Kinetics of penicillin binding to penicillin-binding proteins of *Staphylococcus aureus*

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Reduced affinity of penicillin-binding proteins (PBPs) for binding penicillin has been proposed as a mechanism of β -lactam antibiotic resistance in staphylococci. Penicillin binding by PBPs of three penicillin-susceptible and two penicillin-resistant strains of *Staphylococcus aureus* was studied in kinetic assays to determine rate constants, drug concentrations at which PBPs were bound and the relationship between concentrations that bound PBPs and concentrations that inhibited bacterial growth. PBPs 1 and 2 of the resistant strains exhibited slower acylation and more

rapid deacylation than susceptible strains. In contrast PBP 4, a naturally low-affinity PBP, was modified such that it exhibited a lower rate of deacylation. The concentrations of penicillin at which modified PBPs were bound correlated with concentrations that inhibited growth of the resistant strains. Acquisition of penicillin resistance in these strains of *S. aureus* results, at least in part, from structural modifications affecting binding of multiple PBPs and appears to include recruitment of a non-essential PBP, PBP 4.

INTRODUCTION

The antibacterial activity of penicillin, as with all β -lactam antibiotics, results from its covalent binding to penicillin-binding proteins (PBPs), integral membrane proteins that catalyse cross-linking of cell wall [1]. Susceptible strains of *Staphylococcus aureus* produce four PBPs, 1, 2, 3 and 4, with molecular masses of 85, 81, 75 and 45 kDa respectively [2,3]. PBP 2 and PBP 3 can each be resolved into two components by SDS/PAGE [4,5]. Evidence from radiolabelling experiments indicates that the high-molecular-mass PBPs 1, 2 and 3 are essential for viability and are the targets of β -lactam antibiotics [1,6–8]. The 45 kDa protein, PBP 4, is not essential, nor is it a target for the β -lactam antibiotic effect [1,6,8].

Most methicillin-resistant strains of staphylococci produce PBP 2a, a novel low-affinity PBP that is encoded by *mecA*, which is not found in susceptible strains. Methicillin-resistant strains lacking *mecA* have been reported [9–14]. Similar to resistance in pneumococci, streptococci and *Neisseria* species [15–18], it has been hypothesized that mutations within a normal staphylococcal PBP gene or genes, which then express structurally modified PBPs with lower binding affinity for β -lactam antibiotic, account for methicillin-resistance of non-PBP 2a-producing strains [9]. Although biologically plausible, this hypothesis has not been critically examined. If reduced PBP-binding affinity does account for resistance, then there should be a relationship between the antibiotic concentration at which binding occurs and the concentration at which growth is inhibited.

The association between resistance and altered binding has been based largely on qualitative differences in binding of radiolabelled penicillin to PBPs for susceptible compared with resistant strains. Binding generally has been measured by determination of an IC_{50} value, which is the concentration of a non-radiolabelled antibiotic in a competition assay that blocks radiolabelling of PBP by 50%. Radiolabelling conditions are typically chosen without knowledge of equilibrium conditions for binding reactions of either drug used in the assay. Consequently, although it provides a relative measure of binding, the

IC_{50} cannot be used to quantify the amount of PBP actually bound.

The amount of bound PBP can be calculated from rate constants. We measured rate constants for penicillin-binding reactions of PBPs in susceptible and resistant strains of *S. aureus* to determine which PBPs were altered in resistant strains and how binding reactions were affected. The amount of bound PBP at the drug concentration that inhibited growth was calculated to assess quantitatively whether the modified PBP or PBPs were likely to be mediating the observed resistance.

