Plasmid Location and Molecular Heterogeneity of the L1 and L2 β -Lactamase Genes of *Stenotrophomonas maltophilia*

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An approximately 200-kb plasmid has been purified from clinical isolates of *Stenotrophomonas maltophilia*. This plasmid was found in all of the 10 isolates examined and contains both the L1 and the L2 β -lactamase genes. The location of L1 and L2 on a plasmid makes it more likely that they could spread to other gram-negative bacteria, potentially causing clinical problems. Sequence analysis of the 10 L1 genes revealed three novel genes, L1c, L1d, and L1e, with 8, 12, and 20% divergence from the published strain IID 1275 L1 (L1a), respectively. The most unusual L1 enzyme (L1e) displayed markedly different kinetic properties, with respect to hydrolysis of nitrocefin and imipenem, compared to those of L1a (250- and 100-fold lower k_{cat}/K_m ratios respectively). L1c and L1d, in contrast, displayed levels of hydrolysis very similar to that of L1a. Several nonconservative amino acid differences with respect to L1a, L1b, L1c, and L1d were observed in the substrate binding-catalytic regions of L1e, and this could explain the kinetic differences. Three novel L2 genes (L2b, L2c, and L2d) were sequenced from the same isolates, and their sequences diverge from the published sequence of strain IID 1275 L2 (L2a) by 4, 9, and 25%, respectively. Differences in L1 and L2 gene sequences were not accompanied by similar divergences in 16S rRNA gene sequences, for which differences of <1% were found. It is therefore apparent that the L1 and L2 genes have evolved relatively quickly, perhaps because of their presence on a plasmid.

In recent years there have been major increases in the frequencies with which certain, previously rare bacterial species have been identified as the causes of hospital-acquired bacteremias (26). Three principle factors have combined to bring about this change: (i) increased numbers of hospitalized patients who are severely immunosuppressed; (ii) an increase in complicated surgical procedures, such as transplant and oncology surgery, and the use of intravenous catheters; and (iii) the prophylactic use of antibiotics, particularly β -lactams (26). A prime example of such an emergent pathogen is *Stenotrophomonas maltophilia* (6, 11, 25, 26, 28). Its tolerance to silver-lined catheters (28) and its inherent resistance to many antibacterial drugs, including most, if not all, β -lactams (1, 26, 27), give it a survival advantage over other potential pathogens in the hospital environment. Its incidence as a cause of nosocomial bacteremias caused by gram-negative organisms is now second only to that of bacteremia caused by *Pseudomonas aeruginosa*, and the frequency of its isolation is increasing (25). It is also a significant cause of bacterial infection among young adults with cystic fibrosis (10).

The mechanisms of antibacterial drug resistance in *S. maltophilia* have not been studied in detail, but it is expected that many of the acquired mechanisms found in *P. aeruginosa* and other gram-negative bacteria are likely to be present. Strains that are resistant to all known aminoglycosides, quinolones, β -lactams, chloramphenicol, rifampin, tetracycline, and tri-

methoprim have been reported (1, 26, 27). Resistance to these agents is by a combination of intrinsic and acquired determinants. Resistance to β -lactams is primarily intrinsic, mediated by two inducible β -lactamases, L1 and L2 (10, 18, 21–23). L1 is a Zn^{2+} -dependent metalloenzyme that hydrolyzes virtually all classes of β -lactams, including penicillins, cephalosporins, and carbapenems but excluding monobactams (9, 18, 22, 30), while L2 is a serine active-site cephalosporinase (23, 31). On the basis of the fact that β -lactamase expression in *S. maltophilia* is inducible and intrinsic to the bacterial species, the assumption has been that the L1 and L2 genes are chromosomal, although this has not been rigorously tested.

Recent reports have indicated that the *S. maltophilia* species currently accommodates strains that show significant degrees of evolutionary divergence, as reflected by DNA hybridization studies and 16S rRNA gene (rDNA) sequence analyses (7, 13). In fact, sequence divergence of as much as 30% was found (13). Although strain variations in the amino acid sequences of both L1 and L2 β -lactamases are indicated by isoelectric focusing analysis (10, 19, 20), there is a paucity of information as to how differences in pI values relate to differences in the amino acid sequences. Little is known about allelic variation among L1 and L2 genes. Allelic variation creates a set of natural mutants of a particular gene, and analysis of their products can help us understand the biochemical mechanics of the reaction catalyzed. In the case of the β -lactamases of *S. maltophilia*, it was hoped that such an investigation would facilitate greater understanding of the L1 enzyme in particular.

