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# Indirect Method for Assessing the Penetration of Beta-Lactamase-Nonsusceptible Penicillins and Cephalosporins in *Escherichia coli* Strains

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*Escherichia coli* UB1005 and two mutants of this strain (DC2 and DC3) have been used to assess indirectly the relative ability of various β-lactam antibiotics to penetrate the outer layers of *E. coli*. Benzylpenicillin, ampicillin, methicillin, cloxacillin, cephaloridine, cephalothin, and cephalexin have been examined. The results confirm those obtained with other methods and show that, among the compounds studied here, cephalosporins seem to penetrate more readily than penicillins.

One important feature that affects the activity of β-lactam antibiotics against gram-negative bacteria is their ability to pass through the outer layers of the organisms to their targets in the inner membrane (4, 14). Unfortunately, direct measurements on penetration, for example, by following the uptake of radiolabeled antibiotics, are unreliable because of the high levels of nonspecific adsorption (3, 5, 18). As a result, indirect methods must be used.

One such method depends on a determination of "crypticity." For β-lactam antibiotics, this property is measured as the extent to which the β-lactamase activity of a bacterial culture increases when the permeability barriers in the cell envelope are disrupted (14, 15). The validity of this method for assessing penetration is based on the assumption that the passage of the compound to the β-lactamase in the periplasmic space reflects the properties of the barrier(s) that the antibiotic also encounters on its way to its target in the bacterial inner membrane (15).

Although useful under certain circumstances, crypticity determinations do have a number of important disadvantages (14, 17). Perhaps the most serious in the context of this work is that the method can only be used to evaluate substances against which enzyme used in the test is active. Since many of the latest generation of β-lactam compounds (notably, some cephalosporins [C. H. O'Callaghan, R. B. Sykes, D. M. Ryan, R. D. Foord, and P. W. Muggleton, J. Antibiot., in press] and most cephamycins [11]) are resistant to the enzymes available for use, the crypticity method is unlikely to have much long-term practical value.

This paper describes the evaluation of a series of mutant *Escherichia coli* strains that can be used to provide an alternative method of assessing the penetration of the outer layers of *E. coli* by β-lactam antibiotics, including those resistant to β-lactamases.

## MATERIALS AND METHODS

**Mutant strains and their isolation.** Both mutants used in these studies (DC2 and DC3) were obtained from *E. coli* strain UB1005 (alias EC1005), a nalidixic acid-resistant mutant of *E. coli* W1655 (7), as isolates abnormally susceptible to chloramphenicol. On examination they proved to be abnormally susceptible to penicillins as well (see Results). Mutagenesis was performed with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine under standard conditions (1), and the treated bacteria were selected by penicillin selection in liquid medium containing 1 μg of chloramphenicol and 200 μg of ampicillin per ml. After overnight growth at 37°C, the surviving bacteria in this culture were plated on unsupplemented nutrient agar to give single colonies, and these colonies were then replica plated onto nutrient agar containing 1 μg of chloramphenicol per ml. Colonies growing on the master plates, but not on the replicas, were then picked, and their properties were checked in detail.

**Crypticity measurements.** The theoretical basis of this measurement has been discussed in detail elsewhere (14, 17). In practice, the value is obtained by measuring the activity of a bacterial suspension against a given penicillin or cephalosporin both before and after disruption and dividing the activity of the broken preparation by the unbroken activity (14). Thus, crypticity = activity in milligrams (dry weight) of broken bacteria/activity in milligrams (dry weight) of intact bacteria.

Strain UB1005, and the mutants DC2 and DC3 derived from it, expressed less than 0.1 unit of β-lactamase activity/mg (dry weight) of bacteria when enzyme activity was assayed with either benzylpen-

icillin or cephaloridine. To measure the crypticity of such strains towards β-lactam antibiotics, it was therefore necessary to transfer the gene(s) needed to specify the synthesis of an appropriate periplasmic β-lactamase into them. The enzyme chosen in this case was the type IIIa β-lactamase (6, 15), an enzyme known to have a periplasmic location in *E. coli* (8–10). The necessary genetic information to produce this enzyme was transferred to strain UB1005, and to the mutants DC2 and DC3, by mating with *E. coli* W3110(RP1) as donor under standard conditions for an overnight mating experiment at 37°C (7). After this period, the mating mixture was plated on agar containing 40 μg of nalidixic acid and 50 μg of carbenicillin per ml to select recipient clones that had received the R plasmid.

