Interactions *of Streptococcus suis* with host mucosa

M. Laura Ferrando

Thesis committee

Thesis supervisor
Prof. dr. Jerry M. Wells
Professor of Host Microbe Interactomics
Wageningen University, The Netherlands

Thesis co-supervisors
Dr. Hilde E. Smith
Researcher, Department of Bacteriology - Central Veterinary Institute (CVI) Wageningen UR
Lelystad, The Netherlands

Dr. ir. Peter van Baarlen
Assistant professor, Host Microbe Interactomics (HMI) group
Wageningen University, The Netherlands

Other members
Dr. Marco R. Oggioni
Universita' di Siena, Italy

Dr. Piet Nuijten
Intervet International bv, Boxmeer, The Netherlands

Prof. dr. Tjakko Abee
Wageningen University, The Netherlands

Prof. Jos P.M. van Putten
Utrecht University, The Netherlands

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M. Laura Ferrando

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M. Laura Ferrando
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To Michele and my family

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Chapter 1

General Introduction

M. Laura Ferrando

1. *Streptococcus suis*

1.1 Ecology and taxonomy

Streptococcus suis is a major zoonotic swine pathogen capable of causing serious infections in pigs and persons in close occupational or accidental contact with infected pigs or contaminated pork products. Infections caused by *S. suis* cause significant economic losses to the swine industry worldwide. The natural habitat of *S. suis* is the upper respiratory tract of pigs, particularly the tonsils and nasal cavities, as well as the alimentary and genital tracts [1,2]. Although *S. suis* is considered mainly a pathogen of swine, it has been isolated increasingly frequently from a wide range of mammals (ruminants, cats, dogs, deer and horses) and birds [3,4,5,6] which suggests a capacity to persist in a broad range of very different hosts, leading to high diffusion of this pathogen [7]. *S. suis* can survive for 8 days in faeces and for short periods of exposure to moderate heat (i.e. survival after 60°C for 10 min and 50°C for 2h) and desiccation (24 h in dust at 25°C or 1 month in dust at 0°C) [8].

S. suis is a Gram-positive facultative anaerobe belonging belonging to the phylum Firmicutes, class Lactobacillales that is α- or β-hemolytic, catalase negative and with a low G+C DNA composition. The capsulated bacteria possess cell wall antigenic determinants somewhat related to Lancefield group D [9]. Based on differences in antigenic properties of the polysaccharide capsule, 33 serotypes have been distinguished to date (types 1–33 and 1/2), [10,11]; serotypes 32 and 34 have since been proven to belong to the related species *Streptococcus orisratti* [12]. Phylogenetic analysis of the *S. suis* heat-shock chaperone 60 gene showed the presence of three basal clusters. The majority of serotypes are associated with cluster I; 20, 22 and 26 serotypes with cluster II, and isolates of serotype 33 with cluster III. According to this systematic analysis, *S. suis* was most closely related to *Streptococcus anginosus*, *Streptococcus parasanguinis*, *Streptococcus sanguis*, *Streptococcus oralis* and *Streptococcus mitis* [13].

1.2 Metabolism

The metabolic map of *S. suis* includes more than 80% of the metabolic maps known for other *Streptococcus* species (**Chapter 5**). Like other *Streptococci, S. suis* produces energy mainly through metabolism of glucose via homolactic or mixed-acid fermentations depending on the availability of external oxygen and carbohydrate. Under conditions of excess glucose and in presence of oxygen, glucose is preferentially metabolised by the

glycolytic pathway (or Embden-Meyerhof pathway) to pyruvate that is reduced via oxidative respiration through the tricarboxylic acid (TCA) cycle. However, in *S. suis*, as in most other lactobacilli, the TCA cycle is incomplete, rendering *S. suis* incapable of oxidative respiration. Instead energy is derived from the metabolism of pyruvate using organic compounds as electron acceptors. In homolactic fermentation one molecule of glucose is ultimately converted to two molecules of lactic acid whereas heterolactic fermentation is the production of lactic acid as well as other acids and alcohols. The metabolic map of *S. suis* suggests that pyruvate can be converted into lactic acid but also acetic acid, ethanol and formic acid, but not acetoin (**Chapter 5**). *S. suis* is able to ferment alternative sugars including mono- and di-saccharides such as ribose, L-arabinose, mannose, sorbitol, lactose and raffinose, but also complex carbohydrates such as glycogen, starch and pullulan, to glucose [12]. The metabolism of complex carbohydrates requires the presence of specialized transport systems and catabolic enzymes such amylase, pullulanase and amylopullulanase that permit the utilization of alternative sugars in different environmental niches including those present within the host [14]. These enzymes enable *S. suis* to grow efficiently on complex carbohydrates and colonize the host mucosal epithelia that are rich in such carbohydrates (**Chapter 3**, **Chapter 4**, **Chapter 5**).

1.3 Diagnosis

S. suis can be cultured from nasal, vaginal and tonsillar swabs, cerebrospinal fluid (CSF) or blood samples of infected animals. The solid medium most commonly used to isolate *S. suis* is sheep blood agar on which viridans group streptococci produce an α- hemolysis. This type of hemolysis is due to the production of hydrogen peroxide by *S. suis* which oxidizes hemoglobin to a green colored methemoglobin. However, when cultivated on horse blood agar *S. suis* produces a clear zone of β-hemolysis [15]. Usually the identification is further verified by checking if colonies are catalase negative, negative for the Voges-Proskauer (acetoin) reaction, hydrolysis of esculin, trehalose positive, amylase positive, and if growth inhibition occurs in the presence of 6.5% NaCl. The use of miniaturized biochemical test like the Rapid Strep System is common in microbiology diagnosis. This Analytical Profile Index (API) test is based on the reaction of a panel of chemicals (usually 20) to determine the bacterial species. However, cross-reactivity, a feature of many standard microbiological techniques, often hampers correct identification [16] such that infections may go undiagnosed. It is possible that *S. suis*-positive cultures are sometimes misidentified as other *Streptococcus* species, *Aerococcus viridans*,

Enterococcus faecalis or even *S. sanguinis* [17,18]. Misidentification in diagnostic laboratories has also been influenced by unawareness of the possibility that *S. suis* can lead to meningitis; the result being that *S. suis* colonies have been assumed to be enterococci or (group D) streptococci [19]. Therefore, more accurate molecular methods have been developed to correctly identify *S. suis* from infected tissue samples.

In Asia, molecular techniques to detect *S. suis* in human samples, by real time PCR detection of the *cps2J* gene, improved the rate of pathogen detection over the last few years. Recently a sensitive loop-mediated isothermal amplification (LAMP) technique targeting the *cpn60* gene (encoding chaperonin 60) has been developed that can be used to successfully detect all 33 *S. suis* serotypes [20].

Serotyping of bacteria is an important step in routine diagnostic procedures. In most laboratories, *S. suis* serotyping is usually carried out by scoring different types of agglutination using a panel of 33 specific sera [21]. However this serotyping method presents a low sensitivity due to the fact that some isolates react with more than one antiserum (cross-reactions). A serotype-specific polystyrene bead-based immunomagnetic separation (IMS) technique has been described that permits an better isolation and distinction among the serotypes [16].

1.4 Molecular markers to detect S. suis

Two secreted cell wall located proteins have been proposed as virulence markers, namely the muramidase-released protein (MRP) and the extracellular factor (EF) [22,23]. Both MRP and EF can be detected by monoclonal antibodies and were frequently (77%) produced by strains isolated from pigs with symptoms of meningitis in The Netherlands. However, the majority (86%) of isolates from tonsils of healthy pigs did not produce these proteins [23]. In field isolates of *S. suis* different molecular variants MRP and EF have been characterized by electrophoretic mobility [24]. The variants have been designated as MRP* and EF*. So far several variant types have been described: $MRP^{+}EF^{+}$, $MRP^{+}EF^{*}$, $MRP^{+}EF^{-}$, and $MRP^{-}EF^{-}$. It appears that the EF* strains producing the high-molecular weight variant of EF are avirulent or weakly-virulent. Nevertheless, in the USA, a lower percentage of strains (56%) isolated from the CNS of diseased pigs were $MRP^{+}EF^{+}$ [24] indicating that globally there is no strict correlation between production of MRP and EF and virulence.

2. Porcine infections with *S. suis*

2.1 S. suis epidemiology in pigs

S. suis was first isolated during an outbreak of meningitis among piglets in the Netherlands in 1954 [25]. Since then *S. suis* porcine infection with *S. suis* has been reported worldwide, from North America (United States and Canada) to South America (Brazil), Europe, Asia (China, Thailand, Vietnam, and Japan), Australia, and New Zealand [15]. In the USA alone the economic losses to the swine industry from *S. suis* infection is estimated at over 300 million dollars in the United States [15] and in the Netherlands 12 million Euro [26]. In swine herds, the rates of asymptomatic carriage may be as high as 80%. The incidence of disease varies over time and is generally less than 5%, possibly due to the differential use of antibiotics. Pig morbidity due to infection with *S. suis* has been estimated to be in the range of <1% to >50% [15].

In piglets *S. suis* can be vertically transferred from the sow to the piglet through nasal secretions, during suckling [27,28] or via the vaginal secretions during parturition [29]. In addition horizontal transmission can occur in pig herds, mainly via the respiratory route [30]. Airborne transmission of *S. suis* has been shown over a distance of 40 cm or more, without nose-to-nose contact [31]. In fact, it appears that transmission between herds occurs usually via healthy carrier pigs [32]. The introduction of carriers into a non-infected herd usually results in the subsequent onset of disease in weaning- and/or growing pigs (i.e. pigs 3 to 12 wk of age) [33]. Clinical disease may suddenly develop within a herd of pigs due to predisposing factors, such as co-infection by other swine pathogens. For example, co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) or Aujeszky's disease virus exacerbates *S. suis* infection [34]. PRRSV and Aujeszky's disease are typically enzootic in intensive pig production settings [35] and show a stress-induced susceptibility in pigs [36], suggesting that the association between *S. suis* infection and high-density pig herds is probably multifactorial [8]. Crowding, poor ventilation, sudden weather change, mixing, moving, vaccination, and concurrent disease are all stresses that predispose pigs to *S. suis* infections [15]. In general, *S. suis* most often affects pigs in intensively managed herds, especially herds with a high population density [37].

In pigs all *S. suis* serotypes can potentially cause disease but their virulence differs among and within the serotypes. Serotypes show a different geographical distribution [15]. Serotype 2 is the serotype most commonly associated with disease and is most frequently

isolated from *S. suis*-infected pigs [38,39]. However, not all isolates of type 2 are pathogenic and this serotype, comprises pathogenic, weakly pathogenic, and nonpathogenic strains [40,41,42]. The closely related serotypes 1 and 14 appear highly virulent and have been frequently isolated from piglets or adult pigs [7,15,43]. Lung inflammation has been observed in pigs infected with serotypes 1-5, 7, 8 and 1/2 [44].

Although *S. suis* is widespread, the serotypes seem to have a different geographical location among the 5 continents. The serotypes 1, 2, 7, 9, and 14 are most frequently isolated from diseased pigs in Europe, and serotype 9 in particular, is being increasingly isolated and associated with diseased pigs [45,46].

In Northern America, the percentage of *S. suis* serotype 2 strains isolated from diseased animals decreased from 22% to 15% in the past 10 years [7], while serotypes 3, 5 and 8 have been frequently isolated from diseased animals [30]. Multiple strains or serotypes are often found within a herd during an *S. suis* outbreak, although no significant differences in clinical signs or lesions occur in pigs infected with multiple versus single serotypes [47].

2.2 Clinical signs of disease induced by S. suis

Although pigs of any age can be affected by *S. suis*, the disease mainly is observed in weaning and growing pigs (with peaks at the age of 6 weeks). The first symptoms of the disease include fever, depression, anorexia and lassitude.

The development of the disease is accompanied by a detectable bacteremia or pronounced septicaemia, fluctuating fever (up to 42°C), loss of appetite, depression, and shifting lameness [48].

In pigs, the most important clinical feature associated with *S. suis* is meningitis. Symptoms of meningitis and meningoencephalitis are often preceded by early signs of a nervous disorder including incoordination, adoption of unusual stances, instability, paddling, opisthotonos, convulsions and nystagmus. In case of *S. suis* infections, other pathologies have also been described, such as arthritis, pneumonia, rhinitis, fibrinous polyserositis, abortion, endocarditis, and septicaemia with sudden death [49,50].

2.3 Pathology

Histological features from pigs showing *S. suis* disease symptoms may include lesions with fibrino-purulent exudates in the brain, swollen joints, fibrinous inflammation of the linings of the lungs, and cardiac valvular vegetations (a mass of platelets, fibrin, *S. suis* colonies,

and inflammatory cells). Less frequently, lesions associated with septicaemia or polyarthritis are seen. *S. suis* can also cause lesions associated with pneumonia, but these are often considered to be secondary to other diseases.

2.4 Treatment of S. suis infections

Antimicrobial therapy is an important disease management tool used to treat and/or control streptococcal infections, as current bacterins (vaccines comprising killed suspensions of bacterial strains) provide only serotype-specific protection. Penicillin is the most common antibiotic used in treatment of *S. suis* infection. Although *S. suis* is usually sensitive to penicillin, penicillin resistance has been reported in pig carriers [51]. Antimicrobial susceptibility of *S. suis* strains (Minimum Inhibitory Concentration MIC) differs from country to country [52,53,54]. Most antibiotic resistance determinants identified in *S. suis* are located on plasmids or transposons and are disseminated. Recently, two genetic elements conferring resistance to erythromycin (erm(B)) and tetracycline (tet(W)) were characterized in two human *S. suis* isolates [55].

2.5 Vaccines

Most vaccines used to protect against *S. suis* infections have been based on formalin-killed whole cells. These are called bacterins can confer a good protection only to those serotypes present in the vaccine [56]. Commonly used is a commercial vaccine containing killed *S. suis* serotype 2 (Emulsibac-SS®, MVP Laboratories, Inc., Ralston NE) [57] and an autogenous *S. suis* bacterin (produced by MVP Laboratories, Inc., Ralston NE) [58]. Subunit vaccines based on murein-associated protein (MAP) fraction subunit vaccine have also been compared to an autogenous bacterin [59]. Immunization with bacterin, but not with MAP subunit vaccine, induced opsonizing antibody titres against the serotype 2 strain, and these antibody titres were found to correlate with protection. However cross-protection against a serotype 9 strain was very low for both vaccines [59]. These results emphasize the importance of finding conserved protein antigens for the development of new cross-protective subunit vaccines [60,61,62,63]. The presence of maternal antibodies in the colostrum of sows may present challenges for effective vaccination of young piglets as recently demonstrated by Baums *et al.* [64]. Nevertheless immunization of sows can confer passive immunity against *S. suis* to the young piglets but after weaning those young pigs become more susceptible to infection [64,65].

The use of an avirulent strain as vaccine can give protection in piglets [66]. The protective efficacy of a live and formalin killed non-encapsulated isogenic mutant has been tested in specified pathogen-free (SPF) pigs, housed in boxes at animal facilities [67]. All pigs vaccinated with wild type (capsulated) killed bacteria were completely protected against challenge with the homologous serotype; the killed non-encapsulated strain conferred a partial protection to the pigs [67]. This finding suggested that antigens within the *S. suis* cell wall contribute to induction of an immune response. *S. suis* cell wall proteins could play a role in vaccine protection, but the role of such cell wall proteins in cross-protection is still being actively investigated [60,61,62,63].

3. Zoonotic infections with *S. suis*

3.1 S. suis epidemiology in humans

The first documented case of a human infection by *S. suis* was in Denmark in 1968 [68]. The number of human *S. suis* cases reported in the literature has increased significantly in more than 20 countries and with more than 700 cases over the past few years [69]. Infections with *S. suis* have also caused sporadic human illness in other countries, including Thailand [70,71,72], the United Kingdom [73], Portugal [74], Italy [75], Japan [76], Australia [77], the Netherlands [78] and the United States [79,80,81]. The majority of human cases and outbreaks are reported from Asian countries with intensive pig production, i.e. China, Vietnam, and Northern Thailand. At present, *S. suis* meningitis is considered to be the most common cause of adult human meningitis in some areas of southeast Asia (see below). Sporadic cases have also been reported in European countries, including the Netherlands (Fig. 1.1).

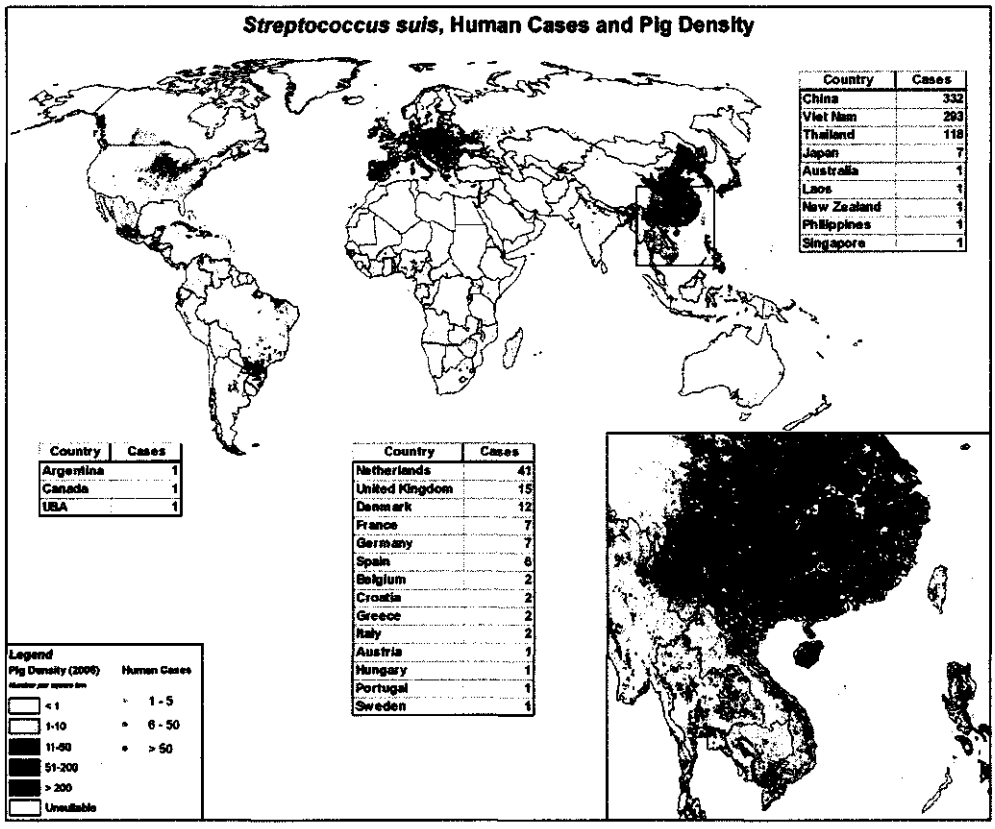

Fig. 1.1 : World map of human *S. suis* cases with background pig density data. Published with permission from the Infectious Diseases Research Foundation (World Atlas of Infectious Diseases Project) [82].

In Southern Vietnam, *S. suis* is the main cause of acute bacterial meningitis in adults (151 meningitis cases in the last 10 years) [83] and it is the third most common cause of meningitis in Hong Kong [84]. In cases of human meningitis in Vietnam, *S. suis* serotype 2 infection was more common than infections with *Streptococcus sanguis* and *Neisseria meningitidis* combined [85]. Most reports of *S. suis* infection are due to sporadic cases of infection but two larger outbreaks of *S. suis* infection occurred in China. One large outbreak was associated with 25 cases and 14 deaths in Jiangsu in 1998, and a second Chinese outbreak involving 204 cases and 38 deaths occurred in the Sichuan province in 2005, emphasizing the importance of *S. suis* as an emerging zoonosis [86,87,88]. *S. suis* serotype 2 is the most common cause of meningitis disease in humans [89] although serotypes 1, 4, 14, and 16 have been linked to severe disease in a limited number of

persons [90]. The relative high mean patient age (47–55 years) and almost complete absence of children in case series, as well as the high male-to-female patient ratio (3.5:1.0 to 6.5:1.0), support the notion that infection with *S. suis* is generally an occupational disease [18,19,69,70,77,78,81,91]. Persons in close occupational or accidental contact with pigs or pork products and those who eat uncooked or undercooked pork may be at higher risk than others. *S. suis* may become an opportunistic pathogen under particular circumstances such as stress or immunodeficiency and the onset of meningitis may manifest itself by clinical signs such as loss of coordination [19,75]. Purulent meningitis may result in permanent hearing loss, septicaemia, and endocarditis, which are commonly fulminant and often fatal [69,70,73,92]. In humans, direct entry of *S. suis* into the blood through skin wounds correlates with a very short incubation phase [87].

3.2 Treatments in humans

Data from Vietnam show that *S. suis* is susceptible to penicillin, ceftriaxone, and vancomycin [69]. Strains isolated from humans are frequently resistant to tetracycline (83.2% of isolates), erythromycin (20% of isolates) and chloramphenicol (3.3%)[69]. Penicillin resistance has been reported in a single human case [92]. The principles of treatment are the same as those for other causes of bacterial meningitis. For empirical treatment, ceftriaxone with or without vancomycin (depending on the local epidemiology of bacterial meningitis and drug resistance) is a good choice until the laboratory diagnosis is confirmed [69]. Penicillin G (24 million U over 24 h for at least 10 days) has been used successfully for the treatment of *S. suis* meningitis [93].

