

Inhibition of *Escherichia coli* K-12 by β -Lactam Antibiotics With Poor Antibacterial Activity: Interaction of Permeability And Intrinsic Activity Against Penicillin-Binding Proteins

NIGEL A. C. CURTIS,* CAROL BROWN, MIKE BOXALL, AND MICHAEL G. BOULTON

Microbial Biochemistry Department, Glaxo-Allenburys Research Ltd., Greenford, Middlesex, England

Received for publication 12 December 1978

The effect of methicillin, cloxacillin, 1078/1/1, penicillin G, and cephaloridine upon the penicillin-binding proteins of a permeability mutant of *Escherichia coli* K-12 and its isogenic wild type have been investigated. Comparison of the 50% inhibition values for the antibiotics against the penicillin-binding proteins of the two strains with the minimal inhibitory concentrations for the same compounds indicates that methicillin, cloxacillin, 1078/1/1, and to a lesser extent penicillin G, owe their poor antibacterial activity to exclusion from the bacterial cell, whereas cephaloridine is not excluded and is equally active against both the mutant and its wild type. The results further suggest that the lesion in the permeability mutant *E. coli* DC2 allows free access of all the compounds tested to the inner membrane target proteins.

The antibacterial activity of a β -lactam antibiotic against *E. coli* K-12 depends on at least three factors. First, the ability of the compound to bind to and inhibit the "lethal" target penicillin-binding proteins (PBPs) located in the inner or cytoplasmic membrane (20, 21). Second, the antibiotic must penetrate the permeability barrier afforded by the outer membrane of the bacterial envelope (17, 18), and third, the compound must be resistant to any periplasmically located β -lactamase (4, 17).

Strains of *E. coli* K-12 not elaborating an R-factor-mediated β -lactamase owe their penicillin and cephalosporin resistance primarily to the first two considerations. The extremely low level of expression of the chromosomal type 1b β -lactamase found in all *E. coli* K-12 strains (2, 18) has little effect upon resistance (13).

Mutants of *E. coli* K-12 and other gram-negative bacteria that have been selected for increased sensitivity to a particular antibiotic are frequently found to exhibit increased sensitivity to a wide range of antibacterial agents (16-18). Such mutants are often regarded as "permeability" mutants having lesions affecting the integrity of the outer membrane and thereby allowing easier access of antibiotics into the bacterial cell.

In one such study, Richmond, Clark, and Wotton isolated a series of permeability mutants of *E. coli* K-12 and suggested their usefulness in elucidating the penetration characteristics of penicillins and cephalosporins through the outer membrane of *E. coli* K-12 (16). The change in

the permeability properties of these mutants was partially substantiated by "crypticity" measurements (16-18, 27) but it is impossible to know how permeable such mutants actually are and, furthermore, the possibility of a change in the sensitivity of the β -lactam target proteins was not excluded.

The inner membrane of the envelope of *E. coli* K-12 has been shown to contain at least six distinct polypeptides which covalently bind [14 C]penicillin G. Some of these proteins have been shown to have distinct roles in determining bacterial elongation, shape, and division (20-26), whereas others appear to be "dispensible" (6, 10).

The purpose of the present study was to investigate the penicillin and cephalosporin sensitivity of the most sensitive permeability mutant (*E. coli* DC2 [16]), isolated by Richmond et al., by comparing the concentration of a number of β -lactam antibiotics required to inhibit the PBPs by 50% with the antibacterial activity of the compounds against *E. coli* DC2 and its parent *E. coli* DC0.

MATERIALS AND METHODS

Bacterial strains. The antibiotic-supersensitive mutant *E. coli* DC2 and its parent *E. coli* DC0 were obtained from M. H. Richmond, of the Bacteriology Department at the University of Bristol. Both strains were grown in Penassay broth (Difco antibiotic medium no. 3) at 37°C with shaking.