MATERIALS AND METHODS

Bacterial strains

Five β -lactamase-negative, *mecA*-negative strains of *S. aureus* were studied. Strains 209P (ATCC 6538) and BB-255 (NCTC 8325) are methicillin-susceptible. Strain 29 is a penicillin-susceptible low-level methicillin-resistant strain that does not have altered PBPs in conventional competitive binding assays [10]. Strains CDC-6 (a gift from Linda McDougal, Centers for Disease Control, Atlanta, GA, U.S.A.) and BB-255R (same as BB-255-P6-M90, a gift from Brigitte Berger-Bächi, Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland; derived by serial passage of strain BB-255 in penicillin) are methicillin-resistant and have modifications of PBPs 1, 2 and 4 and PBPs 2 and 4 respectively [9,12]. The penicillin-binding affinities of PBPs 1 and 2 are reduced, whereas the affinity of PBP 4 appears to be unchanged, but it is present in increased amounts.

Susceptibility tests

Growth inhibition was measured by determination of minimum inhibitory concentrations (MIC). A standard macrobroth dilution method was used in which 1 ml samples were incubated at 35 °C for 24 h in tryptic soy broth at an inoculum of 10^6 colony-forming units of late-exponential-phase organisms/ml. Penicillin concentrations used were 0.06, 0.12, 0.18, 0.24, 0.3, 0.6, 1.2, 2.4, 3, 6, 12, 24, 30 and 36 μ M.

Calculation of rate constants

Rate constants were calculated from densitometry data obtained from fluorographs of radiolabelled PBPs by the method of Frere [19] and Ghuyssen [20]. These reference sources should be consulted for details of the method, which is briefly described below.

The model reaction between PBP and β -lactam antibiotic (Pen) is

obeys Michaelis-Menten kinetics [19–21]. Reaction (1) is association-dissociation in which the penicillin substrate reversibly binds to the enzyme active-site serine to form the Michaelis complex for which the dissociation constant is $K = k_{-1}/k_{+1}$. Reaction (2) is acylation, in which penicillin becomes covalently bound to the active-site serine. Reaction (3) is deacylation, in which the acyl-enzyme intermediate is broken down by hydrolysis, generating inactive β -lactam antibiotic (Pen_i) and regenerating active enzyme. The rate of acyl-enzyme formation is described by the second-order rate constant k_{+2}/K . k_{+2}/K is determined by calculating the pseudo-first-order rate constant, k_a , as follows:

$$k_a = -\ln \frac{1 - ([\text{PBP}_d]/[\text{PBP}_o])}{t}$$

PBP_d is the experimentally derived fluorographic density of the PBP after incubation for time, t , with radiolabelled drug (i.e. [³H]penicillin) at a given concentration, D . PBP_o is the density at which all binding sites have been saturated with radiolabelled penicillin. Each k_a value is plotted against its corresponding drug concentration, either directly or reciprocally, to obtain a linear relationship. The slope of the line, or its reciprocal, is equal to k_{+2}/K .

The deacylation reaction, in which inactive drug is released from the PBP, is described by the first-order rate constant, k_{+3} , which is determined from the following equation:

$$\ln(\text{PBP}_t/\text{PBP}_o) = -k_{+3}t$$

where PBP_t is the density at time t after removal of PBP from radiolabel and PBP_o is the density when the PBP is saturated.

The maximum percentage of PBP covalently bound by drug at a given concentration can be calculated from the Michaelis-Menten constant, K_m . Because PBPs are relatively stable once acylated with penicillin and breakdown is usually rate-limiting (i.e. $k_{+3} \ll k_{+2}$) [20], it follows that:

$$K_m = \frac{k_{+3}}{k_{+2}/K}$$

The maximum fraction of PBP that will be bound at a given drug concentration, D , is calculated as follows:

$$\text{Percentage of bound PBP} = \frac{D \times 100}{K_m + D}$$

Binding assays of PBPs in membranes

Kinetics of PBP binding were assayed with membrane proteins for each strain on two or more separate occasions. Membrane proteins were prepared by differential centrifugation of mechanically disrupted cells harvested during exponential growth at a

cell density of approx. 1×10^8 colony-forming units/ml in tryptic soy broth at 37 °C [5]. Saturating concentrations of penicillin were determined by radiolabelling PBPs in 10 μg of membrane protein (corresponding to picomolar amounts of PBPs) for 10–30 min with [³H]penicillin at concentrations ranging between 0.3 and 60 μM , incremented as described above for determination of MICs. The reaction was stopped by adding sample dilution buffer and heating the sample in a boiling-water bath for 3 min. PBPs were separated by SDS/PAGE in a minigel apparatus (Bio-Rad, Richmond, CA, U.S.A.) in a 0.75 mm gel, either 10% acrylamide/0.26% bisacrylamide or 14% acrylamide/0.13% bisacrylamide (the latter to achieve separation of the two PBP 2 components). Electrophoresis in 10% gels was performed for 1 h at 200 V. Electrophoresis in 14% gels was performed overnight at 60 V.

