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ApuA, a multifunctional α-glucan-degrading enzyme of *Streptococcus suis*, mediates adhesion to porcine epithelium and mucus

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We have identified *apuA* in *Streptococcus suis*, which encodes a bifunctional amylopullulanase with conserved α-amylase and pullulanase substrate-binding domains and catalytic motifs. ApuA exhibited properties typical of a Gram-positive surface protein, with a putative signal sequence and LPKTGE cell-wall-anchoring motif. A recombinant protein containing the predicted N-terminal α-amylase domain of ApuA was shown to have α-(1,4) glycosidic activity. Additionally, an *apuA* mutant of *S. suis* lacked the pullulanase α-(1,6) glycosidic activity detected in a cell-surface protein extract of wild-type *S. suis*. ApuA was required for normal growth in complex medium containing pullulan as the major carbon source, suggesting that this enzyme plays a role in nutrient acquisition *in vivo* via the degradation of glycogen and food-derived starch in the nasopharyngeal and oral cavities. ApuA was shown to promote adhesion to porcine epithelium and mucus *in vitro*, highlighting a link between carbohydrate utilization and the ability of *S. suis* to colonize and infect the host.

INTRODUCTION

Streptococcus suis is a major porcine pathogen of significant commercial importance worldwide. In suckling and weaning pigs, it is the principal cause of acute meningitis, but can infect other organs leading to arthritis, serositis, endocarditis, otitis media and bronchopneumonia (Beineke *et al.*, 2008; Madsen *et al.*, 2002). Healthy pigs asymptomatically colonized with *S. suis* form a reservoir for this disease and play a major role in its epidemiology (Arends *et al.*, 1984). To date, 33 different capsule serotypes of *S. suis* have been identified, but serotype 2 is most commonly associated with disease worldwide (Gottschalk & Segura, 2000; Lun *et al.*, 2007). Serotype 2 strains were also associated with recent large outbreaks of severe human infections in China and elsewhere in Asia (Mai *et al.*, 2008; Tang *et al.*, 2006; Wertheim *et al.*, 2009). The recently obtained genome sequences of two virulent Chinese *S. suis* serotype 2 strains (98HAH12 and 05ZYH33) (Chen *et al.*, 2007) and P1/7, the European reference strain (http://www.sanger.ac.uk/Projects/S_suis/), led to the identification of a large number of potential surface and secreted proteins that might play a role in virulence, including a number of putative carbohydrate-degrading enzymes (Baums & Valentin-Weigand, 2009). Genes that encode carbohydrate-degrading enzymes are common in the genomes of other streptococcal pathogens and play a role in nutrient acquisition for growth and colonization on mucosal surfaces (Rollenhagen & Bumann, 2006; Shelburne *et al.*, 2006, 2008a, b). Dietary sources of highly polymerized α-glycans such as starch and glycogen are abundant in the human colon (Levitt *et al.*, 1987) and oropharynx (Mormann & Muhlemann, 1981; Shelburne *et al.*, 2005, 2007; Virtaneva *et al.*, 2005), as well as the epithelium of the vagina and lung (Gourlay *et al.*, 2009; Gregoire *et al.*, 1971; Santi *et al.*, 2008; van Bueren *et al.*, 2007). Degradation of starch and glycogen proceeds in most organisms via the action of amylases and pullulan-degrading enzymes (e.g. pullulanase and amylopullulanase), which cleave α-(1,4)- and α-(1,6) glycosidic linkages, respectively. Pullulan is a linear polysaccharide of maltotriose repeating units linked via α-(1,6) glycosidic bonds, produced by the ascomycete fungus *Aureobasidium pullulans* (Pouliot *et al.*, 2005). Although pullulan is not found in animals, it is commonly used as a substrate to identify pullulanases with α-(1,6) glycosidase activity (Chen *et al.*, 2007; Morgan *et al.*, 1979; Kanno & Tomimura, 1985).

In group A streptococci (GAS), group B streptococci (GBS) and *Streptococcus pneumoniae*, cell-wall-anchored enzymes that can hydrolyse pullulan have been characterized

Abbreviations: Erm, erythromycin; GAS, group A streptococci; GBS, group B streptococci; Spc, spectinomycin.

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(Bongaerts *et al.*, 2000; Hytönen *et al.*, 2003; Santi *et al.*, 2008). Recent research has shown an additional role for the streptococcal pullulanases in virulence. The GAS pullulanase PulA was shown to function as a strepadhesin, binding to several complex carbohydrate substrates including submaxillary mucin (Hytönen *et al.*, 2003). Additionally, recombinant PulA and the related pneumococcal SpuA have been shown to bind with high affinity to alveolar type II cell glycogen in the lung (van Bueren *et al.*, 2007). Further evidence for the role of SpuA in virulence comes from genome signature-tagged mutagenesis screens in *S. pneumoniae*, using a mouse pneumonia model to identify genes that decreased pathogen fitness *in vivo* (Hava & Camilli, 2002). Recently, it was shown that GAS PulA-deficient mutants were less able to adhere to human epithelial cells (Hytönen *et al.*, 2006). Furthermore, recombinant SAP, a type I pullulanase from GBS, was shown to bind human epithelial cells *in vitro* (Gourlay *et al.*, 2009; Hytönen *et al.*, 2006).

Here, we report on the characterization and mutagenesis of *apuA*, encoding an extracellular bifunctional amylopullulanase that was identified in the genome of *S. suis*. Its potential role in virulence was investigated using binding assays with porcine epithelial cells and mucin.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. suis* strains were grown on Todd–Hewitt broth (THB; Oxoid) or Columbia agar plates with 6 % sheep blood (Oxoid) at 37 °C under 5 % CO_2 for 18 h. An OD_{600} of 1.0 with a 1 cm path length corresponds to approximately 10^9 bacterial c.f.u. ml^{-1}.

Escherichia coli VE7108 (derived from TG1) was cultured in Luria–Bertani (LB) broth or LB agar (Difco) at 37 °C for 18 h. When necessary, antibiotics were added to culture media at the following concentrations: for *E. coli*, 150 μg erythromycin (Erm) ml^{-1} and 50 μg spectinomycin (Spc) ml^{-1}; for *S. suis*, 2 μg Erm $ml^{-1}$and 100 μg Spc ml^{-1}.

The use of pullulan as a sole carbon source for growth was demonstrated using red pullulan agar [1 % peptone, 0.1 % NH_4Cl, 0.1 % red pullulan (Megazyme) and 2 % agar] as described previously (Lai *et al.*, 2005).

