Microbiology (1998), 144, 325–332 Printed in Great Britain

Streptococcus suis serotype 2 mutants deficient in capsular expression

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***Streptococcus suis* serotype 2 is responsible for a wide variety of porcine infections. In addition, it is considered a zoonotic agent. Knowledge about the virulence factors for this bacterium is limited but its polysaccharide capsule is thought to be one of the most important. Transposon mutagenesis with the self-conjugative transposon Tn*916* was used to obtain acapsular mutants from the virulent *S. suis* type 2 reference strain S735. Clones were screened by colony-dot ELISA with a monoclonal antibody specific for a type 2 capsular epitope and clones that failed to react with the antibody were characterized. Two mutants, 2A and 79, having one and two Tn*916* insertions respectively, were chosen for further characterization. Absence of capsule was confirmed by coagglutination, capillary precipitation and capsular reaction tests and by transmission electron microscopy. Absence of capsular polysaccharides correlated with increased hydrophobicity and phagocytosis by both murine macrophages and porcine monocytes compared to the wild-type strain. Furthermore, both mutants were shown to be avirulent in murine and pig models of infection. Finally, mutant 2A was readily eliminated from circulation in mice compared to the wild-type strain, which persisted more than 48 h in blood. Thus, isogenic mutants defective in capsule production demonstrate the importance of capsular polysaccharides as a virulence factor for *S. suis* type 2.**

Keywords: *Streptococcus suis*, Tn*916*, capsule, mutant, virulence

INTRODUCTION

Streptococcus suis is responsible for a wide variety of porcine disease syndromes, such as meningitis, septicaemia, arthritis and endocarditis (Higgins & Gottschalk, 1998). It is also recognized as a human pathogen, causing mainly meningitis (with deafness as a common sequela) and endocarditis (Walsh *et al.*, 1992; Arends & Zanen, 1988; Trottier *et al.*, 1991). To date, 35 serotypes of *S. suis* have been described, with serotype 2 being the most frequently isolated from diseased animals (Higgins & Gottschalk, 1996). Knowledge on virulence factors of *S. suis* is limited. Several virulence factor candidates have been proposed. These include the polysaccharide capsule, cell-wall and extracellular proteins, including muramidase-released protein (MRP), extracellular factor (EF) and adhesins, and a haemolysin (Gottschalk *et al.*, 1995; Jacobs *et al.*, 1994; Tikkanen *et al.*, 1996; Vecht *et al.*, 1991). Recent studies have shown that *S. suis* mutants which produce neither MRP nor EF are as virulent as the wild-type strain (Smith *et al.*, 1996). It has been suggested that these proteins could be used as virulence markers for European strains. On the other hand, most field strains isolated in Canada do not produce these proteins and/or the haemolysin (Gottschalk *et al.*, 1998).

Although the polysaccharide capsule of *S. suis* serotype 2, which contains, among other sugars, a sialic acid moiety (Elliott & Tai, 1978; Katsumi *et al.*, 1996), has been identified as a potential virulence factor, its role in the pathogenesis of the infection can be considered controversial. It has been shown that, with some exceptions (Elliott *et al.*, 1980), both virulent and avirulent strains are encapsulated and are similarly phagocytosed by macrophages, and that the capsule of an avirulent strain does not appear to aid intracellular survival (Brazeau *et al.*, 1996; Williams, 1990). In

Abbreviations: DIG, digoxigenin; EF, extracellular factor; FBS, foetal bovine serum; MRP, muramidase-released protein; NANA, *N*-acetylneuraminic acid (sialic acid); SNA I, *Sambucus nigra* agglutinin; SPF, specific-pathogen free.

addition, sialic acid does not seem to be critical for virulence, since all field strains of *S. suis* serotype 2 tested had the same low sialic acid concentration regardless of their virulence, and blocking or enzymic removal of this sugar did not influence phagocytosis rates and virulence of a virulent strain (Charland *et al.*, 1996). On the other hand, a spontaneous unencapsulated mutant, obtained from a virulent strain by serial passages in presence of anti-capsular antibodies, was shown to be avirulent in mice (Gottschalk *et al.*, 1992) and highly phagocytosed (Brazeau *et al.*, 1996; Salasia *et al.*, 1995). However, this mutant also lacked, at least, a 44 kDa cell-wall protein. The claim that the presence of a capsule is required for virulence awaited testing with isogenic mutants.

