# INTRINSIC RESISTANCE TO β-LACTAM ANTIBIOTICS IN *STAPHYLOCOCCUS AUREUS*

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Received 4 November 1980

## 1. Introduction

Methicillin-resistant strains of *Staphylococcus aureus* were first described in 1961 [1,2]. These strains demonstrate cross-resistance to β-lactam antibiotics other than methicillin [3] and resistance is intrinsic rather than being due to penicillinase activity, as penicillinase-negative variants retain resistance to β-lactam antibiotics [4]. The resistance is unusual in that cell populations are heterogeneous with respect to the level of resistance expressed [5] although all cells in the population retain the ability to express resistance. The degree of resistance is affected dramatically by alterations in the environmental conditions. In particular, resistance is increased by growing cells at <37°C [6] or by incorporating 5% NaCl into the medium [7].

Intrinsic resistance to β-lactam antibiotics has been shown to be due to alteration either in the amounts [8] or the affinities for β-lactam antibiotics [9–11] of the penicillin-binding proteins (PBPs) of certain bacteria other than *S. aureus*. Here we demonstrate that intrinsic resistance to β-lactam antibiotics in *S. aureus* results from an increase in the amount of modified PBP, or the presence of a new PBP, which has a lower affinity for β-lactam antibiotics than PBPs in sensitive strains.

## 2. Materials and methods

### 2.1. *Bacteria*

Methicillin-resistant (13136 $p^-m^+$) and -sensitive (13136 $p^-m^-$) strains of *S. aureus* were provided by Dr R Lacey [12]. Both strains were penicillinase-negative.

### 2.2. *Minimum inhibitory concentrations (MICs)*

MICs of β-lactam antibiotics were determined by the agar dilution method [13] on isosensitest agar (Oxoid CM 471) with an inoculum of $\sim 10^3$ colony-forming units.

### 2.3. *Preparation of membranes*

Cultures were grown under conditions (a) favourable and (b) unfavourable for the expression of resistance.

(a) Overnight cultures incubated at 30°C in isosensitest broth containing 5% NaCl (+100 mg methicillin/l for the resistant strain) were harvested by centrifugation, resuspended in fresh isosensitest broth containing 5% NaCl to an $A_{620}$ ~0.05 and grown with aeration at 30°C.

(b) Overnight cultures incubated at 40°C in isosensitest broth were diluted in fresh isosensitest broth to an $A_{620}$ ~0.05 and were grown with aeration at 40°C. Growth was followed turbidimetrically and cells were harvested by centrifugation when in the exponential growth phase. The bacterial pellet was washed once in 50 mM Tris–HCl buffer (pH 7.5) containing 145 mM NaCl and the cells lysed by incubation for 20 min at 37°C in 50 mM Tris–HCl buffer (pH 7.5) containing 145 mM NaCl, 5 mM $MgCl_2$, lysostaphin (15 mg/l) and DNase (5 mg/l).

Membranes were collected by centrifugation at 50 000 × *g* for 20 min, washed twice in 50 mM Tris–HCl buffer (pH 7.2) containing 5 mM $MgCl_2$, resuspended in the same buffer at 10 mg protein/ml and stored at −20°C.

### 2.4. *Assay of penicillin-binding proteins*

Membranes were treated with various concentrations of [$^{14}$C]benzylpenicillin (The Radiochemical

Table 1
MICs of β-lactam antibiotics for *S. aureus* strains 13136 $p^-m^+$ and 13136 $p^-m^-$

| Strain | Incubation temperature (°C) | MIC (mg/l) of β-lactam antibiotic | | | | |
|---|---|---|---|---|---|---|
| | | Benzylpenicillin | Methicillin | Cloxacillin | Cephaloridine | Cefotaxime |
| 13136 $p^-m^+$ | 30 | 16 | >128 | 64 | 16 | >128 |
| 13136 $p^-m^+$ | 40 | 0.06 | 4 | 0.25 | 0.06 | 8 |
| 13136 $p^-m^-$ | 30 | 0.25 | 4 | 0.5 | 0.25 | 4 |
| 13136 $p^-m^-$ | 40 | 0.0 | 4 | 0.5 | 0.06 | 4 |

