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Staphylococcus saprophyticus Hemagglutinin Is a 160-Kilodalton Surface Polypeptide

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Many strains of *Staphylococcus saprophyticus* cause direct hemagglutination of sheep erythrocytes. For a high proportion of clinical isolates, a surface protein (Ssp) that is apparently not involved in this property has been described. In this study, *S. saprophyticus* CCM883, a hemagglutinating but Ssp-negative strain, was used for the identification, purification, and characterization of a 160-kDa surface polypeptide that appears to be the major component of the hemagglutinin. Expression of the protein required the addition to the growth medium of EDTA in micromolar quantities, suggesting an inhibitory role for some unidentified metal ion. The protein was purified by means of Sephacryl S-300 chromatography, and antisera were raised in rabbits. Antibody against this protein inhibited the hemagglutination of two other, unrelated strains and was used to demonstrate, by electron microscopy, the presence of the protein on the surface of the cells. In a confirmatory experiment, the purified antigen was incubated with erythrocytes and binding was detected by the Western immunoblot technique with the antibody to the 160-kDa polypeptide. These experiments indicate that this surface protein is the hemagglutinin of *S. saprophyticus*.

Staphylococcus saprophyticus is a well-known cause of urinary tract infections (13, 20). A number of properties, such as ureolytic activity (6), adherence to various cell types (1), and agglutination of sheep erythrocytes (12), have been described for this organism, and their contribution to virulence has been discussed. Adherence to uroepithelial cells was found to be partially mediated by lipoteichoic acids (18). The putative hemagglutinin of *S. saprophyticus* apparently recognizes a disaccharide on the erythrocyte membrane (10). In previous reports, we discussed evidence for the role of urease as a virulence factor in urinary tract infections (6, 8). Expression of the enzyme causes tissue damage primarily to the bladders of experimentally infected animals (8). In earlier work, however, we had shown that culture conditions causing the differential expression of *S. saprophyticus* surface properties altered the colonization capabilities of the organism (9). We therefore attempted to identify surface structures of this species. In a recent study, using electron microscopy, we obtained evidence for the abundant presence of a 95-kDa surface protein (Ssp) in some strains of *S. saprophyticus* (7). This protein is not the hemagglutinin, as it does not cause hemagglutination, and neither the protein nor antisera against it inhibit hemagglutination. Moreover, one hemagglutinating strain (CCM883) lacked visible surface structures in our experiments and did not express Ssp. Production of the 95-kDa surface protein required carefully defined culture conditions. Most striking was the observation that this antigen could be obtained from cells only when they were grown on dialysis membranes placed on top of a certain brand of brain heart infusion agar. We therefore surmised that culture conditions that promote the expression and facilitate the preparation of the putatively proteinaceous (12) hemagglutinin of *S. saprophyticus* might be found.

Here we report the identification, purification, and characterization of a surface protein of *S. saprophyticus* that was present in several hemagglutinating strains.

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MATERIALS AND METHODS

Strains. *S. saprophyticus* 7108, 9325, and 9520 were isolated from patients with acute urinary tract infections and were described in detail previously (6–9). CCM883 is the type strain of *S. saprophyticus* and was obtained from F. Götz (Tübingen, Germany). The hemagglutination and adherence properties of the strains have been thoroughly studied (9). During the experiments, the strains were kept on P agar (9); long-term storage was in liquid nitrogen.

Culture media, buffers, and reagents. P broth (9) contained, per liter, 10 g of peptone, 5 g of yeast extract, 5 g of NaCl, 1 g of glucose, and 1 g of Na_2HPO_4 (pH 7.3) (all from Difco). Agar (Bacto Noble agar; Difco) was added when needed (12 g/liter). Phosphate-buffered saline (PBS) contained 10 mM Na_2HPO_4-NaH_2PO_4 and 140 mM NaCl (pH 7.3), and Tris-buffered saline contained 50 mM Tris (pH 7.3) and 150 mM NaCl. All chemicals were from Merck (Darmstadt, Germany) unless stated otherwise and were of the highest available grade.

