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Penicillin-Resistant Mechanisms in *Pseudomonas aeruginosa*: Effects of Penicillin G and Carbenicillin on Transpeptidase and D-Alanine Carboxypeptidase Activities

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A membrane fraction from *Pseudomonas aeruginosa* KM 338 was shown to catalyze in vitro peptidoglycan synthesis from uridine 5'-diphosphate-*N*-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl-D-alanyl-D-alanine and uridine 5'-diphosphate-*N*-acetylglucosamine. Synthesized peptidoglycan was partially cross-linked by transpeptidation, which was accompanied by the release of D-alanine. This reaction was strongly inhibited by 25 and 50 μg of penicillin G and carbenicillin per ml respectively, whereas the intact cells were relatively resistant to penicillins (minimal inhibitory concentration of penicillin G and carbenicillin, 30 and 0.125 mg/ml, respectively). Soluble D-alanine carboxypeptidase present in *P. aeruginosa* KM 338 was studied as well, which was found almost completely inhibited by penicillin G and carbenicillin (10 μg/ml).

In general, most strains of *Pseudomonas aeruginosa* are resistant to penicillins, which inhibit peptidoglycan biosynthesis both in vivo (8, 9, 12, 13, 16, 19, 20, 21) and in vitro (1, 4, 5, 7, 11) in penicillin-susceptible organisms. The peptidoglycan isolated from cell walls of *P. aeruginosa* consists of macromolecular network of covalently linked repeating units (3, 10). This structure of peptidoglycan is similar to that of penicillin-susceptible organisms previously reported (2, 15, 17).

The intrinsic mechanisms of resistance to penicillins in *P. aeruginosa* were investigated.

The present experiment showed that a particulate enzyme system obtained from a strain of *P. aeruginosa* catalyzes peptidoglycan synthesis including the cross-linking reaction. The effect of penicillin G on this system was compared with carbenicillin, to which the growth of *P. aeruginosa* is relatively susceptible (14).

(A summary of this work was presented at the 45th Annual Meeting of the Japanese Society for Microbiology, Sendai, 18 May 1972)

MATERIALS AND METHODS

Organism and preparation of enzyme sources. The organism used was *P. aeruginosa* KM 338 (minimal inhibitory concentration, [MIC] of penicillin G, 30 mg/ml; MIC of carbenicillin, 0.125 mg/ml). This strain was furnished from Culture Collection Room, Research Institute for Microbial Diseases, Osaka University, Osaka, and was derived from *P. aeruginosa* ATCC 17653. It was grown in Trypticase soy broth (BBL) at 37 C with shaking agitation. Cells were harvested at about half-maximal growth which required approximately 3 h from 5% inoculum. Enzyme sources were prepared after alumina grinding as described by Izaki et al. (4, 5, 6). Cell debris was removed by centrifugation at 7,000 × g for 10 min, followed by one centrifugation at 12,000 × *g* for 15 min. The particulate fraction (membrane fraction) was then sedimented by centrifugation at 60,000 × *g* for 1 h. The soluble fraction was then obtained by centrifugation of the resulting supernatant solution at 100,000 × *g* for 2 h.

Chemicals. Uridine 5'-diphosphate (UDP)-*N*-acetylmuramyl-L-alanyl-D-glutamyl-meso-diaminopimelyl D-[^{14}C]alanyl D-[^{14}C]alanine (UDP-MurNAc-pentapeptide) was a gift from Izaki (Tohoku University, Sendai, Japan). It had a specific activity of 66 μCi per μmol. UDP-*N*-acetylglucosamine (UDP-GlcNAc) was obtained from Sigma Pure Chemical Co. Ltd. (St. Louis, Mo.). Penicillin G and carbenicillin were supplied by Meiji Seika Co. Ltd. (Osaka, Japan) and Research Laboratories, Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan), respectively.

