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Biochemical and Immunological Properties of Cell Surface Teichoic Preparations from Encapsulated Strains of *Staphylococcus aureus* (40791)[1]

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Encapsulated strains of *Staphylococcus aureus* have been considered exceptional and their capsules were assumed to be antigenically homologous (1, 2). However, Yoshida (3, 4) serologically differentiated four different capsular-type strains of *S. aureus* by the serum–soft agar (SSA) technique. Using a modification of this technique, he and co-workers (5) easily demonstrated capsular substance even in unencapsulated strains of *S. aureus* and postulated that capsular substance production was a regular phenomenon among *S. aureus* strains.

In addition we (6) reported that the biochemical and immunological heterogeneities of the cell wall teichoic acid preparation were related to the capsular-type determined by the serum–soft agar technique. This paper is concerned with studies undertaken, therefore, to characterize teichoic acid preparations of cell surface fractions isolated from capsule-producing strains from which corresponding unencapsulated variant strains were prepared and reported previously (6).

Materials and methods. Strains. For capsular types A, B, C, and D, we used encapsulated strains of *S. aureus* Smith diffuse, NS58D, NS41D, and NS68D from which corresponding unencapsulated variants, A-28, B-62, C-25, and D-68, respectively (6), had been prepared and characterized by the SSA technique as noted elsewhere (3, 4). The encapsulated strains were positive for coagulase, deoxyribonuclease, mannitol fermentation, phosphatase, and negative for clumping factor reaction; they did produce diffuse-type growth in SSA according to the method of Finkelstein and Sulkin (7), and were phage nontypable. These strains were cultured at 37°C for 18 hr in brain–heart infusion (BHI, Difco) dialysate medium, which was supplemented with 2.5 g sodium chloride and 1.5 g agar (Bactoagar, Difco) per liter.

Preparation of cell surface fraction. The cell surface fraction was prepared according to the method of Brock and Reiter (8) modified as follows: 15 g (wet wt) of organisms were suspended in 0.15 *M* sodium chloride solution and then were treated with a sonic oscillator (Model VK 200 p, Tominaga Co., Ltd., Tokyo) at 10 kcycle for 3 min at 0°C. After this treatment, no decrease in viable cell number was observed. The cells were discarded after centrifugation at 7000*g* for 20 min. After the supernatant was dialyzed against distilled water at 4°C overnight, it was lyophilized and used as the crude cell surface fraction.

Procedure for the extraction of teichoic acid preparation. From the crude cell surface fraction, described above, the surface teichoic acid preparation was obtained according to a method previously described (6).

Methods for chemical analysis. Quantitative determinations of hexose (9) and total phosphorus (10) were made. Amino acid analysis was performed on samples hydrolyzed with 4 *N* methanosulfonic acid (Pierce, Rockford, Ill.) at 100°C for 20 hr in a sealed tube. For the acid hydrolysis of sugar and amino sugar, samples were

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treated with 6 *N* HCl at 105°C for 18 hr in a sealed tube, and then neutralized with $BaCO_3$. Thereafter, liquid was removed by using an evacuated dessicator in the presence of P_2O_5. The hydrolysates were passed through a Dowex 50 column (H^+) (diameter 0.5 cm, height 30 cm), before appropriate fractions were combined and dried in an evacuated dessicator. These were quantitated by an amino acid analyzer (Model JLC-5HA, Nihon Electron Optical Co. Ltd., Tokyo). The ribitol and glycerol contents were determined by a gas chromatographic analyzer (Model GC-4B, Shimazu Co. Ltd., Kyoto) as noted below.

Determination of sugar and amino sugar by paper chromatography. The hydrolysates of the surface teichoic acid preparations were analyzed for sugar and amino sugar by paper chromatography according to the method of Liau *et al.* (11). The samples were stained with ninhydrin (11) and silver nitrate (12). For the controls, standards of focosamine and D-galactosamine were used.