General analytical methods. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out as described by Laemmli (14). Concentrations of running and stacking gels were 12 and 6%, respectively. Samples containing 1 to 5 μg of protein per ml were heated (95°C) for 5 min in sample buffer (62.5 mM Tris [pH 6.8], 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromphenol blue) and applied to individual lanes. Proteins were either silver stained (3) or blotted onto nitrocellulose membranes and processed as described earlier (2, 6). Protein content was assessed by the method of Bradford (4) with bovine serum albumin (BSA) as the standard.

Hemagglutination. For this study, we further standardized the procedure described by Hovelius and Mardh (13) and by Gatermann et al. (9). Bacteria were grown either in liquid medium (25 ml in 100-ml Erlenmeyer flasks; 37°C) with constant agitation or on dialysis membranes (9-cm petri dishes containing 20 ml of agar) for 16 h. For inoculation of these cultures, we used starter cultures grown for 8 h with the same media. Cells were harvested, washed once with

PBS, and resuspended in PBS at an optical density at 600 nm of 6.0. Serial dilutions were prepared in microtiter plates with PBS as the diluent (25-μl final volume). The erythrocyte suspension was added (25 μl of a 1% suspension in PBS), and results were read after 2 h of incubation at an ambient temperature (20 to 22°C). In some experiments, reaction mixtures contained BSA (0.1 to 1% final concentration) or Tween 20 (Serva, Heidelberg, Germany; 0.1 to 0.5% final concentration).

For inhibition studies, 5 μl of the inhibiting agent (antibody or antigen) was added to the bacterial suspension. Controls contained 5 μl of buffer instead of the inhibiting agent. When different antisera were compared, the bacterial suspensions were incubated with the antisera (adjusted to the same protein content for 15 min at room temperature) before the erythrocyte suspensions were added.

Preparation of surface proteins. Strain CCM883 was grown on dialysis membranes (37°C, 16 h) placed on top of P agar containing 64 μmol of EDTA per liter. Growth from 100 plates (9-cm diameter) was harvested in double-distilled water, and the suspension was vortexed for 1 min. Cells were pelleted, the supernatant was saved, and solid ammonium sulfate was added to 40% saturation. After incubation in the cold (4°C, 18 h), the precipitate was collected by centrifugation (30,000 × *g*, 30 min, 4°C), the pellet was resuspended in 8 M urea–50 mM Tris–4 mM EDTA (pH 7.3), and proteins were separated by chromatography on Sephacryl S-300 (see below).

Purification of surface proteins. Procedures for purification of the 160-kDa polypeptide by Sephacryl S-300 chromatography were exactly as described before (7). The 160-kDa protein was prepared from strain CCM883, which is known not to express Ssp (7), whereas the 95-kDa protein was prepared from strain 7108 (7). Samples (2 ml; 2 to 4 mg of protein per ml) were applied to the column and eluted with 8 M urea–50 mM Tris–4 mM EDTA (pH 7.3) (7 ml/h). Fractions containing the 160-kDa polypeptide were dialyzed against PBS and used for immunization or inhibition and binding experiments. Ssp was further purified by chromatography on DEAE–Sepharose CL-6B (Pharmacia, Freiburg, Germany; 20 by 2.6 cm) equilibrated with 50 mM Tris–8 M urea and developed with a linearly increasing LiCl gradient (0 to 0.5 M LiCl; 30 ml/h; gradient volume, 500 ml). Only one protein peak (as assessed by A_{280} measurements) containing Ssp was seen in the eluate.

Electron microscopy. Bacteria were fixed for 60 min at room temperature (2.5% glutaraldehyde in 75 mM sodium cacodylate–2 mM $MgCl_2$ [pH 7.2]). Cells were rinsed with the same buffer and postfixed with 1% osmium tetroxide (30 min). For en bloc staining, the cells were incubated with 1% uranyl acetate in 20% acetone for 30 min. Dehydration was done with a graded series of acetone solutions. Cells were infiltrated with and embedded in Spurr's low-viscosity resin (16). After polymerization, the resin was cut with a diamond knife on a Reichert-Jung Ultracut E ultramicrotome, and the sections were mounted on uncoated copper grids. The sections were poststained with aqueous lead citrate (3%; pH 13).

