Mutation in *Pseudomonas aeruginosa* Causing Simultaneous Defects in Penicillin-Binding Protein 5 and in Enzyme Activities of Penicillin Release and D-Alanine Carboxypeptidase

HIROSHI NOGUCHI,1* MASATOMO FUKASAWA,1 TOSHIKI KOMATSU,1 SUSUMU MITSUHASHI,2 and MICHIRO MITSUHASHI3

Sumitomo Chemical Co., Takarazuka Research Centre, 4-2-1 Takatsukasa, Takarazuka, Hyogo-ken, 6651; Department of Microbiology, School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma-ken, 3712; and Institute of Applied Microbiology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 1133 Japan

Received 1 October 1984/Accepted 28 January 1985

Penicillin-binding protein 5 in *Pseudomonas aeruginosa* had moderately penicillin-sensitive D-alanine carboxypeptidase activity. As in *Escherichia coli*, a defect in this enzyme activity was not lethal.

Penicillin-binding proteins (PBPs) of *Pseudomonas aeruginosa* show an electrophoretic pattern similar to that of *Escherichia coli*, but their enzymatic activities have not been studied. In this paper we briefly report the D-alanine carboxypeptidase activity in pseudomonal PBP-5 and properties of a mutant lacking this activity.

A mutant strain that lacks one of the penicillin-sensitive D-alanine carboxypeptidase activities at 30°C as well as at 40°C was isolated from *P. aeruginosa* PAO2142. The defective enzyme activity in the mutant strain seemed to resemble that of D-alanine carboxypeptidase Ia in *E. coli* (5), judging from its moderate sensitivity to penicillin and its elution on DEAE-cellulose column chromatography. The mutant had thermosensitive PBP-5. The mutant had penicillin-binding activity at 30°C but not the activity to release benzylpenicillin bound to it at the same temperature, and the penicillin-binding activity was lost at 40°C.

*P. aeruginosa* PAO2142, kindly provided by H. Matsu- moto of Shinsu University (Matsumoto, Japan), was derived from strain PA01. Both the parent strain PAO2142 and the mutant strain hs-257 were auxotrophs of isoleucine-valine, lysine, and methionine. Temperature-sensitive mutants were isolated after mutagenesis for 30 min at 30°C with 100 μg of N-methyl-N'-nitro-N-nitrosoguanidine per ml in nutrient broth (Difco Laboratories). A highly penicillin-sensitive, temperature-sensitive mutant strain hs-257 was obtained. Details for the procedure of isolation of the mutant were as described in our previous paper (7). Subsequent in vivo recombination studies with R plasmid R68.45 (3) revealed, however, that the thermosensitivities of growth and of PBP-5 were due to two independent mutations. The relation between the high sensitivity to β-lactam antibiotics and the defect of PBP-5 has not been clearly proved.

The mutant strain hs-257 had thermosensitive PBP-5 (Fig. 1), its activity to bind benzylpenicillin at 30°C being markedly less (lane b) than that of the parent (lane a). On preincubation of the membrane fraction at 40°C for 10 min, the PBP-5 of the mutant strain lost significant activity (lane c), whereas PBP-5 of the parent strain was stable (data not shown). No remarkable abnormalities in the electrophoretic mobilities or thermosensitivities of other PBPs of the mutant were detected. To confirm that the PBP-5 in living mutant cells was also thermolabile, we incubated the parent and mutant cells for 4 h at 44°C and then examined the pattern of their PBPs by electrophoresis. On culture at 44°C, the penicillin binding activity of mutant PBP-5 was lost (Fig. 1, lane e), whereas that of the parent cells was present (lane d).

Like PBP-5 of *E. coli* (9), PBP-5 of *P. aeruginosa* shows weak, β-lactamase-like activity to release the benzylpenicillin bound to it (8). PBP-5 in the mutant hs-257 grown at 30°C did not show this activity even when measured at 30°C (data not shown).

Both the parent and the mutant-type PBP-5s could be

![](http://jb.asm.org/)

**FIG. 1.** Thermosensitivity of penicillin-binding activity of PBP-5. Samples (30 μl) of membrane preparations (total, 600 μg of protein) were preincubated for 10 min at the temperatures indicated below and then tested for binding of [14C]benzylpenicillin. Fluorograms of gels after SDS-polyacrylamide gel electrophoresis are shown. Lanes: a, parent strain PAO2142 cultured at 30°C in nutrient broth (Difco); b and c, mutant strain hs-257 cultured at 30°C in nutrient broth (Difco); for assay of thermosensitivity in vitro, membranes were preincubated for 10 min at 30°C (a and b) or 40°C (c). For assay of thermosensitivity of PBPs in vivo, the parent (d) and mutant (e and f) were cultured at 30°C to 10^7 cells per ml and then for 4 h at 44°C in nutrient broth (Difco), and PBP was assayed after preincubation of the membranes at 40°C (d and f) or 30°C (e). Binding of [14C]benzylpenicillin (54 Ci/mol; Radiochemical Centre, Amersham, England) for 10 min at 30°C was examined. For further details of the preparation of membranes, and separation and detection of PBPs, see reference 8.