A complex medium (CM) comprising 10 g proteose peptone l^{-1}, 5 g trypticase peptone l^{-1}, 5 g yeast extract l^{-1}, 2.5 g KCl l^{-1}, 1 mM urea and 1 mM arginine (pH 7.0) was used to assess growth on different carbon sources by supplementation with different carbohydrates at a final concentration of 1 % (w/v), as described previously (Santi *et al.*, 2008). Growth in CM was determined by measurement of OD_{600} using a SpectraMax M5 reader.

Nucleotide and protein sequence analyses. BLAST searches with the genome sequence of *S. suis* strain P1/7 (serotype 2) were performed using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.nlm.nih.gov). Sequence alignments were performed using the CLUSTAL W program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Signal peptide motifs were identified in protein sequences using the SignalP version 1.1 software (http://www.cbs.dtu.dk/services/SignalP/).

Expression and purification of recombinant α-amylase domain. The nucleotide sequence predicted to encode the mature α-amylase domain of ApuA (amino acids 51–855) was amplified by PCR from *S. suis* strain 10 genomic DNA using Go*Taq* DNA polymerase (Promega) and primers AmyF and AmyR as listed in Table 2. The purified PCR products were ligated to the pTrcHis TOPO TA expression vector

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genetic markers and/or description*	Reference
Strains		
E. coli		
VE7108	*supE hsd*Δ5 *thi* Δ(*lac–proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *repA*$^+$	Mora *et al.* (2004)
VE6838	*supE hsd*Δ5 *thi* Δ(*lac–proAB*) F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]; VE7108 carrying pG$^+$host9	Mora *et al.* (2004)
TOP10	F$^-$ *mcrA* Δ(*mrr–hsdRMS–mcrBC*) *ϕ*80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara–leu*) 7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
S. suis serotype 2		
Strain 10	Virulent serotype 2 strain	Vecht *et al.* (1989, 1992)
apuA::*spc*	Isogenic *apuA*::*spc* mutant of strain 10	This work
Plasmids		
pTrcHis TOPO	Expression vector containing N-terminal His tag	Invitrogen
pTrc-amy	pTrcHis vector containing 2562 bp of *apuA*	This work
pDL282	Replication functions of pUC19 and pVT736-1, Amp^r Spc^r	Sreenivasan *et al.* (1991)
pKUN19-spc	pKUN19 containing Spc^r gene of pDL282	Konings *et al.* (1987)
pG$^+$host9	Erm^r, thermosensitive derivative of pGK12	Maguin *et al.* (1996)
pG9-*apuA*	pGhost$^+$9 derivative containing 3020 bp of *apuA*	This work
pG9*apuA*::*spc*	pG9-*apuA*::*spc* containing a 1.2 kb Spc^r fragment from pKUN19-spc cloned within *apuA*	This work

*Amp^r, Ampicillin-resistant; Erm^r, erythromycin-resistant; Spc^r, spectinomycin-resistant.

(Invitrogen) such that the expressed recombinant α-amylase would be fused to an N-terminal polypeptide containing six histidine residues for affinity purification. After transformation of *E. coli* TOPO 10, several clones were picked and checked for the correct insertion of the α-amylase gene fragment and verified by DNA sequencing. Expression of the amylase protein domain was induced by addition of 1 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG) to an exponential culture (OD_{600} of 0.6) for 3 h at 37 °C with shaking (250 r.p.m.). The cells were harvested by centrifugation (8000 ***g*** for 10 min at 4 °C) and the pellet was resuspended in lysis buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.4) containing a cocktail of protease inhibitors (Roche) and then disrupted using a high-pressure cell disrupter (Constant Systems). The soluble protein extract was recovered after high-speed centrifugation (14 000 r.p.m. for 40 min) and loaded onto a HisTrap affinity chromatography column (Amersham Pharmacia Biotech). Proteins were eluted in a phosphate buffer containing 500 mM NaCl and increasing concentrations of imidazole and checked by SDS-PAGE. Fractions containing purified fusion proteins of the expected size (approx. 105 kDa) were stored at 4 °C in aliquots in the elution buffer. Protein concentration was measured using a BCA protein assay (Thermo Scientific) according to the supplier's instructions.

Assay for α-amylase activity. α-Amylase activity was measured using red starch (Megazyme) according to the manufacturer's instructions. Briefly, red starch [1 % (w/v) in 0.5 M KCl] was incubated for 10 min at 40 °C with 500 μl of each protein fraction in 1 ml buffer B [0.1 M maleic acid, 2 mM calcium chloride, 0.01 % (w/v) sodium azide, pH 6.5]. Red starch is depolymerized by α-amylase to produce low-molecular-mass dye compounds that remain in solution on addition of 2.5 ml ethanol. After centrifugation at 1000 ***g*** for 10 min, the released dye was quantified in the supernatant by spectrophotometry (A_{510}; SpectraMax M5 reader).

Insertional inactivation of *apuA* : : *spc*. Primers used for mutagenesis are listed in Table 2. Chromosomal DNA was isolated using the CTAB extraction method after pretreatment with lysozyme (10 mg ml^{-1}) for 10 min at 37 °C as described previously (Orru *et al.*, 2006). An internal *Eco*RI–*Xho*I fragment (nucleotide positions 3114–6133) of the *apuA* gene was amplified using primers pulF and pulR and ligated into *Eco*RI- and *Xho*I-digested pG^{+}host9, a shuttle vector that is thermosensitive for replication in Gram-positive bacteria (Maguin *et al.*, 1996). The resulting plasmid pG9-*apuA* was introduced into competent *E. coli* strain VE7108 (Mora *et al.*, 2004) by electroporation and transformants were selected on LB agar containing Erm. Plasmid pG9-*apuA* extracted from the transformants was linearized by inverse PCR using *Pfu* polymerase (Promega) and the internal *apuA* primers pulF-*Pvu*I and pulR-*Bgl*II. An *spc* cassette, containing the promoter and transcriptional terminator, was amplified from plasmid pKUN19-Spc (Konings *et al.*, 1987) using primers 5′Spc-*Pvu*I and 3′Spc-*Bgl*II. Both the inverse PCR product of pG9-*apuA* and the *spc* cassette were then digested with *Pvu*I and *Bgl*II enzymes and ligated using T4 DNA ligase (Promega) to generate pG9-*apuA* : : *spc*, which contains an *spc* cassette inserted between nucleotides 4564 and 4624 of *apuA*.

