A Protein Component of the Cell Surface of *Staphylococcus aureus*

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1. Treatment with trypsin of cells of *Staphylococcus aureus* strain Cowan I, known to carry a protein component that includes the Jensen protein A, results in an increase in the negative mobility of the cells at pH values greater than 6. 2. Similar treatment of cells of strain Wood 46, which has no surface protein component, causes no change in the electrophoretic mobility. 3. Electrokinetically heterogeneous populations are observed in two strains of *S. aureus*, one of human and the other of animal origin. Evidence is presented ascribing this to the presence of varying amounts of protein components on the surface of different cells.

In addition to glycopeptide and teichoic acid, the cell wall of *Staphylococcus aureus* contains several other components, some of which are agglutinogens. Among the last is the antigen described by Jensen (1958) and known as protein A (Löfkvist & Sjöquist, 1962). This protein has been obtained in a purified form, and shows a preponderance of basic amino acids (Grov, Myklestad & Oeding, 1964). Until recently it was believed that this protein was an antigen that reacted with antibodies present in all normal human sera, but the discovery that protein A precipitates 45% of the γ -globulins from normal human sera (Forsgren & Sjöquist, 1966) makes it very unlikely that the reaction is a simple one between an antigen and its specific antibody. It has also been shown that part of the protein fraction (fraction B) described by Verwey (1940) is serologically identical with Jensen's protein A (Löfkvist & Sjöquist, 1962).

The electrokinetic studies by Verwey & Frobisher (1940*a,b*) led them to postulate the presence of a protein at the surface of many strains of *S. aureus*, but this protein was not identified further. In a previous paper (James & Brewer, 1968) we demonstrated the presence of teichoic acid at the cell surface of strains of *S. aureus*. The present paper describes the detection of a surface protein component in a number of strains of *S. aureus* and gives evidence that in most strains this is Jensen's protein A.

MATERIALS AND METHODS

Strains of Staphylococcus aureus. In addition to the laboratory strains Cowan I, II, III and Wood 46 (N.C.T.C. 8530, 8531, 8532 and 712 respectively) described by James & Brewer (1968), two further strains of *S. aureus* are discussed

here: strain Colindale 64/3745 (of human origin, phage type 80/81, resistant to penicillin, tetracycline, chloromycetin and erythromycin) and strain Colindale 65/6110 (isolated from a goat, untypable by phage). All cultures were grown on nutrient-agar for 18 hr. at 37° unless otherwise stated.

Electrophoretic-mobility measurements. The electrophoretic-mobility measurements were made on washed suspensions of cells in barbiturate buffer of the required pH and 1.0-0.2 (James & Brewer, 1968; Gittens & James, 1960). For the electrokinetically homogeneous populations the s.d. of the mean (-1.94 to $-2.35 \mu\text{sec./v/cm.}$, depending on the strain) was 0.09. Histograms on heterogeneous populations, showing the distribution of mobility values, were plotted from at least 40 observations on individual and randomly selected cells.

Preparation of partially purified Jensen's protein A. This method is a modification of that described by Grov *et al.* (1964) and Löfkvist & Sjöquist (1963). Cells of strain Cowan I, grown overnight on nutrient agar, were thoroughly washed in 0.89% NaCl and sedimented. The cell mass was boiled in 66 mM-phosphate buffer, pH 6, for 1 hr.; the cells and debris were then sedimented after cooling to 4°. The pH of the supernatant was adjusted to 3 with HCl and the resulting solution kept at 4° overnight. The precipitate was collected by centrifugation and then redissolved in 100 ml. of 66 mM-phosphate buffer, pH 6. The solution was dialysed against phosphate-buffered NaCl solution, pH 7.2, for 2 days at 4° with several changes of buffer, and the volume was then decreased to about 10 ml. by the use of polyethylene glycol. Finally, the solution was dialysed again against the buffered NaCl solution to remove small polymers of polyethylene glycol.

This preparation gave a single precipitation line against normal human serum in gel-diffusion tests.

Test for Jensen's protein A. Jensen's protein A was detected by the gel-diffusion techniques used by Oeding & Haukenes (1963). Acid extracts of cells were prepared by heating 2 ml. of a cell suspension with 2 ml. of 0.2 M-HCl in a boiling-water bath for 10 min. The extracts were neutralized and tested against normal human serum; a partially

purified sample of Jensen's protein A was used as a reference. After the gel had stood for 18 hr. at room temperature, a single precipitin line from the acid extract, continuous with the reference line, showed the presence of protein A.

