Non-Protein Components of the Cell Surface of *Staphylococcus aureus*

By A. M. JAMES AND J. E. BREWER

Department of Chemistry, Queen Elizabeth College, London, W. 8, and The Cross-Infection Reference Laboratory (Public Health Laboratory Service), Colindale Avenue, London, N.W. 9

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1. pH-mobility curves of various laboratory strains of *Staphylococcus aureus* are non-sigmoid in shape, and all pass through a maximum value in the range pH 4–5. 2. The maxima in the curves are not due to incomplete washing of the cells, adsorption of buffer components or irreversible surface damage. 3. Mild oxidation of the cell-surface teichoic acid with sodium metaperiodate gives cells that have typical sigmoid pH-mobility curves, characteristic of either a simple carboxyl surface or a mixed carboxyl-amino surface. 4. The results are discussed in terms of a pH-dependent change in the configuration of the teichoic acid molecules at the surface.

A good deal is now known about the composition of the cell wall of *Staphylococcus aureus*. The structural integrity of the cells is maintained by a three-dimensional polymer of amino sugars and amino acids, the glycopeptide (Strominger, 1965) with which is associated teichoic acid, a linear polymer of ribitol phosphate substituted by *N*-acetylglucosamine (Davison & Baddiley, 1963). Tentative schemes for the arrangement of the constituents of the glycopeptide have been proposed (Ghuysen, Tipper & Strominger, 1965) and possible methods by which teichoic acid may be covalently linked to the cell wall by phosphodiester (Button, Archibald & Baddiley, 1966) or by phosphoramidate links (Hay, Archibald & Baddiley, 1965) have been described. However, the configuration of these polymers *in vivo*, particularly of those present at the cell surface, is still unknown.

The technique of particulate electrophoresis, in association with enzymic and chemical treatments, has been used successfully in investigations of the nature of the surface components of *Streptococcus pyogenes* (Hill, James & Maxted, 1963*a,b,c*). There have been several earlier studies of *Staph. aureus* by electrophoretic methods (Verwey & Frobisher, 1940*a,b*; Dyar & Ordal, 1946; Lerche, 1953), but interpretation of the results in terms of known components of the cell surface was difficult. The present paper describes the results obtained in a study, by similar methods, of the non-protein components of the cell surface of *Staph. aureus*. Information obtained about the cell-surface protein is given by James & Brewer (1968).

METHODS AND MATERIALS

Organisms. The organisms used were obtained as freeze-dried cultures from the National Collection of Type Cultures: N.C.T.C. 8530, Cowan I (phage-typing pattern: 52); N.C.T.C. 8531, Cowan II (3B/3C/5S/71/+); N.C.T.C. 8532, Cowan III (6/7/47/54/75/+); N.C.T.C. 712, Wood 46 (42D/+).

They were maintained by serial subculture on nutrient agar (Oxoid nutrient broth solidified with 1.2% of agar, pH 7.4). Organisms required for electrophoretic measurements were obtained as a confluent growth on this medium after incubation aerobically for 18 hr. at 37°. The strains were periodically tested for purity on blood-agar plates.

Electrophoretic measurements. The cells were harvested from solid medium in buffer solution (pH 7.0 and 10.02). The cell mass was washed twice by resuspension and centrifugation from this buffer solution, and then twice in the buffer solution of required pH (10.02). The washed cells were then suspended in the same buffer solution. Buffer solutions were prepared from sodium barbiturate, sodium acetate and HCl (Michaelis, 1931) with the addition of NaCl to constant 10.02. The electrophoretic mobility was measured by the method of Gittens & James (1960). The technique and precautions described by these authors were rigorously followed. In general the populations were electrophoretically homogeneous, and so only mean values are quoted; the confidence limit for a single mean at *P* 0.05 was $\pm 3\%$; values differing by more than 10% were thus significantly different.