Centre, Amersham) for 10 min at 37°C followed by addition of unlabelled benzylpenicillin (final conc. 2 g/l). The treated membranes were boiled for 2 min in 10 mM Tris–HCl buffer (pH 7.2) containing 10% (w/v) glycerol, 1% (w/v) sodium dodecylsulphate (SDS) and 0.002% bromophenol blue. The proteins were then separated by SDS–polyacrylamide gel electrophoresis and the PBPs detected by fluorography [14]. The sensitivity of PBPs to other β-lactam antibiotics was measured by prelabelling PBPs with various concentrations of the non-radioactive agent followed by excess [$^{14}$C]benzylpenicillin to detect the PBPs still available [15].

## 3. Results and discussion

When grown at 30°C, *S. aureus* 13136 $p^-m^+$ was resistant to all the β-lactam antibiotics tested compared with strain 13136 $p^-m^-$ which was sensitive (table 1). When grown at 40°C both strains had similar sensitivities. Four PBPs were detected in both resistant and sensitive strains (fig.1). PBPs 1, 2 and 4 were similar in amount and in their affinities for benzylpenicillin in 13136 $p^-m^+$ and 13136 $p^-m^-$ whether cultures were grown at 30°C or 40°C. However, when 13136 $p^-m^+$ was grown under conditions allowing expression of resistance there was a considerable