Preparation of antisera. Female rabbits (4 kg; bastard) were immunized by intravenous injection. The animals received the 160-kDa polypeptide (120 μg of protein) on days 0, 5, 15, 30, and 70. Four weeks after the last injection, the animals were bled. Immunoglobulin G antibodies were prepared by chromatography on protein G-Sepharose (Pharmacia) by use of the protocol recommended by the manufacturer. Antibody against Ssp was prepared as described before (7). Purified antibody did not cause agglutination of bacteria.

Immunogold labeling. Cells were grown in liquid or on solid media. Cells were harvested, washed once with Tris-buffered saline, and resuspended to an optical density at 600 nm of 15. To 1 ml of bacterial suspension, antibody was added (125-μg/ml final concentration), and the mixture was incubated for 2 h at room temperature. Cells were washed three times with Tris-buffered saline, and protein A-gold (10 nm; Sigma) was added (dilution, 1:20). After incubation for 1 h at room temperature, the cells were washed as described above and fixed with cacodylate-glutaraldehyde buffer. In some experiments, bacterial cells were treated with proteinase K (Serva; 50 μg/ml; 37°C, 30 min) and washed three times with Tris-buffered saline before being labeled.

Binding of the 160-kDa antigen to erythrocytes. PBS-washed sheep erythrocytes (20-μl packed-cell volume) were resuspended in PBS (80 μl) containing the 160-kDa antigen (75 μg/ml), and the mixture was incubated (45 min) at room temperature. Cells were pelleted and washed three times with PBS. In some experiments, PBS contained Tween 20 (0.1 to 0.5%) to avoid nonspecific binding. Distilled water (40 μl) and Laemmli sample buffer (40 μl) were added, the cells were resuspended, and the mixture was heated (90°C, 5 min). The suspension was centrifuged, and a sample of the supernatant (40 μl) was separated by SDS-PAGE and blotted onto nitrocellulose membranes (0.45-μm-pore size; Schleicher & Schuell, Dassel, Germany). The Western immunoblots were developed with antiserum to the 160-kDa protein (diluted 1:20,000) and an alkaline phosphatase-conjugated secondary antibody (swine anti-rabbit immunoglobulins; DAKO, Hamburg, Germany; 1:1,000 in PBS).

RESULTS

Expression of the hemagglutinin. From earlier studies, it was known that CCM883, when grown on brain heart infusion agar by the dialysis membrane technique (7), does not express surface structures that can be visualized by electron microscopy. In a series of experiments testing different growth media with or without several supplements, we found that surface structures, as assessed by transmission electron microscopy, and hemagglutinating activity could be observed when strain CCM883 was grown on P agar containing micromolar concentrations of EDTA by the dialysis membrane technique or when the strain was grown in P broth with EDTA.

Strains 7108 and CCM883 caused hemagglutination only when grown in the presence of EDTA. In addition, the concentration necessary for the optimal expression of the hemagglutinin was strain dependent. Strain CCM883 required the addition of 64 μmol of EDTA per liter, whereas strain 7108 needed 128 μmol/liter. Although strain 9520 caused hemagglutination when grown with medium not containing EDTA, titers were increased by the presence of 16 μmol of this compound per liter (Table 1). Cells grown on dialysis membranes placed on top of P agar containing EDTA also hemagglutinated sheep erythrocytes at titers of 1:64 to 1:128. Cells grown directly on the agar surface, however, did not cause hemagglutination. Investigated growth conditions did not alter the reaction of strain 9325, a nonhemagglutinating strain (9). With all strains, bacterial growth and, concomitantly, expression of the hemagglutinin were adversely affected by high concentrations of EDTA.

As agglutination caused by a hydrophobic bacterial surface may mimic specific hemagglutination, agglutination

TABLE 1. Hemagglutinating activity of strains of *S. saprophyticus* grown in P broth containing various concentrations of EDTA

Strain	Reciprocal hemagglutination titer in the presence of EDTA at (μmol/liter):						
	0	8	16	32	64	128	256
CCM883	N[a]	N	N	4	32	4	NG[b]
7108	N	N	N	N	1	16	2
9520	32	32	64	32	8	4	NG
9325	N	N	N	N	N	N	NG

[a] N, no hemagglutination observed.
[b] NG, no growth observed.

tests were also conducted in the presence of Tween 20 or BSA. The addition of Tween 20 (0.1 to 0.5%) or BSA (0.1 to 1%) did not alter hemagglutination titers significantly; the maximal difference was 1 dilution step.