The work described here was undertaken, therefore, to determine the extent of allelic variation among L1 and L2 β -lactamase genes from 10 clinical isolates of *S. maltophilia* collected on an oncology ward over a period of several years. The

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TABLE 1. *S. maltophilia* isolates used in the study.

Strain name	API 20NE no.	L1 or L2 gene type	rRNA gene sequence at variable points ^a			
			43/44	55/56	102	104
K279a	1473345	K279a (L1c, L2b)	G/A	T/C	T	T
H33b	1672341	L1c, L2b	G/A	T/C	T	T
J530b	1472345	L1c, L2b	G/A	T/C	T	T
J675Ib	1472341	L1c, L2b	G/A	T/C	T	T
J675a	1472341	J675a (L1d, L2c)	A/G	C/T	C	G
J675b	1472341	L1d, L2c	A/G	C/T	C	G
0132 II	1472341	L1d, L2c	A/G	C/T	C	G
0133	1432340	L1d, L2c	A/G	C/T	C	G
N531	1472341	N531 (L1e, L2d)	G/A	T/C	C	G
P567 II	1472341	L1e, L2d	G/A	T/C	C	G

^a There were only six points of sequence variation among the 10 rRNA gene sequences. The numbers relate to the position within the 500-bp rRNA PCR amplicon (see text for more details).

primary aims were to assess whether the degrees of change of *L1* and *L2* are essentially the same in each isolate and to investigate the effect of consequent amino acid variation on enzyme activity. In addition, the locations of *L1* and *L2* were determined and a comparison of the extent of *L1* and *L2* variation and that seen in the corresponding 16S rRNA genes from the isolates was made.

MATERIALS AND METHODS

Bacterial strains. Ten clinical isolates of *S. maltophilia* were collected over several years from bacteremic oncology patients undergoing treatment at a hospital in Bristol, United Kingdom. The criteria for selection of the isolates were that the patients had recurrent bacteremia which had not responded to piperacillin-tazobactam and ceftazidime therapy. The isolates were plated on nutrient agar (Oxoid plc., Basingstoke, United Kingdom) to confirm their purity, and their identities were validated with API 20NE test strips (BioMerieux, La Balme les Grottes, France). Details about the individual strains are given in Table 1.

Materials. Unless otherwise stated, all media used were either nutrient broth or nutrient agar (Oxoid plc.). PCR primers were purchased from Sigma-Genosys Ltd. (Pampisford, United Kingdom). The β -lactams used were nitrocefin (Beckton-Dickinson, Cockeysville, Md.) and imipenem (Merck Sharpe & Dohme, West Point, Pa.). All other general reagents were from Sigma Chemical Co. or BDH, both of Poole, United Kingdom.

PCR and DNA sequencing. Five microliters of purified plasmid DNA or 20 μ l of genomic DNA was used as a template for PCR analysis. Plasmid DNA was isolated with a Hybaid Plasmid Recovery kit (Hybaid, Teddington, United Kingdom) according to the manufacturer's instructions. Genomic DNA was purified and standard PCR was performed as described previously (14). For multiplex PCR, 1 μ M each primer was used in the same reaction mixture. In all cases an annealing temperature of 60°C was used. The primer sets used in this study were L1-FULL forward (5'-ACCATGCGTTCTACCTGCTCGCCTTCGCC-3') and reverse (5'-TCAGCGGGCCCGGCCGTTTCCTTGGCCAG3'); L2-FULL forward (5'-CGATTCTGCAGTTCAGT3') and reverse (5'-CGGTACCTCATCCGATC-3'); rDNA forward (5'-TCAGA TTTGAACGCTGGCGGCA-3') and reverse (5'-CGTATTACCGCGGCTGCT GCCAC-3'), and D-PEP forward (5'-CGCAACCTGTGGGTGATC-3') and reverse (5'-CCAGATCGTTCTCGACCA-3'). The L1-FULL, L2-FULL, and D-PEP primers flank the first and final codons of the published L1 (30), L2 (31), and dipeptidyl peptidase IV (15) genes respectively. The L2-MID primers amplify an internal 500-bp fragment of the L2 gene sequence. The rDNA primers bind to highly conserved sequences in the 16S rRNA gene and amplify a 500-bp fragment of the gene for which the majority of sequence variation is known to occur in different gram-negative bacteria (17). In some cases, PCR products were purified with a QIAquick PCR purification kit (Qiagen Ltd., Crawley, United Kingdom), and both strands were sequenced with an ABI Prism 377 automatic sequencer (Perkin-Elmer, Warrington, United Kingdom) by use of dye termination chemistry according to the manufacturer's instructions. DNA and protein sequences were compared by using the Lasergene suite of programs (DNASTAR Inc., Madison, Ws.).

