Penicillin-binding proteins of β-lactam-resistant strains of *Staphylococcus aureus*

Effect of growth conditions

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Methicillin-resistant clinical isolates of *Staphylococcus aureus* are intrinsically resistant to β-lactam antibiotics in that the resistance mechanism is unrelated to the possession of β-lactamases. We have demonstrated that a new, high-molecular-mass penicillin-binding protein (PBP) is present in these strains with a low affinity for β-lactams and that its amount is regulated by the growth conditions. The new PBP from all strains that have been examined has an identical mobility on SDS gel electrophoresis and is the only PBP still present in an uncomplexed state with β-lactams (and therefore the only functional PBP) when these strains are grown in media containing concentrations of β-lactam antibiotics sufficient to kill sensitive strains.

Penicillin-binding protein *Methicillin-resistant* S. aureus *Intrinsic resistance* *β-Lactam antibiotic* *Membrane protein* *Polyacrylamide gel electrophoresis*

1. INTRODUCTION

Intrinsic resistance to β-lactam antibiotics in Gram-positive organisms is becoming a clinical problem of considerable significance following the introduction of β-lactamase-resistant penicillins and cephalosporins commencing with the use of methicillin; the first such strain was isolated in 1961 [1]. In some instances mutation of the primary target has been claimed [2–5] in that the affinity of existing penicillin-binding proteins (PBPs) for β-lactams has decreased. One report of methicillin resistance in a strain of *Staphylococcus aureus* [6] referred to the possibility of a different protein with a low affinity for β-lactams being present in large amounts but it was difficult to conclude categorically that a new PBP was present as its molecular mass was similar to those of PBP 2 and PBP 3 in sensitive strains which are not easily resolved by SDS gel electrophoresis. Refinement of the gel separation technique using highly purified chemicals and a prolonged separation time has resulted in an improved and reproducible separation of the high-molecular-mass PBPs found in sensitive and resistant strains.

Here we demonstrate unequivocally that a new PBP is present in methicillin-resistant clinical isolates of *S. aureus*; that its presence and amount depend on the growth conditions; that the new protein is of identical molecular mass in at least 4 different isolates from European sources; and that it is the only high-molecular-mass PBP still present in an uncomplexed state with β-lactams (and therefore probably the only functional PBP) when these strains are cultured in media containing concentrations of β-lactam antibiotics sufficient to kill sensitive strains of *S. aureus*.

2. MATERIALS AND METHODS

2.1. *Bacteria and growth conditions*

Two different pairs of isogenic methicillin-

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sensitive and resistant strains of *S. aureus* were examined, 13136 p^-m^- (sensitive) and 13136 p^-m^+ (resistant) [7]; DU 4916S (sensitive) and DU 4916 K7 (resistant) [8]; and 2 oxacillin-resistant isolates LV 3 and LV 18 obtained from Dr L. Verbist. Strain LV 3 originally possessed β-lactamase but it was cured of the plasmid coding for the enzyme by growth of the organism in the presence of a low concentration of ethidium bromide [9]. The other 5 strains were devoid of β-lactamase.

All strains were grown with aeration in a medium containing 1% tryptone, 0.5% yeast extract, 0.25% K_2HPO_4 and 0.5% glucose, pH 7.2, under conditions favourable (30°C, with 5% NaCl) or less favourable (37°C without NaCl) for the expression of resistance. In some experiments resistant strains were grown for at least 15 generations in the presence of methicillin (10 mg/l), benzylpenicillin (1 mg/l) or cephalothin (1 mg/l) prior to labelling with [^{3}H]benzylpenicillin.

2.2. *Assay of penicillin-binding proteins – labelling of intact cells*

Exponentially growing cells were harvested by centrifugation (25000 × *g* for 30 s), washed once in 50 mM Tris-HCl buffer, pH 7.5, containing 145 mM NaCl and resuspended in the same buffer at 8 mg dry wt/ml. 100 μl samples were incubated with different concentrations of [^{3}H]benzylpenicillin (28.4 Ci/mmol) for 10 min at 30°C. 1 ml lysing buffer containing 50 mM Tris-HCl, pH 7.5, 145 mM NaCl, 5 mM $MgCl_2$, DNase (20 mg/l), benzylpenicillin (5 g/l) and lysostaphin (30 mg/l) was then added and incubation at 30°C continued for 5 min. Lysis was obvious with all strains within 2–3 min but incubation was continued for the complete period to allow total destruction of the DNA. Samples were cooled to 4°C and the membrane fraction collected by centrifugation (45000 × *g*, 20 min). The minute membrane pellet was resuspended in 40 μl of 10 mM phosphate buffer, pH 7, to which was added 10 μl of a solution containing 50 mM Tris-HCl (pH 7.2), 50% (w/v) glycerol, 5% (w/v) SDS, 5% (w/v) 2-mercaptoethanol and 0.01% bromophenol blue. Membrane proteins were solubilised by heating for 2 min at 100°C. The proteins were separated by SDS-polyacrylamide gel electrophoresis [10] using recrystallised bisacrylamide at a final concentration of 0.067%. Electrophoresis was carried out at a constant current of 30 mA after preliminary stacking at 20 mA and was prolonged for 200 V·h after the tracking dye had reached the bottom of the gel to increase separation of protein bands. PBPs were subsequently detected by fluorography [11].