Purified plasmid pG9-*apuA* : : *spc* was transformed into competent *S. suis* strain 10 by electroporation as described previously (Smith *et al.*, 1995). The transformants were selected on agar plates containing 2 μg Erm ml^{-1} at 28 °C, the permissive temperature for replication of pG^{+}host9. Transformants were then grown at 37 °C, the non-permissive temperature of replication, on Colombia agar containing Erm and Spc to select for chromosomal integration. The integrants were serially passaged for 5 days in liquid medium at 28 °C without Erm selection to select for loss of the plasmid via a double crossover event, leaving the *spc* gene insertion in *apuA* : : *spc* (Biswas *et al.*, 1993). Erythromycin-sensitive colonies with the *apuA* : : *spc* phenotype were verified by PCR using primers apuaF and apuaR.

Pullulanase activity of cell wall, intracellular and secreted proteins. The cell-free supernatant (secreted proteins) and cytoplasmic and cell-wall protein fractions of wild-type (wt) and *apuA* : : *spc* mutant were assayed for pullulanase activity. To obtain streptococcal secreted proteins, 90 ml overnight culture grown in THB was pelleted (10 000 ***g*** for 10 min at 4 °C) and 10 ml supernatant was collected and concentrated to a final volume of 1 ml using a 10 kDa filter (Sartorius). To extract the cell-wall proteins, the resultant bacterial pellet was incubated for 1.5 h at 37 °C in 1 ml extraction buffer (30 mM Tris/HCl, pH 8.0, 3 mM $MgCl_2$, 25 % sucrose) containing protease inhibitors (Roche), 1 mg lysozyme ml^{-1} and 125 U mutanolysin ml^{-1} (Sigma). The bacterial suspension was then pelleted by centrifugation (10 000 ***g*** for 10 min at 4 °C) and the supernatant, containing cell-wall proteins, was concentrated using a 10 kDa filter (Sartorius) to a volume of approximately 1 ml. The pellet of osmotically fragile protoplasts was lysed by resuspension in 10 ml buffered saline (pH 7.0) containing 5 mM $MgCl_2$. The suspension was allowed to stand at room temperature for 15 min and then centrifuged at 10 000 ***g*** for 30 min at 4 °C (Law, 1978). The clear supernatant containing the cytoplasmic proteins was concentrated as described above. The concentration of protein in each fraction was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies).

Pullulanase activity was determined by measuring the enzymic release of reducing groups from α-glucans using red pullulan (Megazyme) as substrate. Briefly, red pullulan [1 % (w/v) in 0.5 M KCl] was incubated for 10 min at 40 °C with 1 ml cell-wall protein-extracted cells. The red

Table 2. Oligonucleotide primers used in this study

Sequences in bold and underlined correspond to gene sequence and restriction sites, respectively.

Primer	Sequence (5′–3′)	Purpose
amyF	CTTTCGGAACAGGATGGC	Cloning of α-amylase domain in pTrcHisTopo
amyR	GACGATGTCACCTGCTTCTG	
pulF-*Eco*RI	GCTATCGAATTCTA**TACCGATGGCAATTATGAT**	Cloning of amylopullulanase fragment in pGhost+9
pulR-*Xho*I	TCGAATCTCGAGAT**CTTGTCCAGACGCTTGAG**	
pulF-*Pvu*I	GCTAGTCCATGGTA**CTGCCTCCATGAAGTGATAAT**	Inverse PCR of pG9-*apuA*
pulR-*Bgl*II	TCGTCGATCGCG**CGTCGTGTCTTAGTTGATTCC**	
Spc-*PvuI*	GCTATACCATGGTA**AGGTCGACTCTAGAGGATC**	Insertion of *spc* gene into pG9-*apuA*
Spc-*Bgl*II	TCGTAGCAGATCTCG**TTATAATTTTTTTAATCTGTTATTTA**	
apuaF	TGGGTGTGATTTTGGATGTG	To check for correct mutant clones using colony PCR
apuaR	TAAAGGCCAGCTCAATTGCT	

pullulan substrate was depolymerized by an endo-mechanism to produce dyed fragments which remain in solution on addition of 2.5 ml absolute ethanol. High-molecular-mass material was removed by centrifugation, and the soluble dye was measured in a spectrophotometer (A_{510}; SpectraMax M5 reader). The pullulanase activity in the *S. suis* protein extract was calculated using a standard curve generated with purified pullulanase from *Klebsiella planticola* (Sigma). One unit of activity is the amount of enzyme required to split one micromole of α-(1,6) linkages per minute under the defined assay conditions.

Cell line and culture conditions. Newborn pig tracheal cells (NPTr) (Ferrari *et al.*, 1993) were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (1 : 1), 5 mM glutamine (Gibco) supplemented with 10 % fetal calf serum (Gibco), without antibiotics, at 37 °C and 5 % CO_2. The cells were seeded into new flasks every 4–5 days by detachment with 0.25 % (w/v) trypsin and 1 mM tetrasodium EDTA (trypsin-EDTA; Gibco-Invitrogen) and replacement of the medium (Ferrari *et al.*, 2003). For the adherence assay, approximately 2.3×10^5 cells per well were seeded in antibiotic-free complete medium on 12-well tissue culture plates (Costar) and incubated until they reached confluence.

Adherence assays using NPTr cell line. For the adhesion assay, bacteria were pelleted by centrifugation, washed with PBS and resuspended at 10^9 c.f.u. ml^{-1} in fresh cell culture medium without antibiotics. Bacterial suspensions diluted in cell culture medium (between 1.15×10^5 and 2.3×10^7 c.f.u. ml^{-1}) were added to wells containing a monolayer (2.3×10^5) of epithelial cells in 1 ml medium (m.o.i. ranged from approximately 5 to 100 bacteria per cell). Plates were incubated for 2 h at 37 °C with 5 % CO_2. Cell monolayers were washed three times with PBS and detached by scraping in 800 µl ice-cold MilliQ water. To enumerate viable bacteria, serial dilutions of the cell lysate were plated in triplicate on Columbia sheep-blood agar plates and incubated at 37 °C for 24 h. The number of bacteria recovered in this assay was expressed as a percentage of the original inoculum.

