INFECTION AND IMMUNITY, Sept. 1996, p. 3659–3665 Vol. 64, No. 9
0019-9567/96/$04.00+0

The Galactosyl-(α1-4)-Galactose-Binding Adhesin of *Streptococcus suis*: Occurrence in Strains of Different Hemagglutination Activities and Induction of Opsonic Antibodies

KAARINA TIKKANEN,[1]* SAULI HAATAJA,[2] AND JUKKA FINNE[1,2]

Department of Biochemistry and Biotechnology, A. I. Virtanen Institute, and Veterinarian Research Station, University of Kuopio, FIN-70211 Kuopio,[1] and Department of Medical Biochemistry, University of Turku, FIN-20520 Turku,[2] Finland

Received 22 January 1996/Returned for modification 18 March 1996/Accepted 10 June 1996

The occurrence of the galactose-(α1-4)-galactose-specific adhesin in *Streptococcus suis*, a pig and human pathogen causing sepsis, meningitis, and other serious infections, was studied. Poly- and monoclonal antibodies to the purified adhesin, as well as pigeon ovomucoid, a specific probe for the adhesin activity, detected one single protein band in extracts of *S. suis*. The adhesin was detected in all 23 strains studied, representing pathogenic serotypes (1, 2, 4, 5, 7, 8, and nontypeable) and including several weakly hemagglutinating or nonhemagglutinating strains and phase variants. The amount of adhesin detected was not correlated with the hemagglutination activity of the intact bacteria. Extraction of cells showing no binding of pigeon ovomucoid by ultrasonic treatment resulted in extracts with pigeon ovomucoid binding activity, suggesting that the adhesin was not accessible to the probe on the intact cells. Analysis of the amount of capsular polysaccharide revealed an inverse relationship between the hemagglutination activity and expression of capsular polysaccharide, thus suggesting a factor influencing adhesin accessibility. The purified adhesin was highly immunogenic and induced in preliminary experiments bactericidal activity in mice. Thus, the adhesin, with its specific binding mechanism to host cells and a proposed pathogenic role, is widely expressed among strains of different serotypes and therefore appears to represent a novel promising candidate for the development of a vaccine against *S. suis*.

Streptococcus suis is an important worldwide gram-positive pathogen which causes meningitis, sepsis, and other serious infections in pigs (2, 7, 46, 51) and also in humans who have been in contact with pigs (3, 50). To date 34 different serotypes have been reported (17, 18, 22, 36), and no effective vaccine against *S. suis* infections is available (44). Several candidate vaccines have been proposed, but most of them are restricted to serotype 2 (11, 24, 37, 41). Mouse models are important tools in studying *S. suis* capsular type 2 infections (4, 37–39, 54), and the immunoglobulin G (IgG) responses of mice and pigs to *S. suis* capsular type 2 cellular proteins have been reported to be similar (38).

Adhesins are important virulence factors which mediate the attachment of pathogenic bacteria to host cells and thus represent potential vaccine candidates (10, 34). The binding of *S. suis* to host cells appears to be mediated by an adhesin which recognizes the disaccharide galactosyl-α1-4-galactose (Galα1-4Gal) in cell surface glycolipids (19, 20). Two strains recognizing sialic acid have also been reported (32). Although several strains were found to express Galα1-4Gal-binding activity, many others appeared to lack it, as assayed by the slide hemagglutination method (29). The Galα1-4Gal-specific adhesin was recently purified and was found to correspond to a single 18-kDa protein band with the same hemagglutination specificity as the intact bacteria (48).

In this study, the occurrence of the Galα1-4Gal-binding adhesin of *S. suis* was studied by using immunoblot analysis with antibodies raised to the purified adhesin. The adhesin was detected in all 23 *S. suis* strains of different serotypes studied, including weakly hemagglutinating and nonhemagglutinating strains. Because the adhesin also induced bactericidal activity in the mouse, the adhesin appears to represent a promising novel candidate vaccine against *S. suis* infections.

MATERIALS AND METHODS

Bacterial strains. *S. suis* 628 was obtained from H. C. Zanen, Academic Medical Centre, Amsterdam, The Netherlands; strains TEW/2 and R75/L1 were obtained from F. Clifton-Hadley, Clinical Veterinary School, University of Cambridge, Cambridge, England; strains 598, 652, 663, 752, 825, 836, BA 33/8 (1045), BA 39/3 (1042), BA 41/8 (1033), BA 71/4 (1044), 849 (3031), 851 (3026), 875 (3027), 877 (3028), and 878 (3029) were obtained from J. Hommez, Regional Veterinary Investigation Laboratory, Torhout, Belgium; strains B121, B154, B463, and B565 were obtained from L. Sihvonen, National Veterinary and Food Research Institute, Kuopio Regional Laboratory, Kuopio, Finland; and strain B295 was obtained from S. Pelkonen, National Veterinary and Food Research Institute, Kuopio Regional Laboratory, Kuopio, Finland. The agglutination properties of these strains have been described before (29). The bacteria were maintained in Todd-Hewitt broth at −20°C and grown on sheep blood agar plates overnight at 37°C under anaerobic conditions (Gas-Pak system). The bacteria were harvested from the plates, washed twice, suspended in buffer A (10 mM sodium phosphate, 0.15 M NaCl [pH 7.4]), and adjusted to a concentration that gave an A_{600} of 0.5 at 1:100 dilution.

