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CTX-M Expression and Selection of Ertapenem Resistance in *Klebsiella pneumoniae* and *Escherichia coli*[▽]

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In vitro selection of mutants with decreased susceptibility to ertapenem was performed using *Escherichia coli* and *Klebsiella pneumoniae* clinical strains producing either the $bla_{CTX-M-2}$, $bla_{CTX-M-3}$, $bla_{CTX-M-9}$, or $bla_{CTX-M-15}$ gene. Frequencies of mutants with decreased susceptibilities to ertapenem were similar for all β-lactamases expressed.

Extended-spectrum β-lactamases (ESBLs) of the CTX-M type are emerging worldwide mostly in *Enterobacteriaceae* as a source of community-acquired and nosocomial infections (11).

Most CTX-M-type β-lactamases hydrolyze cefotaxime at a higher level than that of ceftazidime. However, several amino acid substitutions result in an increased hydrolytic activity against ceftazidime, as seen for the CTX-M enzyme that has spread worldwide, CTX-M-15, a variant of CTX-M-3 (14). Specific substitutions at Ambler positions 167 (P167S/T/Q) and 240 (D240G) in CTX-M-2 (21) and in CTX-M-3 (6, 14) conferring resistance to ceftazidime have been selected in vitro after a ceftazidime selection. Although rare ESBLs such as several GES-like enzymes may hydrolyze carbapenems (12), no CTX-M enzyme has been reported to possess carbapenemase activity. Among β-lactam molecules, carbapenems (imipenem, ertapenem, and meropenem) are the drugs of choice for treating infections by ESBL-producing *Enterobacteriaceae* (16, 17, 18).

The objective of this study was to determine if CTX-M β-lactamases with carbapenemase activity may be selected in vitro.

Therefore, the frequency of in vitro selection of mutant strains with reduced susceptibility to ertapenem was evaluated with *Escherichia coli* and *Klebsiella pneumoniae* strains expressing different CTX-M β-lactamases. The risk of selection of mutated bla_{CTX-M} genes and the level of porin expression were investigated also.

The bla_{CTX-M} genes were expressed under the same promoter and in the same genetic background. The bla_{CTX-M} genes ($bla_{CTX-M-2}$, $bla_{CTX-M-3}$, $bla_{CTX-M-9}$, and $bla_{CTX-M-15}$) were amplified from clinical isolates without their promoter sequence (primer sequence in Table 1), cloned into the low-copy-number pACYC184 plasmid (New England Biolabs, Ozyme, Saint-Quentin-en-Yvelines, France), and expressed in clinical isolates of *E. coli* Wi and *K. pneumoniae* M (Hôpital Bicêtre strain collection). Transformants were selected overnight at 37°C on Trypticase soy agar (bioMérieux, Craponne, France) containing chloramphenicol (Euromedex, Souffelweyersheim, France) at 30 μg/ml.

Mutant strains with decreased susceptibilities to ertapenem were selected as described previously (6), on Trypticase soy agar containing ertapenem (Merck Sharp & Dohme-Chibret, Paris, France) at a concentration fourfold higher than the MICs (6). After overnight incubation at 37°C for 18 h, mutation frequencies were calculated, taking plate counts of viable bacteria on drug-free agar (6). Comparison of the means was performed by Student's *t* test on three independent experiments. In *E. coli* as well as in *K. pneumoniae* isolates, mean frequencies of selection of ertapenem-reduced susceptibility were not related to the bla_{CTX-M} content ($P > 0.1$) (Table 2).

Both strands of the bla_{CTX-M} genes were sequenced from five of each bla_{CTX-M}-harboring *E. coli* and *K. pneumoniae* mutant strain. All mutants had a wild-type $bla_{CTX-M-2}$, $bla_{CTX-M-3}$, $bla_{CTX-M-9}$, or $bla_{CTX-M-15}$ sequence, thus indicating that the reduced susceptibility to ertapenem was not due to point mutations located in the bla_{CTX-M} genes.

