Effect of mucoid growth of *Staphylococcus aureus* on its susceptibility to rabbit polymorph bactericidal factors

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1. INTRODUCTION

Enhancements in the virulence properties of staphylococci after growth in vivo in rabbits have been reported earlier [1]. Such organisms subsequently showed increased resistance to killing by crude lysates [2] or soluble bactericidins (SB) of rabbit polymorphonuclear (PMN) leukocytes [3,4] and this was mimicked by growth of the original strain in plasma [4]. These findings suggested a correlation between increased resistance and enhanced virulence. Apart from growth in vivo, other cultural conditions such as growth in the high-salt, high-carbohydrate-modified 110 *Staphylococcus* medium have been shown to enhance the virulence of two strains of staphylococci in bovine skin [5]. The possibility that this medium would also enhance the resistance of staphylococci was therefore investigated.

This paper reports enhancement in the resistance, to killing by SB, of a *Staphylococcus aureus* strain O used in previous studies [1–4] after growth in the modified 110 agar or in the corresponding broth medium.

2. MATERIALS AND METHODS

2.1. Media

The modified 110 medium was prepared from basic ingredients by the method of Yoshida and Ekstedt [6] and Ekstedt and Bernhard [7]. It contained a 48-h dialysate of 1% peptone (Oxoid) and 2.5% yeast extract (Difco) in 3% NaCl, to which were added 1% mannitol, 0.2% lactose and 0.5% K$_2$HPO$_4$ in the presence of 0.05 M phosphate buffer (pH = 7.4) and was divided into two portions. To one portion, agar was added to a concentration of 1.5% and the other was prepared as broth medium. Both were autoclaved at 121°C for 10 min. Brain heart infusion broth (BHI) consisted of infusions from calf brains (2%), beef heart (2.5%), 1% peptone (Difco), 0.2% dextrose, 0.5% NaCl and 0.25% Na$_2$PO$_4$ autoclaved at 121°C for 15 min. Media were stored at 4°C until required.

2.2. Preparation of staphylococci

*S. aureus* strain O as described and used in previous studies [1–4] was used in these tests. The organisms were grown in modified 110 broth or agar as previously described [7]. Growth in BHI was as described for the modified 110 broth. At the end of incubation, organisms were centrifuged and re-suspended by gentle vibration in the appropriate fresh ice-cold broth medium without further washing (except where stated) to prevent loss of extracellular slime.

All bacterial preparations were counted for viability before use to ensure that the inocula in the experiments were comparable. Meanwhile, all suspensions were kept at 0°C in an ice-bath, so that
there were no appreciable changes in viable numbers (< 5%).

2.3. Preparation and measurement of killing by soluble bactericidins (SB) from rabbit PMN

The inducement and collection of PMN leukocytes from the peritoneum of rabbits (New Zealand white) were as described previously [8] with slight modifications. All animal manipulations were carried out after the administration of local anaesthesia by the intradermal injection of lignocaine (Pharmaceutical Manufacturing Company). Only exudates containing > 95% PMN and which were > 99% viable were used. Cells centrifuged from the exudate were washed once, resuspended in 0.34 M sucrose to a known concentration/ml and homogenised for 3–5 min in a tissue grinder (A. Gallenkamp and Co, Ltd) to achieve complete breakage. Homogenates were extracted (16 h) for SB in weak citric acid (BDH Chemicals), and centrifuged at 15000 × g for 30 min at 4°C. The supernatant, which contained the bulk of active SB, was used directly.

A mixture of organisms and SB in phosphate buffer was incubated at 37°C with rotation at 30 rev./min. Samples were taken at 0, 1, 2, and 3 h and plated out in replicates for viable counts. Observations of organisms suspended in a buffer–citric acid–sucrose mixture served as control.

3. RESULTS

Fig. 1 shows the extent of killing, by SB, of S. aureus grown on modified 110 agar, 110 broth or plain BHI. Organisms harvested from 110 agar

![Graph showing the extent of killing by SB](https://example.com/graph.png)

Fig. 1. Polymorph SB killing of S. aureus grown on modified 110 agar (■), modified 110 broth (○) or BHI (○). Test suspensions contained 10⁵ viable organisms and 10⁷ PMN equivalents of SB in phosphate buffer (pH=6.75) and in final volumes of 1 ml. Measurements of viable numbers in buffer–citric acid–sucrose control mixtures are shown with broken lines.

Table 1

<table>
<thead>
<tr>
<th>Treatment of mucoid S. aureus</th>
<th>Reduction in viable numbers</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Viable count at 0 h (×100)</td>
</tr>
<tr>
<td>Unwashed</td>
<td>1200</td>
</tr>
<tr>
<td>Washed 2 ×</td>
<td>940</td>
</tr>
<tr>
<td>Washed 3 ×</td>
<td>876</td>
</tr>
<tr>
<td>Subcultured in BHI</td>
<td>1300</td>
</tr>
</tbody>
</table>

Table 1

Effect of removal of slime layer on the resistance of S. aureus to killing by SB

Bacteria were either washed two or three times in half strength saline in modified 110 broth or subcultured in BHI and then incubated with SB. Test suspensions were as described under Fig. 1. Results are the mean of 2 experiments, each consisting of 2 replicate tests suspensions per organism treatment.
surface or the broth showed viability reductions of approx. 1 and 1.5 logs, respectively, while organisms grown in BHI showed a viability reduction of almost 3 logs. Organisms incubated in the buffer–citric acid–sucrose control mixture showed no appreciable reduction in viable numbers, indicating that the reduction in numbers in the test suspensions was due to killing by SB.

The bacteria harvested from either the 110 agar or broth were covered with slime. On washing in saline broth, the organisms showed progressive susceptibility with increasing number of washings (Table 1). Thus, after three washings, previously mucoid and resistant organisms showed a susceptibility comparable to that of organisms grown in plain BHI. Also, subculture in BHI gave organisms that were devoid of a slime layer and which showed a complete reversal to susceptibility.

4. DISCUSSION

The results show that growth of *S. aureus* strain O in the modified 110 medium enhanced its resistance to killing by SB and appears to confirm a link between factors leading to enhanced virulence and those resulting in increased resistance. However, the resistance reported here appears to have been achieved by a different mechanism from that of the same strain grown in vivo since repeated washing resulted in complete reversal to susceptibility while similar washings during routine purification of in-vivo organisms did not affect their level of resistance. The composition of the slime layer on strain O was not determined but a similar slime layer on other virulent staphylococci has been shown to consist of a complex polysaccharide and series of amino acids which were serologically active [7]. The presence of ribitol teichoic acid, a mannan and a serologically active polypeptide have also been reported [9]. Since washing or subculturing in BHI resulted in loss of slime covering and, subsequently, a reversal to susceptible forms, any attempt to explain the observed resistance must attend primarily to the role of this layer and its components. Two possible explanations are hereby offered. First, the slime layer acted as a physical barrier by blocking possible sites of SB action on the bacterial surface, e.g. the NADH oxidase enzyme which is the target of SB action at the cytoplasmic membrane level [10–12]. Second, various components of the slime layer formed complexes with basic component of SB, thus rendering them ineffective, since it is known that teichoic acid precipitates cationic proteins from PMN [12].

If these mechanisms are applicable in vivo, it would explain, among other factors, the added virulence reported earlier [5], since such organisms would have more chances of survival and hence of establishing an infection in the experimental host despite its natural defence mechanisms.

REFERENCES