Antimicrobial Agents and Chemotherapy, May 2000, p. 1387–1390 Vol. 44, No. 5
0066-4804/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

# Contributions of the AmpC β-Lactamase and the AcrAB Multidrug Efflux System in Intrinsic Resistance of *Escherichia coli* K-12 to β-Lactams

ANNARITA MAZZARIOL,[1,2] GIUSEPPE CORNAGLIA,[1] AND HIROSHI NIKAIDO[2]*

*Institute of Microbiology, University of Verona, Verona, Italy,[1] and Department of Molecular and Cell Biology, University of California, Berkeley, California[2]*

Received 7 October 1999/Returned for modification 19 January 2000/Accepted 1 February 2000

**The roles of the AmpC chromosomal β-lactamase and the AcrAB efflux system in levels of intrinsic resistance and susceptibility of *Escherichia coli* to β-lactams were studied with a set of isogenic strains. MICs of ureidopenicillins, carbenicillin, oxacillin, and cloxacillin were drastically reduced by the inactivation of AcrAB, whereas those of the earlier cephalosporins were affected mostly by the loss of AmpC β-lactamase.**

The β-lactam antibiotics act by inhibiting penicillin-binding proteins. However, MICs seen with wild-type strains of gram-negative bacteria are usually significantly higher than the concentrations needed to bind and inhibit the penicillin-binding proteins because the access of β-lactams to the targets is hindered by the presence of the outer membrane barrier and by their hydrolysis via the chromosomally coded AmpC-type β-lactamase (20). This increase in MIC is called "intrinsic resistance" throughout this paper, although the absolute values of MIC for many compounds fall into the susceptible range for the purpose of clinical use.

AmpC belongs to class C (2) or group 1 (5) in the classification of β-lactamases. Like most *Enterobacteriaceae*, *Escherichia coli* produces the AmpC enzyme (13), but in this case its production is at a low level and is not inducible because there is no *ampR* regulatory gene in this species (3). However, even this low level of the enzyme can contribute to the intrinsic resistance of *E. coli*, as shown earlier by the comparison of β-lactam susceptibility in strains producing this enzyme at different levels (20), a study that concluded that the MICs could be explained quantitatively by the β-lactamase-mediated hydrolysis and the limitation of influx through the outer membrane barrier.

More recently, however, multidrug efflux pumps, and especially the constitutively expressed AcrAB system (17) in *E. coli*, were shown to affect intrinsic β-lactam resistance (14), and comparison of various β-lactam compounds in *Salmonella enterica* serovar Typhimurium, which does not produce the AmpC β-lactamase, showed that β-lactams with lipophilic side chains are pumped out effectively by this system (18). Because these recent results do not fit completely with the conclusion arrived at more than 10 years ago (20), we investigated the role of the AmpC chromosomal β-lactamase and the AcrAB efflux system in intrinsic resistance of *E. coli* to β-lactams.

A set of isogenic K-12 strains lacking the AmpC enzyme and/or the AcrAB transport function was created for this purpose. We first amplified the *ampC* gene by PCR, using CGA GAATTCGGACCCGATGGAATTTTAC and GTCCGGAT CCCATTACCCTGGCGCATCGT as primers. The amplified fragment was cloned between the *Bam*HI and *Eco*RI sites in pUC19. We then inserted the spectinomycin marker (21) in the *Sma*I site within the *ampC* gene. The linearized plasmid was transformed into the *recBC* host strain JC 7623 [K-12 F$^-$ *argE3 hisG4 leuB6 Δ(gpt-proA)62 thr-1 thi-1 ara-14 galK2 lacY1 mtl-1 xyl-1 kdgK51 tsx-33 recB21 recC22 sbcB15 supE44 rpsL31 rac*] (6), and selection for spectinomycin resistance (100 μg/ml) resulted in the isolation of strain AM16, which contained the disrupted, chromosomal *ampC*::*spc* allele, whose presence was confirmed by PCR. All selection procedures for the *ampC* strains were done at 30°C because disruption of this gene has been reported to result in a marginally temperature-sensitive phenotype (4). The absence of detectable β-lactamase activity in AM16 was confirmed by spectrophotometric assay of crude cell extracts with 0.1 mM cephaloridine and cephalothin as substrates. Although the presence of an AmpC homolog, AmpH, in *E. coli* has been described, AmpH totally lacks the ability to hydrolyze nitrocefin and most probably other β-lactams as well (10). The strain JZM120 (14) was also a derivative of JC7623, containing a deletion that covers most of both *acrA* and *acrB* genes and a kanamycin marker inserted in exchange for the deleted sequence. The double mutant AM17 was obtained by P1 transduction of the Δ*acrAB*::*kan* allele from JZM120 into AM16. When a Western blot assay using anti-AcrA rabbit antibodies was done, AcrA could be detected only in JC7623 and AM16, not in JZM120 and AM17.