Treatment of cells with trypsin. Cells grown for 18 hr. were washed twice with 0.89% NaCl and incubated in phosphate buffer (pH 7.8 and 10.11) containing 0.01% of crystallized trypsin (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) for 2 hr. at 37°. The treated cells were washed twice in veronal-acetate buffer, pH 7, before suspension in the buffer solution of the required pH and 1.0-0.2 for use in mobility measurements.

RESULTS

The pH-mobility curves (Figs. 1 and 2 in James & Brewer, 1968) of *S. aureus* cells of strains Cowan I and Wood 46 were taken as the starting point for the present study. The most important difference in the shapes and relative positions of the two curves is attributable to the fact that the cells of strain Wood 46 are more negatively charged in the pH range 6-9. This suggests the absence of amino groups at the cell surface of cells of strain Wood 46. In studies of *Streptococcus pyogenes*, amino groups at the cell surface were shown to be due to the presence of trypsin-labile proteins (Hill, James & Maxted, 1963).

Treatment of cells of strain Cowan I with trypsin resulted in a great increase in the negative charge carried by the cells, particularly in the pH range 6-9, as a result of which the pH-mobility curve became rather similar in shape and position to, although not identical with, that of normal cells of strain Wood 46 (Fig. 1). On the other hand, treatment of cells of strain Wood 46 with trypsin was without effect on the pH-mobility curve. Similar treatment of cells of strain Cowan I with heat-inactivated trypsin caused no change in mobility, suggesting that the effects observed with the unheated trypsin were due to action on the surface protein and not to adsorption of the trypsin. Treatment of cells of strain Cowan I with trypsin for periods exceeding 2 hr. caused no further change in the mobility values measured at pH 4.08 and 7.0 (Table 1). Thus it was not possible to make the pH-mobility curves of the two strains completely coincident by tryptic digestion of the surface components.

The probable identity of the protein removed from the surface of cells of strain Cowan I was investigated by gel-diffusion tests with acid extracts from normal and from trypsin-treated cells of both strains. The extract from normal cells of strain Cowan I showed a line of serological identity with the purified protein A preparation, but no line was obtained with extracts from the trypsin-treated cells of strain Cowan I or with extracts from either normal or trypsin-treated cells of strain Wood 46. Thus it appears that the trypsin-labile protein at

Fig. 1. pH-mobility curves of cells of various strains of *S. aureus* before and after treatment with trypsin (measured in acetate-barbiturate buffer, 1.0-0.2). ○, Strain Wood 46, control cells; △, strain Wood 46, cells after treatment with trypsin; ●, strain Cowan I, control cells; ▲, strain Cowan I, cells after treatment with trypsin.

the surface of cells of strain Cowan I contained the Jensen protein A.

During an investigation into the distribution of protein amongst strains of *S. aureus* isolated from various human and animal sources (A. M. James, J. E. Brewer & M. T. Parker unpublished work) several strains were found to be electrophoretically heterogeneous. This inhomogeneity was shown to be connected with the presence of protein A in these strains. Strains 6110 (goat) and 3745 (human) both gave a positive reaction in the gel-diffusion test for protein A. Typical histograms showing the distribution of mobilities of normal populations of both strains at two pH values are shown in Figs. 2(a), 2(c), 3(a) and 3(c). Both strains gave electrophoretically heterogeneous cell populations on suspension at pH 7. Suspensions of cells of both strains at lower pH values (about pH 4.0) were rather more homogeneous than at pH 7.0, and, in general, the distribution at these low pH values compared favourably with that of cells of strain Cowan I at the same pH value. The mobility distributions of cells of strain 6110 were redetermined at the same two pH values after growth from a single colony (Figs. 2b and 2d) and it was apparent that, irrespective of the mobility value of the parent cell, the progeny had a similarly wide range of mobility values. This suggested that the original heterogeneity was not attributable to contamination, since

Table 1. *Effect of repeated trypsin treatment on cells of S. aureus strain Cowan I*

Time of treatment (hr.)	pH	Mobility (μ /sec./v/cm.)				
		0	1	2	6	24
	7.0	-1.11	-1.94	-2.08	-2.11	-2.12
	4.08	-2.23	-2.38	-2.35	-2.42	-2.36

Fig. 2. Histograms showing distribution of electrophoretic mobilities of *S. aureus* strain 6110 after various treatments (measured in acetate-barbiturate buffer, *I* 0.02). (a) Normal cells, measured at pH 7.0; (b) cells from a single colony, measured at pH 7.0; (c) normal cells, measured at pH 4.22; (d) cells from a single colony, measured at pH 4.22.

