Isolation and characterization of α -enolase, a novel fibronectin-binding protein from *Streptococcus suis*

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Streptococcus suis is an important swine pathogen that causes meningitis, endocarditis, arthritis and septicaemia. As a zoonotic agent, *S. suis* also causes similar diseases in humans. Binding of pathogenic bacteria to extracellular matrix components enhances their adhesion to and invasion of host cells. In the present study we isolated and identified a novel fibronectin-binding protein from *S. suis*. The native protein (designated SsEno) possessed not only high homology with other bacterial enolases but also enolase activity. We cloned, expressed and purified SsEno and showed that it is ubiquitously expressed by all *S. suis* serotypes and we identified its surface localization using immunoelectron microscopy. ELISA demonstrated that SsEno binds specifically to fibronectin and plasminogen in a lysine-dependent manner. Additional surface plasmon resonance assays demonstrated that SsEno binds to fibronectin or plasminogen with low nanomolar affinity. Inhibition experiments with anti-SsEno antibodies also showed that bacterial SsEno is important for the adhesion to and invasion of brain microvascular endothelial cells by *S. suis*. Overall, the present work is the first study, to our knowledge, to demonstrate a fibronectin-binding activity of a bacterial enolase, and shows that, similar to other bacterial fibronectin-binding proteins, SsEno may contribute to the virulence of *S. suis*.

Received 28 January 2008

Revised 5 May 2008

Accepted 29 May 2008

INTRODUCTION

Streptococcus suis is a major swine pathogen that causes septicaemia, meningitis, endocarditis and arthritis (Higgins & Gottschalk, 2005). Of the 35 known serotypes, serotype 2 is the most frequently isolated and associated with disease (Higgins & Gottschalk, 2005). It has been proposed that two serotypes (serotypes 32 and 34) be excluded from *S. suis* and redesignated *Streptococcus orisratti* (Hill *et al.*, 2005). *S. suis*, especially serotype 2, has also been described as an important zoonotic agent that affects people in close contact with infected pigs or pork-derived products (Lun *et al.*, 2007). Indeed, an important number of cases of human disease with a high rate of mortality in China were linked directly to a concurrent outbreak of *S. suis* infection in pigs (Ye *et al.*, 2006).

Little is known about *S. suis* virulence factors. The capsule polysaccharide (CPS) is a critical virulence factor, given that unencapsulated isogenic mutants are completely

avirulent and rapidly cleared from the circulation in pig and mouse infection models (Charland *et al.*, 2000; Smith *et al.*, 1999). However, non-virulent strains are also encapsulated, indicating that the virulence of this pathogen is a multifactorial process (Gottschalk & Segura, 2000). Other potential virulence factors have also been described in *S. suis*, including a haemolysin (suilysin), a 136 kDa muramidase-released protein (MRP), a 110 kDa extracellular factor (EF) protein, a hyaluronidase, a superoxide dismutase, various proteases, a serum opacity factor and different adhesins (Baums *et al.*, 2006; Segura & Gottschalk, 2004).

The pathogenesis of *S. suis* infection is not fully understood and likely involves many steps (Gottschalk & Segura, 2000). Binding between bacterial adhesins and host proteins is an essential step in the colonization of mucosal surfaces, and has also been implicated in bacterial invasion of host cells (Tamura *et al.*, 1994). Indeed, *S. suis* is able to bind to, and in some cases invade, endothelial and epithelial cells of human and porcine origin (Benga *et al.*, 2005; Gottschalk & Segura, 2000; Lalonde *et al.*, 2000; Norton *et al.*, 1999; Vanier *et al.*, 2004). However, the specific mechanisms involved in these interactions are unknown.

Abbreviations: EACA, *L*-amino-*n*-caproic acid; ECM, extracellular matrix; FBPS, fibronectin-fibrinogen-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBMEC, porcine brain microvascular endothelial cell; RU, relative units; SEN, *Streptococcus pyogenes* enolase; SPR, surface plasmon resonance.

Some pathogens use host extracellular matrix (ECM) proteins to potentiate their virulence. The ECM is a stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells (Westerlund & Korhonen, 1993). Its composition varies among different organs, but the main components are fibronectin, collagen, elastin, laminin and glycosaminoglycans (Kreis & Vale, 1993). Many of these proteins can potentially serve as surface receptors for bacterial binding to host cells via their adhesins (Joh *et al.*, 1999; Schwarz-Linek *et al.*, 2004; Westerlund & Korhonen, 1993). *S. suis* is able to adhere to fibronectin and different types of collagens (Esgleas *et al.*, 2005). In fact, a 64 kDa fibronectin-fibrinogen-binding protein (FBPS) with binding capacity for these two host proteins has been described for *S. suis* (de Greeff *et al.*, 2002). The role of FBPS in bacterial pathogenesis is not well understood. However, studies with the *fbps* mutant suggest that this protein could play a role in *S. suis* colonization of various organs (de Greeff *et al.*, 2002).