The outer membrane protein (OMP) profiles of the *E. coli* and *K. pneumoniae* isolates before and after ertapenem selection were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (15). Comparison of the OMP profiles of *E. coli* mutant strains selected on ertapenem-containing plates showed mainly a decrease in OmpC expression (Fig. 1). Similarly, comparison of the OMP profiles of *K. pneumoniae* isolates showed mainly a decrease in OmpK36 expression in ertapenem-selected isolates expressing a CTX-M β-lactamase or not (Fig. 1).

In order to quantify expression of the *ompC* and *ompF* genes in *E. coli* isolates and of the *ompK35* and *ompK36* genes in *K. pneumoniae* isolates, the two-step quantitative reverse transcription-PCR was used as recommended by Fey et al. (3). Expression level results were standardized relative to the transcription level of the constitutively expressed *gapA* (D-glyceraldehyde-3-phosphate dehydrogenase) gene in *E. coli* (19) and the 16S rRNA genes in *K. pneumoniae* (Fig. 2). Transcript quantification was performed by using the LightCycler FastStart DNA Master^{PLUS} kit SYBR-Green I on a LightCycler 1.0 instrument (Roche Diagnos-

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TABLE 1. Nucleotide sequences of primers used for amplification and sequence analysis

Primer	Sequence (5′→3′)	Reference or GenBank accession no.
CTX-M-2F[a]	AATGTATATTGAAGGCCG AGGG	This study
CTX-M-2R	ATACCTCGCTCCATTTATTGC	This study
CTX-M-3F	TCGTCTCTTCCAGAATAAG	This study
CTX-M-3R	TACCTATTACAAACCGTC GGTG	This study
CTX-M-9F	CTGATGTAACACGGATTGAC	This study
CTX-M-9R	AGCGCCCCATTATTGAGAG	This study
CTX-M-15F[b]	TCGTATCTTCCAGAATAAGG	This study
EcOmpC-F	GTTAAAGTACTGTCCCTCCTG	15
EcOmpC-R	TAACCGGTCAGCTGGTCA GTAA	This study
EcOmpF-F	TCGTATCTTCCAGAATAAGG	AM040706
EcOmpF-R	CAGGTACTGCAAACGCTGC	15
GapA-F	TATGACTGGTCCGTCTAAAG ACAA	19
GapA-R	GGTTTTCTGAGTAGCGGTAG TAGC	19
OmpK35-F	TGATCCCTGCCCTGCTGGT	8
OmpK35-R	TCCATGTTGTATTCCCACTGG	This study
OmpK36-F	TTAGACCTGTACGGCAAA ATCG	Z33506
OmpK36-R	AATGCCAGACGAGTCCATGC	Z33506
Kp16S rRNA-F	GGACGGGTGAGTAATGTC	EU048272
Kp16S rRNA-R	TCTCAGACCAGCTAGGGATCG	EU048272

[a] Expected sizes of PCR products with combinations of forward (F) and reverse (R) primers: 943 bp for the $bla_{CTX\text{-}M\text{-}2}$ gene, 916 bp for the $bla_{CTX\text{-}M\text{-}3}$ gene, 932 bp for the $bla_{CTX\text{-}M\text{-}9}$ gene, 904 bp for the $bla_{CTX\text{-}M\text{-}15}$ gene, 215 bp for the *ompC* gene, 204 bp for the *ompF* gene, and 201 bp for the *gapA* gene of *E. coli* and 202 bp for the *ompK36* gene, 222 bp for the *ompK35* gene, and 193 bp for the 16S rRNA gene of *K. pneumoniae*.

[b] The CTX-M-3R primer was also used for the amplification of the $bla_{CTX\text{-}M\text{-}15}$ gene.