Mobility values were determined in buffer solutions of different pH; to ensure that no denaturation or other irreversible surface change had occurred, cells after suspension in solutions of extreme pH were washed three times in buffer solution of pH 7.0 and 10.02, and their mobilities determined in this buffer solution. Results were

accepted and plotted for pH values only when the mobility of the cells on resuspension at pH 7 was the same as the normal control value. For all the strains of *Staph. aureus* studied there were no irreversible changes in the range pH 2.9-11.0.

Oxidation with sodium metaperiodate. The method of Garrett (1965) was used to remove selectively the teichoic acid from the cell wall. Cells, from an 18 hr. growth, were washed in 0.9% NaCl solution and suspended in aq. 0.1M-NH₃ solution to remove ester-linked alanine. After thorough washing to remove NH₃, the cells were resuspended in buffer solution (pH 6 and I 0.02) containing sodium metaperiodate (0.05M) and incubated at 37° for 30 min. The oxidized cells were washed twice in the buffer solution of required pH, before resuspension in the same buffer solution for use in mobility measurements.

RESULTS

Study of variation in the mobility of bacterial cells with the pH of the suspension medium at constant ionic strength provides information on the nature, and often also on the relative amounts, of different components at the cell surface. Thus surfaces containing only carboxyl groups (Gittens & James, 1960) and those containing both carboxyl and amino groups (Hill *et al.* 1963b) can be recognized by their characteristic pH-mobility curves, by the titration of the appropriate ionogenic group.

In marked contrast, the pH-mobility curves for the four strains of *Staph. aureus* were more complicated (Figs. 1 and 2). The mobility of cells of all strains passed through a maximum value between pH 4 and 5 (the value of the maximum being 100% greater than the value at pH 7 for cells of strains Cowan I, II and III and about 10% greater for cells of strain Wood 46). These curves, which have been obtained repeatedly on these and other strains of *Staph. aureus*, were completely unexpected, and cannot be explained on the basis of the titration of acidic or basic groups on the surface. The titration of a weak acid (carboxyl) with a strong base produces a smooth titration curve, a maximum value being inconceivable. Although all strains exhibit a maximum value, there are other small but significant differences between them. Thus free amino groups are present on the surface of cells of strains Cowan I and III, as shown by the positive mobility value at low pH values and the increase in the negative value at pH values greater than 9. This feature of the results is discussed by James & Brewer (1968).

Possible explanations that may be advanced to account for the maxima in the curves include: (a) there is a pH-dependent adsorption of some component either of the buffer solution used for suspension or of some material excreted by the cells; (b) some component of the cell surface is labile in the range pH 4-5; (c) the surface is irreversibly damaged by suspension in buffer solution over this

Fig. 1. pH-mobility curves of cells of various strains of staphylococci (measured in acetate-barbiturate buffer solution, I 0.02). ○, Strain Wood 46; ○ and ●, strain Cowan I (duplicate determinations); ●, strain Cowan II.

Fig. 2. pH-mobility curves of cells of *Staph. aureus* (strain Cowan III) before and after oxidation with sodium metaperiodate (measured in acetate-barbiturate buffer solution, I 0.02). ○, Normal cells; ●, oxidized cells.

range; (d) there is an alteration in the numbers or kinds of surface ionogenic groups indirectly caused by the change in pH in this region. To distinguish between these possibilities various experiments including washing the cells and tests for reversibility were undertaken.

Table 1. *Tests for the stability of the surface of cells of strain Cowan I to change of pH of the suspension medium*

pH of original suspension	Electrophoretic mobility (μ /sec./v/cm.)				
	2.88	3.95	4.46	4.76	7.00
Cells at original pH	-0.28	-1.86	-1.68	-1.34	-1.03
Cells after washing and resuspension at pH 7.00	-1.01	-1.08	-1.01	-1.07	-1.01

Table 2. *Mobility of cells of strain Cowan I after repeated washing and resuspension at pH 7.0 and 4.26*

The arrows indicate the progress of the cells in the washing programme.

