Transformation of *Streptococcus sanguis* to Intrinsic Penicillin Resistance

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A series of step-level penicillin-resistant derivatives of *Streptococcus sanguis* V288 (Challis) were obtained through successive genetic transformations. The DNA donor used was a laboratory-derived, penicillin-resistant multistep mutant of the recipient strain. Detection of the penicillin-binding proteins (PBPs) of wild-type and transformants revealed five major PBPs. While it was found that *S. sanguis* can acquire intrinsic resistance in a stepwise manner and the mechanism was similar to those of some other organisms (changes in penicillin-binding protein affinity and/or in extent of penicillin binding), multiple-PBP changes accompanied a single step-level of resistance. All of the PBPs showed varying degrees of decreased affinity for [³H]benzylpenicillin with increasing penicillin resistance. Of these, the consistent, dramatic and progressive decrease of PBP 4 binding was most notable. After an initial decrease at the first step-level of resistance, PBP 5 was restored to wild-type levels, indicating a possible important role in survival. Genetic linkage of the first two step-levels of resistance was demonstrated by examination of transformation frequencies and by hit-kinetics experiments. A convenient method is described for the quantitative comparison of fluorographs containing PBPs with a wide range of affinities for penicillin.

INTRODUCTION

Penicillin-binding proteins (PBPs), bacterial membrane components that covalently bind penicillin and other β -lactam antibiotics, have in many cases been found to be enzymes involved in cell wall synthesis, assembly and maintenance (for reviews, see Blumberg & Strominger, 1974; Waxman & Strominger, 1983). It is generally considered that the β -lactam class of antibiotics exerts its primary effects through the inhibition of one or more of these enzymic activities. PBPs (three to nine in number) have been found in all bacterial species examined thus far.

Resistance to penicillin usually occurs through one of the following mechanisms: (a) alteration of a cellular membrane to reduce permeability (generally occurring only in Gram-negative organisms); (b) acquisition of a β -lactamase (hydrolysing enzyme); and (c) alterations in the β -lactam targets (presumably one or more of the PBPs) (Dougherty, 1984). The alterations in PBPs are such that the affinity of these proteins for a radiolabelled β -lactam has changed, the quantity of a PBP has changed, or the mobility (apparent molecular mass) has changed as measured by SDS-PAGE fluorography (Spratt, 1977).

While intrinsic resistance in Gram-negative bacteria is in most cases due to inefficient penetration of the antibiotic through the cell membrane (Zimmerman, 1980; Spratt, 1983), Gram-positive bacteria do not possess such a selective barrier and develop resistance through target alterations. For example, after *Staphylococcus aureus* strains became penicillin resistant through the acquisition of a β -lactamase, methicillin (a β -lactamase-resistant antibiotic) was

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Abbreviation: PBP, penicillin-binding protein.

introduced in the treatment of resistant strains. Intrinsic resistance to methicillin soon followed (Brown & Reynolds, 1980). This resistance was accompanied by an increase in this organism's PBP 3 levels or by the appearance of a new PBP (PBP 2a or 2') which was methicillin inducible (Hartman & Tomasz, 1984; Rossi *et al.*, 1985; Ubukata *et al.*, 1985). In both cases, these PBPs exhibited a decreased affinity for all β -lactams.

Studies on intrinsic resistance in *Neisseria gonorrhoeae* showed that a mutation that changed outer membrane penetration only accounted for a two- to fourfold increase in penicillin resistance, whereas the total increase was in the 100- to 200-fold range (Guymon *et al.*, 1978). Examination of the PBPs (three in total) showed an alteration in PBP 2 that accounted for an additional eightfold increase in resistance (Dougherty *et al.*, 1980). An additional mutation affected both PBPs 1 and 2 (Dougherty, 1984).

In *Streptococcus pneumoniae*, genetic transformations of a susceptible laboratory strain with DNA from a highly penicillin-resistant South African clinical isolate yielded a series of step-level resistant (progressively higher increments of resistance) strains. Each step was accompanied by the appearance of new or the disappearance of old PBPs as well as alterations in the binding affinities of the PBPs (Hakenbeck *et al.*, 1980; Zigelboim & Tomasz, 1980). However, the donor and recipient possessed vastly different initial PBP profiles, and while detailed analyses of affinity changes have been described (Handwerger & Tomasz, 1986), the precise number of genetic and/or physiological steps in the acquisition of resistance has not been determined.