Overexpression and purification of L1. The L1a gene, originally cloned from strain IID 1275 on plasmid pUB5811 (30), was amplified by PCR, as described above, by using pUB5811 as the template. The L1c, L1d, and L1e genes were similarly amplified by using *S. maltophilia* isolate K279a, J675a, or N531 genomic DNA, respectively, as the template. The resultant amplicons were individually TA cloned into the pTrcHis2-TOPO vector (Invitrogen, Carlsbad, Calif.), and recombinant molecules were transformed into *Escherichia coli* TOP10 One Shot competent cells (Invitrogen), according to the manufacturer's guidelines, to produce separate clones representing the four L1 isoforms. The presence of L1 in ampicillin-resistant clones was confirmed by PCR, and one of each positive clone was used to inoculate separate broth cultures, which were grown (37°C, with shaking) until an optical density at 600 nm of 0.5 to 0.6 was reached. Isopropyl- β -D-thiogalactopyranoside (IPTG) (final concentration, 1 mM) was then added to each culture to induce L1 overproduction, and growth continued for a further 3 h. The cells were pelleted by centrifugation (4,000 $\times$ g, 20 min, 4°C) and resuspended in buffer A (50 mM cacodylate [pH 6.0] containing 10 μ M ZnCl₂, 0.02% [wt/vol] sodium azide, 1 mM β -mercaptoethanol). Lysozyme was added (to 200 μ g/ml), and each mixture was incubated (10 min, 20°C). CaCl₂ was then added (final concentration, 10 mM) to stabilize the spheroplasts, and cell debris was pelleted by centrifugation (9,000 $\times$ g, 20 min, 4°C). The supernatants were filtered with Stericup filters (Millipore, Watford, United Kingdom) to remove insoluble matter, and in separate purification procedures, each was applied to an SP-Sepharose column (Amersham Pharmacia Biotech) equilibrated with buffer A. In each case, L1 was eluted with a 0 to 1 M NaCl gradient in buffer A, and fractions containing L1 were pooled and concentrated (Centri-con 10 concentrator; Amicon, Stonehouse, United Kingdom). L1 was then further purified with a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) by using buffer A containing 100 mM NaCl.

Production of crude cell extracts for β -lactamase assay. *E. coli* recombinants containing the L1 clones were grown separately in broth. Induction of L1 expression with IPTG was performed as described above. The cells were pelleted, and extracts for β -lactamase assay were prepared as described previously (2).

β -Lactamase assays and steady-state kinetic analysis. Hydrolysis of β -lactam antibiotics was examined by spectrophotometric analysis (Pharmacia LKB Ultraspec III; Pharmacia, St Albans, United Kingdom), with readings recorded at 2-s intervals for 3 min at the wavelength associated with the largest difference in absorbance upon hydrolysis of the β -lactam ring of each drug, i.e., 482 and 299 nm, for nitrocefin and imipenem, respectively. Antibiotic solutions were prepared in 50 mM cacodylate (pH 7.0) containing 100 μ M ZnCl₂. The rate of hydrolysis (v) of each β -lactam was calculated for at least five different concentrations of substrate (S) by using 17,400 and 7,000 AU $\cdot$ M⁻¹ $\cdot$ cm⁻¹ as extinction coefficients for hydrolysis of nitrocefin and imipenem, respectively. A plot of S/v against S was used to calculate V_{max} (1/slope) and K_m (x intercept multiplied by V_{max}). The monomeric k_{cat} (in seconds⁻¹) of L1 for each substrate was determined by dividing V_{max} by the concentration of L1 monomer in the assay. The monomer concentration was determined by using the extinction coefficients (at 280 nm) for a monomer of L1a (38,930 AU $\cdot$ M⁻¹ $\cdot$ cm⁻¹) and L1e (38,930 AU $\cdot$ M⁻¹ $\cdot$ cm⁻¹), which were calculated from their amino acid sequences with the algorithm of Gill and von Hippel (12).