Gas chromatographic analysis and infrared spectrography. Sugar and amino sugar within various materials were determined by using a gas chromatographic analyzer (Model GC-4B, Shimazu Co. Ltd., Kyoto) according to a method described elsewhere (6). Infrared spectra of the samples were examined by infrared spectrography (Model IR-400, Shimazu Co. Ltd., Kyoto) using a commercially prepared pellet containing 2 mg of sample in 100 mg of potassium bromide.

Preparation of rabbit antisera to the strains of S. aureus. The method for preparing hyperimmune rabbit sera to strains Smith diffuse, NS58D, NS41D, and NS68D has been published (3, 4).

Agar diffusion test. Double agar diffusion test was performed according to the method of Ouchterlony (13).

Determination of α- and β-acetylhexosaminyl linkages. Determinations were made by the method of Natheson *et al.* (14). For these experiments α-, β-, and mixed α- and β-acetylglucosaminidase (Biochemical Indust. Co. Ltd., Tokyo) were used.

Results. Chemical composition. Each teichoic acid preparation showed neither absorbancy at 260 nor 280 nm indicating the absence of nucleic acid and protein. By means of gas chromatography and the amino acid analyzer (Table I), we found that the teichoic acid preparation from the Smith diffuse strain (capsular-type A) contained 15.68, 3.68, 8.12, 3.12, 3.72, 7.20, and 12.75 μmol/10 mg of galactose, glucose, glucosamine, ribitol, phosphorus, alanine, and glycine, respectively. The corresponding amounts of the preparation extracted from the strain NS58D (capsular-type B and NS41D (capsular-type C) were similar except for minor variations. However, the preparation from the strain NS68D (capsular-type D) was significantly

TABLE I. QUANTITATIVE ANALYSIS OF HYDROLYSATES OF TEICHOIC ACID PREPARATIONS FROM ENCAPSULATED STRAINS OF *S. aureus*

	Strains (capsular-type) (μmol/10 mg)			
Component	Smith diffuse (A)	NS58D (B)	NS41D (C)	NS68D (D)
Galactose	15.68	21.2	16.80	16.36
Glucose	3.68	7.12	8.40	16.40
Glucosamine	8.12	10.52	12.36	ND
Galactosamine	ND[a]	ND	ND	9.56
Ribitol	3.12	2.72	2.40	ND
Glycerol	ND	ND	ND	1.56
Phosphorus	3.72	3.48	3.32	2.20
Alanine	7.20	5.44	4.60	3.92
Glycine	12.75	16.8	8.60	9.56

[a] Indicates not detected.

different from the strain NS58D (capsular-type B) in that it contained more glucose, 16.36 μmol/10 mg, and was the only strain containing glycerol, 1.56 μmol/10 mg, and D-galactosamine, 9.56 μmol/10 mg. The amounts of ribitol, phosphorus, and alanine were generally similar to each other except for the preparation from the strain NS68D.

Paper chromatographic analysis. Except for the strain NS68D in which alone, besides glycerol, D-galactosamine was detected, paper chromatographic analysis of the acid hydrolysates of all the other preparations (Table II) showed ribitol, glucosamine, glucose, and galactose. D-fucosamine was demonstrated in surface teichoic acid preparations from both strains NS41D (capsular-type C) and NS68D (capsular-type D). Also, L-fucose was found solely in the preparation of strain NS41D. However, neither L-rhamnose nor D-mannose were detected in any of the preparations.

Serological properties. Purified teichoic acid preparations extracted from strains Smith diffuse, NS58D, NS41D, and NS68D produced single precipitation lines only against the homologous hyperimmune rabbit sera. No reaction whatsoever was seen against antisera prepared with heterologous capsular-type strains as shown in Fig. 1. In these experiments the minimal amount of preparation producing precipitin lines against the hyperimmune rabbit sera was 30 μg per ml.