For determination of k_{+2}/K , the lowest penicillin concentrations shown to saturate PBPs were used to radiolabel PBPs for 30, 60 or 120 s. A control sample in which all PBP sites were bound by [³H]penicillin was always included to determine the total amount of PBP, PBP_o .

For determination of k_{+3} , PBPs were radiolabelled with a saturating concentration of [³H]penicillin (15 μM for high-affinity PBPs and 60 μM for low-affinity PBPs) for 15 min at 37 °C. Non-radioactive penicillin (15 mM) was added to the sample and the incubation was continued at 37 °C. Samples were removed at 0, 5, 10, 15, 30, 45, 60, 90 and 120 min. The reaction was stopped by adding SDS sample buffer and heating samples for 3 min in a boiling-water bath. Samples were subjected to SDS/PAGE as described above.

PBPs were detected by fluorography using prefogged film [22]. The film was developed after a 3–6-day exposure on average. The appearance of PBPs in the saturated control sample and the background density were used to determine whether the film was underexposed (indicated by faint or absent PBPs) or overexposed (presence of non-specifically radiolabelled proteins, high background and loss of linear relationship between density and amount of radiolabelled PBP). If necessary, multiple exposures of the same gel were made to obtain a properly exposed film in which the amount of radiolabelled protein and its fluorographic density were linearly related. The amount of radiolabelled PBP at each concentration was measured by scanning densitometry (UVP Gel Documentation System, San Gabriel, CA, U.S.A.). Densitometry values were used to derive rate constants according to the equations shown above. The standard deviation given for each rate constant is that of the slope of the regression line, from which the rate constant was determined.

Competition assays of PBPs in whole cells

Exponential-phase organisms were incubated in 0.5 ml of tryptic soy broth with or without non-radioactive penicillin and cefoxitin at 37 °C for 30 min. Cells were harvested by centrifugation, resuspended in 10 μl of lysis buffer (200 $\mu\text{g}/\text{ml}$ lysostaphin, 50 mM Tris/HCl, 10 mM MgCl₂, 2.5 $\mu\text{g}/\text{ml}$ DNAase, 2.5 $\mu\text{g}/\text{ml}$ RNAase, pH 7.0) containing 60 μM [³H]penicillin, and incubated for 5 min at 37 °C. Sample dilution buffer was added, the sample was placed in a boiling-water bath for 3 min, and then subjected to SDS/PAGE in a 10% gel.

RESULTS

Mean MICs for the penicillin-susceptible strains 209P, BB-255 and 29 ranged between 0.12 and 0.21 μM (corresponding to 0.04–0.07 $\mu\text{g}/\text{ml}$) (Table 1). MICs for the resistant strains CDC-6 and BB-255R were 10 to 100-fold higher.

Table 1 Penicillin MICs

Results are means (range) of three separate determinations.

Strain	MIC (μM)
209P	0.12 (0.12–0.12)
BB-255	0.12 (0.12–0.12)
29	0.21 (0.12–0.24)
CDC-6	2.1 (1.2–2.4)
BB-255R	24 (24–24)

Table 2 Second-order rate constants for acylation of *S. aureus* PBPs by penicillinStandard deviations are for the slope of the regression line. 2_{maj} and 2_{min} are the major higher-molecular-mass and minor components respectively of PBP 2.