Nucleotide sequence accession numbers. The L1c, L1d, and L1e sequences from isolates K279a, J675a, and N531, respectively, were submitted to the EMBL database and have been given accession numbers AJ251814, AJ251815, and AJ272109, respectively. The L2b, L2c, and L2d sequences from the same three

A

L1a	MRSTLLAFAL-----AVALPAAHTSAAEVPLPQLRAYTVDASWLQPMAPLQIADHTWQI	54
L1b	MRSTLLAFAL-----AVALPAAHTSAAEAPLPQLRAYTVDASWLQPMAPLQIADHTWQI	54
L1c	MRSTLLAFAL-----AVALPAVHASAAEAPLPQLRAYTVDASWLQPMAPLQIADHTWQI	54
L1d	MRSTLLAFAL-----VTALPIAHASCAETPLPQLRAYTVDASWLQPMAPLQIADHTWQI	54
L1e	MRSTLLAFALSSSLAIAATLTFTFDGAADASLPQLQAYTVDPSWLQIMAPLQIADHTWQI	59
L1a	GTEDLTALLVQTPDGAVLLDGGMPQMASHLLDNMKARGVTPRDLRLILLSHAHADHAGP	113
L1b	GTEDLTALLVQTAEGAVLLDGGMPQMACHLLDNMKLRGVAPQDLRLILLSHAHADHAGP	113
L1c	GTEDLTALLVQTAEGAVLLDGGMPQMACHLLDNMKLRGVAPQDLRLILLSHAHADHAGP	113
L1d	GTEDLTALLVQTAEGAVLLDGGMPQMACHLLTNMKVRGVAPQDLRLILLSHAHADHAGP	113
L1e	GTEDLTALLVQTAEGAVLLDGGMPQMASHLLTNMKASCTNTGELRMVLLSHAHADHAGP	118
L1a	VAELKRRTGAKVAANAESAVLLARGGSDDLHFGDGITYPPANADRIVMDGEVITVGGIV	172
L1b	VAELKRRTGAKVAANAETAVLLARGGSDDLHFGDGITYPPASADRIIMDGEVITVGGIA	172
L1c	VAELKRRTGAKVAANAETAVLLARGGSDDLHFGDGITYPPASADRIIMDGEVITVGGIA	172
L1d	VAELKRRTGAKVAANAESAVLLARGGSDDLHFGDGITYPPASADRIVMDGEVITVGGIA	172
L1e	VAELKRRTGAKVVVNAETAVLLARGGSDDLHFGDEITTPPVNADRIVMDREVVKLGIA	177
L1a	FTAHFMAGHTPGSTAWTWTDTTRNGKPVRIAYADSLSAPGYQLQGNPRYPRLIEDYRRSF	231
L1b	FTAHFMPGHTPGSTAWTWTDTTRDGKPVRIAYADSLSAPGYQLKGNPRYPRLIEDYKRSF	231
L1c	FTAHFMPGHTPGSTAWTWTDTTRDGKPVRIAYADSLSAPGYQLKGNPRYPRLIEDYKRSF	231
L1d	FTAHFMPGHTPGSTAWTWTDTTRDGKPVRIAYADSLSAPGYQLQGNPRYPRLIEDYRRSL	231
L1e	FTAHFLPGHTPGSTAWTWTDTTRDGKPVRIAYADSLSAPGYQLQGNARYPRFLIEDYRRSF	236
L1a	ATVRALPCDVLLTPHPGASNWDYAAGARAGAKALTCKAYADAAEQRFQDLAKETAGAR	290
L1b	ATVRALPCDLLLTPHPGASNWNYAAGSKASAEALTCNAYADAAEKKFDAQLAARETAGTR	290
L1c	ATVRALPCDLLLTPHPGASNWNYAAGSKASAEALTCNAYADAAEKKFDAQLAARETAGAR	290
L1d	ATVRALPCDVLLTPHPGASNWDYAAGSKASAKALTCKAYADAAEQRFDAQLAARETAGAR	290
L1e	ATVRALPCDLLLTPHPGASNWNYAAGSNASEKVLSCAYADAAEKKFDAQLAARETAGAR	295

B

L1a	L1b	L1c	L1d	L1e	
	12%	11%	8%	19%	L1a
		1%	10%	20%	L1b
			10%	19%	L1c
				21%	L1d
					L1e

FIG. 1. Alignment of L1 amino acid sequences. Both strands of the L1-FULL PCR products from all 10 *S. maltophilia* isolates were sequenced as described in Materials and Methods. The predicted amino acid sequences of L1c, L1d, and L1e are novel, and in panel A they are aligned with the previously published L1 amino acid sequences, L1a (30) and L1b (24). All differences from L1a are shaded. (B) Percent divergence of the L1 proteins compared to each other.

isolates, respectively, were submitted to the EMBL database and have been given accession numbers AJ251816, AJ251817, and AJ272110, respectively.