Binding of *S. suis* to porcine mucus. A modified solid-phase mucin-binding assay was performed with purified porcine gastric mucin (Sigma) as described previously (Ryan *et al.*, 2001). Briefly, a 96-well microtitre plate (Nunc Inc.) was inoculated with 7.5 µg purified porcine gastric mucin in 250 µl $NaHCO_3$ (pH 8.0) and incubated overnight at 37 °C (mucin-coated wells). Overnight bacterial cultures were pelleted by centrifugation (8000 ***g*** for 5 min), washed in PBS and adjusted to an OD_{600} of 1.0. Triplicate wells in both plates were inoculated with 2.5×10^8 and 2.5×10^7 bacteria in a volume of 100 µl in PBS. The microtitre plates were incubated for 2 h at 37 °C and 5 % CO_2. The wells were then washed eight to ten times with sterile PBS. Bound bacteria were desorbed with 250 µl 0.03 % Triton X-100 in sterile PBS for 1 h at room temperature and enumerated by plating on blood agar for 12–14 h at 37 °C. This concentration of Triton X-100 was shown not to affect the viability of *S. suis* under these conditions (not shown).

Statistical analysis. Adherence assays and mucin-binding experiments were performed at least three times using triplicate samples. All numerical data presented here are expressed as means ± SD. Statistical significance was determined using a two-tailed Student's *t*-test. Differences were considered significant at $P\leqslant0.05$.

RESULTS

Identification and analysis of *S. suis* serotype 2 amylopullulanase-encoding gene

A gene designated here *apuA* (6285 bp; 2094 amino acids) and predicted to encode an amylopullulanase was identified in the genome sequence of *S. suis* P1/7 (YP_003027676.1). Based on the presence of a putative signal peptide sequence and a C-terminal LPNTG motif (residues 2059–2064) (Janulczyk & Rasmussen, 2001), ApuA is predicted to be a 230 kDa mature surface protein covalently linked to the cell wall. The *apuA* gene is located upstream of a putative transcriptional regulator gene with homology to the *lacI* family and downstream of genes encoding a putative sugar-specific permease (*sgaT*) classified as component IIC that belongs to a sugar phosphotransferase system (Fig. 1). The ApuA protein was predicted to contain a distinct α-amylase domain (aa 103–860) and a pullulanase domain (aa 921–1962) (Fig. 1). Upstream of each functional domain lie two tandem repeats belonging to carbohydrate-binding motif family 41 (CBM41), which bind tightly to α-glucan polysaccharides containing α-(1,4) glycosidic and α-(1,6) glycosidic linkages (van Bueren *et al.*, 2007). Within the protein, two pairs of four regions highly conserved in α-amylase-like proteins were identified (I, II, III and IV) which form the catalytic triad Asp–Glu–Asp (Doman-Pytka & Bardowski, 2004; Kuriki & Imanaka, 1999).

At the protein level, ApuA shares 47 and 60 % identity with the predicted alkaline amylopullulanase in *Bacillus* sp. KSM-1378 (Hatada *et al.*, 1996) and the putative amylopullulanase in *Streptococcus infantarius*, respectively (Table 3). Additionally, the predicted *S. suis* ApuA pullulanase domain shares 58 and 55 % identity with the well-characterized pullulanases described in pathogenic *S. pneumoniae* (SpuA), GAS (PulA) and *S. agalactiae* (SAP) (Table 3).

Both functional domains of ApuA contain the four highly conserved regions designated I, II, III and IV that are found in α-amylases, pullulanases and amylopullulanases (Fig. 1, Table 4). The two glutamic acid residues that are crucial for catalytic activity of these enzymes (Kuriki & Imanaka, 1999) lie at positions Glu_{661} and Glu_{1598} within conserved region III of the α-amylase and pullulanase domains, respectively (Table 4). Analogously, the catalytic aspartate residues are found at positions Asp_{632} (region I) and Asp_{727} (region IV) of the α-amylase domain and at positions Asp_{1569} (region I) and Asp_{1686} (region IV) within the pullulanase domain. The presence of the LPXTG motif and the distinct α-amylase and pullulanase domains, each possessing conserved catalytic and substrate-binding sites, suggested that ApuA is a cell-wall-anchored bifunctional amylopullulanase.

The α-amylase recombinant domain possesses α-1,4 glycosidic activity

The DNA fragment encoding the predicted α-amylase domain of ApuA was cloned in the IPTG-inducible expression vector pTrcHis to generate pTrc-amy. Induction of expression by IPTG for 3 h at 37 °C resulted in high-level production of a protein of the expected size (Fig. 2a). The recombinant amylase was purified by immobilized metal affinity chromatography using an

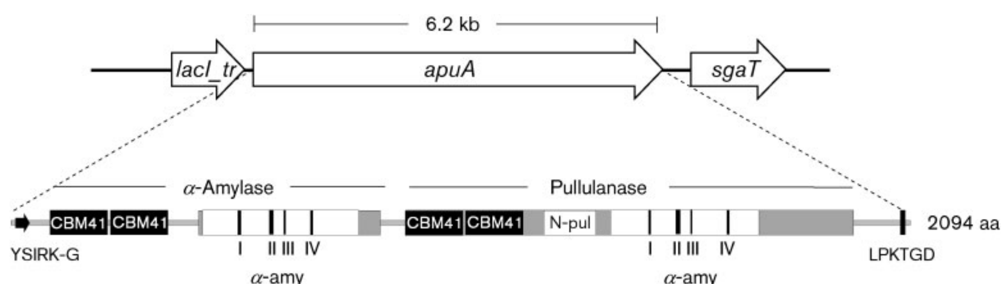

Fig. 1. Schematic representation of the *apuA* locus. ApuA is encoded by a single ORF of 6285 bp, producing a bifunctional amylase/pullulanase protein of 2094 amino acids. Located upstream of the *apuA* locus is a putative transcriptional regulator gene with similarity to *lacI_tr*. Downstream the *apuA* locus is a putative sugar-specific permease, component IIC (*sgaT*), belonging to a sugar phosphotransferase system. The α-amylase and pullulanase domains are respectively located in the N- and C-terminal regions. The N-terminal region includes a signal peptide of 41 amino acids containing a YSIRK-G domain. Within the N-terminal α-amylase domain are two tandem repeats designated CBM41, followed by an α-amylase (α-amy) catalytic domain belonging to glycosyl hydrolase family 13. In the pullulanase C-terminal domain, two tandem CBM41 repeats are followed by a specific pullulanase N-terminal domain (N-pul) associated with an α-amylase catalytic domain. The amylase and pullulanase domains contain a four-motif (I, II, III and IV) region that is highly conserved in α-amylase-like proteins.

imidazole gradient to obtain a fraction highly enriched for the expressed protein (Fig. 2a). The purified recombinant protein tested in the red starch assay comprised a single band on a Coomassie-stained protein gel and was compared to an extract from *E. coli* and 1 mg purified α-amylase from *Aspergillus oryzae* as a positive control. The α-amylase activity of 70 μg purified recombinant protein was 16-fold higher than 130 μg from the *E. coli* expression strain (Fig. 2b). This indicates that the activity of the purified recombinant protein is not due to contamination with residual amounts of *E. coli* glycosidases having an α-1,4 glucosidase activity. Thus, these results confirm that the N-terminal half of ApuA indeed possesses the predicted α-1,4 glucosidase activity.