4. Aspects of Pathogenesis

The progression of disease symptoms caused by *S. suis* are characterized by at least four sequential processes: (i) adherence to host cells, (ii) invasion and crossing of host epithelia, (iii) dispersal and survival in the blood stream, and (iv) infection of downstream organs. During any of these steps, bacteria may proliferate and induce damage leading to exaggerated inflammation (Fig. 1.2). However, there are several open questions concerning the pathogenesis of disease cause by *S. suis* infection that are discussed below and throughout this thesis. These consecutive steps in the pathogenesis of invasive disease caused by S. suis will now be discussed in sequence.

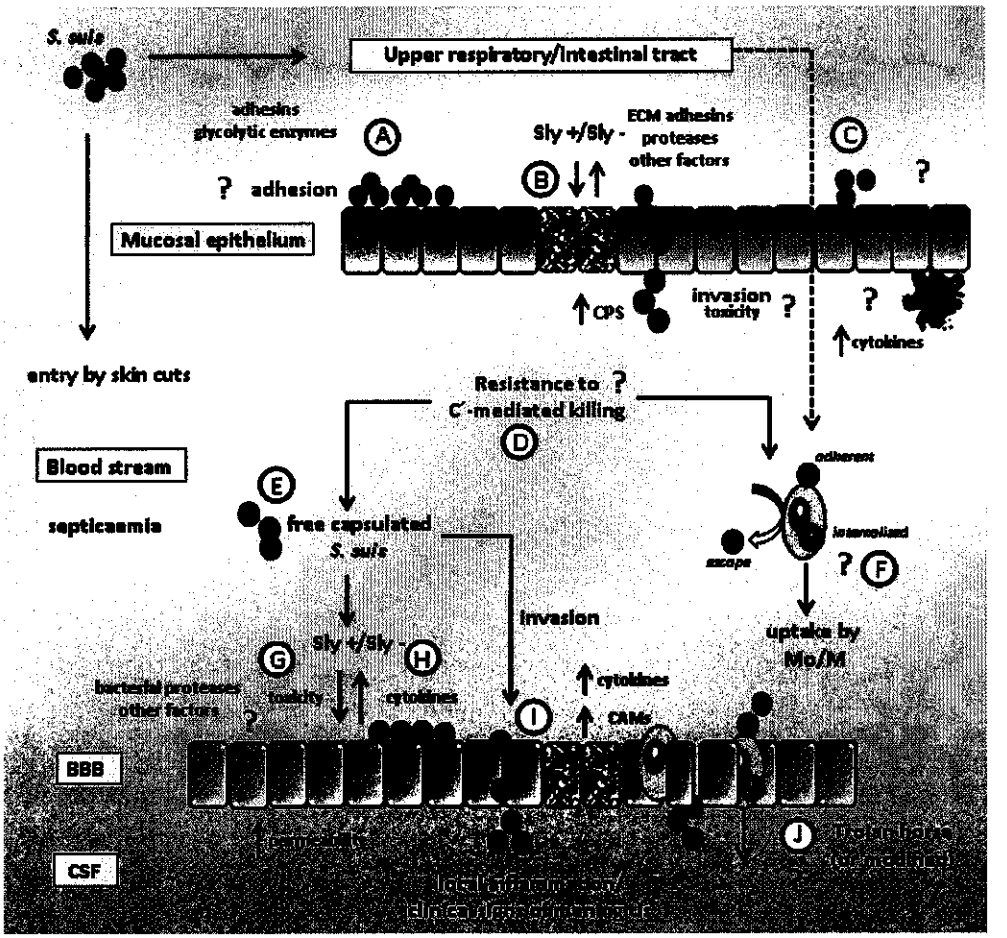

Fig. 1.2 Hypothetical model of the pathogenesis of *S. suis* infection (modified from Gottschalk *et al* 2011) [19]. The description of pathogenesis steps from A to J are found in the text. A Adhesion of mucosal epithelium; B Invasion by epithelia disruption due to Sly toxin production; C Hypothetical dissemination from the mucosal epithelium through the interaction with DC cells; D, E, F *S. suis* survival strategies in the bloodstream as free capsulated or F, J associated with monocytes (adherent or internalized); G, H, I *S. suis* crosses the blood-brain barrier (BBB) by adhesion and penetration of endothelial cells. An increased permeability of BBB due to cytokines or bacterial toxins production can help the bacteria to penetrate across the BBB and gives local inflammation of meninges.
Sly, suilysin; CPS, capsular polysaccharides; CAMs, cellular adhesion molecules, DC dendritic cells, Mo/M Monocytes/Macrophages, BBB brain blood barrier, CFS central nervous system.

4.1 Mucosal adhesion and colonization

Swine can become infected by *S. suis* via both the vertical and horizontal route leading to colonization of the oropharyngeal cavity, in particular the tonsils [94]. In humans, it is thought that people can also become infected through wounded skin by handling of

uncooked pork that is contaminated with *S. suis* [69,94]. Inoculation through cuts and sores in the skin are assumed to be associated with an acute infection because the bacteria may directly multiply in the blood stream. Colonization may lead to asymptomatic carriage or invasion of the mucosal tissues and dissemination in the blood to other organs. In the mucosal epithelium of upper respiratory/intestinal tract are present high concentrations of common forms of α-glucans including dietary starch and glycogen, the storage form of α-glucose in the eukaryotic cell. α-Glucan-binding proteins include the enzymes capable of hydrolysis and synthesis of the α-glucans including starch and cellulose, that can act as substrates for microbial growth [95]. Several adhesins with metabolic enzymatic activity, have been identified *in vitro* and may also contribute to the adhesion to host epithelial cells, by binding to unknown glycoconjugates or host receptors (Fig. 1.2 step A). An example is amylopullulanase (ApuA), a cell wall–anchored α-glucans degrading enzyme, that probably binds different host carbohydrates through the conserved carbohydrate binding domains present in the ApuA protein (**Chapter 3**). Moreover 6-phosphogluconate-dehydrogenase (6-PGD) [96] and glutamine synthetase (GlnA) [97] are cytoplasmic glycolytic enzymes thought to be involved in the adhesion to epithelia. Two different studies showed reduced adherence of *S. suis* lacking 6-PGD- and GlnA-to epithelial HEp-2 cells *S. suis* suggesting the involvement of these proteins in the first steps of the bacterial adhesion to host cells.

The adhesion SadP (SSU0253) was recently shown to bind to galactosyl-α1–4-galactose (Galα1–4Gal) galabiose-containing glycolipids [98,99]. Galabiose occurs as a terminal or internal structure in globo-series glycolipids (GbOs), and in humans they form the blood group P antigen system. Chemical studies have shown GbOs to be expressed in many pig and human tissues [100,101,102]. The SadP was shown to mediate the agglutination of sialidase-treated erythrocytes and binding to glycolipid GbO3 and to participate in the binding of *S. suis* to pig pharyngeal epithelium [99].

S. suis strains that recognize sialylated O-linked carbohydrate (NeuNAcα2–3Galβ1) terminal structures in mucin-like glycoproteins have also been identified [103], but they appear to be less common than the Galα1–4Gal-binding (via SadP) strains.

Electron microscopy studies on *S. suis* revealed the presence of several other external structures similar to pili that may be involved in adhesion [105,106].

4.2 Mucosal invasion

The epithelia provide the first host barrier separating *S. suis* from the circulating blood. The processes leading to *S. suis* invasion of epithelia and from there, translocation through the mucosal tissues into the bloodstream are not clear but as indicated above it may involve altered expression of the capsule, surface adhesins, invasins and the damaging effect of suilysin on the epithelium (Fig. 1.2 step B). Suilysin (Sly) is an extracellular thiol-activated haemolysin produced by *S. suis* which belongs to the cholesterol binding-toxin family. Sly forms pores in host cells by oligomerization [109] and has been shown to be cytotoxic for epithelial [110,111] endothelial [112,113,114] and immune cells *in vitro* [115,116]. Thus production of suilysin is hypothesized to play a role in epithelial disruption, enabling *S. suis* to invade the mucosal tissues and then disseminate in the body, an hypothesis that is tested in **Chapter 5**. However strains not producing suilysin have been associated with invasive disease [114].

Once *S. suis* has crossed the epithelial barrier it may adhere to components of the extracellular matrix (ECM) such as laminin, fibronectin, and plasminogen [117]. The host ECM presents an intermediate interface between the host epithelia and the underlying mucosal tissues. Insynergy with suilysin, the hyaluronate lyase of *S. suis* is thought to degrade hyaluronic acid, one of the major components of the ECM in the loose connective tissue, thereby contributing to invasion and spread of *S. suis* [118].

Furthermore during the interaction of *S. suis* with the host, a broad variety of bacterial extracellular proteins play important roles in binding host cell surface receptors and other possible host adherence sites including ECM molecules and glycoconjugates such as glycosphingolipids and glycoproteins. Potential host cell molecules for adhesion by streptococcal pathogens include surface-associated fibronectin in the ECM [119] and glycosphingolipids and glycoproteins bearing sialic acid, galactose or N-acetylgalactosamine residues [120,121,122]. As in many Gram positive bacteria *S. suis* possesses a sortase A enzyme (SrtA) that anchors the extracellular proteins containing an LPXTG motif to the cell wall. A *S. suis* SrtA$^-$ mutant showed less adherence to ECM proteins [123,124] indicating that LPXTG-motif-containing adhesins are also important for interactions with ECM proteins.

S. suis fibronectin-binding protein (FBPS) was shown to bind human fibronectin and fibrinogen *in vitro* [125]. However experimental infection of pigs with the *fbps* mutant

strain showed that FBPS is not required for colonization of the tonsils but that it may play a role in colonization of specific organs during invasive disease [125].

In *S. suis* two glycolytic enzymes, typically found in the cytosol, have also been identified as anchorless adhesins of ECM, namely, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [126,127], and enolase [128]. A decreased adhesion of *S. suis* to plasminogen of porcine tracheal rings and HEp-2 cells was observed when the cells were pre-incubated with recombinant mutant GAPDH, suggesting its involvement in adhesion [126,127]. The GAPDH gene is highly upregulated during *in vivo* growth in different porcine organs [130] but its contribution to virulence of *S. suis* remains to be demonstrated *in vivo* [129].

Enolase is considered the major plasmin- and plasminogen- binding protein of streptococcal pathogens and the extracellular *S. suis* enolase (Eno) was shown to bind both plasminogen and fibronectin (obtained from commercial companies) and to be important for adhesion and invasion [128]. Plasminogen can be converted, following cleavage by plasminogen activators, to the serine protease plasmin. Plasmin in turn can degrade ECM proteins. Thus, surface-associated plasmin has been proposed to facilitate bacterial invasion and bacterial dissemination through epithelial barriers.

The *S. suis* di-peptidyl peptidase IV (Dpp IV), which interacts with human fibronectin and is required for full virulence in experimental infection studies of pigs [131] and in a similar way to enolase, may contribute to the degradation of fibronectin.

4.3 Avoidance of innate and adaptive immunity in the mucosa

Once present in the mucosal connective tissues, *S. suis* bacteria will be perceived by the mucosal immune system. *S. suis* appears to produce several factors that can interfere with some of the innate immune molecules. Secretory IgA (sIgA) plays an important role in mucosal immunity by immune exclusion mechanisms, toxin neutralization and steric inhibition of adhesion or invasion by binding to bacterial surface antigens [132]. IgA protease-producing bacteria can affect this defence by the cleavage of sIgA, releasing Fab fragments (the regions on antibodies that bind to antigens) [133]. It has recently been reported that *S. suis* produces an IgA1 protease capable of cleaving human IgA1 [134]. However the amount of recombinant IgA1 protein used in the IgA protease activity is very high compared to the physiological condition and it is possible that the presumed IgA1 protease is in fact a metalloprotease family member with a different function to IgA protease. Nevertheless an isogenic mutant of the putative IgA protease was reported to lead to a significantly decreased lethality of *S. suis* in pigs [135].

A secreted DNase has been identified in *S. suis* [136] with an expected role in the breakdown of neutrophil entrapments (NETS) [137] but its contribution to the virulence of *S. suis* remains to be verified. *S. suis* is also able to inhibit neutrophil recruitment by degrading interleukin-8 presumably by the production of a serine protease [138].

Dendritic cells (DCs) are important sentinels in the skin and mucosal surfaces and recognize pathogen-associated molecular patterns (PAMPs) through the binding to specific pathogen recognition receptors (PRRs) like Toll-like receptors (TLRs). Upon microbial invasion, immature DCs are recruited from nearly tissue regions and activated in order to coordinate the adaptive immune responses. Activated or mature DCs then migrate to the adjacent lymphoid organs where they activate T cells producing different cytokines that mediate inflammation and other antimicrobial responses [139,140]. DCs can also initiate phagocytosis to eliminate invasive pathogens. The *S. suis* capsular polysaccharide (CPS) was shown to interfere with phagocytosis and consequently, the level of DC maturation and production of several cytokines was reduced compared to an unencapsulated strain [141].

In **Chapter 1** we reported the effect of different *S. suis* serotypes including an encapsulated mutant on DCs (maturation, cytokines production and phagocytosis) to investigate if capsule composition might differentially modulate the mucosal immune response. In addition we sought if activated DCs could be involved in *S. suis* dissemination from the mucosal site of infection (Fig. 1.2 step C).

Suilysin could also be important in avoidance of innate and adaptive immune responses as it increases the survival of *S. suis* upon phagocytosis by DCs [116]. It has been recently demonstrated that *S. suis* suilysin was partially involved in cytokine release from DCs and also contributed to bacterial escape of phagocytosis [116] and resistance to complement-dependent killing by neutrophils [142,143]. The toxin might activate complement and reduce complement availability for bacterial opsonisation, as has previously shown for pneumolysin, an orthologous toxin produced by *S. pneumonia* [144].

4.4 Survival in the blood and dissemination to the organs

S. suis survival in the blood is thought to largely depend on the production of CPS (Fig. 1.2 steps D-E-F). It has been widely documented that the CPS protects *S. suis* from neutrophil and monocyte/macrophage-mediated phagocytosis and killing [94]. In *Streptococcus agalactiae* capsule sialic acid has been shown to increase the hydrophilic surface properties of the bacteria and to have an inhibitory effect on phagocytosis [145]. Capsular

sialic acid has been shown to be important in preventing the deposition of the complement protein C3 on the surface of GBS, therefore blocking activation of the alternative pathway and allowing for GBS resistance to opsonin-dependent intracellular killing [146] (Fig. 1.2 step 2D). Indeed, several different *in vitro* and *in vivo* experiments using isogenic unencapsulated mutant strains have conclusively shown that the absence of CPS correlates with highly increased phagocytosis and/or killing of these strains by phagocytic cells, with a rapid clearance from circulation [147,148,149,150].

S. suis may travel in the bloodstream to reach the target organs by different means: (i) as free encapsulated bacteria (Fig. 1.2 step 2E), (ii) as internalized by or adherent to monocytes/macrophages (Mo/M) using a "modified Trojan horse" strategy (Fig. 1.2 step F) or (iii) as free bacteria protected by a thick capsule layer, as a high level of bacteremia usually precedes the onset of bacterial meningitis [151]. The possibility that *S. suis* can survive inside professional phagocytes (macrophages and neutrophils) and disseminate to other organs (Trojan horse theory) seems unlikely based on *in vitro* studies (**Chapter 1**) but cannot be ruled out. Sialic acid has been implicated in the "modified Trojan horse" mechanism of *S. suis* adherence (without phagocytosis) to Mo/M [34] (Fig. 1.2 steps F-J).

4.5 Crossing the blood-brain barrier (BBB)

As for other pathogens causing meningitis, *S. suis* must cross the blood-brain barrier (BBB) and/or the blood-cerebrospinal fluid (CSF) barrier in order to cause central nervous system (CNS) infections (Fig. 1.2 steps G,H,I). The main cellular type of the BBB is brain microvascular endothelial cells (BMEC). The BBB is characterised by restricted permeability on both sides of the layer due to the presence of tight junctions between endothelial cells of cerebral vessels and epithelial cells of the choroid plexus. *S. suis* can adhere to, and invade immortalised porcine BMEC, as demonstrated by antibiotic protection assays and electron microscopy [152]. *S. suis* invasion of primary porcine BMEC was confirmed later by Vanier *et al.* who also showed that serum components, possibly fibronectin, played an important role in adherence [153]. As suggested for epithelial cell adhesion, the *S. suis* CPS may partially interfere with the abilities of the pathogen to adhere and invade the BMEC (Fig. 1.2 steps G-H). Cell wall components such as lipoteichoic acid (LTA) have also been implicated in BMEC invasion [153,154]. An *S. suis* mutant impaired in D-alanylation of LTA showed reduced levels of adherence and invasion of porcine BMEC compared to the wild type strain [154]. Other cell wall anchored extracellular proteins may also be involved in BMEC adhesion as indicated by the reduced

adherence capacity of the *S. suis* SrtA mutant to adhere to BMEC, compared to the wild type strain [155]. Suilysin positive strains may also disrupt the BBB through cytotoxic effects, increasing the BBB permeability (Fig. 1.2 steps G-H) although production of suilysin is not essential for invasion [152]. After adherence to BMEC *S. suis* might stimulate production of cytokines resulting in alteration of BBB permeability. This has been suggested because of the high production of cytokines by human BMEC which alter tight junction structure [156]. Apart from alteration of BBB permeability, overproduction of cytokines can activate different leukocyte subpopulations and up-regulate the expression of cell adhesion molecules (CAMs), such as integrins and selectins, that allow trans-endothelial migration of leukocytes [157]. Very recently, using an experimental transwell model, translocation across the blood-CSF barrier of *S. suis*-activated neutrophils was demonstrated [158].

As evidenced by human outbreaks of toxic shock-like syndrome (STLSS) as well as by septic shock cases in Europe and Asia caused by *S. suis* (characterised by short incubation time, rapid disease progression and a high rate of mortality), a substantial release of pro-inflammatory mediators is thought to take place during *S. suis* systemic infections of human [159]. High levels of systemic cytokines induced by *S. suis in vivo* post-infection are thought to be responsible for the death of animals [7,160].

5. Regulation of virulence gene expression

Environmental conditions such pH, temperature, oxygen availability, and organic metabolites may influence the physiology of *S. suis*. The ability of *S. suis* to colonize diverse host niches such mucosal epithelia, the bloodstream and target organs indicates that the bacteria have evolved precise mechanisms to alter, sense and adapt to different environmental conditions, including those within the host.

Like many other bacteria, *S. suis* possess two major types of transcriptional regulators: 1) two-component gene regulatory systems (TCS) and 2) stand-alone regulators. TCS consist of a membrane sensor histidine kinase that influences the phosphorylation state of a cytoplasmatic cognate regulator, which repress or activate gene expression by DNA binding [161]. The TCS regulate the expression of metabolic and virulence genes in response to the external environmental signals. Among the presumptive signals that are thought to be detected by TCS are chemical and physical parameters such as different ions, temperature, pH, oxygen pressure, osmolarity, autoinducer compounds, the redox

state of electron carriers, and the contact with host cells [162]. The regulation systems for other human pathogens (i.e. *Salmonella* and *Staphylococcus aureus*) virulence properties are well characterized, and involve a sophisticated interaction of several TCS [163,164,165]. In *Streptococcus* pathogens, many virulence factors such capsule, hemolysis, and exotoxin have been regulated by TCS [166,167,168]. In total 12 or 13 TCS, depending on strain differences, have been annotated in *S. suis* genomes to date. Despite the importance the two component regulatory systems play in *S. suis* pathogenesis, only few have been studied so far. Mutants lacking TCS CiaR/H or the orphan transcriptional regulators RevSC21 and CovR were impaired in adherence to epithelial cells [169,170,171]. A study on the TCS SalK/SalR has demonstrated that this TCS protects *S. suis* against killing by phagocytes [172,173,174]. This TCS appears not to be present in the European reference strain *S. suis* P1/7. A deletion mutant of *covR* produced a thicker capsule, conferring higher survival compared to the wild type in phagocytosis assays with human monocytes. Inactivation of *covR* gene also increased the lethality of *S. suis* in experimental infection of piglets [175]. These examples show that TCS are important mediators of bacterial virulence and survival in the host, but the precise mechanisms by which each TCS functions are generally unknown and may differ for different bacterial species. In addition to TCSs, transcriptional regulators, e.g. from the LacI/GalR family are important regulators of virulence factor production and of bacterial metabolism such as complex carbohydrate utilization (**Chapter 4**, **Chapter 5**).

As mentioned above, starch α-glycans (large polymers of glucose) are found in high concentrations in the saliva and oropharyngeal cavity [176,177,178]. Glucose, if consumed with the diet may also be present in the oral cavity but concentrations diminish rapidly (within 30 min) after ingestion [179]. In *S. pyogenes* transcript levels of genes involved in carbohydrate metabolism, including those involved in maltodextrin and mannose catabolism, were maximal during the initial colonization phase of primates [180]. These data suggest that in streptococcal pathogens, genes required for α-glucan metabolism were either being induced or derepressed during the *in vivo* growth of the bacteria, suggesting a role for these metabolic genes in bacterial proliferation during the initial stages of colonization of the oropharynx [180].

Generally, host-associated bacteria do not synthesize degradative (catabolic) carbohydrates enzymes unless the substrates of these enzymes are present in the environment. For this reason, bacteria have evolved elaborate regulatory control

mechanisms to utilise the carbon source that allows fastest growth [181]. In the presence of glucose, the sugar that cells use as the primary source of energy, a carbon catabolite control mechanism represses the expression of degradative enzymes, transporters and metabolic pathways for catabolism of other (more complex) sugars. In Firmicutes, the catabolite control protein A (CcpA) is well-conserved among different species and can repress or activate transcription by binding to cis-acting catabolite response element (*cre*) sites (Fig. 1.3).