pH of measurement	Electrophoretic mobility (μ /sec./v/cm.)			
7.0	-1.07	-1.04	-1.06	-1.03
	↓ ↗	↓ ↗	↓ ↗	
4.26	-2.27	-2.24	-2.10	

Suspensions of washed cells of strain Cowan I were prepared in buffer solutions in the range pH 2.9–7.0 as for pH-mobility measurements. The mobility of the cells in part of each suspension was determined and the remainder of the suspension was sedimented, and the cells were washed once in, and finally resuspended in, buffer solution at pH 7.0. The results (Table 1) suggest that no irreversible damage to the cell surface is caused by suspension in buffer solution in this range. The extent of washing in this experiment (five or six times) rendered the adsorption of excreted material unlikely as a possible cause of the maximum in the pH-mobility curve.

In a further experiment, cells were suspended in buffer solution at pH 7.0 and the mobility was determined. The cells were then sedimented and resuspended in buffer solution at pH 4.26 and the mobility was again determined. This alternate suspension at pH 7.0 and 4.26 was repeated several times on the same batch of cells. Again the effects of suspension and washing at pH 4.26 (Table 2) were negligible.

The various components of the buffer solution used for suspension were either omitted or changed in concentration (always at constant ionic strength), but the pH-mobility curves were always coincident, suggesting that specific adsorption of any component of the buffer solution was not occurring. Similar observations were recorded with the other strains studied.

Thus it would seem most likely that the appearance of the maximum in the pH-mobility curve should be explicable in terms of some intrinsic

property of the cell surface. The isopotential point and the pK of the surface of cells of all strains was very low and the only negatively charged groups that can be ionized appreciably below pH 3 are sulphate and phosphate. An excellent source of phosphate groups is teichoic acid, a known component of cell walls of *Staph. aureus*. Garrett (1965) has shown that 80% of the teichoic acid can be removed from the cell walls of *Staph. aureus* by scission of the C₍₃₎-C₍₄₎ bond of the ribitol moiety by oxidation with sodium periodate.

Cells of strains Cowan III and Wood 46 were washed with aq. 0.1M-ammonia solution and oxidized with sodium metaperiodate. The cells were washed again, and mobility measurements were made after resuspension in buffer solution in the range pH 2.9–11.0. The curves (Figs. 2 and 3) were then more nearly comparable with those obtained for more familiar surfaces such as that of *Strep. pyogenes* (Hill *et al.* 1963b) and were indicative of the presence of carboxyl and amino groups. The absence of the maxima in the curves suggests that teichoic acid was in some way responsible for the abnormal pH-mobility curves of normal cells. It is noteworthy also that for both these strains the negative charge in the plateau region of the curve, pH 6–9, was unchanged by the removal of teichoic acid although the charge at pH 4 was greatly diminished. The surface of cells of strain Cowan III still contained amino groups that could not be those of the alanine residue of teichoic acid. Similar results were obtained by the oxidation of cells of strains Cowan I and II.

Treatment of the cells with alkaline phosphatase

Fig. 3. pH-mobility curves of cells of *Staph. aureus* (strain Wood 46) before and after oxidation with sodium meta-periodate (measured in acetate-barbiturate buffer solution, 10-02). ---- Normal cells; O, oxidized cells.

produced a lowering (about 10%) of the pH-mobility curve. Although few sources for phosphomonoester groups in the cell wall have been found, this decrease could possibly be due to loss of groups associated with phospholipids. If the teichoic acid is bound covalently to the mucopeptide through the terminal phosphate group there can be no phosphomonoester linkages present.

The pH-mobility curve of cells of all strains from which the teichoic acid had been removed by acid extraction [5% (w/v) trichloroacetic acid or 0.1M-hydrochloric acid for 20 hr. at 30°] did not contain a maximum. However, as it has already been established that cell-surface disorganization occurs at pH values less than 2.9 any interpretation of these observations on acid-extracted cells must be tentative.