Disruption of *apuA* in *S. suis* strain 10

To study the putative functions and expression of ApuA in *S. suis*, an isogenic *apuA* : : *spc* knockout mutant of strain 10 was constructed using the *E. coli*–Gram-positive shuttle vector pG^{+}host9 that shows thermosensitive replication in *Lactococcus lactis* (Maguin *et al.*, 1996). Plasmid pG^{+}host9 is able to replicate and be maintained episomally in *S. suis* at 30 °C but, at temperatures above 37 °C, it is segregationally unstable and lost in the absence of antibiotic selection. In the presence of antibiotic selection, growth at temperatures above 37 °C promotes recombination between homologous DNA cloned in pG^{+}host9 and the chromosome. A PCR-amplified *apuA* fragment containing the pullulanase domain (positions 3114–6133) was ligated to *Eco*RI- and *Xho*I-digested pG^{+}host9 and transformed in *E. coli* VE7108 to generate pG9-*apuA*. A spectinomycin-resistance cassette was then inserted into the middle of the *apuA* coding region using an inverse PCR strategy to generate the integration construct pG9-*apuA* : : *spc*. This construct was introduced into *S. suis* 10 by electroporation, resulting in ten transformants that were recovered at 28 °C in the presence of Erm (Fig. 3a; step 1). Single crossover events in these transformants were achieved by overnight growth in liquid medium containing Spc and Erm at 37 °C, the non-permissive temperature for plasmid replication in *S. suis* (Fig. 3a; step 2).

Integrant strains were serially passaged for 5 days in liquid medium at 28 °C without Erm selection to facilitate

Table 3. Structural comparison of pullulan-degrading enzymes and their subdomains in different Gram-positive species

Name	Enzyme	Strain	Identity (%)	Reference
ApuA	Amylopullulanase	*S. suis* P 1/7	100	This work
–	(?) Amylopullulanase	*S. infantarius* ATCC BAA-102	60	–
APase	Alkaline amylopullulanase	*Bacillus* sp. KSM-1378	47	Hatada *et al.* (1996)
–	(?) Pullulanase	*S. sanguinis* SK36	69	–
–	(?) Pullulanase	*S. equi* MGCS10565	66	–
SpuA	Pullulanase	*S. pneumoniae* serotype I 3.B	58	Bongaerts *et al.* (2000)
PulA	Pullulanase	*S. pyogenes* NZ131	58	Hytönen *et al.* (2003)
SAP	Type I pullulanase	*S. agalactiae* COH1	55	Santi *et al.* (2008)

Table 4. Conserved sequences of regions I, II, III and IV in the α-amylase and pullulanase domains

Two copies of the four regions highly conserved among α-amylases, pullulanases and amylopullulanases were identified in ApuA from *Bacillus* sp. KSM-1378. Amino acids shown in bold are conserved among all amylolytic enzymes, while the putative catalytic amino acids are underlined.

Strain	Region I	Region II	Region III	Region IV
***α*-Amylase**				
S. suis P 1/7	556**DVVLNH**	628A**FRVDTVKH**	661ETWG	722**FLGSHD**
Bacillus sp. KSM-1378	462**DVVLNH**	546Y**FRVDTVKH**	579EAWG	640**FLGSHD**
Pullulanase				
S. suis P 1/7	1501**DVVYNH**	1565**GFRFDMMGD**	1598**EG**WR	1681**YIAAHD**
Bacillus sp. KSM-1378	1396**DVVFNH**	1460**GFRFDMMGD**	1493**EG**WV	1578**YIEAHD**
S. sanguinis SK36	715**DVVYNH**	779**GFRFDMMGD**	812**EG**WK	895**YIAAHD**
S. equi MGCS10565	734**DVVYNH**	798**GFRFDMMGD**	831**EG**WV	914**YIAAHD**
S. pneumoniae S1 3.B	717**DVVYNH**	781**GFRFDMMGD**	814**EG**WR	897**YIAAHD**
S. pyogenes NZ131	664**DVVYNH**	728**GFRFDMMGD**	761**EG**WR	844**YIAAHD**
S. agalactiae COH1	734**DVVYNH**	798**GFRFDMMGD**	831**EG**WR	914**YIAAHD**

plasmid excision by homologous recombination between flanking duplicated regions. Dilutions of the serially passaged cultures were plated on agar plates and single colonies were tested for erythromycin sensitivity (Erm^s) and spectinomycin resistance (Spc^r) to select for double crossover events resulting in insertion of *spc* into the *apuA* gene (*apuA*::*spc*) (Fig. 3a; steps 3 and 4). Spc^r integrants were confirmed to have the expected genotype by PCR (Fig. 3b), and a single integrant designated *S. suis* 10 *apuA*::*spc* was used for further studies.

Pullulanase activity of the *apuA*::*spc* insertion mutant

A red pullulan plate assay was used first to evaluate the α-(1,6) glycosidic activity of the wt and mutant strains (Lai *et al.*, 2005). Hydrolysis of the red dye-conjugated pullulan resulted in a clear zone on the plate incubated with the wt strain but not the *apuA*::*spc* mutant, indicating that the mutant lacked detectable pullulanase activity (Fig. 4a). The α-amylase activity of the *apuA*::*spc* mutant and wt strain was not assessed, as the *S. suis* genome contains a second predicted α-amylase gene classified as α-(1,4) glucan-branching enzyme (GeneID:8152319). As ApuA was predicted to be anchored to the cell wall in *S. suis*, the pullulanase activity of cell-wall, cytoplasmic and secreted proteins was measured in a spectrophotometric assay for endo-acting pullulanase activity using a purified pullulanase from *Klebsiella planticola* as a reference. The pullulanase activity of the cell-wall fraction from the wt strain was 55 mU (mg protein)$^{-1}$ (Fig. 4b). Only background levels of pullulanase activity were detected for the *apuA* mutant, indicating that ApuA is the sole enzyme responsible for breakdown of α-(1,6) glycosidic linkages found in pullulan, starch and glycogen. No pullulanase activity was measured above background levels in the cell-wall or supernatant fractions (not shown), demonstrating that this enzyme is located on the surface, as predicted.