Materials. Pigeon ovomucoid was obtained from C. François-Gerard, Service d'Immuno-Hematologie, Centre Hospitalier Universitaire de Liège, Liège, Belgium. Sialidase from *Vibrio cholerae* was obtained from Behringwerke AG, Marburg, Germany; phenylmethylsulfonyl fluoride was obtained from Boehringer, Mannheim, Germany. Bovine serum albumin, Freund's complete and incomplete adjuvants, bromochloroindolyl phosphate, nitroblue tetrazolium, and carbonic anhydrase were from Sigma, St. Louis, Mo. Ammonium sulfate was purchased from Riedel-de Haën, Seelze, Germany; $Na^{125}I$ was obtained from Amersham, International, Amersham, England; acrylamide, bisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine, and Bio-Gel P-6DG desalting gel were obtained from Bio-Rad, Richmond, Calif. Kodak X-Omat AR film was from Eastman Kodak, Rochester, N.Y. Todd-Hewitt broth and a Gas-Pak anaerobic system were obtained from Becton Dickinson and Co., Cockeysville, Md.; Mi-

* Corresponding author. Mailing address: Department of Biochemistry and Biotechnology, A. I. Virtanen Institute, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland. Phone: 358-71-163 640. Fax: 358-71-281 1510.

crowell microtiter plates were obtained from Dako, Roskilde, Denmark; polystyrene flat-bottom Microstrips were obtained from Labsystems, Helsinki, Finland. IODOBEADS were purchased from Pierce, Rockford, Ill.; nitrocellulose sheets were purchased from Schleicher & Schuell, Dassel, Germany; PVDF-P and PVDF-N membranes were purchased from Millipore, Bedford, Mass. Rabbit antisera against *S. suis* types 1, 2, 4, and 7 were obtained from Jørgen Henrichsen, WHO Collaborating Centre for Reference & Research on Pneumococci, Statens Seruminstitut, Copenhagen, Denmark. Nonimmune rabbit serum was collected from an adult New Zealand White rabbit. Alkaline phosphatase-conjugated goat anti-rabbit IgG (heavy and light chains) and alkaline phosphatase-conjugated rabbit anti-mouse IgG, IgA, and IgM (heavy and light chains) were from Zymed Laboratories, Inc., San Francisco, Calif.

Preparation of adhesin extracts from *S. suis*. The bacterial suspensions were sonicated with an ultrasonic probe in buffer A five times for 15 s each on ice with a chilling interval of 1 to 2 min between the sonications. After sonication, phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM, and insoluble material was removed by centrifugation at 15,800 × *g* for 20 min at 8°C.

Protein determination. Protein determination was carried out by an adaptation of the method described by Bradford (5) on polystyrene Microstrips, using Bio-Rad protein assay dye reagent concentrate. The A_{595} was measured by an SLT Labinstruments Easy Reader SF Plus enzyme-linked immunosorbent assay reader.

Gel electrophoresis. Proteins from the bacterial sonicates were separated in a 6% polyacrylamide gel electrophoresis (30) without sodium dodecyl sulfate (SDS) addition in a Bio-Rad Mini-Protean II device. The bacterial extracts prepared by sonication in a volume of 75 μl were mixed with 23 μl of sample solution containing 0.65 M sucrose and 4 mM EDTA in 87 mM Tris-HCl (pH 8.0), and 2 μl of 0.1% bromphenol blue and 20 μl of the mixtures were loaded into the sample wells. After electrophoresis, the proteins were transferred electrophoretically to a PVDF-P membrane (60 mA, 30 min) (6) by using 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid buffer (pH 11)–methanol (9:1, vol/vol).

Radiolabeling of pigeon ovomucoid. Pigeon ovomucoid was labeled with ^{125}I by the IODOBEAD method according to the instructions of the manufacturer as described before (48).

Detection of adhesin by pigeon ovomucoid binding. The *S. suis* Galα1-4Gal-binding adhesin activity was identified from whole bacterial cells and electrophoretically separated proteins by dot and blot binding, respectively, using radiolabeled pigeon ovomucoid containing Galα1-4Gal (14). For dot assays, 1-μl dots of bacterial suspensions, extract supernatants, or pellets suspended in the original volume of buffer A were pipetted onto a gridded nitrocellulose sheet. In blot binding analyses, polyacrylamide gel electrophoresis was performed without SDS in order to retain the pigeon ovomucoid-binding activity of the adhesin. Nonspecific binding sites were saturated by incubation of the membranes for 1.5 h in buffer B (0.5% Tween 20 in 0.1 M sodium phosphate buffer–150 mM NaCl [pH 5.3]). The membranes were incubated with ^{125}I-pigeon ovomucoid (6×10^5 cpm/15 cm^2; specific activity, 2.5×10^5 cpm/μg) in buffer B for 1 h at 8°C, washed three times for 10 min in buffer B, dried between filter papers, and exposed to an X-ray film with an intensifying screen at −80°C for 1 to 6 days.

Polyclonal and monoclonal antibodies. The adhesin was purified as described before (48). BALB/c mice were immunized subcutaneously with pure adhesin (6 to 10 μg) twice with complete and once with incomplete Freund's adjuvant at 3-week intervals and then given booster injections monthly. Peripheral blood samples were collected for polyclonal antisera. Monoclonal antibody was produced as described by Köhler and Milstein (27) and Gefter et al. (15). Three days after the first booster injection, spleen cells were removed and fused with myeloma line Sp2/0 in polyethylene glycol 1500. The resulting hybridomas were screened by Western blot (immunoblot) analysis against a sonication extract of *S. suis* 628. The cells were cloned by limiting dilution, grown with hybridoma growth factor supplement, and retested for antibody activity. Culture supernatants from the clones which continued to show a positive reaction were tested in Western blot and hemagglutination inhibition assays.

Antibodies to synthetic peptide. A peptide was constructed according to the N-terminal amino acid sequence of the adhesin (48), with an additional cysteine residue for coupling (Ala-Ser-Pro-Ala-Glu-Ile-Ala-Ser-Phe-Ser-Pro-Ala-Pro-Cys). The peptide was coupled to keyhole limpet hemocyanin by the maleimidobenzoyl-*N*-hydroxysuccinimide method, using 150 μg of peptide and 2.8 mg of hemocyanin (26, 31). Polyclonal antibodies against the synthetic peptide antigen were produced by immunizing a New Zealand White rabbit seven times with 625 μg of the conjugate in Freund's incomplete adjuvant at 3- to 6-week intervals.