a single cell gave rise to similarly heterogeneous cell populations. After treatment with trypsin to remove protein A from the cell surface of both strains, the cell populations were electrophoretically homogeneous at each pH value; Figs. 3(b) and 3(d) show results for strain 3745. This suggested that the original heterogeneity was due to the presence of different amounts of protein A on the surfaces of different cells. Thus a more homogeneous population would be expected at the lower pH value, when the teichoic acid contributed more to the surface charge than did the protein. This theory was further supported by the results after treatment with trypsin, when the population became homogeneous with a high mobility value at pH 7.0. All the cells, irrespective of the amount of protein carried initially, possessed after treatment with trypsin a common protein-free surface. Fig. 4 shows the full pH-mobility curves of cells of strain 6110 before and after trypsin treatment. The average mobility of at least 40 different cells was plotted at each pH for the normal cells; those after trypsin treatment were homogeneous. The curves in Fig. 4

Fig. 3. Histograms showing distribution of electrophoretic mobilities of *S. aureus* strain 3745 after various treatments (measured in acetate-barbiturate buffer, *I* 0.02). (a) Normal cells, measured at pH 7.0; (b) cells after treatment with trypsin, measured at pH 7.0; (c) normal cells, measured at pH 3.38; (d) cells after treatment with trypsin, measured at pH 3.38.

should be compared with the corresponding curves for strain Cowan I (Fig. 1).

It was observed that strain 3754 contained colonial variants that could be recognized by their appearance on milk-agar (Table 2). They were indistinguishable by phage-typing and it was therefore unlikely that either was a contaminant. Their average mobility values at pH 7 differed greatly, but when cells of variant II were treated with trypsin their mobility at pH 7 was increased to that of cells of variant I. Protein A was detected by gel-diffusion in extracts of cells of variant II but

Fig. 4. pH-mobility curves of cells of *S. aureus* strain 6110 before and after treatment with trypsin (measured in acetate-barbiturate buffer, 1.0-0.2). ●, Normal cells (the points plotted represent the average of at least 40 measurements on a heterogeneous population); ○, cells after treatment with trypsin.

Table 2. *Properties of the variants of S. aureus strain 3745*

	Variant I	Variant II
Phage-typing pattern	80/81	80/81
Pigmentation on milk-agar	Golden	Pale buff
Average mobility at pH 7.0 (μ /sec./v/cm.) after growth at 37°	-2.02	-1.34
Average mobility at pH 7.0 (μ /sec./v/cm.) after treatment with trypsin	-2.02	-2.02
Antigen A	Absent	Present
Growth at 44°	+++	±
Mobility (μ /sec./v/cm.) after growth at 44°	-2.02	

not in extracts of cells of variant I. This was additional confirmation that the heterogeneity of the original culture of this strain in mobility tests was attributable to the presence of two distinct populations; only one of these formed protein A, and even then there were different amounts on different cells.

When the original culture of strain 3745 was grown on nutrient-agar plates at 44°, all the colonies consisted of cells with electrokinetic properties

similar to those of cells of variant I, and protein A could not be detected in extracts of them. Subculture on to milk-agar at 37° yielded colonies resembling those of variant I. When cultures of variant I, selected on milk-agar plates of the original culture, were incubated at 44° the cells grew vigorously and were indistinguishable electrokinetically from cells of this variant grown at 37°. It was not possible to obtain sufficient growth of variant II at 44° for measurements of its mobility to be made.

No colonially distinguishable variants were found in strain 6110, but when the original culture was grown at 44° it was found to be devoid of protein A. Populations grown at 44° were electrokinetically homogeneous, and were indistinguishable from cells grown at 37° and subsequently treated with trypsin (Table 3).

DISCUSSION

Although the results of previous electrophoretic studies (Verwey & Frobisher, 1940*a,b*) and serological studies have been interpreted in terms of the presence of protein components at the cell surface of *S. aureus* (Lenhart, Mudd, Yoshida & Li, 1963) the results presented in the present paper constitute more direct evidence for the presence of proteins on the surface of this organism. Unfortunately, although part of this surface protein was contained in the protein fraction B isolated by Verwey (1940), it was not identified further by electrophoretic studies (Verwey & Frobisher, 1940*a,b*). The importance of techniques for the removal of known classes of compounds from cell walls when one is attempting to solve problems of this kind (Hill *et al.* 1963) is obvious. The removal of the protein increased the negative charge on the cell wall at pH 7, the charge at the pH where the maximum mobility was observed remaining unchanged. Thus the mobility of cells of strain Cowan I, and of other strains containing protein A, at pH 7 approached that of cells of strain Wood 46, which do not contain this protein. Trypsin was without effect on strain Wood 46, and gel-diffusion tests on acid extracts of these cells confirmed the absence of protein A.