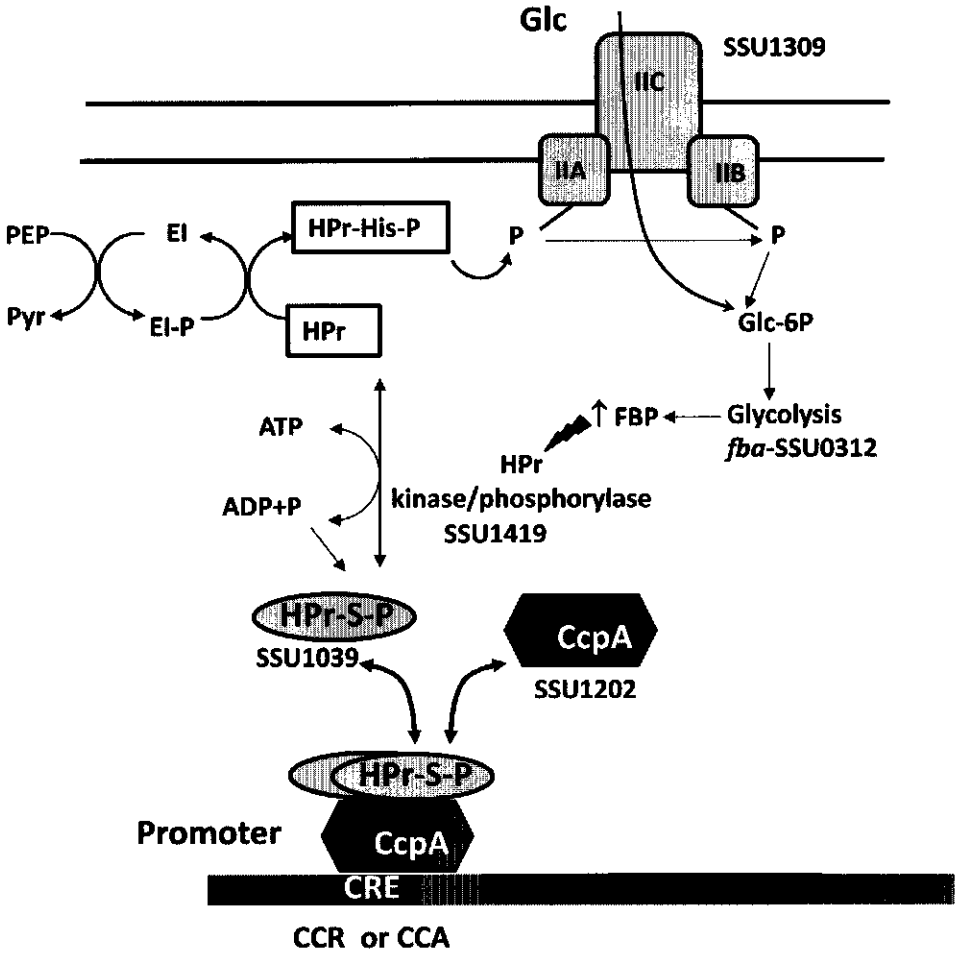

Fig. 1.3: Transport of extracellular glucose is linked to CcpA mediated Carbon Catabolite Repression (CCR) or Activation (CCA).

Consensus *cre* sequences have been determined in several species [181]. Studies in *Bacillus* spp. have shown that the binding of CcpA to DNA *cre* sites is enhanced by interaction of CcpA with the phosphoprotein HPr-Ser-46-P [182,183]. The HPr phosphorylation state is determined by the action of HPr kinase/phosphorylase (HPrK/P), which, in turn, is affected by the intracellular concentration of fructose 1,6-bisphosphate (FBP) that is produced during the glycolysis, the process of degradation of glucose. Orthologues of CcpA, HPr, and HPrK/P from *Bacillus* species are present in the genome sequence of *S. suis* serotype 2 P1/7, the pathogenic European reference strain [84] (Fig. 3). CcpA has been shown to be important for the virulence of several streptococcal species, influencing growth, haemolysin production, biofilm formation and capsule expression [184,185]. Recently, the role of CcpA was investigated by microarray analysis of wild-type *S. suis* and an isogenic *ccpA* mutant grown in THB. Deletion of *ccpA* altered the expression of capsule locus genes encoding surface-associated virulence factors: *arcB*, *sao* and *eno* as well as *ofs* and *cps2A* [186].

In **Chapter 4** we studied if CcpA was involved in the regulation of the virulence factor ApuA. In **Chapter 5** we describe a different approach to study the role of carbohydrate metabolism on metabolism and virulence, by comparing the transcriptomes of *S. suis* grown in complex medium plus α-glucan (pullulan) or glucose in both exponential and early stationary growth phases. Additionally we investigated whether or not carbohydrate composition of the growth medium could influence the virulence of *S. suis* by altering adherence or invasiveness.

6. *S. suis* genomics

Comparative genomics is commonly used in order to study bacterial outbreaks and identification of loci encoding virulence factors [187]. Therefore, several genomics project aimed at sequencing and identifying *S. suis* isolates from human and pig origin have been initiated. One of the first initiatives, started by the Sanger Institute, resulted in the sequencing and annotation of three *S. suis* isolates, two from human origin, and one from pig origin (*S. suis* P1/7, AM946016) [84]. The genome of *S. suis* S10 used in our study, is more than 99% identical to the genome of *S. suis* P1/7. As of January 2012, sixteen genome sequencing project are reported, and thirteen genome sequences have been completed (NCBI Genome and NCBI BioProject http://www.ncbi.nlm.nih.gov/genome/?term=*Streptococcus*%20suis).

S. suis genome sizes are around 2 Mb [84,188], of which 40% is unique for this species in comparison to the genomes of other *Streptococcus* species [84]. Within *S. suis*, the genome content is highly conserved apart from three ca. 90 kb regions, present in the two human sequenced isolates from the Chinese outbreak, that contain insertion sequences (IS) including conjugative elements and transposon sequences, in addition to drug resistance genes, strongly suggestive of horizontal gene transfer [84]. The isolates described by Holden et al. (2009) are of serotype 2, belong to a single lineage and include two highly similar (1 single locus difference) multilocus sequence types. Interestingly, similar numbers of IS were also found in less related strains belonging to different serotypes [188] and horizontal transfer of the 90 kb region between different serotype 2 isolates was demonstrated [188], suggesting that gene transfer, possibly including genes involved in antibiotics resistance, could be a common feature of *S. suis* serotype 2 isolates. For horizontal transfer to occur between serotype 2 isolates, a specific 15 bp sequence was found to be necessary for recombination; this 15 bp sequence was also present in sequenced strains belonging to other (not serotype 2) serotypes [188]. This intriguing observation suggests that genomes of *S. suis* strains could be very dynamic, irrespective of their serotype. Indeed, global comparisons of 13 sequences isolates showed that the numbers of gene gains and losses in *S. suis* genomes was larger compared to other *Streptococcus* genomes [188]. It is clear that further analysis of *S. suis* genomes, from strains isolated from different hosts, different organs, and inducing different symptoms, will eventually reveal loci and genes involved in differential host infection, organ colonisation, and bacterial survival and virulence. Such knowledge will be instrumental in developing therapies against *S. suis* infection, for instance by showing which gene products are necessary for survival in different hosts (organs) and by yielding novel drug targets. One modest example of this is provided in **Chapter 2**, where we mined the *S. suis* genome sequences to find genes encoding surface-anchored, extracellular and thus, exposed proteins that were likely candidates to participate in interactions with the host.

7. The purpose and nature of this PhD thesis

The purpose of this PhD was to characterize a putative surface catabolic enzyme ApuA, in the important zoonotic pathogen *Streptococcus suis* and investigate its role in nutrition acquisition during colonization of the upper respiratory tract. This enzyme was found to have a dual function in adhesion to porcine epithelium and in the degradation of complex dietary or host-derived carbohydrates in the mucosal environment. Studies on the molecular mechanisms of regulation of ApuA revealed that its expression was regulated by carbon catabolite control and a dedicated regulator that induces transcription in the presence of substrates for ApuA. Carbon catabolite control mechanisms were also shown to regulate several virulence factors. In summary the work demonstrates a clear link between virulence and carbohydrate metabolism whereby the pathogen utilises complex carbohydrates as an environmental cue to regulate virulence. In addition the role of different capsule types in phagocytosis and immune response of human dendritic cells was investigated. The thesis contributes substantially to our understanding of the mechanisms of pathogenesis in this major pathogen and has implications for the design of novel vaccines and anti-infective strategies.

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Chapter 2

Immunomodulatory effects of *Streptococcus suis* capsule type on human dendritic cell responses, phagocytosis and intracellular survival

Marjolein Meijerink, M. Laura Ferrando, Geraldine Lammers, Nico Taverne, Hilde E. Smith, Jerry M. Wells

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Summary

Streptococcus suis is a major porcine pathogen of significant commercial importance worldwide and an emerging zoonotic pathogen of humans. Given the important sentinel role of mucosal dendritic cells and their importance in induction of T cell responses we investigated the effect of different *S. suis* serotype strains and an isogenic capsule mutant of serotype 2 on the maturation, activation and expression of IL-10, IL-12p70 and TNF-α in human monocyte-derived dendritic cells. Additionally, we compared phagocytosis levels and bacterial survival after internalization. The capsule of serotype 2, the most common serotype associated with infection in humans and pigs, was highly anti-phagocytic and modulated the IL-10/IL-12 and IL-10/TNF-α cytokine production in favor of a more anti-inflammatory profile compared to other serotypes. This may have consequences for the induction of effective immunity to *S. suis* serotype 2 in humans. A shielding effect of the capsule on innate Toll-like receptor signaling was also demonstrated. Furthermore, we showed that 24 h after phagocytosis, significant numbers of viable intracellular *S. suis* were still present intracellularly. This may contribute to the dissemination of *S. suis* in the body.

Introduction

Streptococcus suis is a major pathogen of swine, causing considerable economic losses and animal health care problems for the pig farming industry worldwide [1]. The natural habitat of *S. suis* is the upper respiratory tract and the intestinal tract [2,3]. In adult pigs carriage of *S. suis* is usually asymptomatic but colonized sows can infect their piglets after nasal or oral contact [4]. Newborn pigs can also become infected during parturition when they contact, swallow or aspirate *S. suis* from sow vaginal secretions [5]. In young pigs *S. suis* infection causes a wide variety of diseases, including meningitis, septicemia which are the main causes of mortality. *S. suis* is also emerging as a serious zoonotic pathogen of humans particularly in South East and East Asia where it is one of the most common causes of human meningitis [6,7]. In 2005 a large outbreak of 215 cases *S. suis* infections occurred in Sichuan, China, resulting in 38 deaths [8]. There are 33 serotypes of S. *suis* of which serotype 2 is most commonly associated with disease in humans and pigs worldwide [9,10]. In addition serotypes 1 to 9 and 14 are responsible for infections in pigs [11] and serotypes 1, 4, 5, 14, 16 and 24 have caused severe disease in a limited number of persons [12,13,14,15]. The capsule is known to be a very important virulence factor in *S. suis* [16] although not all capsulated isolates (including serotype 2) are virulent, highlighting the importance of other virulence factors in the pathogenesis of disease [3]. Dendritic cells (DCs) are important sentinels in the skin and mucosal surfaces that contact the external environment and play a key role in the homoeostatic control tolerance and immunity in the mucosal tissues [17]. Stromal factors such as retinoic acid and thymic stromal lymphopoietin imprint tolerogenic properties on resident DC. However when invading microbes are encountered the homeostatic mechanism are overridden by chemotactic recruitment of DC and their activation by pattern recognition receptor (PRR) binding to pathogen-associated molecular patterns (PAMPs). Upon activation DCs express up to 100x more MHC than monocytes, macrophages and neutrophils other antigen presenting cells (APCs) and migrate to mucosal associated lymphoid tissue to induce antigen-specific T cell responses [18,19]. Thus DCs are instrumental in the orchestration of adaptive immune responses. Cytokines produced by activated DC have a major influence on T cell polarization, differentiation and clonal expansion. Interleukin (IL)-12 and tumor necrosis factor (TNF)-α, are pro-inflammatory cytokines that promote T helper (Th) 1 cell responses, whereas IL-10 is an anti-inflammatory cytokine that can promote induction of Th2 cells or regulatory T cells depending on the expression of other tolerizing factors [20].

DCs recognize different types of PAMPs using pattern recognition receptors (PRRs) of the Toll-like receptor (TLR), nucleotide-binding oligomerisation domain receptor (NLR) and C-type lectin receptor (CLR) protein families, [17,21,22]. PRR signaling is critical to DC maturation and in recent years much emphasis has been given to dissecting the innate signaling pathways involved in pathogen recognition. Each PRR recognizes variants of a specific molecular pattern and can be expressed on the cell surface, in intracellular compartments or in the cytosol. TLR1, 2, 4, 5, 6 and 11 recognize mainly microbial envelope components and are expressed on the cell surface, TLR3, 7, 8 and 9 recognize microbial nucleic acids and are expressed in intracellular compartments such as the endoplasmic reticulum, endosome and phagosome. TLR2 can form heterodimers with TLR1 or TLR6 to detect different, but related ligands. TLR2/1 recognizes tri-acyl lipoproteins found predominantly in Gram-negative bacteria and TLR2/6 the diacyl groups on lipoteichoic acid and lipoproteins of Gram-positive bacteria. NOD1 and NOD2 are cytoplasmic receptors that can detect peptidoglycan fragments produced in the phagosome or phagolysosome of antigen presenting cells although the nature of the transporters involved in translocation to the cytoplasm remains unknown [23]. The CLR family is characterized by the presence of one or more C-type lectin-like domains (CTLDs) and bind mainly sugars including self-antigens. CLRs trigger distinct signaling pathways that induce the expression of specific cytokines which determine T cell polarization fates [24]. Recently the interactions of a virulent serotype 2 strain and its unencapsulated derivative with porcine DC were studied *in vitro*. The capsular polysaccharide was shown to interfere with phagocytosis and consequently the level of DC maturation and production of several cytokines was reduced compared to an unencapsulated strain [25]. Given the emergence of *S. suis* as a significant cause of meningitis in humans we investigated the effect of different serotypes (SS1, SS2, SS4, SS7, SS9 and SS14) and the unencapsulated mutant of *S. suis* serotype 2 (SS2 J28) on the maturation and expression of IL-10, IL-12p70 TNF-α in human monocyte-derived DC. Additionally, we compared the efficiency of the different isolates in DC phagocytosis assays and studied the intracellular survival of internalized *S. suis* serotype 2of internalized *S. suis* serotype 2 S10 and its unencapsulated isogenic mutant . The ability of the different serotype strains to induce TLR signaling via human TLR2/6 was also investigated using a TLR2/6 specific luciferase reporter cell line. To our knowledge this is the first study concerning the interactions of *S.*

suis with human DC and it provides new knowledge of the role of different capsular polysaccharide serotypes in the avoidance of host innate immunity.

Results

S. suis capsule serotype differentially affects DC maturation and activation

Immature monocyte-derived DCs derived from six different human donors were used as *in vitro* model to investigate interactions with *S. suis*. The DCs were stimulated for 48 hours with 6 different *S. suis* serotypes and SS2J28 at MOI 1 and MOI 10 (Fig. 2.1A-C). Expression of the surface expressed co-stimulatory molecule CD86 and maturation marker CD83 were measured to determine the activation and maturation status of the DCs respectively (Fig. 2.1A and B for mean fluorescence intensity and 2.1C for histograms). For all encapsulated strains stimulation of DC with *S. suis* at a MOI 10 resulted in higher maturation and activation marker expression than at MOI 1. The induction of the surface expression CD86 and CD83 differed markedly among the capsule serotypes tested. Significantly higher levels of CD83 and CD86 were observed following DC stimulation with serotypes SS1, SS7 and SS9 and the unencapsulated SS2 mutant than with serotype SS2.

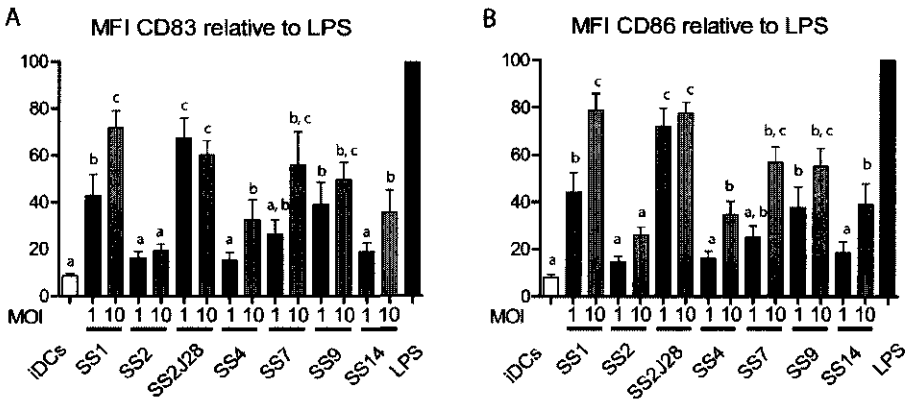

Fig. 2.1. Mean Fluorescence Intensity (MFI) of dendritic cells normalized with LPS.
The MFI of stained cell surface markers by monocyte derived dendritic cells with 6 different *S. suis* strains and SS2J28 mutant, with immature DCs as the negative control and LPS as the positive control. **A.** MFI of CD83 **B.** MFI of CD86. Bars showing unequal letters significantly differ in their surface marker expression ($P<0.05$).

Interestingly, serotype SS2 was the least effective at maturating DC although its unencapsulated variant, SS2J28 was the most effective indicating the importance of the capsule in the avoidance of host innate immunity.

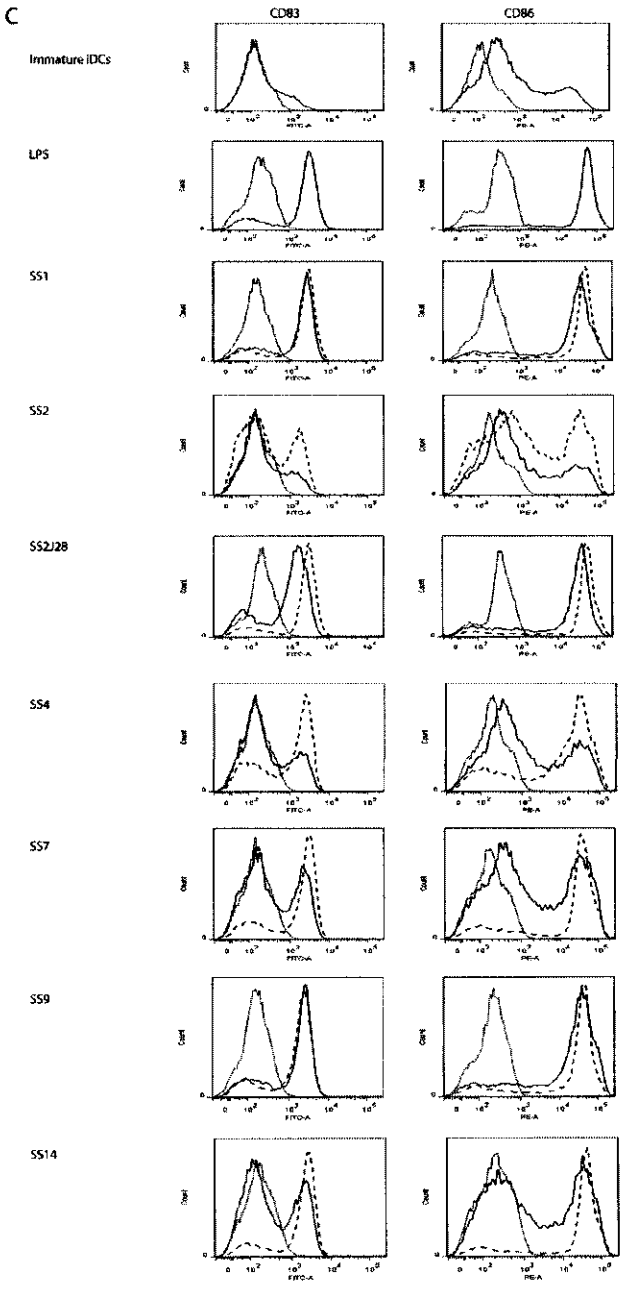

Fig. 2.1. Mean Fluorescence Intensity (MFI) of dendritic cells normalized with LPS.
C. Histograms for expression of surface markers CD83 and CD86. Dotted lines represent the isotype controls and black lines the stimulated samples. In case of bacteria a black line represents a MOI 1 and a dashed line a MOI 10.