Strain	$10^{-3} \times k_{+2}/K \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$				
	1	2_{maj}	2_{min}	3	4
209P	7.8 ± 1.8	14.2 ± 0.3	24.0 ± 6.4	15.4 ± 0.5	0.4 ± 0.1
BB-255	8.9 ± 2.6	5.9 ± 1.6	—	5.5 ± 1.6	1.6 ± 0.3
29	9.7 ± 4.0	10.7 ± 1.8	11.7 ± 2.1	9.0 ± 1.0	1.0 ± 0.3
CDC-6	0.7 ± 1.0	0.7 ± 0.1	1.4 ± 0.2	4.0 ± 0.2	0.8 ± 0.1
BB-255R	6.6 ± 1.4	1.4 ± 0.2	—	$2.5 \pm 0.3^*$	0.3 ± 0.2
				1.0 ± 0.2	

* Two PBPs migrating at the PBP 3 position were present in this strain.

Table 3 First-order rate constant and half-life for deacylation of penicillin by *S. aureus* PBPsNumbers in parentheses are half-lives (min) of the acylated PBP. Standard deviations for the slope of the regression line are shown. 2_{maj} and 2_{min} are the major higher-molecular-mass and minor components respectively of PBP 2.

Strain	$10^4 \times k_{+3} \text{ (s}^{-1}\text{)}$				
	1	2_{maj}	2_{min}	3	4
209P	2.0 ± 0.2 (58)	1.8 ± 0.2 (64)	1.6 ± 0.3 (72)	1.2 ± 0.2 (96)	21 ± 7 (6)
BB-255	1.3 ± 0.2 (88)	1.2 ± 0.2 (96)	—	1.3 ± 0.2 (88)	34 ± 4 (4)
29	1.7 ± 0.2 (68)	2.2 ± 0.3 (52)	2.7 ± 0.2 (43)	1.0 ± 0.2 (115)	18 ± 2 (6)
CDC-6	3.9 ± 0.3 (30)	3.2 ± 0.3 (36)	3.9 ± 0.6 (29)	1.0 ± 0.1 (115)	15 ± 1 (8)
BB-255R	2.3 ± 0.4 (50)	3.3 ± 0.7 (35)	—	$< 1.0, < 1.0^*$ ($> 120, > 120$)	4 ± 0 (29)

* Two PBPs migrating at the PBP 3 position were present in this strain.

Second-order acylation rate constants, k_{+2}/K (Table 2), for PBPs 1, 2 and 3 of the penicillin-susceptible strains were of the order of $10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, in agreement with previously reported values [19]. A PBP 2 minor component was detected in strains 29 and 209P, but was not seen in strain BB-255. PBP 3' was not detected.

k_{+2}/K constants for PBP 1 in strain CDC-6 and PBP 2 in both CDC-6 and BB-255R were approximately one-tenth the values

Table 4 K_m values of *S. aureus* PBPs for penicillin 2_{maj} and 2_{min} are the major higher-molecular-mass and minor components respectively of PBP 2.

Strain	$K_m \text{ (}\mu\text{M)}$				
	1	2_{maj}	2_{min}	3	4
209P	0.02	0.01	0.01	0.01	5.0
BB-255	0.01	0.02	—	0.02	2.0
29	0.02	0.02	0.02	0.01	1.7
CDC-6	0.54	0.45	0.27	0.02	1.9
BB-255R	0.03	0.22	—	$< 0.03^*$	1.7
				< 0.10	

* Two PBPs migrating at the PBP 3 position were present in this strain.

for susceptible strains. PBP 3 appeared as a doublet in strain BB-255R. Both peptides preferentially bound cefaclor, which is a characteristic of PBP 3, and neither bound clavulanic acid, which preferentially binds PBP 2 (results not shown) [5]. The lowest values of k_{+2}/K for PBP 3 were observed in the two resistant strains, suggesting that it may also be modified. For PBP 4, k_{+2}/K ranged between 300 and $1600 \text{ M}^{-1} \cdot \text{s}^{-1}$ for all five strains. k_{+2}/K for PBP 4 of the resistant strain BB-255R was only 20 % of the value observed for the parent strain.

