The susceptibility to lysozyme of β -lactamase-producing and non-producing derivatives of *Staphylococcus aureus* strain 1030

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Summary. The killing of *Staphylococcus aureus* strain 1030 and derived variants of it by lysozyme increased with increased lysozyme concentrations or decreased concentrations of sodium chloride. β -Lactamase-producing and non-producing derivatives of strain 1030 were constructed. The former were less susceptible to lysozyme. Induction of β -lactamase synthesis with 2-2'-carboxyphenyl-benzoyl-6-penicillanic acid increased the resistance of producer strains to lysozyme. These results are discussed in relation to the spread of β -lactamase-producing strains of *S. aureus*.

Introduction

In a previous study (Millar and Lacey, 1984), it was suggested that lysozyme from tears selects for β -lactamase production in *Staphylococcus aureus*. Staphylococcal β -lactamase may be bound to the cell membrane (Neilsen and Lampen, 1982) or ionically associated with the cell wall (Coles and Gross, 1967). It contains a high concentration of polar amino-acids, particularly lysine (Ambler and Meadway, 1969), and has an iso-electric point of 8.9 (Richmond, 1975).

Lysozyme is also a cationic protein (pI 11.0) present in the primary and secondary granules of human neutrophils (Leffell and Spitznagel, 1972; Bretz and Baggiolini, 1974) and also, in high concentration, in tears and, therefore, in nasal secretions. Lysozyme is a muramidase, its action on the peptidoglycan of *Micrococcus lysodeikticus* resulting in cell lysis (Davies *et al.*, 1969). The cell wall of *S. aureus* is resistant to the muramidase activity of lysozyme (Brumfitt *et al.*, 1958; Mandelstam and Strominger, 1961; Morse, 1965; Thorne *et al.*, 1976) but several authors have shown that lysozyme can degrade the cell wall of *S. aureus*, possibly by activating autolytic wall enzymes (Kern *et al.*, 1951; Mitchell and Moyle, 1957; Wecke *et al.*, 1982). Lysozyme has also been shown to inhibit bacterial growth (Iacono *et al.*, 1980) and, in certain conditions, to cause bacterial aggregation (Golub *et al.*, 1985). In this study, the killing of *S. aureus* by lysozyme has been investigated and the hypoth-

esis that lysozyme, derived from tears, selects for β -lactamase production by *S. aureus* has been examined.

Materials and methods

Bacterial strains

A rifampicin-resistant (Rf^R) derivative of *S. aureus* strain 1030 (Novick, 1967) was selected by growth of strain 1030 on nutrient-agar plates containing rifampicin 50 mg/L. This was used to construct two pairs of strains, each pair being isogenic except for the characteristic of β -lactamase production, by the following method. *S. aureus* strain 13136 (Jevons, 1961), which carries a plasmid coding for resistance to cadmium ions and high-level β -lactamase production, was treated with N-ethyl-N'-nitro N-nitrosoguanidine to produce a β -lactamase-negative (βla^-) derivative that was cadmium resistant (Cd^R). Mitomycin C (Lacey, 1971) was used to induce phage lysis of these two strains. The lysate was filtered through an 0.2- μm filter and tested for sterility. Cadmium resistance was transferred to the Rf^R variant of strain 1030 by transduction by the method of Dyke *et al.* (1970), such that two strains were produced differing only with respect to β -lactamase synthesis (strains 1030 $Rf^R, Cd^R, \beta la^+$ and 1030 $Rf^R, Cd^R, \beta la^-$). These two strains were used in subsequent experiments.

Two further strains were derived from them. Cadmium sensitive (Cd^S) derivatives were selected by replicate plating after culture on nutrient-agar plates overnight at 43°C. Mitomycin C lysates of β -lactamase-producing and non-producing strains 13136 were again used to transfer cadmium resistance to these two strains, to produce a further two strains derived from the first pair, i.e., strains 1030' $Rf^R, Cd^R, \beta la^-$ and 1030' $Rf^R, Cd^R, \beta la^+$.