Western blot analysis. Antibody formation was studied on a PVDF-P membrane, with proteins transferred from gels as described above. The membranes were saturated by incubation in 1.5% milk powder and 0.5% Tween 20 in buffer C (25 mM Tris-HCl, 150 mM NaCl [pH 7.8]) for 1.5 h. The membrane was washed three times for 10 min each with washing buffer (0.05% Tween 20 in buffer C) and incubated with antiserum diluted in the washing buffer for 1 h. The membrane was washed five times for 5 min each and incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins diluted 1:1,000 in the washing buffer. The color was developed with nitroblue tetrazolium and bromochloroindolyl phosphate. Stock solutions (5%) of these compounds were prepared in 70 and 100% dimethylformamide, respectively, and 66 and 33 μl of the stock solutions were added to 10 ml of 100 mM Tris-HCl buffer (pH 9.5) containing 5 mM $MgCl_2$ and 100 mM NaCl.

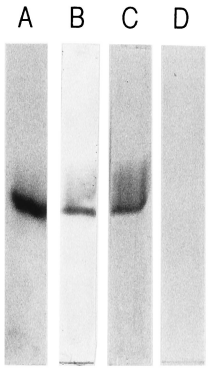

FIG. 1. Detection of *S. suis* adhesin by immunoblot analysis. The soluble extract of *S. suis* 628 was subjected to electrophoresis in a 6% polyacrylamide gel under nondenaturing conditions and transferred to PVDF-P membranes, which were probed with radiolabeled pigeon ovomucoid and subjected to autoradiography (lane A) or immunoblotted with monoclonal antibody E10G3 (1:10) (lane B), polyclonal antibodies to the purified adhesin (1:10,000) (lane C), and no primary antibodies (lane D).

Hemagglutination and hemagglutination inhibition assays. Hemagglutination and hemagglutination inhibition assays were done as described by Korhonen and Finne (28). Erythrocytes from healthy adults were treated with sialidase and used at a 2 or 4% concentration. Hemagglutination inhibition by the immune serum was performed on a microtiter plate. Mouse immune and control nonimmune sera were serially diluted in 25 μl of buffer A, and an equal volume of bacterial suspension at a concentration corresponding to four times the hemagglutinating titer was added to each well and to wells containing buffer A only (positive control). The plate was incubated overnight at 8°C, after which 50 μl of a 4% erythrocyte suspension was added to each well and to wells containing buffer A only (negative control).

Extraction and immunodetection of capsular polysaccharides. Capsular polysaccharides were extracted by sonication as described by Tikkanen et al. (49). Immunoblot analysis after polyacrylamide gel electrophoresis in the absence of SDS was done as described before (49).

Bactericidal assay. Bactericidal assays were done by using an adaptation of the method employed by Agarwal et al. (1) and Elliott et al. (11). BALB/c mice were immunized subcutaneously with 6 μg of adhesin without adjuvant during first injections; Freund's incomplete adjuvant was added to booster injections. Ten days after the last booster, peripheral blood was collected in heparin tubes (15 U of lithium heparin per 0.01 ml of buffer A). Fresh heparin blood (300 μl) was delivered in 1.5-ml screw-cap tubes and inoculated (1:10) with fresh bacterial cultures containing 300 or 3,000 cells of strain 628 in logarithmic growth phase; samples of 80 μl were removed immediately for counting of the initial number of CFU by culture on sheep agar plates overnight at 37°C under anaerobic conditions. In some experiments, the control and immunized blood samples were diluted (1:100) in order to abolish the bacteriostatic action of control blood (8). The vials were rotated at 37°C in an end-over-end shaker for 3.5 h, and samples of 80 μl were taken for counting the number of remaining CFU. Control hyperimmune serum was prepared by immunizing BALB/c mice subcutaneously with 10 μg of carbonic anhydrase once in Freund's complete adjuvant and four times in Freund's incomplete adjuvant at 3- to 7-day intervals.

RESULTS

Characterization of poly- and monoclonal antibodies specific for *S. suis* adhesin. Polyclonal adhesin antibodies induced by immunization with the pure adhesin specifically recognized one single band in extracts of *S. suis* in Western blot analysis (Fig. 1). The band had the same mobility as the band which binds radiolabeled pigeon ovomucoid, a glycoprotein with blood group P_1 (Galα1-4Galβ1-4GlcNAcβ1-) terminals, a specific probe for the Galα1-4Gal-binding adhesin of *S. suis* (13, 48). A monoclonal antibody (E10G3) to the adhesin also recognized a band with the same mobility.

The polyclonal antiserum specifically inhibited the hemagglutination induced by *S. suis* down to a dilution of 1:3,200 (Fig. 2). The E10G3 monoclonal hybridoma culture medium

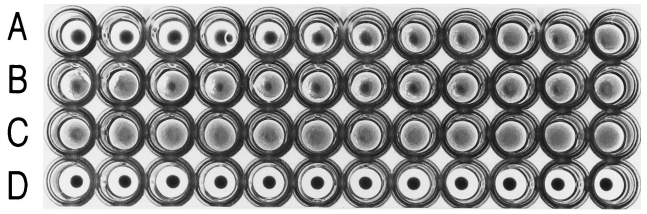

FIG. 2. Inhibition of *S. suis*-induced hemagglutination by polyclonal adhesin antibodies. The microtiter plate wells contained serial dilutions of polyclonal adhesin antibodies (1:100) (A) and serial dilutions of nonimmune serum (1:100) (B). (C) Positive control wells; (D) negative control wells.

exhibited low inhibitory activity with a titer of 1:20 (results not shown).