Using this set of strains, the drug susceptibility was tested by the broth microdilution method in Luria-Bertani medium at 30°C, with a standard inoculum of $10^4$ cells (Table 1). All antibiotics used in this study were obtained from commercial sources.

With certain agents, inactivation of the *ampC* gene made *E. coli* more susceptible. This was observed most prominently with earlier cephalosporins such as cephalothin, cefamandole, and cephaloridine. A marginal (twofold) decrease in MIC was observed with ampicillin, mezlocillin, piperacillin, penicillin G, cefazolin, cefsulodin, cefoperazone, cefuroxime, and cefoxitin. These results can be compared with the rate of hydrolysis of these agents by the *E. coli* AmpC enzyme. However, because the rate varies with the concentration of the drug, a valid comparison requires the knowledge of the $K_m$ and $V_{max}$ for each substrate, as well as a somewhat arbitrary assumption of the drug concentration in the periplasm. We used the kinetic constants of the *E. coli* AmpC enzyme previously reported (8, 9, 20) and used as the periplasmic concentration the concentration at which the targets become inhibited ($C_{inh}$ of reference

* Corresponding author. Mailing address: Department of Molecular and Cell Biology, Room 229, Stanley Hall, University of California, Berkeley, CA 94720-3206. Phone: (510) 642-2027. Fax: (510) 643-9290. E-mail: nhiroshi@uclink4.berkeley.edu.

TABLE 1. β-Lactam MICs for a set of isogenic *E. coli* strains[a]

| β-Lactam | MIC(s) (μg/ml) for *E. coli* strain: | | | |
|---|---|---|---|---|
| | JC7623 (wild type) | JZM120 ($AcrAB^-$) | AM16 ($AmpC^-$) | AM17 ($AcrAB^-$ $AmpC^-$) |
| Penicillin G | 16 | 8 | 8 | 8 |
| Ampicillin | 2 | 1 | 1 | 0.5 |
| Amoxicillin | 4 | 4 | 4 | 4 |
| Carbenicillin | 4 | 1 | 4/2 | 1/0.5 |
| Azlocillin | 16 | 4/2 | 16 | 1 |
| Mezlocillin | 1 | 0.03 | 0.5 | 0.03 |
| Piperacillin | 4 | 0.25 | 2 | 0.25 |
| Cloxacillin | 256 | 2 | 256 | 1 |
| Oxacillin | 256 | 0.5 | 256 | 0.5 |
| Mecillinam | 0.5 | 0.25 | 0.5 | 0.12 |
| Cephalothin | 4 | 4 | 0.5 | 0.5 |
| Cefazolin | 1 | 1 | 0.5 | 0.5 |
| Cephaloridine | 2 | 1 | 0.5 | 0.5 |
| Cefamandole | 0.5 | 0.12/0.06 | 0.12 | 0.06 |
| Cefsulodin | 16 | 16 | 8 | 8 |
| Cefoperazone | 0.03 | 0.015 | 0.015 | 0.0075 |
| Cefuroxime | 2 | 0.12 | 1 | 0.12 |
| Cefoxitin | 4 | 1 | 2 | 1 |
| Cefmetazole | 0.5 | 0.5 | 0.5 | 0.5 |
| Ceftriaxone | 0.015 | 0.015 | 0.015 | 0.015 |
| Ceftazidime | 0.12 | 0.12 | 0.12 | 0.12 |
| Cefepime | 0.0075 | 0.0075 | 0.0075 | 0.0075 |
| Cefpirome | 0.015 | 0.015/0.0075 | 0.015 | 0.0075 |
| Imipenem | 0.12 | 0.12 | 0.12 | 0.12 |
| Spectinomycin[b] | 16 | 16 | >128 | >128 |
| Kanamycin[b] | 4 | >128 | 4 | >128 |
| Puromycin[c] | 128 | 2 | 128 | 2 |
| Novobiocin[c] | 32 | 1 | 32 | 1 |
| Erythromycin[c] | 128 | 8 | 128 | 8 |

