SURFACE PROPERTIES OF CELLS OF SOME METHICILLIN-RESISTANT STRAINS OF *STAPHYLOCOCCUS AUREUS*

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Methicillin-sensitive (MS) cells of *Staphylococcus aureus* had a minimum electrophoretic mobility at pH 4.5, whereas methicillin-resistant (MR) strains showed only a slight plateau effect. Trypsin removed the trough effect of the MS Oxford strain. There was no correlation between surface lipid and resistance in MR strains. Cell walls of MS strains contained much more teichoic acid than walls of MR strains. Lysostaphin lysed all MR and MS strains, and mucopeptide does not appear to be involved in resistance to methicillin.

Methicillin-resistant (MR) strains of *Staphylococcus aureus* have been a problem in chemotherapy for more than 10 years. Surprisingly little attention, however, has been devoted to an understanding of the basic reaction(s) for the high resistance of these strains.

MR strains are characterized by the fact that, at 37°C, a small proportion of the cells is resistant to methicillin and other β-lactam antibiotics, whereas at 25°C the majority of cells in a culture are resistant[1~4]. It is the temperature at which the cells are treated with methicillin and not the pre-treatment growth temperature which is of paramount importance[5].

This paper investigates the surface properties of cells of MR strains of *S. aureus* in relation to those of the methicillin-sensitive (MS) Oxford strain.

Materials and Methods

Strains: The following *S. aureus* strains were kindly supplied by Dr. E. ASHESHOV (Public Health Laboratories, Colindale, London): 7270+, 7270−, 9254+, 9254−. The +ve sign indicates a β-lactamase producer, the −ve sign the absence of β-lactamase. All were received as MR strains, but 9254− was found subsequently to be MS. The Oxford strain, NCTC 6571, of *S. aureus* which is highly sensitive to β-lactam antibiotics was used as a control.

Electrophoretic mobility: Measurement of electrophoretic mobility[6] was made at 25°C in veronal acetate buffer pH 3~7 (I=0.02 M) in a microelectrophoresis apparatus (Rank Brothers Ltd., Cambridge, England).

Surface protein: Cultures grown at 25°C or 37°C were centrifuged, and the cells washed with, and suspended in, 20 ml of 0.85% w/v sodium chloride. The suspension was divided into two portions, centrifuged, and the supernatant fluid removed. One pellet was resuspended in 20 ml SÖRENSEN's phosphate buffer (I=0.11 M) pH 8, and the other in 20 ml of this buffer containing 0.01% crystalline trypsin (Koch-Light Ltd., Colbrook, Berkshire, England). Both portions were incubated at 37°C for 2 hours. The cells were then washed twice with 10 ml veronal acetate buffer (I=0.02 M) pH 7 and subsequently suspended in veronal acetate buffer (I=0.02 M) of the required pH at 25°C. Electrophoretic mobilities were determined, and the T-value calculated: this is the percentage increase in mobility at pH 4.5.

Surface lipid: Cultures grown at 25°C or 37°C were centrifuged. The cells were washed with 20 ml of glass-distilled water and then 20 ml of veronal acetate buffer (I=0.02 M) pH 7. Finally, the

cells were resuspended in 10 ml of this buffer at 25°C. When required 0.5 ml was added to 9.5 ml of buffer containing the appropriate concentration of sodium lauryl sulphate (SLS; B.D.H. Chemicals Ltd., Poole, Dorset, England) at 25°C. Electrophoretic mobilities were determined and the S-value[7] calculated: this is the percentage increase in mobility in the presence of 10^{-4} M SLS.

Surface properties of methicillin-treated cells: When shaken cultures of MR strains are exposed to inhibitory doses of methicillin, confluent growth occurs at 24 hours[5]. The present experiment was undertaken to study the surface properties of cells surviving exposure at 37°C to methicillin concentrations of 1/3, 3/5 and 8/5 times the minimum inhibitory concentration (MIC). Cells were grown in nutrient medium in shaken flasks containing methicillin[5]. After 24 hours, the cells were harvested, washed twice with glass-distilled water and finally resuspended in 20 ml water; 0.4 ml of suspension was added to 10 ml veronal acetate buffer, pH 3~7, previously equilibrated at 25°C, and electrophoretic mobility measured.