Transposon mutagenesis is a powerful tool used to evaluate the involvement of specific factors in the pathogenesis of streptococci (Caparon & Scott, 1991). It has successfully been used to produce unencapsulated mutants of group B streptococci (Rubens *et al.*, 1987). This paper describes the production and characterization of *S. suis* serotype 2 mutants deficient in the expression of the polysaccharide capsule obtained using the self-conjugative transposon Tn*916*.

METHODS

Bacterial strains and culture conditions. The well-encapsulated *S. suis* serotype 2 reference strain S735 (kindly provided by Dr J. Henrichsen, Statens SerumInstitut, Copenhagen, Denmark) was used as the recipient strain for transposon mutagenesis. This strain, which is susceptible to both tetracycline and streptomycin, was rendered resistant to the latter by serial passages in Todd–Hewitt broth (THB; Difco) containing increasing streptomycin concentrations and was named S735-SM. *Enterococcus faecalis* CG110 (kindly supplied by Dr C. E. Rubens, Children's Hospital and Medical Center, Seattle, WA, USA), which contains Tn*916*, a 18 kb transposon encoding tetracycline resistance (Gawron-Burke & Clewell, 1984), was used as the donor strain and grown in THB containing 10 μg tetracycline ml^{-1}. *Escherichia coli* CG120 (kindly provided by Dr D. B. Clewell, University of Michigan, Ann Arbor, MI, USA), possessing plasmid pAM120 encoding ampicillin resistance and containing Tn*916* (Gawron-Burke & Clewell, 1984), was grown in THB with 10 μg tetracycline and 50 μg ampicillin ml^{-1}, and was used for probe preparation.

Transposon mutagenesis. Plate mating between the two strains was done by the method of Caparon & Scott (1991) with the ratio of donor to recipient changed to 1:100. Briefly, *Enterococcus faecalis* CG110, in 5 ml THB containing 10 μg tetracycline ml^{-1}, and *S. suis* S735-SM, in 100 ml THB supplemented with 2 g yeast extract l^{-1}, were grown overnight at 37 °C. Both suspensions were centrifuged, washed three times, resuspended in THB and strain S735-SM mixed with CG110. Spots of the mixed suspension were plated onto bovine blood agar plates and left to incubate overnight at 37 °C. Growth was retrieved by washing the plates with PBS (140 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1·5 mM KH_2PO_4, pH 7·4). The suspension was centrifuged, resuspended in PBS and plated onto Todd–Hewitt agar (THA) plates containing 800 μg streptomycin ml^{-1} and 10 μg tetracycline ml^{-1}. Plates were incubated for 24–36 h at 37 °C and transconjugants transferred on new selective plates. Conjugation frequency was calculated by dividing the number of Tn*916*-containing colonies resistant to both streptomycin and tetracycline by the total number of potential recipient colonies resistant only to streptomycin.

Screening of transconjugants. Screening was done by colony dot of transconjugants as described by Rioux *et al.* (1997) using a monoclonal antibody, mAb Z3, specific for a serotype 2 sialic-acid-containing capsular epitope (Charland *et al.*, 1997). Failure of colonies to react with mAb Z3 could be due either to the absence of sialic acid or to the absence of all capsular polysaccharides.