[a] MIC determination was repeated at least twice. Where different MICs were obtained in different assays, both results are given.
[b] MICs were tested to confirm the presence of interposon units.
[c] Good substrate of the AcrAB pump, used to confirm the absence or presence of the pump.

20), on the assumption that this concentration will be achieved when the external drug concentration is equal to MIC. In Table 2 we list, out of the compounds tested in Table 1, those for which hydrolysis rates could be calculated. A reasonably good correlation is seen between the hydrolysis rate in the cell and the effect of *ampC* disruption. (Although a fourfold decrease in MIC with cefamandole was somewhat larger than that expected from the hydrolysis rate, the MICs determined by twofold serial dilution have an inherent error of twofold. The $k_{cat}$ and $K_m$ values of the K-12 AmpC enzyme, kindly determined for cefamandole by A. Dubus and J.-M. Frère [personal communication], were 5.9 $s^{-1}$ and 40 μM, respectively, close to those reported in reference 20.) Furthermore, with compounds that are hydrolyzed very slowly (with the expected rates of 0.003 pmol $mg^{-1}$ $s^{-1}$ or less), there was no effect of *ampC* disruption on MIC (Table 2), and similar results were obtained with the cephalosporins with quaternary ammonium-containing side chains at the 3 position, such as cefepime

TABLE 2. Apparent contribution of AmpC β-lactamase to MIC

| β-Lactam | $MIC_{wt}$/ $MIC_{ampC}$[a] | AmpC β-lactamase | | |
|---|---|---|---|---|
| | | $k_{cat}$ ($s^{-1}$)[b] | $K_m$ (μM)[b] | Hydrolysis rate[c] |
| Cephalothin | 8 | 250 | 22 | 10.9 |
| Cephaloridine | 4 | 152 | 230 | 1.6 |
| Cefamandole | 4 | 5.9 | 20 | 0.07 |
| Penicillin G | 2 | 65 | 1.9 | 19.2 |
| Ampicillin | 2 | 6.5 | 0.9 | 2.8 |
| Cefazolin | 2 | 850 | 1,900 | 2.05 |
| Cefoxitin | 2 | 0.15 | 0.22 | 0.14 |
| Cefuroxime | 2 | 0.15[d] | 0.15[d] | 0.07 |
| Cefsulodin | 2 | 0.3 | 170 | 0.01 |
| Carbenicillin | 1 | 0.002 | 0.1 | 0.002 |
| Ceftazidime | 1 | 0.34 | 16 | 0.001 |
| Cloxacillin | 1 | 0.003[d] | 0.0005[d] | 0.003 |
| Imipenem | 1 | 0.01[d] | 0.8[d] | 0.001 |

[a] Based on Table 1 data. wt, wild type.
[b] Taken from reference 20.
[c] Calculated rate of hydrolysis by the constitutive level of AmpC lactamase in wild-type cells (20) at the substrate concentration that is expected to inhibit, by 50%, the most susceptible essential penicillin-binding protein (7, 20). The units are picomoles milligram (dry weight)$^{-1}$ second$^{-1}$.
[d] Taken from references 8 and 9.