Purification of the hemagglutinin. For purification of the protein, the techniques described previously (7) were found suitable. The material eluting in the first peak from a Sephacryl S-300 column was used for preparation of the antiserum in our experiments. This material yielded a single band in silver-stained SDS-polyacrylamide gels (Fig. 1). In immunoblots, antisera to the 160-kDa protein did not react with Ssp, and the 160-kDa polypeptide was recognized by antibody to the 160-kDa antigen but not by antisera to the 95-kDa protein.

Electron microscopy. For ultrastructural studies, strain CCM883 was cultured on P agar (on dialysis membranes) with or without the addition of EDTA (128 μmol/liter). Bacteria grown without EDTA showed a rather smooth surface in transmission electron microscopy micrographs (Fig. 2A). When EDTA was present, a fuzzy surface layer was clearly visible (Fig. 2B). This layer appeared to consist of 50- to 100-nm-long fibrillar structures projecting from the cell surface. The surface material could be labeled with immunogold when antibody against the 160-kDa protein was used (Fig. 2D and F), whereas no labeling was seen when antibody against Ssp, the 95-kDa protein, was used (Fig. 2C and E). Apparently, the addition of EDTA also facilitated the expression of surface structures when strain CCM883 was grown in P broth (Fig. 2C and D). Cell surfaces were always less fuzzy when the bacteria were grown in broth; however, the labeling experiments clearly indicated the presence of the 160-kDa peptide on the cells. Appendages of strains grown in the presence of EDTA, either in broth (Fig. 2C and D) or on agar (Fig. 2E and F), could be labeled with antibody against the 160-kDa peptide but not with antibody against Ssp. Cells of strain 9325, a nonhemagglutinating strain (9), showed no or only minor labeling when reacted with antibody against the 160-kDa antigen. Our experiments thus indicate that *S. saprophyticus* possesses at least two different surface proteins. After treatment with proteinase K, cells could no longer be labeled with antibody against the 160-kDa antigen and immunogold.

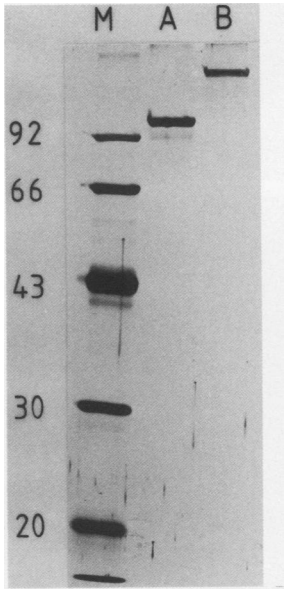

FIG. 1. Purification of the 160-kDa polypeptide. Proteins from dialysis membrane-grown strain CCM883 were separated by size exclusion chromatography and analyzed for the presence of the 160-kDa polypeptide by SDS-PAGE (B). Ssp, the 95-kDa surface protein, was purified from strain 7108 and is shown for comparison only (A). Gels were stained with silver as described by Blum et al. (3). M, molecular size markers (in thousands).

Functional studies. The probable function of the 160-kDa protein was studied by inhibition experiments. When bacteria grown in P broth containing the EDTA concentration necessary for the optimal expression of hemagglutinating activity were preincubated with antibody directed to the 160-kDa peptide (290-μg/ml final concentration), titers were reduced by 3 to 4 dilution steps in the three hemagglutinating strains tested (Table 2). In contrast, antibody directed to Ssp, another surface protein of *S. saprophyticus*, did not reduce hemagglutination titers. Hemagglutination titers with strain CCM883 grown on dialysis membranes (P agar with 64 μmol of EDTA per liter) were reduced from 1:128 to 1:8, i.e., to 1/16, by the 160-kDa peptide antibody. Preparations of the 160-kDa polypeptide containing 8 M urea had to be dialyzed against PBS before use in the assay. This procedure is likely to cause reaggregation of the protein, thereby interfering with its inhibitory activity. Thus, purified protein (15-μg/ml final concentration) was found to inhibit hemagglutination only inefficiently, reducing titers by 0 to 1 dilution step.