The capsule of S. suis serotype 2 differentially modulates the IL-10 to IL-12 ratio

The amounts of IL-10, IL-12 and TNF-α measured in the supernatants of DC co-cultured with the different serotypes was highly variable (Fig. 2.2). The amounts of IL-10 ranged from 5 pg/mL to 56 pg/mL, IL-12p70 from 7 pg/mL to 6948 pg/mL and TNF-α from 5 pg/mL to 3744 pg/mL (Fig. 2.2A-C). As expected, stimulation with an MOI 10 resulted in higher amounts of secreted cytokine than stimulation with an MOI 1. In keeping with the data on maturation markers (Fig. 2.1) serotypes SS1, SS7 and SS9 were the highest inducers of cytokines, whereas serotype SS2 induced the lowest amounts of cytokines (all cytokine < 10pg/mL). In contrast the unencapsulated derivative SS2J28 stimulated the highest amounts of IL-10 and IL-12 and high amounts of TNF-α. The ratio of IL-10 to IL-12 is often used as an indicator of the potential to polarize T cell responses towards Th1 or Th2/Treg [17]. Interestingly all of the *S suis* serotypes except SS2 induce low IL-10 to IL12 ratios (less than 0.08). For SS2 the IL-10 to IL-12 ratio was 0.34 at MOI 10 and almost 1.0 (0.98) at MOI 1. Strikingly, the unencapsulated derivative of SS2 designated SS2J28 has a much lower IL-10 to IL-12 ratio than SS2 (0.03 at MOI 1 and 0.008 at MOI 10) suggesting

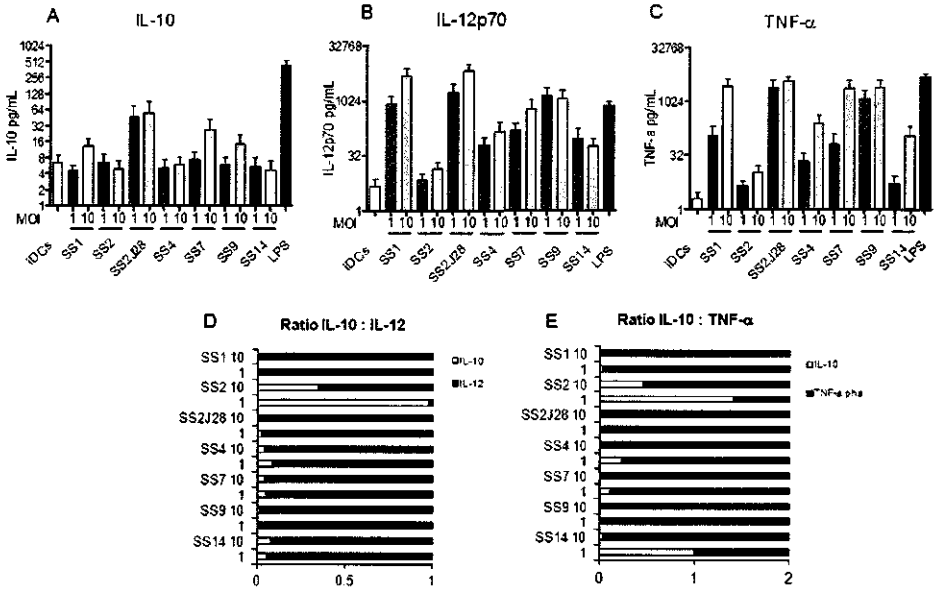

Fig. 2.2. Cytokine secretion by dendritic cells. Cytokine production by monocyte derived dendritic cells with 6 different *S. suis* strains and SS2J28, with immature DCs as the negative control and LPS as the positive control. **A.** IL-10 B. IL-12p70 **C.** TNF-α. **D.** IL-10/IL-12 ratio. **E.** IL-10/TNF-α .

that the type 2 capsule can down-regulate the host cell-mediated response to *S. suis* (Fig. 2D). Similar trend of ratio's were observed for the IL-10 to TNF-α ratio (Fig. 2E).

Effect of capsular polysaccharide on the capacity of DCs to internalize S. suis

The percentage of *S. suis* phagocytosed by DCs varied considerably among the different serotypes tested. After one hour of incubation of the DCs with the bacteria, SS4 and SS9 were more efficiently taken up by the DCs (respectively 23% and 20% of original inoculum (10^7 bacteria) compared to the other strains (Fig. 2.3). In contrast capsule types SS1 and SS2 were relatively resistant to phagocytosis by DCs (0.04% and 2.16% respectively of the original inoculum). The unencapsulated mutant was internalized at significantly higher amounts than its wild type SS2 progenitor (5.29% vs 2.16%; P= 0.0001). Surprisingly however, the unencapsulated strain was less efficiently internalized than serotype strains SS4 and SS9 (Fig 2.3).

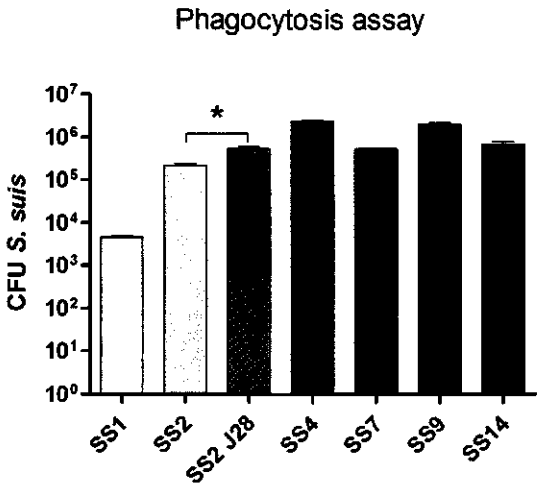

Fig. 2.3. Phagocytosis assay. Phagocytosis by immature DCs with 6 different *S. suis* strains and an unencapsulated mutant at an MOI 10. This is a representative figure from 1 donor, out of 5 donors. *P < 0.05.

Capsular type 2 does not affect intracellular survival of internalized S. suis

To rule out the possibility that differences in internalization of SS2 and SSJ28 by DC might be due to strain variation in intracellular survival we measured the survival of these two strains in DC over time. After one hour of incubation with iDC antibiotics were added for 1 hour to ensure that only internalized bacteria were counted after lysis of the DCs. After a

total 2 hours of incubation the number of viable bacteria inside the DCs decreased considerably, to 39% of the original inoculum for SS2 and 43% of the inoculum for SS2J28 (Fig. 2.4A). Over the first 4 hours the number of viable *S. suis* decreased at a similar rate for both strains indicating that the higher level of internalization measured for SS2 J28 (Fig. 2.4A) could not be due to less rapid killing.

Viable S. suis reside within DC 24 hours after phagocytosis

To examine the survival of the wild type SS2 and its mutant SS2J28 inside the DCs after 24 hours, the DCs (10^6 cells) and bacteria (MOI 10) were incubated for 2 and 24 hours (Fig. 2.4B). After 1 hour of incubation antibiotics were added to the medium kill extracellular and adhered bacteria. After 5 hours, the medium was replaced by RPMI without antibiotics, to prevent the antibiotics from entering the DCs. After 24 hours around 10^3 CFU/mL of live *S. suis* were recovered from the lysed DC suggesting that a small proportion of the bacteria could survive intracellularly. To rule out that these phagocytosed bacteria were released from DC after 5 hours of co-incubation and were growing in the medium the experiment was repeated using an additional antibiotic treatment at 23 hours to kill any extracellular/adherent bacteria that might be present. The results (Fig. 2.4B) showed that the CFU/mL counts present after 24 hour could be attributed to the presence of intracellular *S. suis*.

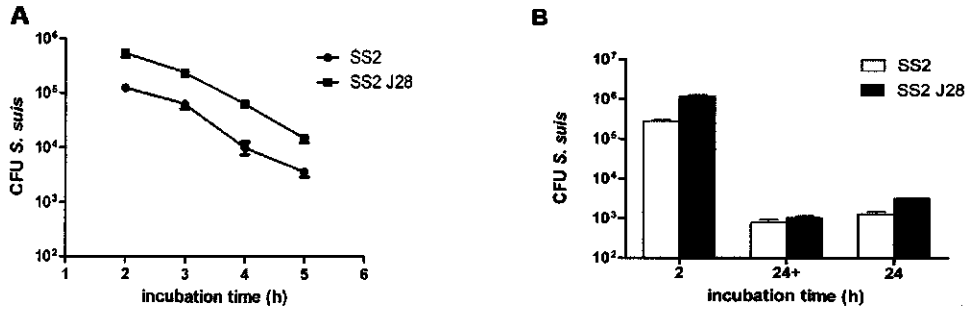

Fig. 2.4. Survival of *S. suis* inside dendritic cells. A. *Phagocytosis kinetics*. Immature DCs were inoculated with SS2 and SS2 J28 at a MOI of 10 bacteria/DC for subsequently 2, 3, 4, 5 and 24 hours of incubation. **B.** *Survival of S. suis inside DCs after 2 and 24 hours*. Immature DCs were inoculated with SS2 and SS2J28 at a MOI10 for 2 and 24 hours of incubation. The last hour the DCs were incubated with (24+) or without (24) antibiotics.

Involvement of TLR2 and TLR6 in cell activation by S. suis

TLR2/6-mediated activation of NF-κB could be one of the major pathways for DC activation via LTA or lipoproteins in the cell envelope of *S. suis*. Therefore we tested the TLR2/6 signaling capacities of all the serotypes in a reporter assay using HEK293 cells expressing human TLR2 and TLR6 heterodimer that recognizes lipoteichoic acid (LTA) and lipoprotein lipid anchors in Gram-positive bacteria (Fig. 2.5). HEK293 cells transformed with only the pNIFTY, a NF-κB luciferase reporter construct did not respond to Pam_2CSK demonstrating the dependency of NF-κB activation on co-expression of hTLR2/6 receptor. Medium was used as a negative control and Pam_2CSK (synthetic agonist of TLR2/6) as a positive control. The results shown in Fig. 2.5 demonstrate that indeed all strains are capable of triggering NF-κB activation via TLR2/6 signaling but there was no correlation between the capacity of the strains to induce TLR2/6 signaling and activate DC (Fig. 2.2). Interestingly the unencapsulated mutant of SS2 induced significantly ($P<0.05$) higher levels of NF-κB than SS2 indicating that the capsule has a shielding effect on TLR activation.

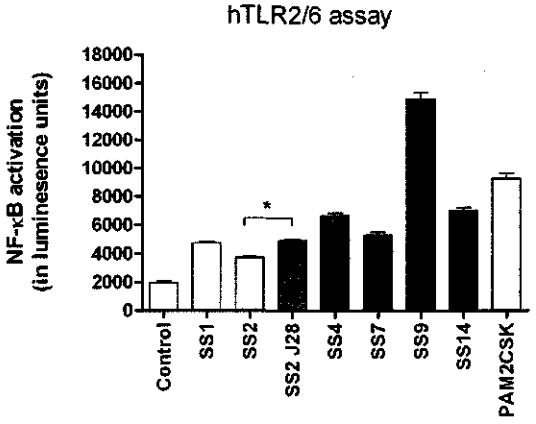

Fig. 2.5. hTLR2/6 assay. HEK293 cells were incubated with 6 different *S. suis* strains and an unencapsulated mutant at a MOI10, PAM2CSK as a positive control and medium as a negative control. This figure is representative out of three hTLR2/6 assays. *$P < 0.05$.

Discussion

Apart from its major economic impact on the mortality and morbidity of young pigs in agro-food production *S. suis* is emerging as a one of the major causes of meningitis in South East Asia. To gain further knowledge on the role of *S. suis* capsule and capsule serotype on the immune response of the human host we compared phagocytosis and immune responses of human immature DC to different serotypes of *S. suis*. DCs are professional antigen presenting cells that play a key role in the induction of adaptive immune responses. Once activated by contact with invading pathogens $CD103^+$ DC can traffic from the sites of mucosal infection to the draining lymph nodes to induce T cell responses. Additionally they play a crucial role in induction of adaptive immune responses in the Peyer's patches of the nasal mucosa and small intestine. Many pathogens have evolved mechanisms to avoid phagocytosis including production of leukotoxins, the inhibition of complement activation and masking of binding sites for endocytic receptors by polysaccharide capsules [37].

The serotypes of *S. suis* differed significantly in their ability to activate DCs and induce cytokine responses. Serotypes SS1, SS7 and SS9 induced expression of significantly higher levels of DC activation and maturation markers than serotypes SS2, SS4 and SS14 (Fig. 2.1). Interestingly SS2, the strain that was the least effective at activating and maturating DC was serotype 2, which is the serotype most commonly associated with invasive disease in pigs and humans [38]. In contrast the unencapsulated variant of SS2 (SS2J28) was the most effective strain in maturating and activating DC, indicating the important role of the capsule in shielding cell wall components that activate DC and induce cytokine responses (Fig. 2.1). The thickness of the capsule may also influence the activation by influencing the release of MAMPs such as lipoproteins and LTA. Capsule serotype 1 has been reported to have a thinner capsule than other serotypes [51] and interestingly this was the most effective of all capsulated strains in activating and maturating DC. The anti-phagocytic effects of SS2 capsule were apparent from the significantly higher phagocytosis of the unencapsulated mutant SS2J28 (5.29% vs 2.16%; P= 0.0001) (Fig. 2.3). To rule out the possibility that differences in internalization of SS2 and SS2J28 by DC might be due to strain variation in intracellular survival we measured survival over period of 4 hours after phagocytosis. The number of viable *S. suis* decreased at a similar rate for both strains indicating that the higher level of internalization measured for the unencapsulated mutant SS2J28 (Fig. 2.3) was not due to less rapid killing (Fig. 2.4A). Similar results were recently

described using porcine DCs and a capsulated and unencapsulated serotype 2 strain [25]. As in our own study an unencapsulated mutant of a serotype 2 strain was phagocytosed at significantly higher levels and once internalized, both the wild-type strain and its non-encapsulated mutant were killed at similar rates.

To investigate the effect of other capsule types on phagocytosis the internalization of several other serotypes were compared to serotype 2 and its unencapsulated mutant SS2J28 (Fig. 2.3). The serotypes differed considerably in their ability to be phagocytosed with around 20% of the inoculum being internalized in the case of SS4 and SS9 but only 2% in the case of SS2 (Fig. 2.3). This might be explained by the difference in composition of the capsules and their charge which is known to be important in the avoidance of phagocytosis. A recent genetic analysis of the capsular polysaccharide synthesis locus of 15 *S. suis* serotypes predicted that capsules of serotypes 1, 2, and 14 may contain sialic acid [39]. In *Streptococcus agalactiae* capsule sialic acid has been shown to increase the hydrophilic surface properties of the bacteria and have an inhibitory effect on phagocytosis [40]. This might be an explanation for the fact that phagocytosis of serotype strains 1, 2 and 14 was significantly lower than for serotype strains 4 and 9. The sialylated capsule of *Streptococcus agalactiae* also inhibits C3 deposition on the bacterial cell surface [41], probably via recruitment of factor H, an anti-activator of the complement alternative pathway [42]. However it is not evident that C3 deposition is inhibited by sialic acid in the serotype 2 capsule of *S. suis* because phagocytosis levels are not significantly different in the presence or absence of serum factors [25].

Interestingly the unencapsulated SS2J28 strain was less efficiently phagocytosed than serotype strains SS4 and SS9 (Fig. 2.3). Thus it is possible that SS2 has other mechanisms that inhibit phagocytosis. A two-component regulatory system (TCS) designated SalK/SalR has been shown to have a protective role in the killing of *S. suis* by granulocytes [16,43,44] but this locus is absent in the genome of SS2 strain and thus cannot be responsible for the lower levels of phagocytosis observed for SS2 compared to SS4, SS7, SS9 and SS14.

A shielding effect of SS2 capsule was investigated using a reporter cell line for TLR2/6 signaling. The TLR2/6 heterodimer is formed by binding of the di-acyl groups present on lipoproteins of Gram-positive bacteria and lipoteichoic acids present in the cell wall [22,45]. This triggers NF-κB activation via a signal kinase cascade involving the adapter protein MyD88 and was detected in our assay by production of luciferase under control of an NF-κB promoter. The unencapsulated mutant of SS2 induced significantly ($P<0.05$)

higher levels of NF-κB than SS2 suggesting that the capsule has a shielding effect on the exposure of TLR agonists that might can activate cells such as DCs (Fig. 2.5). Notably, the level of NF-κB activation obtained with the unencapsulated mutant was significantly lower than for SS9($p<0.0001$), SS14($p<0.0001$) and SS4($p<0.0001$). The highest level of TLR2/6 activation was observed for SS9, something observed in a previous study using the same strains SS2 and SS9 (Fig. 2.5) [46]. Interestingly we found that SS9 was phagocytized more efficiently than the other strains and was highly effective at activating DC. However efficiency of phagocytosis did not correlate with activation of DC as evident for strain SS1 which was phagocytized at relatively low levels compared to the other serotypes but nevertheless strongly activated DC in co-culture.

The amounts of IL-10 IL-12 and TNF-α measured in the supernatants of DC co-cultured with the different serotypes was highly variable (Fig. 2.2). The amounts of IL-10 ranged from 5 pg/mL to 56 pg/mL, IL-12p70 from 7 pg/mL to 6948 pg/mL and TNF-α from 5 pg/mL to 3744 pg/mL (Fig. 2.2A-C). In agreement with the data on maturation and activation markers (Fig. 2.1) the serotypes SS1, SS7 and SS9 were the highest inducers of cytokines, whereas serotype SS2 induced the lowest amounts of cytokines (all cytokine < 10pg/mL). Interestingly all of the *S suis* serotypes except SS2 induce low ratios of IL-10 to IL12 ratios (less than 0.08). For SS2 the IL-10 to IL-12 ratio was 0.34 at MOI 1 and almost 1.0 (0.98) at MOI 10. This qualitative effect on the cytokine response was due to the serotype 2 capsule because the unencapsulated mutant of SS2 induced cytokines with a low IL-10 to IL-12 ratio (0.03 at MOI 1 and 0.008 at MOI 10) as observed for the other serotypes (Fig. 2.2D). A similar trend was seen for the IL-10/TNF-α cytokine ratio (Fig. 2.2E). It is not known whether the immunomodulatory effect of SS2 capsule is also observed with porcine DC as the recent study did not measure IL-10 production by DC [25]. A consequence of increased IL-10 production may be the polarization of T helper cell responses towards Th2 or Treg [47]. In pathogenic species of *Yersinia* for example, the secreted V antigen protein induces IL-10 in macrophages to evade the host's inflammatory response during infection [48]. Pathogens such as *Mycobacterium tuberculosis* and HIV target DC-SIGN on DC to escape immunity. Binding to DC-SIGN cause internalization but not subsequent antigen processing and induces IL-10 expression resulting in suppression of Th1 responses [49]. DC-SIGN binds glycans containing high mannose structures appear not to be present in the published structure of the serotype 2 capsular polysaccharide [50]. However, it is possible that the involvement of other C –type lectin receptors on DC

or other glycan structures may be involved in the immunomodulatory effects of the SS2 capsule.

Over a period of 5 hours after internalization in DC the number of viable *S. suis* were reduced about 100 fold. The rate of killing and overall levels of intracellular survival of *S. suis* after 5 hours was higher than that reported previously using porcine DC [25]. This may have been due the use of a different serotype 2 strain and/or differences in killing capacity of human and pig DCs and warrants further study. Despite the fact that a high proportion of phagocytized *S. suis* were killed by DC in the first 5 hours of incubation we were able to recover around 10^3 CFU/ml of SS2 and the unencapsulated mutant after 24 hours incubation (Fig. 2.4B). In these experiments antibiotics were added to the medium for 5 hours to kill extracellular and adhered bacteria then the medium was replaced by RPMI without antibiotics, to prevent the antibiotics from entering the DCs. Prior to lysis antibiotics were added a second time to some of the samples to kill any extracellular bacteria that might have been released from DC. The results (Fig. 2.4B) showed that the CFU counts present after 24 hour could indeed be attributed to the presence of viable intracellular *S. suis*. This has important consequences for pathogenesis because activated DCs eventually undergo apoptosis and may release viable *S. suis*. As DC traffic from the mucosa travel via the bloodstream to lymphoid tissue such a mechanism may enable *S. suis* to rapidly disseminate in the body during invasive disease.

Materials and Methods

Bacterial strains

Six different serotypes (SS1, SS2, SS4, SS7, SS9 and SS14) and the unencapsulated mutant of SS2 (SS2 J28) were obtained from Central Veterinary Institute, Lelystad NL (Table 2.1). The genome of *S. suis* S10 is more than 99% identical to the genome of *S. suis* P1/7, a sequenced reference strain of which the genome had been annotated previously (**Chapter 1**). In the table are indicated for each strain the expression of three virulence markers: two secreted cell wall located proteins namely the muramidase-released protein (MRP) and the extracellular factor (EF) [26,27], and secreted hemolytic toxin suilysin (SLY) [28]. MRP and EF variants have been designated as MRP^s and EF*. All *S. suis* strains were cultured overnight at 37°C in Todd Hewitt broth (Oxoid). The bacteria were then recovered by centrifugation, washed twice in phosphate buffered saline (PBS, pH=7.4), resuspended at approximately 1×10^9 colony forming units (CFU)/mL in PBS containing 20% glycerol, and stored in aliquots at -80°C prior to use. The exact number of bacterial CFU in a thawed aliquot was determined by plating serial dilutions on Columbia blood agar plates (BD) containing 5% sheep blood in presence of 5% CO_2.