Crypticity measurements were carried out as follows. A culture of the strain under test was grown exponentially in nutrient broth at 37°C with shaking until a bacterial density of about $10^8$ organisms/ml had been reached. The culture was then centrifuged at 5,000 × *g* for 15 min to collect bacteria, and these were resuspended to a density of about 10 mg (dry weight)/ml in 0.1 M $KH_2PO_4/K_2HPO_4$ buffer, pH 7.0. A sample was removed from this suspension for assay of β-lactamase activity of the intact bacteria, and the remainder of the suspension was disrupted for 5 min in an ultrasonic disintegrator (25Kc/s, Mullard Instruments, Crawley, England). After disruption, the suspension was stored on ice until required for assay. The assay of the intact bacteria was carried out as soon as possible to minimize leakage of enzyme from the bacteria.

**β-Lactamase assays.** All β-lactamase assays were carried out by the iodometric method (12) using substrate concentrations of 2.5 mg/ml dissolved in 0.1 M $KH_2PO_4/K_2HPO_4$ buffer, pH 7.0. The results of these assays were calculated on the assumption that, after hydrolysis, 1 mol of a penicillin reacts with eight equivalents of iodine solution, whereas the equivalent value for a hydrolyzed cephalosporin is four (2).

## RESULTS

**Comparative properties of UB1005 and the mutants DC2 and DC3.** Although DC2 and DC3 were isolated as abnormally susceptible mutants on agar containing chloramphenicol, they also showed reduced minimum inhibitory concentration (MIC) values for a range of other antibiotics when compared with strain UB1005. Among these were several penicillins, notably, ampicillin and cloxacillin. The only β-lactam compounds tested that were not more active against the mutants than against the parental strain were cephaloridine, cephalothin, and cephalexin. These results are summarized in Table 1 (see also Fig. 1).

**Crypticity determinations on UB1005 and on DC2 and DC3.** Since the mutants DC2 and DC3 were more susceptible than UB1005 to a range of antibiotic substances, it seemed possible that the mutants were altered in a broadly nonspecific way to allow freer access of these substances to their targets in the bacterial cell. One method of confirming that this was so for β-lactam compounds was to determine the crypticity values of the mutants towards an appropriate penicillin and to compare it with the equivalent value for strain UB1005.

Crypticity determinations suffer a number of shortcomings, but their accuracy is greatest when used for comparative purposes with an enzyme that has an affinity for the antibiotic being used in the test of greater than $5 \times 10^{-4}$ M and when the enzyme activity of the unbroken bacteria is high enough to be measured accurately (17). In *E. coli* K-12 these conditions are best met when the plasmid RP1 is used with benzylpenicillin as substrate. Accordingly, the crypticity values for strain UB1005 (RP1-1) and for the mutants DC2 (RP1-1) and DC3 (RP1-1) were determined with benzylpenicillin as substrate. Table 2 shows the results of these measurements. Strain UB1005 (RP1-1) gave a crypticity value of 55 with benzylpenicillin in this test, whereas DC2 (RP1-1) and DC3 (RP1-1) gave values of 2.5 and 20, respectively. These results suggest, therefore, that the abnormal susceptibility of DC2 and DC3 to benzylpenicillin arises from a reduced ability to prevent this antibiotic from passing through the outer layers of the cell envelope to its target in the bacterial inner membrane.

