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

Maria Laura Ferrando,1 Susana Fuentes,1 Astrid de Greeff,2 Hilde Smith2 and Jerry M. Wells1

1Host-Microbe Interactomics, Wageningen University and Research Centre, Marijkeweg 40, 6709 PG Wageningen, The Netherlands
2Central Veterinary Institute of Wageningen UR, Edelhertweg 15, 8219 PH Lelystad, The Netherlands

We have identified *apuA* in *Streptococcus suis*, which encodes a bifunctional amylopolullanase 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, sepsis, endocarditis, otitis media and bronchopneumonia (Beineke et al., 2008; Madsen et al., 2002). Healthy pigs asymptptomatically 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, 2008; 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 (98HAI12 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 & Valentim-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* (Poulhiet 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...
(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., 2009; Hytönen & Camilli, 2002). Recently, it was shown that GAS PulA-ulanase PulA was shown to function as a strepadhesin, streptococcal pullulanases in virulence. The GAS pullulanase was identified in the genome of S. suis (Strr), 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 amylpullulanase 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% CO2 for 18 h. An OD600 of 1.0 with a 1 cm path length corresponds to approximately 109 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% NH4Cl, 0.1% red pullulan (Megazyme) and 2% agar] as described previously (Lai et al., 2005).

A complex medium (CM) comprising 10 g proteose peptone F−1, 5 g trypticase peptone F−1, 5 g yeast extract F−1, 2.5 g KCl F−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 OD600 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 GoTag 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.

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**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genetic markers and/or description*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
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<td><em>E. coli</em> VE7108</td>
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<td>Mora et al. (2004)</td>
</tr>
<tr>
<td>VE6838</td>
<td>supE hisΔ5 thi Δ(lac–proAB) F (traD36 prophA lac8 lacZΔM15); VE7108 carrying pGp9 host9</td>
<td>Mora et al. (2004)</td>
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<td>TOP10</td>
<td>F− mcrA Δ(mrr–hsdRMS–mcrBC) 680lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara–leu) 7697 galU galK rpsL (Strr) endA1 nupG</td>
<td>Invitrogen</td>
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<td><strong>S. suis</strong> serotype 2</td>
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<td>Strain 10</td>
<td>Virulent serotype 2 strain Isogenic apuA::spc mutant of strain 10</td>
<td>Vecht et al. (1989, 1992)</td>
</tr>
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<td>apuA::spc</td>
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<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<td>pTrcHis TOPO</td>
<td>Expression vector containing N-terminal His tag</td>
<td>Invitrogen</td>
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<td>pTrc-amy</td>
<td>pTrcHis vector containing 2562 bp of apuA</td>
<td>This work</td>
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<tr>
<td>pDL282</td>
<td>Replication functions of pUC19 and pVT736-1, Ampr, Spc+</td>
<td>Sreenivasan et al. (1991)</td>
</tr>
<tr>
<td>PG4 host9</td>
<td>Ermr, thermostable derivative of pGK12</td>
<td>Maguin et al. (1996)</td>
</tr>
<tr>
<td>pG9-apuA</td>
<td>pGhost + 9 derivative containing 3020 bp of apuA</td>
<td>This work</td>
</tr>
<tr>
<td>pG9apuA::spc</td>
<td>pG9-apuA::spc containing a 1.2 kb Spc+ fragment from pKUN19-spc cloned within apuA</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Amp+, Ampicillin-resistant; Ermr, erythromycin-resistant; Spc+, spectinomycin-resistant.*

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On: Sun, 02 Dec 2018 01:56:28
(Invitrogen) such that the expressed recombinant x-amylase would be fused to an N-terminal polypeptide containing six histidine residues for affinity purification. After transformation of E. coli TOP10, several clones were picked and checked for the correct insertion of the x-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 (OD600 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/HC1, 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 column (Amersham Pharmacia Biotech). Proteins were eluted in a phosphate buffer containing 500 mM NaCl and (14 000 r.p.m. for 40 min) and loaded onto a HisTrap affinity 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 (A510; 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 1 % (w/v) in 0.5 M KCl) for 10 min at 37 °C as described previously (Ortu et al., 2006). An internal EcoRI–Xhol fragment (nucleotide positions 3114–6133) of the apuA gene was amplified by using primers pulF and pulR and ligated into EcoRI– and Xhol-digested pG9–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 V7108 (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-PvuII and pulR-BglII. An spc cassette, containing the promoter and transcriptional terminator, was amplified from plasmid pKUN19-Spc (Konings et al., 1987) using primers 5′-Spc-PvuII and 3′-Spc-BglII. All the inverse PCR product of pG9-apuA and the spc cassette were then digested with PvuII and BglII 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 electroperoration 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 pG9–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 allow 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 apuF and apuR.