Reagents. Methicillin and cloxacillin were purchased from Beecham Pharmaceuticals Ltd., and

[¹⁴C]penicillin G was from the Radiochemical Centre, Amersham. Unlabeled penicillin G, cephaloridine, and 1078/1/1 [(3S, 5R, 6R)-6-benzoylmethyl-2,2-dimethylpenam-3-carboxylic acid] were prepared in our own laboratories. Sarkosyl NL97 (sodium lauryl sarcosinate) was a gift from Ciba-Geigy Ltd. Acrylamide and methylene bisacrylamide, specially purified for electrophoresis were purchased from BDH Chemicals Ltd. Sodium lauryl sulfate (SDS) was purchased from Sigma Chemical Co. and was not "Analar" quality (8). All other reagents were obtained from standard commercial sources.

Determination of MIC. Minimal inhibitory concentrations (MICs) were determined by serial dilution of antibiotics in Penassay broth, inoculating the tubes with exponentially growing bacteria to a density of 10⁸ organisms per ml. The tubes were incubated at 37°C overnight, and MICs were recorded as the lowest concentration to significantly impair growth.

Preparation of bacterial envelopes and assay of PBPs. Envelopes were prepared from exponentially growing broth cultures of *E. coli* DC0 and DC2 by sonic oscillation as described by Spratt (21) and were stored in 10 mM sodium phosphate buffer (pH 7), at a protein concentration of 10 mg/ml at -70°C until required.

The binding of the various antibiotics to the PBPs of *E. coli* DC0 and DC2 was determined by competition with [¹⁴C]penicillin G exactly as described by Spratt (20, 21). Inner membrane proteins were solubilized by Sarkosyl treatment (5) and separated on a 10% SDS-polyacrylamide gel according to the method of Laemmli and Favre (7) as modified by Spratt (21). Gels were prepared for fluorography by the procedure of Bonner and Laskey (1) and exposed to prefogged Kodak X-Omat X-ray plates (8) at -70°C for 50 days. After development, 50% inhibition (I₅₀) values were obtained by densitometry on a Joyce Loebel scanning densitometer 3CS with peak integration.

RESULTS

Resistance of *E. coli* DC0 and DC2 to β -lactam antibiotics. The mutant *E. coli* DC2 was originally isolated on the basis of increased sensitivity to chloramphenicol (16). Table 1 shows that the mutant was considerably more sensitive than its parent to all the β -lactam antibiotics tested, with the exception of cephaloridine.

Comparison of the PBPs of *E. coli* DC0 and DC2. Figure 1 shows microdensitometer

TABLE 1. MICs of a number of β -lactam antibiotics against *E. coli* DC0 and DC2

Antibiotic	MIC (μ g/ml) against:	
	<i>E. coli</i> DC0	<i>E. coli</i> DC2
Methicillin	>250	4
Cloxacillin	>250	4
1078/1/1	>250	16
Penicillin G	16	1
Cephaloridine	2	2

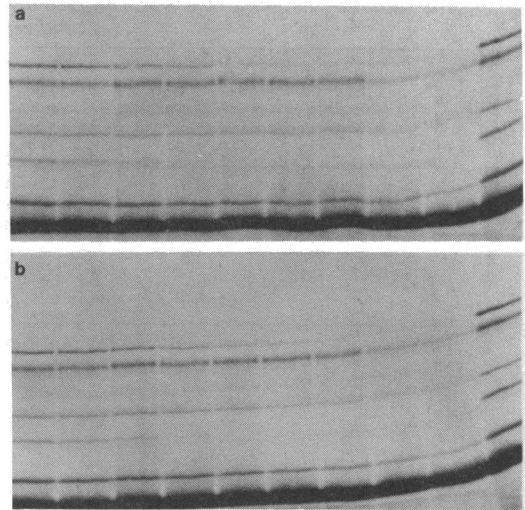


FIG. 1. Microdensitometer traces of the PBPs of *E. coli* DC0 (a) and DC2 (b).

traces of the PBPs of *E. coli* DC0 and DC2. No significant difference could be seen between the PBPs of the two strains.