Another *S. suis* adhesin that is able to bind to different host proteins such as plasminogen and albumin is the 39 kDa glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Jobin *et al.*, 2004; Quessy *et al.*, 1997). Evidence that this protein is involved in the binding of *S. suis* to host tissues has been provided by the finding that mutants deficient in the surface expression of GAPDH have decreased binding to tracheal cells and porcine tracheal rings (Brassard *et al.*, 2001, 2004). GAPDH is also a cytosolic enzyme that participates in the degradation of carbohydrates via glycolysis and other catabolic pathways, as well as in glucose synthesis via gluconeogenesis. Another enzyme essential for glycolysis and gluconeogenesis is enolase (Marks, 1998). This cytosolic enzyme catalyses the conversion of 2-phosphoglycerate (2-PG) into phosphoenolpyruvate (PEP) via the elimination of a water molecule (Wold & Ballou, 1957). Enolase has also been identified as a novel plasminogen receptor on the surface of many eukaryotic cells (Miles *et al.*, 1991; Nakajima *et al.*, 1994). This unexpected feature has also been described in several bacteria (such as *Staphylococcus aureus* and many streptococci), fungi and nematodes (Bergmann *et al.*, 2001; Jolodar *et al.*, 2003; Jong *et al.*, 2003; Molkanen *et al.*, 2002; Pancholi & Fischetti, 1998; Sharma *et al.*, 2006). While the role of plasminogen as an important component in streptococci adhesion is well established (Pancholi *et al.*, 2003), the role of enolase in bacterial adherence and host cell invasion has not, to our knowledge, been investigated previously.

In addition to identifying and inducing the expression of *S. suis* α -enolase (SsEno), we now demonstrate for the first time, to our knowledge, that this protein not only has plasminogen-binding activity but also has important fibronectin-binding properties. Finally, we also demonstrate that surface SsEno, which is present in all the described serotypes of *S. suis*, may play an important role in the processes of adhesion and invasion of host endothelial cells.

METHODS

Bacterial strains and growth conditions. Reference strains from the 35 different *S. suis* serotypes came from our collection. Highly virulent strain 166' of *S. suis* serotype 2 (Berthelot-Herault *et al.*, 2005) was kindly provided by Dr M. Kobisch (AFSSA, Ploufragan, France). *S. suis* strains were grown in Todd-Hewitt broth (THB) (BD) as described previously (Al-Numani *et al.*, 2003). Late-exponential-phase bacteria were washed in PBS, pH 7.3. *Escherichia coli* strain BL21DE3 was used for expression experiments. *E. coli* was grown in Luria-Bertani broth or on agar plates. Ampicillin (100 μ g ml⁻¹; Roche) was used in growth media when required.

Isolation of 52 kDa protein by fibronectin-affinity chromatography. Strain 166' was harvested from THB by centrifugation at 5500 g for 15 min, washed twice in PBS (0.01 M, pH 7.4), and resuspended in HEPES buffer (10 mM, pH 7.4). Bacteria were disrupted by sonicating the bacterial suspension with an ultrasonic probe (Sonics & Materials) on ice for 30 min with 15 s intervals of cooling on ice at 80 % duty cycle. Cell debris and non-lysed bacteria were removed by centrifugation. Supernatant proteins were concentrated to a volume of <1.0 ml using Amicon Ultra-15 concentrators (Millipore) and applied to a 5 ml fibronectin-coupled CNBr-activated Sepharose 4B column (Amersham Biosciences) pre-equilibrated with 0.1 M Tris/HCl buffer, pH 8.0. After washing with five column-volumes of this buffer, bound proteins were eluted with 30 ml 8 M urea. Starting lysates and fractionated samples were analysed by SDS-PAGE (12.5 % polyacrylamide) under reducing conditions, followed by Coomassie blue staining. Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951). Samples were maintained at -70 °C until analysis.

N-terminal sequencing was carried out with chromatographic fractions containing fibronectin-binding proteins after being electro-blotted onto PVDF membranes (Immobilon-P; Millipore) and visualized by staining with 0.1 % Ponceau S (Sigma-Aldrich) in 1 % acetic acid. Bands of interest were excised, destained with distilled water, and subjected to peptide sequencing at the Biotechnology Research Institute (Montreal, Canada).

Cloning, expression and purification of recombinant SsEno (rSsEno). The peptide sequence identified by N-terminal sequencing of the predominant fibronectin-binding protein was subjected to a homology search using the BLAST program against the strain P 1/7 genome sequence of *S. suis* (http://www.sanger.ac.uk/Projects/S_suis/). A database search revealed 100 % homology to the N-terminal region of a 1308 bp ORF that corresponds to the *S. suis* enolase (SsEno). The sequence was used to design primers for PCR amplification of the SsEno gene. The primers used were the Eno1-forward primer 5'-TATAAGGATCCTTGTCAATTATTACTGATGTTTACGC-3', introducing a *Bam*HI site (bold and underlined letters), and the Eno2-reverse primer 5'-TATAAAGCTTTTATTTTTCAGTTGTAGAATGAGTTCAAGCC-3', introducing a *Hind*III site (bold and underlined letters). Automated sequencing was then used to check the amplified enolase gene. The verified complete gene was cloned into pET-32a (Novagen) using the *Bam*HI and *Hind*III sites. This plasmid contains a His-tag-encoding sequence of about 25 kDa. The plasmid pET-32a-SsEno was introduced into *E. coli* BL21DE3 for IPTG-inducible expression of recombinant SsEno. Under native conditions, the His-tagged fusion protein was purified by HisTrap chromatography according to the manufacturer's protocols (Amersham Biosciences). Protein-containing fractions were determined by SDS-PAGE (12.5 % polyacrylamide) followed by Coomassie blue staining. Purified rSsEno was used to produce polyclonal antibodies to SsEno in rabbits, as described previously (Li *et al.*, 2006). To prepare affinity-purified anti-SsEno IgG, the polyclonal

serum was subjected to purification on protein-A Sepharose CL-4B (Amersham Pharmacia) (Pancholi & Fischetti, 1998).