Carbohydrate utilization assays

S. suis 10 wt and the *apuA*::*spc* mutant were analysed for the ability to grow on glycogen, pullulan and maltotriose as major sources of carbohydrate. In CM, both strains grew to a low density (OD_{600} 0.25–0.3) after 13 h incubation (Fig. 5a). Supplementation of the medium with maltotriose or glycogen supported growth of both the wt and *apuA*

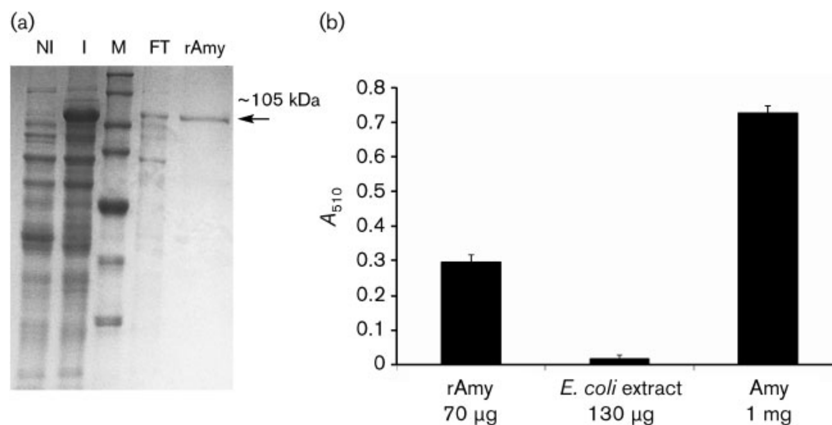

Fig. 2. Expression and enzymic activity of rAmy recombinant protein. (a) SDS-PAGE (12 % gel) of rAmy purification steps. NI, Total *E. coli* cellular protein, not induced; I, cleared cell lysate of *E. coli* after 3 h induction at 37 °C; M, size markers; FT, Ni-NTA flow-through; rAmy, recombinant amylase domain of the expected size which has been eluted from a Ni-NTA column. (b) Enzyme activity of proteins using the red starch assay. rAmy, Recombinant amylase domain; *E. coli* extract, soluble total protein from non-induced *E. coli*; Amy, α-amylase positive control from *A. oryzae*.

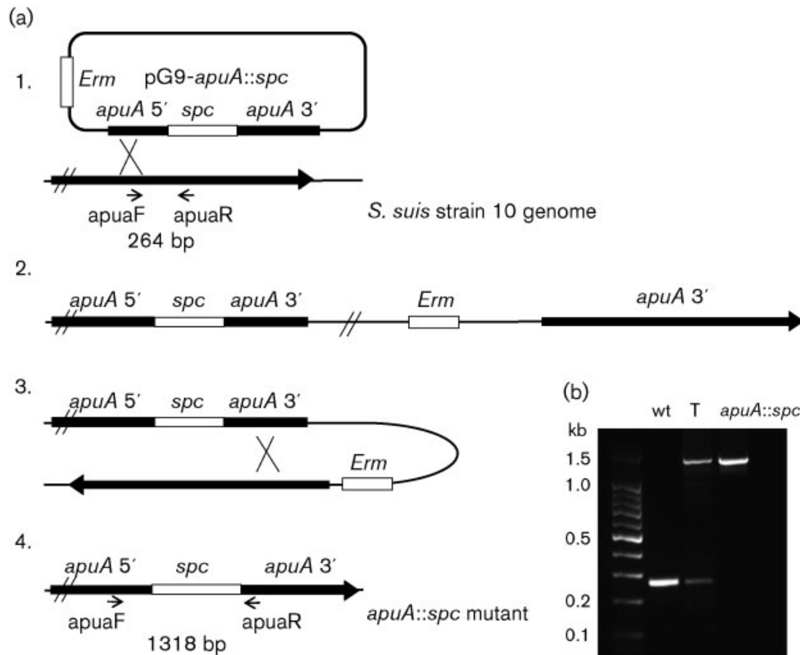

Fig. 3. Strategy for mutagenesis of the *apuA* gene to generate the knockout mutant *apuA* : : *spc* via a double crossover event. (a) Step 1, *S. suis* strain 10 transformed with pG9-*apuA* : : *spc*. The first recombination event (integration) occurs by a single cross-over (SCO) event through sequences homologous to *apuA* (only one possible SCO indicated); step 2, the resulting SCO integrants; step 3, a shift of the SCO integrants to 28 °C stimulates the second (double) crossover event (DCO) and leads to plasmid excision; step 4, genotype of the verified *apuA* : : *spc* mutant. (b) PCR validation of the expected genotype using primers apuaF and apuaR, which flank the insertion region of *spc*. A 264 bp PCR product is detected in *S. suis* strain 10 (wt) and a 1318 bp product in the *apuA* : : *spc* mutant. As expected, the SCO transformant (T) contained both PCR products.

mutant strains to a higher density (OD_{600} 0.4–0.6) than in CM alone (Fig. 5c, d). However, in the presence of pullulan, growth of the mutant was decreased compared with the wild-type strain, and it reached the same optical density as for CM alone (Fig. 5a, b).

Adhesion to a porcine tracheal cell line

The newborn porcine tracheal cell line NPTr (Ferrari *et al.*, 1993) was chosen to investigate the role of ApuA in adhesion to the epithelium. In agreement with previous studies on serotype 2 strains of *S. suis* in porcine and human kidney and lung adenocarcinoma cell lines, we found that the wt was adherent but not invasive within 2 h of incubation (results not shown). In contrast to previous adhesion studies with the same *S. suis* strains and a human laryngeal carcinoma cell line, we found that the wt strain adhered strongly to the porcine epithelium, with a maximum percentage of adherence of 19 % using an m.o.i. of 75 for the wt (Fig. 6a). The adhesion of wt and mutant strains were tested over a range of m.o.i.; in all cases, the mutant was significantly ($P<0.05$) less adherent than the wt strain (Fig. 6a).