RESULTS

Allelic variation among L1 β -lactamase genes in *S. maltophilia*. The L1 genes in 10 clinical isolates of *S. maltophilia*, recovered as PCR amplicons, were sequenced. Three distinct sequences were recovered (Fig. 1; Table 1), typified by L1 from isolates K279a (L1c), J675a (L1d), and N531 (L1e). The predicted amino acid sequences of the L1 variant proteins and their alignments with two previously published L1 sequences, L1a (30) and L1b (24), are shown (Fig. 1). The L1c, L1d, and L1e genes are 12, 8, and 20% divergent from L1a, respectively,

and the encoded proteins are 11, 8, and 19% divergent from L1a, respectively. There was a similar range of divergence among all of the proteins (Fig. 1B).

Allelic variation among L2 β -lactamase genes in *S. maltophilia*. An analysis of the L2 genes of the same isolates also gave three distinct sequences, again typified by the sequences obtained from isolates K279a, J675a, and N531, named L2b, L2c, and L2d, respectively (Fig. 2; Table 1). Isolates with the same L2 allele also have common L1 alleles (Table 1). The L2b, L2c, and L2d genes are 9, 4, and 25% divergent from L2a (31), respectively, and the L2 proteins are 7, 5, and 32% divergent from L2a, respectively, with a similar range of divergence among all of the L2 proteins (Fig. 2B).

A

L2a	MLARRRFLQFSGAAVASSLALPLLARAAGKTAASAPTDAAALTAATDFAALEKAVRGRFGVT	61
L2b	MLARRRFLQFSGAAVASSLALPLLARAAGKATANAPTDAAITTAASDFAALEKACAGRLGVT	61
L2c	MLARRRFLQFSGAAVASSLALPLLARAAGKTASAPTDAAALTAATDFAALEKACAGRLGVT	61
L2d	MLARRRFLQFSGAAVASSLAFBALVQPVSASSGTPAANAATAGAADFAALEKASGGRLGVT	61
L2a	LLDTASGRRIG-HRQDERFPMCSTFKSVLAATVLSQAERQPALLDTRVPVRDADLLSHAPV	121
L2b	LLDTASGRRIG-HRQDERFPMCSTFKSMLAATVLSQAERMPPALLDTRVPVGEADLLSHAPV	121
L2c	LLDTASGRRIG-HRQDERFPMCSTFKSVLAATVLSQAERQPALLDTRVPVRDADLLSHAPV	121
L2d	VLLNTGNGRRIGCHRQDERFPMCSTFKSMVAHVLSLADAGRVS LDTRVPIAGKDLLSHAPV	122
L2a	TRRHAGKDMTVRDLCRATIIITSDNTAANLLFGVVGPPAVTAFLRSIGDAVSRTDRLEPEL	182
L2b	TRRHAGKDMTVRDLCRATIIITSDNTAANLLFGVVGPPAVTAFLRASGDTVSRSRDRLEPEL	182
L2c	TRRHAGKDMTVRDLCRATIIITSDNTAANLLFGVVGPPAVTAFLRTSGDAVSRSRDRLEPEL	182
L2d	ARRHV GKDLTVRDLCRATIIITSDNTAANLLLEVVGGPSATTAFLRGQGD SITRNDRNEPDV	183
L2a	NSFAKGDPRDTPAAMAATLQRVVLGEVLQLASRQQLADWLI DNETGDACL RAGLGKLWR	243
L2b	NSFAKGDPRDTPAAMAATLQRVVLGEVLQFASRQQLADWLI DNETGDACL RAGLGKRW	243
L2c	NSFAKGDPRDTPGAMAATLQRVVLGEVLQFASRQQLADWLI DNETGDACL RAGLGKRW	243
L2d	NLFAKGDPRDTPAAMATSLARFAVGNGLQFASRQQLADWLI DNETGDACL RAGLGKRW	244
L2a	VRDKTGSNGEDARNDIAVLWPVAGGAPWVLTAYLQAGAI SYEQRATVLAQVGR IADRLIG	303
L2b	VGDKTGSNGEDARNDIAVLWPVAGGAPWVLTAYLQAGAI SYEQRASVLAQVGR IADRLIG	303
L2c	VGDKTGSNGEDARNDIAVLWPVAGGAPWVLTAYLQAGAI SYEQRATVLAQVGR IADRLIG	303
L2d	VGDKTGSNGDDTRNDIAVLWPHAGGTAWVLTAYLQ GASVDDQRAAVLARV GADADAMIG	304