Enolase activity. α -Enolase activity was determined by measuring the transformation of $\text{NADH}\cdot\text{H}^+$ to NAD^+ , as described elsewhere (Pancholi & Fischetti, 1998) with some modifications. Briefly, the enzymic reactions were performed at 37 °C in 100 mM HEPES buffer, pH 7.0, containing 5.0 mM MgSO_4 , 0.2 mM NADH (Roche), 0.25 mM 2-phosphoglycerate (2-PGE) (Sigma), 1.2 mM ADP (Roche), 10.7 IU lactate dehydrogenase (Roche) and 2.5 IU pyruvate kinase (Roche), in a final volume of 1 ml. The reaction was started by adding 100 μl of test solution containing rSsEno (0–20 μg). The α -enolase activity was measured in terms of the rate of reduction in the A_{340} (i.e. increase in the production of NAD from $\text{NADH}\cdot\text{H}^+$). Rabbit muscle enolase (Sigma) was used as a positive control and buffer without enolase was used as a negative control.

Localization of SsEno in *S. suis*. To evaluate the presence of SsEno in different bacterial compartments, *S. suis* strain 166' was grown overnight at 37 °C with agitation in 30 ml THB. The subcellular components (supernatant, cell wall, cytoplasmic and membrane fractions) were fractionated with mutanolysin by using the method of Yother & White (1994). Equivalent amounts of all fractions were analysed by SDS-PAGE (12.5% polyacrylamide) and Western blotting using optimally diluted rabbit anti-SsEno IgG.

The surface exposure of SsEno was examined by using immunoelectron microscopy. Briefly, *S. suis* 166' was grown in 5 ml THB until late exponential phase, centrifuged, and resuspended in PBS (pH 8.0). A 20 μl aliquot of the bacterial suspension was placed on nickel-Formvar grids (INRS, Institut Armand Frappier, Laval, Canada) and allowed to partially air dry. After blocking for 30 min with 10% normal donkey serum in dilution buffer (PBS, pH 8.0, 1% BSA, 1% Tween 20), the grids were incubated with optimally diluted rabbit anti-SsEno IgG for 2 h at room temperature. The grids were washed three times with PBS, pH 8.0, 1% Tween 20 and incubated for 1 h at room temperature with 12 nm colloidal gold-Affinipure donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories). After several washes with PBS, 1% Tween 20, bacteria were stained with 1% phosphotungstic acid and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV. Bacteria coated with normal rabbit antibodies served as a negative control.

rSsEno binding assays for plasminogen and fibronectin. Purified rSsEno binding to immobilized fibronectin (Roche) and plasminogen (Sigma) was first determined by ELISA. Maxisorp flat-bottomed microtitre 96-well plates (Nunc) were coated with 100 μl of serial dilutions of fibronectin or plasminogen, depending on the experiment, in 0.1 M carbonate coating buffer [0.15% (w/v) Na_2CO_3 , 0.1% (w/v) $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$, 0.3% (w/v) NaHCO_3 (pH 9.6)] and incubated overnight at 4 °C. Casein-coated wells served as a control for non-specific adhesion of rSsEno. The plates were washed three times with PBS, pH 7.3, containing 0.05% (v/v) Tween 20 (PBST), and 200 μl 3% (w/v) non-fat dried milk in PBST was added to each well to prevent non-specific binding. After 1 h at 37 °C, the wells were washed three times with PBST. Next, 100 μl 5 μg rSsEno ml^{-1} was added and the plates were incubated for 2 h at 37 °C. After several washes with PBST, optimally diluted rabbit anti-SsEno IgG was added to each well and the plates were incubated for 1 h at 37 °C. The wells were washed three times with PBST and specific horseradish peroxidase-labelled IgG (Jackson ImmunoResearch Laboratories) was added. Plates were then incubated for 1 h at 37 °C with the secondary antibody and 3,3',5,5'-tetramethylbenzidine (Zymed) was used as the enzyme substrate according to the manufacturer's instructions. The reactions were stopped by adding 25 μl H_2SO_4 (0.5 M) and were read at 450 nm using a microplate reader (Uvmax; Molecular Devices).

Binding interactions between rSsEno (75 kDa) and fibronectin (440 kDa) or plasminogen (92 kDa) were also analysed in real-time using Biacore 2000/3000 instrumentation. Surface plasmon resonance (SPR) assays were performed on research-grade CM4 sensor chips at 25 °C using filtered (0.2 μm) and degassed HBS-EP running buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% (v/v) Tween 20]. Prior to all experiments, the purity of protein preparations (>95%) was assessed by SDS-PAGE (12.5% polyacrylamide) under non-reducing and reducing [5% (v/v) 2-mercaptoethanol] conditions, followed by Coomassie blue and silver staining. Protein concentrations were determined using the Lowry method (Lowry *et al.*, 1951). The zwitterionic detergent Empigen was from Calbiochem; all other chemicals were of reagent-grade quality.

Fibronectin or plasminogen (10 μg ml^{-1} in 10 mM sodium acetate, pH 4.5) was immobilized to sensor chip surfaces using the Amine Coupling kit (Biacore) according to the manufacturer's recommendations. Similarly, corresponding reference surfaces were prepared in the absence of ligand addition. rSsEno (0–250 nM diluted in HBS-EP) was injected in-tandem over reference (no ligand) and active [290 relative units (RU) fibronectin or 140 RU plasminogen-immobilized] surfaces at 25 μl min^{-1} (600 s association + 600 s dissociation). Surfaces were regenerated at 50 μl min^{-1} using two 30 s pulses of solution I [HBS-EP containing 1 M NaCl, 5 mM NaOH, and 0.03% (v/v) Empigen] and solution II (HBS-EP), followed by EXTRACLEAN and RINSE procedures. Additional assay controls to test for binding specificity (BSA negative control), surface performance (consistent replicate injections), and lack of mass transport were performed as recommended by the manufacturer.

