Inhibition of Peptidoglycan Cross-Linking in Growing Cells of *Escherichia coli* by Penicillins and Cephalosporins, and Its Prevention by R Factor-Mediated Beta-Lactamase

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The degree of peptidoglycan cross-linking has been studied in growing cells of a Dap⁻ Lys⁻ auxotroph of *Escherichia coli* K-12 by following the incorporation of [³H]diaminopimelic acid into the lysozyme digestion products of crude, isolated peptidoglycan. The percentage of inhibition of cross-linking increases with increasing concentrations of penicillin G, cephaloridine, and cefuroxime. When the R factor R1drd 19 was introduced into the strain by conjugation, it was found that the type IIIa, β -lactamase specified by the plasmid was able to protect the cross-linking target against inhibition by penicillin G but not against cephaloridine, even though the β -lactamase hydrolyzes this substrate 50% faster than penicillin G. Cefuroxime, which is completely resistant to hydrolysis by the type IIIa β -lactamase, inhibited the peptidoglycan cross-linking target in both the R⁺ and R⁻ variants of the assay strain. A mutant plasmid, R1drd19amp², which specified no type IIIa β -lactamase synthesis, could not provide protection of the cross-linking target against penicillin G. The significance of these results, in relation to the ability of the antibiotics to pass the permeability barrier of the bacterial envelope, is discussed.

The lytic effect of penicillins and cephalosporins upon growing cells of *Escherichia coli* is believed to be due to inhibition of the transpeptidation or cross-linking reaction that occurs between neighboring oligosaccharide strands of the cell envelope peptidoglycan (6). The enzyme(s) responsible for this reaction is located in the cytoplasmic membrane of *E. coli* (12), and, to inhibit cross-linking, penicillin and cephalosporin molecules must permeate the various surface layers to this inner part of the bacterial envelope.

In an attempt to quantify the ability of a penicillin or cephalosporin to penetrate the permeability barrier of the cell envelope, the concept of "crypticity" has been used (13, 14). One way of measuring crypticity is to insert a gene specifying the synthesis of a β -lactamase into the strain, usually by R-factor transfer and then to determine the accessibility of the intracellular (periplasmic) enzyme to its extracellular substrate by comparing the β -lactamase activity of intact and broken bacteria. However, such measurements can only be performed with substrates that are susceptible to β -lactamase action and, at best, only give an indication of the ease with which a penicillin or cephalosporin can reach the periplasmic space, and not

necessarily the target located in the cytoplasmic membrane.

This study was intended to investigate in vivo the availability of the cross-linking target to penicillin G, cephaloridine, and cefuroxime (sodium (6R, 7R)-3-carbamoyloxymethyl-7-[(2z)-2-methoximino(fur - 2 - yl)acetamido] - ceph - 3 em-4-carboxylate) and the ability of a given R factor-mediated β -lactamase to prevent inhibition of cross-linking.

MATERIALS AND METHODS

Unlabeled diaminopimelic acid (Dap) (a mixture of LL, DD, and *meso* isomers), lysozyme, and trypsin were obtained from the Sigma Chemical Co. Ltd. [³H]Dap was obtained from the Radiochemical Centre, Amersham, and was assumed to contain LL, DD, and *meso* isomers in equal proportions (2). Rifampin was purchased from Lepetit Pharmaceuticals Ltd., Slough.

Bacterial strains. The strain used to measure peptidoglycan cross-linking in vivo was *E. coli* W7(W) (Dap⁻ Lys⁻) (4) kindly provided by J. P. Beard, Department of Bacteriology, The Medical School, University of Bristol.

The R factors R1drd19 (Ap Sm Cm Km Su) (8) and R1drd19amp² (Sm Cm Km Su) (3) were maintained in *E. coli* 1005 (Met⁻ Nal^r) (laboratory stock strain, University of Bristol). All strains were maintained

on nutrient agar slopes supplemented with *meso*-Dap (10 μ g/ml) in the case of *E. coli* W7(W) and with chloramphenicol (25 μ g/ml) for both R factor-carrying derivatives of *E. coli* 1005.

Media. The liquid and solid media were Oxoid nutrient broth no. 2 and Oxoid blood base agar, respectively. Supplements of Dap and antibiotics were added, where appropriate, directly to the media and dissolved in a minimal volume of sterile distilled water.