The contribution of the deacylation reaction to penicillin binding was examined. PBPs 1, 2 and 3 of susceptible strains formed relatively stable acyl derivatives with half-lives ranging between 43 and 115 min (Table 3). The longest half-lives for the acyl-PBP were observed for PBP 3, which is consistent with previously reported results [19,21,23]. Penicillin-bound PBP 4 in susceptible strains was rapidly deacylated with half-lives of 4–6 min, also consistent with previously reported results [21,24].

PBPs 1 and 2 of the resistant strains deacylated bound penicillin more rapidly than the PBPs of susceptible strains, with half-lives of the acyl-PBP ranging between 29 and 50 min compared with 43–96 min. Deacylation rates of the two PBP 3 peptides of strain BB-255R were similar, with relatively long half-lives for both, as in susceptible strains. In contrast with the increased rates of deacylation observed for PBPs 1 and 2 in the resistant strains, PBP 4 of strain BB-255R exhibited a lower rate of deacylation with a half-life of 29 min.

K_m values for the high-molecular-mass high-affinity PBPs 1, 2 and 3 of susceptible strains were of the order of $10^{-2} \mu\text{M}$ (Table 4). K_m values for PBPs 1 and 2 of strain CDC-6 and PBP 2 of strain BB-255R were 10–20 times higher than that of the susceptible strains. K_m values for PBP 3 were similar in both susceptible and resistant strains. PBP 4 exhibited low-affinity binding in all strains.

The maximum percentage of each PBP bound by penicillin was calculated from the K_m value to determine which PBPs would be bound at MIC for each strain. Binding at $0.1 \times \text{MIC}$ was also calculated to demonstrate which PBPs had an incremental increase in percentage binding as the drug concentration approached the MIC. For the susceptible strains, maximum binding of PBPs 1, 2 and 3 was approx. 90 % at MIC compared with 2–11 % binding of PBP 4 (Table 5). Binding of PBPs 1, 2 and 3 increased from an average of 50 % of maximum at $0.1 \times \text{MIC}$ to 90 % at MIC. These data are consistent with results of other studies [1,12–14] showing that PBPs 1, 2 and 3, but not PBP 4, are the antibiotic targets in susceptible strains.

Table 5 Maximum percentage of bound PBP at 0.1 $\times$ and 1 $\times$ MIC for penicillin

2_{maj} and 2_{min} are the major higher-molecular-mass and minor components respectively of PBP 2.

Strain	$\times$ MIC	1	2 _{maj}	2 _{min}	3	4
209P	0.1	54	38	54	54	< 1
	1	92	86	92	92	2
BB-255	0.1	54	38	—	38	< 1
	1	92	86	—	86	6
29	0.1	51	51	51	68	1
	1	91	91	91	95	11
CDC-6	0.1	28	32	44	91	10
	1	88	82	88	99	52
BB-255R	0.1	99	92	—	96, 99*	65
	1	99	99	—	99, 99	95

* Two PBPs migrating at the PBP 3 position were present in this strain.

Table 6 Effect of 0.05 μ g/ml cefoxitin on penicillin MICs

Results are means $\pm$ S.D. for three separate determinations. MICs for cefoxitin were ≥ 4 μ g/ml for all strains. $P > 0.05$ for all comparisons except $P < 0.001$ for strain BB-255R (two-sample t test).

Strain	Penicillin MIC (μ M)	
	— Cefoxitin	+ Cefoxitin
209P	0.12 $\pm$ 0.00	0.12 $\pm$ 0.00
BB-255	0.12 $\pm$ 0.00	0.09 $\pm$ 0.03
29	0.21 $\pm$ 0.06	0.15 $\pm$ 0.06
CDC-6	2.1 $\pm$ 0.6	1.2 $\pm$ 0.00
BB-255R	24 $\pm$ 0	5.1 $\pm$ 0.6

The data also suggest that approx. 90% binding may be the physiologically relevant threshold for inhibition of a target PBP.