B

L2a	L2b	L2c	L2d	
	7%	5%	32%	L2a
		5%	30%	L2b
			32%	L2c
				L2d

FIG. 2. Alignments of L2 amino acid sequences. Both strands of the L2-FULL PCR products from all 10 *S. maltophilia* isolates were sequenced as described in Materials and Methods. The predicted amino acid sequences of L2b, L2c, and L2d are novel, and in panel A they are aligned with the previously published L2a amino acid sequence (31). All differences from L2a are shaded. (B) Divergence among the L2 proteins.

Allelic variation among 16S rRNA genes in *S. maltophilia*. To determine the phylogenetic divergences among the 10 strains, 500 bp of the hypervariable section of the 16S rRNA gene in each strain was amplified by PCR and sequenced. The chosen sequence is one in which maximum variation would be expected (16). All 10 sequences were found to be essentially the same and showed less than 1% divergence from each other and from the type sequence in the EMBL database (accession number AB008509) (Table 1). In fact, only six variant nucleotides were detected (Table 1), and the exact distribution of alleles divided the isolates into the same three groupings that were defined by the L1 and L2 alleles (Table 1).

Kinetic analysis of the novel L1 β -lactamases. The L1a gene and the three novel L1 genes were separately cloned into an *E. coli* expression vector (see Materials and Methods), and the level of imipenem hydrolysis in extracts of *E. coli* transformed with the recombinant plasmids was analyzed (i.e., clones of either L1a, L1c, L1d, or L1e). The specific activity of imipenem hydrolysis in extracts of the L1c and L1d clones was approxi-

mately equal to that in extracts of the L1a clone, but extracts of the L1e clone displayed markedly lower rates of imipenem hydrolysis. Because of these differences in hydrolytic activity and the degree of sequence divergence between L1a and L1e, some of the kinetic parameters of L1e were studied in more detail. The cloned L1a and L1e were overexpressed, purified, and analyzed separately but in parallel for comparison. The purity (>99%) and monomeric molecular size (29 kDa) of each enzyme was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition, electrospray mass spectrometry was performed, and this revealed a monomeric molecular mass of 28,726 Da for L1a (predicted mass, 29,122 Da) and 29,613 Da for L1e (predicted mass, 29,974). In both cases, the L1 peak was sharp and represented more than 95% of the total protein. The K_m and k_{cat} values for hydrolysis of nitrocefin and imipenem determined for the two enzymes, which were markedly different, are shown in Table 2.

Locations of the L1 and L2 β -lactamase genes in *S. maltophilia*. Each of the 10 *S. maltophilia* isolates was found to

TABLE 2. Kinetic properties of L1 β -lactamases

Enzyme	Nitrocefin kinetic parameters			Imipenem kinetic parameters		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \mu\text{M}^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{s}^{-1} \cdot \mu\text{M}^{-1}$)
L1e	15 ± 2.5^a	0.5 ± 0.2	0.03 ± 0.01	76 ± 19	6.1 ± 2.2	0.08 ± 0.01
L1a	4.3 ± 0.7	35 ± 3	8.1 ± 0.5	47 ± 8	384 ± 5	8.2 ± 1.1

^a Values are means $\pm$ standard errors of the means ($n = 3$).