Plasmid DNA, extracted from these strains by a

modification of the method of Birnboim and Doly (1979) in which lysostaphin replaced lysozyme, was separated by slab gel electrophoresis. When stained with ethidium bromide and seen by UV transillumination, the plasmid DNA from β la⁺ and β la⁻ strains had similar electrophoretic mobility. Both pairs of derivatives of strain 1030 were used in experiments to compare the susceptibility of β la⁺ and β la⁻ strains to lysozyme.

By use of Mitomycin-C lysates of recent clinical isolates of *S. aureus*, strains 37810, 54353 and 778, further derivatives of strain 1030 were constructed as above—strains 1030/37810 Rf^R, Cd^S, β la⁺; 1030/54353 Rf^R, Cd^R, β la⁺; and 1030/778 Rf^R, Cd^S, β la⁺.

All the derivatives of strain 1030 appeared identical after growth on milk and mannitol salt agar and on Baird-Parker medium (1962) and had similar phage typing patterns (see the table).

Susceptibility to lysozyme

Stationary-phase cultures of all seven derivatives of *S. aureus* strain 1030 were prepared by seeding 10 ml of nutrient broth with material from an overnight nutrient-broth culture to a concentration of 10^6 staphylococci/ml and incubating at 37°C for 18 h. To induce β -lactamase synthesis, methicillin or 2-2' carboxyphenyl-benzoyl-6-aminopenicillanic acid (CBAP) was added at concentrations of 0.1–5 mg/L before incubation. The cells from a 1 ml sample of each overnight broth culture were washed twice and resuspended in 0.1M Tris-HCl buffer, pH 7.0. To measure the effect of removing extra-cellular β lactamase on the susceptibility of cocci to lysozyme, β -lactamase producing cells were incubated in 0.2M phosphate buffer, pH 7.0, for 0.5 h after the first wash. The influence of time of incubation in nutrient-broth cultures on the susceptibility of strain 1030 Rf^R, Cd^R, β la⁻ to lysozyme was also examined. For these experiments, 10-ml volumes of warmed nutrient broth were seeded from a single overnight broth culture kept at 4°C and incubated for intervals of up to 6 h. The cells from each broth culture were then washed as described above.

The washed-cell suspensions were adjusted to the same optical density in a spectrophotometer (Pye Unicam SP 6)

at 680 nm, by adding 0.1M Tris-HCl buffer, pH 7.0. Chicken egg-white lysozyme (Sigma Chemical Co., Poole, Dorset) was added in a range of concentrations from 50 to 1000 mg/L to Tris-HCl or phosphate buffer or saline, 0.01–0.2M. In experiments to compare the susceptibility to lysozyme of derivatives of strain 1030, lysozyme was prepared in 0.03M Tris-HCl buffer, pH 7.0, at a concentration of 500 mg/L. To limit differences in susceptibility due to small differences in growth rate, stationary-phase cultures were used in all these experiments. Tubes (10 × 1 cm) containing 1 ml of buffer or buffer plus lysozyme were seeded with the derivatives of strain 1030 to give a final concentration of viable organisms of 5×10^6 /ml. The tubes were then incubated unshaken for 1.5 h at 37°C in aerobic conditions.

Viable bacterial counts

Microbiological method. Tenfold dilutions of each live-cell suspension were made in 0.1M buffer and 0.1 ml volumes of each were spread on to two nutrient-agar plates which were incubated overnight at 37°C. The mean viable counts were obtained by counting, with a colony counter, the colonies on plates carrying 30–300 colonies.