Table 2.1: List of strains used in this study

Serotype	Strain	Virulence in pigs	MRP	EF	Suilysin	CPS	Reference
SS1	6388	HV	MRP^s	EF^+	SLY^+	$Cps1^+$	[29,30]
SS2	S10	V	MRP^*	EF^+	SLY^+	$Cps2^*$	[16]
SS2 J28	10cpsΔEF[a]	AV	MRP^*	EF^+	SLY^+	$Cps2^-$	[16]
SS4	S213	ND	MRP^s	-	ND	$Cps4^*$	[31]
SS7	8039	ND	-	-	-	$Cps7^+$	[30,32]
SS9	8067	AV	-	-	SLY^-	$Cps9^*$	[30,33]
SS14	13730	ND	-	EF*	ND	$Cps14^+$	-

[a] The isogenic unencapsulated mutant strain 10cpsΔEF parts of the cps2E and cps2F gene were replaced by an antibiotic resistance gene. HV high virulent, V virulent, AV avirulent. MRP muraminidase-released protein. EF extracellular factor. SLY suilysin. CPS capsular polysaccharide synthesis *, higher MW protein expressed; s, smaller MW protein expressed

Differentiation and maturation of dendritic cells

The study was approved by the Wageningen University Ethical Committee and was performed according to the principles of the Declaration of Helsinki. Buffy coats from four blood donors were obtained from the Sanquin Blood bank Nijmegen, Netherlands. A written informed consent was obtained before the sample collection. Human monocytes were isolated from blood using a combination of Ficoll density centrifugation and cell separation using CD14-specific antibody coated magnetic microbeads (Miltenyi Biotec). The purity of isolated CD14+ cell fraction was greater than 90% and viability >95% in all experiments. To generate immature DC (iDCs), the purified CD14+ cells were cultured for 6 days in RPMI 1640 medium (Invitrogen), supplemented with 100 units/ mL penicillin G (Invitrogen), 100 µg/mL streptomycin (Invitrogen), IL-4 (R&D systems) and granulocyte-macrophage colony-stimulating-factor (GM-CSF) (R&D systems). GM-CSF and IL-4 were added to

differentiate the monocytes into myeloid DCs. At day 6 the iDCs (1 x 10^6/mL) were stimulated with LPS (1 μg/mL) or the different *S. suis* serotypes at multiplicities of infection (MOI) of 1 bacterium per DC or 10 bacteria per DC for 48 hours. Unstimulated iDCs were used as a negative control.

Analysis of cell surface markers and measurement of cell death by flow cytometry

During the 8 day culture period of the CD14+ cells (6 days of differentiation of monocytes into immature dendritic cells and two days of stimulation), cells were stained on days 3, 6 and 8 with fluorescence-conjugated monoclonal antibodies specific for CD83, CD86 or their isotype-matched controls (BD biosciences, San Diego, USA) and analyzed by flow cytometry (FACSCanto II, BD, San Diego, USA) to check the maturation and activation status of the cells. CD86 and CD83 were not expressed on immature dendritic cells (d3 and 6) but were highly expressed on DCs after activation with known maturation factors (e.g. LPS). The magnitude of the response from different human donors can vary considerably so for comparison the data was normalized to the LPS control sample data (100%) for each donor. On days 3, 6 and 8 the percentage of viable cells was measured by flow cytometry (FACSCanto II, BD, San Diego, USA). Live, apoptotic and necrotic cells were discriminated by staining with Annexin V and propidium iodide on days 3, 6 and 8 according to the manufacturer's protocol. The cells were analyzed on a flow cytometer (FACSCanto II, BD, San Diego, USA). Cells that are negative for both Annexin V and PI are not apoptotic or necrotic as translocation of the membrane phospholipid phosphatidylserine has not occurred and the plasma membrane is still intact. Therefore, Annexin V and PI double negative cells were considered as viable cells, whereas both single and double positive cells were regarded as non-viable [29]. The flow cytometry data was analyzed using the BD FACSDiva software. On days 3 to 8 the viability of the cells was between 60 and 95%. There were no significant differences in cell death between *S. suis* co-cultures or compared to the medium and LPS controls.

Cytokine assay

Supernatants from the DC stimulation assays were collected after stimulation for 48 hours, and analyzed for the presence of cytokines (IL-10, IL-12p70 and TNF-α) using a cytometric bead-based immunoassay that enables multiplex measurements of soluble cytokines in the same sample [30], according to the manufacturer's protocol (BD biosciences). The limits of sensitivity for detection were as follows: 0.13 pg/mL, 0.6 pg/mL and 0.7 pg/mL. The flow cytometry data were analyzed using the BD FCAP software.

Phagocytosis assay

The iDCs (10^6 cells) were inoculated with the different *S. suis* serotypes (MOI 10) and incubated for one hour in antibiotic-free RPMI 1640 at 37°C and the presence of 5% CO_2. The DCs were further incubated for one hour in RPMI 1640 containing 56.2 μg/mL penicillin G and 100 μg/mL gentamicin. Subsequently the DCs were collected and the centrifuged for 5 minutes at 845 g. The pellet was

washed with PBS to remove the antibiotics and the DCs lysed and vigorously vortex in ice-cold milliQ water. The cell lysate was then serial plated on Columbia blood agar plates (BD) containing 5% sheep blood to enumerate the CFU of *S. suis*.

Adhesion and Phagocytosis Assay

In Fig. 3 the iDCs (10^6 cells) were inoculated with SS2 and SS2 J28 (MOI10) and incubated for 1 hour in antibiotic-free RPMI1640. To count the adherent and phagocytosed bacteria DCs were washed after one hour twice with PBS to remove the unbound bacteria, lysed with ice-cold milliQ water and plated on Columbia blood agar plates (BD) containing 5% sheep blood.

Kill Curve

Phagocytosis of *S. suis* was performed as described above and then the DCs were incubated in RPMI 1640 containing 56.2 µg/mL penicillin G and 100 µg/mL gentamicin to kill extracellular bacteria. The killing of phagocytosed *S. suis* was determined after 1, 2, 3 and 4 h by removing the antibiotics with PBS washes, lysis in ice-cold milliQ water and serial plating on Columbia blood agar plates (BD) containing 5% sheep blood (Fig 4A)

Survival of S. suis inside DCs after 2 and 24 hours

The iDCs (10^6 cells) were inoculated with SS2 and SS2 J28 (MOI10) and incubated for one hour in antibiotic-free RPMI1640. After one hour of incubation antibiotics (100 µg/mL gentamicin and 56.2 µg/mL of penicillin G) were added to kill all the extracellular bacteria. After a further one hour incubation in the presence of the antibiotics DCs samples were collected and plated in the same way as described in the phagocytosis assay (2h time point). After a further 4 hours, the medium was replaced by RPMI lacking antibiotics to prevent the antibiotics from entering the DCs and killing intracellular bacteria. After a total of 23 hours incubation the DCs were incubated for one hour in RPMI with or without antibiotics washed twice with PBS, lysed with ice-cold milliQ water and plated on Columbia blood agar plates (BD) containing 5% sheep blood (24 h time point).

TLR2/6 assay

The TLR2/6 signaling assay was performed essentially as previously described [31]. Briefly, HEK293 cells (Invivogen, Toulouse, France) were transformed with human TLR2/6 and pNIFTY, a NF-κB luciferase reporter construct (Invivogen). The cells were plated a concentration of 6×10^4 cells per well in DMEM medium. Cells were then stimulated with the different *S. suis* strains, Pam2CSK as a positive control and with medium alone (negative control) and incubated at 37°C and 5% CO_2 for 24 hours. After this incubation period the medium was replaced with Bright glow (Promega), the plate was vortexed and the luminescence was measured using a Spectramax M5 (Molecular Devices). Human embryonic kidney (HEK)293 cells not expressing TLR receptors but harbouring pNIFTY, a NF-

κB luciferase reporter construct (Invivogen, Toulouse, France) were used as the negative control in the NF-κB assays.

Statistical analysis

Dixon's Q test was applied for the evaluation of differences in the values of the immune- and cytokine assays. Datasets contained values of six different donors. P values of <0.05 were considered significant. Independent sample t-test was applied for the evaluation of differences between SS2 and SS2J28 in the phagocytosis assay and the kill curve. $P<0.05$ were considered significant.

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Chapter 3

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

M. Laura Ferrando, Susanna Fuentes, Astrid de Greeff, Hilde Smith and Jerry M. Wells

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Summary

We have identified *apuA* in *Streptococcus suis* which encodes a bifunctional amylopullulanase with conserved α-amylase and pullulanase binding domains and catalytic motifs. ApuA exhibited properties typical for a Gram-positive surface protein with a putative signal sequence and LPKTGE cell wall anchoring motif. The α-1,4 glycosidic activity has been showed by the production of an recombinant protein of the α-amylase domain. The predicted α-(1,6) glycosidic activity of a pullulanase was found in the cell surface protein extract of *S. suis*. ApuA was required for normal growth in complex medium containing pullulan as the major carbon source suggesting that *in vivo* this enzyme plays a role in nutrient acquisition 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 [1,2]. Healthy pigs asymptomatically colonized with *S. suis* form a reservoir for this disease and play a major role in the epidemiology [3]. To date 33 different capsule serotypes of *S. suis* have been identified but serotype 2 is most commonly associated with disease worldwide [4,5]. Serotype 2 strains were also associated with recent large outbreaks of severe human infections in China and Asia [6,7,8]. The recently obtained genome sequences of two Chinese virulent *S. suis* serotype 2 (SS2) strains (98HAH12 and 05ZYH33) [9] and P1/7 the European reference strain [10] 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 [11]. Genes encoding 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 [12,13,14,15]. Dietary sources of highly polymerized α-glycans such as starch and glycogen are abundant in the human colon [16], oropharynx [17,18,19,20], epithelium of the vagina and lung [21,22,23,24]. Starch and glycogen degradation in most organisms proceeds via the action of amylases and pullulan degrading enzymes (such pullulanase and amylopullulanase) which cleave α-(1,4) and α-(1,6) glycosidic linkages. Pullulan is a linear polysaccharide of maltotriose repeating units linked via α-(1,6) glycosidic bonds, produced by the ascomycete fungus *Aureobasidium pullulans* [25]. Although pullulan is not found in animals it is commonly used as substrate to identify pullulanases with α-(1,6) glycosidase activity [9,26,27].

In Group A streptococci (GAS), Group B streptococci (GBS) and *Streptococcus pneumoniae*, cell wall anchored enzymes that can hydrolyze pullulan, have been characterized [23,28,29]. 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 submaxillar mucin [28]. 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 [24]. 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* [30]. Recently, it was shown that GAS PulA deficient mutants were less able to adhere to human epithelial cells [31]. Furthermore, recombinant SAP, a type I pullulanase from GBS, was shown to bind human epithelial cells *in vitro* [21,31]. Here we report on the characterization and mutagenesis of *apuA*, encoding an extracellular bifunctional amylase/pullulanase with C-terminal pullulanase activity 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.

Results

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

A gene designated here as *apuA* (6285 bp and 2094 amino acids (aa)) 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) [32] ApuA is predicted to be a 23 kDa mature surface protein covalently linked to the cell wall (Fig. 3.1).

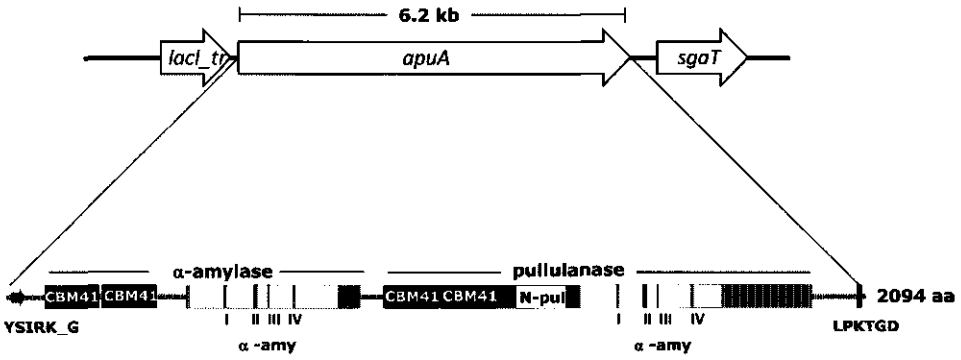


Fig. 3.1 Schematic representation of the *apuA* locus. ApuA is encoded by a single open reading frame of 6,285 bp, producing a bifunctional amylase/pullulanase protein of 2,094 amino acids. Located upstream of the *apuA* locus is a putative sugar-specific permease, component IIC (*sgaT*) belonging to a sugar phosphotransferase system (PTS). Downstream the apuA locus is a putative transcriptional regulator gene with similarity to *lacI_tr*. The α-amylase and pullulanase domains are located in the amino-terminal and carboxy-terminal regions, respectively. The N-terminal region includes a signal peptide (SP) 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 alpha-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 alpha-amylase (α-amy) 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.

The *apuA* gene is located upstream of genes encoding a putative sugar-specific permease (*sgaT*) classified as component IIC that belongs to a sugar phosphotransferase system

(PTS) and downstream of a putative transcriptional regulator gene with homology to the *lacI* family (Fig. 3.1).

The ApuA protein was predicted to contain a distinct α-amylase domain (103 to 860 aa) and a pullulanase domain (921 to 1962 aa) (Fig. 3.1). Upstream of each functional domain lie two tandem repeats belonging to carbohydrate binding motif family 41 (CBM41) which binds tightly to α-glucan polysaccharides containing α-(1,4) glycosidic and α-(1,6) glycosidic linkages [24]. 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 [33,34].

At the protein level ApuA shares 47 % and 60 % identity to the predicted alkaline amylopullulanase in *Bacillus sp. KSM*-1378 [35] and the putative amylopullulanase in *Streptococcus infantarius* respectively (Fig. 3.2A). 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) (Fig. 3.2A). 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, Fig. 3.2). The two glutamic acid (Glu) residues that are crucial for the catalytic activity of these enzymes [33], lie at positions Glu_{661} and Glu_{1598} within conserved region III of the α-amylase and pullulanase domains respectively (Fig. 3.2B).

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.

A.

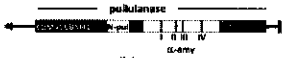
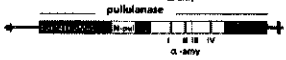
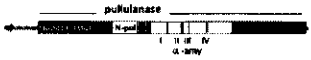
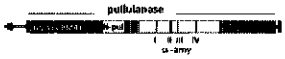
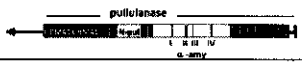
Name	Enzyme	Strain		Identity	Reference
ApuA	amylopullulanase	*S. suis P 1/7*		100%	This work
—	(?) amylopullulanase	*S. infantarius*		60%	—
APase	alkaline amylopullulanase	*Bacillus sp. KSM 1378*		47%	20
—	(?) pullulanase	*S. sanguinis SK36*		69%	—
—	(?) pullulanase	*S. equi MGCS10565*		66%	—
SpuA	pullulanase	*S. pneumoniae serotype I 3.B*		58%	7
PulA	pullulanase	*S. pyogenes NZ131*		58%	23
Sap	Type I pullulanase	*S. agalactiae COH1*		55%	43

Fig.3.2A. Structural comparison of pullulan-degrading enzymes and their subdomains in different Gram-positive species Amylopullulanase/pullulanase enzymes in *S. suis* P1/7, *Streptococcus infantarius*, *Bacillus spp. KSM* 1378, *Streptococcus sanguinis SK36*, *Streptococcus equi* MGCS10565, *Streptococcus pneumoniae* serotype I 3.B, *Streptococcus pyogenes* NZ131 and *Streptococcus agalactiae* COH1.

B.

	Consensus	Region I		Region II		Region III		Region IV	
				*		*		*	
amy	P 1/7	556	**DVVLNH**	628	**AFRVDTVKH**	661	**ETWG**	722	**FLGSHD**
	KSM-1378	462	**DVVLNH**	546	**YFRVDTVKH**	579	**EAWG**	640	**FLGSHD**
				*		*		*	
pul	P 1/7	1501	**DVVYNH**	1565	**GFRFDMMGD**	1598	**EGWR**	1681	**YIAAHD**
	KSM-1378	1396	**DVVFNH**	1460	**GFRFDMMGD**	1493	**EGWV**	1578	**YIEAHD**
	SK36	715	**DVVYNH**	779	**GFRFDMMGD**	812	**EGWK**	895	**YIAAHD**
	MGCS10565	734	**DVVYNH**	798	**GFRFDMMGD**	831	**EGWV**	914	**YIAAHD**
	S1 3.B	717	**DVVYNH**	781	**GFRFDMMGD**	814	**EGWR**	897	**YIAAHD**
	NZ131	664	**DVVYNH**	728	**GFRFDMMGD**	761	**EGWR**	844	**YIAAHD**
	COH1	734	**DVVYNH**	798	**GFRFDMMGD**	831	**EGWR**	914	**YIAAHD**

Fig. 3.2B. Conserved sequences of the 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 spp.* KSM-1378. The amino acids shown in bold are conserved among all amylolytic enzymes, while the putative catalytic amino acids are denoted by (*).

The α-amylase recombinant domain contains 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 hours at 37°C resulted in high level production of a protein of the expected size (Fig. 3.3A). The recombinant amylase was purified by Immobilized Metal Affinity Chromatography (IMAC) using an imidazole gradient to obtain a fraction highly enriched for the expressed protein (Fig. 3.3A). The purified recombinant protein tested in the Red Starch assay comprises of a single band on a Coomassie stained protein gel and was compared to an extract from *E. coli* and 1 mg of the purified α-amylase from *Aspargillus oryzae* as a positive control. The α-amylase activity of 70 ug of the purified recombinant protein was 16 fold higher than 130 ug from the *E. coli* expression strain (Fig. 3.3B). 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 glycosidase activity. Thus these results confirm that the N- terminal half of ApuA indeed possesses the predicted α-1,4 glycosidase activity.

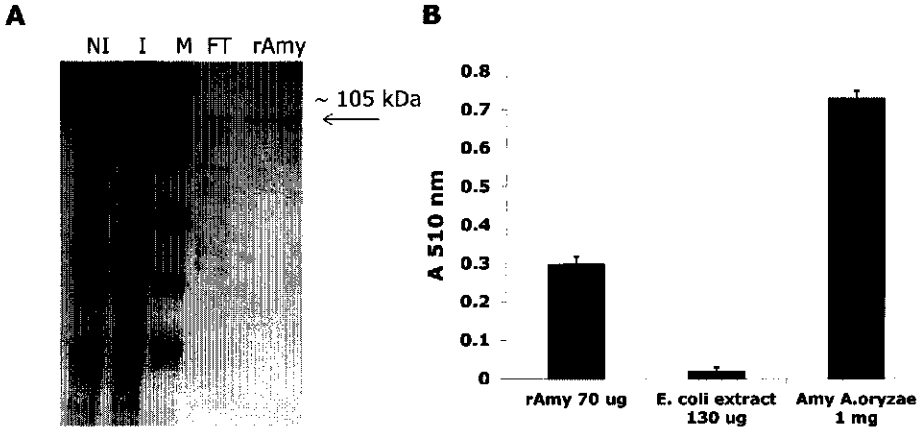


Fig. 3.3 α-amylase domain purification and activity **A.** SDS-PAGE (12% gel) of Amy purification steps. NI: total *E. coli* cellular protein no inducted; I: cleared cell lysate of *E. coli* after 3 h at 37 °C of induction; M marker; FT: Ni-NTA flow-through.; rAmy elution from an Ni-NTA column containing the recombinant Amy with the expected size. **B.** Amylase activity measured with Red Starch as substrate.

Disruption of apuA in S. suis S10

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 *L. lactis* [36]. 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. PCR *apuA* fragment containing the pullulanase domain (from 3114 bp to 6133 bp) was ligated to the *EcoRI* and *XhoI* digested pG$^+$host9 and transformed in *E. coli* VE7108 to generate pG9-*apuA::spc*. 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* by electroporation resulting in 10 transformants that were recovered at 28°C in the presence of Erm (Fig. 3.4A 1st step). Single cross-over 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. 3.4A 2nd step). Integrant strains were then serially passaged for 5 days in liquid medium at 28°C without erythromycin selection to facilitate plasmid excision by homologous recombination between flanking duplicated regions. Dilutions of the serially passaged cultures were plated on agar plates and single colonies 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. 3.4A 3rd, 4th step). Spc^R integrants were confirmed to have the expected genotype by PCR (Fig. 3.4B) and a single integrant designated *S. suis* 10 *apuA::spc* was used for further studies.

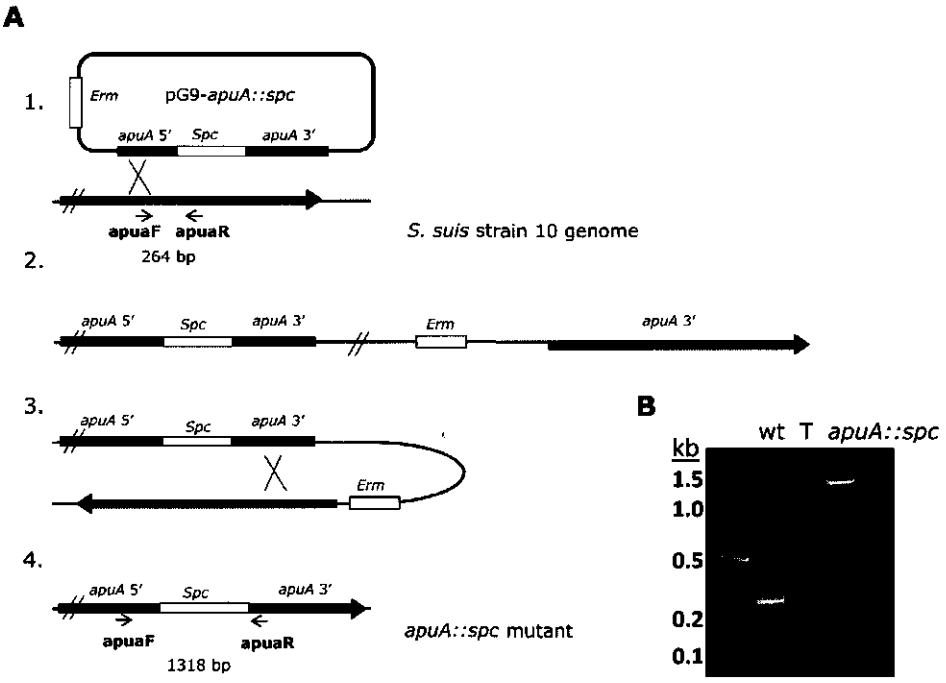


Fig. 3.4 ApuA mutant construction A. Strategy for mutagenesis of the *apuA* gene to generate the knockout mutant *apuA::spc* via a double cross-over event. 1st step: *S. suis* strain 10 transformed with pG9-*apuA::spc*. The first recombination event (integration) occurs by a single cross-over (SCO) event through sequences homologous to *apuA* (only one possible SCO indicated); 2nd step: the resulting SCO integrants; 3rd step: a shift of the SCO integrants to 28 °C stimulates the second (double) cross over event (DCO) and leads to plasmid excision; 4th step: 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.