and cefpirome, which are hydrolyzed extremely slowly by the class C chromosomal enzymes (19) (Table 1). Penicillin G, which is expected to be hydrolyzed even faster than cephalothin, showed only a twofold decrease in MIC in a reproducible manner. We earlier observed a 16-fold decrease in MIC even in a mutant still producing about 10% of the wild-type activity of AmpC (20), and the MIC observed in the Δ*acrAB ampC* strain AM17 (Table 1) was far above the 50% inhibitory concentration earlier determined for *E. coli* penicillin-binding proteins (7). We believe that the most likely explanation is the overexpression of an efflux pump(s) other than AcrAB in the heavily mutagenized parent strain JC7623.

Inactivation of the AcrAB efflux pump often produced a significant (more than twofold) decrease in MIC (Table 1). This was observed with oxacillin, cloxacillin, mezlocillin, piperacillin, azlocillin, carbenicillin, cefamandole, cefuroxime, and cefoxitin. Table 3 lists mostly those compounds that were tested in *S. enterica* serovar Typhimurium earlier (18). The extent of decrease in MIC due to the deletion of *acrAB* genes in *E. coli* was very similar to what has been observed in the *S. enterica* serovar Typhimurium *acr* mutant SH7616 and generally showed a correlation with the lipophilicity of the side chains (18). Although ureidopenicillins (azlocillin, mezlocillin, and piperacillin) were not included in the serovar Typhimurium study, these compounds contain lipophilic phenyl branches in their side chains, and for this reason their efflux by the AcrAB system was not unexpected. A large discrepancy was again found with penicillin G. Inactivation of AcrAB in *E. coli* produced only a twofold change in MIC, a surprisingly small change in contrast to the 32-fold decrease found in serovar Typhimurium (18); a possible explanation is presented above. In contrast to these compounds with lipophilic side chains, compounds with much less lipophilic side chains, such as cefmetazole and cefazolin, showed no evidence of significant efflux (Table 3), in agreement with our previous conclusion that efflux through AcrAB requires the presence of lipophilic substituents on the substrate molecules. Imipenem is also a poor substrate of AcrAB-type pumps (Table 3); this is consistent with the finding obtained with *Pseudomonas aeruginosa*. Thus, although the overproduction of the MexEF OprN system

TABLE 3. Apparent contribution of multidrug efflux to MIC

| β-Lactam | $MIC_{wt}/MIC_{\Delta acrAB}$ in *E. coli* K-12[a] | $MIC_{wt}/MIC_{acr}$ in *S. typhimurium*[b] | Side chain lipophilicity[c] |
|---|---|---|---|
| Cloxacillin | 128 | 256 | 890 |
| Oxacillin | 512 | ND | ND |
| Mezlocillin | 32 | ND | ND |
| Piperacillin | 16 | ND | ND |
| Cefuroxime | 16 | ND | 55[d] |
| Carbenicillin | 4 | 4 | 80 |
| Penicillin G | 2 | 32 | 270 |
| Cefoxitin | 4 | 4 | 130 |
| Cephaloridine | 2 | 2 | 130 |
| Ceftriaxone | 1 | 2 | 6 |
| Cefsulodin | 1 | 1 | 80[e] |
| Cefmetazole | 1 | 1 | 2 |
| Cefazolin | 1 | 1 | 0.5 |
| Cefepime | 1 | ND | 6 |
| Cefpirome | 1 | 1 | 6 |
| Imipenem | 1 | 1 | 0.3 |

[a] Based on Table 1 data. wt, wild type.
[b] From reference 18.
[c] Expressed as the calculated octanol-water partition coefficient. From reference 18.
[d] Calculated as described in reference 18.
[e] Although the phenyl group shows a moderate lipophilicity, insertion of this side chain may be prevented by the presence of a negatively charged group next to it (18).

and increased resistance to imipenem occur simultaneously, the latter was shown to be the result of the decreased level of a basic amino acid channel OprD (11), through which imipenem permeation predominantly takes place.

