study suggests that the L1 and L2 genes are differentially regulated during induction.

Nucleotide sequence accession numbers. The nucleotide sequences of the L1 and L2 PCR amplicons have been deposited in the GenBank database under accession numbers EF601224 and EF601225, respectively.

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