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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.**

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.

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).

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.

**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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## REFERENCES

1. **Aktas, Z., L. Poirel, M. Salcioğlu, P. E. Ozcan, K. Midilli, C. Bal, O. Anğ, and P. Nordmann.** 2005. PER-1- and OXA-10-like ß-lactamases in ceftazidime-resistant *Pseudomonas aeruginosa* isolates from intensive care unit patients in Istanbul, Turkey. Clin. Microbiol. Infect. **11:**193–198.
2. **al Naiemi, N., B. Duim, and A. Bart.** 2006. A CTX-M extended-spectrum ß-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. J. Med. Microbiol. **55:**1607–1608.
3. **Ambler, R. P., A. F. W. Coulson, J.-M. Frère, J. M. Ghuysen, B. Joris, M. Forsman, R. C. Levesque, G. Tiraby, and S. G. Waley.** 1991. A standard numbering scheme for the class A ß-lactamases. Biochem. J. **276:**269–270.
4. **Aubron, C., L. Poirel, N. Fortineau, P. Nicolas, L. Collet, and P. Nordmann.** 2005. Nosocomial spread of *Pseudomonas aeruginosa* isolates expressing the metallo-ß-lactamase VIM-2 in a hematology unit of a French hospital. Microb. Drug Resist. **11:**254–259.