Probing for the presence of adhesin in *S. suis* strains by using pigeon ovomucoid and adhesin antibodies. To probe for the presence of the adhesin in different *S. suis* strains, both pigeon ovomucoid and polyclonal adhesin antibodies were used. A selection of hemagglutination-negative strains or strains with low hemagglutination activity was chosen for analysis to investigate whether the adhesin would also be present in strains other than previously analyzed hemagglutinating strains (48). Western blot analysis with the adhesin antibodies of the extracts of all *S. suis* strains investigated revealed a single band with the mobility of the adhesin (Fig. 3A). Probing of the membranes with radiolabeled pigeon ovomucoid indicated that the same band also exhibited Galα1-4Gal-binding activity (Fig. 3B) and thus confirmed its identity as the adhesin. Interestingly, slight differences in the mobilities of the adhesin band in different strains were observed, which suggests possible differences in the primary structure or posttranslational modification of the adhesin.

Comparison of hemagglutination activity and the amount of adhesin. To evaluate whether the differences in the agglutination activities of different *S. suis* strains could be due to differences in the expression of adhesin, the amounts of adhesin detected with polyclonal antibodies in the Western blots were compared with the hemagglutination activities (Table 1). In three of the four strains analyzed during a strong and a weak hemagglutination phase, the amount of adhesin appeared to correlate with the hemagglutination activity. However, in one case (strain TEW/2), the extracts exhibited a high amount of adhesin even under a nonhemagglutinating phase. Furthermore, analysis of an additional 19 weakly hemagglutinating or nonhemagglutinating strains revealed several strains with a high amount of adhesin as detected with the polyclonal adhesin antibodies in Western blots. When polyclonal antibodies to the synthetic N-terminal peptide were used, the amounts correlated with those determined with the polyclonal adhesin antibodies, which suggested that the N termini did not antigenically differ significantly in the different strains (results not shown).

TABLE 1. Hemagglutination activities and adhesin expression in *S. suis* strains of different serotypes and under different stages of phase variation

Strain[a]	Serotype	Hemagglutination[b]	Adhesin[c]
836	1	256	++++
		32	+++
628	2	256	++++
		32	++
877	2	8	++++
		2	++
TEW/2	2	64	++++
		0	++++
652	1	1	+++
B565	1	2	++++
663	2	2	++++
BA 41/8	2	4	+
R75/L1	2	1	+++
825	4	1	+
BA 71/4	4	4	+
849	4	2	+
598	5	0	++
B121	7	0	++++
B463	7	0	+++
752	7	1	+++
BA 33/8	8	0	+
BA 39/3	8	4	++
B154	NT[d]	1	+
B295	NT	0	+
851	NT	0	+++
875	NT	0	+
878	NT	8	+

[a] The first four strains were analyzed under a high- and a low-hemagglutinating phase.
[b] Reciprocal of the hemagglutination titer.
[c] The amount of adhesin was estimated from duplicate Western blots, using polyclonal adhesin antibodies; synthetic peptide antibodies against the N terminus of the adhesin gave similar results.
[d] NT, nontypeable.

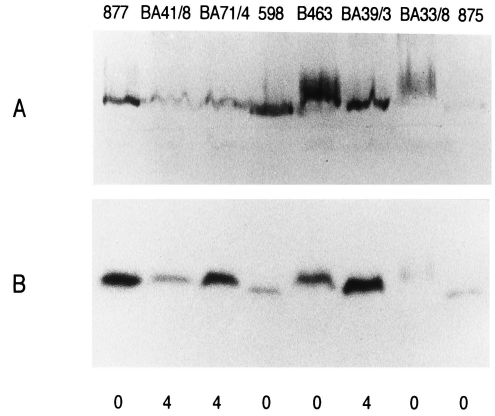

FIG. 3. Detection of adhesin in weakly hemagglutinating and nonhemagglutinating strains of *S. suis* by immunoblot analysis. Soluble extracts of the *S. suis* strains indicated at the top were subjected to electrophoresis in 6% polyacrylamide gels under nondenaturing conditions and transferred to PVDF-P membranes, which were analyzed by immunoblotting with polyclonal antibodies to the purified adhesin (1:10,000) (A) or probed with radiolabeled pigeon ovomucoid and subjected to autoradiography (B). The hemagglutination activities of the strains are given as the reciprocals of the titers (bottom).

The adhesin was detected by Western blotting in all 23 strains examined, including several nonhemagglutinating strains and strains examined under a weak hemagglutination or a nonhemagglutination phase. The adhesin was also present independent of the serotypes, which included the main pathogenic types.

Analysis of the presence of capsular polysaccharides. To investigate whether the variation in adhesin expression could be related to differences in the amount of capsular polysaccharide, bacterial cells were extracted by sonication under conditions leading to the solubilization of the polysaccharide and the adhesin (49), and the remaining cellular pellets and supernatants were analyzed by dot binding assay. Two strains with hemagglutination activity (628 and 836) and two with no or weak activity but significant amounts of adhesin in Western

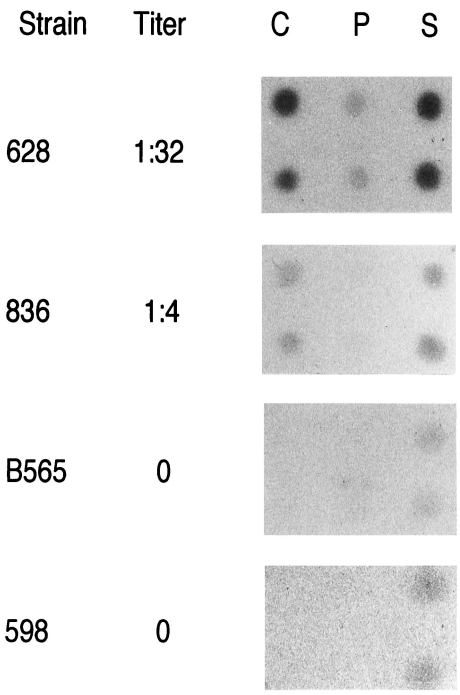

FIG. 4. Binding of pigeon ovomucoid to *S. suis* cells and ultrasonic extracts in a dot assay. Samples of intact bacterial cells (lane C), bacterial pellets after ultrasonic treatment (lane P), and supernatant extracts (S) of the strains with the hemagglutinating titers indicated were pipetted onto a nitrocellulose membrane and probed with radiolabeled pigeon ovomucoid for adhesin activity.