These conclusions were further substantiated by the investigations on the electrokinetically heterogeneous strains. Most previous electrokinetic studies on bacterial cells have been concerned entirely with homogeneous populations, or with populations that have been treated as such. Although Verwey & Frobisher (1940*a*) observed that some strains produced populations that were unusually heterogeneous, they did not discover the source of this phenomenon. One of the main advantages of the technique of particulate electro-

Table 3. pH-mobility relationship for *S. aureus* strain 6110 after growth at 44° or after growth at 37° followed by trypsin treatment

Temp. of growth	pH ... Treatment of cell	Mobility (μ /sec./v/cm.)						
		3.30	4.23	4.48	6.04	7.0	9.40	
37°	—	—2.28	—2.34	—1.90	—1.56	—1.48	—1.47	
44	—	—2.27	—2.20	—2.07	—2.07	—2.04	—2.05	
37	Trypsin	—2.43	—2.20	—2.08	—2.05	—2.03	—2.06	

phoresis lies in the ability of the observer to detect and investigate such heterogeneous populations, but until now little use has been made of this facility. The fact that electrokinetically heterogeneous populations can arise from apparently pure strains was shown by the behaviour of populations of cells of strain 6110 derived from a single colony (Fig. 2), which was comparable with similar observations by James & Barry (1954) on cells of *Aerobacter aerogenes* trained to grow in proflavine. The heterogeneity of the *S. aureus* cultures must have been due to the presence of different amounts of protein A on individual cells. This is apparent since when the cells were treated with trypsin the populations had a unimodal distribution, displaced to a slightly lower mobility value than the distribution of normal cells measured at pH 4 (i.e. the pH where the maximum mobility was observed), characteristic of a surface lacking protein A (Figs. 3 and 4). The protein cannot have been present as a continuous layer over the cell surface, since variations in the amounts of protein present in such a layer would have affected only the thickness of the layer, leaving the surface charge density unaffected. The existence of cell-wall components as discrete patches on the surface has already been put forward by Haydon (1961) to explain the effects of ions of different valence on the surface charge density of *Escherichia coli*.

The growth of cells of strains 6110 and 3745 at higher temperatures resulted in the loss of protein A and the production of an electrokinetically homogeneous population from an initially heterogeneous parent population.

It is significant that treatment of cells with trypsin to remove the surface protein did not leave a basal surface common to many strains of *S. aureus*. This observation is in marked contrast with that of Hill *et al.* (1963), who found that, after the removal of the M and T proteins from the cell walls of *Strep. pyogenes* by trypsin, a common electrokinetic and serological basal surface was attained whatever the serological type of the organism. In *Strep. pyogenes* it is possible that this common surface consisted of a layer of the group antigen specific to Lancefield's

(1940*a,b*) group A. The failure to obtain evidence for the presence of any such common layer in *S. aureus* is therefore not surprising, since, with the exception of the teichoic acids, which do not contribute to the surface charge above pH 6, and the glycopeptide, there does not appear to be a similar compound or group of compounds common to the cell walls of all strains of this organism. The removal of the protein A by trypsin treatment leaves a surface that consists essentially of the glycopeptide to which is still attached the teichoic acid (since the maximum in the pH-mobility curves is still present; see James & Brewer, 1968). Slight differences in the polymerization or structure of the glycopeptide might result in a difference in the carboxyl/amino group ratio and hence in a different mobility value.

It is now possible, in conjunction with the previous paper (James & Brewer, 1968), to give a tentative picture of the ionic structure of the cell surface of *S. aureus*. The components of the surface overlying the glycopeptide, which can be detected by microelectrophoresis, are teichoic acid and a protein. The protein component may not be composed entirely of Jensen's protein A but it certainly contains it as a major constituent. The teichoic acid contributes phosphate groups to the surface charge only below pH 5–6, when a rearrangement of the teichoic acid molecules at the cell surface occurs and brings these groups into the plane of shear. The higher value of the isoelectric point of the surface after the removal of teichoic acid with sodium periodate (James & Brewer, 1968) suggests that the groups responsible for this pH-dependent change in configuration are carboxyl groups associated with the cell-wall glycopeptide. In some strains, the difference between the net negative charges at pH 4 and pH 7 is greatly increased, owing to the contribution from the positively charged amino groups of the protein component. The maximum is always present in the pH-mobility curves of all strains, irrespective of whether protein A is present or not; the presence of the protein merely emphasizes the maximum. This means, of course, that the maximum cannot be

attributed to any interaction between teichoic acid and a protein. The existence of electrokinetically heterogeneous strains in which the amounts of protein A on individual cells varies suggests that this protein was distributed not as a continuous layer but in discrete patches.

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