Bioluminescence method. Cells were centrifuged at 800g for 10 min to a pellet and 0.8 ml of supernate removed by pipette before 0.2 ml of trichloroacetic acid 5% w/v with 4 mM EDTA was added to each tube to release intracellular ATP. After mixing, 10 μ l were removed and added to a cuvette containing 240 μ l of Tris-acetate buffer, pH 7.75, with 2 mM EDTA and 50 μ l of luciferase (LKB Wallace, Croydon, Surrey). The resulting light output was measured for 10 s in an LKB Wallace luminometer. An ATP standard was used to allow adjustment for differences in luciferase activity. There was less than 5% variation in luminometer values on repeated sampling from the same specimen.

Because lysozyme may cause aggregation of bacterial particles, particularly in solutions of low ionic strength (Golub *et al.*, 1985), the viable counts were performed microbiologically and repeated by the luminometer method.

Table. Phage typing patterns (at RTD) of strains derived from *S. aureus* strain 1030

Strains providing Mitomycin C lysate	Characteristics of derived strains	Lytic reactions with phages																								
		29	52	52A	79	80	3A	3C	55	71	95	6	42E	47	53	54	75	77	83A	84	85	81	94	96	88A	90
13 136 β la+	*Rf ^R , Cd ^R	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
13 136 β la-	*Rf ^R , Cd ^R	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
54 353 β la+	Rf ^R , Cd ^R	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
778 β la+	Rf ^R , Cd ^S	++	++	+	+	++	++	++	++	+	+	±	++	++	±	++	++	±	++	++	++	++	±	++	±	+
37 810 β la+	Rf ^R , Cd ^S	++	++	...	...	++	++	++	+	...	...	++	++	++	...	++	++	...	++	++	±	++	...	...	...	++

* 1st pair: The 2nd pair had an identical phage-typing pattern. ++ = 50 plaques or more; + = 20–49 plaques; ± = <20 plaques. β la⁺ = β -lactamase producing; β la⁻ = β -lactamase non-producing; Rf = Rifampicin; Cd = Cadmium; R = Resistant; S = Sensitive.

Percentage survival figures

These were calculated from the formula:

$$\frac{\text{Viable count in buffer + lysozyme}}{\text{Viable count in buffer}} \times 100$$

β -lactamase activity

This was assayed by the iodometric method of Perret (1954).

Results

When the killing of *S. aureus* by lysozyme was investigated, reducing the molarity to below 0.04M in Tris-HCl, phosphate buffers or saline increased the susceptibility of strain 1030Rf^R, Cd^R, β la⁻, and cells prepared from broth cultures incubated aerobically were more susceptible in solutions of low molarity than those from broth cultures incubated anaerobically (fig. 1). The pH of a nutrient-broth culture of *S. aureus* strain 1030 grown aerobically for 18 h was 7.3 compared with 6.7 for a culture grown in anaerobic conditions. Increasing the concentration of lysozyme over the range 50–1000 mg/L increased the killing of *S. aureus* strain 1030 Rf^R, Cd^R, β la⁻ (fig. 2). When the influence of the age of nutrient-broth cultures was studied (fig. 3), fewer cells from stationary-phase cultures incubated for >6 h were killed than were cells from broth cultures incubated for <2 h.

Fig. 1. The influence of sodium chloride concentration on survival of strain 1030 β la⁻ from aerobic and anaerobic stationary-phase cultures exposed to lysozyme 1000 mg/L for 1 h at 37°C; ● = aerobic broth culture, ○ = anaerobic broth culture.

Fig. 2. The influence of lysozyme concentration in 0.02M phosphate buffer, pH 7, acting for 3 h at 37°C, on the viability of strain 1030 β la⁻, prepared from aerobic 1-h nutrient-broth cultures.