TABLE 2. Mean frequencies of mutation to ertapenem decrease susceptibility for *Escherichia coli* and *Klebsiella pneumoniae* strains producing no CTX-M (wild type) and producing the ESBLs CTX-M-2, CTX-M-3, CTX-M-9, and CTX-M-15

Strain	Ertapenem selection (μg/ml)[b]	Frequency of selection of mutants with decreased susceptibility to ertapenem (10^{-8})[a]
K. pneumoniae		
M (wild type)	0.032	4 ± 1.7
M (CTX-M-2)	0.064	7 ± 6
M (CTX-M-3)	0.064	5 ± 3
M (CTX-M-9)	0.048	1.8 ± 1.2
M (CTX-M-15)	0.064	4.2 ± 2.5
E. coli		
Wi (wild type)	0.032	0.8 ± 0
Wi (CTX-M-2)	0.092	3 ± 1.4
Wi (CTX-M-3)	0.092	2.4 ± 1.3
Wi (CTX-M-9)	0.064	2.7 ± 0.6
Wi (CTX-M-15)	0.092	1.2 ± 0.5

[a] Mutation frequencies are arithmetic means from three independent experiments, each performed in triplicate.

[b] Ertapenem selection was performed at concentrations of four times the MICs.

tics, Neuilly, France) at an annealing temperature of 57°C. The calibration curves were generated with serially diluted cDNA from in vitro-obtained RNA standards (10) for each gene with primers listed in Table 1. The slope of each calibration curve was used to compare the number of copies of each *omp* gene in *E. coli* and *K. pneumoniae* isolates before and after ertapenem selection. Real-time quantitative reverse transcription-PCR experiments showed that the *E. coli* isolates with decreased susceptibilities to ertapenem had similarly decreased expression of the *ompC* and *ompF* genes whatever the CTX-M expressed (Fig. 2), the converse of what was observed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Similarly, *K. pneumoniae* isolates with decreased susceptibilities to ertapenem had decreased expression of the *ompK35* and *ompK36* genes whatever the CTX-M expressed (Fig. 2). The level of *ompK35* transcript was 10-fold lower than that of *ompK36* in the same RNA extracts, and the decrease in transcript level upon ertapenem selection was then less significant for *ompK35* (two- to sixfold) than for *ompK36* (up to 30-fold) (Fig. 2).

Resistance to carbapenems in *Enterobactericeae* may be related to carbapenemases (13, 20) or to dual mechanisms associating the outer membrane permeability defect and β-lactamases such as AmpCs and ESBLs (2, 5, 7). In the absence of β-lactamase, no defect in outer membrane permeability is sufficient to lead to carbapenem resistance (1). Previous studies reported ertapenem resistance in CTX-M-producing *K. pneumoniae* (2, 9) and CTX-M-producing *E. coli* (7) isolates exhibiting a permeability defect. Here, we show that ertapenem selects for mutant strains with decreased susceptibility by modification of porin expression whatever the content in CTX-M β-lactamases.

Finally, this study may indicate that the frequency of selection of ertapenem resistance is not higher in isolates expressing CTX-Ms. In additional to the alteration of membrane permeability, CTX-M β-lactamases most probably contribute to the decreased ertapenem susceptibility by binding with a high affinity to this molecule. Indeed, even if poorly hydrolyzed by CTX-Ms, ertapenem has been shown to have a strong inhibitory effect (low K_i) on these β-lactamases as exemplified with CTX-M-15 (4). This finding shall be added to the current debate on usage of ertapenem for treating infections due to ESBL-producing *K. pneumoniae* and *E. coli* isolates.

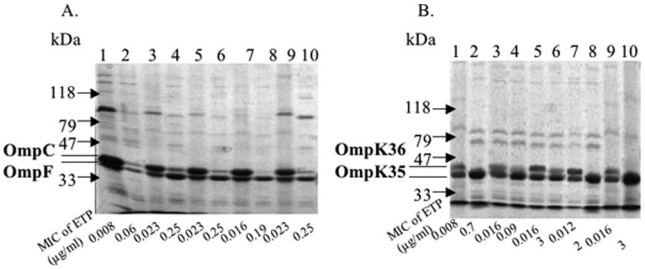

FIG. 1. OMP profiles of *E. coli* Wi (A) and *K. pneumoniae* M (B) isolates expressing no CTX-M (lanes 1 and 2) or expressing CTX-M-2 (lanes 3 and 4), CTX-M-3 (lanes 5 and 6), CTX-M-9 (lanes 7 and 8), or CTX-M-15 (lanes 9 and 10). Lanes 1, 3, 5, 7, and 9 correspond to OMPs extracted from isolates cultured without ertapenem (ETP); lanes 2, 4, 6, 8, and 10 correspond to OMPs extracted after a single-step ertapenem selection (4× MIC for each).