Cell wall preparation: Twenty liters of nutrient broth no. 2 (Oxoid Ltd., London) in a large fermentor were inoculated with 250 ml of a culture and incubated at the appropriate temperature for 18 hours. In some experiments, methicillin was included in the media. The cells were harvested by centrifugation. Cell walls were prepared by means of the X-press[8]. Examination by ultraviolet absorption at 260 nm and by electron microscopy indicated that the walls were free from nucleic acid contamination and from whole cells and other visible contaminants.

Quantitative extraction and purification of teichoic acid: The method used was based upon that of BADDILEY *et al*[9], using 10% trichloroacetic acid. Chromatographic identification of teichoic acid components was made with an acid (2 N hydrochloric) hydrolysate on activated silica gel plates[10].

Removal of surface teichoic acid from cells: Cultures were grown at 25°C or 37°C, and their cells washed once in 20 ml glass-distilled water and then in 20 ml of 0.85% w/v sodium chloride. Twenty ml of 0.1 M ammonia were added to remove any ester-linked alanine and left for 5 minutes at 20°C. The suspension was centrifuged and the cells washed with 20 ml water. After centrifugation, the cells were suspended in 20 ml of MICHAELIS buffer (I=0.02 M) pH 6 containing the oxidising agent sodium metaperiodate (for removal of teichoic acid), and held for 30 minutes at 37°C.

The cells were centrifuged, washed twice in 20 ml water and finally resuspended in 15 ml water, then 0.7 ml of this suspension was added to 9 ml of each buffer solution at 25°C for the measurement of electrophoretic mobilities.

Effect of lysostaphin: Lysostaphin[11] was tested against whole cells, cell walls and mucopeptide obtained from walls after removal of teichoic acid. Changes in extinction were measured at 620 nm in the SP 600 spectrophotometer. Minimum inhibitory concentrations (MIC's) of lysostaphin against the strains were determined at 25°C and 37°C.

Results

Electrophoretic Studies

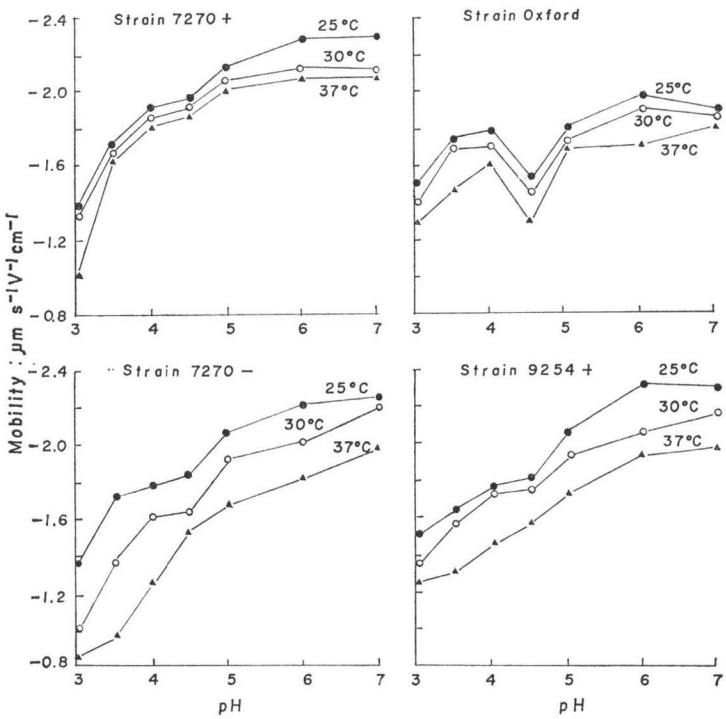
Fig. 1 indicates that, in nearly all cases, as the pH was decreased the mobility of the cells decreased. MS strains grown at 37°C had a minimum mobility (trough effect) at pH 4.5, whereas the MR strains showed only a slight plateau effect. Between pH 6 and 7, there was usually a decrease in mobility or a plateau effect. Cells of a particular strain grown overnight at 25°C tended to have a higher mobility than those likewise grown at 37°C, possibly the result of difference in the configuration of the teichoic acid. Treatment of cells with trypsin (not shown) did not remove the plateau or trough effect, except with the Oxford strain. The increases in mobility of the strains at pH 4.5 are shown as the T-value in Table 1; this is highly significant only with the Oxford strain and with strain 9254− grown at 37°C.