Selection of rifampin-resistant mutants of *E. coli* W7(W). An overnight culture of *E. coli* W7(W), grown in nutrient broth containing 10 μ g of Dap per ml, was subcultured into the same medium and incubated with shaking at 37 C, until the density was approximately 10^8 bacteria/ml. The bacteria were washed twice in broth by centrifugation and finally suspended at a density of 10^8 to 10^{10} bacterial/ml. Aliquots (0.1 ml) of this suspension were seeded onto nutrient agar plates containing Dap (10 μ g/ml) and rifampin (100 μ g/ml) and were then incubated at 37 C overnight. Colonies growing after this period were picked into broth containing Dap (10 μ g/ml) and grown at 37 C to a density of about 10^8 bacteria/ml, and their rifampin resistance and Dap dependence were checked. One isolate showing both required characters was retained.

Transfer of plasmids to *E. coli* W7(W). The plasmids R1drd19 and R1drd19amp2 were transferred by conjugation from their host strain, *E. coli* 1005 (Met⁻ Nal^R), to the *E. coli* W7(W) Rif^R recipient as follows: the donor and recipient strains were grown overnight in 10 ml of nutrient broth [containing 10 μ g of Dap per ml in the case of *E. coli* W7(W)] in universal bottles at 37 C, with shaking. The cultures were diluted 50-fold in identical, prewarmed media and grown to a density of approximately 10^8 bacteria/ml.

At this point, volumes of the donor and recipient cultures were mixed in a ratio of 2:1, respectively, and the cultures were maintained statically at 37 C to allow mating to proceed.

After 3 h, the cultures were vortexed briefly, to separate mating pairs, and then serially diluted in fresh broth containing Dap (10 μ g/ml). Aliquots (0.1 ml) of the dilutions were spread over the surface of nutrient agar plates containing Dap (10 μ g/ml), rifampin (100 μ g/ml), and chloramphenicol (25 μ g/ml). These plates were then incubated at 37 C overnight, and transcient colonies growing after this time were purified once more on the selective media and retained.

Preparation of crude β -lactamase. An overnight broth culture of *E. coli* W7(W), with or without the appropriate R factor, was diluted 100-fold in fresh, prewarmed nutrient broth containing Dap (10 μ g/ml) and incubated at 37 C, with shaking, until the optical density (675 nm) was 1.0 to 1.3 (1 mg/ml [dry weight]). A volume (100 ml) of *E. coli* W7(W) (R1drd19) was disrupted by ultrasound treatment for 4 min at 20 kHz with ice-water cooling (100-W MSE Ultrasonic generator). The other two cultures were concentrated 10-fold before disruption, and the resulting broken bacterial cultures were assayed for

β -lactamase activity without further purification.

Measurement of β -lactamase activity. The iodometric assay of Perret (11) as modified by Novick (9) was used to determine the level and substrate profile of the β -lactamase specified by R1drd19 in *E. coli* W7(W) and to obtain the crypticity values for penicillin G and cephaloridine in this strain. The assay was performed at 37 C, and the substrates (2.5 mg/ml) were dissolved in phosphate buffer, pH 7.0.

The spectrophotometric assay (10) with the chromogenic cephalosporin substrate 87/312 was used to determine the amount of β -lactamase activity produced by *E. coli* W7(W) and also that specified by the mutant plasmid R1drd19amp2 in this strain.

Determination of crypticity. The crypticity value of a given β -lactamase to a given substrate is defined as " β -lactamase activity of broken bacteria/ β -lactamase activity of unbroken bacteria" (13, 14) and was measured by performing standard iodometric assays with penicillin G or cephaloridine as substrates, using appropriate volumes of an unbroken and ultrasonically disrupted culture of *E. coli* W7(W) (R1drd19).

Determination of minimum inhibitory concentration. Minimum inhibitory concentrations for penicillin G, cephaloridine, and cefuroxime against the various R⁺ and R⁻ derivatives of *E. coli* W7(W) were determined by the tube dilution technique, in nutrient broth containing Dap (5 μ g/ml) at inoculum of 10^8 bacteria/ml. The conditions used were kept as close as possible to those used in the in vivo assay of peptidoglycan cross-linking.