All binding data were double-referenced (Myszka, 1999) and representative of triplicate injections acquired from three independent trials. Due to the slow off-rates observed (less than 10% dissociation over 10 min), it was not possible to evaluate the data using standard kinetic (K_A , K_D) analysis in the BIAevaluation 4.1 software (Biacore). Instead, the steady-state binding responses were averaged (540–580 s plateau) and then analysed globally as a function of rSsEno concentration. For non-linear regression analysis (R_{eq} versus C), overall affinity constants were derived by fitting the data to a steady-state binding model:

$$R_{\text{eq}} = (R_{\text{max}} \cdot C) / (K_D + C)$$

where R_{eq} is the binding response at equilibrium, R_{max} is the maximal specific binding to the ligand surface, C is the concentration of analyte in solution, and K_D is the equilibrium dissociation constant. For linear regression analysis, K_D values were derived as the negative inverse of the slope generated by Scatchard transformations (R_{eq}/C versus R_{eq}).

Lysine-dependent rSsEno binding to fibronectin and plasminogen. To address the importance of lysine residues in the binding of rSsEno with fibronectin, competition binding assays were performed using the lysine analogue ε -amino-*n*-caproic acid (EACA). Briefly, fibronectin and the positive control plasminogen (10 and 1 μg ml^{-1} , respectively) were incubated overnight at 4 °C in Maxisorp flat-bottomed microtitre 96-well plates, and an ELISA was performed as described above using 20 μg rSsEno ml^{-1} mixed with different concentrations of EACA (1, 5, 10 and 50 mM; Sigma) and rabbit anti-SsEno IgG as a primary antibody. Binding of SsEno to fibronectin and plasminogen in the absence of EACA competitor was considered to be 100%.

Role of SsEno in adhesion and invasion of *S. suis* to PBMEC. The porcine brain microvascular endothelial cell (PBMEC) line PBMEC/C1-2 (Teifel & Friedl, 1996), already tested with *S. suis* in our laboratory (Vanier *et al.*, 2004), was used. The adhesion assay to quantify total cell-associated (intracellular plus surface-adhered) bacteria was performed as described previously (Nizet *et al.*, 1997;

Vanier *et al.*, 2004), with some modifications. Briefly, *S. suis* strain 166' (10^7 c.f.u. ml^{-1}) was incubated with equal concentrations of either normal rabbit IgG or purified rabbit anti-SsEno IgG for 1 h at 37 °C in fresh cell culture medium without antibiotics. Confluent cell monolayers were inoculated with 1 ml aliquots of either bacterial suspension. The plates were centrifuged (800 g) and incubated for 2 h at 37 °C with 5 % CO_2 . The monolayers were then washed vigorously five times to eliminate non-specific bacterial attachment, incubated for 10 min at 37 °C in the presence of 200 μl 0.05 % trypsin, 0.03 % EDTA, and disrupted by scraping the bottom of the well and by repeated pipetting to liberate cell-associated bacteria in the presence of ice-cold deionized water. Serial dilutions of this cell lysate were plated onto THB agar and incubated overnight at 37 °C, after which the bacteria were counted. The invasion assay to quantify intracellular bacteria was performed in a similar manner. However, to kill extracellular and surface-adhered bacteria after the initial infection period, cells were washed twice with PBS, and 1 ml cell culture medium containing 100 μg gentamicin ml^{-1} and 5 μg penicillin G ml^{-1} (Sigma) was added to each well. After incubation for 1 h at 37 °C with 5 % CO_2 , monolayers were washed three times with PBS and processed as described above. Results obtained with bacteria incubated with normal rabbit IgG were considered as 100 % adhesion or invasion.

Statistical analysis. All experiments were performed at least three times with samples in triplicate. All numerical data presented here are expressed as means $\pm$ SD. Statistical significance was determined using Student's *t* test. Differences were considered significant at $P \leq 0.05$.

RESULTS

Identification of a major *S. suis* fibronectin-binding protein of 52 kDa as an α -enolase

Loading of *S. suis* lysates onto a fibronectin-affinity column resulted in the specific retention and elution of a predominant native 52 kDa protein along with very few faint bands of additional proteins (Fig. 1). N-terminal amino acid sequencing of the 52 kDa protein revealed the following 14 residues: MIITDVYAREVLDS. Comparison of this 14 aa N-terminal sequence with the genomic sequence database of *S. suis* (http://www.sanger.ac.uk/Projects/S_suis/) revealed 100 % homology to the N-terminal region of a 1308 bp ORF that encodes a putative 435 aa protein with a deduced molecular mass of 47.09 kDa. A protein BLAST search with the putative protein sequence in the NCBI sequence database (<http://www.ncbi.nlm.nih.gov/BLAST/>) revealed significant homology to bacterial α -enolases, a family of proteins involved in carbohydrate transport and metabolism. The identity between the putative *S. suis* protein and the α -enolase from the closely related streptococci was more than 93 % (data not shown). These results indicated that this *S. suis* 52 kDa protein is most likely an α -enolase (SsEno). Sequence analysis showed that the SsEno contains neither a signal peptide nor an LPXTG motif. This protein also does not contain choline-binding repeats. Immunoblot analysis confirmed the cross-reactivity of SsEno with polyclonal antibodies against *Streptococcus pyogenes* enolase (SEN; data not shown).

