Characterization of the charge variants of L2 \(\beta\)-lactamase in *Stenotrophomonas maltophilia*

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*Stenotrophomonas maltophilia* KH has two acid \(\beta\)-lactamas with isoelectric points (pl) of 4.6 and 5.4, and several basic \(\beta\)-lactamas (pl > 7.0) that produce a ladder-shaped pattern by IEF. An isogenic L2 mutant, KHL2\(_{xyE}\), was constructed by gene replacement. From IEF and native PAGE zymograms of strains KH and KHL2\(_{xyE}\), it was demonstrated that the basic \(\beta\)-lactamas and the acid \(\beta\)-lactamase with pl 5.4 are encoded by the same L2 gene and that the active types of these L2 charge variants were dependent on the buffer pH. The \(\beta\)-lactamase activities of these L2 charge variants in phosphate buffer at pH 7.0 and 8.0 were 1075 ± 29 and 1114 ± 81 U mg\(^{-1}\), respectively. These results indicate that L2 charge variants give *S. maltophilia* a better chance of adapting and surviving in response to changes in the environment.

INTRODUCTION

*Stenotrophomonas maltophilia*, an aerobic, non-fermentative, Gram-negative bacillus, is ubiquitous in environments such as water, soil and sediments (Bollet et al., 1995; Papapetroupolou et al., 1994). Recently, it has emerged as a significant nosocomial pathogen capable of causing respiratory, urinary and bloodstream infections in hospitalized patients (Denton & Kerr, 1998).

*S. maltophilia* intrinsically produces at least two types of \(\beta\)-lactamase, known as L1 and L2 (Saino et al., 1982, 1984), giving rise to resistance against virtually the entire spectrum of \(\beta\)-lactams (Alonso & Martinez, 1997). The isoelectric point (pl) of a \(\beta\)-lactamase is an important property used for characterization and classification (Huovinen, 1988). In general, L1 and L2 can readily be discriminated by their pl values, as determined by IEF, where L1 has a pl < 7.0 and L2 has a pl > 7.0 (Denton et al., 1999). However, minor \(\beta\)-lactamase-active bands have been reported in IEF gels that have not yet been classified precisely (Bicknell et al., 1985; Paton et al., 1994). In this study, a clinical *S. maltophilia* isolate, KH, was found to display a peculiar IEF zymogram pattern, with two acid \(\beta\)-lactamas and several basic \(\beta\)-lactamas that formed a ladder-shaped pattern on the gel. Genetic and phenotypic characterization of the latter was also undertaken.

**Abbreviation:** pl, isoelectric point.

The GenBank/EMBL/DDBJ accession number for the sequence of the *ampR* gene, intergenic region and L2 \(\beta\)-lactamase gene is EU032534.

METHODS

**Bacterial strains and media.** Twenty isolates of *S. maltophilia* were investigated in this study. They were identified using an ID32 GN system (bioMérieux) and confirmed by PCR amplification of a 531 bp fragment in the 16S rRNA gene region (Whitby et al., 2000). Unless otherwise specified, *Escherichia coli* and *S. maltophilia* were routinely cultured in Luria–Bertani (LB) medium and on L agar at 37 °C. The growth of *S. maltophilia* was monitored by measuring the OD\(_{450}\) of the cultures.

**Preparation of \(\beta\)-lactamase extracts.** Twenty millilitres of fresh LB broth was inoculated with bacteria at a turbidity of 0.15 OD\(_{450}\) after overnight growth at 37 °C. The culture was further incubated for 0.5 h. Cefoxitin (50 \(\mu\)g ml\(^{-1}\)) was then added and the incubation process was continued for 2.5 h. Bacterial cells from 20 ml of the culture were harvested by centrifugation (5 min, 4 °C, 12 000 g) and washed with 50 mM sodium phosphate buffer at pH 7.0 or 8.0. Following resuspension of the pellet in 2 ml of the same buffer, the cells were disrupted by sonication. Cell debris was pelleted by centrifugation (30 min, 4 °C, 12 000 g) and the supernatant was transferred to a clean tube, ready for IEF, native PAGE and \(\beta\)-lactamase activity assays.