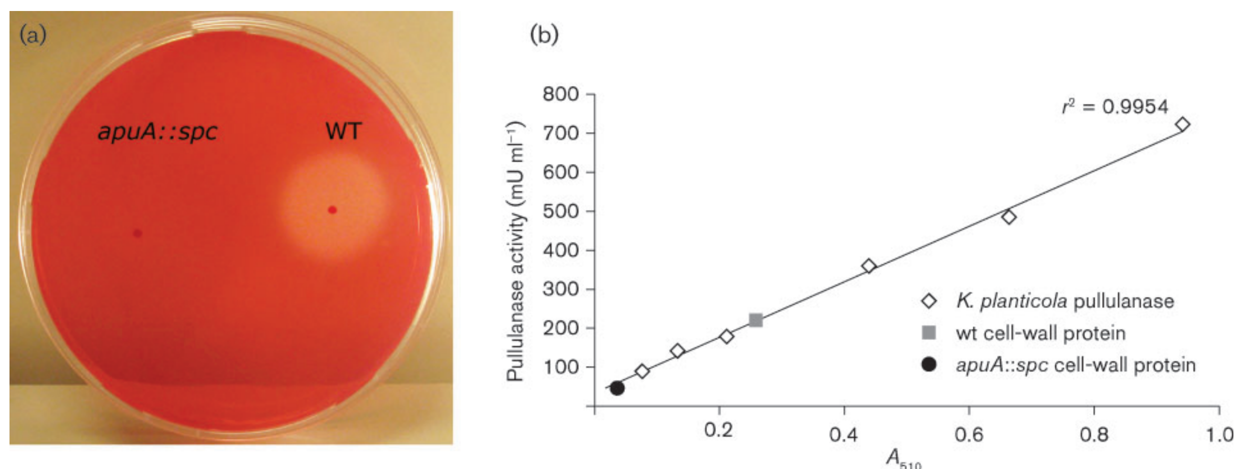

Fig. 4. Characterization of wt and *apuA* : : *spc* mutant phenotypes. (a) Pullulanase activity of *apuA* : : *spc* mutant (left) and wt strain 10 (right) on red-pullulan plate. The light zone indicates pullulan degradation by the wt isolate. (b) Pullulanase activity of extracted cell-wall proteins from wt and *apuA* : : *spc* mutant as determined by linear regression on a standard curve obtained with *K. planticola* pullulanase.

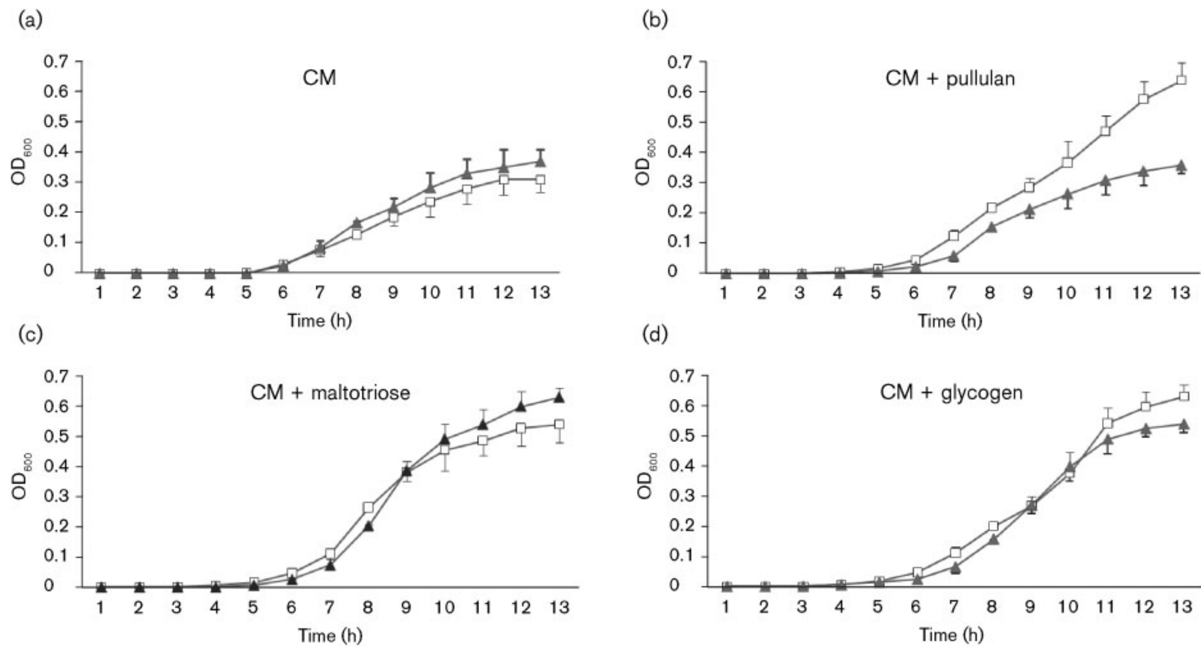

Fig. 5. Growth curves of wt (□) and *apuA* : : *spc* (▲) mutant strains grown in complex medium (CM), without additions (a) or with the addition of 1 % pullulan (b), maltotriose (c) or glycogen (d).

Binding to porcine mucin

The ability of *S. suis* strains to adhere to porcine mucin was examined in a solid-phase assay using mucin-coated microtitre wells. As shown in Fig. 6(b), the binding of the wt strain to porcine mucin was significantly higher than binding of the *apuA* : : *spc* mutant using inocula of 10^8 and 10^7 c.f.u. ml^{-1}. At the higher inoculum, approximately 2-fold more cells of the wt strain were recovered after 2 h of incubation.

DISCUSSION

A gene, *apuA*, encoding a cell-wall-anchored amylopullulanase was identified in the genome of *S. suis* strain 10. *apuA*

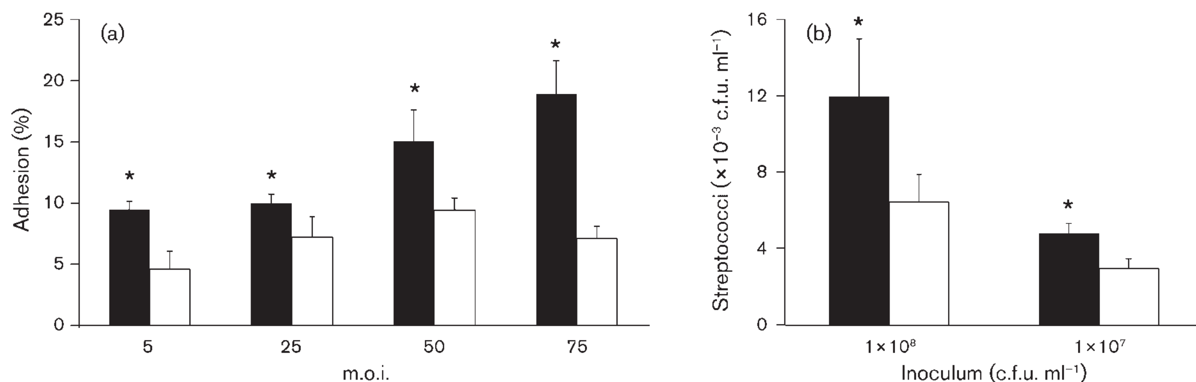

Fig. 6. Adhesion assay to porcine epithelium and mucus. (a) Percentage adhesion of wt (filled bars) and isogenic mutant *apuA* : : *spc* (open bars) to the epithelial cell line NPTr. Results were determined after 2 h of co-incubation of epithelial cells with wt and mutant strains at 37 °C with four different m.o.i. followed by extensive washing, lysis and viable plate counts. *, $P<0.05$ compared with adherence of the *apuA* : : *spc* mutant. (b) Binding of *S. suis* wt (filled bars) and *apuA* : : *spc* mutant bacteria (open bars) (at doses of 10^8 and 10^7 c.f.u. ml^{-1}) to microtitre plate wells coated with porcine mucin at 37 °C. Means and SD (error bars) were derived from triplicate wells in two independent experiments. *, $P<0.05$ compared with binding of the mutant strain *apuA* : : *spc*.