Fig. 1. Isolation of a 52 kDa protein by fibronectin-affinity chromatography. *S. suis* lysate was applied to a fibronectin-coupled CNBr-activated Sepharose 4B column pre-equilibrated with 0.1 M Tris/HCl buffer, pH 8.0. After washing, bound protein was eluted with 30 ml 8 M urea elution buffer. Elution profiles were analysed by SDS-PAGE (12.5 % polyacrylamide) with Coomassie blue staining. Lanes: 1, Molecular markers; 2, *S. suis* total proteins; 3, *S. suis* fibronectin-binding proteins. Large arrow, 52 kDa enolase; small arrows, other fibronectin-binding proteins.

rSsEno has enolase activity

rSsEno was successfully cloned and expressed as a His-tagged fusion protein of ~75 kDa in *E. coli*. The higher molecular mass of the cloned protein compared with the native protein (52 kDa) is due to the N-terminal sequence of about 25 kDa containing the His-tag added by the plasmid. The recombinant protein was purified to homogeneity (>95 %) using HisTrap affinity chromatography (Fig. 2), and it presents cross-reactivity identical to that of the native protein, as shown by immunoblot with antibodies against SEN (results not shown). To confirm that the fibronectin-binding protein candidate was an α -enolase, its activity was assayed in a coupled enzyme assay for enolase. Similarly to the positive control used, we demonstrated that the recombinant protein was able to convert NADH to NAD, resulting in a change in A_{340} (Fig. 3). This indicated that pyruvate was converted to lactate and NADH by lactate dehydrogenase, confirming the conversion of phosphoglycerate to phosphoenolpyruvate by α -enolase, and finally to pyruvate in the presence of externally provided pyruvate kinase and ADP in a sequential manner.

SsEno has fibronectin- and plasminogen-binding activities

Prior to all ELISA and SPR analyses, the purity (>95 %) of commercially available fibronectin and plasminogen (data not shown) and rSsEno preparations was determined by scanning densitometry of the protein on an SDS-PAGE gel stained with Coomassie blue (data not shown) and on a

Fig. 2. Expression and purification of recombinant SsEno. The *SsEno* gene was cloned into pET-32a, which was introduced into *E. coli* BL21DE3 for IPTG-inducible expression of rSsEno (recombinant *S. suis* enolase). The His-tagged fusion protein was purified by chromatography on a HisTrap column according to the manufacturer's protocols. Lanes: 1, molecular mass markers; 2, non-induced *E. coli*; 3, induced *E. coli*; 4, French press control; 5, HisTrap non-adhered proteins; 6, HisTrap-purified SsEno.

silver-stained SDS-PAGE gel (Fig. 4a). Qualitatively, ELISA assays provided the initial demonstration that rSsEno interacts specifically with immobilized fibronectin and plasminogen (positive control) in a saturable, dose-dependent manner (Fig. 4b, c). Under similar assay conditions, rSsEno failed to interact with immobilized casein used as a negative control (Fig. 4d). Competition experiments with the lysine analogue EACA showed lysine-dependent interactions between rSsEno and fibronectin or

plasminogen (Fig. 5). Notably, the inhibition of rSsEno binding by EACA was more pronounced with plasminogen than with fibronectin.

To complement the ELISA datasets with more detailed quantitative kinetic analysis, rSsEno binding interactions were also examined in real-time by SPR. Specific, dose-dependent binding of rSsEno to immobilized fibronectin or plasminogen (positive control) was characterized by slow association (K_A) and slow dissociation (K_D) rates in both cases (Fig. 6a, c). Under similar assay conditions, BSA failed to interact with the immobilized surfaces used as a negative control (data not shown). Due to the slow off-rates observed for rSsEno binding to fibronectin or plasminogen (less than 10% dissociation over 10 min), it was not possible to evaluate the data using standard kinetic (K_A , K_D) analysis in the BIAevaluation 4.1 software (Biacore). Instead, steady-state binding responses were averaged at the end of the association phase (540–580 s plateau) and then analysed globally as a function of rSsEno concentration. Linear and non-linear regression analyses (Fig. 6b, d) indicated similarly high-affinity interactions between SsEno and fibronectin ($K_D \sim 21$ nM) and SsEno and plasminogen ($K_D \sim 14$ nM).

SsEno is present both intracellularly and at the bacterial surface

Western blot results showed that SsEno is present in *S. suis* supernatant, cell wall and cytoplasmic fractions, with negligible amounts in the membrane fraction (data not shown). Immunoelectron microscopy using the same antibody confirmed the surface location of SsEno on *S. suis* (strain 166') cells (Fig. 7a). In contrast, no labelling was observed for the negative control (Fig. 7b).

Fig. 3. Enolase activity assay of recombinant SsEno. α -Enolase activity of the purified rSsEno was measured using a coupled enzyme assay catalysing 2-phosphoglycerate to phosphoenolpyruvate, as described in Methods. (a) Enzyme kinetics of the purified rSsEno were determined, using 1 μ g purified rSsEno (▲) or 1 μ g rabbit muscle enolase as positive control (■) as the enzyme source. As a negative control, enolase was omitted (◆). α -Enolase activity of SsEno was also measured as described in Methods. (b) Complementary assay, in which different SsEno concentrations were used as enzyme source and the enzyme activity in the reaction mixture was measured at the end of 1 min incubation. Data represent results of one individual experiment. The enolase assay was done at least in triplicate.

