Cross-linking and *O*-acetylation of peptidoglycan in *Staphylococcus aureus* (strains H and MR-1) grown in the presence of sub-growth-inhibitory concentrations of β -lactam antibiotics

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Staphylococcus aureus H was grown for 4 generation times with various sub-growth-inhibitory concentrations of β -lactam antibiotics specific for particular penicillin-binding proteins (PBPs) – PBP2, clavulanic acid; PBP3, methicillin; PBP4, cefoxitin – and also with the non-specific benzylpenicillin. Isolated cell walls were digested with *Chalaropsis* muramidase and the resulting peptidoglycan fragments were fractionated by HPLC into disaccharide-peptide monomers and cross-linked dimers, trimers, tetramers and greater oligomers. The pattern of relative fragment concentrations with increasing amounts of drug was roughly the same regardless of the antibiotic used, monomers and dimers increasing while trimers and tetramers changed little and oligomers decreased rapidly. The patterns resembled closely those predicted by the ‘random addition’ model for multiple cross-link formation and not at all those predicted by the ‘monomer addition’ model. The *O*-acetylation of the peptidoglycan remained essentially unaffected under all these conditions. *S. aureus* MR-1, a constitutive producer of PBP2', gave similar results when treated with methicillin.

Introduction

Concentrations of β -lactam antibiotics too small to affect bacterial growth or the overall synthesis of peptidoglycan may nevertheless influence its composition. Thus in *Neisseria gonorrhoeae*, sub-growth-inhibitory drug concentrations profoundly affect the degree of *O*-acetylation of the peptidoglycan and the cellular morphology (Blundell & Perkins, 1981). Similarly, in *Staphylococcus aureus* SG511 ‘Berlin’ sub-lytic doses of benzylpenicillin, which nonetheless had some long-term inhibitory effect on growth, produced within 60 min a marked decrease in *O*-acetylation and a near-maximum effect on the degree of cross-linking of the peptidoglycan (Sidow *et al.*, 1990; Labischinski *et al.*, 1988).

Our previous work has shown the rapidity with which cross-linking and *O*-acetylation occur during the synthesis of peptidoglycan in *S. aureus* H (Snowden *et al.*, 1989) and also that a random mechanism is used to achieve the high degree of cross-linking observed in this species (Snowden & Perkins, 1990). Some β -lactam antibiotics are known to have a fair degree of specificity for particular penicillin-binding proteins (PBPs) and this is

true in *S. aureus* (cf. Beise *et al.*, 1988). In the present work we have examined the effect of sub-growth-inhibitory concentrations of β -lactams specific for PBP2, 3 and 4 upon the *O*-acetylation of the peptidoglycan and also upon the pattern of monomers and cross-linked fragments (dimers, trimers, etc.) found therein. Earlier work by Qoronfleh & Wilkinson (1986) used this kind of approach but employed rather imprecise methods of fractionating the peptidoglycan.

The correspondence between our results and the predictions of the random addition model for cross-linking is also considered.

Methods

Organisms and growth conditions. *Staphylococcus aureus* H (NCIB 6571) and MR-1, a constitutive producer of the β -lactam resistant PBP2', (Hayes *et al.*, 1981) were grown in nutrient broth (Oxoid no. 2, 50 g l⁻¹) at 37 °C with rotatory shaking at 180 r.p.m. from a 1% (v/v) inoculum of a 16 h culture. Growth was followed by measuring OD₅₈₅; during the exponential phase, mean generation time was 30–35 min.

Preparation and fractionation of peptidoglycan. Peptidoglycan was selectively solubilized from trypsin-treated SDS-insoluble cell wall with *Chalaropsis* B muramidase in 50 mM-sodium citrate buffer (pH 4.7) as described previously (Snowden & Perkins, 1990). Soluble teichoic-acid-linked peptidoglycan fragments were removed by anion-exchange

Abbreviation: PBP, penicillin-binding protein.

chromatography and the remaining material separated into its component peptide-linked oligomers by HPLC gel filtration on a 60×0.75 cm TSK SW2000 column (Snowden & Perkins, 1990). The column was eluted with 50 mM-sodium phosphate buffer (pH 7.0) at 0.3 ml min^{-1} with detection at 214 nm.

Chemical measurement of O-acetyl content. The O-acetyl and N-acetyl content of the peptidoglycan was calculated by measuring the amount of acetate liberated from solubilized peptidoglycan after mild alkali or acid treatment, essentially as described by Fromme & Beilhartz (1978). Duplicate samples of peptidoglycan that had been digested with muramidase, equivalent to about 250 μg , were hydrolysed in sealed ampoules with either 0.05 M-NaOH for 3 h at room temperature or 0.02 M-HCl for 18 h at 105 °C. The former procedure converted O-acetyl groups and the latter both O- and N-acetyl groups, to acetate. The acetic acid content of the hydrolysed samples was measured by using a Dionex high pressure ion chromatograph, and the degree of O-acetylation of the muramic acid was calculated as the ratio O-acetyl/(N-acetyl/2), it being assumed that both hexosamine components were N-acetylated.