It is of interest to compare our results on *acrAB* inactivation with the recently reported data on the inactivation of the homologous *mexAB oprM* system in *P. aeruginosa* (15, 16). Both previous studies showed that the deletion of efflux genes results in a drastic decrease in the MIC of carbenicillin, cefoperazone, and piperacillin, in agreement with the present results. However, some of the *P. aeruginosa* data are surprising. Nakae et al. (16) report that their efflux mutant is strongly hypersusceptible to cefpirome, and Masuda et al. (15) report that after the removal of the β-lactamase, further inactivation of the MexAB OprM system results in a 500-fold decrease in the MIC of cefmetazole, which contains a very hydrophilic side chain and does not appear to be a substrate of the AcrAB system. Possibly these differences are due to the differences in the substrate-binding sites of AcrB and MexB. An alternative explanation is that both *P. aeruginosa* studies (15, 16) used mutants in which *oprM* was inactivated. The OprM outer membrane channel was suggested to participate also in efflux systems other than MexAB (23) and was indeed shown recently to participate in the aminoglycoside efflux through MexXY (1). Thus, the *P. aeruginosa* studies are not strictly examining the effect of the AcrAB homolog MexAB.

In retrospect, the earlier MIC prediction scheme (20) included only those β-lactams that are relatively hydrophilic (and thus cross the outer membrane rapidly) and are readily hydrolyzed by β-lactamases, because these two conditions were prerequisites for obtaining reliable values of permeability coefficient across the outer membrane by the Zimmermann-Rosselet assay. Because lipophilic compounds were thus excluded (unintentionally) from the earlier study (20), most of the compounds tested were not good substrates for the AcrAB efflux system, and the calculated MICs were in reasonable agreement with the observed values without the inclusion of the efflux term. However, for compounds such as cloxacillin or oxacillin, hydrolysis is very slow but the efflux is quite efficient, and neglecting efflux would produce an extremely incorrect prediction. Thus, the MIC prediction scheme would certainly be improved if we could include efflux. Although an equation with an efflux term has been explicitly formulated (12), the difficulty at present is that kinetic constants of the efflux system are unknown, although some idea of the affinity of several substrates could be obtained based on the recent AcrB reconstitution study (22).

Which factor is more important in increasing MIC, efflux or β-lactamase? The answer of course depends on the type of β-lactam and the nature and activity of the β-lactamase present. Thus, for *E. coli*, which produces only a low, constitutive level of class C β-lactamase, efflux generally plays a significant role. Especially for these compounds that are hydrolyzed poorly by the AmpC enzyme and in addition contain lipophilic substituents (cloxacillin, oxacillin, carbenicillin, and ureidopenicillins), the effect of efflux is predominant. A similar situation is likely to exist for these compounds for wild-type strains of other *Enterobacteriaceae* and *P. aeruginosa*, which produce inducible class C enzymes that hydrolyze these compounds only very slowly (8, 9). In contrast, for those compounds that are rapidly hydrolyzed by chromosomal or plasmid-coded β-lactamases, hydrolysis tends to be a more important mechanism, and in strains producing high levels of such enzymes, the contribution of efflux will be hardly visible.

This study was supported in part by a grant from the U.S. Public Health Service (AI-09644).

