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Purification and Properties of *Myxococcus xanthus* Cell Surface Antigen 1604

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A cell surface antigen complex from Zwittergent-solubilized *Myxococcus xanthus* has been purified by immunoaffinity chromatography with monoclonal antibody (MAb) 1604 and by subsequent gel filtration. We propose that the cell surface antigen (CSA) 1604 complex participates in intercellular interactions. The apparent total molecular mass of the CSA 1604 complex is 200 kilodaltons (kDa), as determined by gel filtration and by electrophoresis and Western immunoblot probing with MAb 1604. The antigen epitope recognized by MAb 1604 is on a 51-kDa polypeptide. The CSA complex also contains 14% neutral carbohydrate and a 23-kDa polypeptide that lacks the 1604 epitope. The carbohydrate is most likely part of a lipopolysaccharide (LPS) associated with the CSA, because an MAb recognizing an O antigen epitope from the LPS of *M. xanthus* also reacted with CSA 1604 on Western immunoblots. Thus, the 200-kDa CSA complex consists of 97 ± 6 kDa of protein and many associated LPS molecules. The LPS evidently produces the multiplicity of bands observed on Western immunoblots between 100 and 200 kDa. The association with LPS may contribute to the negative charge of the CSA 1604 complex, which has a pI of 4.3. The CSA was clustered on the surface of intact *M. xanthus* cells after labeling with MAb 1604 and immunogold. Furthermore, fractionation studies indicated that cells grown on a plastic surface had 50% of their total CSA 1604 in the cytosol, 39% in the membrane fraction, and 8% in the periplasm. Saturable binding studies with ^{125}I-MAb 1604 indicated that there were 2,400 CSA 1604 sites per cell. The K_d for MAb 1604 binding to the cell was 9 nM.

Myxococcus xanthus is a gram-negative soil procaryote that goes through an intricate life cycle. In response to starvation, *M. xanthus* cells exhibit social behavior involving aggregation, sporulation, and fruiting body formation (50). Some of these interactions are mediated by cell-to-cell contact and may involve the exchange of signals between the cells (9, 24, 26). Any attempt to understand fully the mechanism of signal transduction will, of course, involve those cell surface molecules that receive or transmit the signal. We have previously shown that monoclonal antibody (MAb) 1604, which is directed against cell surface antigen (CSA) 1604 of *M. xanthus* (17), will block the submerged development of fruiting bodies in *M. xanthus* (16). One explanation of this effect is that the normal function of the corresponding antigen that mediates the contact-initiated signal process has been impaired.

The proof that the antigen is, in fact, playing a direct role in the reception or transmission of the signal must ultimately depend on the properties and function of the isolated and, preferably, purified cell surface molecule. The development-blocking effect of MAb 1604 suggests that the corresponding CSA 1604 may belong to a small subset of cell surface molecules—those molecules involved with intercellular interactions. Thus, it has been our intention to isolate, purify, and examine the properties of CSA 1604, which we believe may play a direct role in the contact-mediated cell-to-cell interactions that occur during the development of *M. xanthus*. In an accompanying paper (27), we describe the effect on development of the isolated antigen complex and of additional MAbs directed against other domains of the purified CSA 1604 complex.

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MATERIALS AND METHODS

Bacterial growth and enumeration. *M. xanthus* DK1622 (28) was used throughout this work. The cells were grown each week from a stock of glycerol spores (57) stored at −70°C and inoculated into CT medium (44). In liquid suspension, vegetative cells were grown in CT medium in flasks shaken at 300 rpm at 32°C. Obtaining maximum amounts of CSA 1604 on the cell surface and in the cytosol required growing the cells on a surface. This was crucial for cells used for Western immunoblotting, CSA purification, and saturable-binding studies. Such surface-grown cells were obtained by incubation without shaking for 24 h in 23 ml of CTT liquid medium (24) in 75-cm^2 tissue culture flasks (Costar, Cambridge, Mass.) at an initial density of 10^7 cells per ml. After 24 h at 32°C, during which time the cell numbers increased 10-fold and the cells became attached to the plastic surface, the CTT medium was decanted, 2 ml of 10 mM Tris (Sigma Chemical Co., St. Louis, Mo.) buffer (pH 7.6) was added, and the cells were scraped free.

Cells were counted in liquid suspension by means of a Petroff-Hausser counting chamber and a Zeiss microscope equipped with phase contrast optics. Numbers of viable spores (26) were determined by disrupting mixtures of spores and cells for 55 s at 50 W with a Sonifier cell disruptor (Heat Systems-Ultrasonics, Plainview, N.Y.). The disrupted suspension was then heated at 51°C for 2 h in a water bath, and serial dilutions were plated on CTT plating medium (1.5% agar). The combination of sonic oscillation and high temperature killed >99% of the vegetative cells but had no effect on myxospore viability (19).

The procedure for inducing development in submerged culture was adapted from that of Kuner and Kaiser (29) as modified by Gill and Dworkin (15) for use with MAbs. The cells (10^7 per ml) were grown in CTT liquid in 96-well tissue culture plates (100 μl/well) (Corning Glass Works, Corning, N.Y.) for 24 h, during which time they became attached to

the plastic surface. They were then rinsed with distilled water and induced with a MOPS-salts buffer (10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid; Sigma], pH 7.2, 2 mM $CaCl_2$, 4 mM $MgSO_4$, and 50 mM NaCl).

Hybridoma growth, MAb purification, and ELISA procedures. Hybridoma growth, MAb purification, and enzyme-linked immunosorbent assay (ELISA) procedures were done by the methods of Gill and Dworkin (15), Ey et al. (10), and Gill et al. (16), respectively. MAb purity was monitored by visualization of protein bands on polyacrylamide gel electrophoresis (PAGE). However, the standard ELISA was modified when CSA 1604 was to be detected in the presence of Zwittergent 3-14 detergent (*N*-tetradecyl-*N*,*N*-dimethyl-3-amonio-1-propanesulfonate; Calbiochem-Behring, La Jolla, Calif.). The CSA was spotted onto 6-mm-diameter disks of nitrocellulose (0.2 μm pore size; Schleicher & Schuell, Keene, N.H.), dried at 23°C, and probed with the Young and Davis (61) Western immunoblotting method and buffers. Substrate for the ELISA alkaline phosphatase conjugate (15) was added to wells of 96-well plates, the disks of nitrocellulose plus CSA were added and incubated as usual, the disks were removed, and absorbance values were read on a Minireader II (Dynatech Laboratories, Alexandria, Va.) against blanks from areas of the nitrocellulose free of CSA.

PAGE, Western immunoblotting, and MAb probing. The procedures for PAGE, electroblotting, and probing Western immunoblots with MAbs were as described by Gill and Dworkin (15). Silver staining of gels was done by combining the methods of Oakley et al. (43) and Wray et al. (60). Protein bands on Western immunoblots were visualized with India ink by the method of Hancock and Tsang (20). The presence of carbohydrate in gels was identified with periodate acid-Schiff stain (11) with orosomucoid as a positive control. Lysed cell suspensions were made from cells grown in tissue culture flasks as described above under "Bacterial growth." In each lane of the 5 to 15% polyacrylamide gradient gel (30), there were 2×10^8 cells, which had been lysed by boiling for 2 min in 2× sample buffer (30) containing 5% 2-mercaptoethanol. Proteinase K (Sigma) treatment of the lysed cells (20 μg of proteinase K per 2×10^8 cells) was done for 1 h at 37°C after the cells were boiled in sample buffer and cooled. When lipopolysaccharide (LPS) was electrophoresed, 4 μg of LPS from *M. xanthus* was loaded per lane.

Zwittergent solubilization of CSA 1604. Zwittergent has been used successfully to solubilize bacterial membrane proteins (55). The optimal concentration (0.1%) of Zwittergent 3-14 for solubilizing CSA 1604 was determined by the method of Hjelmeland and Chrambach (22); 8×10^8 cells were lysed and stirred in various Zwittergent concentrations (0, 0.03, 0.1, and 0.3%) in 500 μl of 10 mM Tris (pH 7.6)–100 mM KCl–4 mM $MgCl_2$–2 mM $CaCl_2$ for 2 h at 4°C. After centrifugation, the supernatant suspensions were assayed by modified ELISA for the amount of soluble CSA.