Streptococcus faecium can possess two types of intrinsic resistance. The first, as described above, involves alterations in PBP affinity. The second appears to be novel, detected as changes in the staining intensity of membrane proteins and by an increase in PBP 5 binding, possibly reflecting increasing amounts of a slow-binding, low-affinity penicillin-binding enzyme (Fontana *et al.*, 1983; Daneo-Moore *et al.*, 1983).

In an effort to better understand the alterations in PBPs that accompany the acquisition of penicillin resistance, we have created a series of penicillin step-level resistant transformants of *Streptococcus sanguis* V288 (Challis strain) using the DNA of a laboratory-derived penicillin-resistant multistep mutant of the same strain. By kinetic analysis we could identify the number of transformation events required to achieve any one level of resistance (Goodgal, 1961). Using single-step transformants, we could then deduce whether a single transformation event results in the alteration of one, or more than one, PBP.

METHODS

Strains. *S. sanguis* V288 (Challis, biotype I), kindly provided by F. Macrina, Medical College of Virginia - VCU, Richmond, Va., USA, was used as the wild-type strain. The minimum inhibitory concentration (MIC) of penicillin for this strain was 0.015 $\mu\text{g ml}^{-1}$. Successive platings of *S. sanguis* V288 (approx. 10^9 cells) on increasing concentrations of penicillin G yielded *S. sanguis* S.55D (MIC 0.80 $\mu\text{g ml}^{-1}$). In each successive plating, the concentration of penicillin G was increased by 0.02 $\mu\text{g ml}^{-1}$ and the plate was incubated for 48 h, after which the entire surface of the plate was harvested using an inoculating loop, and plated on the next penicillin concentration. No mutagens were used - S.55D was a multistep spontaneous mutant. The steps were not characterized.

Media. Organisms were routinely grown in Todd-Hewitt broth (Difco) supplemented with 2% (w/v) glucose (THG) or additionally with 0.1% (w/v) cysteine (THGc). All strains were stored in THGc with 30% (v/v) glycerol at -80°C .

DNA isolation. We used a glycine-enhanced lysis procedure to isolate bacterial DNA (Reider & Macrina, 1976). Prewarmed THGc (200 ml) was added to a 50 ml overnight culture. After 2 h incubation, 7.5 g glycine was added. Cells were harvested, washed, and resuspended in 25% (w/v) glucose in ET buffer (5 mM-EDTA, 10 mM-Tris, pH 8.5). Lysozyme (25 mg in 2.5 ml ET) was added and the suspension incubated for 1 h at 37°C . Pronase (15 mg in 2.5 ml ET, self-digested, 37°C , 1 h) was added and incubation continued for 30 min, followed by the addition of 0.4 mg RNAase and 0.7 ml 20% (w/v) SDS. The mixture was heat-shocked at 60°C for 5 min and incubated for an additional 2 h at room temperature. One volume of ice-cold 95% (v/v) ethanol was added. The DNA was wound on a glass rod, washed successively with 80%, 70% and 50% (v/v) ethanol and resuspended overnight in $1 \times \text{SSC}$ (0.015 M-sodium citrate, 0.15 M-sodium chloride). Deproteination was completed through three phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) extractions. The final solution was dialysed against $1/10$ TES (50 mM-Tris, 5 mM-EDTA, 50 mM-sodium chloride, pH 8.0) and the concentration of DNA determined spectrophotometrically.

Genetic transformation. This was basically by the method of Lawson & Gooder (1970). A frozen glycerol culture of the recipient (100 µl) was appropriately diluted (usually 1:10⁴) and grown for 1 h in THGc supplemented with 10% (v/v) horse serum to induce competence. The standard transformation procedure used 4 µg DNA, but subsequent hit-kinetics experiments used a range of 0.01–4.0 µg DNA. The DNA (in 100 µl) was added to 330 µl of the recipient culture. After 2 h, portions were diluted and plated on penicillin plates. Transformants were isolated after 24 h incubation in a candle jar and characterized by MIC replica plating (see below). Transformation frequency was calculated as the number of transformant c.f.u. divided by the number of viable cells at the time of DNA addition.

In preliminary experiments (data not shown) it was found that 2 h was sufficient to express resistance, and that the addition of DNAase 15 min after DNA addition had no substantial effect on the kinetics of transformation.

MIC determinations. Three methods were used to determine MIC.