possess a large plasmid, although the yield of plasmid DNA obtained varied considerably from one isolate to another (Fig. 3A). The plasmids were all estimated to be approximately 200 kb. When 5 μl of these plasmid preparations was used as the template DNA in PCR with a primer set designed to amplify the L1 gene, a product of a size consistent with that of the target sequence was obtained in each case (Fig. 3B) and the intensity of the product band was proportional to the amount of plasmid DNA in each preparation (cf. Fig. 3A and B). Similarly, in plasmid-primed reactions with a primer set designed to amplify the L2 gene, products of a size consistent with that targeted were also obtained (Fig. 3C). To eliminate the possibility that the results simply reflected chromosomal

contamination in the plasmid preparations, multiplex PCR analysis with two sets of primers in the same reaction was performed. In the first multiplex system, one primer pair was that used to amplify the L1 gene; the second targeted a sequence encoding part of the 16S rRNA molecule (Fig. 4A). When the template provided was total genomic DNA from one of the isolates, both the expected PCR products were obtained (Fig. 4A, lane G). In contrast, when the genomic DNA was replaced by plasmid DNA from the same strain, only the L1 gene PCR product was obtained (Fig. 4A, lane P). The same pattern of results was found with DNA from all 10 isolates (data not shown). In the second multiplex system, one primer pair amplified part of the L2 gene, while the second primer pair was directed to the *S. maltophilia* dipeptidyl peptidase IV gene (15). When the template provided for the PCR was genomic DNA from one of the isolates, two products with sizes consistent with those of the expected amplicons were clearly visible in the agarose gel (Fig. 4B, lane G). Again, when genomic DNA was replaced by plasmid DNA in the reaction mix, only one product, that expected as the result of L2 gene amplification, was obtained (Fig. 4B, lane P). The results indicate that both the L1 and L2 β -lactamases are encoded on

FIG. 3. PCR to show that the L1 and L2 genes of *S. maltophilia* are plasmid encoded. Plasmids were isolated from 1.5 ml of an overnight culture of *S. maltophilia* strains 1 to 10 (lanes 1 to 10, respectively, in each panel). (Table 1), (A) A total of 5 μl of the plasmid preparation was resolved by using a 0.8% agarose gel. (B and C) A total of 5 μl of the plasmid preparation was used as a template for PCR with the L1-FULL (B) or L2-FULL (C) primer sets. Primer sequences and PCR conditions are described in Materials and Methods. (B and C) A total of 10 μl of the PCR product was resolved by using a 1.2% agarose gel. DNA molecular size markers (1 kb plus ladder; Life Technologies Ltd., Paisley, United Kingdom) were run in parallel (lanes M) to check the sizes of the PCR products. The figures are photographs of the resultant ethidium bromide-stained gels under UV irradiation.

FIG. 4. Assessment of chromosomal DNA contamination of plasmid preparations by multiplex PCR. (A) Multiplex PCR was performed with 5 μl of plasmid preparation (lane P) or 20 μl of genomic DNA (lane G) (see Materials and Methods) with the L1-FULL and rDNA primer sets (Materials and Methods). (B) Multiplex PCR was performed with the L2-MID and D-PEP primer sets. The L2-MID primers were used instead of the L2-FULL primers because of the similarity in size between the L2-FULL and D-PEP amplicons. The PCR was performed as described in Materials and Methods, and PCR products were separated and visualized as described in the legend to Fig. 3. Lanes M, DNA molecular size markers.

the DNA in each of the plasmid preparations, while the 16S rRNA and dipeptidyl peptidase IV genes are not.

DISCUSSION

The L1 and L2 β -lactamase genes of *S. maltophilia* are encoded on a plasmid-like element. Acquired β -lactamase production is a common feature of clinical isolates of gram-negative bacteria, a reflection of the high levels of consumption of β -lactam antibiotics both in hospitals and in the community. Many of the genes for these enzymes are not intrinsic to the species in which they have been found; rather, they have been acquired by horizontal transfer on plasmids, transposons, and integrons (3, 4). In general, production of such enzymes is not regulated (5, 8). In contrast, several gram-negative bacteria of clinical importance produce at least one β -lactamase intrinsic to the species (5, 8). Expression of these enzymes is often regulated, and inducible β -lactamase expression by a gram-negative species has been considered indicative of chromosomally encoded systems (5). In the present study we have shown that the L1 and L2 β -lactamases of *S. maltophilia* are encoded on plasmid-like elements in all isolates examined. It remains to be seen if the control system(s) is also encoded on the same elements. We refer to the DNA molecules carrying the L1 and L2 genes as plasmids simply because they were isolated by a standard plasmid isolation technique. However, since all the isolates examined in this study carry the plasmid-like molecule and all these molecules encode β -lactamase genes that are considered to be intrinsic to *S. maltophilia* (8, 10, 18, 24, 30, 31), it may be more accurate to consider these elements as part of a fragmented chromosome. This interpretation is consistent with another report that *S. maltophilia* possesses a fragmented chromosome (16). We have not determined if the plasmid-like elements in the isolates examined are related, although this seems probable.