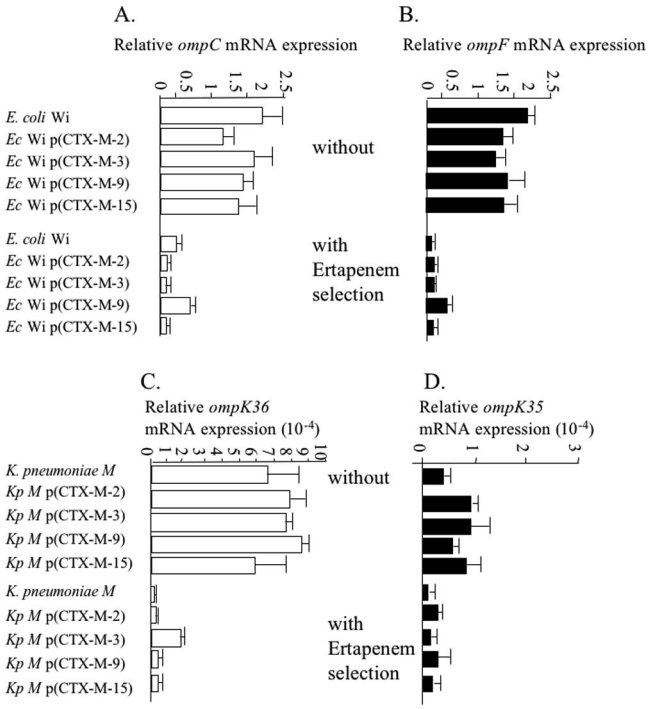

FIG. 2. Effects of one-step selection of ertapenem (4× MIC) on transcription of *ompC* (A) and *ompF* (B) genes in *E. coli* and of *ompK36* (C) and *ompK35* (D) genes in *K. pneumoniae*. Isolates expressing no CTX-M, CTX-M-2, CTX-M-3, CTX-M-9, or CTX-M-15 were used as templates. Data are expressed as the amount of target mRNA relative to the control value, i.e., the *gap* gene of *E. coli* and the 16S rRNA genes of *K. pneumoniae*. Upper lanes of each diagram correspond to the *omp* transcript level in isolates cultured without ertapenem, and lower lanes correspond to the *omp* transcript level after one-step selection of ertapenem.