3. RESULTS

3.1. *Presence of additional penicillin-binding protein in resistant strains: effect of growth conditions*

Membrane fractions obtained from intact cells of *S. aureus* strains DU 4916S and DU 4916 K7 that had been labelled with [^{3}H]benzylpenicillin at 30 mg/l contained 3 and 4 high-molecular-mass PBPs, respectively (fig.1B). The additional PBP in

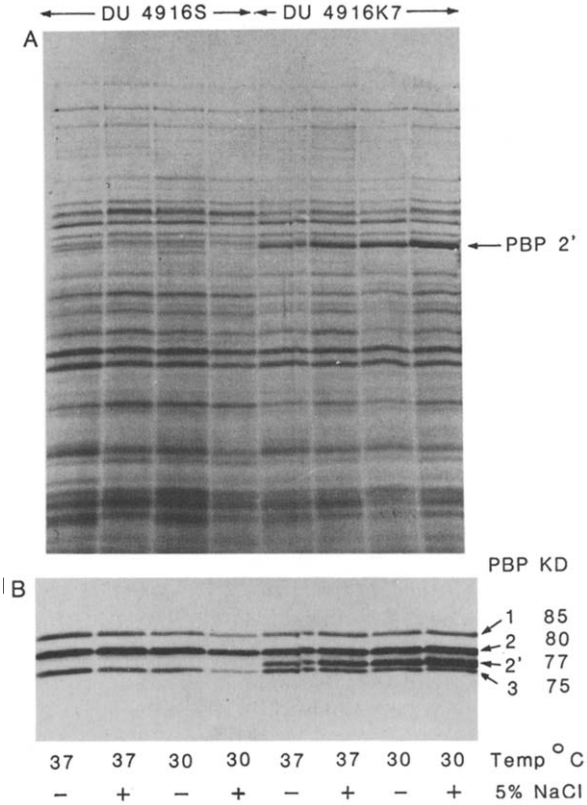

Fig.1. Effect of growth conditions on the PBPs of methicillin-sensitive (DU 4916S) and resistant (DU 4916 K7) strains of *S. aureus*. Intact cells were labelled with [^{3}H]benzylpenicillin as in section 2 before separation of membrane proteins by SDS gel electrophoresis. (A) Membrane proteins stained with Coomassie brilliant blue; (B) PBPs revealed by fluorography.

the resistant strain (M_r 77000) had a mobility between that of PBP 2 (M_r 80000) and PBP 3 (M_r 75000). It was clearly resolved from both these PBPs largely as a result of prolonging the electrophoretic separation for 200 V · h after the tracking dye had reached the bottom of the gel, but also as a result of using high specific activity benzylpenicillin (and thus less protein) and the method of labelling which incorporated a rapid lysis step. In common with the other strains used in this investigation DU 4916 K7 demonstrates the phenomenon of heteroresistance in that it is highly resistant when grown at 30°C in the presence of 5% NaCl but has increased sensitivity to β-lactam antibiotics when grown at 37°C in the absence of 5% NaCl [12], and is virtually as sensitive as the sensitive strain when grown at 42°C. There appears to be a correlation between the expression of resistance and the presence of the additional PBP in the resistant strain. At 37°C in the absence of salt a small amount of the additional PBP is present whereas when the strain is grown at 30°C in the presence of salt a large amount of the extra protein can be seen both on the Coomassie-stained gel (fig.1A) and also on the fluorogram (fig.1B): apparently both temperature and the presence of the osmotic stabiliser have an effect on the production of this new PBP which is totally absent from the sensitive strain when grown under all conditions.