Inhibition of protease activity in lysed cells. Degradation of bovine serum albumin (BSA) as monitored by gel electrophoresis was used as an indicator of protease activity. BSA (12 μg) was incubated for 2 h at 32°C with 8×10^8 cells which had been lysed in 100 μl (total volume) of 100 mM Tris (pH 7.5)–100 mM NaCl–0.05% Zwittergent. Protease inhibitors, such as EDTA, freshly prepared phenylmethylsulfonyl fluoride (PMSF), and leupeptin (all from Sigma), were added before incubation. After incubation, each sample (with 3 μg of BSA and 2×10^8 cell equivalents) was loaded on a 9% polyacrylamide gel.

Preparation of the immunoaffinity and gel filtration columns. An immunoaffinity column with a covalent linkage of purified MAb 1604 to cyanogen bromide-activated Sepharose 4B (Sigma) was prepared with 40 mg of MAb 1604 protein. The MAb was purified on protein A-Sepharose as described above. Protein concentration was determined by the bicinchoninic acid (BCA) method of Smith et al. (53) with BSA as a standard. After the MAb 1604 solution was concentrated to 4 ml with a Minicon B15 concentrator (Grace and Co., Danvers, Mass.), the MAb was dialyzed twice against 500 ml of 100 mM $NaHCO_3$ (pH 8.5) with 50 mM NaCl. Following the instructions in "Affinity Chromatography: Principles and Methods" (Pharmacia Fine Chemicals, Uppsala, Sweden, 1983), the CNBr-activated Sepharose was incubated with 4 ml (40 mg) of MAb 1604 solution. After completion of MAb binding, unreacted crosslinker esters were blocked with the addition of 0.16 ml of 1 M ethanolamine (Sigma), pH 8.0. The Sepharose was then rinsed with 50 mM Tris (pH 7.5)–50 mM NaCl–0.05% Zwittergent–0.005% Thimerosal (sodium ethylmercurithiosalicylate; Sigma) and stored at 4°C.

A gel filtration column was prepared following the guidelines in "Gel Filtration: Theory and Practice" (Pharmacia). The G-200-120 (Sigma) beads were swollen over the course of several days in 25 mM Tris (pH 7.5)–25 mM NaCl–0.025% Zwittergent–0.005% Thimerosal and poured into a buret to form a column 1 cm by 38 cm. After 2 bed volumes (60 ml) of buffer had passed through the column, the void volume, V_0, was determined by measuring the volume at which 0.3 mg of blue dextran with a molecular mass of 2 megadaltons (MDa) eluted from the column, as detected by absorbance at 280 nm. Similarly, the elution volumes, V_e, were determined for three proteins in the presence of Zwittergent: 0.1 mg of carbonic anhydrase (29 kDa), 3 mg of BSA (66 kDa), and 0.2 mg of alcohol dehydrogenase (150 kDa). A semilog plot was drawn of relative molecular mass versus V_e/V_0 for these proteins and used to estimate the mass of the CSA.

CSA 1604 purification. *M. xanthus* cells (10^{10}) were grown without shaking on the surface of five 75-cm^2 tissue culture flasks as described above and scraped into 10 ml of 2 M NaCl at 4°C. The cell slurry was centrifuged for 5 min at 12,000 × *g* at 4°C. The cell pellet was then osmotically lysed by suspension in 25 ml of 10 mM Tris (pH 7.5) with three protease inhibitors at final concentrations of 10 mM EDTA, 1 mM PMSF, and 0.1% leupeptin at 4°C. To the suspension was added 25 ml of 50 mM (Tris pH 7.5)–50 mM NaCl–0.1% Zwittergent plus the three inhibitors. The clarified suspension of lysed cells was filtered through a Stervifex-GS filter unit (0.22-μm pore size; Millipore) and loaded onto the 1604 MAb affinity column. As recommended in "Affinity Chromatography: Principles and Methods" (Pharmacia), the affinity column had been rinsed with the elution buffer (50 mM diethylamine [Sigma], 10 mM Tris [pH 11.3], 50 mM NaCl, 0.05% Zwittergent) (8). Before sample loading, the pH of the column was restored to pH 7.5 by rinsing with binding buffer (50 mM Tris [pH 7.5], 50 mM NaCl, 0.05% Zwittergent).

After the lysed cell suspension had been loaded (over 4 to 6 h) onto the MAb affinity column, the column, which was packed in ice, was rinsed with about 40 ml of binding buffer until the A_{280} of the effluent reached that of the buffer. The CSA was eluted with about 16 ml of elution buffer and collected in 2-ml fractions. The fractions that absorbed at 280 nm were pooled and dialyzed twice in Spectrapor 4 tubing (Fisher Scientific, Pittsburgh, Pa.) each time against 1 liter of 25 mM Tris (pH 7.5)–25 mM NaCl–0.01% Zwittergent at 4°C for 12 h. After dialysis, the CSA was concentrated to 200 μl with Centricon 30 microconcentrators (Grace and

Co.) by repeated centrifugation at 3,000 × *g*. The concentrated CSA was stored at −70°C.

The CSA 1604 was further purified by gel filtration after the immunoaffinity procedure. The frozen CSA 1604 was thawed and loaded onto the G-200 column, which was maintained at 8°C. The CSA was eluted in 25 mM Tris (pH 7.5)–25 mM NaCl–0.025% Zwittergent and collected in 0.6-ml fractions with a Gilson FC-80 Microfractionator (Gilson Medical Electronics, Middleton, Wis.) until 1 bed volume (30 ml) had eluted. By monitoring the A_{280} of the fractions and by determining the V_e for the CSA, the mass of the eluted CSA size categories was found as previously described. Only the 200-kDa gel filtration fractions were pooled and used for subsequent work.

When fractions were assayed by ELISA for MAb 1604 binding, 15 μl of each fraction was spotted onto disks of nitrocellulose and probed with MAb 1604 as described for immunoblot probing. The substrate (2.5 mM *p*-nitrophenyl phosphate in ELISA dilution buffer [20 mM Tris, 500 mM NaCl, 2 mM $MgCl_2$, 1 mM $ZnCl_2$, 0.005% Tween 20, pH 8.5]) was incubated with the disks for 60 min at 37°C. Before the A_{410} was read, the disks were removed from the 96-well plate. The desired fractions were then pooled, concentrated with the Centricon 30, and frozen to −70°C.

Protein and hexose content of CSA 1604. The protein concentration of CSA 1604 was determined by the BCA assay (53), in which BSA was used as a standard. Correction was made for the A_{532} of the G-200 column buffer in which the CSA was concentrated and stored.

The total neutral hexose content of the CSA 1604 was determined by the phenol-sulfuric acid method, with glucose used as a standard (14). The A_{488} was read against a water blank, and correction was made for the absorbance of the G-200 column buffer. This method also detected pentoses and heptoses but not amino sugars (14).

LPS purification and spot blots. LPS from vegetative cells of *M. xanthus* was extracted and purified by the method of Panasenko (45). LPS (6 μg) was spotted onto nitrocellulose, dried, and probed with MAbs by the method of Young and Davis (61).

Proteolysis and oxidation of CSA 1604 to define the epitope. CSA 1604 was dialyzed against 100 mM sodium bicarbonate (pH 7.5)–100 mM NaCl–1 mM $CaCl_2$ and exposed to Amberlite XAD beads for 15 min with shaking (38). Next, 4 μg of trypsin per 10 μg of CSA was added and incubated for 1 h at 37°C. An equivalent amount of CSA was treated similarly but without trypsin.

Periodate oxidation of the CSA was carried out as described by Panasenko (45). Oxidation was stopped by adding ethylene glycol to a final concentration of 250 mM to both treated and untreated samples. The oxidized, proteolyzed, and untreated CSA samples were spotted onto nitrocellulose in duplicate, dried at 23°C, and probed with MAb 1604 as described for the ELISA procedure.

Isoelectric focusing. Before isoelectric focusing, 30 μg of CSA 1604 was dialyzed overnight at 4°C against 100 ml of 0.1% Triton X-100 (Beckman Instruments, Palo Alto, Calif.). The acrylamide gel was prepared by the method of Winter et al. (58), and the CSA was applied to filter paper strips placed 1 cm from the anode. Focusing occurred after 2 h at 20 A and 80 W. The pH gradient in the gel was determined at intervals by excising pieces of gel perpendicular to the electrodes, soaking the pieces in water, and measuring the pH.