DISCUSSION

The interpretation of the results of previous electrokinetic studies of *Staph. aureus* was largely handicapped by a lack of knowledge of the chemical composition and structure of the cell walls. Organisms that have been studied in the light of such knowledge, e.g. *Strep. pyogenes* (Hill *et al.* 1963a,b,c), have been altogether more rewarding as experimental subjects. The interpretation of the present results is made possible by knowledge recently obtained of the composition and possible structure of the components of the cell walls of staphylococci.

The most obvious difference between the surface properties of these strains of *Staph. aureus* and those of other organisms is the shape of the pH-mobility curve. A non-sigmoid titration curve was not only unexpected but, at first sight, inconceivable. It was observed, however, to a greater or smaller extent, with a very large number of strains of staphylococci that the pH-mobility curve always passed through a maximum value in the range pH 4-5. This cannot be accounted for by incomplete removal of growth medium from the cell surface, adsorption of buffer components on to the surface or irreversible denaturation of the surface in this pH range. The maximum in the curve is eliminated, with the production of a typical sigmoid curve, only when the teichoic acid is removed by mild oxidation at pH 6. The new curve is coincident (within the limits of experimental error) with the original curve at pH values greater than 6. This suggests (i) that teichoic acid is in some way responsible for the peak in the curve, (ii) that oxidation has not affected the remaining surface groups and (iii) that in the normal cells teichoic acid does not contribute to the net charge at pH values greater than 6. Further evidence of the implication of teichoic acid is that the surface pK of the oxidized cells is higher than that of the normal cells; loss of phosphate groups could account for this. Complete removal of teichoic acid by acid extraction, although causing surface disorganization, also results in a loss of the maxima in the pH-mobility curves.

It may be argued that the teichoic acid on the normal cells is in some way covering ionogenic groups that after oxidation will be exposed and thereby contribute to the charge. This is not acceptable, since these groups would also produce an alteration of the charge at pH values greater than 6; it would be difficult to envisage how such a situation could produce a maximum in the curve. Since teichoic acid cannot contribute to the charge on the normal cells above pH 6, this must be due only to carboxyl groups (strain Wood 46) or carboxyl and amino groups (strain Cowan III). Teichoic acid is a highly charged polymer whose precise configuration in the cell wall depends largely on the electrostatic and hydrogen-bonds it is capable of forming with other cell-wall components. It is apparent, from molecular models, that the positive configurations are many and varied. The possibilities for both hydrogen-bonding and electrostatic-bond formation are obviously governed by the nature and quantity of other charged groups present, and when changes in these occur changes in the configuration of the teichoic acid might be expected to follow. Thus it could be envisaged that when the carboxyl groups are fully dissociated, above pH 6, the configuration of the

teichoic acid is such that it makes no contribution to the net surface charge. As the pH decreases in the range 6-4 the number of free carboxyl ions begins to decrease, which results in a change of configuration such that the ionogenic phosphate groups of the teichoic acid come into the plane of shear and contribute to the charge.

It is conceivable that if teichoic acid is linked to the cell wall by an extremely acid-labile link (e.g. Button *et al.* 1966) then the reversible breakage and re-formation of such a bond could also provide a reason for the maximum in the pH-mobility curve. However, the probability that the bond could be rapidly and quantitatively reformed under our conditions is small.

Various attempts finally to establish the presence of free phosphate groups, e.g. by complex-formation with uranyl ions, produced inconclusive results.

As the pH decreases still further, i.e. below 4, the decrease in the number of ionized carboxyl or phosphate groups continues as in a normal titration curve. The maximum in the pH-mobility curve therefore is the result of a pH-dependent effect that is related only indirectly to the titration of charged groups at the surfaces.

This behaviour of teichoic acid may account for the observation of Hofstad (1965) that antisera to the teichoic acids, polysaccharide A and polysaccharide 263, failed to agglutinate strains containing these antigens, since at the pH at which the agglutinations were carried out the antigens may either not be at the cell surface or be in the wrong stereochemical configuration.

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