blot analysis (B565 and 598) (Table 1) were chosen for analysis. Radiolabeled pigeon ovomucoid bound efficiently to the intact cells of the two hemagglutinating strains but not to the two weakly hemagglutinating or nonhemagglutinating cells (Fig. 4, lane C). In contrast, pigeon ovomucoid binding to the soluble extracts of all four strains was observed (Fig. 4, lane S). This result suggested that the adhesin was present but not accessible to pigeon ovomucoid on the intact bacterial cells of low hemagglutination activity.

The presence of capsular polysaccharide in the differentially hemagglutinating strains was examined by immunoblotting with the capsular serotype-specific antisera after polyacrylamide gel electrophoresis, which displays the capsular polysaccharides in typical ladder-like patterns (35, 49). Each strain studied was also immunoblotted with nonimmune control serum. Strains with low hemagglutination activity revealed the presence of capsular polysaccharide (Fig. 5A), whereas strains with high hemagglutination activity contained essentially no polysaccharide (Fig. 5B). A reciprocal relationship between the hemagglutination activity and amount of capsule was also observed in samples of the same strain analyzed under a high and a low hemagglutination phase (Fig. 5C).

Induction of bactericidal activity by the adhesin. The adhesin was immunogenic in mice and induced antisera with titers of the order of 1:10,000 in Western blot analysis (Fig. 1), and the antibodies also inhibited the hemagglutination induced by the bacteria (1:3,200) (Fig. 2). To evaluate the induction of bactericidal activity by the adhesin, samples of blood from mice immunized with the purified adhesin were inoculated with different amounts of *S. suis* 628 cells and analyzed for bacterial growth after incubation at 37°C. Blood from mice vaccinated with 6 μg of pure adhesin and given one booster injection with Freund's incomplete adjuvant exhibited opsonizing activity against the bacteria, whereas blood from control nonimmunized mice did not exhibit such activity (Table 2). A control hyperimmune serum against carbonic anhydrase (antibody titer, 1:10,000) did not develop opsonic action. Opsonic activity was also induced against other strains of *S. suis* (836 and BA 41/8). A 10-fold opsonic response was observed compared with those in control hyperimmune and nonimmunized mice (dilution, 1:100).

DISCUSSION

To probe for the presence of the Galα1-4Gal-binding adhesin in different strains of *S. suis*, polyclonal antibodies to the

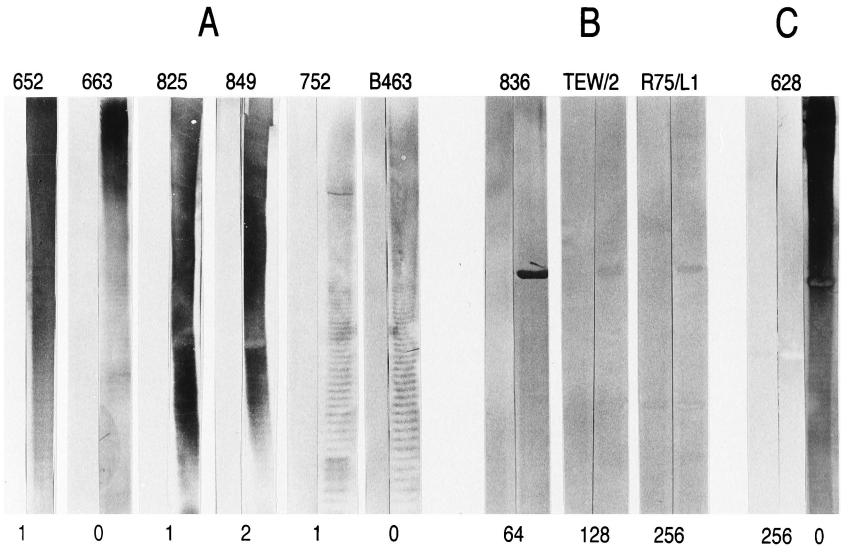

FIG. 5. Immunoblot analysis of capsular polysaccharides. The extracts of weakly hemagglutinating or nonhemagglutinating strains (A), strongly hemagglutinating strains (B), or a strain in a high and a low hemagglutinating phase (C) were subjected to nondenaturing polyacrylamide gel electrophoresis, transferred to a PVDF-N membrane, and probed with serotype-specific antibodies (1:15,000). The hemagglutination activities of the strains (top) are given as the reciprocals of the titers (bottom). For each strain, the left lanes are control blots with nonimmune serum (1:15,000).

TABLE 2. Bactericidal activity of blood of mice immunized with *S. suis* adhesin[a]

Mouse	CFU/100 μl		
	Inoculated	At 0 h[b]	At 3.5 h[b]
Nonimmunized			
1	10^3	1,295	>2,000
2	10^3	1,290	>2,000
3	10^2	231	>2,000
4	10^2	238	>2,000
Immunized			
1	10^3	748	105
2	10^3	898	56
3	10^2	86	16
4	10^2	185	80

[a] Blood from nonimmunized mice or mice immunized with the purified *S. suis* adhesin (with a serum antibody titer of 1:1,000) was collected and mixed with living *S. suis* 628 cells. Samples were taken immediately after inoculation and after incubation at 37°C for 3.5 h.