To measure released acetic acid, samples were suitably diluted to give a concentration of between 10 and 100 nmol per 100 μl sample.

Each sample was applied to a Dionex Ionpac ion exclusion ASI column coupled to an ASF-2 suppression system, and eluted with 1.25 mM-tridecafluoroheptanoic acid and 1.5% (v/v) 2-propanol at a flow rate of 0.7 ml min^{-1} . Sample peaks were detected with a Dionex A1 conductivity meter and integrated by using a Trilab Trio dedicated system. The system was calibrated against a standard solution of 0.5 mM-sodium acetate and showed a linear response in the range of 1–1000 nmol per 100 μl sample.

Results

Culture of *S. aureus* H and MR-1 were tested with concentrations of β -lactam antibiotics up to 0.6 times the minimum growth inhibitory concentration (MIC). The later was determined as the concentration required to inhibit overnight growth at 37 °C from an inoculum of $10^5 \text{ cells ml}^{-1}$ (Curtis *et al.*, 1980), giving in our hands the following results for *S. aureus* H and MR-1 respectively ($\mu\text{g ml}^{-1}$): benzylpenicillin, 0.02, 200; cefoxitin, 5.0, 350; clavulanic acid, 8.0, 1000; methicillin, 1.25, 900.

Experiments in which growing cultures of *S. aureus* H were treated with β -lactams at 0.5 MIC showed that neither the rate of growth up to 4 h, during which time OD_{585} increased about 50-fold, nor the incorporation of N-acetyl[^{14}C]glucosamine (Snowden *et al.*, 1989) into peptidoglycan was appreciably affected by the presence of benzylpenicillin, cefoxitin, clavulanic acid or methicillin.

Apart from benzylpenicillin, to which *S. aureus* H is extremely sensitive, the MIC value being two orders of magnitude smaller than for any other β -lactam (Wyke *et al.*, 1981), the β -lactams used were chosen on the basis of their selective affinity for particular PBPs. By using conventional methods, the binding of the various drugs to PBPs was studied both in whole cells (Reynolds & Brown, 1985) and in isolated membranes (Frere & Joris, 1985) by competition for the binding of [^3H]benzylpeni-

Fig. 1. Ion exclusion chromatography of acetate liberated from solubilized peptidoglycan after acid and mild alkali hydrolysis. Acetate eluted at between 13.5 and 14 min. The large peak at 7.4 min contains peptidoglycan fragments and Cl^- and OH^- ions present in the acid and alkali hydrolysates respectively.

Table 1. Antibiotic-binding affinities of PBPs in *S. aureus* H

Affinities are expressed as the concentrations of antibiotic ($\mu\text{g ml}^{-1}$) required in the growth medium to decrease the subsequent binding of [^3H]benzylpenicillin by 50% (I_{50}) or 90% (I_{90}). PBP4 was not detected by this technique because of its low affinity for penicillin.

Antibiotic	PBP1		PBP2		PBP3	
	I_{50}	I_{90}	I_{50}	I_{90}	I_{50}	I_{90}
Clavulanic acid	>5.0	>5.0	0.2*	3.0*	>5.0	>5.0
Methicillin	0.55	>0.6	>0.6	>0.6	0.03*	0.2*
Cefoxitin†	>2.0	>2.0	1.0	>2.0	0.15	0.75
Benzylpenicillin	0.005	0.01	0.006	>0.01	0.002	0.006

* Indicates selective binding affinity.

† Cefoxitin binds selectively to PBP4, I_{90} being $0.01 \mu\text{g ml}^{-1}$ (Hayes *et al.*, 1981).

cillin (Table 1). These methods did not show PBP4, but it is already known that cefoxitin binds very specifically to this component, concentrations of $0.01 \mu\text{g ml}^{-1}$ added to whole cells being sufficient to bind more than 90% of PBP4 (Hayes *et al.*, 1981). As Table 1 shows, no other PBP would be bound appreciably even by five times that concentration.

Fig. 2. Effects of sub-MIC concentrations of β -lactam antibiotics on peptidoglycan cross-linking. The bacteria were grown for 4 generations in the presence of antibiotic before preparation of cell walls, muramidase treatment and fractionation of peptidoglycan fragments by HPLC (see Methods). The proportion of total peptidoglycan found as monomers (●), dimers (○), trimers (□), tetramers (■) and higher oligomers (▲) is shown. (a) *S. aureus* H; (b) *S. aureus* MR-1.