However, when a dialyzed preparation of the 160-kDa antigen was incubated with sheep erythrocytes, binding of the protein to the erythrocytes could be demonstrated by the Western blot technique (Fig. 3). One distinct band appeared in the expected range. Two additional minor bands (of 50 to 60 kDa) were also observed. We do not yet know if these are degradation products of the 160-kDa polypeptide or are due to unidentified contaminants present in our preparation. Contamination, however, is unlikely, as blots of the antigen preparation developed with the antibody to the 160-kDa polypeptide never showed a band within that range. No reactions were seen with a control incubated with PBS instead of the antigen, and binding of the antigen was also observed in the presence of Tween 20 (0.1 to 0.5%) (data not shown).

DISCUSSION

Hemagglutination of sheep erythrocytes by *S. saprophyticus* was first observed by Hovelius and Mardh (12). Since then, several workers have tried to associate the expression of this surface property with the colonization capabilities or virulence of the organism (9, 11, 19). In one study, with electron microscopy, surface structures were found in a

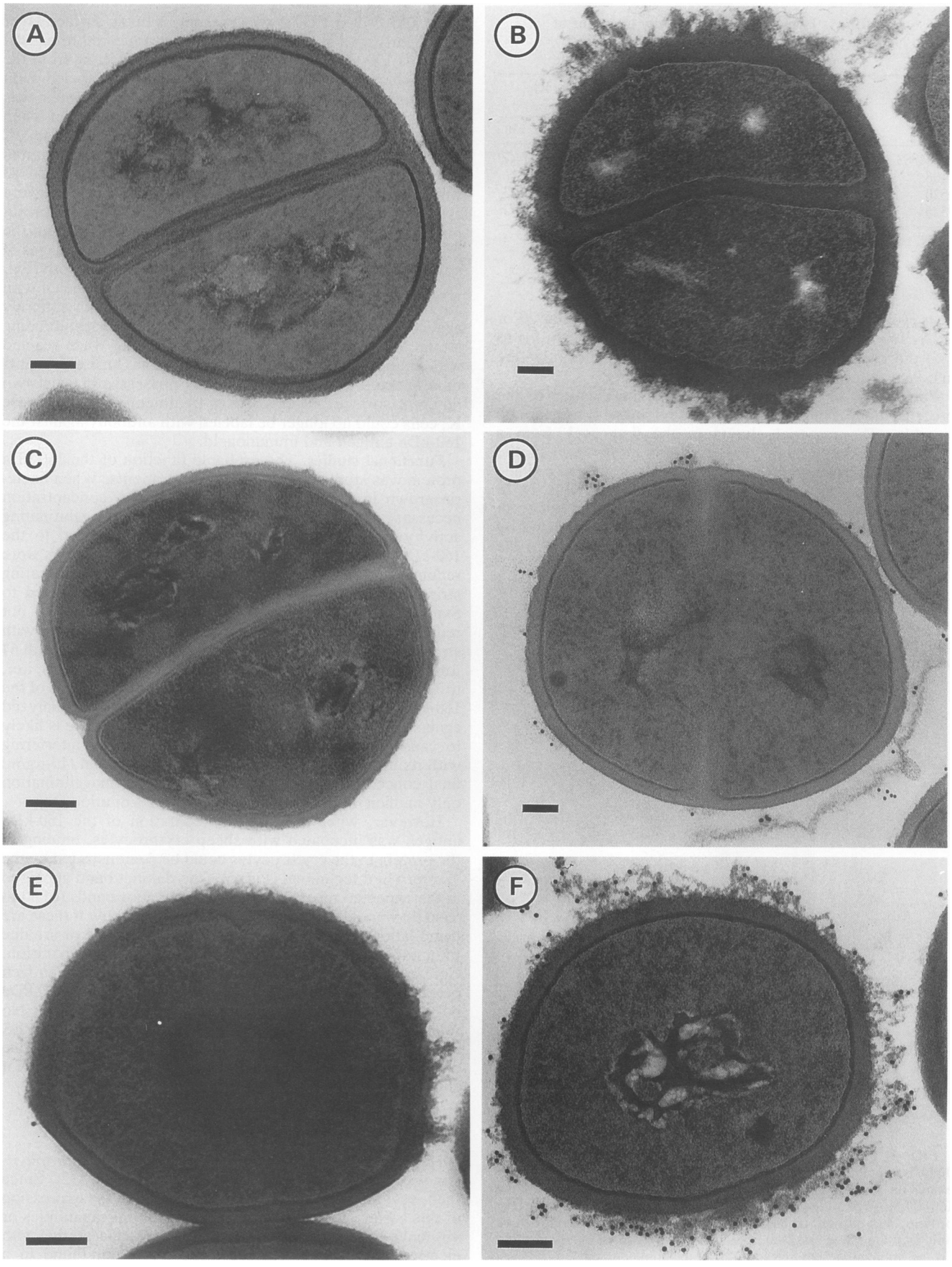
A
B
C
D
E
F

FIG. 2. Surface structures and immunogold labeling of *S. saprophyticus* cells grown under various conditions. (A) Strain CCM883 grown on dialysis membranes placed on top of P agar without EDTA. (B) Same as A, but with EDTA at 128 μmol/liter. (C) CCM883 grown in P broth with EDTA and labeled with antibody to Ssp. (D) CCM883 grown in P broth with EDTA and labeled with antibody to the 160-kDa antigen. (E and F) CCM883 grown on dialysis membranes placed on top of P agar containing EDTA and labeled with antibody to either Ssp (E) or the 160-kDa polypeptide (F). EDTA enhanced the expression of the surface structures, and the 160-kDa polypeptide was present when cells were grown in broth as well as on dialysis membranes. Bars, 100 nm.

strain hemagglutinating sheep erythrocytes at a neutral pH (5); however, no attempt was made to isolate the protein responsible for hemagglutination. We describe culture conditions that allowed the identification and characterization of a 160-kDa surface protein of *S. saprophyticus* that appears to be involved in direct hemagglutination of sheep erythrocytes by many strains of this species. In two strains, the hemagglutinin was expressed only when EDTA was added to P broth at concentrations of 8 to 128 μmol/liter. It is not uncommon that defined culture conditions are necessary for the expression of bacterial surface proteins (7, 15). Expression of the hemagglutinin reportedly (9, 12) requires culturing in broth, whereas Ssp, a 95-kDa surface protein of *S. saprophyticus*, is only produced when certain strains are grown on dialysis membranes placed on top of brain heart infusion agar (7). In the latter