Fig. 4. Qualitative binding of SsEno to fibronectin and plasminogen. (a) Silver nitrate staining of SsEno used for ELISA and SPR tests. (b–d) Representative ELISA analysis for the binding of SsEno ($5 \mu\text{g ml}^{-1}$) to immobilized plasminogen (b), fibronectin (c) and casein (d, negative control). Binding was evaluated after 2 h. Data are expressed as mean $\pm$ SD of at least three experiments with samples in triplicate.

SsEno participates in *S. suis* adhesion to and invasion of PBMEC

To evaluate the potential role of SsEno in *S. suis* adhesion to and invasion of PBMEC, bacterial SsEno was blocked with affinity-purified rabbit anti-SsEno IgG. As shown in Fig. 8, anti-SsEno IgG treatment decreased the adhesion of *S. suis* serotype 2 strain 166' to PBMEC to $\sim 46\%$ compared with the control. The invasion of PBMEC by

S. suis was also decreased to $\sim 79\%$ compared with the control.

DISCUSSION

Over the past 15 years, *S. suis* infections have emerged as a major problem for the swine industry worldwide, and it is also considered an important zoonotic agent (Higgins & Gottschalk, 2005; Tang *et al.*, 2006; Ye *et al.*, 2006). The pathogen is able to spread systemically from the nasopharynx, resulting in either general septicaemia or infections of specific organs (e.g. endocarditis, meningitis and arthritis), followed frequently by death (Gottschalk & Segura, 2000; Madsen *et al.*, 2002). The palatine and pharyngeal tonsils are both potential portals of entry for *S. suis*, although the mechanisms and virulence factors that enable the pathogen to disseminate throughout the animal and colonize different tissues are not well understood.

Adhesion of pathogens to host tissues is a critical early step in the process of infection. The ability to bind to fibronectin is a characteristic that has been reported for many pathogens (Joh *et al.*, 1999). As fibronectin plays an important role in diverse normal physiological processes, its targeting appears to be an example of the exploitation of a host cell process in the establishment, maintenance or dissemination of infection (Knodler *et al.*, 2001). Although a previously reported FBPS has been described for *S. suis* (de Greeff *et al.*, 2002), a *fbps* mutant has been shown to adhere to fibronectin at similar levels to those of the wild-

Fig. 5. Lysine-dependent binding of SsEno to fibronectin and plasminogen. Different EACA concentrations were used to evaluate the role of the lysine residues in the binding of SsEno to plasminogen (black bars) and fibronectin (grey bars) by ELISA, as described in Methods. * $P < 0.05$ in comparison with the level of adhesion without competitor (considered to be 100 % adhesion). Data are expressed as mean $\pm$ SD of at least three experiments with samples in triplicate.

Fig. 6. Quantitative binding of SsEno to fibronectin and plasminogen. Representative SPR analysis of rSsEno (0, 3.9, 7.8, 15.6, 31.3, 62.5, 125 and 250 nM) injected (600 s association + 600 s dissociation) over amine-coupled fibronectin [290 RU; (a)] or plasminogen [140 RU; (c)] at $25 \mu\text{l min}^{-1}$. Equilibrium binding responses (R_{eq}) were averaged (540–580 s) and then evaluated according to the steady-state affinity model [R_{eq} versus concentration (C); fibronectin (b); plasminogen (d)]. Subsequent Scatchard transformations (R_{eq}/C versus R_{eq} ; insets) indicate similar high-affinity interactions between SsEno and fibronectin ($K_D \sim 21$ nM) and SsEno and plasminogen ($K_D \sim 14$ nM).

type parent strain (Vanier *et al.*, 2007a), suggesting that other major adhesins are also involved.

In the present study, using fibronectin-coupled affinity chromatography, we have identified a predominant *S. suis* fibronectin-binding native protein of 52 kDa. Subsequently, the protein was identified as an α -enolase based upon the high similarity of its sequence with other bacterial enolases (93% with other streptococcal α -enolases) and our biochemical tests confirmed enolase activity. Furthermore, SsEno is also cross-reactive with anti-SEN antiserum, which has been shown to react with α -enolase-like molecules on streptococci (Pancholi & Fischetti, 1998). This is the first time, to our knowledge, that the fibronectin-binding activity of an α -enolase has been described. These results were confirmed using rSsEno cloned and expressed in the present study. In fact, Antikainen *et al.* (2007) have recently reported that

α -enolases from other Gram-positive bacteria, such as *S. pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Lactobacillus crispatus* and *Lactobacillus johnsonii*, do not possess the ability to bind to fibronectin. Thus, the interaction of enolase with fibronectin may serve different purposes in *S. suis* from those in the pathogenic and commensal bacteria studied in that work. Interestingly, after a complete proteome analysis, Jing *et al.* (2008) have recently proposed the enolase as one of two putative virulence-associated factors for *S. suis* serotype 2. However, the hypothetical plasminogen binding of the *S. suis* enolase (based on earlier studies performed with other streptococci) is proposed in that study as the mechanism by which this virulence factor participates in the pathogenesis of the infection caused by this pathogen.

To possess fibronectin-binding activity, it was anticipated that SsEno should be exposed at the surface of bacteria.

Fig. 7. Surface localization of SsEno on *S. suis*. (a) Immunogold electron microscopic detection of the location of SsEno ($\times 45\,000$ magnification) on *S. suis* pretreated with rabbit anti-SsEno IgG, followed by colloidal-gold-labelled anti-donkey IgG (gold particle sizes are 12 nm; indicated by the arrows). (b) Normal rabbit IgG used as a negative control. Bars, 250 nm.