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 wild type and mutant strains [37]. Hydrolysis of the red dye-conjugated pullulan resulted in a clear zone on the plate incubated with the wild type strain but not the *apuA::spc* mutant indicating that the mutant lacked detectable pullulanase activity (Fig. 3.5A). The α-amylase activity of the *apuA::spc* mutant and wild type 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 cell wall anchored 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 wild type strain was 55 mU mg^{-1} protein. 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 and supernatant fractions (data not shown) demonstrating that this enzyme is surface located as predicted.

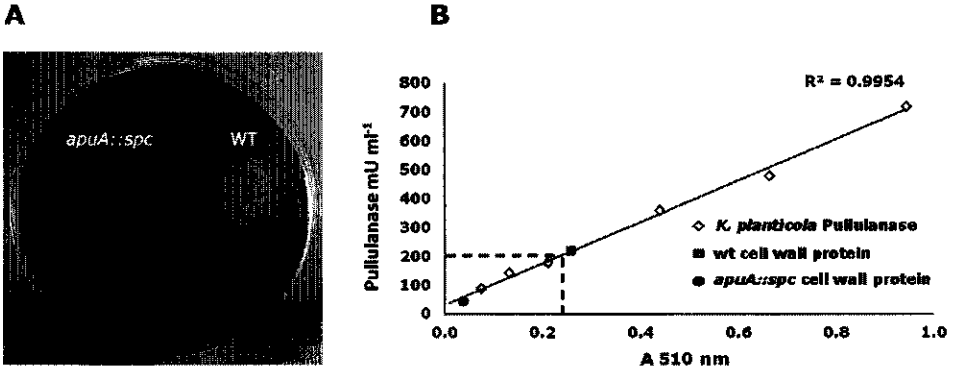


Fig. 3.5. Characterization of wild type and *apuA::spc* mutant phenotype A. Pullulanase activity of *apuA::spc* mutant (left) and wt strain 10 (right) on Red-Pullulan plate. The light zone indicates pullulan degradation by the wt isolate. **B.** Pullulanase activity of extracted cell wall proteins from wt and *apuA::spc* mutant as determined by linear regression on a standard curve obtained with *K. planticola* pullulanase

Carbohydrate utilization assays

S. suis wt and the *apuA::spc* mutant were analyzed for the ability to grow on glycogen, pullulan and maltotriose as the major sources of carbohydrate. In complex medium both strains grow to a low density (OD_{600} 0.25 – 0.3) after 13 hours incubation (Fig. 3.6A). Supplementation of the medium with maltotriose or glycogen supported growth of both the wt and *apuA* mutant strains to a higher density (OD_{600} 0.4 to 0.6) than in complex medium alone (Fig. 3.6C and D). However, in the presence of pullulan growth of the mutant was decreased compared to the wild type strain and it reached the same optical density as for complex media alone (Fig. 3.6A and B).

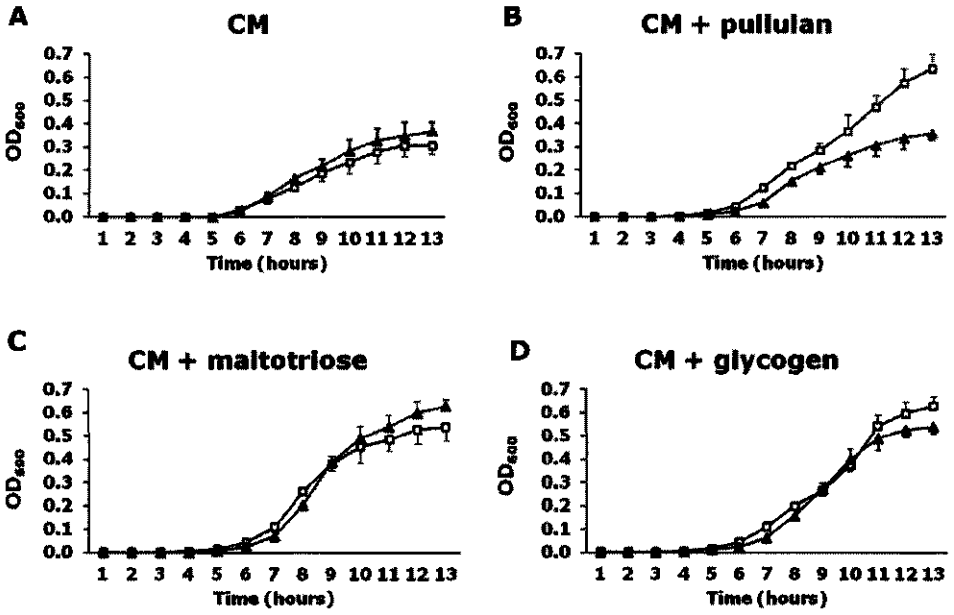


Fig. 3.6. Growth curves of wild type (-□-) and *apuA::spc* (- ▲-) mutant strains grown in complex medium A. alone, or **B.** with the addition of 1 % of pullulan **C.** maltotriose and **D.** glycogen.

Adhesion to a porcine tracheal cell line

The newborn porcine tracheal cell line NPTr was chosen to investigate the role of ApuA in adhesion to the epithelium [38]. 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 wild type was adherent but not invasive within 2 hours 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 wild type strain adhered strongly to the porcine epithelium with maximum percentage of adherence of 19% using a multiplicity of infection (m.o.i.) of 75 for the wild type (Fig. 3.7A). 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. 3.7A).

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 microtiter wells. As shown in Fig. 3.7B, the binding of the wild

type strain to porcine mucin was significantly higher than binding of the *apuA::spc* mutant using inoculums of 10^8 and 10^7 c.f.u. ml^{-1}. At the higher inoculum dosages, approximately 2 fold higher numbers of the wild type strain were recovered after 2h of incubation.

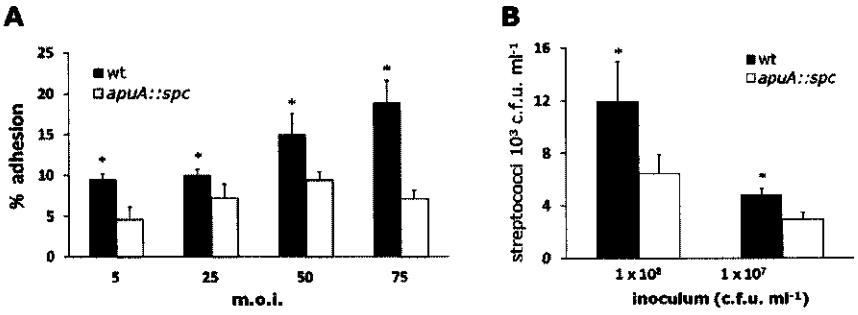


Fig. 3.7. A. Percentage adhesion of wild type and isogenic mutant *apuA::spc* to the epithelial cell line NPTr. Results were determined after 2h co-incubation of epithelial cells with wt and mutant strains at 37ºC with 4 different multiplicities of infection (m.o.i) followed by extensive washing, lysis and viable plate counts. *, $P < 0.05$ compared to adherence of the *apuA::spc* mutant. **B.** Binding of *S. suis* wt and *apuA::spc* mutant bacteria (at doses 10^8 and 10^7 c.f.u. ml^{-1}) to microtiter plate wells coated with porcine mucin at 37 °C. The mean standard deviation (error bars) was derived from triplicate wells in two independent experiments. *, $P < 0.05$ compared to binding of the mutant strain *apuA::spc*.

Discussion

A gene, *apuA*, encoding a cell wall anchored amylopullulanase was identified in the genome sequence 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. 3.1). 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 [24,33] (Fig. 3.2B). The pullulanase domain of ApuA shares high identity with α-(1,6) glycosidic pullulanases identified in other pathogenic streptococcal species such as *S. infartarius, S. sanguinis, Streptococcus equi, S. pneumoniae* (*pulA*) [29], GAS (*spuA*) [24,28,31] and GBS (*sap*) [23]. The last three proteins have been well characterized and have 58-55 % identity with the pullulanase domain of *S. suis* ApuA (Fig. 3.2A). Pullulanase activity was found solely in the cell fraction of *S. suis* wild type 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 wild type, growth of the *apuA::spc* mutant was significantly impaired in complex medium 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 encoding 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 infartarius* (60 %) and the characterized alkaline amylopullulanase (APase) from *Bacillus sp. KSM-1378* (47 %; Fig. 3.2A) which was shown to hydrolyze both α-(1,4) and α-(1,6) glycosidic bonds from two distinct domains and catalytic sites within the same protein [35].

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 bifunctional enzymes, such as enzymes with endo- and exoglucanase activities from *Caldocellum saccharolyticum* [39].

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 the type I pullulanase of Group A streptococci mediates adherence to eukaryotic carbohydrate residues [28] 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, but not invade, eukaryotic cells [40,41]. We found, for the first time, that this was also the case for the porcine NPTr cell line which has not previously been used for *S. suis* virulence studies. The adherence to the porcine epithelial cells was substantially (twenty fold) stronger than that reported previously for the human laryngeal carcinoma cell line HEp-2 using similar assay conditions [41]. Strikingly, in our cell line model, the adherence of *apuA::spc* mutant was around 2 fold lower than the wt strain over a range of multiplicities of infection suggesting that ApuA may play an important role in colonization and virulence *in vivo*. As pullulan is not found in humans and pigs ApuA presumably binds to similar carbohydrate structures on porcine epithelium and mucus.

Colonization of the nasopharyngeal cavity by *S. suis* is an important risk factor for the infection of young pigs [42,43]. Recent evidence suggests that the nasopharynx and palatine tonsils may be the routes of entry in invasive disease [2]. Adhesion of *S. suis* to mucus in the oral cavity is likely to be important for colonization as demonstrated for other opportunistic pathogens colonizing the nasopharyngeal cavity [44,45,46]. 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*.

In vivo, ApuA is likely to play a role in nutrient acquisition 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 cavity. Our results demonstrate an important role for ApuA in adhesion to porcine epithelial cells and to mucus *in vitro*. These results also point at a link between carbohydrate utilization by *S. suis* and the ability to colonize and infect hosts.

Materials and Methods

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1. *S. suis* strains were grown in Todd-Hewitt broth (THB) (Difco) or on Columbia agar plates with 6 % sheep blood (Oxoid) at 37°C under 5 % CO_2 for 18 h. The genome of *S. suis* S10 is more than 99% identical to the genome of *S. suis* P1/7, a sequenced reference strain of which the genome had been annotated previously (**Chapter 1**). An optical density (OD) of 1.0 at 600 nm with a 1 cm path length corresponds to approximately 10^9 bacterial colony forming units per milliliter (c.f.u. ml^{-1}). *Escherichia coli* VE7108 (derived from TG1) was cultured in Luria-Bertani (LB) broth or LB agar (Difco Laboratories) at 37°C for 18 h. When necessary, antibiotics were added to culture media at the following concentrations: for *E. coli*, erythromycin (Erm) 150 µg ml^{-1}; and spectinomycin (Spc) 50 µg ml^{-1}; for *S. suis*, Erm at 2 µg $ml^{-1}$and Spc at 100 µg 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 previously described [37]. A complex medium (CM) comprising of 10 g l^{-1} proteose peptone, 5 g l^{-1} trypticase peptone, 5 g l^{-1} yeast extract, 2.5 g l^{-1} KCl, 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 previously described [23]. Growth in complex medium was determined by measurement of turbidity OD_{600} using a SpectraMax M5 reader.

TABLE 3.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genetic markers and/or description [a]	Reference
E. coli VE7108	*supE hsdΔ5 thi Δ(lac-pro AB) F'[traD36 proAB+ lacIq lacZΔM15] repA*$^+$	[48]
E. coli VE6838	*supE hsdΔ5 thi Δ(lac-pro AB) F'[traD36 proAB+ lacIq lacZΔM15]* VE7108 carrying pG$^+$host9	[48]
E. coli TOP10	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
S. suis 2 S10	Virulent serotype 2 strain	[55,56]
S. suis apuA::spc	Isogenic *apuA::spc* mutant of strain S10	This work
Plasmids		
pTrcHis TOPO2	Expression vector containing C-Terminal His-tag	Invitrogen
pTrc-ApuaR	pTrcHis2 vector containing 936 bp of apuA	This work
pDL282	Replication functions of pUC19 and pVT736-1, Amp^r Spc^r	[57]
pKUN19-spc	pKUN19 containing Spc^r gene of pDL282	[49]
pG$^+$host9	Erm^r, a thermosensitive derivative of pGK12	[36]
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

[a] Erm^r, erythromycin resistant; Amp^r, ampicillin resistant; Spc^r, spectinomycin resistant.

Nucleotide and protein sequence analyses

Blast searches with *S. suis* strain P1/7 (serotype 2) genome sequences 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 ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Signal peptide motifs were identified in protein sequences using the SignalP V1.1 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 to 855) was amplified by PCR from *S. suis* 2 10 genomic DNA using *GoTaq* (Promega) and primers AmyF and AmyR as listed in Table 3.2.

TABLE 3.2. Oligonucleotide primers used in this study

Primer	Sequence[a]	Purpose
amyF	CTTTCGGAACAGGATGGC	Cloning of α-amylase
amyR	GACGATGTCACCTGCTTCTG	domain in pTrcHisTopo
pulF-*EcoRI*	GCTATCGAATTCTA**TACCGATGGCAATTATGAT**	Cloning of amylopullulanase
pulR-*XhoI*	TCGAATCTCGAGA**TCTTGTCCAGACGCTTGAG**	fragment in pGhost'9
pulF-*PvuI*	GCTAGTCCATGGTA**CTGCCTCCATGAAGTGATAAT**	Inverse PCR of pG9-*apuA*
pulR-*BglII*	TCGTCGATCGCG**CGTCGTGTCTTAGTTGATTCC**	
Spc-*PvuI*	GCTATACCATGGTA**AGGTCGACTCTAGAGGATC**	Insertion of *spc* gene into
Spc-*BglII*	TCGTAGCAGATCTCG**TTATAATTTTTTTAATCTGTTATTTA**	pG9-*apuA*
apuaF	TGGGTGTGATTTTGGATGTG	To check for correct mutant
apuaR	TAAAGGCCAGCTCAATTGCT	clones using colony PCR

[a] The sequences in bold and underlined correspond to gene sequence and restriction sites respectively.

The purified PCR products were ligated to the pTrcHis TOPO TA expression vector (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 Isopropil β-D-1-tiogalattopiranoside (IPTG) to an exponential culture (OD_{600} of 0.6) for 3 hours at 37°C with shaking (250 rpm). The cells were harvested by centrifugation (8 000 g x 10 min at 4°C) and the pellet was resuspended in lysis buffer (50 mM Tris-Cl; 150 mM NaCl [pH 7.4]) containing a cocktail of protease inhibitors; Roche), and then disrupted using a high pressure cell disrupter (Constant Systems, U.K.). The soluble poteins extract was recovered after high speed centrifugation (14 000 rpm 40 min) and loaded onto a HisTrap affinity chromatography column (Amersham Pharmacia Biotech, Freiburg, Germany). Proteins were eluted in a phosphate buffer containing 500

mM NaCl and increasing concentrations of imidazole and checked by SDS-PAGE gel electrophoresis. Fractions containing purified fusion proteins of the expected size (approx. 110 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

Alpha 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 fractions in 1 ml of buffer B (maleic acid 0·1 M, calcium chloride 2 mM, sodium azide 0.01% w/v pH 6.5). Red-Starch is depolymerized by α-amylase to produce low-molecular weight dye compounds that remain in solution on addition of 2.5 ml ethanol. After centrifugation at 1 000 g x 10 min the released dye was quantified in the supernatant by spectrophotometer (510 nm) (SpectraMax M5 reader).

Insertional inactivation of apuA::spc

All the primers used for mutagenesis have been listed in Table 3.2. Chromosomal DNA was isolated using the CTAB extraction method after pretreatment of the bacteria with lysozyme (10 mg ml^{-1}) for 10 min at 37°C as previously described [47]. An internal *EcoRI-XhoI* fragment (nucleotide position 3114 to 6133) of the *apuA* gene was amplified using primers pulF and pulR and ligated into *EcoRI* and *XhoI* digested pG^{+}host9, a shuttle vector that is thermosensitive for replication in Gram-positive bacteria [36]. The resulting plasmid pG9-*apuA::spc* was introduced into competent *E. coli* strain VE7108 [48] by electroporation and the transformants selected on LB agar containing erythromycin. Plasmid pG9-*apuA::spc* extracted from the transformants was linearized by inverse PCR using *Pfu* Taq polymerase (Promega) and the internal *apuA::spc* primers pulF-*PvuI* and pulR-*BglII*. A *spc* cassette, containing the promoter and transcriptional terminator, was amplified from plasmid pKUN19-Spc [49] using primers 5'Spc-*PvuI* and 3' Spc-*BglII*. Both the inverse PCR product of pG9-*apuA::spc* and the *spc* cassette were then digested with *PvuI* and *BglII* enzymes and ligated using T4 DNA ligase (Promega) to generate pG9-*apuA::spc* which contains a *spc* cassette inserted between nucleotides 4564 and 4624 of *apuA*.

Purified plasmid pG9-*apuA::spc* was then transformed into competent *S. suis* strain 10 by electroporation as described previously [50]. The transformants were selected on agar plates containing 2 µg ml^{-1} Erm 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 cross-over event, leaving the *spc* gene insertion in *apuA::spc* [51]. 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 of overnight culture bacteria grown in THB was pelleted (10 000 g x 10 min at 4 °C) and 10 ml of 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 of extraction buffer (30 mM Tris-HCl pH 8.0; 3 mM $MgCl_2$, 25 % sucrose) containing protease inhibitors (Roche), 1 mg ml^{-1} lysozyme and 125 U ml^{-1} mutanolysin (Sigma). The bacterial suspension was then pelleted by centrifugation (10 000 g x 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 of buffered saline (pH 7·0) containing 5 mM $MgCl_2$. The suspension was allowed to stand at room temperature for 15 min and centrifuged at 10 000 x g for 30 min at 4°C [52]. The clear supernatant containing the cytoplasmatic proteins was concentrated as described above. The concentration of the protein in each fraction was quantified using NanoDrop Spectrophotometer (NanoDrop Technologies, USA).

The pullulanase activity was determined by measuring the enzymatic release of reducing groups from α-glucans using red-pullulan as substrate (Megazyme). Briefly, Red-Pullulan (1 % w/v in 0.5 M KCl) was incubated for 10 min at 40°C with 1 ml of cell wall protein extracted cells. The red-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 weight material was removed by centrifugation, and the soluble dye measured in a spectrophotometer at 510 nm (SpectraMax M5 reader). The amount of 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) [38] were maintained in Dulbecco's modified eagle 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, 1 mM Na-EDTA (trypsin- EDTA, Gibco-Invitrogen) and replacement of the medium [53]. For the adherence assay, approximately 2·3 x 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 x 10^5 and 2·3 x 10^7 c.f.u. ml^{-1}) were added to wells containing a monolayer (2·3 x 10^5) of epithelial cells in 1 ml of medium (multiplicities of infection (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. The cell monolayers were washed three times with PBS, and detached by scrapping in 800 µl of ice-cold milli-Q water. To enumerate the 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 [54]. Briefly, a 96-well microtiter plate (Nunc Inc., Roskilde, Denmark) was inoculated with 7·5 µg of 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 (8 000 x g for 5 min), washed in PBS, and adjusted to an OD of 1·0 at 600 nm. Triplicate wells in both plates were inoculated with 2·5 x 10^8 and 2·5 x 10^7 bacteria in a volume of 100 µl in PBS. The microtiter plates were incubated for 2 h at 37°C and 5 % CO_2. The wells were washed 8 to 10 times with sterile PBS. Bound bacteria were desorbed with 250 µl of 0·03 % Triton X-100 in sterile PBS for 1 h at room temperature and enumerated by plating on blood agar for 12 to 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

The adherence assays and the binding mucin experiments were performed at least three times using triplicate samples. All numerical data presented here are expressed as means ± standard deviation (sd). Statistical significance was determined using a two-tailed Student's t test. Differences were considered significant at $P \leq 0{\cdot}05$.

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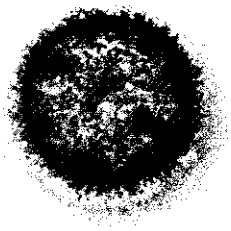
Chapter 4



Catabolite regulation of a bifunctional adhesin and α-glucan utilization enzyme in *Streptococcus suis*

M. Laura Ferrando, Peter van Baarlen, Germano Orrù, Hilde E. Smith and Jerry M. Wells

Submitted in part

Summary

Streptococcus suis is the major pathogen of young pigs and an emerging zoonotic pathogen. In humans and pigs it causes a variety of diseases including meningitis, septicemia and endocarditis. During colonization, dietary or host sources of α-glucans are likely to be a key source of nutrients for *S. suis*. A cell wall anchored amylopullulanase (ApuA) is necessary for the degradation of α-glucans and contributes to the adhesion of *S. suis* to porcine epithelium. ApuA expression was induced in the presence of pullulan and maltotriose and repressed in the presence of glucose. The promoter of *apuA* contains two transcription factor binding motifs. The motif located upstream of the -35 box is bound by ApuR, a transcriptional activator we propose to be allosterically regulated by maltotriose. Additionally CcpA, global carbon catabolite regulator, specifically binds a catabolite responsive element (*cre*) overlapping the -35 box. Based on these findings we propose a model for the regulation of α-glucan utilization in *S. suis* during colonization and invasive disease. Here we demonstrate a link between virulence and carbohydrate metabolism whereby the degradation products of complex carbohydrates serve as an environmental cue to induce ApuA expression.