(i) Transformants were initially characterized by being plated to a non-selective medium (THG with 2%, w/v, agar) and incubated for 24 h, after which they were velvet replica-plated to penicillin-containing plates. Isolates with the desired MIC were then picked from the non-selective plate, diluted in broth, single-colony passaged three times on the 'characteristic' concentration of penicillin, and tested for maintenance of the desired MIC.

(ii) The strain was grown in THGc to a density of 10⁷ cells ml⁻¹, and then dilutions were plated on various concentrations of penicillin G. Survivors were scored as a percentage of the total c.f.u. ml⁻¹ on non-selective plates. The concentration supporting the growth of 90% of the organisms is approximately 0.5 × the MIC.

(iii) The MIC was thrice verified by the macro-broth dilutions method using THGc medium (Washington & Sutter, 1980).

The MICs obtained for all strains did not vary with procedure.

Membrane (envelope) preparation. Cells were grown in 1 litre of THGc to an adjusted optical density, AOD, of 600 at 675 nm measured in a Coleman model 14 spectrophotometer [1 AOD unit ≡ 0.39 µg dry wt of cells ml⁻¹ (Toennies & Gallant, 1949)], at which time they were harvested, washed, and resuspended in 10 ml 10 mM-sodium phosphate buffer (pH 7.0) and frozen in a dry ice/ethanol bath for storage. After defrosting for 5 min at room temperature, the cell suspension was combined with 20 ml (35 g) of glass beads (0.10–0.12 mm) and the cells were disintegrated in a Bead-Beater with an ice-water jacket (Biospec Products). The procedure involved seven 20 s bursts with 5 min intervals to ensure sufficient cooling. The lysate was treated with 200 µl DNAase (0.5 mg ml⁻¹) and passed through a coarse-grade sintered glass filter to remove the beads. The filtrate was centrifuged (81 000 g, 30 min, Beckman Instruments 50Ti rotor) and resuspended in 1.5 ml buffer. Microscopic examination revealed no whole cells. Portions were frozen in a dry ice/ethanol bath and stored at -20 °C until needed for the PBP assay.

Penicillin-binding assay. Membranes (60 µl) were added to 20 µl of appropriately diluted [³H]benzylpenicillin and incubated for 15 min at 37 °C. Preliminary experiments (data not shown) indicated that the binding of penicillin by *S. sanguis* V288 was linear for 20–30 min. The reaction was stopped by the addition of 5 µl unlabelled penicillin G (100 mg ml⁻¹). The sample was immediately prepared for SDS-PAGE by the addition of 50 µl sample buffer [120 mM-Tris/HCl buffer, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 0.001% bromophenol blue], 2-mercaptoethanol (10 µl) and 10% (w/v) SDS (10 µl). The samples were boiled for 2 min and centrifuged for 5 min at 12 000 g (Fisher Micro-centrifuge 235).

SDS-PAGE. The procedure was basically that of Laemmli & Favre (1973). Supernatants from the binding assay (approx. 200 µg protein) were loaded on a 7.5% (w/v) acrylamide separating gel with a 4.0% stacking gel. The gel was run at room temperature for 21.0 h at 10 mA, then fixed in acetic acid/2-propanol/water (1:1:8, by vol.), stained with 0.275% Coomassie blue, and destained with acetic acid/methanol/water (1:1:8, by vol.). The gel was then prepared for fluorography by the method of Laskey & Mills (1975). Dehydration was accomplished through washes with DMSO and the gel was impregnated with PPO (25%, w/v, in DMSO) and rehydrated. Alternatively, the gel was treated with Amplify (Amersham). A Hoeffer slab dryer was used to vacuum-dry the gel. Kodak X-Omat AR film was placed over the gel in a light-tight cassette and exposed for various periods of time at -70 °C. The developed film was scanned with a soft-laser densitometer (Biomed Instruments) and areas under the resulting peaks were measured and plotted after corrections for protein loaded on the gel and comparison to a wild-type standard which was included in every experiment (see below).

Quantification of binding. Gel and fluorograph results were standardized from experiment to experiment in the following manner. The amount of protein loaded on the gel was determined by the method of Lowry. Every gel run contained a lane with V288 wild-type membranes bound with 12.5 µg [³H]benzylpenicillin ml⁻¹. The densitometric data were corrected for the protein content, and PBP 2 of the wild-type lane was assigned a densitometric value of 4.00. All sample values were then related to this value.

It was found that a 6 d exposure of the gel to film allowed optimum visualization of the five major PBPs of *S. sanguis* V288 wild-type with minimal background from non-specific binding as determined by measurements with the soft-laser densitometer.