Electron micrograph of *M. xanthus* labeled with MAb 1604-gold. Exponentially growing cells were chilled, centrifuged, and suspended in MOPS-sodium (10 mM MOPS [pH 7.2], 50 mM NaCl) at a concentration of 3×10^9 cells per ml. MAb 1604 was added at a final concentration of 800 μg/ml, and the cells were incubated for 30 min at 32°C. The cells were removed by centrifugation and suspended in MOPS-sodium at 3×10^9 cells per ml, and a 10-μl drop was applied to the surface of CTT agar. The drop was allowed to dry, and the cells were then incubated for 30 min at 32°C. A 10-μl amount of 40-nm-diameter immunogold beads (each bead coated with several goat anti-mouse immunoglobulin G molecules; Janssen Life Sciences, Beerse, Belgium) was added directly to the cells on the agar surface and incubated for an additional 30 min at 32°C. Finally, 100 μl of 0.2% glutaraldehyde was added to the agar surface, the cells were scraped off and collected, and 10 μl was added to the surface of a Parlodion-coated grid. The grid was examined with a Phillips 201C electron microscope set at 80 kV.

Radioiodination of MAbs. MAbs 1514 and 1604 (600 μg of each), which had been affinity-purified on protein A-Sepharose CL 4B, were iodinated with $Na^{125}I$ (Du Pont-NEN, Cambridge, Mass.) by the lactoperoxidase and glucose oxidase method (31, 52). Before iodination, one vial of Enzymo Beads (Bio-Rad Laboratories, Richmond, Calif.) containing lactoperoxidase and glucose oxidase was activated for 2 h in 2 ml of 100 mM imidazole (pH 6.9) at 4°C, centrifuged at 1,000 × *g* for 2 min, and suspended in 250 μl of imidazole. The 400 μl of reaction buffer consisted of final concentrations of 100 mM imidazole (pH 6.9), 140 μM KI, 50 μM $CaCl_2$, 100 μl of Enzymo Bead slurry, 500 μCi of $Na^{125}I$, and 600 μg of MAb. The reaction was started by adding glucose (760 μM final concentration) and incubated at 25°C for 40 min with intermittent agitation of the capped tube.

After the incubation, two samples of 5 μl each were taken for dilution and counting. The reaction mixture was loaded onto a Biogel P-2 (Bio-Rad) gel filtration column (8-ml bed volume preequilibrated with 10 mM imidazole [pH 6.9]–100 mM NaCl) to separate iodine bound to the MAb from free iodine. Fractions (250 μl) were collected, and 10 μl of each was counted in a Biogamma II counter (Beckman Instruments, Palo Alto, Calif.). The fractions of greatest radioactivity were pooled, and the amount of protein was estimated from the number of pooled counts per minute (cpm) relative to the total cpm eluted before the free iodine. The specific activity of the ^{125}I-MAb was determined as cpm per microgram of MAb on the assay date for $Na^{125}I$. The specific activities were 1.06×10^5 cpm/μg for MAb 1604 and 1.15×10^5 cpm/μg for MAb 1514, where 1 nmol of MAb is 160 μg. The ^{125}I-MAb was stored at 4°C.

Saturable binding of ^{125}I-MAbs to cells. *M. xanthus* cells were grown for 24 h in 75-cm^2 tissue culture flasks as described above, harvested in 10 mM MOPS (pH 7.2) with 50 mM NaCl, and centrifuged. The cells were suspended in 100 μl of 10 mM MOPS (pH 7.2)–50 mM NaCl–1% BSA. The ^{125}I-MAb was added, and the mixture was incubated at 4°C for 30 min. Longer incubations were avoided to prevent MAb proteolysis (J. S. Gill and M. Dworkin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, I-130, p. 202). For ^{125}I-MAb 1604, duplicate tubes of 2×10^8 cells per tube were used at each MAb concentration: 1, 5, 10, 20, 30, 50, and 100 nM. For ^{125}I-MAb 1514, duplicate tubes of 5×10^6 cells per tube were incubated at each MAb concentration: 10, 25, 50, 100, 250, 500, and 750 nM. After incubation, the cells were twice centrifuged and resuspended, and the radioactivity in the pellets was counted in a Biogamma II counter.

Nonspecific binding was quantitated with ^{125}I-MAb B_{10} at the same specific activities as ^{125}I-MAbs 1604 and 1514. MAb B_{10} binds to human placental alkaline phosphatase

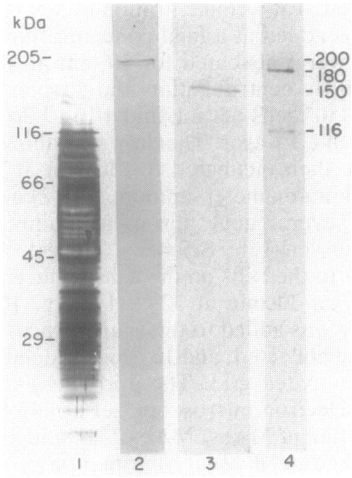

FIG. 1. Western immunoblots of 2×10^8 *M. xanthus* cells per lane. Immunoblots were stained with ink or probed with MAb 1604. The first lane is an ink stain of total proteins. In the second lane, the cells were reduced but not boiled before electrophoresis and probing with MAb 1604. In the third and fourth lanes, the cells were reduced and boiled before electrophoresis and probing.

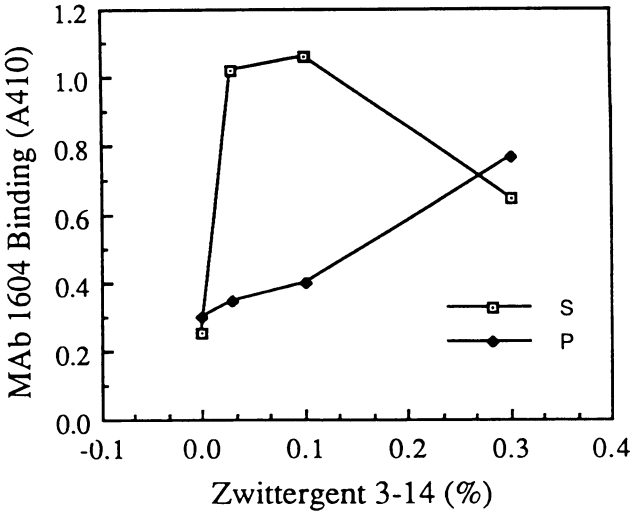

FIG. 2. Solubility of CSA 1604 in various concentrations of Zwittergent 3-14. The soluble supernatant (S) and insoluble pellet (P) forms of the CSA were detected by ELISA with MAb 1604. The ELISA A_{410} was proportional to the MAb 1604 binding to CSA 1604. The amounts of Zwittergent used to dissolve samples of cells were 0, 0.03, 0.10, and 0.3%.

(36). Specific binding was calculated as the difference between the average of bound cpm for ^{125}I-MAb 1604 or 1514 at each concentration and the average of bound cpm for ^{125}I-MAb B_{10}. Each binding study was repeated four times in duplicate.

Distribution of the CSA 1604 in cell fractions. *M. xanthus* cells were separated into four fractions: filtered medium, periplasm, cytosol, and envelope (inner and outer membrane). Cells were scraped from tissue culture flasks (2.3×10^9 cells per 75-cm^2 flask), as described above, and centrifuged at $11,000 \times g$ for 3 min. The CTT medium in which the cells had been grown was filter sterilized, and volumes previously containing 5×10^7 cells were saved. Equal volumes of uninoculated CTT were also saved to control for absorbance of the medium. Cells were resuspended in 2 ml CTT, the suspension was centrifuged as above, and the cells were suspended in 20% sucrose–5 mM EDTA–10 mM Tris (pH 7.6) at 4°C for 10 min. Cells were centrifuged and resuspended at 4°C (after rinsing the pellet) in 0.5 mM $MgCl_2$ to shock the cells. The cells were centrifuged, leaving the periplasmic fluid in the supernatant; amounts equivalent to 5×10^8 cells were saved. This was the osmotic shock procedure of Nossal and Heppel (42) as first applied to the myxobacteria by Burchard (3).

The cell pellets were suspended in 2 M NaCl at 4°C for 5 min. The solutions were centrifuged as above, the supernatant fluids were discarded, and the pellets were rinsed with water and suspended in water at 4°C. The lysed contents of both tubes were centrifuged at $100,000 \times g$ for 2 h at 4°C. The supernatant fluid contained the cytosol fraction, while the pellet contained the envelope fraction. Volumes of each fraction were pipetted into triplicate wells of a 96-well plate. The plate was dried overnight at 23°C and probed with MAb 1604 by the standard ELISA procedure. The A_{410} was read with the substrate solution as a blank. All data were normalized to 5×10^7 cell equivalents per well.