[b] Means from two plates.

adhesin were used. In contrast to monoclonal antibodies, polyclonal antibodies were expected to give a more reliable indication of the presence of the adhesin among strains in which the adhesin exhibits slight differences in electrophoretic mobility, indicating structural differences. In spite of the mobility differences, immunoblot analysis with the polyclonal antibodies, monoclonal antibody, and antibodies to the synthetic N-terminal peptide, as well as probing with radiolabeled pigeon ovomucoid, all gave comparable estimates on the amount of the adhesin in different strains, which indicated that the Galα1-4Gal-binding adhesins are closely related in structure and antigenicity.

A striking finding was that the Galα1-4Gal-binding adhesin was found in immunoblot analysis to be present in all *S. suis* strains examined, even in strains with no hemagglutinating activity. Furthermore, the adhesin was present in all serotypes studied representing major pathogenic types. The number of *S. suis* capsular types defined to date is 34 and could be even higher (17, 18, 22, 36). However, most *S. suis* isolates belong to the capsular types 1 to 9 (21). In many countries, capsular type 2 has been isolated from diseased pigs (7), but in Denmark and Finland, capsular type 7 is most prevalent (36, 46). In the United States, types 1/2, 1, and 2 predominate in young pigs showing clinical signs of *S. suis* infection (12). In addition to being important porcine pathogens *S. suis* serotype 2 as well as serotype 4 and untypeable strains also cause infections, mainly meningitis, in humans (3, 50).

Although the hemagglutinating activity of the phase variants of some of the strains seemed to correlate inversely with the amount of adhesin detected by immunoblotting, examination of all the strains studied clearly indicated that these two properties were not correlated, and other factors must therefore influence the agglutination property of the bacterial cells. As seen in the dot assays (Fig. 4), ultrasonic treatment rendered the adhesin accessible to pigeon ovomucoid binding. The binding of pigeon ovomucoid to the supernatant fluid of highly encapsulated strains (598 and B565) is weaker than to unencapsulated strains (628 and 836) (Fig. 4, lane S) because of the presence of capsular polysaccharides released by the ultrasonic treatment (49). This finding indicates that in *S. suis* cells, the polysaccharide capsule may prevent the interaction of the adhesin with pigeon ovomucoid or erythrocytes and thus inhibit hemagglutination. The capsular layer of *S. suis* has been suggested to mask an IgG-binding protein on the surface of *S. suis* strains (45). In the present study, the possibility that polysaccharides restrict the availability of the Galα1-4Gal binding is indicated by the reciprocal relationship between the amount of capsule and the hemagglutination activity of the *S. suis* strains (Fig. 5). Thus, hemagglutination activity may depend on both the amount of adhesin expressed and its availability on the bacterial surface. There is no further information on the molecular properties of the adhesin. The mechanism of polysaccharide interference with adhesin activity therefore remains unknown.

Capsular polysaccharides have been suggested to play a role in the pathogenesis of infections caused by *S. suis*, and serotype 2 is considered to be the most virulent serotype (3). On the other hand, a reference strain of serotype 2 studied by transmission electron microscopy was not covered by a thick layer of capsular material (25), and Clifton-Hadley et al. (9) did not demonstrate any correlation between the capsular thickness and the virulence of *S. suis* serotype 2 strains. Capsule expression may also vary with growth conditions (16). As in many other bacteria (33), hemagglutination of *S. suis* undergoes spontaneous phase variation. The highest hemagglutination activity was observed in strains of serotype 2 (628, TEW/2, and R75/L1) and in a strain of serotype 1 (836), which was in correlation with the amount of the adhesin (Table 1). As shown in Fig. 5, these strains were unencapsulated during the high agglutination phase. Thus, the high virulence of serotype 2 (3) is possibly due to a high expression of the adhesin.

The *S. suis* adhesin contains a relatively high proportion of hydrophobic amino acids (40%) (48). According to Wibawan and Lämmler (53), an unencapsulated variant of *S. suis* had a more hydrophobic surface and adhered significantly more to HeLa cells. It was proposed that the unencapsulated form initiates the first steps of bacterial infection, adherence and colonization, and the encapsulated form might then resist the host's immune defense. Accordingly, it has been reported that an avirulent strain may produce more capsular material in vitro than virulent strains (40). There was a significant increase in vivo in the capsular thickness of virulent strains, whereas the capsular thickness of an avirulent strain did not increase.

Being essential virulence factors, adhesins are potential vaccine candidates (10). Among *Streptococcus pneumoniae* strains, a 37-kDa surface protein with a possible role in bacterial adhesion (43) has been detected from 24 *S. pneumoniae* capsular types (42). Immunization with this protein could protect mice against challenge (47). Several candidate vaccines against *S. suis* infections have been examined. These include capsular polysaccharides (11), whole-cell vaccines (23), and a streptomycin-dependent mutant of *S. suis* type 1/2 (12). Vecht et al. (52) have reported that both a membrane-bound protein of 136 kDa and a 110-kDa extracellular factor are virulence markers for *S. suis* capsular type 2 isolates. Also, a virulence marker protein of 110 kDa, which induced an IgG response and protected against experimental infection in mice with virulent homologous and heterologous *S. suis* strains, has recently been reported by Quessy et al. (37). In addition, a thiol-activated hemolysin (24) has been shown to protect against a lethal *S. suis* type 2 challenge in a mouse model.

The Galα1-4Gal-binding adhesin of *S. suis* appears to differ from most other candidate vaccines proposed for gram-positive bacteria in that it represents a molecule with a defined binding specificity and role in the pathogenic process and occurs in all strains examined independent of serotype. The purified adhesin is highly immunogenic and induces bactericidal activity in mice. Further studies are indicated to evaluate the possible protection achieved by the adhesin against *S. suis* diseases.

ACKNOWLEDGMENTS

We thank H. C. Zanen, F. Clifton-Hadley, J. Hommez, L. Sihvonen, and S. Pelkonen for bacterial strains, C. François-Gerard for pigeon ovomucoid, and T. Taskinen for technical assistance.