Preparation of digoxigenin-labelled Tn*916* probe and DNA–DNA hybridization. Total cellular DNA was first isolated by the method of Sambrook *et al.* (1989). To perform Southern blot DNA–DNA hybridization analysis (Nizet *et al.*, 1996), a Tn*916* probe labelled with digoxigenin (DIG) was prepared using the Boehringer Mannheim DIG DNA labelling and detection kit. The *Eco*RI fragment of plasmid pAM120 containing Tn*916* was isolated from low-melting-point agarose as described by Sambrook *et al.* (1989). The DIG-labelled Tn*916* was used to probe *Eco*RI and *Sca*I digests of whole-cell DNA as described by the manufacturer.

Characterization of mutants

Biochemical identification of mutants. Mutants were confirmed biochemically to be *S. suis* by standard identification techniques (Higgins & Gottschalk, 1990).

Production of haemolysin, MRP and EF. Production of haemolysin was tested as described previously (Gottschalk *et al.*, 1995). Production of MRP and EF was tested as described by Vecht *et al.* (1991).

Sialic acid determination tests. An agglutination test with a sialic-acid-binding lectin from *Sambucus nigra*, SNA I, known to react with the sialic acid moiety of *S. suis* serotype 2 capsule (Charland *et al.*, 1995), was used as a screening test to identify capsule deficient mutants. Presence of *N*-acetylneuraminic acid (NANA) was shown by coarse aggregates. Absence of NANA was confirmed by the thiobarbituric acid assay (Warren, 1963).

Serotyping of mutants. Serotyping was carried out by three different techniques. Coagglutination, capillary precipitation and capsular reaction tests utilized specific type 2 antiserum as previously described (Higgins & Gottschalk, 1990).

Transmission electron microscopy. Transmission electron microscopy after immunostabilization was carried out as described by Jacques *et al.* (1990). Briefly, wild-type as well as mutant strains were mixed with specific type 2 antiserum or with normal serum as a negative control. Bacterial suspensions were adjusted to OD_{540} 1·8 and exposed to undiluted polyclonal or normal sera for 1 h at 4 °C. Bacterial cells were then suspended in 0·1 M cacodylate buffer, pH 7·0, containing 5% (v/v) glutaraldehyde and 0·15% (w/v) ruthenium red. Fixation was for 2 h at 20 °C. Cells were then immobilized in 4% (w/v) agar, washed five times in cacodylate buffer plus 0·05% ruthenium red, and postfixed with 2% (v/v) osmium tetroxide for 2 h. Samples were washed as above and dehydrated in a graded series of acetone washes containing 0·05% ruthenium red. Specimens were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV.

Hydrophobicity test. Hydrophobicity was measured using the n-hexadecane method (Rosenberg, 1984).

Phagocytic assays. Phagocytic cells were retrieved from the peritoneal cavity of mice or from pig blood and left to adhere on microscope slides as described by Brazeau *et al.* (1996) and Charland *et al.* (1996). Bacteria were added to a ratio of 10 bacteria to 1 phagocyte and incubated for 1 h at 37 °C, 5% CO_2. After incubation, cells were stained with the vital dye acridine orange and counterstained with crystal violet, so that only engulfed bacteria were visible under UV light (Williams, 1990). The assay was repeated in three independent experiments. Percentages of phagocytosis were calculated as: (no. of phagocytes with engulfed bacteria divided by 200 examined phagocytes) × 100 (Charland *et al.*, 1996).

Virulence assays. Virulence was assessed in mice and piglets. To measure the LD_{50} in mice, bacteria were grown for 6 h in THB supplemented with 10% heat-inactivated foetal bovine serum (FBS). For each strain, five groups of five BALB/c mice 4–6 weeks old were injected intraperitoneally with 1 ml per mouse of undiluted bacterial suspension or tenfold dilutions in THB-FBS of the original suspension. Mortality was monitored over a week and the LD_{50} of each strain calculated by the method of Reed & Muench (1938). An experimental infection in swine was also carried out. Three groups of seven specific-pathogen-free (SPF) piglets 6–7 weeks old, naturally born from hysterectomy-derived sows, were used (one group per strain) and kept in high-security barns. Bacteria were grown in THB-FBS for 5 h at 37 °C and the OD_{600} of the suspension adjusted to 0·15, which corresponds to about 10^8 c.f.u. ml^{-1}. Piglets were injected intravenously with 1 ml of the suspension. Clinical signs were monitored daily and pigs were killed either if severely sick or, if they presented no signs, one week after infection. Autopsies were performed on each piglet with macroscopic, bacteriological and/or histological examinations of affected tissues.