Fig. 8. Decrease in the adhesion to and invasion of PBMEC by *S. suis* strain SS166' caused by antibodies against SsEno. *S. suis* strain 166' was preincubated with the appropriate concentrations (10 μ g) of antibodies at 37 °C for 60 min prior to infection. Adhesion was determined after exposure of PBMEC to *S. suis* (10^7 c.f.u. ml $^{-1}$), followed by extensive washing of non-adherent bacteria and cell lysis to obtain *S. suis* viable plate counts on THB. Invasion was determined as described above, except that after washing the bacteria, the cells were exposed to antibiotics to kill extracellular bacteria. * $P < 0.05$ in comparison with the level of adhesion/invasion with control antibodies (considered to be 100 % adhesion/invasion).

Fractionation of *S. suis* strain 166' showed that SsEno is present in all the subcellular components, including the bacterial surface, as demonstrated for other pathogens, including *S. pyogenes* (Pancholi & Fischetti, 1998). ELISA tests carried out with whole bacteria and anti-SsEno also showed a clear recognition of this protein at the bacterial surface (M. Esgleas and others, unpublished observations). Electron microscopy corroborated this surface localization and also showed that SsEno is present at the surface in low abundance, a feature that has also been described for *S. pneumoniae* enolase (Kolberg *et al.*, 2006). Moreover, such a relatively low amount has proven sufficient for contributing to bacterial pathogenesis (Bergmann *et al.*, 2003). Interestingly, in addition to serotype 2, all reference strains of other serotypes of *S. suis* were found to express surface-exposed SsEno (M. Esgleas and others, unpublished observations). The mechanisms by which this protein is translocated to the bacterial surface are still unknown. In fact, the amino acid sequence of SsEno shows the absence of an N-terminal signal sequence and of an anchor C-terminus LPXTG motif. It has been demonstrated that this LPXTG motif is necessary for the translocation of proteins to the bacterial surface because it is recognized by a transpeptidase (sortase A) that cleaves between the Thr and Gly residues (Schneewind *et al.*, 1995). Once cleaved, the carboxyl group of the Thr residue is linked to a free amino group of a branch peptide within the peptidoglycan cell wall, permitting the anchorage of these LPXTG-containing proteins to the bacterial cell wall (Schneewind *et al.*, 1995). Interestingly, we have shown recently that disruption of the *srtA* gene in *S. suis* results in only a slight decrease in adhesion to fibronectin (Vanier *et al.*, 2007b), confirming that anchorless (such as the enolase) rather than LPXTG-motif proteins play a major role in the adhesion to this ECM protein.

To date, the major function described for surface enolases is a strong plasminogen-binding activity (Pancholi &

Fischetti, 1998; Pancholi, 2001). In this study we confirmed that, in addition to a previously described GAPDH (Jobin *et al.*, 2004), SsEno is also an *S. suis* plasminogen-binding protein. Although it has been demonstrated recently that enolases of other Gram-positive bacteria bind to laminin and/or collagen (Antikainen *et al.*, 2007), we could not identify any adhesion activity of rSsEno to either of these two proteins (M. Esgleas and others, unpublished observations). Interestingly, in addition to the fibronectin and plasminogen-binding activities of the rSsEno, a heat-shock protein activity [as described previously for other enolases (Iida & Yahara, 1985; Prasad *et al.*, 2003)] and an IgG-binding property of this protein have also been observed (M. Esgleas and others, unpublished observations).

The binding of purified rSsEno to both plasminogen and fibronectin was demonstrated qualitatively by ELISA and quantitatively by SPR. Interestingly, a higher amount of fibronectin, compared with that used for plasminogen, had to be used in the ELISA test to obtain similar absorbances. Although this might be interpreted as a significantly higher affinity (which was not confirmed by SPR), this is not the case. The ELISA test was used in this study as a qualitative test, since plasminogen and fibronectin have different molecular masses and their attachment to plastic wells may vary with the conditions used for the ELISA. In particular, SPR analysis indicated clearly that rSsEno interactions with plasminogen and fibronectin were of similarly high affinity (K_D ~14 nM and ~21 nM, respectively). High-affinity binding between SsEno and fibronectin is consistent with other bacterial fibronectin-binding adhesins, including F1 and F2 of *S. pyogenes* (Ensenberger *et al.*, 2001; Kreikemeyer *et al.*, 2004). These results support our hypothesis that the specific interaction between SsEno and fibronectin is biologically significant. Both ELISA and SPR also demonstrated, as described for other pathogens, that the interaction between rSsEno and plasminogen is specific, suggesting that this interaction is also a biologically significant event. This provides further evidence to support the earlier finding that bacterial enolases interact with plasminogen with high affinity (Antikainen *et al.*, 2007; Kinnby *et al.*, 2008). In fact, the low nanomolar affinity constant between SsEno and plasminogen obtained in this study agrees with values obtained for the adhesion to plasminogen of enolases from *S. pyogenes* (Pancholi & Fischetti, 1998) and *S. pneumoniae* (Bergmann *et al.*, 2003).