The mobility of cells containing surface lipid is altered by treatment with a surface-active agent such as SLS[12]. S-values are listed in Table 1, a value >10 indicating the presence of surface lipid[7].

The two MS strains contained significantly more surface lipid than the MR strains and in only one MR strain (7270−, grown at 37°C) was the S-value >10.

The electrophoretic mobilities of methicillin-treated cells are shown in Fig. 2. For most strains the mobility curves of cultures exposed to 3/5 × MIC of methicillin were very similar to those of untreated cells. However, the curves of cultures exposed to 8/5 × the MIC were markedly different, indicating that the surfaces of those cells differed from those of control (untreated) cells.

Fig. 1. pH-Mobility curves of cells of strains of *S. aureus* grown at different temperatures.



The effects of pH on the electrophoretic mobilities of cells from which surface teichoic acid had been removed by means of sodium periodate are shown in Fig. 3. Every strain, irrespective of growth temperature, had an increased mobility with increasing pH, but the trough and plateau responses see in "normal" MS or MR cells (Fig. 1) were not observed.

Table 1. 'T' and 'S' values of some MS and MR strains of *S. aureus*

Strain	Growth temperature (°C)	'T' value*	'S' value*
7270+	25	6.8	7.0
	37	3.6	2.0
7270−	25	7.0	2.5
	37	6.6	17.5
9254+	25	4.7	2.6
	37	6.5	1.6
9254−	25	7.8	15.0
	37	12.3	18.8
Oxford	25	38.0	14.0
	37	45.0	8.0

* See 'Materials and Methods' for definitions.

Cell-Wall Composition

Chromatographic analyses of teichoic acid hydrolysates demonstrated the presence of ribitol, N-acetylglucosamine, D-alanine and glucosamine in all strains; no glycerol was found, indicating that the extracted teichoic acid was of the ribitol moiety.

Quantitative studies on the amount of teichoic acid present in the walls of each strain gave the results presented in Table 2. The amount of teichoic acid in the walls of cells grown at 25°C was always somewhat higher than in the walls of 37°C grown cells, and the walls of MS cells contained considerably greater contents of teichoic acid than did those of MR cells. Methicillin caused a reduction in the teichoic acid content of the walls of the two MS strains.

Effect of Lysostaphin

MIC values (μg/ml) of lysostaphin at, respectively, 25°C and 37°C against the various strains were:

7270+, 12.8 and 6.4; 7270−, 12.8 and 6.4; 9254+, 12.8 and 6.4; 9254−, 12.8 and 6.4; Oxford, 6.4 and 3.2. Rates of lysis of four of these strains exposed to lysostaphin are provided in Fig. 4. Lysis was most rapid with whole cells, and least with isolated mucopeptide (possibly because of some degradation during preparation). With all strains, lysis tended to be somewhat greater with cells or their components from cultures grown at 37°C.

Discussion

As the pH decreased, the electrophoretic mobility of the cells decreased (Fig. 1). At low pH values, protonation of the negatively charged phosphate groups and the positively charged amino groups of teichoic acids occurs; as the pH increases, the amount of protonation decreases and the negative surface charge increases.

Although a relationship between surface or cell wall lipid and resistance to antibiotics has been found in *S. aureus*[18], the results in the present paper suggest that wall lipid is not involved in resistance to methicillin. Likewise, studies involving lysostaphin indicate that mucopeptide appears to be unimportant in this content, and this agrees with the earlier findings of DYKE[2].

Rather more teichoic acid has been found in the walls of MR strains grown at 25°C than in those grown at 37°C, but it is to be wondered how significant this is. Our results differ somewhat in this context from those of JAMES and his colleagues[7,14,15]. Nevertheless, there are significant differences in the relative amounts of teichoic acid in the walls of MR and MS cells. It is also

Fig. 2. pH-Mobility curves of cells of strains of *S. aureus* treated with inhibitory and subinhibitory doses of methicillin at 37°C.