Assay for peptidoglycan synthesis and D-alanine carboxypeptidase. The methods used were the same as description in the studies (4, 5, 6) with *Escherichia coli* with the following exceptions. Both assays were carried out in 0.2 M of tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, containing 10^{-3} M Mg^{2+} without 2-mercaptoethanol at 37 C. The reaction mixture contained in a total volume of 25 μliters, 0.26 mg of particulate fraction protein, or 0.11 mg of soluble fraction protein, 5 μmol of tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, 1 μmol of $MgCl_2$, and 0.92 nmol of UDP-MurNAc-pentapeptide (labeled with D-

[^{14}C]alanine) with or without 9 nmol of UDP-GlcNAc. After incubation, the reaction was terminated by addition of 10 μliters of isobutyric acid and the reaction mixture was then spotted on Whatman 3MM filter paper. After this was developed on isobutyric acid-1 N ammonia (5:3, vol/vol) descending system, a radioautogram was prepared. Areas of the paper corresponding to the origin (peptidoglycan), alanine, and lipid intermediate were cut out and counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer.

RESULTS

Utilization of UDP-MurNAc-pentapeptide for peptidoglycan synthesis. When the cell wall precursor, UDP-MurNAc-pentapeptide (labeled with D-[^{14}C]alanine) and UDP-GlcNAc were incubated with the particulate fraction prepared from *P. aeruginosa* KM 338, incorporation of radioactivity into both lipid intermediate and peptidoglycan was observed (Fig. 1). Peptidoglycan was synthesized more slowly than the lipid intermediate and its synthesis ceased at about 60 min of incubation. The release of D-alanine paralleled the formation of peptidoglycan during the early stages of incubation. It, however, continued after completion of peptidoglycan synthesis and the amount of free [^{14}C]alanine liberated was greatly in excess of the amount incorporated into peptidoglycan. The amount of the residual nucleotide substrate decreased sharply and it had virtually disappeared after 1 h of incubation.

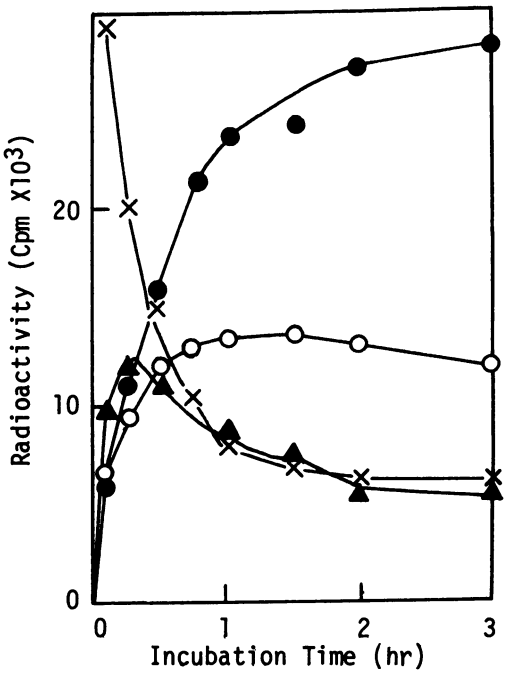

FIG. 1. *Time course of peptidoglycan synthesis by a particulate fraction from P. aeruginosa KM 338. Assays were carried out as described in the methods. Incubation was at 37 C for the times shown. Data are expressed as counts per minute incorporated into products. Symbols: peptidoglycan* (○), *lipid intermediate* (▲), *released* D-*alanine* (●), *UDP-MurNAc-pentapeptide* (×).

Effects of addition of penicillin G and carbenicillin on peptidoglycan synthesis. When the substrates were UDP-MurNAc-pentapeptide and UDP-GlcNAc, release of alanine was markedly inhibited by 25 and 50 μg of penicillin G and carbenicillin, respectively, in spite of MIC of 30 mg/ml and 0.125 mg/ml in *P. aeruginosa* KM 338 (Fig. 2). In case of the addition of these antibiotics to the particulate fraction from *P. aeruginosa*, the inhibition of alanine release was accompanied by the increased amount of D-[^{14}C]alanine incorporated into the peptidoglycan.