To compare the susceptibility of different strains to lysozyme, inocula were prepared from stationary-phase broth cultures grown aerobically. After incubation for 18 h at 37°C, the β la⁻ derivatives of strain 1030 reached viable counts half as high again as those of the β la⁺ strains, the viable counts by either microbiological or bioluminescence methods being comparable. All the β la⁺ derivatives of strain 1030 were more resistant to lysozyme in the concentration range 100–500 mg/L than were the β la⁻ derivatives (fig. 4). When CBAP in concentrations of 0.1–5 mg/L was used to induce an increase in β -lactamase synthesis in the 1030 and 1030' Rf^R, Cd^R, β la⁺ derivatives, the cells produced 6–12 Perret units of β lactamase/10⁸ viable bacteria and viable counts of these preparations either did not fall or rose after incubation for 1.5 h at 37°C in the presence of lysozyme 500 mg/L in 0.03M Tris-HCl buffer. The survival of the β la⁻ derivatives of strain 1030 in the presence of lysozyme did not differ from that of the Rf^R derivative of strain 1030 from which they were constructed, nor did addition of CBAP in concentrations of 0.1–5 mg/L influence survival.

When concentrations of methicillin, from 0.06 mg/L to the MIC for both β la⁺ and β la⁻ derivatives of strain 1030 of 2 mg/l, were added to broth cultures before incubation in lysozyme, the washed cells were more susceptible to lysozyme, despite the induction of β -lactamase synthesis in the β la⁺ strains by these concentrations of methicillin.

Incubation of the β la⁺ derivatives of strain 1030 in 0.2M phosphate buffer for 0.5 h at 37°C resulted in up to 50% reduction in the cell-associated β lactamase (Coles and Gross, 1967) but did not

Fig. 3. The influence of the age of nutrient-broth cultures on the survival of strain 1030 Rf^R, Cd^R, βla⁻ — exposed for 1.5 h at 37°C to lysozyme 300 mg/L in Tris-HCl buffer, pH 7.0; ● = mean survival and standard deviation (SD), ○ = viable count.

influence the susceptibility of these strains to lysozyme.

Discussion

Before penicillin was used in clinical trials in 1942 some strains of *S. aureus* were noted to be resistant to it (Segalove, 1947; Parker and Lapage, 1957; Altmeier *et al.*, 1981). Since then, the percentage of diagnostic laboratory isolates of *S. aureus* producing β lactamase has risen to more than 80. In early studies, β-lactamase-producing strains of *S. aureus* were acquired by patients and nursing staff not exposed to therapeutic penicillin (Barber and Rozwadowska-Dowzenko, 1948; Rountree and Thomson, 1949), possibly as a result of exposure to environmental penicillin (Gould, 1958), but the number of β-lactamase-producing strains also rose in the community outside the hospital. The majority of clinical isolates of *S.*

aureus are now resistant to penicillin, yet resistance to other antibiotics is far less common despite the widespread use of tetracyclines, erythromycin and other antibiotics with anti-staphylococcal activity. Methicillin-resistant strains produce large amounts of β lactamase (Richmond *et al.*, 1964), despite being penicillin-resistant at 30°C, the temperature of the anterior nares, the carrier site for *S. aureus* (O'Grady and Wittstadt, 1963; Polakoff *et al.*, 1967).

Plasmid-mediated β-lactamase synthesis in *S. aureus* may be lost *in vitro* on non-selective media but since 1966 there have been several reports of chromosomally mediated β-lactamase synthesis in *S. aureus* (Asheshov, 1966; Sweeney and Cohen, 1968) and chromosomal β-lactamase synthesis may be becoming more common (Townsend *et al.*, 1985).

The concentration of sodium chloride in nasal mucus is not known, but is probably similar to that of tracheo-bronchial mucus, which has been reported from animal studies to be <0.02M (Boyd *et*

Fig. 4. The influence of β-lactamase production on the survival of derivatives of strain 1030 incubated for 1.5 h at 37°C in 0.03M Tris-HCl buffer, pH 7, containing lysozyme 500 mg/L. ● = mean and SD; a = strain 1030 Rf^R, Cd^R, βla⁻ (6); b = strain 1030/778 Rf^R, Cd^R, βla⁺ (3); c = strain 1030/37810 Rf^R, Cd^R, βla⁺ (3); d = strains 1030 and 1030' Rf^R, Cd^R, βla⁺ (6); e = strain 1030/54353 Rf^R, Cd^R, βla⁺ (3); f = strains 1030 and 1030' Rf^R, Cd^R, βla⁺ (6) after induction of β-lactamase synthesis with CBAP; () indicates number of experiments. * Range 6.3–12.2 units.