To ensure proper quantification of the binding of radiolabelled penicillin, a calibration gel was made as follows. From a standard binding assay using a final concentration of radiolabelled penicillin of 12.5 µg ml⁻¹, different

volumes of the final supernatant (and therefore different amounts of protein: 50, 100, 150, 200, 250 and 300 μg , as determined by the Lowry method) were loaded on a standard-type SDS-PAGE gel. After a 6 d exposure of film to the gel, PBPs 1, 2, 3, and 5 showed a linear relationship of protein concentration versus fluorographic density measurement in the 150–250 μg protein range. However, the major binding protein, PBP 4, did not respond in a linear fashion. To account for a possible undermeasurement of PBP 4 values, the film was exposed for 1 d. In such an exposure, PBP 4 did respond linearly, as did PBP 2, but the values for PBPs 1, 3, and 5 were too low for precise measurement. Therefore, PBP 4 binding values were determined as follows. A fluorograph from a 6 d exposure was subjected to soft-laser densitometry to obtain values for PBPs 1, 2, 3, and 5, and a fluorograph from a 1 d exposure was then measured to obtain values for PBPs 2 and 4. The ratio of PBP 4 values to PBP 2 values from the 1 d exposure must hold true for the 6 d exposure; hence the PBP 4 value was calculated from the product of the 1 d ratio and the 6 d PBP 2 value. Pre-flashing of films was found unnecessary for quantification of bound tritium radioactivity.

Only protein amounts from 150 to 250 μg were used in any experiment. In most cases, approximately 200 μg of protein was loaded on the gels.

Chemicals. Penicillin G and pronase were obtained from Calbiochem, lysozyme from Boehringer-Mannheim, DNAase and RNAase from Millipore, electrophoresis materials from BRL, and miscellaneous chemicals from Fisher Scientific. Tritiated penicillin G ($[^3\text{H}]$ benzylpenicillin ethylpiperidinium salt, 851 GBq mmol $^{-1}$) was a generous gift of Merck and Co., Inc., Rahway, NJ, USA.

RESULTS

Transformation of S. sanguis

DNA from the laboratory-derived resistant organism, S.55D (MIC 0.80 μg penicillin G ml $^{-1}$), was used to transform the wild-type *S. sanguis* V288 (MIC 0.01 μg ml $^{-1}$) (Table 1). Transformants were isolated on 0.03 and 0.10 μg penicillin G ml $^{-1}$. Two hundred isolates from the 0.03 μg ml $^{-1}$ plate (the first step) were replica-plated on concentrations of penicillin ranging from 0.02 to 0.25 μg ml $^{-1}$. This experiment yielded a bimodal distribution of transformant MICs (Fig. 1), with peaks of MICs at 0.04 and 0.125 μg penicillin ml $^{-1}$. According to this analysis, about half of the transformants isolated on 0.03 μg penicillin ml $^{-1}$ exhibited a resistance level associated with a second step (at approximately 0.10 μg ml $^{-1}$).

Transformants that grew with 0.03 μg penicillin ml $^{-1}$ but not with higher concentrations (designated ss.03T), were purified (see Methods), and were transformed again with S.55D DNA to a resistance level of 0.10 μg ml $^{-1}$ (ss.10T, second step) (Table 1). Transformation of ss.10T yielded organisms resistant to 0.25 μg ml $^{-1}$ (ss.25T, third step) (Table 1). Similarly, an ss.55T isolate was obtained from transformation of ss.25T with S.55D DNA (Table 1). Survival curves

Fig. 1. Distribution of resistance levels of transformants isolated on 0.03 μg penicillin G ml $^{-1}$. Two hundred transformants from the 0.03 μg ml $^{-1}$ plate were velvet replica-plated to increasing concentrations of penicillin G (0.02–0.25 μg ml $^{-1}$). The concentration at which an individual isolate no longer showed growth was designated as its MIC. The value plotted at any concentration of penicillin G excludes isolates with any other MIC. The abscissa units are expressed as [(number of isolates with a particular concentration for an MIC)/200] $\times$ 100.

Fig. 2. Survival curves of transformation step isolates. Dilutions of exponentially growing cultures (approx. 10^7 c.f.u. ml $^{-1}$) of transformant isolates (in THGc with no antibiotic) were plated onto increasing concentrations of penicillin G. Survival is expressed as [(number of c.f.u. ml $^{-1}$ on penicillin)/(number of c.f.u. ml $^{-1}$ on non-selective plates) $\times$ 100]. ●, V288 wild type; ○, ss.03T; ▽, ss.10T; □, ss.25T; ◇, ss.55T.