Inhibition of cross-linked peptidoglycan synthesis by penicillin G and carbenicillin. The appearance on the radioautogram of the peptidoglycan product was quite different in the presence or absence of penicillin. Izaki et al. (4, 5) reported that peptidoglycan product formed in the presence of penicillin could be eluted from the paper with water, but the material formed in its absence could not be eluted under the similar circumstance, so that peptidoglycan formed in the presence of penicillin is considered soluble and not cross-linked, whereas that formed in its absence is an insoluble and cross-linked polymer. After areas of the paper corresponding to the origin (peptidoglycan product) were cut out and then washed with adequate amounts of distilled water for 2 h, comparison of the remaining amounts of peptidoglycan synthesized by the particulate fraction from *P. aeruginosa* KM 338 in the absence and in the presence of penicillin G or carbenicillin was shown in Table 1. When either penicillin G or carbenicillin (10 μg/ml) was contained in the incubation mixture, at least 50% inhibition of cross-linked peptidoglycan synthesis was observed by both antibiotics.

Inhibition by penicillin G and carbenicillin of particulate or soluble D-alanine carboxypeptidase from P. aeruginosa KM 338. In the case of both particulate and soluble fractions from *E. coli* (6), release of D-alanine from UDP-MurNAc-pentapeptide was also observed in the absence of UDP-GlcNAc, the second substrate, required for peptidoglycan synthesis. The particulate and soluble D-alanine carboxypeptidases from *P. aeruginosa* were almost completely inhibited by penicillin G or carbenicillin (10 μg/ml) (Fig. 3).

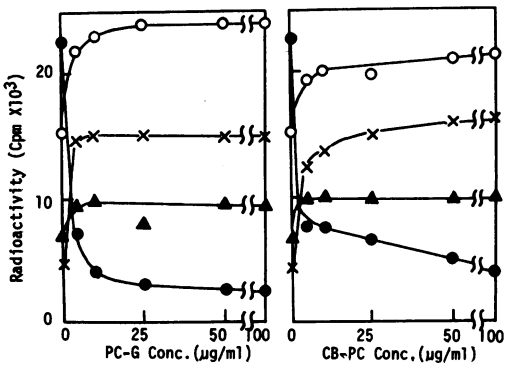

FIG. 2. *Effects of addition of penicillin G and carbenicillin on peptidoglycan synthesis. Assays were carried out as described in the methods with the addition of penicillin G (left) or carbenicillin (right) as indicated. Incubation was at 37 C for 1 h. Data are expressed as counts per minute incorporated into products. Peptidoglycan* (○), *lipid intermediate* (▲), *released* D-*alanine* (●), *UDP-MurNAc-pentapeptide* (×).

TABLE 1. *Inhibition of cross-linked peptidoglycan synthesis by penicillin G and carbenicillin*[a]

Antibiotics	Cross-linked peptidoglycan (counts/min)	Inhibition (%)
None	3,153	
Penicillin G (10 μg/ml)	1,583	49.8
Carbenicillin (10 μg/ml)	1,708	45.8

[a] Reaction mixtures were carried out as described in the methods with or without the addition of penicillin G or carbenicillin (10 μg/ml). Incubation was at 37 C for 1 h. After incubation, the reaction mixtures were spotted as a 1-cm zone on Whatman 3MM filter paper. After descending paper chromatography as described above, radioautogram was prepared. Areas of the paper corresponding to the peptidoglycan (origin) were cut out and washed with adequate amounts of distilled water for 2 h with stirring and then counted in a liquid scintillation counter.

DISCUSSION

Martin et al. (10) and Heilman (3) reported that the peptidoglycan from cell walls of *P. aeruginosa* consists of a macromolecular bag-shaped network of covalently linked repeating units. That is to say, the peptidoglycan builds up polyacetylhexosamine and peptide chains. Schematically, the linear polysaccharide chains are composed of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The carboxyl groups of the muramic acid residues of the repeating units of the peptidoglycan are cross-linked to other units.