RESULTS

Western immunoblots of lysed *M. xanthus* cells. Figure 1 presents the results of Western immunoblots in which lysed cells were either electrophoresed directly or boiled before electrophoresis. The first lane is an India ink stain of total *M. xanthus* protein from lysed cells. The second lane is an immunoblot of lysed cells that were reduced but not boiled in the 2× electrophoresis sample buffer. MAb 1604 bound to a double band of antigen at 200 kDa during subsequent probing of the immunoblot. The third and fourth lanes are immunoblots of lysed cells reduced and boiled in 2× sample buffer before electrophoresis. After boiling, electrophoresis, and probing, the CSA exhibited heat lability. All of the material in the 200-kDa antigen band shifted to 150 kDa in lane 3. In lane 4 some of the CSA remained at 200 kDa, while the rest shifted to 180 and to 116 kDa. Long delays in loading boiled samples onto the gel resulted in more numerous bands lower than 200 kDa, such as those seen in lane 4.

Zwittergent solubilization of CSA 1604. Three detergents were tested for their ability to solubilize CSA 1604. Neither the detergent recommended by Helenius et al. (21), octylglucoside, nor that recommended by Hjelmeland et al. (23), CHAPS(3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate), solubilized the antigen. Because the dipolarionic detergent Zwittergent 3-14 had been reported to solubilize surface antigens from *Streptococcus faecalis* (55), Zwittergent was tested with CSA 1604 and found to solubilize it. During ELISA procedures, there was no apparent decrease in MAb binding to CSA 1604 in the presence of Zwittergent.

The optimal concentration of Zwittergent 3-14 for solubilization of CSA 1604 from 8×10^8 cells was determined. A concentration of 0.1% Zwittergent dissolved the maximal amount of CSA, which was subsequently found in the supernatant fraction (Fig. 2). For fewer than 8×10^8 cells, 0.1% Zwittergent also gave optimal solubilization. When no Zwittergent was added, about one-half of the total amount of antigen was detected in the sum of the pellet and supernatant fractions, because 50% of the total antigen remained in the cytosol (see Table 3) and was not released into the supernatant solution. A similar varying solubility of membrane proteins at different detergent concentrations has also been observed in solubility studies of other cell membrane proteins (22).

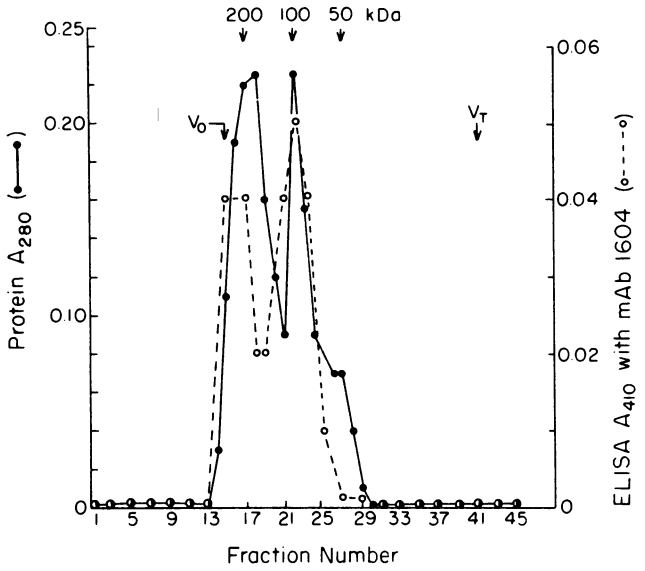

FIG. 3. Elution profile from a Sephadex G-200 column loaded with immunoaffinity-purified CSA 1604 from 10^{10} cells. After elution, fractions were assayed for protein by A_{280} and for MAb 1604 binidng as measured by ELISA A_{410}. Peaks of absorbance due to protein and peaks of MAb binding overlap. V_T, Included volume.

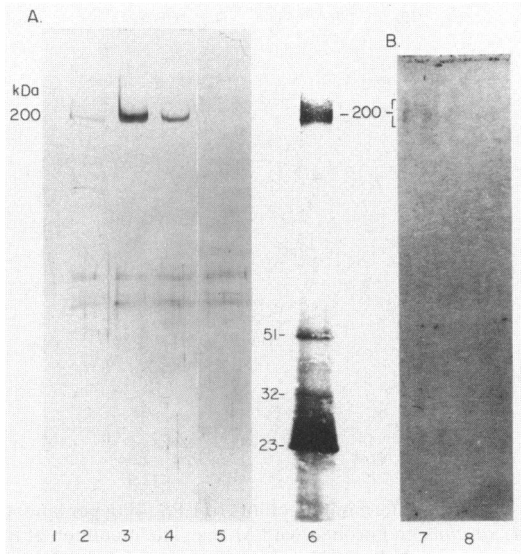

FIG. 4. (A) Silver-stained SDS electrophoretic gel (5 to 15% acrylamide gradient) of immunoaffinity- and gel filtration-purified CSA 1604. The CSA samples (<1 μg) were reduced but not boiled in lanes 1 to 5. In lane 6, the CSA (25 μg) was not reduced. Lanes: 1, buffer alone; 2, 3, and 4, fractions 14 to 16, 17 and 18, and 19 and 20, respectively, of a 200-kDa CSA peak from the gel filtration column; 5, buffer alone. Lanes 2 through 5 contained the reductant 2-mercaptoethanol; lane 1 did not. The double row of bands at about 60 kDa is an artifact of the reduction. Lane 6 contained fractions 21 to 25 of a 100-kDa CSA peak from gel filtration. (B) Periodate acid-Schiff-stained SDS gel of gel filtration-purified CSA 1604. Lane 7 contained 25 μg of CSA 1604 from the 200-kDa peak, while lane 8 contained 25 μg from the 100-kDa peak from gel filtration.

Immunoaffinity and gel filtration chromatography. In the presence of the protease inhibitor EDTA, the CSA 1604 complex could be solubilized with less proteolysis and subsequently retained on an immunoffinity column consisting of MAb 1604 covalently bound to Sepharose beads. The bound CSA 1604 was rinsed and selectively eluted from the MAb affinity column. Then the CSA was size-fractionated on a G-200 Sephadex column to isolate the high-molecular-mass form of the CSA that had been observed on Western immunoblots (Fig. 1) and reported previously (15). Figure 3 is the G-200 elution profile of CSA 1604. There were two major A_{280} peaks: one peak at 200 kDa and one at 100 kDa. The A_{410} peaks in the ELISA due to MAb 1604 binding overlapped the A_{280} peaks. The 200-kDa peaks at A_{280} and A_{410} are offset slightly because there is evidently a smaller protein, which lacks the 1604 epitope, present in the 200-kDa antigen complex.

In a preliminary experiment in which cells were grown in a mixture of ^{3}H-amino acids, the gel filtration elution profile looked very similar to that in Fig. 3. Peaks of ^{3}H radioactivity coincided with the other two peaks. This was further evidence that the CSA contained protein. The CSA from only the 200-kDa peak was routinely used for subsequent work. When the material from the 200-kDa gel filtration peak was electrophoresed after reduction but without prior boiling (Fig. 4, lanes 2 to 4), there was one diffuse band at 200 kDa on a silver-stained sodium dodecyl sulfate (SDS) gel. When the 100-kDa gel filtration material was electrophoresed without reduction or prior boiling (lane 6), there was one diffuse band at 200 kDa and three lower-mass bands at 51, 32, and 23 kDa. These three proteins were not disulfide-linked, because they were separated under nonreducing conditions. In lane 7, periodate acid-Schiff staining of the gel showed that there was carbohydrate present in the material from the 200-kDa gel filtration peak. Less than 1 μg of carbohydrate (no staining) was found in the material from the 100-kDa gel filtration peak (lane 8). Therefore, both gel filtration peaks contained the diffuse band, in which carbohydrate was present, but the 100-kDa peak material apparently had less carbohydrate. The 100-kDa gel filtration peak was generated by dissociation of some of the 200-kDa material, because rechromatographing the 200-kDa material on the column yielded more 100- and less 200-kDa material.

Protein and hexose content of CSA 1604. The A_{280} (Fig. 3) suggested that the CSA contained protein. We also tested for the presence of hexose and lipid. The lipid determinations were inconclusive, because there was not enough antigen available. As described later, lipid in the form the LPS was present and was detected with an MAb. Hexose was also detected in the CSA. The hexose assay (which also measures pentoses and heptoses [14]) was sensitive enough to measure antigen hexose. Table 1 contains the data about the amounts of protein and neutral hexose in CSA 1604 after purification by immunoaffinity and gel filtration. From three separate samples of CSA, the mean neutral carbohydrate content was 14 ± 2%.