Amino acid composition. The amino acid composition of the four teichoic acid preparations are listed in Tables I and III. Among the strains there was a marked difference in the measurable amount of taurine, alanine, glycine, and phenylalanine. Likewise, depending upon the preparation, only trace amounts of lysine, histidine, hydroxyproline, aspartic acid, proline, serine, valine, methionine, leucine, isoleucine, threonine, and glutamic acid were detected.

Infrared spectrogram. When teichoic acid preparations were examined by infrared spectroscopy, specific absorbancies were observed at 2380, 1750, 1520, 1440, 1390, 1300, 1200, 1140, 930, and 790 nm depending upon the strain. However, no specific absorbancies were observed at other bands as shown in Table IV.

Identification of α- and/or β-acetylhexosaminyl linkages. Upon treatment of the first three surface teichoic acid preparations separately with α- and β-acetylglucosamidase, the α-linked glucosaminyl residues were zero, 80, and 0% in the strains Smith diffuse, NS58D, and NS41D, respectively. Conversely, the β-linked acetylglucosaminyl residues correspondingly were 100, 20, and 100%. With capsular-type B strain, *N*-acetylgalactosaminyl residues were both α- and β-type in the ratio of 30 and 70%, respectively, as shown in Table V. In these experiments, 100% of the α-acetylglucosamine was released within 4 to 5 hr from the surface teichoic acid preparations of the strains Smith diffuse and NS41D.

TABLE II. QUALITATIVE ANALYSIS OF HYDROLYSATES[a] OF TEICHOIC ACID PREPARATIONS FROM ENCAPSULATED STRAINS OF *S. aureus* BY PAPER CHROMATOGRAPHY[b]

Component	Strain (capsular-type)			
	Smith diffuse (A)	NS58D (B)	NS41D (C)	NS68D (D)
D-Glucosamine	+	+	+	−
D-Galactosamine	−	−	−	+
Fucosamine	−	−	+	+
D-Glucose	+	+	+	+
D-Galactose	+	+	+	+
L-Rhamnose	−	−	−	−
L-Fucose	−	−	+	−
D-Mannose	−	−	−	−

[a] 6 *N* HCl.
[b] Solvent system: *n*-butanol–pyridine–water (6:4:3); *n*–butanol–ethanol–water (4:1:1).

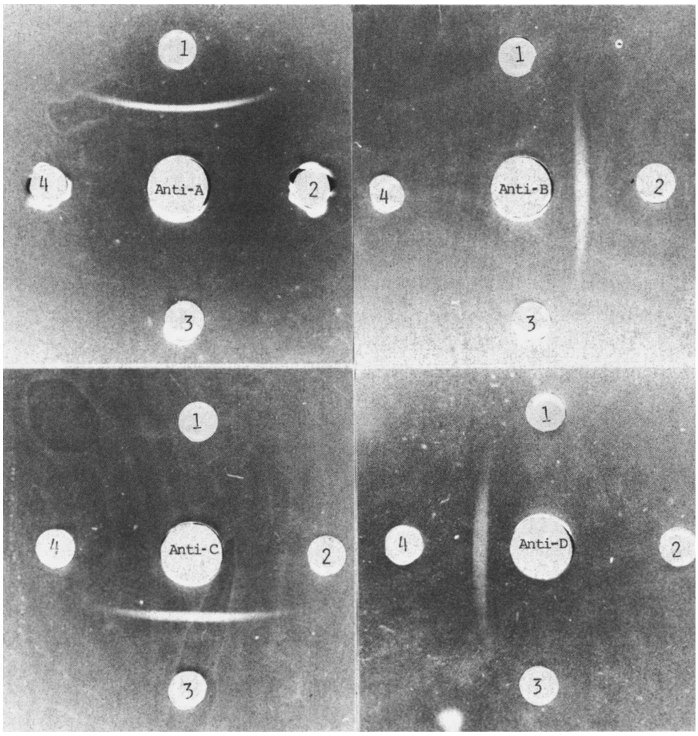

FIG. 1. Gel diffusion analysis of teichoic acid preparations from the four strains (Smith diffuse, NS58D, NS41D, and NS68D) against each of the hyperimmune rabbit sera prepared from the corresponding four encapsulated strains. Anti-A, -B, -C, and -D represent wells filled with hyperimmune rabbit sera prepared with the strains Smith diffuse, NS58D, NS41D, and NS68D, respectively. The peripheral wells 1, 2, 3, and 4 contain teichoic acid preparations extracted from the four corresponding homologous strains: Smith diffuse, NS58D, NS41D, and NS68D.