TABLE 1. *MIC values obtained with E. coli UB1005 and with the mutants DC2 and DC3 against a range of antibacterial substances*

| Antibiotic | MIC (μg/ml)[a] | | |
|---|---|---|---|
| | UB1005 | DC3 | DC2 |
| Chloramphenicol | 8 | 1 | 0.5 |
| Benzylpenicillin | 25 | 10 | 1.2 |
| Ampicillin | 8 | 4 | 0.5 |
| Cloxacillin | 500 | 32 | 2.0 |
| Cephaloridine | 2 | 2 | 2 |
| Cephalothin | 2 | 2 | 1 |
| Novobiocin | 200 | 25 | 5 |
| Rifampin | 16 | 8 | 4 |
| Tetracycline | 4 | 2 | 1 |

[a] MIC values were obtained by plating bacteria as single colony-forming units on the surface of agar containing the antibiotic in question.

TABLE 2. *Crypticity values for E. coli UB1005 (RP1) and the mutant strains DC2 (RP1) and DC3 (RP1)*

| Substrate | Crypticity value | | |
|---|---|---|---|
| | UB1005 (RP1) | DC2 (RP1) | DC3 (RP1) |
| Benzylpenicillin | 55 | 20 | 2.5 |
| Cephaloridine | 1.7 | 1.2 | 1.2 |

Table 1 also shows that, unlike the results with penicillins, DC2 and DC3 were no more susceptible to cephaloridine than was strain UB1005, and it was therefore important to see whether the crypticity of the mutants to this antibiotic was altered or not. The crypticity values for strains DC2 (RP1-1) and DC3 (RP1-1) were therefore measured with cephaloridine as substrate, and the results were compared with that obtained with strain UB1005 (RP1-1). With this substrate no difference in crypticity between the parental and either of the mutant strains could be detected (Table 2), and this observation was consistent with the similar MIC values obtained with the three strains in the presence of cephaloridine.

**Use of DC2 and DC3 to compare the penetration of various penicillins and cephalosporins.** The implication of these results is that certain β-lactam antibiotics (notably, cephaloridine) can pass freely through the envelope of unmutated *E. coli* strains, whereas penicillins are impeded, in some cases severely. Moreover, substrates that show a range of different MIC values with UB1005, DC2, and DC3 must also be impeded by this barrier, whereas, in contrast, those that show a similar MIC against these three strains are indifferent to the barrier. Mutants DC2 and DC3 may therefore be used, in conjunction with strain UB1005, in a standard test to determine whether penicillins and cephalosporins are impeded in their entry into *E. coli*.

Figure 1 shows the results obtained with the three test strains and a range of penicillins and cephalosporins. The crypticity values of the test strains towards benzylpenicillin has been used as an index of the extent to which the cells' penetration barrier has been impaired by mutation. In practice, two broad types of response have been obtained (see Fig. 1a and b). Figure 1a shows the results obtained with penicillins. With methicillin and cloxacillin the difference in MIC between UB1005 and the mutant DC2 is about 250-fold, and this argues that these compounds are powerfully excluded from unmutated *E. coli*. Furthermore, the high level of activity of the penicillins against DC2 argues that both these penicillins would be very active against naturally occurring *E. coli* strains if the antibiotics were able to reach their target in the bacterial inner membrane. Figure 1a also contains results for benzylpenicillin and ampicillin. These compounds are also substantially more active against DC2 than against UB1005, although the difference is not as marked as with methicillin and cloxacillin.

Figure 1b shows the results obtained with cephaloridine, cephalexin, and cephalothin. In this case, the mutant strains were no more than twice as susceptible as strain UB1005, and even this minimal difference was not observed with cephaloridine. In contrast to the penicillins, therefore, these three cephalosporins seem to exercise a similar effect on *E. coli* whether or not the barrier to penicillin entry is damaged. Furthermore, this behavior argues that cephaloridine, cephalexin, and cephalothin express their full potential in inhibiting cell wall synthesis in *E. coli* when tested against naturally occurring strains.