TABLE 1. Protein and hexose content of CSA 1604[a]

CSA sample	Yield (μg)		% Hexose[b]
	Protein	Hexose	
1	90	15	17
2	28	4	14
3	150	16	11

[a] CSA was purified by immunoaffinity and gel filtration. Only material from the 200-kDa gel filtration peak was used.

[b] The hexose assay also detects pentoses and heptoses. The mean hexose percentage for three different samples ± SEM was 14 ± 2%.

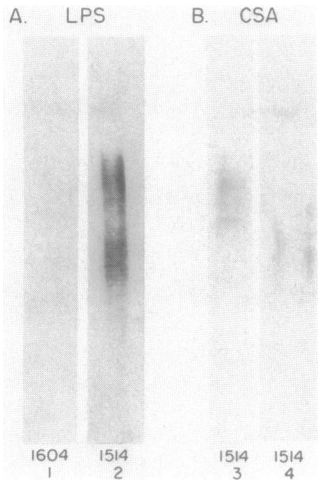

FIG. 5. (A) Western immunoblots of LPS (4 μg per lane) purified from *M. xanthus* and probed with MAbs. The immunoblot in lane 1 was probed with MAb 1604, which recognized CSA 1604 but not LPS. The immunoblot in lane 2 was probed with MAb 1514, which recognized LPS O antigen and gave a typical ladderlike pattern. (B) Western immunoblots of affinity-purified CSA 1604. In lane 3, the CSA was probed with MAb 1514 against LPS O antigen. In lane 4, the CSA was treated with proteinase K, subjected to electrophoresis, and then probed with MAb 1514.

Identification of LPS copurifying with CSA 1604. Before detecting any LPS that might be associated with the CSA, control Western immunoblots of LPS (4 μg per lane) were probed with an MAb that recognized *M. xanthus* LPS. MAb 1514 recognized an O antigen epitope of the LPS, as expected (Fig. 5, lane 2) (13, 15). The pattern of O antigen units from LPS had a ladderlike appearance on the electrophoretic gel. The multiple bands were in discrete increments due either to the sequential addition of units of O antigen saccharides to each LPS molecule or to the addition of multiple LPS molecules. As expected, MAb 1604 did not bind to the purified LPS (lane 1). There was also evidence that LPS is associated with the affinity-purified CSA 1604 (Fig. 5, lane 3). MAb 1514 reacted with the CSA. The reactivity remained but shifted to a lower position on the gel after thorough proteolysis of the CSA 1604 polypeptides by proteinase K treatment. Thus, the LPS is not merely coinciding in migration in the gel with the proteins of the CSA but is actually attached to the proteins.

Western immunoblots of CSA 1604. After purification on the immunoaffinity column, the CSA continued to be recognized by MAb 1604. Affinity-purified CSA 1604 still contained the 1604 epitope (Fig. 6, lanes 5 to 7). Lane 2 of Fig. 6 is a control for the nonspecific adherence of protein to the immunoaffinity column matrix; very little adhered. The dark bands between 50 and 60 kDa are due to the reductant. Lane 3 has the banding pattern from proteins coeluting with LPS from an MAb 1514 immunoaffinity column. There were four prominent bands at 116, 62, 52, and 45 kDa and many minor bands, as expected for proteins associated with LPS. Lane 4 shows the proteins that were stained after eluting from the MAb 1604 immunoaffinity column. There was a prominent band at 23 kDa and a minor band at 21 kDa. Lanes 5 to 7 show CSA 1604 bands reacting with MAb 1604. Lane 5 had material (bands at 200, 180, 130, 123, and 116 kDa) from one antigen preparation and lanes 6 and 7 had material from another. The shift to lower-mass bands (51, 33, 21, and 19 kDa) in lanes 6 and 7 was caused by both a longer time for sample preparation, indicating the heat lability of the protein-LPS association, and by proteolysis. Lane 8 is a control showing no detectable leakage of antibody heavy and light chains from the MAb 1604 immunoaffinity column after elution of the CSA complex at pH 2.2. (The low pH rendered the CSA less immunoreactive but would not have prevented reactivity of the conjugate with antibody protein had any been present [18].)

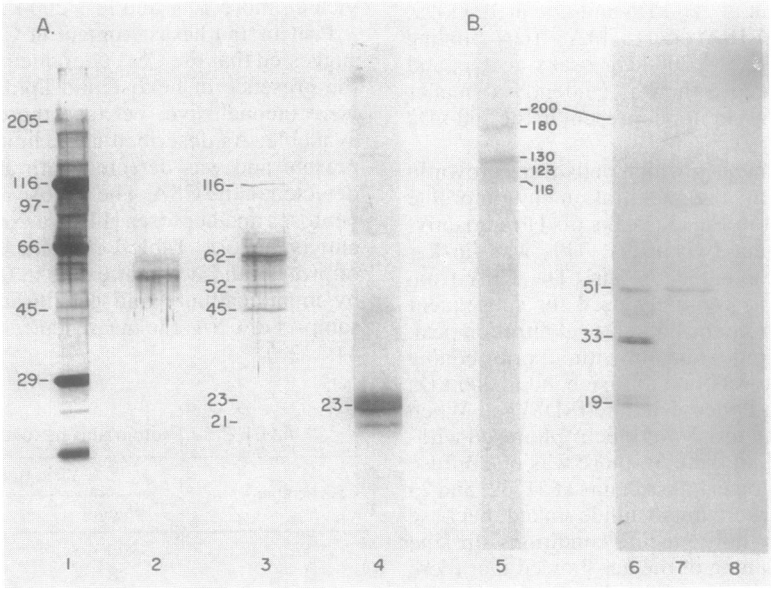

FIG. 6. Western immunoblots (15 μg of protein per lane) from reducing SDS gels (A) stained with India ink or (B) probed with MAb 1604. Lanes: 1, standards; 2, material eluting from a blank immunoaffinity column (lacking MAb); 3, material eluting from an MAb 1514 affinity column; 4 to 8, material eluting from an MAb 1604 affinity column. Lane 5 is a different preparation of CSA 1604 than in lanes 6 and 7. Lane 8 is a control immunoblot showing no detectable leakage of antibody heavy and light chains from the MAb 1604 immunoaffinity column after elution of the CSA complex at pH 2.2.

TABLE 2. MAb 1604 binds to protein epitope on CSA 1604

Treatment[a]	ELISA A_{410} with MAb 1604		Mean % of untreated CSA A_{410} ± SEM[b]
	Untreated CSA	Treated CSA	
Periodate oxidation	0.04	0.04	118 ± 13
	0.11	0.13	
	0.13	0.18	
Trypsin digestion	0.65	0.37	51 ± 9
	0.22	0.08	
	0.85	0.51	

[a] For periodate oxidation, CSA (5 to 10 μg) was added to 50 mM sodium acetate–25 mM periodate (pH 4.5) and incubated at 4°C for 22 h in the dark. Untreated CSA was incubated with sodium acetate alone. Both received 250 mM ethylene glycol to stop oxidation. For trypsin digestion, CSA was dialyzed into 100 mM sodium bicarbonate (pH 7.5)–100 mM NaCl–1 mM $CaCl_2$ and adsorbed with Amberlite XAD resin. Treated CSA (10 μg) was incubated with 4 μg of trypsin for 1 h at 37°C. Results are shown for three different experiments.

[b] Mean of three experiments with different amounts of CSA. The A_{410} for ELISA conjugate alone was subtracted from other readings.

Proteolysis and periodate oxidation of MAb 1604 epitope. The carbohydrate moieties of complex molecules tend to be the preferred immunogens (59). Results of a phenol-sulfuric acid assay indicated that the CSA 1604 contained about 14% neutral carbohydrate (Table 1), which was probably LPS (Fig. 5). Nevertheless, MAb 1604 recognized a protein epitope on CSA 1604 (Table 2). In three experiments, trypsin digestion of different amounts of CSA 1604 decreased subsequent binding of MAb 1604 by an average of 49%. However, periodate oxidation of CSA monosaccharide rings containing 1,2-dihydroxy substituents (32) did not decrease MAb 1604 binding. Apparently, proteolysis of the CSA damaged the epitope, while periodate oxidation did not. The periodate-treated and untreated samples as a group showed lower A_{410} intensities than the trypsin-treated samples and controls. The addition of ethylene glycol to stop the periodate oxidation evidently decreased the MAb binding.