Introduction

Streptococcus suis is the major pathogen of young pigs causing a variety of diseases including meningitis, septicaemia and endocarditis. Additionally *S. suis* is a zoonotic agent which can be transmitted to humans through contact with contaminated swine products or infected animals [1]. Recently *S. suis* emerged as one of the most common causes of adult human meningitis in South East and East Asia [2,3]. Pigs colonized by *S. suis* harbor the organism in the nasal-oropharynx [4] which may lead to asymptomatic carriage, invasion or dissemination in the bloodstream and eventually, infections of the central nervous system (CNS) or meningitis [2,3,5].

In the oropharynx dietary α-glucans are likely to be a key nutrient source for *S. suis* and non-pathogenic commensals [6,7]. Alpha-glucans such as starch and glycogen are polysaccharides comprising of repeating glucose monomers linked by alpha-glycosidic bonds (α-1,4 and α-1,6 linked) and are present at concentrations sufficient to support bacterial growth in the saliva and oropharyngeal cavity of pigs and humans [8,9,10,11]. Additionally, animal glycogen stores released from damaged or lysed cells may be an important substrate for pathogen growth during the early stages of infection. The genomes of commensals inhabiting the digestive tract of mammals frequently encode amylases and pullulanases to degrade α-glucans via the cleavage of α-1,4 and α-1,6 glycosidic bonds respectively. The concerted activity of these enzymes primarily generates α-1,4 linked polymers of glucose containing two (maltose), three (maltotriose) or four up to nineteen glucose units (maltodextrins). In *Streptococcus pyogenes* (Group A Strep-GAS), maltose and maltotriose are transported by a phosphoenolpyruvate-dependent phosphotransferase system (PTS) (M5005_Spy_1692) [12], a homologue of which is also present in both *Streptococcus pneumoniae* (SP_0758) [13] and *Streptococcus mutans* (SMU2047) [14]. Additionally, maltodextrins with degrees of glucose polymerization of 3 to 7 are taken up via a maltodextrin-specific ATP-binding cassette (ABC) transport system that is conserved in other streptococci capable of degrading α-glucans [13,15,16]. In GAS, mutants affected in maltose and maltodextrin transport are significantly attenuated in their growth in human saliva and their ability to colonize the mouse oropharynx compared to wild-type [12,17].

Recently, a cell wall anchored amylopullulanase (ApuA) was characterized in *S. suis* and shown to be necessary to support growth on glycogen or pullulan (α-1,6 linked glucose

polymer) as a sole carbon source [18]. Additionally, ApuA was shown to promote adhesion of *S. suis* to porcine tracheal cells suggesting that it has a dual role in virulence and colonization [18]. Recently, inactivation of pullulanases (SpuA) and *S. pneumoniae* (PulA) were also shown to significantly reduce virulence in mouse models [17,19]. Several other recent studies have highlighted a link between α-glucan degrading enzymes and virulence in opportunistic pathogens [20,21,22,23]. In *S. pneumoniae* for example, several signature-tagged mutagenesis screens identified genes known to be involved in carbohydrate metabolism including several enzymes involved in the breakdown of α-glucans [24,25,26,27].

To date, the mechanism and regulation of α-glucan catabolism in *S. suis* is only partly understood although this is important in virulence and oropharyngeal colonization. Generally, bacteria do not synthesize degradative (catabolic) enzymes unless the substrates for these enzymes are present in their environment and have evolved elaborate control mechanisms to select the carbon source that allows the fastest growth [28]. Bacteria have also developed diverse mechanisms for the control of biosynthetic (anabolic) pathways when the end product of the pathway is not needed or is readily obtained by uptake from the environment. Control of biosynthetic pathways in microbes is often performed at the level of DNA transcription, by transcriptional regulators that activate or repress transcription of metabolic genes.

Catabolite control protein A (CcpA) is a global transcriptional regulator of carbon catabolite control (CCC) in *Firmicutes* and can repress or activate transcription by binding to cis-acting catabolite responsive element (*cre*) sites. Consensus *cre* sequences have been determined in several species [28]. In the model organism *Bacillus subtilis*, growth on a preferred, easily metabolized sugar leads to high concentrations of fructose-1,6-bisphosphate (FBP) and ATP and phosphorylation of histidine-containing phosphocarrier protein (HPr) at Ser46 by HPr kinase/phosphorylase (HPrK). Phosphorylated HPr(Ser-P) then binds to the CcpA protein inducing a conformational change that enhances binding to *cre* sites on the DNA. Under conditions of poor nutritional supply HPrK is also responsible for dephosphorylation of HPr(Ser-P). Depending on the position of the *cre* sites in the promoter, binding of the HPr-P/CcpA complex can cause carbon catabolite repression (CCR) or carbon catabolite activation (CCA). If the *cre* is located upstream of the -35 promoter element it may mediate CCA via interaction with the RNA polymerase. In contrast *cre* elements located downstream of the -35 typically lead to CCR by repression

of transcription. Apart from CcpA, several other transcriptional repressors or activators play a role in the regulation of carbon catabolism and anabolism in Gram-positive bacteria. For example in different streptococci, MalR, a member of the LacI/GalR family of transcriptional regulators regulates the maltodextrin transport operon and a pullulanase in GAS [12]. We hypothesized that in *S. suis* ApuA was regulated by an upstream transcription factor designated here as ApuR. ApuR shares low sequence homology with MalR but high similarity to the maltodextrin regulators Lmo2128 and MdxR in *Listeria monocytogenes* and *B. subtilis* respectively. Herein we report on studies designed to unravel the role of ApuR in the regulation of ApuA. Our results provide the first detailed insights into the control of α-glucan utilization in *S. suis* and further expand our understanding of the links between metabolic processes and virulence gene regulation.

Results

Description of the apuR gene locus and its homology to known transcriptional regulators

Microbial genes encoding degradative (catabolic) enzymes for carbohydrates are usually regulated depending on the availability of the substrates. We hypothesized that this may also be the case for the amylopullulanase *apuA* (SSU1849) and that an upstream gene designated here as *apuR* (SSU1850) was involved in its regulation. The gene encoding ApuR was predicted to possess its own promoter with a rho-independent terminator (ΔG -10 kcal/mol) downstream of the stop codon (Fig. 4.4.1A). Homology searches indicated that ApuR is a member of the LacI/GalR family of regulators containing a conserved two domain structure. The ApuR N-terminus contains a DNA binding domain which is capable of dimerization and DNA binding through a helix-turn-helix motif. The C-terminus contains a conserved ligand-binding domain that can be bound by a specific sugar(s), conferring specificity to the regulator by modulating its DNA binding activity.

Comparison of ApuR with protein sequences in the UniProt database revealed similarities to several regulators of operons comprising clusters of carbohydrate catabolic enzymes. Highest homologies were to *Lactobacillus casei* BL23 YvdE (53% identity), *B. subtilis* 168 MdxR (49% identity) (also referred to as YvdE) and *L. monocytogenes* EGD-e Lmo2128 (47% identity) which are all transcriptional regulators in the maltodextrin utilization cluster of genes [29,30,31,32]. Genes in these clusters encode predicted maltogenic amylases or neopullulanases as well as ABC transporters and permeases for maltodextrin uptake (Fig. 4.4.1B). MdxR of *B. subtilis* has been proposed to be the cognate transcriptional regulator of the maltodextrin-utilization cluster but so far there is no experimental evidence to support this hypothesis [29]. However in *L. monocytogenes,* YvdE (Lmo2128) was shown to be a transcriptional activator of the maltodextrin gene cluster [32].

Within the streptococcal species highest homology of ApuR was found to MalR (25% identity-not shown), the negative transcriptional regulator of genes involved in maltodextrin uptake and degradation [12,15,16,33,34]. In *S. suis* the genes downstream of *apuR* include *apuA* but not the maltodextrin transport gene cluster which is present at a different locus in *S. suis* (from SSU1914 to SSU1920). Located downstream of *apuA* are a cluster of genes predicted to be involved in uptake and fermentation of ascorbate; *sgaT* (SSU1848, a predicted phosphotransferase system (PTS) IIC component), *sgaB* (SSU1847,

PTS IIB component), *ptsN* (SSU1846, a PTS IIA component), *sgbH* (SSU1845, hexulose-6-phosphate synthase), *sgaU* (SSU1844, putative hexulose-6-phosphate isomerase), *ulaD* (SSU1843, L-ribulose-5-phosphate 4-epimerase) (Fig. 4.1A). The size of the intergenic region between *apuA* and *sgaT* and presence of a predicted terminator (ΔG -15 kcal/mol) suggested that the genes for ascorbate metabolism were transcribed as a separate operon. This gene organization is well conserved in the genomes of 5 sequenced strains of *S. suis* genomes (Fig. 4.1B) although in the Chinese strain 05ZYH33 the *apuA* gene was annotated as two genes that encode an amylase and a pullulanase. Homologues of ApuR were not found upstream of amylases or pullulanases in other pathogenic streptococci indicating that the genetic organization of the *apu* locus is unique to *S. suis*.

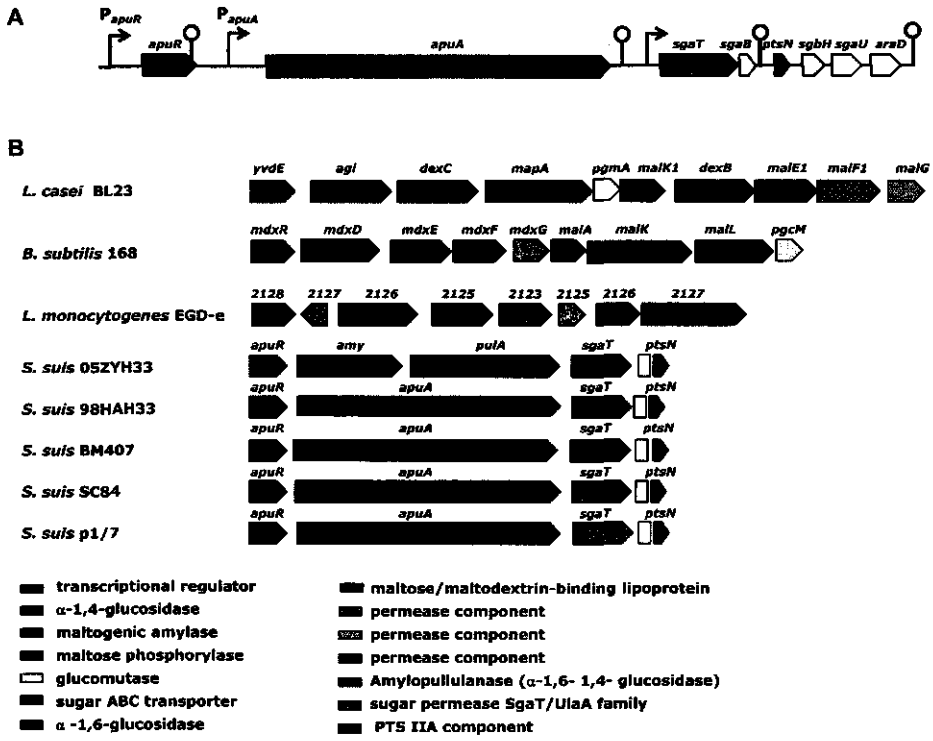


Fig. 4.1. Organization and homology of *apuA* locus in *S. suis*. A. The 6 kb amylopullulanase gene *apuA* is located downstream of *apuR* which encodes a putative transcriptional regulator of the LacI/GalR family. Downstream of *apuA* are cluster of genes predicted to be involved in uptake and fermentation of ascorbate (*sgaT*, *sgaB*, *ptsN*, *sgbH*, *sgbH* and *ulaD*). For each gene, the direction of transcription is indicated by an arrow, whose size is proportional to the length of the corresponding open reading frame. Putative promoters are represented by arrows and transcription terminators by loops. **B.** Gene homologues in other Gram positive bacteria that share ≥ 45% of protein identity with *apuR* gene (black arrows). The annotations of the genes downstream of *apuR* are also indicated and colored to show functional relatedness. Gene names are indicated above the arrows.

Transcriptional regulation of the apuA gene cluster is induced by pullulan and maltotriose

To investigate the substrate dependent regulation of the *apuA* gene cluster including *apuR*, its putative transcriptional regulator (Fig. 4.1A), we performed qPCR analysis of gene transcripts during growth in different sources of carbohydrate (see below). Addition of 1% (w/v) of glucose, lactose, maltotriose or pullulan resulted in high density growth of *S. suis* S10 compared to complex medium alone, demonstrating that these four sugars can be metabolized (Fig. 4.2).

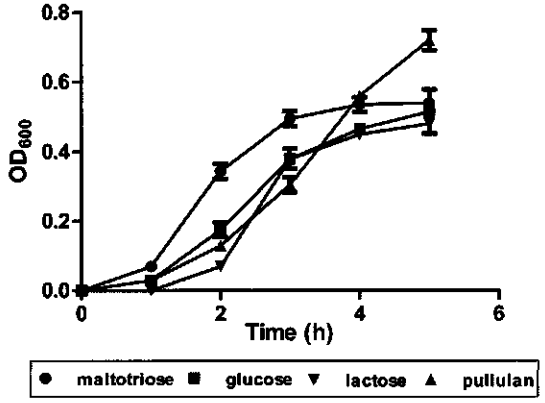


Fig. 4.2 *S. suis* S10 growth curve at 37°C in different media. OD_{600} readings were taken at indicated times to measure growth in complex media (CM) supplemented with 1% (w/v) of maltotriose, glucose, pullulan and lactose. The graph shows the means and standard deviations from two independent experiments.

Compared to growth in glucose or lactose, *apuA* transcription was strongly induced (up to 4 fold) by growth in pullulan and maltotriose (Fig. 4.3A). Highest induction of *apuA* was seen after 4 hours in the presence of pullulan while this was at 2.5 hours in maltotriose (Fig. 4.3A). This most likely reflects the fact that after 4 hours growth in pullulan the bacteria were still in late exponential phase whereas the bacteria had already reached stationary phase in the presence of maltotriose (Fig. 4.2). Relative expression levels of *sgaT* and *ptsN* were significantly induced by growth in pullulan compared to the other three sugars (Fig. 4.3B and C). However, fold changes were low (0.1 to 0.06 fold) compared to *apuA*, suggesting these genes were independently regulated (Fig. 4.1). Additionally we measured the relative transcript level of *apuR* to determine if transcription was constitutive or induced by different carbohydrate sources. Expression of

a*puR* was significantly induced after 2.5 hrs growth in maltotriose, and after 4 hrs growth both in maltotriose and pullulan compared to growth in glucose (Fig. 4.3D). A similar low induction was observed for the homologous transcriptional regulator (*lmo2128*) of the maltodextrin gene cluster in *L. monocytogenes* [32].

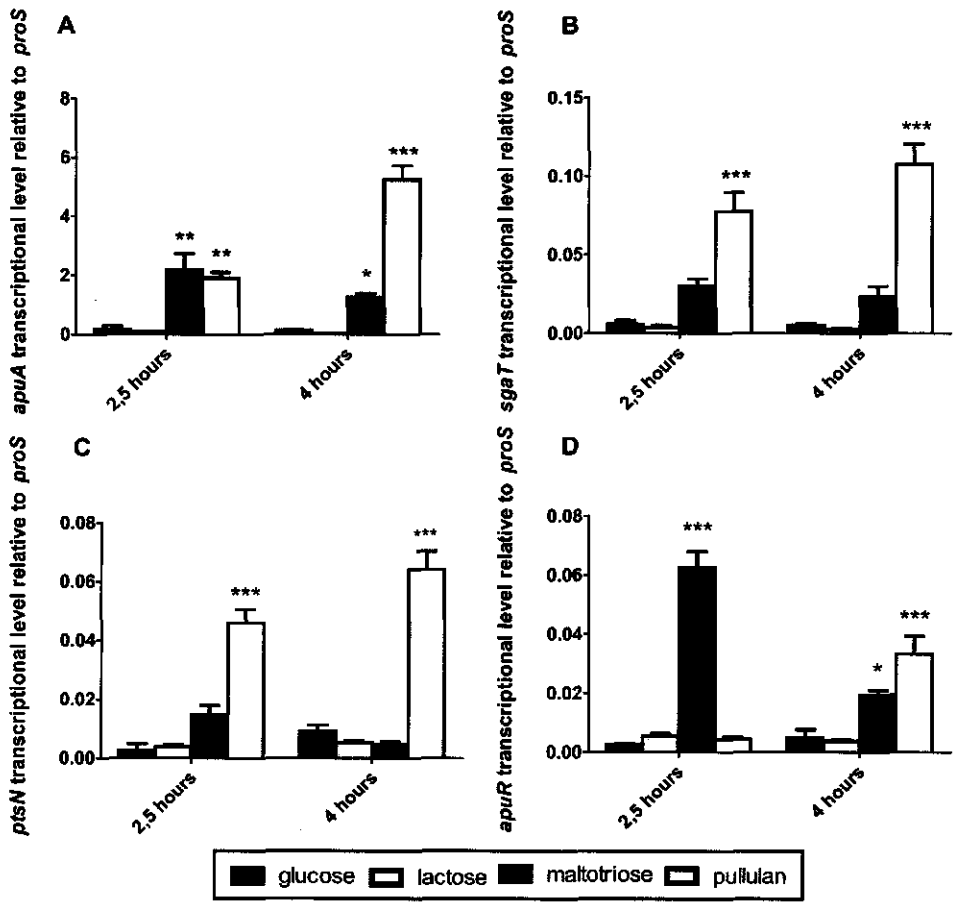


Fig. 4.3. Relative expression of *apu* locus in CM containing different sugars. Transcript levels of **A.** *apuA*, **B.** *sgaT*, **C.** *ptsN* and **D.** *apuR* were measured after 2.5 hours and 4 hours of growth in CM containing 1% glucose, lactose, maltotriose or pullulan, relative to the reference gene *proS* which is constitutively expressed at similar levels during growth in different sugars (data not shown). The height of the bars represent mean values for the relative expression data ± SEM from two independent experiments (n = 3). Statistical significance was calculated using a two-way ANOVA test followed by Bonferroni's post hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Taken together these results show that *apuA* transcription is induced by maltotriose and pullulan. Maltotriose is an end product of the enzymatic degradation of pullulan, starch or glycogen by α-glucan degrading enzymes such as pullulanases or amylopullulanases like

ApuA [18]. Owing to its proximity to *apuA* and homology with other regulators of amylases/pullulanases and maltodextrin utilization genes in *Listeria* and *Bacillus*, we hypothesized that ApuR was a regulator of *apuA* and that its activity was modulated by maltotriose and potentially other transported maltodextrins. To investigate the regulation of *apuA* we determined the transcription start site, performed homology searches for binding motifs of known regulators, and carried out promoter binding assays with purified transcription factors (TFs).

Transcription start site and bioinformatics analysis of the apuA *promoter*

During growth in pullulan the transcriptional start site of the *apuA* transcript was determined experimentally by 5′-RACE to be 31 nt (T nt of 5′-TAC-3′ codon) upstream of the start codon ATG (described in Materials and Methods) (Fig. 4.4A).

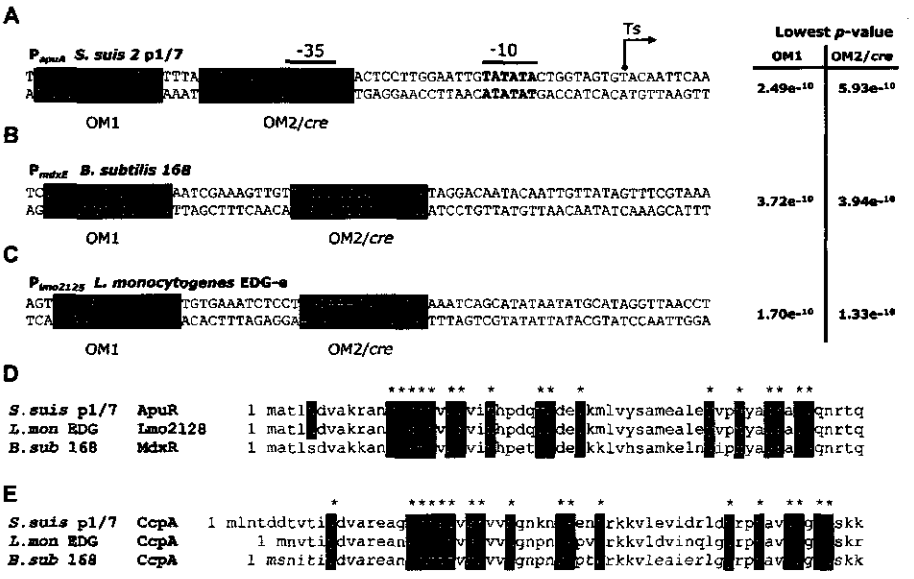


Fig. 4.4. Identification of conserved operator binding motifs for ApuR (OM1) and CcpA (OM2) in *S. suis* P1/7, *B. subtilis* 168 and *L. monocytogenes* EDG-e. The promoter regions and the relative p-values were determined using the MEME Suite software, which calculates the probability of match score with the given sequence. **A.** The -10 and -35 regions (indicated) were deduced based on the transcription start site (Ts arrow) that was determined experimentally by 5′-RACE. **B.** The OM1 and OM2 putative binding sites (black boxes) were identified in the *B. subtilis* promoter of the *mdxE* gene (BSU34610) that encodes a maltodextrin-binding periplasmic protein and in **C.** the promoter of the *L. monocytogenes lmo2125* gene that encodes a maltose/maltodextrin ABC-transporter. **D.** and **E.** Sequence alignment of the DNA binding domains of the ApuR and CcpA proteins of *S. suis, L. monocytogenes* and *B. subtilis*. Conserved amino acid sequences are indicated in black.