A similar relationship between MIC and binding of PBPs 1 and 2, but not PBP 3, was observed for strain CDC-6, i.e. these PBPs displayed maximum binding at MIC and a significant

incremental increase in binding occurred between 0.1 $\times$ MIC and MIC. PBP 3 was 90% bound at 0.1 MIC, making it an unlikely mediator of the resistance in this strain. However, PBP 4 showed a significant incremental increase in binding from 10% at 0.1 $\times$ MIC to 52% at MIC, suggesting that it could contribute to the resistance in strain CDC-6. The association of binding of PBP 4 with resistance effect was even stronger for strain BB-255R. PBPs 1, 2 and 3 of strain BB-255R were calculated to be 90% or more bound at 0.1 $\times$ MIC, whereas PBP 4 was 65% bound at 0.1 $\times$ MIC and 95% bound at MIC. Thus, unlike the other strains in which binding of high-molecular-mass PBPs was associated with an antibacterial effect, inhibition of strain BB-255R seemed to require binding of PBP 4.

To test the hypothesis that PBP 4 was an essential PBP and an antibiotic target in the resistant strains, penicillin MICs were determined with cefoxitin, a β -lactam antibiotic which at low subinhibitory concentrations preferentially binds PBP 4. Whole-cell competition assays were performed to examine PBP binding in growing cells exposed to penicillin with and without cefoxitin. Addition of 0.05 μ g/ml cefoxitin (approx. 0.01 $\times$ MIC or less) had little effect on the penicillin MIC of the three susceptible strains (Table 6), and the whole-cell assay confirmed that the antibacterial effect was independent of PBP 4 binding (see Figure 1 for results with strain BB-255; results were identical for strains 209P and 29, but are not shown). With resistant strain CDC-6, addition of cefoxitin to penicillin reduced the mean penicillin MIC from 2.1 to 1.2 μ M, although this difference did not reach statistical significance. However, the only difference in PBP binding between 1.2 μ M of penicillin alone and in combination with cefoxitin was binding of PBP 4 (Figure 1 and Table 7), suggesting that increased binding of this PBP by cefoxitin did contribute to the lower MIC of strain CDC-6. With strain BB-255R cefoxitin reduced the penicillin MIC from 24 to 5.1 μ M, and this enhanced antibacterial effect was specifically associated with cefoxitin binding of PBP 4. PBP 4 was only 17% bound by 5.1 μ M penicillin alone, whereas it was 76% bound when cefoxitin was added. Percentage binding of PBPs 1, 2 and 3 did not change with the addition of cefoxitin (Figure 1 and Table 7). The principle difference in PBP binding in whole cells for penicillin at MIC compared with sub-MIC penicillin was also binding of PBP 4, with 46% bound by penicillin at 24 μ M compared with 17% at 5.1 μ M. PBP 4 was further implicated in resistance by increased production of PBP 4 by resistant strains [9,12]. When radiolabelled under saturating conditions, the density of PBP 4 in susceptible strains ranged between 9 and

Figure 1 Fluorographs of whole-cell competition assays for strains BB-255, CDC-6 and BB-255R

Lane 1 in each panel is the sample radiolabelled with 60 μ M [3 H]penicillin; lane 2, sample preincubated with 0.05 μ g/ml cefoxitin before radiolabelling; lane 3, sample preincubated with penicillin alone at 0.12, 2.1 or 24 μ M for strains BB-255, CDC-6 or BB-255R respectively; lane 4, sample preincubated with penicillin alone at 0.09, 1.2 or 5.1 μ M for strains BB-255, CDC-6 and BB-255R respectively; lane 5, penicillin in concentrations used for lane 3 samples plus 0.05 μ g/ml cefoxitin; lane 6, penicillin in concentrations used for lane 4 samples plus 0.05 μ g/ml cefoxitin.