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 MgCl2, 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 MgCl2. 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Purpose</th>
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<tr>
<td>amyF</td>
<td>CTTCGGAAACAGGATGCC</td>
<td>Cloning of x-amylase domain in pTrcHisTopo</td>
</tr>
<tr>
<td>amyR</td>
<td>GAGCAGTGTCACTGCCTCCTG</td>
<td>Cloning of x-amylase domain in pTrcHisTopo</td>
</tr>
<tr>
<td>pulF-EcoRI</td>
<td>GCTATCGAATTTCTAACCAGTGGGATATGAT</td>
<td>Cloning of x-amylase domain in pTrcHisTopo</td>
</tr>
<tr>
<td>pulR-Xhol</td>
<td>TGACTGTTTCTGGCGATCCTTACGCAGCGGTTGAG</td>
<td>Inverse PCR of pG9-apuA</td>
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<tr>
<td>pulF-PvuII</td>
<td>GCTATGTTTCTGGCGATCCTTACGCAGCGGTTGAG</td>
<td>Insertion of spc gene into pG9-apuA</td>
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<tr>
<td>pulR-BglII</td>
<td>TCTGGCGTTTCTGGCGATCCTTACGCAGCGGTTGAG</td>
<td>To check for correct mutant clones using colony PCR</td>
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<tr>
<td>Spc-PvuII</td>
<td>TCTGGCGTTTCTGGCGATCCTTACGCAGCGGTTGAG</td>
<td>To check for correct mutant clones using colony PCR</td>
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<td>Spc-BglII</td>
<td>TCTGGCGTTTCTGGCGATCCTTACGCAGCGGTTGAG</td>
<td>To check for correct mutant clones using colony PCR</td>
</tr>
<tr>
<td>apuA</td>
<td>TGGTGTTGATTTTCTGGGATG</td>
<td>To check for correct mutant clones using colony PCR</td>
</tr>
</tbody>
</table>
pullulan substrate was depolymerized by an endo-mechanism to produce fragmented material which remains 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 (A350 SpectraMax M5 reader). The pullulanase activity in the S. suis 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 x-(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 % CO2. 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), supplemented with 10 % fetal calf serum (Gibco), without antibiotics, at 37 °C and 5 % CO2. 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 \times 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 10^7 c.f.u. ml^-1 in fresh cell culture medium without antibiotics. Bacterial suspensions diluted in cell culture medium (between 1.15 \times 10^8 and 2.3 \times 10^8 c.f.u. ml^-1) were added to wells containing a monolayer (2.3 \times 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 % CO2. 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 NaHCO3 (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 OD600 of 1.0. Triplicate wells in both plates were inoculated with 2.5 \times 10^7 and 2.5 \times 10^6 bacteria in a volume of 100 μl in PBS. The microtitre plates were incubated for 2 h at 37 °C and 5 % CO2. 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 \pm SD. Statistical significance was determined using a two-tailed Student’s t-test. Differences were considered significant at P \leq 0.05.

RESULTS

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

A gene designated here apuA (6285 bp; 2094 amino acids) and predicted to encode an amylomucinase 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 \( \alpha \)-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 \( \alpha \)-glucan polysaccharides containing \( \alpha -(1,4) \) glucosidic and \( \alpha -(1,6) \) glycidosidic linkages (van Bueren et al., 2007). Within the protein, two pairs of four regions highly conserved in \( \alpha \)-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 amylomucinase in Bacillus sp. KSM-1378 (Hatada et al., 1996) and the putative amylomucinase 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 \( \alpha \)-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 Glu646 and Glu1598 within conserved region III of the \( \alpha \)-amylase and pullulanase domains, respectively (Table 4). Analogously, the catalytic aspartate residues are found at positions Asp632 (region I) and Asp727 (region IV) of the \( \alpha \)-amylase domain and at positions Asp1569 (region I) and Asp1686 (region IV) within the pullulanase domain. The presence of the LPXTG motif and the distinct \( \alpha \)-amylase and pullulanase domains, each possessing conserved catalytic and substrate-binding sites, suggested that ApuA is a cell-wall-anchored bifunctional amylomucinase.