**IEF.** IEF analysis was performed using an ampholine polyacrylamide gel with a pH range of 3.5–9.5 (Ampholine PAGplate; Amersham Pharmacia Biotech) for 90 min at 1500 V, 50 mA and 25 W in a Multiphore II Electrophoresis System (Amersham Biosciences; Matthew & Harris, 1976; Mathew et al., 1975). The separated \(\beta\)-lactamas were detected by overlaying the gel with 100 \(\mu\)M nitrocefin solution (Oxoid). The pl of each \(\beta\)-lactamase was determined using a pH 3–10 broad-range pl calibration kit (Amersham Biosciences).

**Construction of the gene replacement plasmid pKHL2\(_{xyE}\).** According to *S. maltophilia* K279a genome sequences (www.sanger.ac.uk/Projects/S_maltophilia; Crossman et al., 2008), an *ampR* homologue gene, denoted here as *ampR*, was located separately
upstream of the L2 gene. We designed the primers AmpRL2-F (5'-CGCACAACACCTGGACC-3') and AmpRL2-R (5'-ATGCCGAT-GATGCCGAAC-3') to amplify these two genes together (ampR-L2) from S. maltophilia KH. PCRs were conducted in a reaction volume of 50 µl containing PCR buffer [500 mM KCl, 1% Triton X-100, 100 mM Tris/HCl (pH 9.0), 2.5 mM MgCl2], 200 µM each dNTPs, 10% DMSO, 2 U SuperTaq XL (ProTech), 1.5 µM primer for each reaction and 2 µl DNA template. The PCR conditions were 94 °C for 5 min, followed by 30 cycles of 60 s at 94 °C, 60 s at 58 °C and 90 s at 72 °C, with a final 10 min extension step at 72 °C. A 2.8 kb PCR product was ligated into T-vector (Yeastern Biotech), giving rise to pTKHR2. A catechol 2,3-dioxygenase gene cassette, xylE, was retrieved from pX1918GT (Schweizer & Hoang, 1995) by PCR with the primer pair XylE-F (5'-GAATTCCGGCCGCGATCAGACCATGACC-3') and XylE-R (5'-GCGGCCGCAAGTCGTACCGGAC-CATCAG-3') and was inserted into the Stul site of the L2 gene. This disrupted the reading frame of the L2 gene in pTKHR2L2. The orientation of disrupted the reading frame of the L2 gene in pTKHRL2. The xylE gene encoded a 303 aa polypeptide whose N-terminal sequence exhibited features typical of prokaryotic signal peptides (http://www.cbs.dtu.dk/services/SignalP/).

**RESULTS AND DISCUSSION**

**IEF assay of S. maltophilia isolates**

Two major discrete IEF patterns were observed among the 20 isolates: pattern I with two major bands and pattern II composed of multiple bands forming a ladder-shaped pattern (see Fig. 1, lane 1, as a representative example of pattern II). Among the 20 isolates tested, 17 isolates exhibited pattern I and three had pattern II.

Pattern I revealed for these 17 isolates was typical of S. maltophilia β-lactamases, with one band of pI <7.0 (referred as L1) and the other band of pI >7.0 (assumed to be L2). In contrast, pattern II was rather unexpected and consisted of two acidic β-lactamases (pI <7.0) and a series of β-lactamases with pIs >7.0. We suggested two hypotheses for the formation of this ladder-like band pattern: (i) the different proteins were encoded by the same gene (presumed to be L2) and had undergone amino acid modifications, which, in turn, produced a series of charge variants; or (ii) this pattern of β-lactamases resulted from separate β-lactamase genes, implying that, in addition to the L1 and L2 genes, S. maltophilia contains other β-lactamase genes.

To test these hypotheses, an isolate with IEF pattern II, denoted S. maltophilia KH, was chosen for further study.

**Sequence analysis of the L2 gene and ampR of S. maltophilia KH**

Two divergently transcribed genes, L2 and ampR, were determined in the 2.8 kb PCR amplicons and were found to be separated by a 174 bp intergenic region. The G+C content of ampR, the intergenic region and the L2 gene was 70, 52 and 70 mol%, respectively. The G+C content of the intergenic region was notably low compared with the overall G+C content of S. maltophilia (66 mol%). The L2 gene encoded a 303 aa polypeptide whose N-terminal sequence exhibited features typical of prokaryotic signal peptides (http://www.cbs.dtu.dk/services/SignalP/).