Bacterial clearance test. Bacteria were grown as for the virulence assay except that suspensions were adjusted to a concentration of 3×10^6 c.f.u. ml^{-1} in THB-FBS to prevent mortality. Groups of seven mice were injected intraperitoneally with 1 ml bacterial suspension. Mice were killed by cervical dislocation at various time intervals after injection, blood samples taken from hearts and viable counts performed on blood agar plates. The assay was repeated in three independent experiments.

RESULTS AND DISCUSSION

Characterization of mutants deficient in capsular expression

Tn*916* was transferred from *Enterococcus faecalis* CG110 to S735-SM at a frequency of 1 per $1{\cdot}5 \times 10^6$ recipient cells, which is in the range of conjugation frequency obtained with *Streptococcus pyogenes* (Caparon & Scott, 1987). More than 8000 clones were screened and, of these, seven mutants did not present any reaction with mAb Z3.

After preliminary testing of these clones based on the number of transposon insertions and capsule production, two mutants, named 2A and 79, were selected for further characterization. Both mutants were shown to be biochemically identical to the wild-type strain. In addition, the production of other putative virulence factors of *S. suis* such as haemolysin, MRP and EF was not affected by transposition (Table 1). Since mAb Z3 is specific for a sialic-acid-containing epitope, mutants were tested for the presence of NANA. Fine aggregates were seen in the reaction between both mutants and the lectin SNA I compared to coarse ones with the wild-type strain. In addition, NANA concentration was shown to be very low in both mutants (Table 1). Serotyping, which is indicative of the presence of a capsule, was carried out by three different techniques. Mutants reacted only weakly by coagglutination with specific type 2 antiserum compared to the wild-type strain S735-SM, indicating little capsule expression or reaction with other surface antigens. Capillary precipitation and capsular reaction tests, which are more specific for the detection of capsular antigens, were negative for both mutants (Table 1).

To confirm the absence or altered expression of capsule on mutants, both of the mutants and the wild-type strain were examined by transmission electron microscopy. No or very little capsular material seemed to be present on cell surfaces of mutants 2A (Fig. 1c) and 79 (not shown) after immunostabilization of the capsule. The absence of sialic acid implies the probable absence of the complete capsule since it has been suggested that, after complete removal of the sialic acid moiety, a reaction with anti-type 2 serum is not detected (Katsumi *et al.*, 1996). This would also correlate with the absence of immunostabilization. Katsumi *et al.* (1996) mentioned that the sialic acid residue in the capsule of *S. suis* serotype 2 is probably the exact epitope component.

Molecular characterization of mutants

To determine the number of Tn*916* insertions in each mutant, total DNA was extracted and cut with *Eco*RI and *Sca*I. *Eco*RI does not cut within Tn*916* whereas *Sca*I cuts only once (Flannagan *et al.*, 1994). Southern blot DNA–DNA hybridization analysis, using DIG-labelled Tn*916* to probe *Eco*RI and *Sca*I chromosomal digests of mutants, shows that mutant 2A contained one (Fig. 2 lanes B and C) and mutant 79 two (lanes D and E) Tn*916* insertions. No background hybridization was seen between wild-type DNA and the probe (Fig. 2, lane A). DNA adjacent to these insertions, after digestion with *Sca*I, was of 11·6 kb for mutant 2A, and of 11·6 and 12·7 kb for mutant 79. As shown in Fig. 2, although mutant 79 seemed to have only one Tn*916* insertion after digestion with *Eco*RI, *Sca*I digestion showed another transposon insertion. It seems that only one transposon insertion is sufficient to alter capsular expression as seen for group B streptococci (Rubens *et al.*, 1993, 1987).