3.2. *Affinity of the additional penicillin-binding protein (PBP 2') for benzylpenicillin*

All the high-molecular-mass PBPs of sensitive strains of *S. aureus* bind benzylpenicillin maximally at a relatively low concentration of the antibiotic (approx. 0.1 mg/l) and PBPs 1, 2 and 3 of resistant strains have a similar high affinity (fig.2A and B). The extra PBP (labelled PBP 2' because of its intermediate position between PBPs 2 and 3) does not bind benzylpenicillin below 10 mg/l and is not saturated at 60 mg/l (fig.2, B–D). Although PBP 2' is present in substantial amounts when the organism is grown at 30°C with salt, its production is increased markedly by pre-growth of the resistant strain in the presence of a sub-inhibitory concentration of methicillin (fig.2D). In this particular experiment *S. aureus* DU 4916 K7 was grown with methicillin (10 mg/l) for 15 generations before the antibiotic was removed and growth continued in

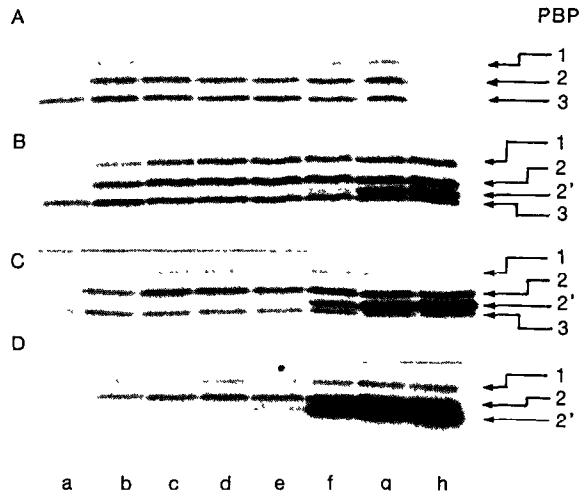

Fig.2. Effect of [^{3}H]benzylpenicillin concentration on the formation of PBP-penicillin complexes. Intact cells of DU 4916S (A) and DU 4916 K7 (B–D) were incubated with different concentrations of [^{3}H]benzylpenicillin, lysed and the membrane proteins separated as in section 2. PBPs were revealed by fluorography. The growth conditions prior to labelling were: (A) and (C) 30°C, + 5% NaCl; (B) 37°C without NaCl; (D) 30°C, + 5% NaCl + methicillin (10 mg/l) for 15 generations followed by 5 generations in the same medium lacking methicillin. The concentrations of benzylpenicillin were (mg/l): (a) 0.03, (b) 0.1, (c) 0.3, (d) 1, (e) 3, (f) 10, (g) 30, (h) 60.

the absence of methicillin for 5 generations prior to labelling the cells. Apart from the large increase in the amount of PBP 2' the amount of the normal PBP 3 has been severely depressed (fig.2D) and in some experiments it appears to be totally absent suggesting that prior growth in the presence of methicillin has inhibited its synthesis.

3.3. *Presence of PBP 2' in other methicillin-resistant strains*

When methicillin-resistant strains are grown in sub-inhibitory concentrations of methicillin (10 mg/l) the antibiotic saturates PBPs 1, 2 and 3 and only PBP 2' is available to form a covalent complex with [^{3}H]benzylpenicillin during the labelling procedure (fig.3A). The small amount of PBP 2 seen on the fluorograph results from the rapid dissociation of the PBP 2-methicillin complex (half-life 7 min) during the labelling period with [^{3}H]benzylpenicillin. The complexes of PBP 2