Table 1. Transformation of V288 wild-type and step-level isolates to penicillin resistance

Horse-serum-induced competent recipients were incubated in THGc with 4.0 µg donor DNA for 2 h prior to selection on penicillin plates. The DNA donor in all cases was a laboratory-derived penicillin-resistant (MIC 0.8 µg penicillin G ml $^{-1}$) *S. sanguis* Challis strain designated S.55D. The recipients were representative strains of each step-level of resistance (unable to survive at a concentration of penicillin greater than that on which they were isolated). See Results for a complete explanation of the derivation of step-level isolates.

Recipient	Concn of penicillin G (µg ml $^{-1}$)	Transformation frequency*
V288	0.03	2.5×10^{-2}
	0.10	2.6×10^{-2}
	0.25	0
ss.03T	0.10	1.1×10^{-2}
	0.25	1.6×10^{-4}
	0.55	0
ss.10T	0.25	3.0×10^{-3}
	0.55	0
ss.25T	0.55	1.9×10^{-3}

* Transformation frequency was calculated as the number of transformants per number of viable cells at the time of DNA addition. The values presented are means of three replicate determinations on separate occasions.

of V288, step transformants, and S.55D organisms on various concentrations of penicillin G are shown in Fig. 2. The steep nature of the curves suggests that each transformant represents one step in the acquisition of penicillin resistance.

Comparison of transformation frequencies with a different marker

The decrease in transformation frequencies that accompanied the acquisition of penicillin resistance (Table 1) posed an interesting question. Was there a general decrease in the transformability of these organisms or was the lowered frequency associated with the penicillin resistance marker itself? To answer this question, a rifampicin-resistant derivative of S.55D was obtained by spontaneous mutant selection (MIC > 1.0 µg rifampicin ml $^{-1}$). Its DNA was

Table 2. *Transformation of V288 wild-type and step-level isolates to penicillin and rifampicin resistance*

Horse-serum-induced competent recipients were incubated in THGc with 4.0 µg donor DNA for 2 h prior to selection on penicillin or rifampicin plates. The DNA donor was a rifampicin-resistant derivative of S.55D created through spontaneous mutant selection (MIC > 1.0 µg rifampicin ml⁻¹).

Recipient	Concn of penicillin G (µg ml ⁻¹)	Transformation frequency*		Ratio b/a†
		Pen ^r (a)	Rif ^r (b)	
V288	0.03	1.4×10^{-1}	4.2×10^{-2}	0.3
ss.03T	0.10	1.3×10^{-1}	2.2×10^{-1}	1.7
ss.10T	0.25	1.3×10^{-2}	9.4×10^{-2}	7.2
ss.25T	0.55	2.5×10^{-4}	5.6×10^{-2}	223.0

* Calculated as described in Table 1. The values are means of two replicate determinations on separate occasions.

† The ratio of the transformation frequencies of the two markers was calculated to determine whether there was a decrease in transformability of the strains or whether reduced transformation frequencies were associated with the penicillin resistance marker (see Results).

extracted and used to transform the step-level isolates and the V288 wild-type to the next penicillin step as well as to rifampicin resistance (Table 2). Once again, the transformation of ss.10T and ss.25T to higher levels of penicillin resistance occurred at a lower frequency than the transformation of the V288 wild-type and ss.03T. However, transformation to rifampicin resistance of all of these organisms occurred at similar frequencies (4.2×10^{-2} to 2.2×10^{-1}).

Transformation hit-kinetics analysis

The bimodal distribution of penicillin resistance levels in the original 0.03 µg ml⁻¹ isolates (Fig. 1) suggested the possibility that at least two genes were being transformed (one for each level of resistance). In addition, the similar frequency in the transformation of the wild-type to resistance levels of 0.03 and 0.10 µg ml⁻¹ (2.5×10^{-2} and 2.6×10^{-2} , respectively; see Table 1) raised the possibility of linkage between these genes. To investigate this possibility further, transformations of *S. sanguis* V288 were conducted using various amounts of S.55D DNA (0.01–4.0 µg). Analysis of the slope of the log-log plot of transformants per ml versus amount of DNA gives the number of DNA fragments taken up by the cells that yield successful transformational events (Goodgal, 1961). Transformation of V288 to the first step requires a single 'hit' as evidenced by a slope of 1 (Table 3). Transformation of V288 to the second step gives a slope greater than 1, indicating that more than one hit is required for expression of this level of resistance. That the slope is 1.26 to 1.58 and not 2 suggests that both genes are, in some cases, on the same piece of DNA (as would be the case if the two genes were linked).