An enzymatic system has been obtained from cells of *P. aeruginosa*, which catalyzes the terminal cross-linking reaction in bacterial cell wall synthesis. The closure of the interpeptide bridge is accompanied by release of D-alanine from carboxyl terminal end of the UDP-MurNAc-pentapeptide as described in the studies with *E. coli* (4, 5) and *Bacillus subtilis* (7). This fact indicates that this reaction is a transpeptidation. The transpeptidase from *P. aeruginosa* is inhibited by low concentration of penicillin G as in the case of *E. coli* (4, 5). This reaction is inhibited by the same amount of carbenicillin, which affects growth of *P. aeruginosa*.

On the other hand, both particulate and soluble fractions from *P. aeruginosa*, as well as these fractions from *E. coli* (6), contain D-alanine carboxypeptidase which can successively remove each of D-alanine residues from the carboxyl terminus of the UDP-MurNAc-pentapeptide. However, this carboxypeptidase from *B. subtilis* (7) was only found entirely in particulate fraction. Both particulate and soluble carboxypeptidases were inhibited by lower concentration of penicillins compared to transpeptidase. The particulate carboxypeptidase might well be considered uncoupled transpeptidase. The physiological significance of the occurrence of this soluble carboxypeptidase is still unclear.

A cell-free system from *P. aeruginosa*-synthesizing peptidoglycan is almost as susceptible to penicillin G as to carbenicillin, whereas the intact cells are unsusceptible to penicillin G.

β-Lactamase produced from *P. aeruginosa*

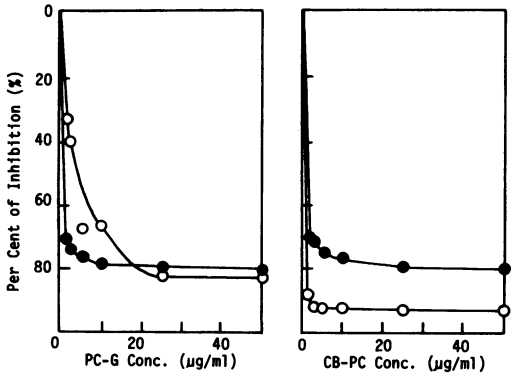

FIG. 3. *Effects of penicillin G and carbenicillin on particulate and soluble* D-*alanine carboxypeptidases. Assays were carried out as described in the methods with the use of UDP-MurNAc-pentapeptide, except that UDP-GlcNAc was omitted. Incubation was at 37 C for 2 h with particulate* (○) *or soluble* (●) *fractions in P. aeruginosa KM 338. Data are expressed as total counts per minute of* D-[^{14}*C*]*alanine released.*

might be considered as a resistant factor to penicillin G. This organism, however, has an extremely low activity of penicillinase compared to penicillinase-producing *Staphylococcus aureus* 235 and *E. coli* 3032 (MIC of penicillin G, 2 and 16 mg/ml, respectively) (unpublished data).

On the other hand, it has been suggested that penicillin is irreversibly bound to transpeptidase in the cytoplasmic membrane of penicillin-susceptible organisms (7, 17, 18). Penicillin G and carbenicillin were also irreversibly bound to the particulate fraction derived from *P. aeruginosa* KM 338, which catalyzed peptidoglycan synthesis. This binding followed saturation-type kinetics (specific binding), as had been shown earlier (7, 17, 18), whereas the binding of penicillin G to the whole cells was entirely nonsaturation type (nonspecific binding) which was linearly related to the penicillin concentrations (unpublished data). The β-lactamase and the binding of penicillins to *P. aeruginosa* KM 338 will be described in the following paper (manuscript in preparation).

These results appear to suggest that intrinsic resistance to penicillin G is due to predominantly permeability barrier of the cell surface in *P. aeruginosa* KM 338.

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