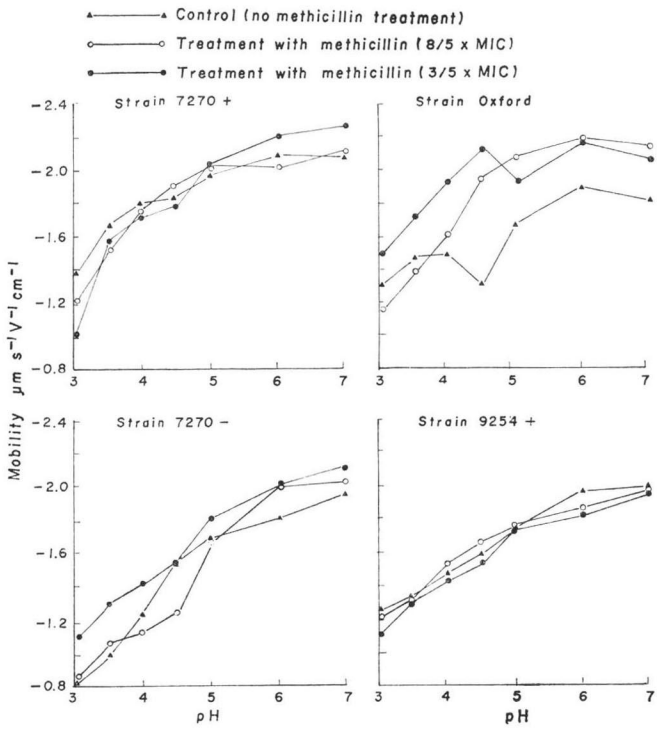


Fig. 3. pH-Mobility curves of *S. aureus* strains grown overnight at 25°C or 37°C and then treated with sodium metaperiodate.

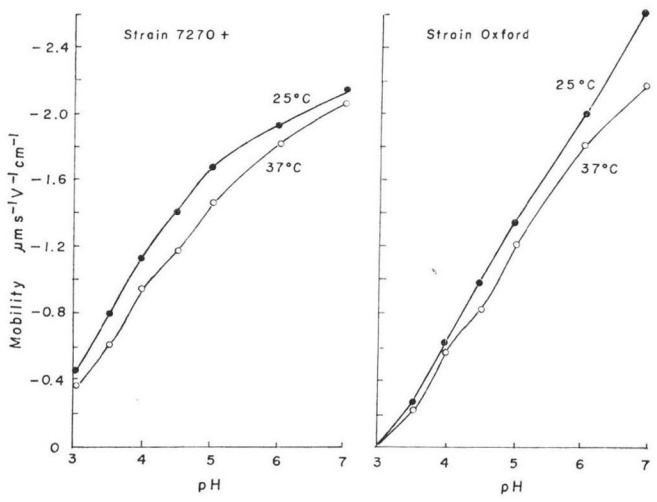
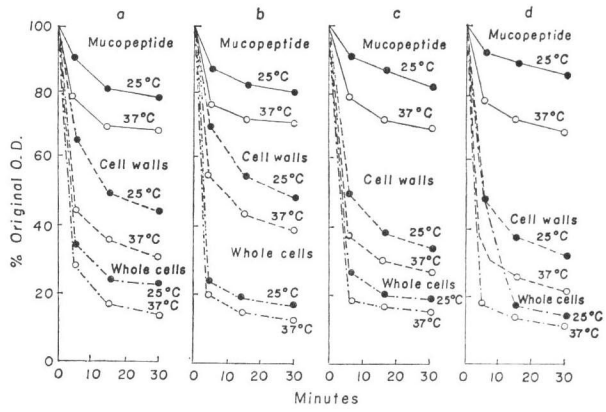


Table 2. Teichoic acid content of the cell walls of *S. aureus* strains

Strain	Growth temperature (°C)	Teichoic acids as % of cell wall
7270+	25	19.8
	37	18.4
7270−	25	19.2
	37	15.8
9254+	25	20.5
	37	17.6
9254−	25	42
	37	28
	37*	21
Oxford	25	32
	37	28.4
	37*	20

* Growth in presence of methicillin, 8/5 ×MIC (see Ref. 5)

Fig. 4. Effect of lysostaphin (50 μg/ml) at 20°C on whole cells, cell walls and mucopeptide of *S. aureus*: (a) strain 7270+, (b) strain 7270−, (c) strain 9254+, (d) strain Oxford.



pertinent to note, however, that there are also considerable differences between the cell wall protein content of the MS Oxford strain and the MR strains.

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