This work was supported by grants from the High Technology Foundation of Savo, Finland, the North Savo Fund of Finnish Cultural Foundation, the Sigrid Jusélius Foundation, and the Academy of Finland.

REFERENCES

1. **Agarwal, K. K., S. D. Elliott, and P. J. Lachman.** 1969. Streptococcal infection in young pigs. III. The immunity of adult pigs investigated by the bactericidal test. J. Hyg. Camb. **67:**491–503.
2. **Alexander, T. J. L.** 1992. *Streptococcus suis*: an update. Pig Vet. Soc. Proc. **21:**50–60.
3. **Arends, J. P., and H. C. Zanen.** 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131–137.
4. **Beaudoin, M., R. Higgins, J. Harel, and M. Gottschalk.** 1992. Studies on a murine model for evaluation of virulence of *Streptococcus suis* capsular type 2 isolates. FEMS Microbiol. Lett. **99:**111–116.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Burnette, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulphate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
7. **Clifton-Hadley, F. A.** 1983. Epidemiology of *Streptococcus suis* type 2 infections, p. 33–39. *In* J. R. Walton, E. G. White, and S. A. Hall (ed.), Agriculture. Some diseases of emerging importance to Community trade. Report EUR 85 15 EN. Commission of the European Communities, Luxembourg, Luxembourg.
8. **Clifton-Hadley, F. A.** 1984. Studies of *Streptococcus suis* type 2 infection in pigs. Vet. Res. Commun. **8:**217–227.
9. **Clifton-Hadley, F. A., T. J. L. Alexander, and M. R. Enright.** 1986. The epidemiology, diagnosis, treatment and control of *Streptococcus suis* type 2 infection, p. 471–491. *In* Proceedings of the Annual Meeting of the American Association of Swine Practitioners, Kansas City.
10. **Collett, M. S.** 1989. The development of biosynthetic vaccines. Adv. Vet. Sci. Comp. Med. **33:**109–172.
11. **Elliott, S. D., F. Clifton-Hadley, and J. Tai.** 1980. Streptococcal infection in young pigs. V. An immunogenic polysaccharide from *Streptococcus suis* type 2 with particular reference to vaccination against streptococcal meningitis in pigs. J. Hyg. Camb. **85:**275–285.
12. **Foster, N., J. J. Staats, and M. M. Chengappa.** 1994. Isolation, characterization and protection studies in mice of a streptomycin-dependent mutant of *Streptococcus suis* type 1/2. Vet. Res. Commun. **18:**155–163.
13. **François-Gerard, C., and J. Brocteur.** 1980. Application à la sérologie transfusionelle du caractère antigénique P_1 du blanc dœuf de pigeon. Rev. Fr. Transfus. **23:**95–102.
14. **François-Gerard, C., C. Gerday, and J. G. Beeley.** 1979. Turtle-dove ovomucoid, a glycoprotein proteinase inhibitor with P_1-blood-group antigen activity. Biochem. J. **177:**679–685.
15. **Gefter, M. L., D. H. Margulies, and M. D. Scharff.** 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell. Genet. **3:**231–236.
16. **Gottschalk, M., R. Higgins, and M. Jacques.** 1993. Production of capsular material by *Streptococcus suis* serotype 2 under different growth conditions. Can. J. Vet. Res. **57:**49–52.
17. **Gottschalk, M., R. Higgins, M. Jacques, M. Beaudoin, and J. Henrichsen.** 1991. Isolation and characterization of *Streptococcus suis* capsular types 9-22. J. Vet. Diagn. Invest. **3:**60–65.
18. **Gottschalk, M., R. Higgins, M. Jacques, M. Beaudoin, and J. Henrichsen.** 1991. Characterization of six new capsular types (23 through 28) of *Streptococcus suis*. J. Clin. Microbiol. **29:**2590–2594.
19. **Haataja, S., K. Tikkanen, J. Liukkonen, C. François-Gerard, and J. Finne.** 1993. Characterization of a novel bacterial adhesion specificity of *Streptococcus suis* recognizing blood group P receptor oligosaccharides. J. Biol. Chem. **268:**4311–4317.
20. **Haataja, S., K. Tikkanen, U. Nilsson, G. Magnusson, K.-A. Karlsson, and J. Finne.** 1994. Oligosaccharide-receptor interaction of the Galα1-4Gal binding adhesin of *Streptococcus suis*. J. Biol. Chem. **269:**27466–27472.
21. **Higgins, R., and M. Gottschalk.** 1994. Distribution of *Streptococcus suis* capsular types in 1993. Can. Vet. J. **35:**245.
22. **Higgins, R., M. Gottschalk, M. Boudreau, A. Lebrun, and J. Henrichsen.** 1995. Description of six new capsular types (29–34) of *Streptococcus suis*. J. Vet. Diagn. Invest. **7:**405–406.
23. **Holt, M. E., M. R. Enright, and T. J. L. Alexander.** 1988. Immunization of pigs with live culture of *Streptococcus suis* type 2. Res. Vet. Sci. **45:**349–352.
24. **Jacobs, A. A. C., P. L. W. Loeffen, A. J. G. van den Berg, and P. K. Storm.** 1994. Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect. Immun. **62:**1742–1748.
25. **Jacques, M., M. Gottschalk, B. Foiry, and R. Higgins.** 1990. Ultrastructural study of surface components of *Streptococcus suis*. J. Bacteriol. **172:**2833–2838.
26. **Kitakawa, T., and T. Aikawa.** 1976. Enzyme coupled immunoassay of insulin using a novel coupling reagent. J. Biochem. **79:**233–236.
27. **Köhler, G., and C. Milstein.** 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature (London) **256:**495–497.