The L1 and L2 β -lactamase genes of *S. maltophilia* show considerable allelic variations. The existence of different active isoforms of an enzyme arising from allelic variation is a well-established phenomenon and provides the basis of multilocus enzyme electrophoresis bacterial typing systems (19). Isoelectric focusing analysis of both the L1 and the L2 β -lactamases of different isolates of *S. maltophilia* has indicated allelic variation of both the L1 and the L2 β -lactamase genes of *S. maltophilia* (10, 20), and two published sequences for the L1 gene show more than 10% divergence (24, 30). While DNA hybridization studies and 16S rRNA gene analysis of *S. maltophilia* isolates indicate that the species, as presently constituted, contains strains that show up to 30% divergence (7, 13), there was no information as to whether the observed and implied sequence variation seen in previous studies of the L1 and the L2 genes and their products reflects the genetic drift within the species as a whole.

In the study described here we have shown that in some strains of *S. maltophilia*, the L1 gene has undergone a considerable degree of sequence alteration (between 8 and 20% from L1a). A similar degree of change is also seen in the L2 genes of the same strains (between 4 and 25% from L2a), indicative of significant strain divergence. Surprisingly, these high levels of sequence variation were not reflected as differences between the 16S rRNA genes from the same isolates for which the

extent of genetic drift was less than 1% (Table 1). Importantly, these rRNA gene sequencing data have confirmed the species identification of each isolate used (Table 1).

The discrepancy between the degrees of divergence among the L1 and L2 genes compared with that seen for their associated 16S rRNA genes was unexpected. If the sequence changes recorded in this study have occurred by a step-by-step process, then the extremes for the L1 and L2 genes recorded here suggest that the allelic variants have been diverging for some considerable time. In contrast, the 16S rRNA sequence data mean that there has been little genetic drift among the isolates examined. Accordingly, the variation seen among the β -lactamase genes may reflect some form of accelerated evolution, and this could argue for an involvement of horizontal gene transfer in the process. To this end, it may be significant that the β -lactamase genes are located on a DNA molecule whose location is different from the location of the 16S rRNA gene.

Effect of allelic drift on activities of the L1 and L2 β -lactamases. Comparison of kinetic data for L1a and L1e shows that L1e is a less efficient enzyme with a significantly lower k_{cat}/K_m for both nitrocefin and imipenem (Table 2). It is important that the L1a kinetics of nitrocefin hydrolysis have been reported previously (using a different expression-purification procedure) (9), and within error, they are equivalent to the data reported in Table 2.

Structural determination of L1a (29) has led to the putative identification of key residues involved in binding of substrate and enzyme catalysis, including an extended loop between residues 116 and 137 (in particular, F124 and I128) (29, 30). Changes in residues adjacent to these amino acids are likely to distort their position and/or orientation, affecting the kinetic parameters of the enzyme. Interestingly, the amino acid sequence of L1e highlights such changes, namely, G127E and Y130F (Fig. 1), which are predicted to affect substrate docking and therefore lead to weaker binding of substrates. In addition to these changes, the L1e sequence reveals a change (noted in boldface) in one of the zinc binding motifs, from HAHADH (residues 84 to 89 [29, 30]) to HAHTDH (Fig. 1). This A87T substitution may distort the geometry of the histidines, affecting the coordination to one or both of the zinc ions. This in turn is likely to affect the coordination of water molecules, particularly WAT1, responsible for the nucleophilic attack on the β -lactam ring (29) and will inevitably have some bearing on the k_{cat} of the enzyme. In contrast to the kinetic differences seen among the L1 enzymes, preliminary analysis suggests that the four L2 enzymes have similar K_m and k_{cat} values with respect to nitrocefin hydrolysis (data not shown). Even though the sequences of the four enzymes are divergent (Fig. 2), the key residues (STFK and SDN) are conserved and changes in the residues surrounding these motifs are conservative (Fig. 2). Thus, it is probable that several complementary changes have taken place to allow two similar structures to evolve, but without a published L2 crystal structure, this hypothesis cannot be tested at present.

Conclusions. The driving force behind the accelerated evolution of β -lactamase genes in *S. maltophilia* is unknown, but it is possible that it represents, in part, the use of β -lactam antibiotics. The result might be the selection of enzyme variants

better able to provide protection for the cell, and this process may continue in the future.

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