Transformation of ss.03T to a resistance level of 0.10 µg ml⁻¹ (first step to second step) using DNA from ss.10T should represent a single step, and should be a single-hit event. As expected, the slope was approximately 1 (0.94). However, using the same ss.10T DNA to transform V288 to 0.10 µg ml⁻¹ (two steps) gave a slope greater than 1 (1.15) (Table 3). The difference in these values is statistically significant ($P = 0.006$).

To further check the hypothesis that the genes giving resistance to 0.03 and 0.10 µg ml⁻¹ are linked, we did an experiment using mechanically sheared and unsheared S.55D DNA (Fig. 3). Unsheared DNA gave a slope of 1.41, whereas sheared DNA gave a slope of 1.80 (much closer to the theoretical maximum value of 2.0), consistent with the involvement of two linked genes which were separated by the shearing process.

Penicillin-binding proteins

S. sanguis possesses five major PBPs of molecular masses 86, 83.5, 78.5, 72 and 47 kDa as determined by mobility in SDS-PAGE. We number the PBPs 1 to 5 in order of decreasing size (Fig. 4). PBPs 3, 4, and 5 show a major band and a minor band, of slightly lower apparent

Fig. 3. Transformation hit-kinetics analysis. Various amounts of sheared ($\bullet$) or unsheared ($\circ$) S.55D DNA were used to transform V288 wild-type (see Methods). Transformants were selected on penicillin G, $0.03 \mu\text{g ml}^{-1}$. Shearing of DNA was accomplished by passing it 30 times through a 25-gauge needle. Slopes, determined by linear-regression analysis of a log-log plot of transformant c.f.u. ml^{-1} versus μg DNA used, were: unsheared, 1.41; sheared, 1.80 ($P < 0.01$).

Table 3. Results of transformation hit-kinetics experiments

Competent cells were incubated with various amounts of donor DNA (0.01 – $4.0 \mu\text{g}$). The slope of a log-log plot of the number of transformants ml^{-1} versus the amount of DNA yields the number of DNA fragments taken up by the cell resulting in a successful transformation event (see Results).

DNA donor	Recipient	Concn of penicillin G ($\mu\text{g ml}^{-1}$)	Expected slope*	Observed slope†
S.55D	V288	0.03	1.00	1.04
S.55D	V288	0.03	1.00	1.02
ss.10T	ss.03T	0.10	1.00	0.94
S.55D	V288	0.10	> 1	1.26
S.55D	V288	0.10	> 1	1.47
S.55D	V288	0.10	> 1	1.58
ss.10T	V288	0.10	> 1	1.15

* Expectation based on the hypothesis that two genes exist, one conferring resistance to $0.03 \mu\text{g ml}^{-1}$, one to $0.10 \mu\text{g ml}^{-1}$.

† The slope was obtained by linear regression analysis using seven points.

molecular mass. There was no change in the molecular mass of any of the PBPs with increasing level of resistance. The fluorograph shown in Fig. 4 is presented for qualitative not quantitative analysis. Quantification of the fluorographs involved consideration of fluorograph incubation time and protein content as well as comparison to standardized samples. The minor bands of PBPs 3, 4, and 5 detected with very long film exposure varied in intensity from preparation to preparation and were shown in a series of experiments to be breakdown products of the respective major bands. They were therefore pooled in densitometric analysis.

Fig. 4. Fluorograph of *S. sanguis* strains. PBPs were detected by incubation of membrane-wall preparations with [3 H]benzylpenicillin, SDS-PAGE and subsequent fluorography (see Methods). All lanes contain approximately equal amounts of protein (200 μ g). The apparent molecular masses of the five major PBPs are 86, 83.5, 78.5, 72 and 47 kDa. The donor, S.55D, possesses PBPs of the same molecular mass as the recipients (lane from different gel). Quantitative differences are described in the text and Fig. 5.

Fig. 5 presents the results obtained by the quantification procedure described in Methods. In the wild-type, V288, PBP 2 binds at the lowest concentration of antibiotic and becomes saturated at 12.5 μ g penicillin ml $^{-1}$. PBP 4 has the highest saturation value by far of all of the PBPs (note different scale for the PBP 4 plot in Fig. 5). Comparison of the PBPs of *S. sanguis* V288 and of the resistant DNA donor S.55D (Figs 4 and 5) shows a decrease in affinity of binding of penicillin by PBPs 1, 2 and 3 in the resistant strain. PBP 4 has a great decrease in affinity and saturation value. There is no apparent alteration in PBP 5.