**Conjugation.** An overnight culture of each bacterium to be mated was prepared using LB broth. The cultures of donor strain [E. coli S17-1(ampRL2)] and recipient strain (S. maltophilia KH) were mixed at a ratio of 10:1. These mixed cultures were spread onto LB agar containing tetracycline (40 µg ml⁻¹) and incubated at 37 °C overnight. The merodiploids from homologous recombination were selected on LB agar antibiotics added and incubated at 37 °C for 5 min, followed by 30 cycles of 60 s at 94 °C, 60 s at 58 °C and 90 s at 72 °C, with a final 10 min extension step at 72 °C. A 2.8 kb PCR product was ligated into T-vector (Yeastern Biotech), giving rise to pTKHR2. A catechol 2,3-dioxygenase gene cassette, xylE, was retrieved from pX1918GT (Schweizer & Hoang, 1995) by PCR with the primer pair XylE-F (5'-GAATTCCGGCCGCGATCAGACCATGACC-3') and XylE-R (5'-GCGGCCGCAAGTCGTACCGGAC-CATCAG-3') and was inserted into the Stul site of the L2 gene. This disrupted the reading frame of the L2 gene in pTKHR2L2. The orientation of disrupted the reading frame of the L2 gene in pTKHRL2. The xylE gene encoded a 303 aa polypeptide whose N-terminal sequence exhibited features typical of prokaryotic signal peptides (http://www.cbs.dtu.dk/services/SignalP/).

**Charge variants of L2 β-lactamase**

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Characteristic elements of Amber class A β-lactamases were identified in the L2 protein, including the consensus residues STXK (aa 83–86), SDN (aa 143–145) and KTG (aa 247–249), and a glutamic acid residue (aa 179) located 34 residues downstream from the SDN loop (Walsh et al., 1997). A sequence homology search using BLAST (http://www.ncbi.nlm.nih.gov/) revealed that L2 exhibited a sequence identity of 69–95% with other L2 proteins from different S. maltophilia strains, and 55–58 and 52% identity with β-lactamases of the genera Xanthomonas and Bordetella, respectively. The 288 aa AmpR protein shared 98% identity with AmpR of S. maltophilia R551-3, 68–72% identity with the known AmpR proteins from the genus Xanthomonas and 32–72% identity with those of the genus Pseudomonas. AmpR had the characteristics of a typical LysR transcriptional regulator protein (Schell, 1993), with a helix–turn–helix DNA-binding motif in the N-terminal region (aa 23–43).

**Genotypic and phenotypic confirmation of the isogenic L2 mutant KHL2xyIE**

The isogenic L2 mutant of isolate KH, KHL2xyIE, was obtained by conjugation between E. coli S17-1(pKHL2xyIE) and S. maltophilia KH by two-step antibiotic/10% sucrose selection. The mutant KHL2xyIE was genetically verified by PCR/sequencing analysis and phenotypically confirmed by a susceptibility test against cefepime, which is known to be a susceptible substrate for the L2 enzyme. The pI values of the active L2 charge variants also varied from 7.0 to 8.0. At pH 7.0, the pl values of the active L2 charge variants ranged from 5.4 to 7.9. More basic active L2 charge variants (pl 5.4–8.5) were revealed when the buffer pH was shifted to basic conditions. This trend was further confirmed by 10% native PAGE followed by in-gel activity staining with nitrocefin (Fig. 2). Because the resolution of native PAGE for pl discrimination is inferior to that of IEF, all L2 charge variants in the native polyacrylamide gel were focused in a single band.

Nevertheless, it was still obvious that the active L2 charge variants in the pH 8.0 buffer (Fig. 2, lane 2) exhibited lower mobility than those in the pH 7.0 buffer (Fig. 2, lane 1). The pl values of the active L2 charge variants also varied with the pH of the buffer system. Higher pl L2 charge variants appeared in more basic buffers. This phenomenon implies that some differential modification, folding and/or multimerization of the L2 enzyme occurred, either during gene expression or in response to the pH of the buffer.