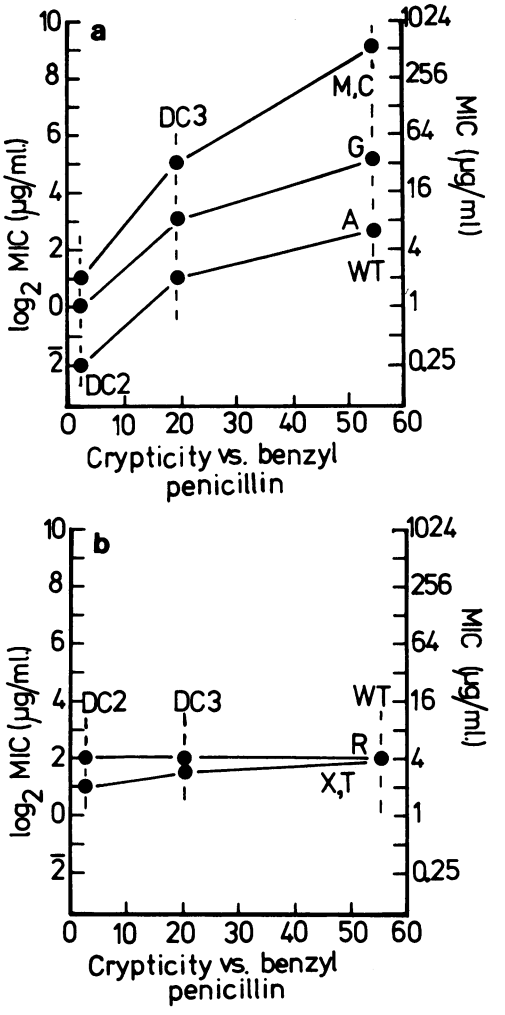


FIG. 1. *Relative MIC values obtained with β-lactam antibiotics acting on UB1005 and the mutants DC2 and DC3. WT, strain UB1005. (a) Penicillins: A, ampicillin; G, benzylpenicillin; C, cloxacillin; M, methicillin. (b) Cephalosporins: R, cephaloridine; T, cephalothin; X, cephalexin. The crypticity values used in these figures were determined using UB1005 (RP1), DC2 (RP1), and DC3 (RP1).*

## DISCUSSION

The activity of β-lactam antibiotics on a gram-negative bacterial strain depends on three main properties: first, its inherent activity against the enzymes in the bacterial inner membrane that are related to peptidoglycan biosynthesis (D,D-transpeptidase and carboxypeptidase); second, its ability to pass through the outer layers of the bacterial cell envelope to reach its target; and third, its ability to resist hydrolysis by β-lactamases that the antibiotic may encounter on the way to its target. Any full evaluation of a new β-lactam antibiotic, therefore, requires measurements to be made on each of these three crucial processes.

Methods for assessing the effect of β-lactamases on new β-lactam antibiotics have already been well worked out (16), and effective in vitro test systems are also available to evaluate the action of these compounds directly on transpeptidase and carboxypeptidase enzyme systems from the inner membrane (13). Only with the measurements of penetration are methods still less than adequate, largely because the β-lactam group of antibiotics are so reactive chemically that attempts to measure their uptake by conventional radiolabeling methods give levels of nonspecific adsorption so high that interpretation of the results is very difficult on a routine basis (3, 5, 18).

The method for assessing penetration described here is an advance on the "crypticity method" used hitherto (4, 14, 15, 17). Not only is it less sensitive to the growth conditions of the culture, but it can also be used for β-lactam antibiotics completely nonsusceptible to β-lactamase hydrolysis, and this ability is likely to be of increasing importance as more β-lactamase-nonsusceptible penicillins and cephalosporins are developed. Another advantage of the method described here is that the determination only involves the measurement of MIC values on agar plates, a technique widely available in microbiology laboratories. The main disadvantage of the method is that the measurement is indirect, and compounds are evaluated in comparison with antibiotics whose properties must be known in detail in advance. With β-lactam actibiotics this limitation is not too severe, since a number of penicillins and cephalosporins (such as ampicillin, cephaloridine, and cephalothin) are widely available and have already been studied extensively.

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