Isoelectric focusing of CSA 1604. In contrast to MAb 1640, which has an isoelectric point of 6.0, the affinity-purified CSA 1604 complex had a pI of 4.3 (Fig. 7). The absence of any bands in the CSA lane at pI 6.0 indicates that the CSA 1604 preparation was free from detectable MAb contamination. The pI of the CSA is probably affected somewhat by the presence of LPS, which has some negatively charged carboxyl groups (54). For example, 3-deoxy-D-manno-octulosonic acid has been found in *M. xanthus* LPS (44). LPS in its native form is anionic and thus has a low pI value. However, to produce the native form, LPS must be deionized by electrodialysis (47).

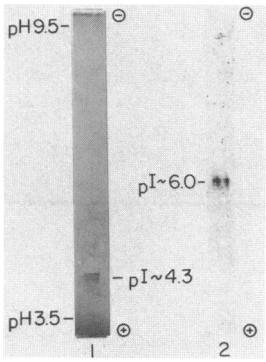

FIG. 7. Isoelectric focusing of 30 μg each of immunoaffinity-purified CSA 1604 (lane 1) and of protein A-purified MAb 1604 (lane 2). The ampholytes established a pH gradient from pH 3.5 to 9.5. The CSA 1604 complex migrated to net charge neutrality at approximately pI 4.3, while MAb 1604 migrated to pI 6.0. The proteins were visualized with Coomassie blue stain.

Electron micrograph of *M. xanthus* labeled with MAb 1604-gold. Figure 8 is an electron micrograph of an *M. xanthus* cell treated with MAb 1604 that was then labeled with second antibodies, goat anti-mouse immunoglobulin G (IgG) conjuated to 40-nm gold particles. Each electron-dense gold particle corresponds to one or more 1604 antigen sites on the cell surface. The antigen sites appeared to be distributed over the cell surface. Not evident in this micrograph is the observation that the gold particles seemed to be associated with a thin, amorphous layer over the cell surface. The precise nature of this layer is at present unknown.

Saturable binding of ^{125}I-MAbs to vegetative cells. Saturable-binding studies provide two types of information: the number of sites bound and the dissociation constant, which is a measure of binding affinity. The number of CSA 1604 sites per vegetative cell was calculated from the saturable binding data (Fig. 9). At saturation, 8.0×10^{-13} mol of ^{125}I-MAb 1604 bound to 2×10^8 cells. Assuming monovalent binding, as predicted by Roe et al. (49), there were 2,400 CSA 1604 sites per cell. A Scatchard transformation (51) of the binding data provided an estimate of the dissociation constant (K_d) for MAb 1604 binding to cells of 9.1×10^{-9} M. The K_d was calculated from the negative inverse of the slope of the Scatchard plot. The slope was determined by linear regression and had a correlation coefficient (r^2) of −0.98.

For comparison with MAb 1604, Fig. 9 also contains saturable binding data for MAb 1514. As shown in Fig. 5, the epitope for MAb 1514 is LPS (13, 15), a common cell surface molecule on gram-negative bacteria. The MAb 1514 epitope was more numerous (54,000 sites per cell) than that of MAb 1604 (2,400 sites per cell). The K_d (r^2, −0.99) for MAb 1514 was 6.4×10^{-8} M, an order of magnitude lower than that of MAb 1604.

Localization of CSA 1604 in vegetative cell fractions. From the results of the saturable-binding studies (Fig. 9), it is clear that MAb 1604 bound to the intact cell. However, CSA 1604 was not limited to the cell surface. The cells were fractionated into three parts: the cytosol, the total membrane fraction (inner and outer membrane), and the periplasm. Of the total CSA 1604, 50% was in the cytosol, 39% in the total membrane fraction, and 8% in the periplasmic space of vegetative cells (Table 3). The culture medium contained only 3% of the total amount of antigen when the cells were grown on a plastic surface. Nonspecific binding of the antibodies during ELISA was quantitated and subtracted from the other readings.

When the cells were grown in liquid suspension, less CSA was found in the membrane fraction and very little antigen

TABLE 3. Localization of CSA 1604 in cell fractions

Fraction[a]	Mean ELISA A_{410} with MAb 1604 ± SEM[b]	% of total
Cell envelope	0.15 ± 0	39
Periplasm	0.03 ± 0.01	8
Cytosol	0.19 ± 0.01	50
Filtered medium	0.01 ± 0.01	3

[a] 5×10^7 vegetative cells or cell equivalents. Vegetative cells were grown on a surface.

[b] Mean of three experiments, each in triplicate.

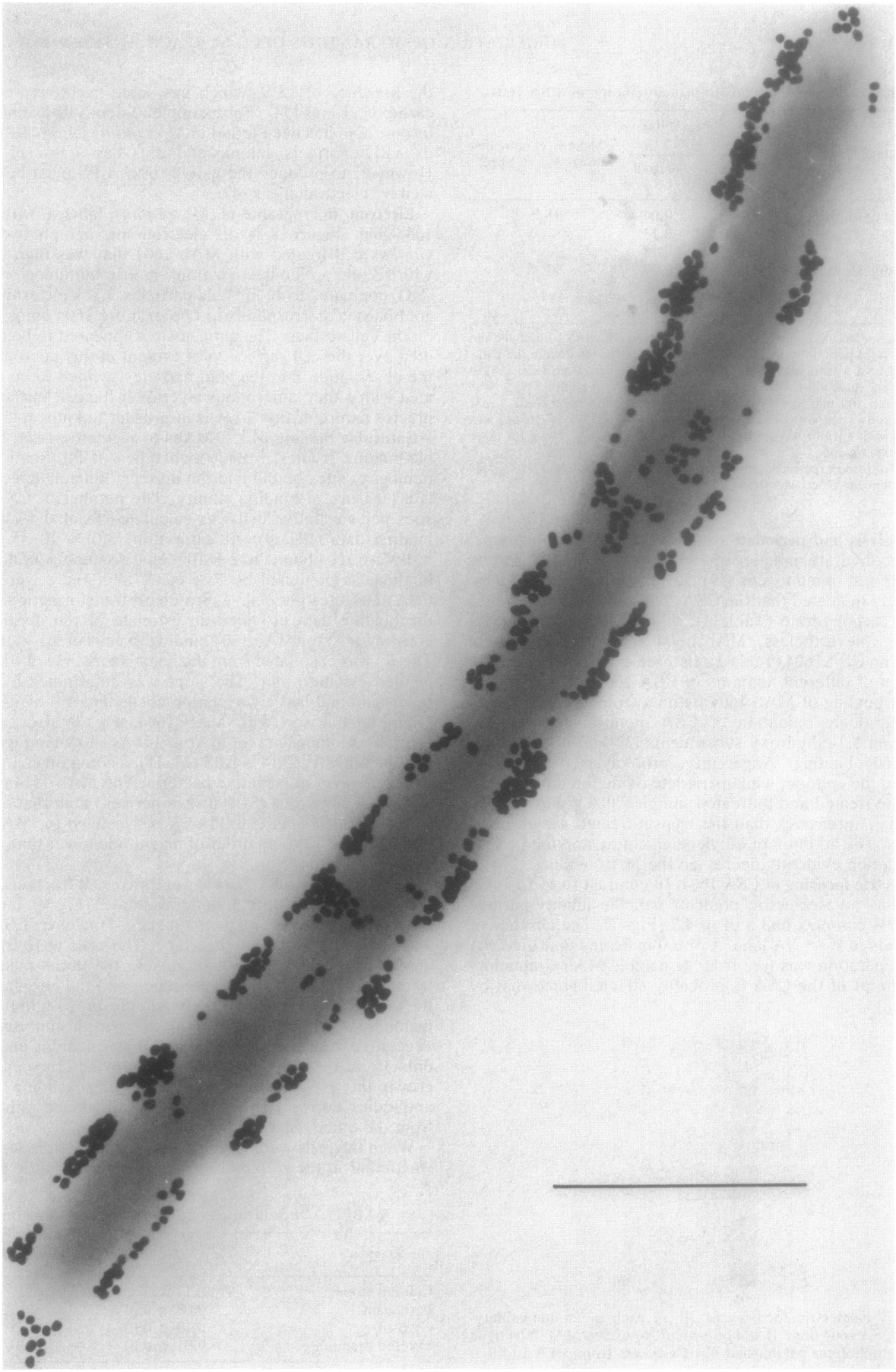

FIG. 8. Electron micrograph of an *M. xanthus* cell labeled with MAb 1604 and goat anti-mouse IgG conjugated to 40-nm gold particles. Bar, 1 μm.

