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BEL-2, an Extended-Spectrum β-Lactamase with Increased Activity toward Expanded-Spectrum Cephalosporins in *Pseudomonas aeruginosa*▽

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A *Pseudomonas aeruginosa* isolate recovered in Belgium produced a novel extended-spectrum ß-lactamase, BEL-2, differing from BEL-1 by a single Leu162Phe substitution. That modification significantly altered the kinetic properties of the enzyme, increasing its affinity for expanded-spectrum cephalosporins. The *bla*$_{BEL-2}$ gene was identified from a *P. aeruginosa* isolate clonally related to another *bla*$_{BEL-1}$-positive isolate.

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Extended-spectrum ß-lactamases (ESBLs), such as TEM, SHV, PER, VEB, GES, and more recently, CTX-M variants, are reported increasingly to be found in *Pseudomonas aeruginosa* in various areas (1, 2, 7, 8, 10–12, 15, 17, 21, 23, 27, 28, 30). The BEL-1 ß-lactamase, distantly related to other ESBLs, was identified from a *P. aeruginosa* isolate from Roeselare, Belgium, which interestingly shows resistance to ticarcillin and ceftazidime but only reduced susceptibility to piperacillin, cefepime, cefpirome, and aztreonam (24). The *bla*$_{BEL-1}$ determinant was found as a gene cassette in the chromosome-borne class 1 integron, In120, that includes other resistance genes (*aacA4*, *aadA5*, and *smr2*) and that was part of a Tn*1404*-type transposon structure (24). Very recently, Bogaerts et al. (5) reported on the diffusion of BEL-1-producing isolates in various hospital centers of Belgium and also found that BEL-1 could be associated with other relevant β-lactamases, such as the VIM-1 metallo-β-lactamase (5).

P. aeruginosa isolate 531 (this study) was recovered from a urine sample of a patient hospitalized in Roeselare, Belgium, in February 2007 for pneumonia and was resistant to all β-lactams but imipenem (Table 1). A synergy between aztreonam or ceftazidime and clavulanic acid-containing disks suggested the synthesis of an ESBL (19). PCR followed by sequencing using ESBL gene-specific primers (24) identified a novel gene encoding BEL-2, which differs from BEL-1 by a single amino acid substitution (Leu to Phe at Ambler position 162) (3). Transfer of a ß-lactam resistance marker from *P. aeruginosa* 531 to *Escherichia coli* or to *P. aeruginosa* reference strains was unsuccessful by either conjugation or transformation (25). Plasmid extraction performed as described previously (14) did not identify any plasmid, suggesting a chromosomal location of the *bla*$_{BEL-2}$-like gene in *P. aeruginosa* 531. A pulsed-field gel electrophoresis (PFGE) analysis (4) showed that isolates 531 (BEL-2 positive) and 51170 (BEL-1 positive), recovered from the same geographical area, were clonally related. A PCR mapping approach confirmed the presence of a class 1 integron whose structure was identical to that of In120 of *P. aeruginosa* 51170 (24) and identified an identical structure in *P. aeruginosa* 531 (data not shown). Overall, these data suggest that the *bla*$_{BEL-2}$ sequence likely resulted from a mutational event that had occurred in In120-carrying *P. aeruginosa* strains.

In order to compare the contributions of BEL-1 and BEL-2 to ß-lactam resistance, the corresponding genes (amplified using primers PreBEL-A [5′-AGACGTAAGCCTATAATCTC] and PreBEL-B [5′-GCGAATTGTTAGACGTATG]) were cloned in the pCR-BluntII-TOPO vector (Invitrogen, Cergy-Pontoise, France) and subsequently introduced into *E. coli* TOP10, giving rise to recombinant strains *E. coli* TOP10(pSB-1) and *E. coli* TOP10(pSB-2), producing BEL-1 and BEL-2, respectively. MICs of ß-lactams were determined by solid agar dilutions following the guidelines of the CLSI (9). *E. coli* TOP10(pSB-2) had MICs of piperacillin, cephalothin, and cefuroxime that were lower than those of *E. coli* TOP10(pSB-1), but its cefotaxime, ceftazidime, ceftriaxone, and cefepime MICs were higher than those of TOP10(pSB-1), while MICs of carbapenems were the same (Table 1).

E. coli TOP10(pSB-2) produced a ß-lactamase with a pI value of 7.1 (identical to that of BEL-1) (18). Approximately 1.5 mg of BEL-2 was purified (>95% as estimated by SDS-PAGE analysis; data not shown) from an *E. coli* MCT236(pET-BEL-2) crude extract by using a two-step chromatography process (an anion exchange at pH 7.5 using a Q Sepharose Fast Flow column followed by a cation exchange at pH 6.2 using a 1-ml Resource S column). (The specific activity was 8,800 nmol/min · mg of protein with 100 μM of cephalothin as the substrate, purified 95-fold.) BEL-2 had a broad-

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TABLE 1. MICs of β-lactams[a]