**REFERENCES**


1. **Aires, J. R., T. Köhler, H. Nikaido, and P. Plésiat.** 1999. Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrob. Agents Chemother. **43:**2624–2628.
2. **Ambler, R. P.** 1980. The structure of β-lactamases. Philos. Trans. R. Soc. Lond. B **289:**321–331.
3. **Bennett, P. M., and I. Chopra.** 1993. Molecular basis of β-lactamase induction in bacteria. Antimicrob. Agents Chemother. **37:**153–158.
4. **Bishop, R. E., and J. H. Weiner.** 1993. Complementation of growth defect in an *ampC* deletion mutant of *Escherichia coli*. FEMS Microbiol. Lett. **114:** 349–354.
5. **Bush, K., G. A. Jacoby, and A. A. Medeiros.** 1995. A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39:**1211–1233.
6. **Cohen, A., and A. J. Clark.** 1986. Synthesis of linear plasmid multimers in *Escherichia coli* K-12. J. Bacteriol. **167:**327–335.
7. **Curtis, N. A. C., D. Orr, G. W. Ross, and M. G. Boulton.** 1979. Affinities of penicillins and cephalosporins for the penicillin-binding proteins of *Escherichia coli* K-12 and their antibacterial activity. Antimicrob. Agents Chemother. **16:**533–539.
8. **Galleni, M., and J.-M. Frère.** 1988. A survey of the kinetic parameters of class C β-lactamases: penicillins. Biochem. J. **255:**119–122.
9. **Galleni, M., G. Amicosante, and J.-M. Frère.** 1988. A survey of the kinetic parameters of class C β-lactamases: cephalosporins and other β-lactam compounds. Biochem. J. **255:**123–129.
10. **Henderson, T. A., K. D. Young, S. A. Denome, and P. K. Elf.** 1997. AmpC and AmpH, proteins related to the class C β-lactamases, bind penicillin and contribute to the normal morphology of *Escherichia coli*. J. Bacteriol. **179:** 6112–6121.
11. **Köhler, T., M. Michéa-Hamzehpour, U. Henze, N. Gotoh, L. K. Curty, and J. C. Pechère.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa. Mol. Microbiol. **23:**345–354.
12. **Lakaye, B., A. Dubus, S. Lepage, S. Groslambert, and J.-M. Frère.** 1999. When drug inactivation renders the target irrelevant to antibiotic resistance: a case story with β-lactams. Mol. Microbiol. **31:**89–101.
13. **Livermore, D. M.** 1995. β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8:**557–584.
14. **Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido, and J. E. Hearst.** 1995. Genes acrA and acrB encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16:**45–55.
15. **Masuda, N., N. Gotoh, C. Ishii, E. Sakagawa, S. Ohya, and T. Nishino.** 1999. Interplay between chromosomal β-lactamase and the MexAB-OprM efflux system in intrinsic resistance to β-lactams in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**400–402.

16. **Nakae, T., A. Nakajima, T. Ono, K. Saito, and H. Yoneyama.** 1999. Resistance to β-lactam antibiotics in *Pseudomonas aeruginosa* due to interplay between the MexAB-OprM efflux pump and β-lactamase. Antimicrob. Agents Chemother. **43:**1301–1303.
17. **Nikaido, H.** 1996. Multidrug efflux pumps of gram-negative bacteria. J. Bacteriol. **178:**5853–5859.
18. **Nikaido, H., M. Basina, V. Nguyen, and E. Y. Rosenberg.** 1998. Multidrug efflux pump AcrAB of *Salmonella typhimurium* excretes only those β-lactam antibiotics containing lipophilic side chains. J. Bacteriol. **180:**4686–4692.
19. **Nikaido, H., W. Liu, and E. Y. Rosenberg.** 1990. Outer membrane permeability and β-lactamase stability of dipolar ionic cephalosporins containing methoxyimino substituents. Antimicrob. Agents Chemother. **34:**337–342.
20. **Nikaido, H., and S. Normark.** 1987. Sensitivity of *Escherichia coli* to various β-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic β-lactamases: a quantitative predictive treatment. Mol. Microbiol. **1:**29–36.
21. **Prentki, P., A. Binda, and A. Epstein.** 1991. Plasmid vectors for selecting IS-1 promoted deletions in cloned DNA: sequence analysis of the omega interposon. Gene **103:**17–23.
22. **Zgurskaya, H. I., and H. Nikaido.** 1999. Bypassing the periplasm: reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **96:**7190–7195.
23. **Zhao, Q., X.-Z. Li, R. Srikumar, and K. Poole.** 1998. Contribution of outer membrane efflux protein OprM to antibiotic resistance in *Pseudomonas aeruginosa* independent of MexAB. Antimicrob. Agents Chemother. **42:** 1682–1688.