The \( \alpha \)-amylase recombinant domain possesses \( \alpha -1,4 \) glucosidic activity

The DNA fragment encoding the predicted \( \alpha \)-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
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 *a*-amylase from *Aspergillus oryzae* as a positive control. The *a*-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 *a*-1,4 glucosidase activity. Thus, these results confirm that the N-terminal half of ApuA indeed possesses the predicted *a*-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 ℃ but, at temperatures above 37 ℃, it is segregationally unstable and lost in the absence of antibiotic selection. In the presence of antibiotic selection, growth at temperatures above 37 ℃ 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 EcoRI- and XhoI-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 ℃ 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 ℃, 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 ℃ without Erm selection to facilitate

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

<table>
<thead>
<tr>
<th>Name</th>
<th>Enzyme</th>
<th>Strain</th>
<th>Identity (%)</th>
<th>Reference</th>
</tr>
</thead>
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<td>ApuA</td>
<td>Amylopullulanase</td>
<td><em>S. suis</em> P 1/7</td>
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<td>This work</td>
</tr>
<tr>
<td>–</td>
<td>Amylopullulanase</td>
<td><em>S. infantarius</em> ATCC BAA-102</td>
<td>60</td>
<td>–</td>
</tr>
<tr>
<td>APase</td>
<td>Alkaline amylpullulanase</td>
<td><em>Bacillus</em> sp. KSM-1378</td>
<td>47</td>
<td>Hatada et al. (1996)</td>
</tr>
<tr>
<td>–</td>
<td>Pullulanase</td>
<td><em>S. sanguinis</em> SK36</td>
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<td>–</td>
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</tr>
<tr>
<td>PulA</td>
<td>Pullulanase</td>
<td><em>S. pyogenes</em> NZ131</td>
<td>58</td>
<td>Hytönen et al. (2003)</td>
</tr>
<tr>
<td>SAP</td>
<td>Type I pullulanase</td>
<td><em>S. agalactiae</em> COH1</td>
<td>55</td>
<td>Santi et al. (2008)</td>
</tr>
</tbody>
</table>
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, pullulans 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Region I</th>
<th>Region II</th>
<th>Region III</th>
<th>Region IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Amylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. suis P 1/7</td>
<td>556DVVLNH</td>
<td>628FRYDVTYKH</td>
<td>661ETWG</td>
<td>722FLGSHD</td>
</tr>
<tr>
<td>Bacillus sp. KSM-1378</td>
<td>462DVVLNH</td>
<td>546YFRYDVTYKH</td>
<td>579EAWG</td>
<td>640FLGSHD</td>
</tr>
<tr>
<td>Pulululanase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. suis P 1/7</td>
<td>1505DVYNYNH</td>
<td>1566GFRFDMMGD</td>
<td>1598EGRWR</td>
<td>1681YIAAHDD</td>
</tr>
<tr>
<td>Bacillus sp. KSM-1378</td>
<td>1396DVYFNYNH</td>
<td>1460GFRFDMMGD</td>
<td>1493EGWV</td>
<td>1578YIAAHDD</td>
</tr>
<tr>
<td>S. sanguinis SK36</td>
<td>715DVYVNYHN</td>
<td>779GFRFDMMGD</td>
<td>812EGWK</td>
<td>895YIAAHDD</td>
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<tr>
<td>S. equi MGCS10565</td>
<td>734DVYNNHN</td>
<td>798GFRFDMMGD</td>
<td>831EGWV</td>
<td>914YIAAHDD</td>
</tr>
<tr>
<td>S. pneumoniae S1 3.B</td>
<td>717DVYNYNN</td>
<td>781GFRFDMMGD</td>
<td>814EGW</td>
<td>897YIAAHDD</td>
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<td>S. pyogenes NZ131</td>
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<tr>
<td>S. agalactiae COH1</td>
<td>734DVYVNNHN</td>
<td>798GFRFDMMGD</td>
<td>831EGW</td>
<td>914YIAAHDD</td>
</tr>
</tbody>
</table>

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<sup>+</sup>) and spectinomycin resistance (Spc<sup>+</sup>) to select for double crossover events resulting in insertion of spc into the apuA gene (apuA::spc) (Fig. 3a; steps 3 and 4). Spc<sup>+</sup> 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 (GenelD: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)<sup>−1</sup> (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<sub>600</sub> 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.](http://mic.sgmjournals.org)
mutant strains to a higher density (OD₆₀₀ 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).
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 amylpullulanase was identified in the genome of S. suis strain 10. apuA
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, insertion 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 domain contains conserved α-glucan-binding 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 Caldoccum 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 NPTri. 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 NPTri 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 Immengluck 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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REFERENCES


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