β-Lactam(s)[b]	MIC (μg/ml)				
	P. aeruginosa 531	*P. aeruginosa* 51170	*E. coli* TOP10 (pSB-2) (BEL-2)	*E. coli* TOP10 (pSB-1) (BEL-1)	*E. coli* TOP10
Amoxicillin	>512	>512	>512	>512	4
Amoxicillin and CLA	>512	>512	64	64	4
Ticarcillin	>512	>512	>512	>512	4
Ticarcillin and CLA	>512	128	64	64	4
Piperacillin	16	16	32	128	1
Piperacillin and TZB	8	8	8	32	1
Cephalothin	>512	>512	256	256	2
Cefuroxime	>512	>512	32	128	2
Cefoxitin	512	512	2	2	2
Ceftazidime	256	32	128	16	0.06
Cefotaxime	256	32	32	4	0.12
Cefepime	16	4	1	0.25	0.06
Cefpirome	64	16	0.5	0.25	0.06
Aztreonam	64	32	16	16	0.12
Imipenem	1	1	0.12	0.12	0.12

[a] MICs of β-lactams for *P. aeruginosa* 531 and 51170 clinical isolates, producing ESBLs BEL-2 and BEL-1, respectively, *E. coli* TOP10 harboring recombinant plasmid pSB-2 expressing BEL-2, *E. coli* TOP10 harboring recombinant plasmid pSB-1 expressing BEL-1, and the *E. coli* TOP10 reference strain.

[b] CLA, clavulanic acid at a fixed concentration of 4 μg/ml; TZB, tazobactam at a fixed concentration of 4 μg/ml.

TABLE 2. Kinetic parameters of purified ß-lactamase BEL-2, in comparison with previously reported values of BEL-1 (22)[a]

ß-Lactam	BEL-2			BEL-1		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Benzylpenicillin	1.2	4	300	3	20	150
Piperacillin	1.1	0.3	3,700	2	15	130
Cephalothin	4.3	2	2,200	150	280	540
Cephaloridine	1.2	8	150	30	130	230
Cefoxitin	<0.01	ND	ND	<0.01	ND	ND
Cefuroxime	0.88	12	73	10	40	250
Ceftriaxone	0.2	0.1	2,000	25	30	830
Cefotaxime	0.13	0.45	290	30	250	120
Ceftazidime	0.03	0.64	47	>1.5	>700	ND
Cefepime	0.003	0.3	10	1	150	7
Aztreonam	0.1	0.36	280	10	100	100
Imipenem	<0.01	ND	ND	<0.01	ND	ND

[a] Standard deviations were below 15%. ND, not determinable, due to the initial rate of hydrolysis being too low.

spectrum hydrolysis profile, including penicillins and expanded-spectrum cephalosporins but not cephamycins and carbapenems (Table 2). BEL-2 overall showed higher catalytic efficiencies (k_{cat}/K_m) than BEL-1 for aztreonam and most oxyiminocephalosporins (cefotaxime, ceftazidime, ceftriaxone, and cefepime but not cefuroxime). This was due to a significant alteration of the K_m values for these substrates with BEL-2, which were decreased relative to those of BEL-1 by 300-fold (ceftriaxone) to up to three orders of magnitude (ceftazidime) (Table 2). Interestingly, a decrease of the K_m value was also observed with all the other substrates (though the variation was less important), likely reflecting a modification of the active site structure and thus substrate recognition. Overall, BEL-2 k_{cat} values were also lower but to a lesser extent (Table 2). The values of catalytic efficiency toward expanded-spectrum cephalosporins for BEL-2 may explain the higher MICs observed for the BEL-2-producing recombinant *E. coli* strains and *P. aeruginosa* clinical isolate. Position 162 is located at the beginning of the Ω loop, which bears the functionally important Glu166 residue, which is conserved in class A enzymes, and where mutations conferring extended-spectrum properties have been extensively reported in natural TEM and SHV variants (13). The presence of a bulky Phe residue in BEL-2 might modify the orientation of the Ω loop and the overall geometry of the active site. The further extension of the substrate profile as a consequence of a single substitution in the Ω loop observed with the BEL-2 variant may parallel that of other enzymes, e.g., CTX-M-19 (CTX-M-14 Pro167Ser variant) (26) or GES-2 (GES-1 Gly170Asn variant) (28). Inhibition studies showed that BEL-2 and BEL-1 are similarly inhibited by clavulanic acid, tazobactam, and sulbactam (50% inhibitory concentrations of 0.1, 2, and 3 μM, respectively).

Conclusion. This study emphasizes the spread and evolution of BEL-type ESBLs in *P. aeruginosa* in Belgium. A novel integron-encoded BEL-type ß-lactamase with enhanced hydrolytic properties was identified. This evolution of an ESBL determinant expressed in a *P. aeruginosa* strain is similar to that reported for GES-1 in South Africa, where *P. aeruginosa* isolates expressing ESBL GES-2 or GES-5 with expanded-spectrum activities toward carbapenems have been reported (16, 29), and also in Brazil, where clonally related *P. aeruginosa* isolates expressing either GES-1 or GES-5 coexist (22).

It is tempting to hypothesize that a BEL-2 producer has been selected under antibiotic selective pressure and especially by expanded-spectrum cephalosporins. This work shows that ESBL BEL-1 has the potential to evolve with significantly altered biochemical properties. It remains to evaluate whether BEL-type ß-lactamases could further evolve to gain activity on cephamycins and/or carbapenems (as observed for several GES-type ESBLs [20]) or resistance to ß-lactamase inhibitors (as observed for several TEM and SHV variants [6]).

Nucleotide sequence accession number. The nucleotide and protein sequences corresponding to the BEL-2 enzyme have been registered in GenBank under accession number FJ666063.

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