Table 7 Percentage of bound PBPs in whole-cell assays of penicillin with and without 0.05 µg/ml cefoxitin

MIC is the inhibitory concentration for penicillin alone and sub-MIC is the penicillin inhibitory concentration when 0.05 µg/ml cefoxitin is present. MIC and sub-MIC concentrations (µM) respectively were 0.12 and 0.12 for strain 209P, 0.21 and 0.15 for strain 29, 0.12 and 0.09 for strain BB-255, 2.1 and 1.2 for strain CDC-6 and 24 and 5.1 for strain BB-255R. The values of percentage bound for PBP 2 and the PBP 3 doublet of strain BB-255R are for both components.

Strain	PBP	Percentage of bound PBPs			
		Cefoxitin	Penicillin (MIC)	Penicillin (sub-MIC)	Penicillin (sub-MIC) + cefoxitin
209	1	17	> 90	> 90	> 90
	2	8	83	85	87
	3	26	> 90	> 90	> 90
	4	> 90	22	29	> 90
BB-255	1	19	> 90	88	> 90
	2	26	70	60	85
	3	22	> 90	> 90	> 90
	4	> 90	15	19	> 90
29	1	< 10	> 90	> 90	> 90
	2	< 10	84	84	85
	3	26	> 90	> 90	> 90
	4	> 90	< 10	21	> 90
CDC-6	1	27	> 90	> 90	> 90
	2	36	> 90	> 90	> 90
	3	25	> 90	> 90	> 90
	4	80	23	15	83
BB-255R	1	46	> 90	> 90	> 90
	2	32	86	76	79
	3	48	> 90	> 90	> 90
	4	76	46	17	76

18 % of total PBP density compared with 42 and 32 % for strains CDC-6 and BB-255R respectively.

DISCUSSION

The results of kinetic binding studies were consistent with the conventionally held view that, in susceptible strains, high-molecular-mass PBPs 1, 2 and 3, but not the low-molecular-mass PBP 4, are the primary targets for penicillin. The high-molecular-mass PBPs of susceptible strains were 90 % bound at MIC and the major incremental increase in binding of these PBPs occurred as the concentration approached MIC, whereas binding of PBP 4 was 11 % or less at MIC. Growing cells are obviously a more dynamic system than the membranes used for the kinetic assays. Synthesis of PBPs is on-going in growing cells and whether a kinetic model of PBP inactivation based on assays performed with membranes can account for growth inhibition in whole cells is a valid question. Growing cells, for example, might produce target PBPs faster than they can be acylated by antibiotic. The second-order rate constant, k_{+2}/K , can be used to calculate the time required to acylate PBPs from the following equation [20]:

$$t_{90} = \frac{-\ln 0.1}{(k_{+2}/K)[D]}$$

where t_{90} is the time required at a given drug concentration $[D]$ to achieve 90 % acylation (a value chosen on the basis of binding studies associating this degree of PBP binding with growth

inhibition). Interestingly, when this calculation is performed for a drug concentration equal to MIC, then PBPs 1, 2 and 3 of susceptible strains are 90 % bound after 13–58 min compared with 3 h or more for 90 % acylation of PBP 4. As the doubling time for *S. aureus* (and presumably the PBPs as well) is typically 45–60 min, at MIC only 10 % or less of the essential target PBPs will not be acylated one generation later. At sub-MIC, cells can produce target PBPs faster than they can be acylated and inactivated. Thus the biochemical reaction of PBP binding appears to be quantitatively and directly correlated with growth inhibition measured by MIC.

Evidence for modification of each of the four PBPs was obtained in resistant strains. Reduced binding of PBPs 1 and 2 resulted from both slower acylation and more rapid deacylation. The concentrations of penicillin at which PBP 3 was bound were not significantly different for resistant strains compared with susceptible strains, but PBP 3 may also have been modified because its rates of acylation were slowest in resistant strains. An extra PBP similar to PBP 3 was also present in strain BB-255R.