We also looked for differences associated with each step level of resistance (Fig. 6). The first step resulted in a decrease in binding affinity of all five PBPs. The decreases in PBPs 4 and 5 were quite pronounced. The second step (ss.10T) showed no further decreases in PBP affinity, although PBP 5 increased back to levels similar to the wild-type. The third (ss.25T) and fourth (ss.55T) steps were characterized by further decreases in the binding of penicillin by PBP 4. In the final step, PBP 4 was barely detectable, and only at the highest concentrations of antibiotic.

In a time course assay, there was an average increase of 30% in the amount of radiolabelled penicillin bound to individual PBPs when the binding incubation period was increased from 15 to 60 min (data not shown). The data did not suggest any mechanisms of resistance due to a slow-kinetic binding PBP such as that found in *S. faecium*, where differences are about 500%.

Fig. 5. Densitometric analyses of V288 wild-type (a) and S.55D (b) PBP banding on fluorographs versus concentration of $[^3\text{H}]$ benzylpenicillin. Density units were determined by measuring the areas under peaks in a densitometric scan of the fluorographs, correcting for amount of protein loaded, and standardizing against the wild-type results (see Methods). Error bars represent the standard deviation of values from three separate preparations and gels. Data points are staggered horizontally to allow for clear presentation of error bars (PBP 2 points are on actual values). $\circ$, PBP 1; $\bullet$, PBP 2; $\triangle$, PBP 3; $\square$, PBP 4 (note different scale); $\diamond$, PBP 5.

Membrane protein patterns

PBPs rarely constitute more than 1% of the total membrane protein of the cell (Blumberg & Strominger, 1974), preventing their visualization without radiolabelling. Examination of the Coomassie-blue-stained SDS-polyacrylamide gels of our step-resistant organisms failed to show consistent differences in membrane proteins.

Fig. 6. Densitometric analyses of the PBP binding for each PBP of V288 wild-type and transformant isolates (see Fig. 5 for explanation). ●, V288 wild-type; ○, ss.03T; ∇ , ss.10T; $\square$, ss.25T; $\diamond$, ss.55T.

DISCUSSION

In this report, we describe the transformation of a penicillin-susceptible strain of *S. sanguis* V288 to step-levels of penicillin resistance using a resistant DNA donor. That resistance to β -lactam antibiotic in streptococci occurs in increments had already been described by Shockey & Hotchkiss (1970) and by Butler & Smiley (1970). As in those studies using *S. pneumoniae*, penicillin resistance in *S. sanguis* appears to be multigenic.

The present report suggests that at least two of the genes responsible for the observed transformation of *S. sanguis* V288 to step-levels of penicillin resistance are linked. The initial evidence for linkage of resistance to 0.03 and 0.10 $\mu\text{g penicillin ml}^{-1}$ was that the transformation frequencies for both levels were similar (approx. 2.5×10^{-2}) (Table 1). Further evidence of possible linkage was obtained from transformation hit-kinetics experiments, which showed that transformation to a resistance level of 0.03 $\mu\text{g penicillin G ml}^{-1}$ required a single 'hit' or DNA fragment (Table 3), whereas transformation to a resistance level of 0.10 $\mu\text{g ml}^{-1}$ required more than one hit (a range of 1.25 to 1.58 hits). The observation that this requirement was not for two fragments (as would be expected with two separate unlinked genes) further supported the linkage hypothesis (since in some transformation events, both genes resided on a single fragment taken up by the cell). Additional hit-kinetics experiments using DNA from ss.10T (the second step-level isolate) gave similar results: single-step transformants of resistance (ss.03T to 0.10 $\mu\text{g ml}^{-1}$) required one hit (0.94 fragments) and two-step transformations (V288 wild-type to 0.10 $\mu\text{g ml}^{-1}$) required more than one hit (1.15 fragments) (Table 3). Finally, mechanically shearing the donor DNA prior to transformation increased the fragment requirement from 1.41 to 1.80, closer to the 'ideal' value of 2.0 expected from two different fragments providing the two steps of resistance (Fig. 3).