study, we had found that Ssp is not the hemagglutinin, as neither the protein nor antibody against it inhibited hemagglutination. In an earlier study (9), in agreement with others (12), we had found that culturing in broth was necessary for the expression of hemagglutinating activity in *S. saprophyticus*. From that study (9), we knew that strain CCM883, which was found not to express Ssp in our more recent report (7), was able to hemagglutinate sheep erythrocytes. We therefore surmised that culture conditions that promote the expression and facilitate the preparation of the hemagglutinin might be found. Culturing on dialysis membranes was chosen for the preparation of surface proteins, because this technique avoids contamination of the preparations with high-molecular-weight proteins originating from the growth medium, a situation that has been described for streptococci grown in Todd-Hewitt broth (17). When strains causing hemagglutination in broth were grown on dialysis membranes, hemagglutination was also observed, and EDTA increased the hemagglutination titers. We conjecture that some as-yet-unidentified contaminating ion that inhibits the expression of the hemagglutinin is neutralized by the addition of EDTA. These experiments thus identified and characterized the growth conditions conducive to the expression of the hemagglutinin.

Purification of the hemagglutinin was achieved by Sephacryl S-300 column chromatography with 8 M urea. As described earlier (7), this process yields rather pure preparations. Antisera raised against the purified protein reacted with the 160-kDa polypeptide but not with another surface protein, Ssp. In addition, antisera raised against purified Ssp did not recognize the 160-kDa antigen. When both types of antisera were used in hemagglutination inhibition studies, only those raised against the 160-kDa polypeptide reduced the hemagglutination titers of all three strains by 3 to 4 dilution steps. This inhibition was not dependent on the growth conditions. It is not surprising that the purified protein did not inhibit hemagglutination efficiently, i.e., that it reduced the titers by 1 dilution step only. As reported earlier (7), this antigen is not soluble unless chaotropic reagents, such as urea, are present. For inhibition experiments, however, urea must be removed by dialysis. It is therefore likely that the dialyzed antigen will reaggregate, a process that is likely to interfere with its neutralizing activity in the hemagglutination assay. Inhibition by soluble purified immunoglobulin G antibodies against this surface structure but not by antibodies against other staphylococcal proteins suggests that the 160-kDa polypeptide is involved in the hemagglutination caused by *S. saprophyticus*. This hypothesis is further supported by the observation that the 160-kDa antigen bound to sheep erythrocytes in vitro.