Influence of defective capsular expression on hydrophobicity and phagocytosis

It has been shown that alteration of capsular expression can modify surface hydrophobicity and affect adherence on cells (Absolom, 1988). Influence of altered capsular expression on hydrophobicity and phagocytosis was tested (Table 1). Mutants 2A and 79 demonstrated

Table 1. Characterization of two *S. suis* serotype 2 mutants produced by transposition from reference strain S735-SM

For agglutination experiments, the capsular reaction test and the capillary precipitation test, the strength of the reaction is indicated as follows: + + +, strong reaction; +, weak reaction; −, no reaction. For phagocytosis experiments, pig monocyte-derived macrophages were used. Data for NANA concentration, hydrophobicity and phagocytosis are the means of three independent experiments ± SD; results for the mutant strains were significantly different ($P < 0.05$) compared to the wild-type strain.

Test	S735-SM	2A	79
MRP protein	Yes	Yes	Yes
EF protein	Yes	Yes	Yes
Haemolysin	Yes	Yes	Yes
SNA I agglutination	+ + +	+	+
NANA concentration	4.07 ± 1.42 μg mg^{-1}	0.11 ± 0.04 μg mg^{-1}	0.10 ± 0.03 μg mg^{-1}
Coagglutination test	+ + +	+	+
Capsular reaction test	+ + +	−	−
Capillary precipitation test	+ + +	−	−
Hydrophobicity	0.9 ± 0.4%	22.0 ± 0.9%	17.7 ± 0.8%
Phagocytosis	23 ± 9%	70 ± 8%	70 ± 4%
LD_{50}	7×10^5 c.f.u. g^{-1}	$> 7 \times 10^7$ c.f.u. g^{-1}	$> 7 \times 10^7$ c.f.u. g^{-1}