In vivo assay of peptidoglycan cross-linking. The procedure used to follow peptidoglycan cross-linking in vivo was based on that of Kamiryo and Strominger (7). *E. coli* W7(W), with or without the appropriate plasmid, was grown overnight in nutrient broth containing Dap (5 μ g/ml). A sample (30 ml) was spun down in universal bottles in a bench centrifuge and washed twice with fresh, prewarmed nutrient broth. The bacteria were finally resuspended in fresh broth (20 ml) and incubated at 37 C with shaking. Aliquots (0.05 ml) of this stock culture were used to inoculate 2-ml broth cultures that contained [³H]Dap (5 μ g/ml) and incorporated appropriate concentrations of the various antibiotics. At the same time, two control cultures, one containing cold Dap (5 μ g/ml) and the other no Dap, were inoculated and all cultures were incubated at 37 C with shaking. Examination of the control culture lacking added Dap showed that lysis occurred once the endogenous pool of Dap has been exhausted (1), usually after about 1 h. After 2 to 3 h of incubation, the experimental cultures were precipitated with 50% (wt/vol) trichloroacetic acid (0.25 ml) and heated in a boiling-water bath for 5 min. The precipitates were spun down and washed twice with 2 ml of 0.1 M ammonium acetate (pH 7.1) and, after the addition of 60 μ l of trypsin (2 mg/ml in 0.1 M ammonium acetate-0.1 mM ethylenediaminetetraacetate, pH 7.9), incubated statically at 37 C for 2 h. The suspensions were then boiled in a water bath for 5 min to destroy trypsin activity, and, after cooling to room temperature, 40 μ l of lysozyme (10

mg/ml in the ammonium acetate-ethylenediamine-tetraacetate buffer) was added and incubation at 37 C was continued overnight. This material was then loaded onto Whatman 3MM filter paper and developed overnight in isobutyric acid-1 N NH₄OH (5:3, vol/vol) by descending chromatography. The chromatograms were dried and fractionated into 1-cm units, which were counted in a Nuclear-Chicago scintillation counter using Triton-toluene-water scintillant (Triton X-100, 500 ml, and distilled water, 120 ml, were added to 1 liter of toluene containing 6 g of butyl-PBD). The *R_f* values of the dimer (*N*-Ac-glucosamine-*N*-Ac-muramyl-peptide-muramyl-Ac-*N*-glucosamine-Ac-*N*) and monomer (*N*-Ac-glucosamine-*N*-Ac-muramyl-tetrapeptide) peaks were 0.15 and 0.35, respectively (7).

RESULTS

β -Lactamase activity of *E. coli* W7(W) with and without the plasmids R1drd19 and R1drd19-amp². The rates of hydrolysis of selected β -lactam substrates by the β -lactamase specified by R1drd19 in *E. coli* W7(W) are as follows: penicillin G, 100; ampicillin, 175; carbenicillin, 10; cephaloridine, 146; cefuroxime, 0 (rates expressed as a percentage of penicillin G hydrolysis). These rates confirm that the enzyme is a type IIIa β -lactamase (14).

The level of β -lactamase activity (expressed as units per milligram [dry weight] of bacteria) present in *E. coli* W7(W) carrying the plasmid R1drd19 was determined by the iodometric assay. This assay could detect no activity in either the R⁻ parent or in the derivative carrying the mutant plasmid R1drd19-amp², but the spectrophotometric assay (10) is a much more sensitive procedure and was used to measure the extremely low levels expressed by these strains (Table 1). This low level of activity is likely to be the chromosomally mediated type Ib β -lactamase detectable in all strains of *E. coli* K-12 (14), especially since it could be inhibited by 10⁻⁵ M carbenicillin (N. Curtis, personal observation).

Resistance of strains to penicillin G, cephaloridine, and cefuroxime. The plasmid R1drd19 conferred considerable resistance to penicillin G but not to cephaloridine or cefuroxime (Table 2). As expected, the mutant plasmid R1drd19-amp², which lacks a functional β -lactamase gene, provided no additional resistance to penicillin G in *E. coli* W7(W).

Crypticity values. The crypticity values for penicillin G and cephaloridine against the β -lactamase specified by R1drd19 in *E. coli* W7(W) are 21 and 1, respectively. Penicillin G has a high crypticity value, whereas cephaloridine shows virtually no crypticity at all. The value for cefuroxime was not determinable, since this compound is stable to the type IIIa β -lactamase.

Peptidoglycan cross-linking, its inhibition by penicillin G, cephaloridine, and cefuroxime and its protection by β -lactamase. Strain W7(W) of *E. coli* K-12 is a Dap- and lysine-requiring auxotroph (2, 5) and thus incorporates [³H]Dap exclusively into peptidoglycan. The in vivo assay of Kamiryo and Strominger (7), which has been adapted for this study, allows determination of the amount of [³H]Dap incorporated into the principal lysozyme digestion products of peptidoglycan, i.e., monomer and dimer.

Under normal growth conditions, the ratio of dimer to monomer was 0.98 (itself highly reproducible); this ratio decreased with increasing concentrations of penicillin G, cephaloridine, or cefuroxime. The percentage of inhibition of cross-linking at a given antibiotic concentration is thus the dimer-to-monomer ratio observed at that concentration expressed as a percentage of the ratio observed in the uninhibited control.