Inhibition of the PBPs of *E. coli* DC0 and DC2 by β -lactam antibiotics. The concentration of the various β -lactam antibiotics required to reduce [¹⁴C]penicillin G binding to each of the PBPs of *E. coli* DC0 and DC2 by 50% is shown in Table 2. A typical example of the inhibition of the PBPs of the two strains by one of the compounds, methicillin, is shown in Fig. 2. The fluorograms for the other compounds are not shown. The nomenclature of the various PBPs follows that of Spratt (20), and it can be seen (Table 2) that no difference was observed between the mutant *E. coli* DC2 and its parent DC0 for any of the compounds tested. The different compounds showed different primary affinities for the different "essential" binding proteins. The essential PBP for which methicillin and cloxacillin had highest affinity was PBP3, the protein involved in cell division (22), and the antibiotics bound to this protein at a concentration correlating with the MIC of these derivatives for *E. coli* DC2 (Table 1). Penicillin G bound to all the essential PBPs, again at a concentration agreeing with the MIC against DC2.

The novel penicillin derivative 1078/1/1 showed highest primary affinity for PBP4, but since this protein is "dispensable" (6, 10), the compound acts through inhibition of PBP2, the essential protein involved in determining rod morphology (20, 25) and which is specifically inhibited by mecillinam (24). Once more, the I₅₀ for binding was comparable to the MIC of the

TABLE 2. Inhibition of the PBPs of *E. coli* DC0 and DC2 by cloxacillin, methicillin, 1078/1/1, penicillin G, and cephaloridine

PBP	Concn ($\mu\text{g/ml}$) of: ^a									
	Cloxacillin		Methicillin		1078/1/1		Penicillin G		Cephaloridine	
	DC0	DC2	DC0	DC2	DC0	DC2	DC0	DC2	DC0	DC2
1a	2	1.5	9	10	10	8	0.5	0.4	0.25	0.25
1b	23	22	250	250	240	250	3	4	2.5	3
2	15	13	27	30	6	5	0.8	1.1	50	64
3	3	2	5.5	7	75	82	0.9	1.2	8	5
4	125	110	60	64	8	6	1.0	1.3	17	13
5/6	64	70	>250	>250	60	70	30	22	>250	>250

^a Values indicate concentration of β -lactam antibiotic required to reduce [^{14}C]penicillin G binding by 50%.

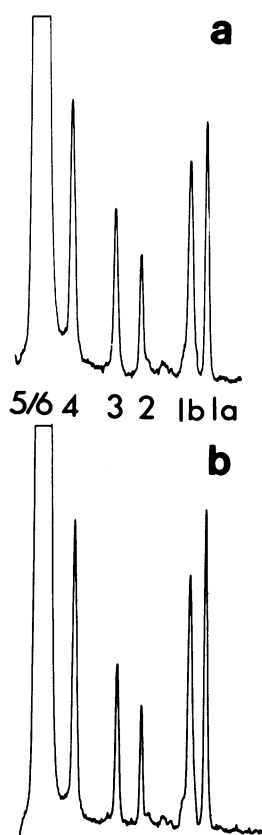


FIG. 2. Competition of methicillin for the PBPs of *E. coli* DC0 (a) and *E. coli* DC2 (b). The methicillin concentration was a doubling dilution from 1 to 250 $\mu\text{g/ml}$, and the last gel slot contained no methicillin in both cases.

compound against *E. coli* DC2 (Table 1). Cephaloridine had highest affinity for PBPs 1a and 1b, the proteins determining cell elongation (21, 25) and whose joint inhibition leads to rapid lysis (26). As with the other compounds, good correlation was found between the I_{50} for binding and the MIC against *E. coli* DC2.

The data in Table 2 indicate that the "target" proteins in both *E. coli* DC0 and DC2 are identical and show, moreover, that the compounds which are antibacterially inactive against the wild-type *E. coli* strain owe this inactivity to an inability to penetrate the envelope to the PBPs located in the cytoplasmic membrane (16, 17, 21). Cephaloridine, on the other hand, is equally active against the PBPs of both *E. coli* strains and has the same activity against intact bacteria of both strains, indicating that this derivative has little difficulty penetrating to the target, a result substantiated by other work (3, 16, 17).

DISCUSSION

The results described in this paper show that the lack of antibacterial activity of methicillin, cloxacillin, 1078/1/1 and, to a lesser extent, penicillin G against non- β -lactamase-producing strains of *E. coli* K-12 is due to impenetrability of the compounds through the outer membrane of the bacterial envelope.