TABLE III. AMINO ACID ANALYSIS OF THE TEICHOIC ACID PREPARATIONS FROM ENCAPSULATED STRAINS OF *S. aureus*

Composition	Strains (capsular-type) (μmol/10 mg)			
	Smith diffuse (A)	NS58D (B)	NS41D (C)	NS68D (D)
Lysine	2.10	1.30	Trace	Trace
Histidine	1.10	0.92	Trace	1.60
Taurine	2.80	1.95	3.10	0.19
Hydroxyproline	0.09	0.08	0.05	0.06
Asparatic acid	0.35	0.92	0.42	Trace
Glutamic acid	0.08	0.92	0.32	Trace
Proline	0.12	0.33	Trace	Trace
Threonine	0.10	Trace	0.29	Trace
Serine	Trace	Trace	Trace	Trace
Valine	0.92	Trace	0.65	Trace
Methonine	Trace	Trace	Trace	Trace
Leucine	Trace	Trace	Trace	Trace
Isoleucine	Trace	Trace	Trace	Trace
Phenylalanine	1.30	1.92	2.15	3.30
Nitrogen	21.5	19.3	20.3	22.9

TABLE IV. INFARED ADSORPTION SPECTRAL PATTERNS OF TEICHOIC ACID PREPARATIONS FROM ENCAPSULATED STRAINS OF *S. aureus*

Absorption band (cm^{-1})	Strains (capsular-type)			
	Smith diffuse (A)	NS58D (B)	NS41D (C)	NS68D (D)
3400	+[a]	+	+	+
2940	+	+	+	+
2380	–[b]	–	–	+
1750	+	+	–	–
1650	+	+	+	+
1520	+	+	+	+
1440	+	+	+	+
1400	+	+	+	+
1390	–	+	+	–
1310	+	+	–	–
1300	+	–	–	–
1240	+	+	+	+
1200	+	–	–	–
1140	–	–	–	–
1090	+	+	+	+
930	+	–	–	–
790	+	–	–	–

[a] Indicates positive absorption.
[b] Indicates negative absorption.

Discussion. Recently, Brock and Reiter (8) and Seltman and Beer (15) purified capsular materials from several strains of *S. aureus* grown on media high in both carbohydrate and sodium chloride and found that the capsular substance contained ribitol teichoic acid, and small quantities of uronic acid and glucose. Liau *et al.* (11) found aminogalacturonic acid and D-fucosamine in the cell surface material from strain M of *S. aureus*. Also, Reeder and Ekstedt (16) isolated a teichoic acid composed of *N*-acetylgalactosamine residues in addition to aminoglucuronic acid; these had been reported in cell surface material of various staphylococci (17). In our experiments, D-fucosamine and D-galactosamine were also found in the two strains, capsular-type C and D. However, 100% of the *N*-acetylglucosaminyl residues in the surface teichoic acid preparation from the strains, capsular type A and C, were β-linked. Also, the bulk (80%) of the residues from the capsular-type B strain were α-linked. In the case of capsular-type D strain, *N*-acetylgalactosaminyl residues were composed of both α- and β-type but the latter (70%) was predominant. These results closely paralleled those obtained from the cell wall teichoic acid preparations of the same strains (6). Also, these data matched the typing results of capsular-type strains determined by the serum–soft agar tech-

TABLE V. DETERMINATION OF ACETYLGLUCOSAMINYL AND ACETYLGALACTOSAMINYL LINKAGE (α OR β) TO RIBITOL AND GLYCEROL IN TEICHOIC ACID PREPARATIONS FROM ENCAPSULATED STRAINS OF *S. aureus*[a]