Plasminogen-binding interactions were originally described as being mediated by recognition of the C-terminal lysine residues of eukaryotic and prokaryotic enolases by the lysine-binding sites of plasminogen (Redlitz *et al.*, 1995). Later, Bergmann *et al.* (2003) identified another internal plasminogen-binding site in the pneumococcal enolase (FYDKERKVVY) that is located on the outer surface of the octameric molecule (Ehinger *et al.*, 2004), in which the lysines and glutamic acid are important for plasminogen binding (Bergmann *et al.*, 2005). Importantly, SsEno possesses both plasminogen-binding motifs (M. Esgleas and others, unpublished observations), which

could explain the similar nanomolar affinity constant of both *S. pneumoniae* (Bergmann *et al.*, 2003) and *S. suis* enolase to plasminogen. Binding of plasminogen to *S. suis* enolase is inhibited by the lysine analogue EACA, which indicates involvement of lysine residues in this binding activity. Interestingly, SsEno adhesion to fibronectin is also decreased by EACA, indicating that lysine residues are also important for its fibronectin-binding activity. However, further research is required to determine the specific domain(s) and the individual amino acids that participate in the binding of SsEno to fibronectin.

As mentioned above, the pathogenesis of *S. suis* infection is poorly understood at present. Once in the bloodstream, *S. suis* resists phagocytosis and killing by neutrophils and monocytes (Chabot-Roy *et al.*, 2006; Charland *et al.*, 1998; Segura *et al.*, 1998; Smith *et al.*, 1999). In the event that *S. suis* fails to cause acute fatal septicaemia, bacteria are able to reach the CNS via different mechanisms that are only partially elucidated, such as adhesion to, with or without toxicity, and invasion of brain microvascular endothelial cells (BMEC) (Benga *et al.*, 2005; Charland *et al.*, 2000; Vanier *et al.*, 2004) and/or choroid plexus epithelial cells (Tenenbaum *et al.*, 2005, 2006). In fact, interactions of *S. suis* with both fibronectin and plasminogen may play a role in some of these mechanisms. For example, fibronectin-binding proteins of streptococci and staphylococci have been reported to mediate bacterial adhesion to and invasion of host cells (Oehmcke *et al.*, 2004; Talay *et al.*, 2000; Valentin-Weigand *et al.*, 1993). In the case of *S. suis*, it has been demonstrated recently that *S. suis* adhesion to and intracellular invasion of PBMEC increases more than 500 and 700 %, respectively, when bacteria are precoated with fibronectin (Vanier *et al.*, 2007a). Although the mechanisms by which fibronectin-binding proteins trigger the internalization of bacteria by mammalian cells are not completely understood, it has been suggested that binding of fibronectin to a host integrin might initiate a complex cascade of cell signalling that leads to reorganization of cytoskeletal components and consequent internalization of the bacteria (Schwarz-Linek *et al.*, 2004). Accordingly, *S. suis* invasion of endothelial cells is actin-dependent (Vanier *et al.*, 2004). In the present study, a role for SsEno in adhesion to and invasion of these cells has been shown by blocking the protein using affinity-purified anti-SsEno IgG. Since these studies have been carried out with cell monolayers, the fibronectin-binding activity of SsEno might explain this inhibition. It might be hypothesized that *S. suis* enolase is an important receptor for plasma fibronectin, which may increase bacterial adhesion to host cells. Another mechanism involved in adhesion of *S. suis* enolase to cellular fibronectin present in endothelial cells cannot be completely ruled out. However, further studies have to be carried out to demonstrate such an interaction. Although no increase in adhesion to/invasion of PBMEC was observed when increased concentrations of plasminogen were added (M. Esgleas and others, unpublished observations), a role for the plasminogen-binding activity

of SsEno (and GAPDH) *in vivo* cannot be discounted. In fact, it has also been demonstrated that plasminogen adhered to *S. suis* is able to acquire plasmin activity when co-incubated with human BMEC (Jobin *et al.*, 2005). As a consequence of plasminogen activation on bacterial surfaces, bacteria become armed with the broad-substrate-spectrum proteolytic potential of plasmin that is not susceptible to regulation by host-derived inhibitors (Lottenberg *et al.*, 1994). The capture of plasminogen by adhesins such as SsEno and GAPDH and its conversion to plasmin has been described for other pathogens (Antikainen *et al.*, 2007; Kinnby *et al.*, 2008) and can be used to facilitate bacterial penetration through biological membranes such as the blood-brain barrier, and therefore could represent an important determinant of virulence. The role of SsEno in studies using endothelial-cell monolayers in a trans-well system is currently under investigation in our laboratory.

In conclusion, we have demonstrated for the first time, to our knowledge, that *S. suis* α -enolase has not only a high affinity for plasminogen, as demonstrated for other bacterial enolases, but also a similarly high affinity for fibronectin. Moreover, we have shown that this protein is a novel *S. suis* adhesin that participates in bacterial adhesion to and invasion of endothelial cells. Our results indicate that SsEno of this human and porcine pathogen could be a key molecule in pathogenesis by facilitating bacterial interactions with host cells. This is in agreement with two recent studies that describe *S. suis* enolase as an antigenic protein and a putative virulence factor of *S. suis* serotype 2 (Jing *et al.*, 2008; Zhang & Lu, 2007). Mutants with defective export of enolase to the surface of the bacteria may give some insights into the role of this adhesin in the pathogenesis of *S. suis* infection.

ACKNOWLEDGEMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) grant 0680154280. Sheldon Biotechnology Centre is supported by a Research Resource Grant from the Canadian Institutes of Health Research (CIHR). Canada Foundation for Innovation (CFI) provided infrastructure for the SPR instrumentation. We thank Dr Vijay Pancholi (The International Center for Public Health, Newark, NJ) for providing the polyclonal antibodies against SEN. We also thank M. Mourez and N. Fittipaldi for useful discussions.

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Edited by: T. J. Mitchell