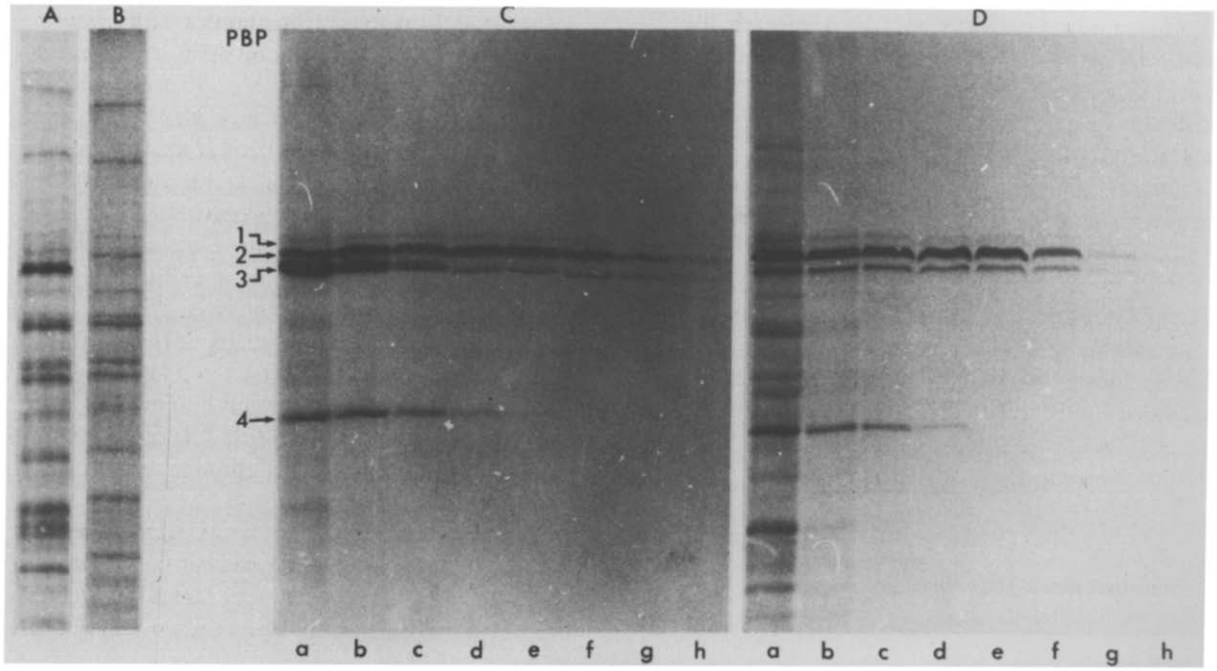


Fig.1. Membrane proteins of *S. aureus* strains 13136 $p^-m^+$ (A) and 13136 $p^-m^-$ (B) stained with Coomassie brilliant blue, and PBPs of strains 13136 $p^-m^+$ (C) and 13136 $p^-m^-$ (D) as revealed by fluorography. Membranes were incubated with various concentrations of [$^{14}$C]benzylpenicillin for 10 min at 37°C and separated by SDS–polyacrylamide gel electrophoresis. The concentrations of [$^{14}$C]benzylpenicillin used were (mg/l): (a) 36; (b) 10; (c) 3.6; (d) 1; (e) 0.36; (f) 0.1; (g) 0.036; (h) 0.01.

increase in the amount of label running in the position of PBP 3 and this protein was not fully labelled until >10 mg [$^{14}$C]benzylpenicillin/l was reached. In 13136 $p^-m^-$ PBP 3 was fully labelled by <1 mg [$^{14}$C]benzylpenicillin/l. In 13136 $p^-m^+$ it appears that in addition to the resistant PBP which has the same mobility as PBP 3 of 13136 $p^-m^-$ there is a PBP produced in similar amounts with similar mobility and sensitivity to benzylpenicillin as the PBP 3 of 13136 $p^-m^-$. Therefore PBP 3 as seen on the fluorogram of [$^{14}$C]benzylpenicillin-treated membranes of 13136 $p^-m^+$ (fig.1C) may be a composite of the 'normal' PBP 3 and a new resistant PBP. The gel stained with Coomassie blue also showed the presence of a large amount of a protein with a mobility coincident with that of PBP 3, in the resistant strain only (fig.1A,B). When 13136 $p^-m^+$ was grown under conditions in which resistance was not expressed the prominent protein band was not seen, neither was the additional resistant PBP present. Therefore the expression of resistance to benzylpenicillin in 13136 $p^-m^+$ appears to correlate with the production of large amounts of a protein resistant to the binding of benzylpenicillin, and this protein is present in addition to the normal PBP 3 but has the same $M_r$.

Three other strains of penicillinase-negative, methicillin-resistant *S. aureus* and their methicillin-sensitive variants were investigated. The PBPs detected in membrane preparations were similar in their affinities for [$^{14}$C]benzylpenicillin as PBPs of *S. aureus* strains 13136 $p^-m^+$ and 13136 $p^-m^-$. Also the SDS–polyacrylamide gels stained with Coomassie blue showed the large amount of protein with mobility coincident with that of PBP 3 in the methicillin-resistant strains only.

In membrane preparations of *S. aureus* strains 13136 $p^-m^+$ and 13136 $p^-m^-$ in which resistance is not expressed, the affinities of PBPs 1, 2 and 3 for benzylpenicillin were similar and were of the same order as the concentrations required to inhibit growth. When 13136 $p^-m^+$ was grown under conditions that allowed expression of resistance, the additional protein corresponding to PBP 3 had an affinity for benzylpenicillin similar to the concentration necessary to inhibit growth. These findings are different from those in [11] where the PBPs of resistant and sensitive non-isogenic strains of *S. aureus* were compared and large differences found in the affinities of the PBPs for benzylpenicillin but no apparent difference in the amount of a particular PBP. Surprisingly, the affinities of the PBPs of the resistant strain were unaltered in cells grown under conditions in which resistance was not expressed [11].