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REFERENCES

1. **Cornaglia, G., K. Russell, G. Satta, and R. Fontana.** 1995. Relative importances of outer membrane permeability and group 1 beta-lactamase as determinants of meropenem and imipenem activities against *Enterobacter cloacae*. Antimicrob. Agents Chemother. **39:**350–355.
2. **Elliott, E., A. J. Brink, J. van Greune, Z. Els, N. Woodford, J. Turton, M. Warner, and D. M. Livermore.** 2006. In vivo development of ertapenem resistance in a patient with pneumonia caused by *Klebsiella pneumoniae* with an extended-spectrum β-lactamase. Clin. Infect. Dis. **42**(11)**:**e95–e98.
3. **Fey, A., S. Eichler, S. Flavier, R. Christen, M. G. Höfle, and C. A. Guzmán.** 2004. Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. Appl. Environ. Microbiol. **70:**3618–3623.
4. **Girlich, D., L. Poirel, and P. Nordmann.** 2008. Do CTX-M beta-lactamases hydrolyse ertapenem? J. Antimicrob. Chemother. **62:**1155–1156.
5. **Jacoby, G. A., D. M. Mills, and N. Chow.** 2004. Role of β-lactamases and porins in resistance to ertapenem and other β-lactams in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **48:**3203–3206.
6. **Karisik, E., M. J. Ellington, R. Pike, D. M. Livermore, and N. Woodford.** 2006. Development of high-level ceftazidime resistance via single-base substitutions of $bla_{\text{CTX-M-3}}$ in hyper-mutable *Escherichia coli*. Clin. Microbiol. Infect. **12:**803–806.
7. **Lartigue, M. F., L. Poirel, C. Poyart, H. Réglier-Poupet, and P. Nordmann.** 2007. Ertapenem resistance of *Escherichia coli*. Emerg. Infect. Dis. **13:**315–317.
8. **Lee, C. H., C. Chu, J. W. Liu, Y. S. Chen, C. J. Chiu, and L. H. Su.** 2007. Collateral damage of flomoxef therapy: in vivo development of porin deficiency and acquisition of $bla_{\text{DHA-1}}$ leading to ertapenem resistance in a clinical isolate of *Klebsiella pneumoniae* producing CTX-M-3 and SHV-5 beta-lactamases. J. Antimicrob. Chemother. **60:**410–413.
9. **Livermore, D. M., K. J. Oakton, M. W. Carter, and M. Warner.** 2001. Activity of ertapenem (MK-0826) versus *Enterobacteriaceae* with potent β-lactamases. Antimicrob. Agents Chemother. **45:**2831–2837.
10. **Maillet, S., T. Naas, S. Crepin, A. M. Roque-Afonso, F. Lafay, S. Efstathiou, and M. Labetoulle.** 2006. Herpes simplex virus type 1 latently infected neurons differentially express latency-associated and ICP0 transcripts. J. Virol. **80:**9310–9321.
11. **Mena, A., V. Plasencia, L. García, O. Hidalgo, J. I. Ayestarán, S. Alberti, N. Borrell, J. L. Pérez, and A. Oliver.** 2006. Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to in vivo carbapenem resistance development. J. Clin. Microbiol. **44:**2831–2837.
12. **Naas, T., L. Poirel, and P. Nordmann.** 2008. Minor extended-spectrum β-lactamases. Clin. Microbiol. Infect. **14:**42–52.
13. **Nordmann, P., and L. Poirel.** 2002. Emerging carbapenemases in Gram-negative aerobes. Clin. Microbiol. Infect. **8:**321–331.
14. **Poirel, L., M. Gniadkowski, and P. Nordmann.** 2002. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum beta-lactamase CTX-M-15 and of its structurally related beta-lactamase CTX-M-3. J. Antimicrob. Chemother. **50:**1031–1034.
15. **Poirel, L., C. Héritier, C. Spicq, and P. Nordmann.** 2004. In vivo acquisition of high-level resistance to imipenem in *Escherichia coli*. J. Clin. Microbiol. **42:**3831–3833.
16. **Raveh, D., A. M. Yinnon, E. Broide, and B. Rudensky.** 2007. Susceptibilities of ESBL-producing *Enterobacteriaceae* to ertapenem, meropenem and piperacillin-tazobactam with and without clavulanic acid. Chemotherapy **53:**185–189.
17. **Tamayo, J., B. Orden, J. Cacho, J. Cuadros, J. L. Gomez-Garces, and J. I. Alos.** 2007. Activity of ertapenem and other antimicrobials against ESBL-producing enterobacteria isolated from urine in patients from Madrid. Rev. Esp. Quimioter. **20:**334–338.
18. **Teng, C. P., H. H. Chen, J. Chan, and D. C. Lye.** 2007. Ertapenem for the treatment of extended-spectrum beta-lactamase-producing Gram-negative bacterial infections. Int. J. Antimicrob. Agents **30:**356–359.
19. **Toshima, H., A. Yoshimura, K. Arikawa, A. Hidaka, J. Ogasawara, A. Hase, H. Masaki, and Y. Nishikawa.** 2007. Enhancement of Shiga toxin production in enterohemorrhagic *Escherichia coli* serotype O157:H7 by DNase colicins. Appl. Environ. Microbiol. **73:**7582–7588.
20. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-beta-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18:**306–325.
21. **Welsh, K. J., M. Barlow, F. C. Tenover, J. W. Biddle, J. K. Rasheed, L. A. Clark, and J. E. McGowan, Jr.** 2005. Experimental prediction of the evolution of ceftazidime resistance in the CTX-M-2 extended-spectrum β-lactamase. Antimicrob. Agents Chemother. **49:**1242–1244.