Table 2. *O*-Acetylation of peptidoglycan from β -lactam-treated *S. aureus*

Values represent the percentage of muramic acid *O*-acetylated. All results are for *S. aureus* H except where stated. The control values (mean $\pm$ SD) were 62.7 ± 1.9 for *S. aureus* H and 63.7 ± 2.5 for strain MR-1. Concentrations of β -lactam at or above I_{90} for PBP2 (clavulanic acid), PBP3 (methicillin) and PBP4 (cefoxitin), are in **bold** type.

Clavulanic acid		Cefoxitin		Benzylpenicillin		Methicillin		Methicillin* (<i>S. aureus</i> MR-1)	
$\mu\text{g ml}^{-1}$	% <i>O</i> -acet.	$\mu\text{g ml}^{-1}$	% <i>O</i> -acet.	$10^2 \times$ $\mu\text{g ml}^{-1}$	% <i>O</i> -acet.	$\mu\text{g ml}^{-1}$	% <i>O</i> -acet.	$\mu\text{g ml}^{-1}$	% <i>O</i> -acet.
0.25	64.5	0.2	56.2	0.05	59.7	0.01	64.6	1	63.5
0.5	59.5	0.4	61.3	0.1	65.5	0.05	65.5	2	62.6
0.75	62.7	0.5	59.9	0.2	62.9	0.125	63.5	4	59.5
1.0	64.3	1.25	61.2	0.4	61.4	0.25	61.0	8	58.8
2.0	63.6	1.5	60.4	0.6	63.7	0.375	57.0	10	61.1
3.0	61.5	2.0	59.0	0.8	62.1	0.5	65.6	12	62.3
4.0	60.0	2.5	61.5	1.0	61.9	0.625	63.0	20	66.4
								60	60.8
								100	61.1
								120	59.7

* Concentrations in **bold** type are above those needed to saturate PBPs 1, 2 and 3 (Brown & Reynolds, 1985).

Effects on O-acetylation of peptidoglycan

The peptidoglycan of bacteria grown for 4 generations in the presence and absence of various non-growth-inhibitory concentrations of β -lactams was prepared and its *O*-acetyl content was measured chemically by high-pressure ion chromatography (see Methods; Fig. 1). None of the four β -lactams used had any appreciable effect on the degree of *O*-acetylation in *S. aureus* H and a similar lack of effect was observed in *S. aureus* MR-1 exposed to methicillin (Table 2). The concentrations of antibiotic used included ones both below and above those required to saturate PBP2, 3 and 4 selectively or all PBPs collectively in *S. aureus* H.

Effects on cross-linking of peptidoglycan

Samples of peptidoglycan from the type of experiment just described were exhaustively digested with *Chalaropsis* muramidase and separated into monomers (i.e. uncross-linked) and dimers, trimers, tetramers and higher oligomers by HPLC gel filtration (Snowden & Perkins, 1990). At all concentrations of the drugs <0.6 MIC present for 4 generations of growth, profound changes were observed in the cross-linking (Fig. 2*a, b*). The proportion of monomers always increased, and in *S. aureus* H at 0.5 MIC cefoxitin produced the greatest effect, whereby about 50% of all the peptidoglycan was present as monomers, corresponding values being 46% (clavulanic acid), 39% (methicillin) and 37% (benzylpenicillin).

The effects on the proportion of cross-linked fragments also varied somewhat with the antibiotic. Thus from a control value for dimer of about 11–13%, the presence of 0.5 MIC β -lactams produced increases to the following values: cefoxitin, 30%; benzylpenicillin, 26%; clavulanic acid, 25%; and methicillin, 28%. These increases were presumed to be as a direct consequence of the inhibition of higher oligomer synthesis rather than by a specific stimulation of dimer production. For the trimers there were slight increases in the presence of benzylpenicillin and clavulanic acid and no change with cefoxitin or methicillin. Tetramers (10%) remained unchanged with clavulanic acid but declined slightly in the presence of the other β -lactams. At 0.5 MIC the proportion of oligomers larger than tetramers decreased sharply: controls, 54–57%; benzylpenicillin, 12.5%; methicillin, 14%; clavulanic acid, 6%; and cefoxitin, 4%.

The pattern of cross-linking inhibition in *S. aureus* MR-1 caused by methicillin (Fig. 2*b*) was similar to the pattern observed with *S. aureus* H with all β -lactams. Although in the resistant strain equivalent levels of cross-linking inhibition were only attained at much higher

concentrations of the antibiotic, these concentrations represented a far smaller proportion of the MIC. Thus, whereas in *S. aureus* H the monomers and dimers increased respectively to about 39% and 28% at 0.5 $\mu\text{g ml}^{-1}$ methicillin (0.5 MIC), comparable increases in these fractions in *S. aureus* MR-1 were seen only at 9–10 $\mu\text{g ml}^{-1}$ (0.01 MIC). Methicillin concentrations above 20 $\mu\text{g ml}^{-1}$ had little further effect on the extent of cross-linking. At the final plateau the fragment proportions were: monomers, 44%; dimers, 32%; trimers, 14%; and tetramers 5%. The oligomers, originally representing 58% of all peptidoglycan units, decreased to only 1–2%. These relative proportions corresponded to a cross-linking index of about 0.28.