---

* Corresponding author.
solubilized from the membrane with 2% Triton X-100 at 0°C and separated from other PBPs on a column of DEAE-cellulose in the presence of 2% Triton X-100. Both proteins appeared in the flow-through fraction, whereas other PBPs were adsorbed to the column and eluted with buffer containing 0.2 M NaCl and 2% Triton X-100. Figure 2 shows the separation of the mutant PBPs (A) and their sensitivities to heat (B). The mutant PBP-5 in the flow-through fraction (no. 3 and 4) on the DEAE-cellulose column chromatography again showed thermosensitive penicillin-binding activity, whereas the PBP-5 in a similar fraction from the parent strain was thermostable (data not shown).

The membrane fraction of *P. aeruginosa* possessed strong activity of d-alanine carboxypeptidase, which was supposed to be due mainly to the activities of PBP-4 and PBP-5, by analogy to *E. coli* PBP. On DEAE-cellulose chromatography of a Triton X-100 extract of membranes of the parent cells, d-alanine carboxypeptidase activity was eluted in two peaks (Fig. 3), one in the flow-through fraction and the other in the 0.2 M NaCl eluate (A), but a similar extract from the mutant gave only one peak in the 0.2 M NaCl eluate (B). The d-alanine carboxypeptidase activity in the flow-through fraction from the parent membrane was moderately sensitive to benzylpenicillin, its 50% inhibitory dose being 5 ng/ml, whereas that in the 0.2 M NaCl eluate from both the parent and mutant membranes was very sensitive to benzylpenicillin, the 50% inhibitory dose being less than 1 ng/ml. As PBP-5 was found solely in the flow-through fraction, whereas other PBPs (PBP-1A, 1B, 2, 3, and 4) were mainly found in the NaCl eluate (Fig. 2), the moderately penicillin-sensitive d-alanine carboxypeptidase activity in the flow-through fraction was concluded to be due to PBP-5. The very penicillin-sensitive activities in the NaCl eluate seemed to be due to PBP-4 by analogy to *E. coli* PBP, but this possibility needs further study.

The present results indicated that PBP-5 in *P. aeruginosa* is responsible for the moderately penicillin-sensitive d-alanine carboxypeptidase activity, and that the weak β-lactamase-like activity of PBP-5 is due to the d-alanine carboxypeptidase activity. This enzyme activity may correspond to the d-alanine carboxypeptidase Iα activity in *E. coli*, which has been proved to be a property of *E. coli* PBP-5 (5, 6). In our mutant, this enzyme activity in vitro was lost at either 30 or 40°C (data not shown). At 30°C, only the activity binding substrate (penicillin) was active, but the activity transferring the substrate seemed to be defective. The mutant therefore appeared to be analogous to the dacA11191 (PBP-5') mutation of *E. coli* isolated and described by Matsuhashi et al. (5, 6).
This E. coli mutant has subsequently been studied in detail in Strominger's laboratory (1; personal communication) and sequenced by Broome-Smith et al. (2).

In E. coli, three genetically independent PBPs, 4, 5, and 6, have D-alanine carboxypeptidase activities. Double mutants lacking the D-alanine carboxypeptidase activities of both PBP-4 (dacB mutation) and 5 (dacA mutation) have been obtained (5), but no triple mutant lacking all three D-alanine carboxypeptidase activities has yet been constructed. As the P. aeruginosa strains investigated have no PBP corresponding to E. coli PBP-6 (8), the mutant obtained may correspond to a double mutant of E. coli with defective PBP-5 and 6.

For this reason it is very interesting that at 44°C, the nonpermissive temperature, the mutant of P. aeruginosa with thermostable PBP-5 produced an excessive amount of PBP-4, whereas the parent formed a large amount of PBP-5 but little PBP-4 (Fig. 1, lanes d and e). There was no increased level of PBP-4 in the mutant cells grown at 30°C. It is unknown whether PBP-4 in the mutant cells grown at 44°C was identical to that at 30°C. Isolation of a mutant of P. aeruginosa with defective PBP-4 and preparation of a double mutant defective in PBP-4 and 5 may be necessary to establish the functions of these proteins.

LITERATURE CITED