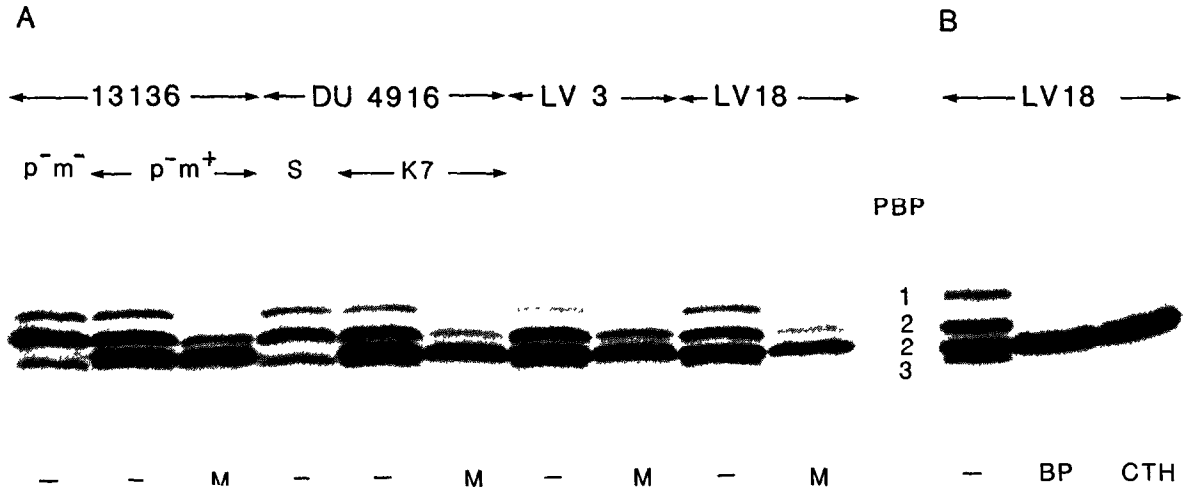

Fig.3. The PBPs of 4 β-lactam-resistant strains of *S. aureus* grown in the presence and absence of β-lactam antibiotics. Intact cells were labelled, the membrane proteins fractionated and PBPs revealed by fluorography as in section 2. (A) All resistant strains were grown with and without methicillin (M) at 10 mg/l prior to labelling. The isogenic sensitive strains 1316 p^-m^- and DU 4916S are included for comparison. (B) Strain LV 18 was grown in the absence and presence of unlabelled benzylpenicillin (BP) or cephalothin (CTH), both at 1 mg/l, prior to counterlabelling with [^{3}H]benzylpenicillin at 30 mg/l.

with benzylpenicillin or cephalothin are more stable (half-lives 30–40 min) and when resistant strains are grown for 15 generations in the presence of unlabelled benzylpenicillin or cephalothin (both at 1 mg/l) followed by labelling with [^{3}H]benzylpenicillin (30 mg/l), PBP 2′ is the sole PBP seen on fluorographs (fig.3B). Consequently it appears that PBP 2′ can replace the functions of all the essential PBPs of *S. aureus* when β-lactam antibiotics are present above a certain threshold concentration. PBP 2′ in all 4 organisms examined has the same mobility on SDS gels and appears to be identical (fig.3A). Although the methicillin-resistance determinant is apparently located chromosomally [13,14] it is a completely new gene without any corresponding allele in sensitive strains [15]. The identical mobility of PBP 2′ in all strains examined lends weight to the hypothesis that all intrinsically methicillin-resistant strains of *S. aureus* have been derived from a single clone [16]. Examination of recent clinical isolates from Japan (2), Australia (2), the London Hospital (2), St. Thomas' Hospital (3) and Addenbrookes Hospital, Cambridge (4) has also revealed the additional PBP of identical mobility in all strains (P.E. Reynolds, submitted).

4. DISCUSSION

The inherent difficulties involved in separating PBP 2, PBP 3 and PBP 2′ by SDS-polyacrylamide gel electrophoresis have resulted in a number of conflicting proposals for the mechanism of methicillin resistance in intrinsically resistant isolates of *S. aureus* [5,6,17]. Re-examination of one of these strains has indicated that an additional, low-affinity, PBP is present [18] and strain MR 1, a non-heteroresistant strain which was believed to have an altered PBP 3 [5] has now also been shown to possess the new PBP (P.E. Reynolds, submitted). The strains examined here originally contained β-lactamase and removal of the naturally occurring β-lactamase without affecting methicillin resistance indicates the separate nature of these 2 factors. Nevertheless in strains possessing β-lactamase there is considerable induction of the new PBP and of β-lactamase at concentrations of β-lactam antibiotics less than the MIC for the sensitive strain (P.E. Reynolds and D.F.J. Brown, unpublished) [19–21], suggesting that the synthesis of both these proteins is controlled in a similar manner.

If the additional PBP is the main factor deter-

mining methicillin resistance it would be expected to be involved in a late stage of peptidoglycan biosynthesis, probably catalysing a transpeptidation reaction, and that process should be relatively insensitive to β-lactam antibiotics. This has been shown in strain 13136 p^-m^+ in which the attachment of nascent peptidoglycan to the existing cell wall peptidoglycan under non-growing conditions has a low sensitivity to benzylpenicillin, commensurate with the MIC for the organism [22].

The large amount of the new PBP (20% of the total membrane protein) suggests that its catalytic efficiency may be low. Although there is no equivalent sensitive allele and therefore one cannot invoke mutation of a PBP in the sensitive strains to explain a change in structure, the geometry of the active site of a PBP that has such a low affinity for β-lactams may also be considerably suboptimal in relation to substrate binding; consequently a large amount may be necessary to accomplish balanced peptidoglycan synthesis. Furthermore PBP 2′ presumably functions as the sole active PBP under certain growth conditions and therefore may have to substitute for more than one of the PBPs in sensitive strains, so more of it might be required than of any one normal PBP. Although resistant strains can survive in media containing high concentrations of β-lactam antibiotics, growth is not as rapid as that of sensitive strains and the cells of the resistant strain are enlarged suggesting that not all the enzymic activities catalysed by PBPs 1, 2 and 3 can be compensated by the activity of PBP 2′.

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