Discussion

In our experiments, in which the degree of *O*-acetylation of *S. aureus* H and MR-1 peptidoglycans was studied after 4 generations of growth in the presence of sub-MIC concentrations of β -lactams, no appreciable changes were observed (Table 2). This result contrasts with those of Sidow *et al.* (1990) and Labischinski *et al.* (1988), who used *S. aureus* SG 511 'Berlin' and a growth period of only 60 min (approx. 2 generations), and with those of Qoronfleh & Wilkinson (1986), who studied methicillin-resistant and susceptible strains of *S. aureus* (strains DU 4916 and derivatives) grown for 18 h in the presence of β -lactam antibiotics. These authors detected reductions in the degree of *O*-acetylation in some circumstances. At the moment, apart from the differences of strain and experimental design noted, no obvious explanation for this discrepancy presents itself.

Since *S. aureus* lacks carboxypeptidase activity (Matsushashi *et al.*, 1967), all the PBPs are presumed to have transpeptidase activity (Wyke *et al.*, 1983; Georgopapadakou *et al.*, 1986). Our observation that peptidoglycan cross-linking was decreased by inhibition of PBP2, 3 and 4 either separately (by clavulanic acid, methicillin or cefoxitin respectively) or together (by benzylpenicillin) was unsurprising, though one might not have expected that increasing drug concentrations from values as low as 0.05 MIC, or even less for strain MR-1, would produce smoothly cumulative effects (Fig. 2). Still more surprising was the fact that selective inhibition of the various PBPs yielded a remarkably similar pattern of cross-linking inhibition. There was no evidence that the pattern of inhibition changed as different PBPs were successively bound by increasing drug concentrations. The similarities between the inhibition curves could possibly reflect the fact that PBP2 and 3 in *S. aureus* have similar catalytic properties, whereby each is capable of forming peptide oligomers with a similar size range.

Fig. 3. Predictions of proportions of peptidoglycan fragments that should occur in benzylpenicillin-treated *S. aureus* H, as calculated from the random addition (a) and monomer addition (b) models. The observed values for each drug concentration (Fig. 2a) were converted to crosslinking indices (Snowden & Perkins, 1990) and the equations of Flory (1953) were used to calculate the theoretical proportion of each component (for details see text). Symbols are as in Fig. 2.

Such a proposal would not preclude different PBPs having different physiological roles (Georgopapadakou *et al.*, 1986) but would suggest that if they had, the distinguishing features would lie in properties other than the formation of specific polymer types. In contrast, Kraus & Hölte (1987) proposed that *Escherichia coli* had two transpeptidation systems (PBPs), one of which preferentially synthesized dimers and the other larger oligomers. In *S. aureus* circumstantial evidence for co-ordinated PBP activities derives from the work of Reynolds & Brown (1985), who found that all the essential PBPs can be replaced by one enzyme (PBP2') and still allow normal growth of the bacteria. Our results could well support the view of Tuomanen (1986) that only the relatively small proportion of newly-made PBP is critical in determining the effects of antibiotics. This PBP could be PBP1 (Beise *et al.*, 1988; Reynolds, 1988; Sidow *et al.*, 1990).

Snowden & Perkins (1990) produced evidence for the operation of a random addition model for the synthesis of the observed population of peptidoglycan oligomers in *S. aureus*. It was therefore possible to ask whether the patterns of change in relative oligomer proportions wrought by increasing β -lactam concentrations (Fig. 2) were consistent with this model. Theoretical curves for both the random addition and monomer addition models (Snowden & Perkins, 1990) were constructed as follows. First the values for the cross-linking index (CI) achieved

in practice with *S. aureus* H under the influence of β -lactam concentrations up to 0.5 MIC were calculated from the observed proportions of monomers, dimers, etc., shown in Fig. 2(a). Then the theoretical equations of Flory (1953) were used to calculate the proportions of monomer, dimer, etc., that either model would predict for each observed value of CI (for details of this type of calculation see Snowden & Perkins, 1990). The resulting values were plotted against benzylpenicillin concentration (Fig. 3). It was immediately apparent that the random addition model produced curves closely similar to the observed values (Fig. 2a), whereas the monomer addition model did not. Thus the present experiments support our proposal that a random addition model operates in the formation of multiply cross-linked peptidoglycan in *S. aureus* H. The similarity of the corresponding curves for *S. aureus* MR-1 (Fig. 2b) suggests that the same mechanism operates in that strain too.

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