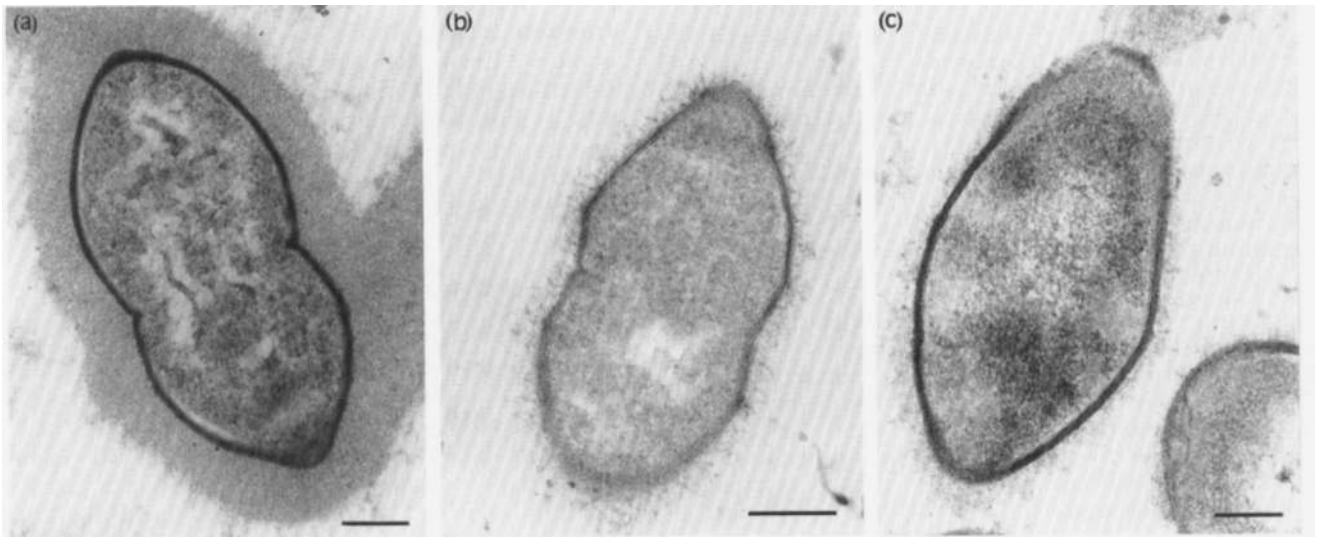

Fig. 1. Transmission electron micrographs of thin sections of *Streptococcus suis* serotype 2: (a) strain S735-SM stabilized with specific type 2 rabbit polyclonal antiserum, (b) strain S735-SM stabilized with normal rabbit serum (negative control) and (c) mutant 2A stabilized with type 2 rabbit polyclonal antiserum. Results similar to (c) were obtained with mutant 79, and results similar to (b) with both mutants. Bars, 200 nm.

hydrophobicity at 22% and 17·7% respectively, compared to 0·9% for S735-SM, indicating that capsular polysaccharides are important to surface hydrophobicity of *S. suis* (Salasia *et al.*, 1995). Percentages of phagocytosis by murine macrophages and porcine monocyte-derived macrophages were evaluated for the three strains. These phagocytic cells were chosen to provide a comparison of phagocytosis rates between the natural host's phagocytes and phagocytes from the animal model of choice for *S. suis* infections (Beaudoin *et al.*, 1992; Williams *et al.*, 1988). Table 1 shows that both mutants were significantly more phagocytosed than the wild-type strain by porcine phagocytic cells, with a mean number of 6 (S735-SM), 16 (mutant 2A) and 18 (mutant 79) phagocytosed organisms per phagocyte. Similar results were obtained with murine cells (data not shown). These results show that the presence of capsular polysaccharides prevents phagocytosis, which may help bacterial pathogenesis. Since capsular polysaccharide material confers a net negative charge on a bacterium, it is rendered hydrophilic (Moxon & Kroll, 1990). Removal of capsular material increases the hydrophobic index, thereby increasing adherence and phagocytosis (Absolom, 1988). This seems to be the case for *S. suis*

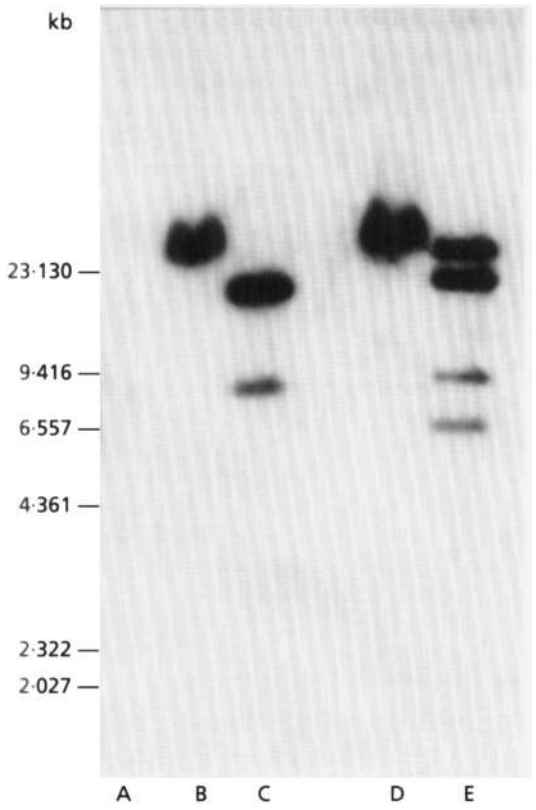

Fig. 2. Southern blot with DIG-labelled Tn*916* of total cellular DNA. Lane A, DNA of strain S735-SM digested with *Eco*RI; lanes B and C, DNA of mutant 2A digested with *Eco*RI (lane B) and *Sca*I (lane C); lanes D and E, DNA of mutant 79 digested with *Eco*RI (lane D) and *Sca*I (lane E). Molecular sizes of standards in kb are indicated. Image generated using a HP ScanJet IIc with PhotoFinish 2.0 software on a PC 486 DX.