The mutant strain *E. coli* DC2 is sensitive to these compounds and is at a level which correlates with the I_{50} against the essential PBPs. This in turn suggests that the permeability barrier present in the wild-type is lacking in the mutant. Obviously, an understanding of the chemical basis of the lesion in the mutant envelope would be extremely useful in designing penicillins and cephalosporins with favorable permeability properties in wild-type strains. However, at present this remains obscure. Preliminary results (N. Curtis and M. Boxall, unpublished data) indicate that the defect does not involve major outer membrane proteins, which have been shown to determine the rate of entry of certain molecules into gram-negative bacteria (11, 12, 14, 15), or envelope phospholipids (P. Lambert, personal communication). The mutation does, however, map near *purA* (D. C. Clark, personal communication). Whatever its basis, the sensitive mutant is extremely useful in the development of new β -lactam antibiotics. As

described above, at least three factors are involved in determining the antibiotic activity of β -lactam compounds against *E. coli*. Effective test systems are available for monitoring β -lactamase stability (19), and the PBP procedure can be used to study the inherent activity, or lack of it, of β -lactam compounds against the target proteins in the absence of permeability barriers. Methods for assessing penetrability are, however, less than adequate (16, 18). A comparison of the I_{50} values for a compound against the PBP_s of *E. coli* with the MIC gives a good indication of the penetration of the compound in intact bacteria, but the somewhat lengthy fluorography time renders the technique unsuitable for rapid screening of large numbers of novel penicillins and cephalosporins. A comparison of the MICs of such compounds against *E. coli* DC0 and DC2 gives a good estimation of permeability by a single MIC test and, moreover, the MIC against *E. coli* DC2 itself indicates the "intrinsic" activity, or lack of it, of β -lactam antibiotics against their "lethal" target binding proteins. Furthermore, by monitoring the morphological response of *E. coli* DC2 induced by β -lactam antibiotics, the "primary" PBP target can be determined. That is, spheroplasting indicates binding to PBP_s 1a/1b, round-form production indicates binding to PBP₂, and filamentation implies competition for PBP₃ (20.)

However, in the search for new semisynthetic β -lactam antibiotics with improved therapeutic efficacy, it may be desirable to rationally design compounds with increased affinity for specific PBP_s. To do this, an understanding of the structure-activity relationships governing affinity for the various PBP_s is necessary.

In this context, screening of new types of β -lactam compounds must provide information on the binding of the compounds to all the PBP_s of *E. coli*, for example, as well as to their primary target. This complete information can only be obtained by use of the PBP assay itself.

ACKNOWLEDGMENTS

We thank A. Wright and D. Kedgley for assistance in the development of fluorograms and M. H. Richmond and G. W. Ross for suggestions during the preparation of this paper.