Although not a target of β -lactam antibiotics in susceptible strains, PBP 4 seems to have an important role in non-PBP 2a-mediated resistance. As has been previously reported [9,12], PBP 4 was overexpressed in resistant strains. Net binding affinities measured by K_m were similar for both resistant and susceptible strains, yet the rate of deacylation of PBP 4 rather than being increased was severalfold less rapid in strain BB-255R than in other strains. The higher MIC of strain BB-255R compared with CDC-6 probably reflects structural modifications of PBP 4 allowing it to function in the former but not in the latter. Analysis of binding kinetics, the enhancement of penicillin antibacterial activity by cefoxitin and whole-cell PBP-binding assays indicated a role for PBP 4 in the resistant phenotype. In support of its role in resistance, transposon insertion that disrupts production of PBP 4 in a resistant strain overproducing PBP 4 resulted in loss of resistance (B. Berger-Bächi, personal communication).

The molecular and biochemical basis by which modified PBP 4 might confer resistance is not clear. We speculate that modifications have resulted in a remodelled PBP 4 that can replace an essential function of one or more high-molecular-mass PBPs. It is not known what this essential function is, but, as an example, it could be that, by increasing production of a PBP 4 remodelled into a more efficient transpeptidase that can substitute for the essential transpeptidase activity of one or more other PBPs, strain BB-255R might then be able to survive exposure to β -lactam antibiotic. Such a model could account for the reduced rate of deacylation observed for PBP 4 of strain BB-255R. As penicillin is a structural analogue of the natural substrate of PBPs, the penicillin-binding reaction may be a surrogate for reactions with cell-wall substrate [1,25]. The deacylation reaction with penicillin is analogous to the carboxypeptidase reaction with cell-wall substrate, with water as the acyl acceptor in both reactions. In the transpeptidase reaction, the critical cross-linking reaction of cell-wall synthesis, glycine is the acceptor. Structural features that permit rapid deacylation of PBP with donation of the acyl moiety to water may make for relatively poor transpeptidase activity. Conversely, structural features that enable a PBP to perform the essential transpeptidase reaction of high-molecular-mass PBPs may impose a constraint on how rapidly deacylation can occur. PBP 4, which has both carboxypeptidase and transpeptidase activity *in vitro* [1,24], is thought to function as a non-essential transpeptidase *in vivo* [26]. If appropriately modified, its transpeptidase activity might be able to sustain cell growth in susceptible cells when high-molecular-mass PBPs are bound by penicillin.

Staphylococcal resistance to penicillin caused by modified target PBPs is analogous to penicillin-resistance in pneumococci and *Neisseria* sp., but there are probably differences in how the mutations occur. The proposed mechanism for acquisition of mutations in the non-staphylococcal species is transformation with DNA from closely related species resulting in mosaic PBP genes and chimaeric proteins [27–32]. Although staphylococci are transformable, transformation with chromosomal DNA is inefficient and probably is not an important mechanism of genetic transfer among staphylococci in nature. The PBP alterations that occur clinically in staphylococci are probably the result of selection for successful mutations arising spontaneously within critical domains of resident PBP genes, rather than from recombination events with PBP genes from related species. If so, then staphylococci might be expected to show some variability in their PBP genes as a consequence of persistent low-level penicillin-selective pressure which began with the introduction of β -lactam antibiotics into clinical medicine. If mosaic PBPs were found in staphylococci, suggesting that horizontal genetic transfer can occur, this would alter our understanding of staphylococcal genetics as well as have important clinical implications because rapid acquisition of resistance may be possible by such a mechanism.

These kinetic studies provide insight into the structure–activity relationships of PBPs. As there is no single target PBP for penicillin even in susceptible strains [6], it is not surprising that multiple mutations involving more than one PBP are required to produce resistance. PBPs 2 and 4 appear to be particularly important for expression of resistance [9,12–14]. Modifications that reduce penicillin binding by slowing down acylation or increasing deacylation are intuitively advantageous mechanisms of resistance. Reduced binding alone, however, may not be sufficient for resistance. PBP 4 exhibits reduced binding relative to the high-molecular-mass PBPs in both susceptible and resistant strains, but seems to need remodelling for it to assume the necessary functions of cell-wall synthesis. Molecular characterization of staphylococcal PBPs combined with biochemical studies to determine structure–activity relationships would greatly enhance understanding of PBP functions and their role in resistance.

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