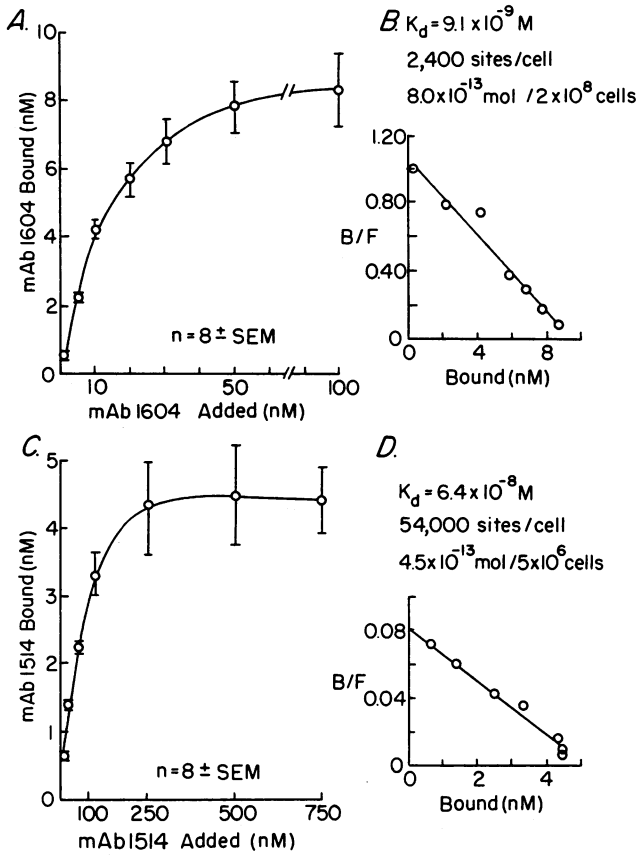

FIG. 9. Saturable binding of ^{125}I-MAbs to whole *M. xanthus* vegetative cells grown on a surface. (A and C) Binding curves for MAb 1604 and 1514, respectively. The amount of ^{125}I-MAb bound to the cells was plotted against the amount added. The data points are the mean of four experiments, each done in duplicate, ± SEM. (B and D) Scatchard plots of binding data for MAbs 1604 and 1514, respectively. The ratio of the amount of ^{125}I-MAb bound to MAb free (B/F) is plotted against the amount bound. The dissociation constants, K_d, and number of antigen sites per cell are listed. The K_d values were calculated from the negative inverse of the slope of the Scatchard plot. The slope was calculated by linear regression. The number of sites per cell was determined by dividing the number of antibody molecules bound at saturation by the number of cells present.

was found in the cytosol. Instead, one-half of the total antigen was found in the growth medium. Lower amounts of antigen on the surface and in the cytosol were found in cells from late log phase than in cells from the early log phase of growth (unpublished observations). These fluctuations prevented reproducible determinations of the antigen distribution from cells grown in liquid suspension. Because the cells in the wild normally grow on a surface, we decided that growth on a surface was the more realistic situation for the localization assays.

DISCUSSION

We have used a combination of immunoaffinity and gel filtration chromatography to purify the CSA 1604, a cell surface antigen complex we believe to be involved in contact-mediated intercellular interactions in *M. xanthus*. The purified antigen complex consisted of two proteins and 14% (by mass) neutral carbohydrate. LPS copurified with the CSA and probably accounted for the carbohydrate (Fig. 5). Using the ratio of neutral to amino sugars found for *M. xanthus* LPS (54), we estimate the amount of LPS in the antigen complex to be 19%. One of the proteins in the complex migrated in electrophoretic gels with a relative mass of 51 kDa and the other at 23 kDa. (The accuracy of 5 to 15% gradient SDS-PAGE between ca. 90 and 200 kDa is ±3 kDa and below ca. 90 kDa it is ±2 kDa.)

Mass determination by Western immunoblots. The 51-kDa protein contained the 1604 epitope, while the 23-kDa protein did not (Fig. 6). The difference in staining intensity of the two proteins suggested that more of the 23-kDa protein was present. Thus, a heat-labile, noncovalent association of two proteins of 23 kDa and one of 51 kDa would yield a total mass of 97 ± 6 kDa of protein (the ±2 kDa of uncertainty for each protein must also be summed). Bands of protein identified by MAb 1604 on the Western immunoblots of lysed cells in Fig. 1 appear to range from 116 to 200 kDa. The simplest explanation for these multiple bands is that the bands arise from various amounts of LPS associated with the 97-kDa protein heterotrimer.

There are three factors leading to the mutiple bands of CSA 1604: (i) heat lability of the association of CSA components, (ii) proteolysis of the protein components, and (iii) the presence of LPS associated with the CSA proteins. There is also a regularity to the bands between 116 and 200 kDa, respectively the bands of lowest and highest mass observed on Western immunoblots of lysed cells. In Fig. 6 (lanes 6 and 7), the Western immunoblots of affinity-purified CSA 1604 provide evidence that the 51-kDa band identified by MAb 1604 is the native mass of that polypeptide. All four bands of protein in lane 6 at 51, 33, 21, and 19 kDa are recognized by the MAb 1604. Only the protein band at 51 kDa appears in lane 7, containing CSA that eluted later in the same preparation that was loaded in lane 6. Thus, the 33-, 21-, and 19-kDa proteins have lost some mass, probably from partial proteolysis, but retained the 1604 epitope, as if they were part of a larger common progenitor. (Proteolysis after boiling in the presence of SDS has also been noted for *Vibrio* [7] and for *Erwinia* [56]). The 51-kDa protein is unlikely to be an aggregate of the 33- and 19-kDa proteins, because the data from gel filtration and refiltration of the 200-kDa material demonstrated that this higher-mass form of the CSA always produced more of the lower-mass forms and less of the 200-kDa form.

In Fig. 1 we noted that MAb 1604 recognized a double band at 200 kDa when the lysed cell suspension was not boiled but was reduced. However, when the lysed cells were boiled and reduced just prior to electrophoresis, MAb 1604 identified a band at 150 kDa. The loss of two heat-labile, noncovalently associated 23 ± 2-kDa proteins would explain this shift to 150 kDa. Long delays in loading boiled samples onto the gel resulted in more numerous bands lower than 200 kDa (Fig. 1, lane 4). The lowest band with the 1604 epitope observed in Fig. 1 with lysed cells is 116 ± 3 kDa (12). Some of the bands in Fig. 1 are reminiscent of the bands at 200, 170, and 140 kDa reported by Gill and Dworkin (15) under similar conditions. They suggested that the loss of 30-kDa subunits may explain the pattern of bands. In this case, boiling may have dislodged heat-labile 30-kDa subunits that did not have epitopes for MAb 1604. A similar result was reported previously by Louis et al. (33), who found that when an endoplasmic reticulum membrane protein was heated in SDS, three smaller subunits were produced. Subsequent freezing of the subunit mixture forced them to reassociate into the original multimer.

We propose that the 30-kDa subunit observed by Gill and Dworkin (15) was the 23-kDa protein associated with LPS that had an apparent mass of 7 kDa. This value for the apparent mass for an *M. xanthus* LPS molecule with one O antigen unit was suggested by Fink and Zissler (13), who reported that the LPS consisted of a 5-kDa lipid A core plus 1 to 30 units of O antigen. The 30 O antigen units spanned a 55-kDa range from 15 to 70 kDa. Thus, each O antigen unit differed from the adjacent unit by 1.8 kDa (1.8 = 55/30) apparent mass. For simplicity, the 1.8-kDa value is rounded to 2 kDa. We believe that bands from the CSA 1604 complex that differed by multiples of 7 ± 2 kDa on Western immunoblots in Fig. 1 and 6 and in the report of Fink et al. (12) are explained by sequential additions of LPS in 7-kDa units to the 97 ± 6-kDa protein trimer of the CSA 1604 complex. These bands from CSA 1604 that occur in 7-kDa multiples are arranged in a stepladder pattern characteristic of LPS.

Mass determinations by gel filtration. Two size classes of antigen molecules (200 and 100 kDa) were found after gel filtration of the immunoaffinity-purified CSA 1604 complex (Fig. 3). The fractions from the 200-kDa gel filtration peak contained one diffuse band at 200 kDa on a silver-stained SDS gel when the sample was reduced but not boiled before electrophoresis (Fig. 4). When the 200-kDa material from gel filtration was reduced and boiled, a diffuse band at 200 and a tight band at 116 kDa were found on SDS-PAGE (unpublished observation). This result suggested that the association of the antigen protein with carbohydrate was heat labile. When the material from the 100-kDa gel filtration peak was electrophoresed (Fig. 4, lane 6), there was one diffuse band at 200 kDa and bands at 51, 32, and 23 kDa. These same bands have been identified on Western immunoblots of the CSA 1604 complex (Fig. 6), as discussed above.