**The β-lactamases forming the ladder-shaped pattern are encoded by the same L2 gene**

A pH 7.0 β-lactamase extract of mutant KHL2xyIE was prepared as described above. IEF was carried out using the pH 7.0 extracts of KH and KHL2xyIE (Fig. 1, lanes 1 and 3). The basic ladder-like pattern of β-lactamase bands and the band of pl 5.4 were absent in the extracts from mutant KHL2xyIE, indicating that mutation of the L2 gene resulted in the loss of these additional β-lactamases. Therefore, hypothesis (i) proposed above appeared to be plausible in S. maltophilia.

Multiple β-lactamases on IEF gels have been described in the literature. When purifying the L1 protein by chromatofocusing, Bicknell et al. (1985) found three charge variants of L1 β-lactamases of S. maltophilia IID 1275. However, a genetic approach has not been attempted to elucidate the relationship of the charge variants. Four different β-lactamases were also reported from seven S. maltophilia isolates: an L1 of pl 6.8 and three β-lactamases with pl values of 6.2, 5.55 and 5.3 (Paton et al., 1994). Other than their acidic pl values, these three β-lactamases share several similar features with the L2 enzyme. Nevertheless, in-depth exploration using a molecular approach is still lacking in the literature. This study shows for the first time, to our knowledge, that the different β-lactamase charge variants are products of the same L2 gene.

**The active types of the L2 charge variants are pH-dependent**

In general, pH value is crucial for the occurrence of charge variants of a protein. To evaluate the effects of pH shift on the active types of the L2 charge variants, the β-lactamase extracts of isolate KH and mutant KHL2xyIE were prepared in 50 mM sodium phosphate buffer at pH 7.0 or 8.0. As can be seen in Fig. 1, the active charge variants of L2 β-lactamase differed markedly when the buffer pH was shifted from 7.0 to 8.0. At pH 7.0, the pl values of the active L2 charge variants ranged from 5.4 to 7.9. More basic active L2 charge variants (pl 5.4–8.5) were revealed when the buffer pH was shifted to basic conditions. This trend was further confirmed by 10% native PAGE followed by in-gel activity staining with nitrocefin (Fig. 2). Because the resolution of native PAGE for pl discrimination is inferior to that of IEF, all L2 charge variants in the native polyacrylamide gel were focused in a single band. Nevertheless, it was still obvious that the active L2 charge variants in the pH 8.0 buffer (Fig. 2, lane 2) exhibited lower mobility than those in the pH 7.0 buffer (Fig. 2, lane 1). The pl values of the active L2 charge variants also varied with the pH of the buffer system. Higher pl L2 charge variants appeared in more basic buffers. This phenomenon implies that some differential modification, folding and/or multimerization of the L2 enzyme occurred, either during gene expression or in response to the pH of the buffer.
/β-Lactamase activity assay of the different L2 charge variants

To elucidate whether the different L2 charge variants in 50 mM phosphate buffer at different pH values displayed different /β-lactamase activity, the induced /β-lactamase activities of strains KH and KHL2-xylE were determined. The assumed L2 /β-lactamase activity was obtained by subtracting the activity of KHL2-xylE from that of KH. The /β-lactamase activities of L2 in pH 7.0 and 8.0 buffers were approximately equal (1075 ± 29 vs 1114 ± 81). Thus the L2 charge variants exhibited a broad optimal pH range, i.e. 7.0–8.0. In previous reports, the purified L2 enzymes from S. maltophilia IID1275 and GN12873 displayed a sharp pH optimum at pH 7 and pH 7.5, respectively (Cartwright & Waley, 1984; Saino et al., 1984). The pI values of the L2 enzymes from strains IID1275 and GN12873 are 9.3 and 8.4 (Cartwright & Waley, 1984; Saino et al., 1984), suggesting that the L2 forms of strains IID1275 and GN12873 are likely to belong to pattern I of the IEF assay. In addition to pattern I, this study identified an additional type of L2 /β-lactamase, denoted pattern II (Fig. 1, lanes 1 and 2), which exhibited different active types of charge variants under different pH conditions. These L2 charge variants of pattern II displayed a wider optimal pH range than that of pattern I.

S. maltophilia is widely distributed in different environments and geographical regions. The L2 enzyme shows a wide substrate profile for most /β-lactam antibiotics, effectively protecting S. maltophilia from the effect of /β-lactams. The existence of multiple L2 charge variants under different pH conditions gives the L2 enzyme high activity under different pH conditions, thus allowing S. maltophilia to adapt to fluctuating environments.

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REFERENCES