In conjunction with the results of our earlier experiments (7), these results indicate that *S. saprophyticus* possesses at least two distinct surface proteins: Ssp, a 95-kDa polypeptide that is expressed by many strains when cells are grown on dialysis membranes placed on brain heart infusion agar,

TABLE 2. Results of inhibition experiments[a]

Strain	Reciprocal hemagglutination titer in the presence of the following antibody:		
	None	95-kDa peptide	160-kDa peptide
CCM883	32	32	4
7108	16	16	2
9520	64	64	8

[a] Cells were grown in the presence of the optimal concentration of EDTA. The final antibody concentration was 290 μg/ml.

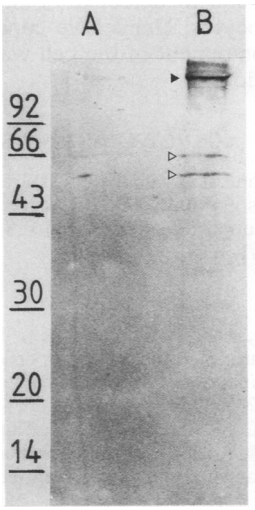

FIG. 3. Binding of the 160-kDa polypeptide to erythrocytes. Sheep erythrocytes were incubated with purified and dialyzed 160-kDa antigen and washed with PBS, and proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes. The blot was developed with antibody to the 160-kDa antigen and alkaline phosphatase-conjugated antiantibody. Lanes: A, control, erythrocytes incubated with buffer; B, erythrocytes incubated with antigen. In lane B, a distinct band that was within the expected range was seen (closed arrowhead); additional faint bands were seen at 50 to 60 kDa (open arrowheads). Molecular sizes (in thousands) are shown on the left.

and a 160-kDa polypeptide that is expressed in liquid media and is involved in the direct agglutination of sheep erythrocytes. In hemagglutinating strains of *S. aureus*, a surface protein of similar size (145 kDa) was found to interact with erythrocytes (15).

To prove that the protein is surface associated in hemagglutinating strains, we used electron microscopy. Strain CCM883 was devoid of surface structures when grown on unsupplemented P agar. On P agar containing EDTA, however, surface projections were visible. This material seemed to consist of very thin, 50- to 100-nm-long fibrillar structures that tended to clump. These surface structures were not recognized by antibody against Ssp but were recognized by antibody against the 160-kDa polypeptide. The antigen was present on cells grown in broth as well as on those cultured on dialysis membranes. In broth, however, the structures were less pronounced. Thus, it is possible that the surface projections consist of more than one component or that agar-grown cells retain the protein more efficiently in their walls. After treatment with proteinase K, cells could no longer be labeled with immunogold. Our experiments with electron microscopy and our functional studies therefore indicate that the 160-kDa polypeptide is present on the surface of *S. saprophyticus* and that it is necessary for the hemagglutinating activity found in this species. It was suggested earlier by Hovelius and Mardh (12) that a protein was involved in hemagglutination by *S. saprophyticus*. In a subsequent study (5), Christiansen and Mardh reported filamentous surface protrusions on hemagglutinating strains of *S. saprophyticus*. In that study, however, no attempt was made to characterize the protein associated with these structures. We purified a protein from whole cells of *S. saprophyticus* and raised antisera against it by using rabbits. These antisera were used to demonstrate the presence of the protein on the surface of the organism. In addition, the antisera were used to inhibit the hemagglutination caused by *S. saprophyticus* and to detect the binding of the purified protein to erythrocytes. Hence, we conclude that this protein is a major component of the cell wall and is necessary for hemagglutination.

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