unencapsulated mutants. In addition, Tikkanen *et al.* (1996) recently showed that the variable agglutinating activity of some *S. suis* strains was inversely proportional to the amount of capsular polysaccharides, which the authors suggest could mask adhesins.

Influence of defective capsular expression on virulence and blood clearance

Both mutants were more than 100-fold less virulent for mice than the wild-type strain as shown by their LD_{50} of more than 7×10^7 c.f.u. g^{-1}, compared to 7×10^5 c.f.u. g^{-1} for S735-SM (Table 1). For pig infection, Table 2 shows that all piglets injected with S735-SM presented with hyperthermy (i.e. at least 1 °C over the normal body temperature of 39·5 °C for an SPF pig) and locomotive difficulties for at least 3 d post-infection. Forty-eight hours after injection, three piglets showing opisthotonos and paddling signs with elevated temperature were killed. These pigs showed suppurative meningitis at autopsy. *S. suis* serotype 2 was recovered from brains and/or from other organs of all seven pigs. Histological examinations of synovial membranes of some pigs showed an early inflammatory reaction. Piglets injected with both mutants showed no clinical signs. Animals of these groups were killed one week after infection. Autopsies did not reveal significant macroscopic or microscopic lesions. *S. suis* serotype 2 was isolated only from the synovial fluid of a knee in a pig injected with mutant 79. The isolate was still tetracycline-resistant. No bacterium was isolated in any tonsils of these two groups. Isolation of the wild-type strain from many different organs of all infected pigs shows that the presence of capsular polysaccharides may help bacteria to disseminate and survive more easily. In fact, S735-SM could be isolated from its natural reservoir in pigs, the tonsils (Higgins & Gottschalk, 1998), in all animals, whereas mutants could not be recovered from this site. Thus, the presence of capsular polysaccharides contributes to the virulence of *S. suis* serotype 2 in both mice and pigs.

Table 2. Experimental infection of SPF piglets with *S. suis* serotype 2 reference strain S735-SM and two serotype 2 mutants produced by transposition from strain S735-SM

Strain	Dose [10^{-8} × (c.f.u. ml^{-1}) ± SD]	Clinical signs	Lesions	Isolation
S735-SM	5·3 ± 1·2	Fever, locomotive difficulties*	Suppurative meningitis, early inflammatory reaction of synovial membranes†	7/7‡
Mutant 2A	5·7 ± 1·3	None	None	0/7
Mutant 79	3·6 ± 1·4	None	None	1/7§

* All seven pigs of the group were affected.

† Three pigs with severe locomotive difficulties and paddling signs showed meningitis; two other pigs shows inflammation.

‡ Strain isolated from liver, spleen, lungs, heart, tonsils, brains and/or joints from all seven infected pigs.

§ Isolated in synovial fuid from a knee of one pig.

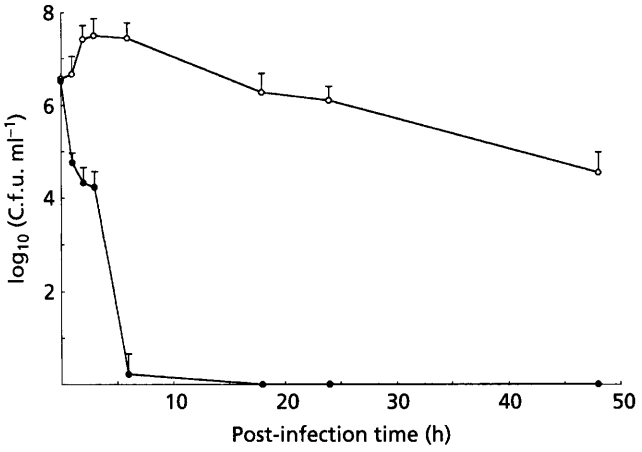

Fig. 3. Bacterial clearance in mice injected with *S.* suis serotype 2 strain S735-SM (○) and acapsular mutant 2A (●). The data are means of three experiments; error bars represent SD.