The paradoxical result that both gel filtration peaks contained a diffuse band at 200 kDa on SDS-PAGE can be explained as follows. As shown by the periodate acid-Schiff stain, the diffuse 200-kDa band contained carbohydrate (Fig. 4, lane 7). The relative amounts of LPS carbohydrate that copurified with the CSA during gel filtration (Fig. 3, 4, and 5) apparently affected the elution behavior of the CSA and thereby produced the two peaks. Upon SDS gel electrophoresis, the heat-labile, noncovalently associated proteins separated en masse from most of the LPS (resulting in the 116-kDa band) and sometimes also from each other (resulting in the 51-, 32-, and 23-kDa forms). Because both gel filtration peaks contained material that reacted with MAb 1604, the proteins found in both peaks were probably the same proteins in either the associated 116-kDa or dissociated 51-, 32-, and 23-kDa forms. To summarize, it seems most likely that the native, intact mass of the CSA 1604 protein-LPS complex is 200 kDa (ca. 97 kDa of protein and the remainder of the apparent mass from the associated LPS), as demonstrated by both electrophoresis and gel filtration.

Protease activity. The myxobacteria are known for their predatory life style, in which they surround and kill other bacteria by the secretion of lytic factors and proteases (50). These proteases complicate any attempt to purify cellular proteins from *M. xanthus*. EDTA alone among a group of five protease inhibitors (EDTA, PMSF, leupeptin, pepstatin, and diisopropyl fluorophosphate) was found to inhibit proteolysis in an assay with BSA as a substrate (unpublished observation). Apparently, these proteases were metalloproteases, requiring divalent cations (4). Nevertheless, in addition to EDTA, 1 mM PMSF and 0.1% leupeptin were also included with the lysed cells during CSA purification.

Copurifying LPS. The LPS that copurified with the CSA 1604 remained tightly associated with the antigen during boiling in the presence of a reductant in preparation for electrophoresis (Fig. 5). This is not unusual. Others have been unable to separate LPS and outer membrane proteins from *Escherichia coli* and *Pseudomonas aeruginosa* after the boiling and reduction that accompany electrophoresis (46, 48). Nonetheless, Rietschel (47) claimed that such data do not prove that LPS is covalently bound to the proteins.

There is a close association between LPS and surface proteins in gram-negative bacteria. It is thought that proteins are translocated along with LPS to the outer membrane. Some porin proteins do not function unless LPS is present (47). LPS can also block binding to molecules on the cell surface. With a panel of 21 MAbs produced against *E. coli* porins, Bentley and Klebba (2) have found that intact O antigen completely blocked MAb binding to porin surface epitopes. When mutants lacking various amounts of LPS were tested, the number of MAbs able to recognize each mutant increased with the number of O antigen units lost. It has also been demonstrated that a mutation in *Salmonella enteritidis* (Mu resistant) that removed O antigen saccharides from LPS exposed the cells to bacteriophage Mu and rendered them Mu sensitive (37). Clearly then, the bacterial surface proteins and surface interactions depend upon LPS for some structural and functional properties. How CSA 1604 from *M. xanthus* depends upon LPS is at present unknown, although it is likely that the LPS somehow attaches the CSA complex to the cell surface.

MAb 1604 epitope. MAb 1604 recognized a peptide epitope on CSA 1604 (Table 2). Proteolysis with trypsin decreased the MAb binding to the CSA. Incubation for longer than 1 h would probably have reduced MAb binding even more. Periodate oxidation for 24 h did not decrease MAb binding.

The decrease in MAb 1604 binding after proteolysis is attributed to cleavage and disruption of the structure of a peptide epitope. An alterative interpretation is that the structure of a carbohydrate epitope attached to a peptide was cleaved. This is unlikely, because polysaccharides are linked to proteins by one residue in a chain of sugars. That one residue is covalently bound to an amino acid (Ser, Thr, or Asn) in the polypeptide (32). Cleavage of the polypeptide on either side of the amino acid bound to the carbohydrate would not disrupt the structure of the carbohydrate per se. However, if the epitope consisted of both peptide and carbohydrate, then proteolysis would disrupt MAb binding, but so would periodate oxidation. There was no evidence for this. The most reasonable interpretation is that the eitope is a peptide.

Isoelectric focusing. The isoelectric point for CSA 1604 was 4.3 (Fig. 7). Acidic pI values have been found for other complex surface molecules from bacteria. For example, Maeba (34) described a putative glycoprotein with a pI of 3.2 from vegetative *M. xanthus* cells. In addition, a surface glycoprotein of 200 kDa and 12% carbohydrate was reported from *Halobium salinarium*. Although no pI value was given, the amino acid profile predicted a low pI (35). The negatively charged LPS (54) associated with the CSA 1604 complex probably affects the pI, but because the amino acid composition of CSA 1604 proteins is not yet known, the relative contribution of amino acids and of LPS to the pI is also unknown.

Electron micrograph. The electron micrograph in Fig. 8 shows about 300 to 400 40-nm gold particles per cell. However, one cannot see every particle in each cluster, and some clusters are probably hidden on the far side of the cell. The gold particles localized the CSA 1604 on the cell surface.

Each gold particle is attached to several goat anti-mouse IgG antibodies that were bound to the MAb 1604 that had been bound to the CSA on the cell. Therefore, each gold particle identifies one or more antigen sites. Unlike the myxobacterial hemagglutinin (MBHA), which is one of the two best-characterized cell surface molecules of *M. xanthus* and is polar in location (39), the CSA 1604 seems to be distributed in about 30 clusters along an amorphous layer around the cell periphery. Before labeling with immunogold, the cells were grown on an agar surface. Gliding of the cells on agar seemed to cause the immunogold to redistribute to the cell periphery, in contrast to cells grown in liquid suspension.

Binding studies with MAbs. Saturable-binding studies with the radioiodinated MAbs indicated that the K_d values for the two MAbs were 6×10^{-8} M for 1514 and 9×10^{-9} M for 1604 (Fig. 9). A nanomolar dissociation constant is very strong binding affinity for an antibody (18). MAb 1604 bound more tightly to the cell than did 1514, but MAb 1604 bound to relatively few protein epitope sites (2,400) in contrast to 1514 binding to LPS (54,000 sites per cell). For comparison with these two molecules, an outer membrane protein, OmpA, from *E. coli* has 10^5 sites per cell (41). Another comparison is the MBHA, which has 21,000 sites per cell (6), 10 times more numerous than the CSA 1604 sites.

CSA 1604 localization. A large proportion of the total CSA 1604 was found in the cytosol of the vegetative cells when they were grown on a solid surface (Table 3). It is not known how much CSA was on the cell surface of starved cells prior to development. Attempts to use cells grown in liquid suspension resulted in very little CSA in either the membrane fraction or the cytosol. Most of the CSA had been released into the medium. When cells were grown on a plastic surface, the CSA 1604 found in the cytosol was probably nascent molecules not yet exported to the cell surface. A similar finding has been described for the MBHA, another cell surface molecule from *M. xanthus*. Cumsky and Zusman (5) reported that no hemagglutination activity could be found in extracts from vegetative cells, cells starved in liquid, or glycerol-treated cells. Only when the cells were starved on the solid medium on which aggregation occurred did they find hemagglutination activity. Clearly then, some cell surface molecules require growth on a surface for maximal presence of the molecule on the surface.

The distribution of other surface molecules into both membrane and cytosol fractions has been reported. For the outer membrane protein LamB fused to the LacZ protein of *E. coli*, about 40% of the total fusion protein (although not a naturally occurring protein) LamB-LacZ was found in the cytosol and 60% in the membrane fraction (1). Moreover, a temporal distribution has been found in *M. xanthus* for protein S, which has been found to accumulate in the cytosol between 3 and 15 h of development. After 15 h, protein S is translocated through the cytoplasmic membrane and appears on the cell surface (25, 40).

Previous data have indicated that MAb 1604 directed against CSA 1604 interfered with normal development (16). We believe, therefore, that the CSA 1604 protein-LPS complex may play a role in mediating a cell-cell interaction involved in *M. xanthus* development. An accompanying paper (27) describes some of the biological properties of this complex.

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