LITERATURE CITED

- Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83-88.
- Bowman, H. G., K. Nordstrom, and S. Normark. 1974. Penicillin resistance in *Escherichia coli* K-12: synergism between penicillins and a barrier in the outer part of the envelope. *Ann. N.Y. Acad. Sci.* **235**:569-585.
- Curtis, N. A. C., J. M. Hughes, and G. W. Ross. 1976. Inhibition of peptidoglycan cross-linking in growing cells of *Escherichia coli* by penicillins and cephalosporins, and its prevention by R-factor-mediated beta-lactamase. *Antimicrob. Agents Chemother.* **9**:208-213.
- Curtis, N. A. C., M. H. Richmond, and R. B. Sykes. 1972. "Periplasmic" location of a β -lactamase specified either by a plasmid or a chromosomal gene in *Escherichia coli*. *J. Bacteriol.* **112**:1433-1434.
- Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J. Bacteriol.* **115**:717-722.
- Iwaya, M., and J. L. Strominger. 1977. Simultaneous deletion of D-alanine carboxypeptidase 1B-C and penicillin-binding component IV in a mutant of *Escherichia coli* K-12. *Proc. Natl. Acad. Sci. U.S.A.* **74**:2980-2984.
- Laemmli, U. K., and M. Favre. 1973. Maturation of the head of bacteriophage T4. 1. DNA packaging events. *J. Mol. Biol.* **80**:575-599.
- Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56**:335-341.
- Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen. 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* into four bands. *FEBS Lett.* **58**:254-258.
- Matsuhashi, M., Y. Takagaki, I. N. Maruyama, S. Tamaki, Y. Nishimura, H. Suzuki, U. Ogino, and Y. Hirota. 1977. Mutants of *Escherichia coli* lacking in highly penicillin-sensitive D-alanine carboxypeptidase activity. *Proc. Natl. Acad. Sci. U.S.A.* **74**:2976-2979.
- von Meyenburg, K., and H. Nikaido. 1977. Outer membrane of gram-negative bacteria. XVII. Specificity of transport process catalysed by the λ -receptor protein of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **78**:1100-1107.
- Nakae, T. 1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. *Biochem. Biophys. Res. Commun.* **71**:877-884.
- Nguyen-Disteche, M., J. J. Pollock, J. M. Ghuysen, J. Puig, P. Reynolds, H. R. Perkins, J. Coyette, and M. R. Salton. 1974. Sensitivity to ampicillin and cephalothin of enzymes involved in wall peptide cross-linking in *Escherichia coli* K-12, strain 44. *Eur. J. Biochem.* **41**:457-463.
- Nikaido, H. 1976. Outer membrane of *Salmonella typhimurium*. Transmembrane diffusion of some hydrophobic substances. *Biochim. Biophys. Acta* **433**:118-132.
- Nikaido H., S. A. Song, L. Shalitel, and M. Nurminen. 1977. Outer membrane of salmonella. XIV. Reduced transmembrane diffusion rates in porin-deficient mutants. *Biochem. Biophys. Res. Commun.* **72**:324-330.
- Richmond, M. H., D. C. Clark, and S. Wotton. 1976. Indirect method of assessing the penetration of β -lactamase-nonsusceptible penicillins and cephalosporins in *Escherichia coli* strains. *Antimicrob. Agents Chemother.* **10**:215-218.
- Richmond, M. H., and N. A. C. Curtis. 1974. The interplay of β -lactamases and intrinsic factors in the resistance of gram-negative bacteria to penicillins and cephalosporins. *Ann. N.Y. Acad. Sci.* **235**:533-567.
- Richmond, M. H., and R. B. Sykes. 1973. The β -lactamases of gram-negative bacteria and their possible physiological role. *Adv. Microb. Physiol.* **9**:31-88 1973.
- Ross, G. W., and C. H. O'Callaghan. 1975. β -Lactamase assays. *Methods Enzymol.* **43**:69-85.
- Spratt, B. G. 1975. Distinct penicillin binding proteins involved in the division, elongation and shape of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **72**:2999-3003.
- Spratt, B. G. 1977. Properties of the penicillin binding proteins of *Escherichia coli*. *Eur. J. Biochem.* **72**:341-352.
- Spratt, B. G. 1977. Temperature-sensitive cell division mutants of *Escherichia coli* with thermolabile penicillin

- lin-binding proteins. *J. Bacteriol.* **131**:293-305.
23. **Spratt, B. G.** 1977. Penicillin-binding proteins of *Escherichia coli*: general properties and characterization of mutants, p. 182-190. In D. Schlessinger (ed.), *Microbiology—1977*. American Society for Microbiology, Washington, D.C.
 24. **Spratt, B. G., and A. Pardee.** 1975. Penicillin-binding proteins and cell shape in *E. coli*. *Nature (London)* **254**: 516-517.
 25. **Suzuki, H., Y. Nishimura, and Y. Hirota.** 1978. On the process of cell division in *Escherichia coli*: a series of mutants of *E. coli* altered in the penicillin-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* **75**:664-668.
 26. **Tamaki, S., S. Nakajima, and M. Matsuhasi.** 1977. Thermosensitive mutation in *Escherichia coli* simultaneously causing defects in penicillin-binding proteins 1B's and in enzyme activity for peptidoglycan synthesis in vitro. *Proc. Natl. Acad. Sci. U.S.A.* **74**:5472-5476.
 27. **Zimmermann, W., and A. Rosselet.** 1977. Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. *Antimicrob. Agents Chemother.* **12**:368-372.