It is possible that the 'normal' PBP 3 operates in the absence of β-lactam antibiotics but in the presence of these agents the additional protein takes over the function of PBP 3. Larger amounts of the additional protein may be necessary because it is less efficient than the normal PBP 3 and/or because it also takes over some of the functions of the other PBPs. Methicillin-resistant staphylococci grow more slowly in the presence of methicillin than in its absence, as might be expected if cell wall peptidoglycan synthesis is less efficient under these conditions.

Table 2
Affinities of β-lactam-binding proteins of *S. aureus* strains 13136 $p^-m^+$ and 13136 $p^-m^-$ for β-lactam antibiotics

| Strain | PBP | Concentration of β-lactam (mg/l) resulting in 50% binding | | | | |
|---|---|---|---|---|---|---|
| | | Benzylpenicillin[a] | Methicillin[b] | Cloxacillin[b] | Cephaloridine[b] | Cefotaxime[b] |
| 13136 $p^-m^+$ | 1[c] | 0.1 | – | – | – | – |
| | 2 | 0.1 | 0.1 | 0.1 | 1 | 0.1 |
| | 3 | >10 | >100 | >100 | >100 | >100 |
| | 4 | 1 | 0.1 | 3 | 10 | 3 |
| 13136 $p^-m^-$ | 1[c] | 0.1 | – | – | – | – |
| | 2 | 0.1 | 0.3 | 0.3 | 1 | 1 |
| | 3 | 0.3 | 0.1 | 0.3 | 1 | 3 |
| | 4 | 1 | 0.3 | 3 | 10 | 10 |

[a] Determined by direct labelling; [b] Determined by pre-labelling technique (see section 2)
[c] PBP 1 was not detectable in sufficient amounts to determine 50% binding values except by the direct labelling technique

Cells were grown under conditions allowing the expression of resistance, membranes prepared and the affinities of membrane proteins for β-lactam antibiotics determined (see section 2)

In addition to benzylpenicillin, 13136 $p^-m^+$ was also resistant to a wide range of β-lactam antibiotics including methicillin, cloxacillin, cephaloridine and cefotaxime (table 1). As with benzylpenicillin the expression of resistance was dependent on the conditions of growth. As radioactively-labelled forms of these antibiotics were not available, their binding to PBPs was followed by means of a prelabelling technique (see section 2). The concentrations of antibiotics at which ~50% of the individual PBPs were labelled are shown in table 2. At >10 mg [$^{14}$C]benzylpenicillin/l, (used to saturate available PBPs after prelabelling with cold antibiotic) there was some non-specific binding of radioactivity to membrane proteins. This rendered the visualisation of PBP 1 difficult since this protein was detected in only small amounts in all experiments. Therefore no estimation of the affinity of β-lactam antibiotics for PBP 1 is given in table 2. The major difference between the PBPs of 13136 $p^-m^+$ and 13136 $p^-m^-$ was that a PBP corresponding to PBP 3, but with a much reduced affinity for the β-lactam antibiotics, was present in large amounts in the resistant strain only.

To determine whether the resistant cells were less permeable to β-lactam antibiotics than the sensitive strain, cells were grown exponentially, under conditions allowing expression of resistance, with different concentrations of [$^{14}$C]benzylpenicillin followed by the rapid preparation of membranes and detection of PBPs by SDS–polyacrylamide gel electrophoresis and fluorography. In these in vivo experiments the PBPs had similar affinities for benzylpenicillin as had been determined with isolated membranes so it is unlikely that an altered cell wall is presenting a permeability barrier to β-lactam antibiotics in the resistant cells.

We conclude that the main factor determining the intrinsic resistance to β-lactam antibiotics in *S. aureus* is the presence of an additional protein, having the same mobility as the PBP 3 in sensitive cells, but which has a much reduced affinity for benzylpenicillin and other β-lactam antibiotics. The concentration of benzylpenicillin required to inhibit growth of the resistant strain is similar to that which binds to 50% of the available new protein in the membrane.

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