28. **Korhonen, T. K., and J. Finne.** 1985. Agglutination assays for detecting bacterial binding specificities, p. 301–313. *In* T. K. Korhonen, E. A. Dawes, and P. H. Mäkelä (ed.), Enterobacterial surface antigens: methods for molecular characterization. Elsevier, Amsterdam.
29. **Kurl, D. N., S. Haataja, and J. Finne.** 1989. Hemagglutination activities of group B, C, D, and G streptococci: demonstration of novel sugar-specific cell-binding activities in *Streptococcus suis*. Infect. Immun. **57:**384–389.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
31. **Liu, F.-T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18:**690–697.
32. **Liukkonen, J., S. Haataja, K. Tikkanen, S. Kelm, and J. Finne.** 1992. Identification of *N*-acetylneuraminyl α2-3 poly-*N*-acetyllactosamine glycans as the receptors of sialic acid-binding *Streptococcus suis* strains. J. Biol. Chem. **267:**21105–21111.
33. **Nowicki, B., J. Vuopio-Varkila, P. Viljanen, T. K. Korhonen, and P. H. Mäkelä.** 1986. Fimbrial phase variation and systemic *E. coli* infection studied in the mouse peritonitis model. Microb. Pathog. **1:**335–347.
34. **Ofek, I., and E. H. Beachey.** 1980. General concepts and principles of bacterial adhesion, p. 1–29. *In* E. H. Beachey (ed.), Bacterial adherence. Receptors and recognition, ser. A, vol. 6. Chapman and Hall, London.
35. **Pelkonen, S., J. Häyrinen, and J. Finne.** 1988. Polyacrylamide gel electrophoresis of the capsular polysaccharides of *Escherichia coli* K1 and other bacteria. J. Bacteriol. **170:**2646–2653.
36. **Perch, B., K. B. Pedersen, and J. Henrichsen.** 1983. Serology of capsulated streptococci pathogenic for pigs: six new serotypes of *Streptococcus suis*. J. Clin. Microbiol. **17:**993–996.
37. **Quessy, S., J. D. Dubreuil, M. Caya, and R. Higgins.** 1995. Discrimination of virulent and avirulent *Streptococcus suis* capsular type 2 isolates from different geographical origins. Infect. Immun. **63:**1975–1979.
38. **Quessy, S., J. D. Dubreuil, M. Caya, R. Létourneau, and R. Higgins.** 1994. Comparison of pig, rabbit and mouse IgG response to *Streptococcus suis* serotype 2 proteins and active immunization of mice against the infection. Can. J. Vet. Res. **58:**220–223.
39. **Quessy, S., J. D. Dubreuil, and R. Higgins.** 1994. Immunization of mice against *Streptococcus suis* serotype 2 infections using a live avirulent strain. Can. J. Vet. Res. **58:**299–301.
40. **Quessy, S., J. D. Dubreuil, M. Jacques, F. Malouin, and R. Higgins.** 1994. Increase of capsular material thickness following in vivo growth of virulent *Streptococcus suis* serotype 2 strains. FEMS Microbiol. Lett. **115:**19–26.
41. **Ripley, P. H.** 1983. Vaccines against streptococcal meningitis. Pig Vet. Soc. Proc. **10:**24–39.
42. **Russell, H., J. A. Tharpe, D. E. Wells, E. H. White, and J. E. Johnson.** 1990. Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*. J. Clin. Microbiol. **28:**2191–2195.
43. **Sampson, J. S., S. P. O'Connor, A. R. Stinson, J. A. Tharpe, and H. Russell.** 1994. Cloning and nucleotide sequence analysis of psaA, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp. adhesins. Infect. Immun. **62:**319–324.
44. **Sandford, S. E., and R. Higgins.** 1992. Streptococcal diseases, p. 588–590. *In* D. J. Taylor (ed.), Diseases of swine. Iowa State University Press, Ames, Iowa.
45. **Serhir, B., R. Higgins, B. Foiry, and M. Jacques.** 1993. Detection of immunoglobulin-G-binding proteins in *Streptococcus suis*. J. Gen. Microbiol. **139:**2953–2958.
46. **Sihvonen, L., D. N. Kurl, and J. Henrichsen.** 1988. *Streptococcus suis* isolated from pigs in Finland. Acta Vet. Scand. **29:**9–13.
47. **Talkington, D. F., A. Koenig, and H. Russell.** 1992. The 37-kDa protein of *Streptococcus pneumoniae* protects mice against fatal challenge, abstr. E-29, p. 149. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
48. **Tikkanen, K., S. Haataja, C. François-Gerard, and J. Finne.** 1995. Purification of a galactosyl-α1-4-galactose-binding adhesin from the gram-positive meningitis-associated bacterium *Streptococcus suis*. J. Biol. Chem. **270:**28874–28878.
49. **Tikkanen, K., J. Häyrinen, S. Pelkonen, and J. Finne.** 1995. Immunoblot analysis of bacterial polysaccharides: application to the type-specific polysaccharides of *Streptococcus suis* and *Streptococcus agalactiae*. J. Immunol. Methods **187:**233–244.
50. **Trottier, S., R. Higgins, G. Brochu, and M. Gottschalk.** 1991. A case of

human endocarditis due to *Streptococcus suis* in North America. Rev. Infect. Dis. **13:**1251–1252.
51. **Vecht, U., L. A. M. G. van Leengoed, and E. R. M. Verheijen.** 1985. *Streptococcus suis* infections in pigs in the Netherlands (part I). Vet. Q. **7:**315–321.
52. **Vecht, U., H. J. Wisselink, M. L. Jellema, and H. E. Smith.** 1991. Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect. Immun. **59:**3156–3162.
53. **Wibawan, I. W. T., and C. Lämmler.** 1994. Relation between encapsulation and various properties of *Streptococcus suis*. J. Vet. Med. B **41:**453–459.
54. **Williams, A. E., W. F. Blakemore, and T. J. L. Alexander.** 1988. A murine model of *Streptococcus suis* type 2 meningitis in the pig. Res. Vet. Sci. **45:** 394–399.

Editor: V. A. Fischetti