The frequency of transformation of ss.03T to penicillin resistance levels of 0.10 $\mu\text{g ml}^{-1}$ and 0.25 $\mu\text{g ml}^{-1}$ (10^{-2} versus 10^{-4}) indicated that each level of resistance appeared to require an additional unlinked gene. The isolation of ss.25T and ss.55T at decreasing frequencies (3×10^{-3} and 1.9×10^{-3} , Table 1) was shown to be due to the expression of the penicillin resistance phenotype, rather than to a general decrease in transformability of the cells (Table 2). A decrease in the frequency of transformation to high levels of penicillin resistance has also been observed in *S. pneumoniae* (Tomasz *et al.*, 1984) and *Bacillus subtilis* (Buchanan & Strominger, 1976). The difficulties in the expression of the penicillin resistance phenotype may be due to problems associated with the insertion of an altered PBP into the membrane, to growth-inhibiting changes in membrane structure, or to the presence of other copies of the susceptible genotype acting in a dominant manner. Possibly, the expression of high-level penicillin resistance requires a longer phenotypic lag.

A most important observation in this study was that single-step changes in the levels of penicillin resistance were associated with changes in binding affinities of more than one PBP. This suggests that the various transformants may not have mutations in the structural genes coding for the PBPs themselves. The mutations may have affected gene expression, antibiotic accessibility, membrane composition, or the processing and insertion of PBPs into the membrane, all of which can affect PBP binding affinities or levels. A similar observation has been made in *Pseudomonas aeruginosa*, where reduction in binding affinities of several PBPs accompanied penicillin resistance acquired after a single gene mutation (Godfrey & Bryan, 1982). That the multi-PBP alteration at a single step of resistance is not due to more than one DNA molecule taken up during transformation was demonstrated by the hit-kinetics analysis.

The decrease in PBP 4 that accompanied the development of penicillin resistance in *S. sanguis* is particularly notable, for it was consistent and dramatic. The PBP 4 of the high-level transformant (ss.55T) was barely detectable. Due to the limitations of the assay system, this decrease may be due to either reduced numbers of PBP 4 molecules or production of a modified molecule which binds penicillin poorly. The modified protein may or may not be able to function at a level of activity corresponding to wild-type levels. A decrease in PBP 4 activity may be compensated by increased synthetic activities of other PBPs, since there appears to be redundancy in PBP activity (Brown & Reynolds, 1980). Severely decreased PBP 4 activity does not result in substantial reduction in the growth rate of ss.55T in THGc medium (doubling time

36 min, versus 32 min for V288 wild-type and for transformants ss.03T, ss.10T and ss.25T). It may be interesting in future studies to attempt to detect low-affinity PBPs by utilizing anti-PBP antibodies (Hakenbeck *et al.*, 1986).

The alterations in PBP 5 did not follow the expected gradual decrease (or increase) with increased penicillin resistance. There did appear to be a decrease in affinity associated with resistance, but saturation values increased across the final three step-levels. After the initial decrease in ss.03T, PBP 5 was apparently restored to wild-type levels. This may indicate that there is an absolute requirement for a certain level of PBP 5 activity for growth and survival of cells in higher concentrations of penicillin. Alternatively, PBP 5 may be a protein which is able to compensate for reduced activities in other PBPs. Thus the increase in resistance from 0.03 to 0.10 µg ml⁻¹ may involve a deregulation or 'amplification' of an altered PBP 5 serving to compensate for the reduced PBP 4.

A further point of interest was the fact that the highest step-level resistant transformant, ss.55T, although having the same level of penicillin resistance as the DNA donor S.55D, had a different PBP pattern. In theory, the series of steps of resistance obtained in this study may be one of several possible series. Acquisition of a different first-step 'alteration' may potentiate the development of further resistance via an alternative series of transformable mutations. It is probable that the series of mutations that led to the isolation of the DNA donor S.55D is not reflected in the step-level resistances of the transformants, since the PBPs of the transformants do not approach the pattern of S.55D (Fig. 4). A less likely possibility is that the altered binding levels of S.55D are due to its very slow growth (doubling time 64 min, versus 32 min for the wild-type V288).

In summation, we have described a series of genetic and biochemical changes associated with increasing penicillin resistance, and have shown that these changes result in multiple alterations in binding activities of the PBPs at each step. The novel aspect of this study is the demonstration that a 'single-hit' transformation event can result in alterations in the affinity of more than one PBP. In addition, a progressive decrease in the penicillin-binding affinity and in penicillin binding was found to occur through a series of presumably single transformation events.

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