Strain (Capsular type)	Type linkage (%)	
	α	β
Smith diffuse (A)	0	100
NS58D (B)	80	20
NS41D (C)	0	100
NS68D (D)	30	70

[a] In the total volume of 5 ml of the sample containing 0.03 *M* citrate buffer and 0.2 *M* NaCl, pH 7.5, there were included 2.0 mg of the surface teichoic acid preparation combined with 2.0 mg of either α-, β-, or mixed α- and β-acetylglucosaminidases. Each sample was incubated at 37°C and an aliquot of the sample was taken every 6 hr during a 24-hr period. The acetylglucosamine liberated at different intervals was measured by the gas chromatographic procedure described above. In a similar manner the linkage of the acetylgalactosamine residues was determined.

nique (3, 4). However, greater amounts of galactose and much lesser amounts of glucose were found in the present surface teichoic acids than in those from cell walls (6).

Concerning the immunological properties of teichoic acid preparations from the cell surface of *S. aureus* strains, Ekstedt and Bernhard (18) reported significantly high proportions of galactose and galacturonic acid in a teichoic acid prepared from the slime material of a strain of *S. aureus* and suggested that antigenic specificity was related to the nature of the polymer of sugar and the configuration of amino acids. Wiley and Wonnacott (19), Brock and Reiter (7) and Wu and Park (20) also postulated a correlation between chemical structure and immunological properties. In our experiments, however, *N*-acetylgalactosamine was the major amino sugar in the surface teichoic acid preparation of capsular-type D strain while the amino sugar of the cell wall teichoic acid preparation was *N*-acetylglucosamine; nevertheless, immunological specificities of both paralleled each other. These studies indicate that the concept of the relationship between immunological specificity and chemical structure requires further consideration as suggested by Coley *et al.* (21).

The infrared spectroscopic findings of the teichoic acid preparations were similar to those of Bohden and Charles (22) and to those in our previous report (6) on the cell wall teichoic acid preparations. However, in spite of certain specific absorbancies in various fractions, differential interpretation remains unclear.

With regards to the amino acids in the capsule of *S. aureus* strains, Liau *et al.* (11) detected taurine besides the other known amino acids within bacterial cells. In our experiments, hydroxyproline was observed in the cell surface fraction in addition to taurine. These two amino acids were also detected in corresponding similar fractions from the organisms grown in a synthetic medium (unpublished observations). Also, while comparing our present analytical results on the surface teichoic acids to the cell wall ones (6), we found that the molar ratios of alanine to glycine and glucose to galactose were inverted. As a consequence we are continuing our investigation on the significance of both the amino acid and sugar molar inversions and on the possible role of taurine and hydroxyproline in the teichoic acid preparations.

Summary. The biochemical and immunological properties of cell surface teichoic acid preparations were investigated by using strains Smith diffuse, NS58D, NS41D, and NS68D of *Staphylococcus aureus*, capsular-types A, B, C, and D, respectively, as determined by the serum–soft agar technique. D-fucosamine, D-galactosamine, taurine, and hydroxyproline were detected in these preparations. The surface teichoic acid preparations from strains Smith diffuse, NS58D and NS41D were of the ribitol-type but the glycerol-type was found in strain NS68D. The major acetylglucosaminyl and acetylgalactosaminyl residues of surface teichoic acid preparations from strains Smith diffuse and NS41D were attached to the polyribitol phosphate by the β-linkage, but attachment for strain NS58D was by the α-linkage, and for strain NS68D the attachment to the polyglycerol phosphate was by both the acetylgalactosaminyl α- and β-linkages. In the agar diffusion test, the teichoic acid preparations of all strains produced a single precipitin line only against hyperimmune rabbit sera prepared from corresponding homologous capsular-type strain; however, no precipitin line was produced against heterologous antisera. These results confirmed the serological classification of the capsular-types determined by the serum–soft agar technique.

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