encodes a protein with distinct α-amylase [α-(1,4) glycosidic] and pullulanase [α-(1,6) glycosidic] domains (Fig. 1). Each domain contains conserved α-glucan-binding domains and four highly conserved regions, designated I, II, III and IV. These regions are predicted to confer catalytic activity by comparison to a wide range of α-amylases, pullulanases and amylopullulanases (Kuriki & Imanaka, 1999; van Bueren *et al.*, 2007) (Table 4). The pullulanase domain of ApuA shares high identity with α-(1,6) glycosidic pullulanases identified in other pathogenic streptococcal species such as *Streptococcus infantarius*, *S. sanguinis*, *S. equi* and *S. pneumoniae* (PulA) (Bongaerts *et al.*, 2000), GAS (SpuA) (Hytönen *et al.*, 2003, 2006; van Bueren *et al.*, 2007) and GBS (SAP) (Santi *et al.*, 2008). The last three proteins have been well characterized and have 58–55 % identity to the pullulanase domain of *S. suis* ApuA (Table 3). Pullulanase activity was found solely in the cell-wall fraction of *S. suis* wt, indicating that this enzyme is surface-located. Furthermore, insertional inactivation of *apuA* (by generating the interrupted locus *apuA*::*spc*) resulted in loss of pullulanase activity.

Compared to the wt, growth of the *apuA*::*spc* mutant was significantly impaired in CM containing pullulan but not glycogen or maltotriose as major carbon sources. The ability of the *apuA*::*spc* mutant to grow efficiently on glycogen may be due to the fact that the *S. suis* genome contains a second gene (GeneID:8152319) that encodes a predicted α-amylase domain. Hydrolysis of pullulan with an α-(1,6) pullulanase yields maltotriose as the main product. The failure of the mutant to utilize pullulan as a carbon source strongly suggests that ApuA is necessary for hydrolysis of pullulan and the release of maltotriose, which is used as a carbon source for growth. ApuA shows highest protein sequence similarity to a putative amylopullulanase STRINF_01787 of *Streptococcus infantarius* ATCC BAA-102 (60 %) and the characterized alkaline amylopullulanase (APase) from *Bacillus* sp. KSM-1378 (47 %; Table 3), which was shown to hydrolyse both α-(1,4) and α-(1,6) glycosidic bonds from two distinct domains and catalytic sites within the same protein (Hatada *et al.*, 1996).

The evolution of this type of bifunctional enzyme could have resulted from recombination between α-amylase and type I pullulanase genes. Similar events are thought to be responsible for the origin of genes encoding other bifunctional enzymes, such as enzymes with endo- and exoglucanase activities from '*Caldocellum saccharolyticum*' (Saul *et al.*, 1990).

The upper respiratory pharyngeal mucosa is the primary site of adherence and colonization by *S. suis*, but the factors involved in these key virulence mechanisms have not been identified. Given that PulA, the type I pullulanase of *Streptococcus pyogenes*, mediates adherence to several mammalian glycoproteins including thyroglobulin, submaxillary mucin and asialofetuin (Hytönen *et al.*, 2003), we investigated the potential role of ApuA in adhesion to the porcine epithelial cell line NPTr. Previous studies with human and porcine kidney epithelial cell lines showed that *S. suis* serotype 2 strains are able to adhere to, but not invade, eukaryotic cells (Benga *et al.*, 2004; Lalonde *et al.*, 2000). We found, for the first time, that this was also the case for the porcine NPTr cell line, which has not been used previously for *S. suis* virulence studies. The adherence to the porcine epithelial cells was substantially (20-fold) stronger than that reported previously for the human laryngeal carcinoma cell line HEp-2 using similar assay conditions (Benga *et al.*, 2004). Strikingly, in our cell line model, the adherence of the *apuA*::*spc* mutant was around 2-fold lower than the wt strain over a range of m.o.i., suggesting that ApuA may play an important role in colonization and virulence *in vivo*. As pullulan is not found in humans or pigs, ApuA presumably binds to similar carbohydrate structures on the porcine epithelium and mucus. Streptococcal proteins combining glycoprotein-binding and carbohydrate-degrading activities, such as *S. pyogenes* PulA and SpeB, have been described previously (Hytönen *et al.*, 2003, 2006). Another explanation for the role of ApuA in adhesion could be that the loss of this protein alters the surface charge of *S. suis* and affects adhesion indirectly via electrostatic forces such as cation bridging.

Colonization of the nasopharyngeal cavity by *S. suis* is an important risk factor for the infection of young pigs (Cloutier *et al.*, 2003; Gottschalk *et al.*, 1991). Recent evidence suggests that the nasopharynx and palatine tonsils may be the routes of entry in invasive disease (Madsen *et al.*, 2002). Adhesion of *S. suis* to mucus in the oral cavity is likely to be important for colonization, as demonstrated for other opportunistic pathogens that colonize the nasopharyngeal cavity (Cheng Immergluck *et al.*, 2004; Faden, 1998; Melles *et al.*, 2007). The demonstration that the *apuA*::*spc* mutant binds less well to porcine gastric mucus is an indication that this surface enzyme may also promote adhesion to mucus *in vivo*.

ApuA is likely to play a role in nutrient acquisition *in vivo* via the degradation of glycogen, the major carbohydrate storage protein in animals, as well as in the degradation of food-derived starch or glycogen in the nasopharyngeal and oral cavities. Our results demonstrate an important role for ApuA in adhesion to porcine epithelial cells and to mucus *in vitro*. These results also point to a link between carbohydrate utilization by *S. suis* and the ability to colonize and infect hosts.

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