al., 1944). The dilution of tears by nasal mucus could produce lysozyme concentrations of 100–500 mg/L with a molarity of $<0.04\text{M}$ sodium chloride. The lysis of the cell wall of *Micrococcus lysodeikticus* (Davies *et al.*, 1967), the aggregation of streptococci (Laible and Germaine, 1982; Golub *et al.*, 1985) and the killing of *Streptococcus sanguis* (Laible and Germaine, 1985) by lysozyme depend upon a reduction in ionic strength. The present study has shown that lysozyme kills *S. aureus* in conditions which could result from dilution of tears by nasal mucus. The mechanism for this killing may be through lysis of staphylococci following the activation of cell wall autolysins by lysozyme, after adsorption of lysozyme to the bacterial cell wall.

S. aureus strain 1030 R^r, Cd^r, β la[–] was less susceptible to lysozyme when grown in broth incubated anaerobically rather than aerobically. Small differences in pH influence the alanyl ester content of cell-wall teichoic acids (Rogers *et al.*, 1980) and may thereby alter the susceptibility of *S. aureus* to lysozyme through an alteration in polymer charge.

The β -lactamase-producing derivatives of *S. aureus* strain 1030 were more resistant to lysozyme than non-producing derivatives. However, removal of the extracellular β lactamase by incubation in 0.2M phosphate buffer did not increase the susceptibility of strains to lysozyme, suggesting that the membrane-bound β lactamase may be responsible for inhibiting lysozyme killing, perhaps by inhibiting the binding of lysozyme to the cytoplasmic membrane or to lipoteichoic acids, which are important in stabilising cell wall autolytic enzymes.

β -Lactam antibiotics at sub-inhibitory concentrations enhance the lysis of *S. aureus* by lysozyme

(Warren and Gray, 1965; Ginsburg *et al.*, 1982) and, in this study, methicillin at sub-inhibitory concentrations enhanced the killing of *S. aureus* strain 1030 by lysozyme. This effect may be due to alterations in cell-wall structure (Tomasz, 1979). CBAP, which at concentrations of $<5\text{ mg/L}$ does not damage the cell wall of *S. aureus* (Leggatt and Holms, 1968), did not influence the susceptibility to lysozyme of the β la[–] derivatives of strain 1030. Growth of the β la⁺ strains in the presence of CBAP at concentrations up to 5 mg/L, however, resulted in increased resistance of those strains to lysozyme, perhaps as a result of the induction of β -lactamase synthesis, in the absence of cell wall damage.

Penicillin therapy, by selecting for β -lactamase production, may have selected strains of *S. aureus* with increased resistance to host-derived cationic proteins. Lysozyme may help to maintain the β -lactamase-producing strains of *S. aureus* that have been selected at the carrier site by antibiotic therapy. Apart from lysozyme, other cationic proteins may select for β -lactamase synthesis by *S. aureus*. Neutrophil granules contain several cationic proteins that have potent bactericidal activity (Olsson *et al.*, 1978). Neutrophils from patients with chronic granulomatous disease will kill *S. aureus*, despite a defect in the oxygen-dependent killing system (Elsbach and Weiss, 1981; Quie *et al.*, 1967) and neutrophils will also kill *S. aureus* in anaerobic conditions (Mandell, 1974). The exposure of *S. aureus* on the mucosa of the anterior nares to lysozyme will vary with time and between individuals and may provide a less potent selection pressure than the granular cationic proteins present in an inflammatory exudate (Noble, 1977).

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