5. **Bogaerts, P., C. Bauraing, A. Deplano, and Y. Glupczynski.** 2007. Emergence and dissemination of BEL-1-producing *Pseudomonas aeruginosa* isolates in Belgium. Antimicrob. Agents Chemother. **51:**1584–1585.
6. **Cantón, R., M. I. Morosini, O. M. de la Maza, and E. G. de la Pedrosa.** 2008. IRT and CMT ß-lactamases and inhibitor resistance. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**53–62.
7. **Castanheira, M., R. E. Mendes, T. R. Walsh, A. C. Gales, and R. N. Jones.** 2004. Emergence of the extended-spectrum ß-lactamase GES-1 in a *Pseudomonas aeruginosa* strain from Brazil: report from the SENTRY antimicrobial surveillance program. Antimicrob. Agents Chemother. **48:**2344–2345.
8. **Celenza, G., C. Pellegrini, M. Caccamo, B. Segatore, G. Amicosante, and M. Perilli.** 2006. Spread of $bla_{\text{CTX-M}}$-type and $bla_{\text{PER-2}}$ beta-lactamase genes in clinical isolates from Bolivian hospitals. J. Antimicrob. Chemother. **57:**975–978.
9. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial susceptibility testing; 18th informational supplement M100–S18. Clinical and Laboratory Standards Institute, Wayne, PA.
10. **Dubois, V., L. Poirel, C. Marie, C. Arpin, P. Nordmann, and C. Quentin.** 2002. Molecular characterization of a novel class 1 integron containing $bla_{\text{GES-1}}$ and a fused product of *aac3-Ib*/*aac6″-Ib″* gene cassettes in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **46:**638–645.
11. **Empel, J., K. Filczak, A. Mrówka, W. Hryniewicz, D. M. Livermore, and M. Gniadkowski.** 2007. Outbreak of *Pseudomonas aeruginosa* infections with PER-1 extended-spectrum beta-lactamase in Warsaw, Poland: further evidence for an international clonal complex. J. Clin. Microbiol. **45:**2829–2834.
12. **Girlich, D., T. Naas, A. Leelaporn, L. Poirel, M. Fennewald, and P. Nordmann.** 2002. Nosocomial spread of the integron-located *veb-1*-like cassette encoding an extended-spectrum ß-lactamase in *Pseudomonas aeruginosa* in Thailand. Clin. Infect. Dis. **34:**175–182.
13. **Gniadkowski, M.** 2008. Evolution of extended-spectrum ß-lactamases by mutation. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**11–32.
14. **Kieser, T.** 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12:**19–36.
15. **Kolayli, F., G. Gacar, A. Karadenizli, A. Sanic, H. Vahaboglu, and the Study Group.** 2005. PER-1 is still widespread in Turkish hospitals among *Pseudomonas aeruginosa* and *Acinetobacter* spp. FEMS Microbiol. Lett. **249:**241–245.
16. **Labuschagne, C. J., G. F. Weldhagen, M. M. Ehlers, and M. G. Dove.** 2008. Emergence of class 1 integron-associated GES-5 and GES-5-like extended-spectrum ß-lactamases in clinical isolates of *Pseudomonas aeruginosa* in South Africa. Int. J. Antimicrob. Agents **31:**527–530.
17. **Libisch, B., L. Poirel, Z. Lepsanovic, V. Mirovic, B. Balogh, J. Pászti, Z. Hunyadi, A. Dobák, M. Füzi, and P. Nordmann.** 2008. Identification of PER-1 extended-spectrum ß-lactamase producing *Pseudomonas aeruginosa* clinical isolates of the international clonal complex CC11 from Hungary and Serbia. FEMS Immunol. Med. Microbiol. **54:**330–338.
18. **Mammeri, H., L. Poirel, and P. Nordmann.** 2003. In vivo selection of a chromosomally encoded β-lactamase variant conferring ceftazidime resistance in *Klebsiella oxytoca*. Antimicrob. Agents Chemother. **47:**3739–3742.
19. **Naas, T., L. Philippon, L. Poirel, E. Ronco, and P. Nordmann.** 1999. An SHV-derived extended-spectrum ß-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**1281–1284.
20. **Naas, T., L. Poirel, and P. Nordmann.** 2008. Minor extended-spectrum ß-lactamases. Clin. Microbiol. Infect. **14**(Suppl. 1)**:**42–52.
21. **Pagani, L., E. Mantengoli, R. Migliavacca, E. Nucleo, S. Pollini, M. Spalla, R. Daturi, E. Romero, and G. M. Rossolini.** 2004. Multifocal detection of multidrug-resistant *Pseudomonas aeruginosa* producing the PER-1 extended-spectrum ß-lactamase in Northern Italy. J. Clin. Microbiol. **42:**2523–2529.
22. **Picão, R. C., L. Poirel, A. C. Gales, and P. Nordmann.** 2009. Further identification of CTX-M-2 extended-spectrum ß-lactamase in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **53:**2225–2226.
23. **Picão, R. C., L. Poirel, A. C. Gales, and P. Nordmann.** 2009. Diversity of ß-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolates causing bloodstream infections in Brazil. Antimicrob. Agents Chemother. **53:**3908–3913.
24. **Poirel, L., L. Brinas, A. Verlinde, L. Ide, and P. Nordmann.** 2005. BEL-1, a novel clavulanic acid-inhibited extended-spectrum ß-lactamase, and the class 1 integron In120 in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **49:**3743–3748.
25. **Poirel, L., M. Guibert, S. Bellais, T. Naas, and P. Nordmann.** 1999. Integron- and carbenicillinase-mediated reduced susceptibility to amoxicillin-clavulanic acid in isolates of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 from French patients. Antimicrob. Agents Chemother. **43:**1098–1104.
26. **Poirel, L., T. Naas, I. Le Thomas, A. Karim, E. Bingen, and P. Nordmann.** 2001. CTX-M-type extended-spectrum ß-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob. Agents Chemother. **45:**3355–3361.
27. **Poirel, L., G. F. Weldhagen, C. De Champs, and P. Nordmann.** 2002. A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum ß-lactamase GES-2 in South Africa. J. Antimicrob. Chemother. **49:**561–565.
28. **Poirel, L., G. F. Weldhagen, T. Naas, C. De Champs, M. G. Dove, and P. Nordmann.** 2001. GES-2, a class A ß-lactamase from *Pseudomonas aeruginosa* with increased hydrolysis of imipenem. Antimicrob. Agents Chemother. **45:**2598–2603.
29. **Weldhagen, G. F.** 2004. Rapid detection and sequence-specific differentiation of extended-spectrum ß-lactamase GES-2 from *Pseudomonas aeruginosa* by use of a real-time PCR assay. Antimicrob. Agents Chemother. **48:**4059–4062.
30. **Weldhagen, G. F., L. Poirel, and P. Nordmann.** 2003. Ambler class A extended-spectrum ß-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. Antimicrob. Agents Chemother. **47:**2385–2392.