Bacterial clearance of the wild-type strain and mutant 2A was tested in mice. Fig. 3 shows that the mutant is cleared rapidly from circulation compared to the wild-type strain. These results support those obtained from the virulence experiments where the mutants were reisolated unfrequently. In addition, clearance results are in agreement with those obtained by Clifton-Hadley (1981) who showed that a less encapsulated variant of a *S. suis* type 2 strain was readily cleared from pig blood.

Capsules have been shown to have a key role in host–pathogen interactions (Moxon & Kroll, 1990). Unencapsulated mutants of bacteria involved in invasive infections, such as group A and B streptococci (Wessels *et al.*, 1991; Rubens *et al.*, 1987) or *Escherichia coli* K-1 (Kim *et al.*, 1992), have demonstrated the critical role of the capsule in virulence. One means by which a bacterium disseminates through a host is by evading the immune system (Moxon & Kroll, 1990) and many encapsulated bacteria have been shown to possess antiphagocytic properties (Dale *et al.*, 1996; Kasper, 1986; Martin *et al.*, 1992). Previous studies with spontaneous, genetically uncharacterized *S. suis* serotype 2 mutants suggested that loss or alteration of capsule expression could influence the fate of the bacterium in a host (Brazeau *et al.*, 1996; Gottschalk *et al.*, 1992). Results reported here show that isogenic mutants with specific mutations which preclude capsular expression are avirulent and cleared rapidly from circulation compared to the wild-type strain, clarifying the ambiguous role of the capsule in the virulence of *S. suis* for both mice and pigs. To our knowledge, the capsule of *S. suis* serotype 2 is the first critical virulence factor described so far for this bacterium.

It is clear, however, that the capsule of *S. suis* serotype 2 cannot be the only factor for virulence since it is known that serotype 2 avirulent field strains are encapsulated (Beaudoin *et al.*, 1992; Quessy *et al.*, 1995). In fact, a monoclonal antibody against a capsular epitope of *S. suis* serotype 2 reference strain could not protect mice completely from a lethal dose of bacteria (Charland *et al.*, 1997). Other factors, such as extracellular or cell-wall-associated proteins, are probably required for successful establishment of the bacteria in the host. The role of the capsule may be, for example, to protect *S. suis* from phagocytosis while it produces its toxic components.

Concluding remarks

Isogenic mutants of *S. suis* serotype 2 deficient in capsule expression were produced by transposon mutagenesis. These mutants were more hydrophobic and more susceptible to phagocytosis by mouse and pig phagocytes than the wild-type strain. In addition, they were avirulent for both mice and piglets in challenge experiments and cleared from circulation rapidly. Characterization of the insertion site is currently in progress.

ACKNOWLEDGEMENTS

We are grateful to Dr J. Henrichsen for confirmation of the capsular reaction test and to Dr M. Jacques for the transmission electron micrographs. We also wish to thank S. Lacouture, B. Chevallier, A. Mesplède, A. Keranflec'h, R. Cariolet and the Laboratoire Départemental d'Analyses of Ploufragan for excellent technical assistance and Dr C. E. Rubens for interesting discussions. This work was supported by Grant 0GP0154280 from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Grant 98-NC-1037 from the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche. N.C. is the recipient of an NSERC scholarship.

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Received 17 June 1997; revised 29 September 1997; accepted 1 October 1997.