The results plotted in Fig. 1, 2, and 3 show that the β -lactamase mediated by the plasmid R1drd19 is able to protect the sensitive peptidoglycan cross-linking target of *E. coli* W7(W) against inhibition by penicillin G but not against cephaloridine or cefuroxime. The mutant plasmid R1drd19-amp², which specifies no

TABLE 1. β -Lactamase activity specified by R1drd19, R1drd19-amp², and the R⁻ derivatives of *E. coli* W7(W)

Strain	β -Lactamase activity (U/mg dry wt of cells) ^a	
	Iodometric	Spectrophotometric
<i>E. coli</i> W7(W)	ND	0.11
<i>E. coli</i> W7(W) (R1drd19)	26	NT
<i>E. coli</i> W7(W) (R1drd19-amp ²)	ND	0.10

^a ND, Not detectable; NT, not tested.

TABLE 2. Minimum inhibitory concentrations (MICs) of *E. coli* W7(W) with and without the plasmids R1drd19 and R1drd19-amp² against penicillin G, cephaloridine, and cefuroxime

Strain	MIC (μ g/ml)		
	Penicillin G	Cephaloridine	Cefuroxime
<i>E. coli</i> W7(W)	20	2	4
<i>E. coli</i> W7(W) (R1drd19)	500	4	4
<i>E. coli</i> W7(W) (R1drd19-amp ²)	20	2	2

FIG. 1. Percentage of inhibition of peptidoglycan cross-linking, as a function of penicillin G concentration, in *E. coli* W7(W), in the R^+ (β -lactamase-producing) variant *E. coli* W7(W)(R1drd19), and in the derivative carrying the mutant plasmid R1drd19amp2, which produces no β -lactamase.

β -lactamase synthesis, cannot provide protection against penicillin G and further indicates that this plasmid, unlike others (3), does not increase the intrinsic resistance of its host strain to penicillins.

DISCUSSION

Cephaloridine has the ability to lyse growing cells of β -lactamase-producing strains of *E. coli*, for example, *E. coli* W7(W) (R1drd19), at a concentration nearly identical to that required to inhibit the R^- parent. In contrast, the β -lactamase specified by R1drd19 can provide marked protection of *E. coli* strains against penicillin G. This situation appears anomalous, since cephaloridine is hydrolyzed nearly 50% faster than penicillin G by the β -lactamase *in vitro*. However, this study has shown that the efficacy of cephaloridine against β -lactamase-producing strains of *E. coli* is due to its ability to

enter the bacterium rapidly and to inhibit the peptidoglycan cross-linking target. Under these conditions, the periplasmic β -lactamase can provide little protection. The reverse seems to be the case for penicillin G, where high envelope impermeability restricts the entry of the drug to a rate at which the β -lactamase can deal with it. The good antibiotic activity of cefuroxime against both R^+ (β -lactamase producing) and R^- strains of *E. coli* is due to the complete stability of the drug to the type IIIa β -lactamase. Unfortunately, this precludes estimation of penetrability by crypticity determination, but it is believed (R. B. Sykes, personal communication) that the antibiotic enters *E. coli* cells less rapidly than cephaloridine but more rapidly than penicillin G.

At minimum inhibitory concentrations of all three antibiotics against all strains, the percentage of inhibition of peptidoglycan cross-

Fig. 3. Percentage of inhibition of peptidoglycan cross-linking as a function of cefuroxime concentration. The strains are as Fig. 1.

Fig. 2. Percentage of inhibition of peptidoglycan cross-linking as a function of cephaloridine concentration. The strains are as Fig. 1.

linking was about 50 to 60% under the described assay conditions. Our results suggest that the peptidoglycan of *E. coli* W7(W) is cross-linked by about 50 to 60% more than is required to prevent osmotic lysis of the cell. If, during normal growth, the peptidoglycan is cross-linked by about 30% as this study suggests, and which agrees with the findings of Braun et al. (2), then 12 to 15% is the minimum cross-linking required to maintain the integrity of the *E. coli* bacterium. Under different growth conditions, for example, in poorer media, the lethal percentage of inhibition of cross-linking seemed to be about 35%. This finding may correlate with the results of Kamiryo and Strominger (7), who reported that, in a series of *E. coli* temperature-sensitive mutants showing hypo-cross-linked peptidoglycan at the restrictive temperature, none was found to show more than a 30% reduction in cross-linking. The results of this work predict that such a mutation would be lethal.

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