The consensus –35 element (5′-TTGCAA-3′) and –10 element (5′-TATATA-3′) required for interaction with the RNA polymerase and transcription initiation were found at the expected positions upstream of the transcription start site.

The –10 element differs at one nucleotide from the consensus for prokaryotic promoters; the –35 element is similar to the consensus sequence for RpoD, the main σ factor in prokaryotes (Fig. 4.4A).

Visual inspection of the DNA region bound by ApuR for consensus binding motifs of *B. subtilis mdxR* or the LacI/GalR family of regulators revealed two potential operator motifs (OMs) (RegPrecise database) [35]. Using the MEME informatics tool [36], these same two OMs were identified in alignments of the *apuA* promoter sequence with sequences of promoter regions of *mdxR* and *lmo2128* (i.e. P_{mdxE} and $P_{lmo2125}$ respectively) (Fig. 4.4B and C). The first sequence, similar to operator motifs for TFs belonging to the LacI/GalR family and designated here as OM1 was found at position –50 nt upstream of the *apuA* start codon. OM1 has homology to the predicted binding sites of *mdxR* and the transcription activator *lmo2128* and is located 13 bp upstream of the –35 element. The amino acid sequences of the helix-turn-helix domains of MdxR and Lmo2128 that are predicted to interact with OM1 are highly conserved and are also present in ApuR (Fig. 4.4D), suggesting that ApuR might also be a transcriptional activator.

A second operator motif (designated OM2) was found at position –27 nt overlapping the –35 region that is identical to the catabolite responsive element (*cre*) consensus sequence determined in *Bacillus* species (WTGNAANCGNWNNCWW where W = A or T and N = any base). The *cre* motif is used as a binding site by CcpA [37,38,39]. The amino acid sequence of the region of CcpA that was predicted to interact with *cre* was highly conserved in *S. suis, Listeria* and *Bacillus* (Fig. 4.4E). The location of OM1 and OM2/*cre* suggested that *apuA* is subject to dual regulation: i) by a transcriptional activator, ApuR and ii) via carbon catabolite repression by CcpA.

Binding of recombinant ApuR and CcpA to the apuA promoter

Recombinant ApuR (rApuR) and CcpA (rCcpA) were expressed in *E. coli* with C terminal-His-tag using the IPTG inducible *trc* promoter at 18°C to optimize solubility. Both proteins were purified by HPLC affinity chromatography using extensive washing of the column bound protein with a buffer containing low concentrations of imidazole to remove weakly binding proteins. His-tagged rApuR and rCcpA were eluted using an imidazole gradient and

fractions containing the highest purity were obtained using an imidazole concentration of around 100 mM (Fig. 4.5A and C). Western blotting with a monoclonal antibody to the His-tag confirmed that soluble eluted rApuR was of the predicted molecular weight and not degraded by proteolysis (Fig. 4.5B).

To investigate whether rApuR did bind to the promoter of *apuA,* three overlapping fragments of the P_{apuA} promoter region (Pr 1-3; approx. 120 bp in length) were amplified using fluorescent dye labeled primers (IRDye 800 nm) and tested in an electrophoresis mobility shift assay (EMSA). A single rApuR-DNA complex was observed with promoter fragments Pr2 and Pr3 suggesting the binding motif for ApuR lies within the 64 bp overlapping region (Fig. 4.6A and B).

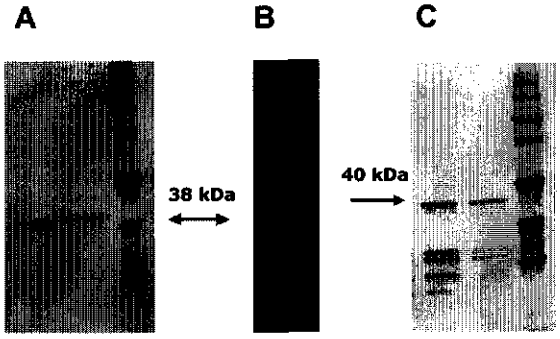


Fig. 4.5. SDS polyacrylamide electrophoresis of purified transcriptional regulators A. A Coomassie stained SDS-PAGE gel (12%), showing purified fraction His-ApuR at the expected size of 38 kDa. **B.** Western Blotting of the gel from A. with a monoclonal His-tag antibody to the recombinant His-ApuR protein. **C.** A Coomassie stained SDS-PAGE gel (12%) of purified His-CcpA at expected size of 40 kDa.

The binding of ApuR to Pr2 was shown to be concentration dependent in the range from 0.5 to 4.0 µM of rApuR (see Fig. 4.6C). The specificity of rApuR binding to Pr2 was shown by the use of non-fluorescent competitor DNA fragments (the fragment Pr2 and a non-specific DNA fragment not containing the two predicted OM1/OM2 operator binding motifs) in a competition EMSA.

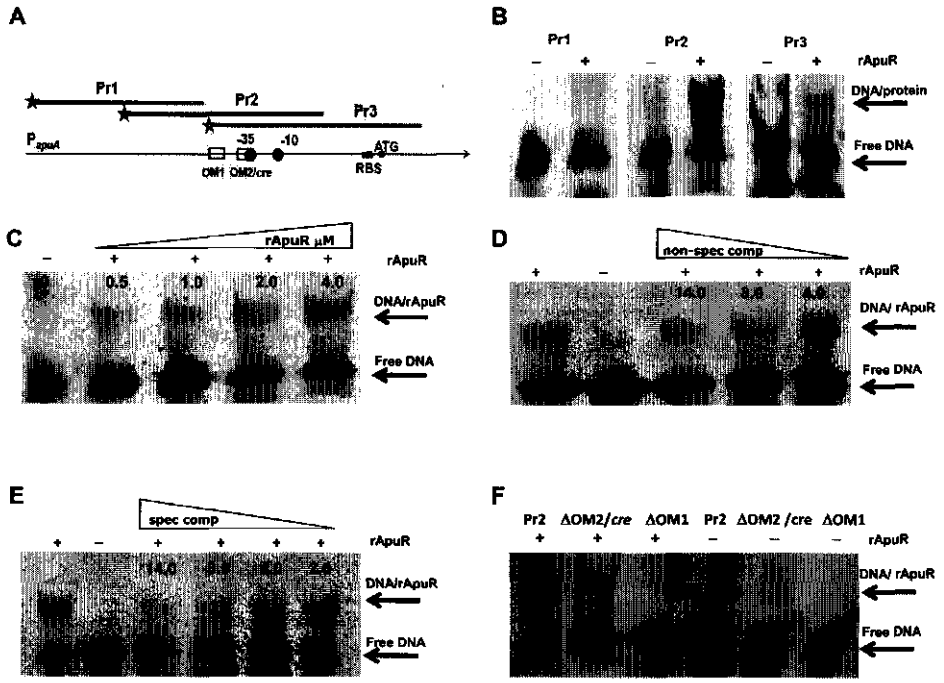


Fig. 4.6. Binding of recombinant ApuR protein to P_{apuA} promoter region A. Schematic representation of the *apuA* promoter (P_{apuA}) and fluorescently labeled PCR amplified DNA fragments (Pr1, Pr2 and Pr3) used for EMSA. **B.** to **F.** Native 5% TBE electrophoresis gels of DNA amplicons and DNA/rApuR protein complexes visualized using the Odyssey Imager. In these EMSA assays the concentration of each DNA amplicon was around 4-6 ng **B.** EMSA of 6 µM rApuR binding to Pr1, Pr2 and Pr3. **C.** Increasing DNA/rApuR complex formation in presence of 4 ng Pr2 DNA amplicon and an increasing amount of rApuR (~ 0,5 - 4 µM as indicated) **D.** Competitive EMSA using increasing concentrations of non-fluorescent non-specific competitor DNA (lacking OM1 binding motifs). **E.** Competitive EMSA using increasing concentrations of non-fluorescent Pr2 as a specific competitor. The amounts of competitor DNA added are indicated (2 – 14 ng). **F.** Identification of specific ApuR binding sites in P_{apuA}. PR2: native promoter region fragment 2. ΔOM1 and ΔOM2/*cre* are synthetic DNA fragments of P_{apuA} that lack the predicted binding sites. + rApuR recombinant present – rApuR recombinant absent.

The non-specific competitor DNA fragment had no effect on Pr2 complex formation whereas non-fluorescent Pr2 substantially reduced rApuR binding (Fig. 4.6D and E). To investigate whether ApuR binds to OM1 or OM2/*cre*, promoter fragments lacking these motifs were synthesized and tested in the EMSA (ΔOM1 and ΔOM2/*cre*, Table 2). In several independent experiments we observed a lower amount of the rApuR-DNA complex with fragments lacking ΔOM2/*cre* suggesting that ApuR binds strongly to this motif. In some experiments complex formation was slightly reduced when ΔOM1 was deleted, possibly due to low affinity binding of ApuA to ΔOM2/*cre* at higher concentrations (Fig. 4.6F).

We hypothesized that CcpA could directly repress *apuA* expression through binding to the P_{apuA} OM2/*cre* that overlaps with the predicted -35 promoter element. Recombinant rCcpA was tested for DNA binding in an EMSA using a fluorescent IRdye-Pr2 fragment containing the putative *cre* element (Fig. 4.7). rCcpA binding was enhanced upon addition to the binding reaction mixture of glucose-6-phosphate, a putative cofactor for CcpA [40,41,42]. An increasing amount of a rCcpA-DNA complex was observed with an increasing concentration of purified rCcpA (1.5 to 5 µM) (Fig. 4.7A). The complex could be outcompeted by addition of unlabeled Pr2 but not with non-specific competitor DNA fragment lacking the *cre*, indicating that CcpA binds specifically to P_{apuA} (Fig. 4.7B and 7C). Interestingly, the complex DNA/rCcpA appears to possess residual binding to ΔOM2/*cre* suggesting that CcpA may also bind OM1 or other sequences in Pr2 (Fig. 4.7D).

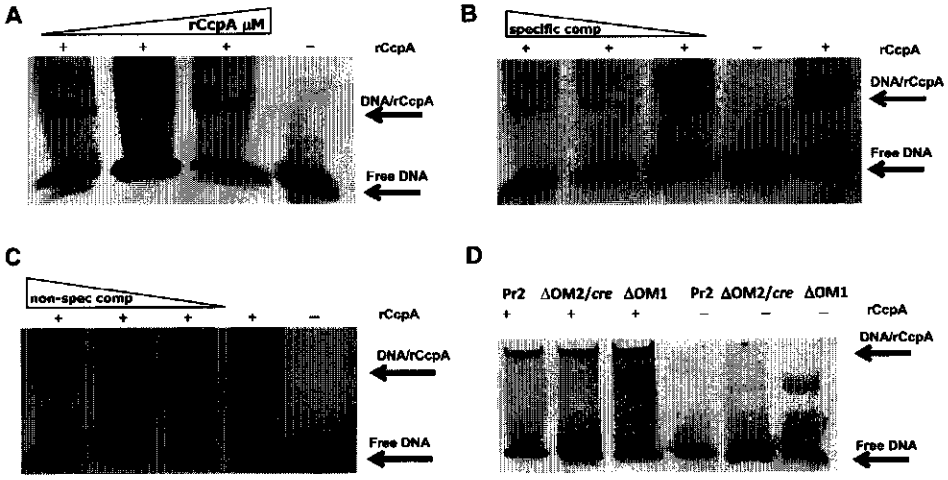


Fig. 4.7. Binding of CcpA recombinant protein to the *apuA* promoter. Native 5% TBE electrophoresis gels of DNA and DNA/rCcpA protein complexes visualized using the Odyssey Imager. In the EMSA assays the concentration of each Pr2 DNA amplicon was around 4-6 ng **A.** DNA/rCcpA complex formation in presence of in presence of 4 ng Pr2 DNA amplicon and an increasing amount of rCcpA (~ 1.5 - 5 µM as indicated). **B.** Competitive EMSA using increasing concentrations of non-fluorescent Pr2 as a specific competitor. **C.** Competitive EMSA using increasing concentrations of non-fluorescent, non-specific competitor DNA lacking OM1 and OM2 binding motifs. **D.** Identification of specific binding sites for CcpA in P_{apuA}. ΔOM1 and ΔOM2 are synthetic DNA fragments of P_{apuA} that lack the predicted binding sites. + rCcpA recombinant present – rCcpA recombinant absent.

ApuA is co-regulated by carbon catabolite repression

Given that OM2/*cre* specifically binds CcpA we hypothesized that *apuA* was co-regulated by carbon catabolite repression. To verify this finding we performed qPCR assays on *apuR*,

apuA, *sgaT* and *ptsN*. We first obtained growth curves where putative inducers were added to *S. suis* growing in exponential phase (Fig. 4.8A and B).

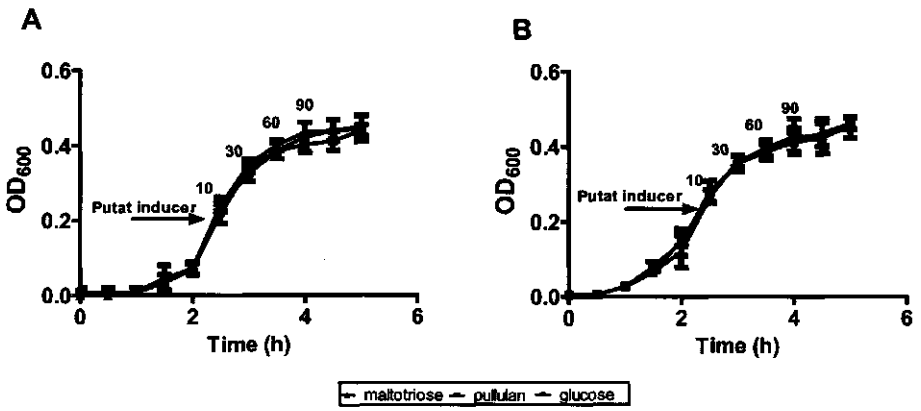


Fig. 4.8. Growth curves of *S. suis* S10 in CM containing lactose or glucose before and after addition of putative inducers. *S. suis* bacteria were grown in CM plus 1% lactose **(A)** or 1% glucose **(B)** to which pullulan or one of three putative inducers (maltotriose, pullulan or glucose) were added (0.25% w/v) during exponential growth (arrow). The graphs show the means and standard deviations from two independent experiments.

The growth curves for *S. suis* were very similar in lactose and glucose and were not influenced by addition of 0.25% (w/v) inducer. In medium containing lactose, both maltotriose and pullulan strongly induced expression of *apuA* (4.5 fold and 2.2 fold respectively; Fig. 4.9A and B). Transcription of *sgaT* and *ptsN* which lie downstream of *apuA* were not significantly modulated by addition of maltodextrin or pullulan.

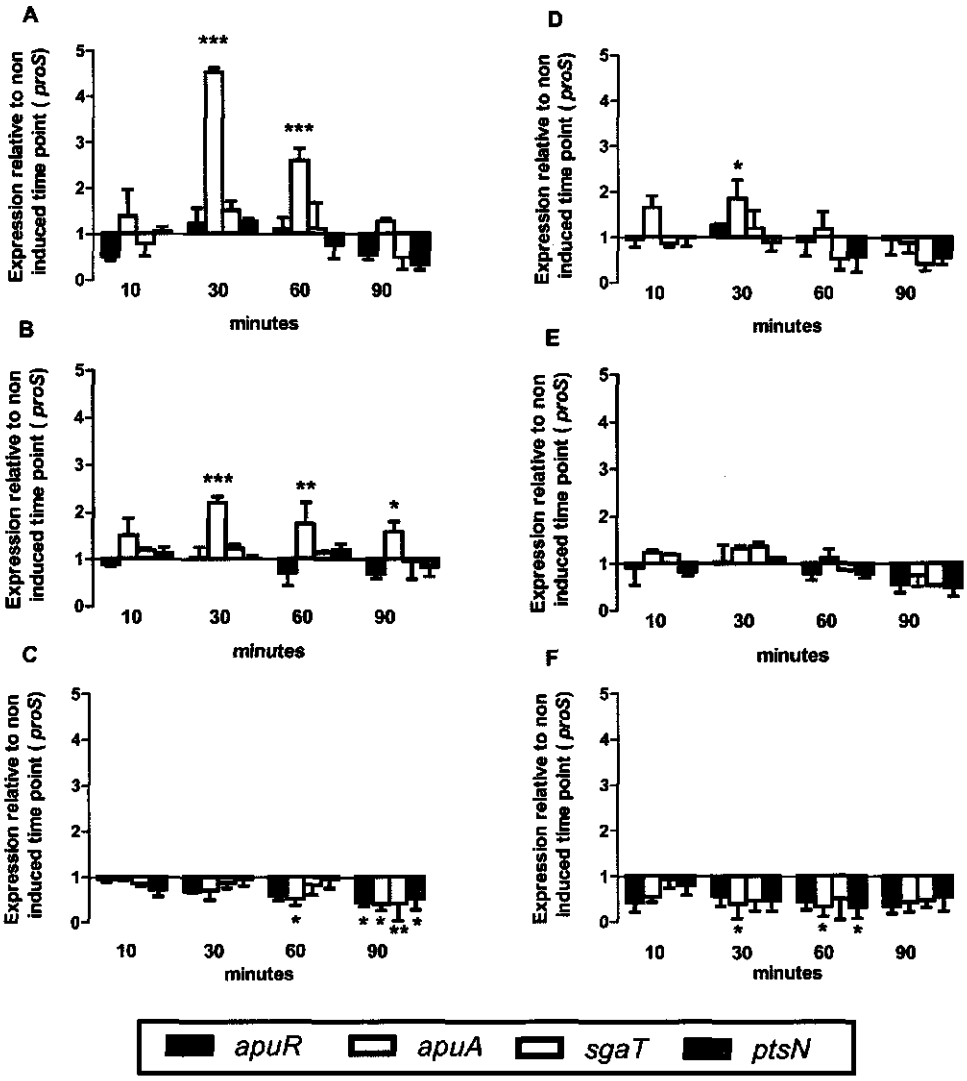


Fig. 4.9. Relative expression of *apu* locus genes following addition of putative inducers. *S. suis* was grown in CM with 1% of lactose and in the exponential phase, 0.25% (w/v) putative inducers of *apuA* expression were added as follows: **A.** Maltotriose, **B.** Pullulan and **C.** Glucose. Similarly **D.**, **E.** and **F.** represent expression data of *S. suis* in CM containing 1% w/v glucose after addition of the putative inducers **D.** Maltotriose, **E.** Pullulan and **F.** glucose. At time zero (immediately prior to addition of the putative inducers) and after 10, 30, 60 and 90 minutes, the relative expression of *apuR, apuA, sgaT* and *ptsN* were measured by qPCR. The height of the bars shows the mean (n = 3) fold change in expression ± SEM from two independent experiments. Statistical significance was calculated using a two-way ANOVA test followed by Bonferroni's post hoc test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

In medium containing glucose the addition of maltotriose (Fig. 4.9D) or pullulan (Fig. 4.9E) had no significant effect on the expression of *apuA, apuR, sgaT*, and *ptsN;* only a slight

increase (1.6 fold) of *apuA* expression was observed 30 min after addition of maltotriose (Fig. 4.9D).

Furthermore, when bacteria exponentially growing in complex medium containing glucose or lactose were supplemented with additional glucose, the relative transcript levels of all the genes were lowered (Fig. 4.9C and F).

Taken together these results suggest that *apuA* expression is repressed by growth in glucose. These results, together with the results shown in Fig. 4.3B and C, suggests that *sgaT* and *ptsN* are not co-transcribed with *apuA*. Supporting this is the finding that the 328 nt intergenic region upstream of *sgaT* and *ptsN* does not contain the operator motifs OM1 or OM2/*cre*.

binding motif for ApuR is also found in the predicted promoter for *malQ* but further work is needed to confirm the precise regulatory mechanisms for these operons.

On mucosal surfaces α-glucans may not always be abundant but other sugars apart from glucose could support growth. Under these conditions ApuA is presumably expressed at low levels but in sufficient amounts to initiate enzymatic degradation of α-glucans, thereby generating inducers of ApuR binding and activation. After invasion of the mucosal tissues and dissemination of *S. suis* via the bloodstream, our model predicts that expression of *apuR* (and potentially the *mal* operon) would be repressed by blood glucose and the lack of maltotriose as an inducer (Fig. 4.10B).

These complex intertwined mechanisms of regulation would allow fine tuning of ApuA production (energy-demanding because of its large size of 2094 amino acids, ca. 230 kDa), according to the availability of carbohydrates and sugars in order to optimize proliferation in the host.

Here we demonstrate a link between virulence (adhesion to host epithelial cells) and carbohydrate metabolism whereby the degradation products of complex carbohydrates serve as environmental cues to regulate expression of proteins like ApuA which play a role in both virulence and carbohydrate catabolism. This concept is an emerging theme in the biology of opportunistic pathogens such as *S. pyogenes* and *S. pneumoniae* [43]. The ability of a pathogen to efficiently utilize different carbohydrates and sugars during infection and to adapt rapidly to its environment is crucial to infectivity. In the future, it may be possible to devise strategies to combat pathogens by interfering with these regulatory mechanisms or pathways.

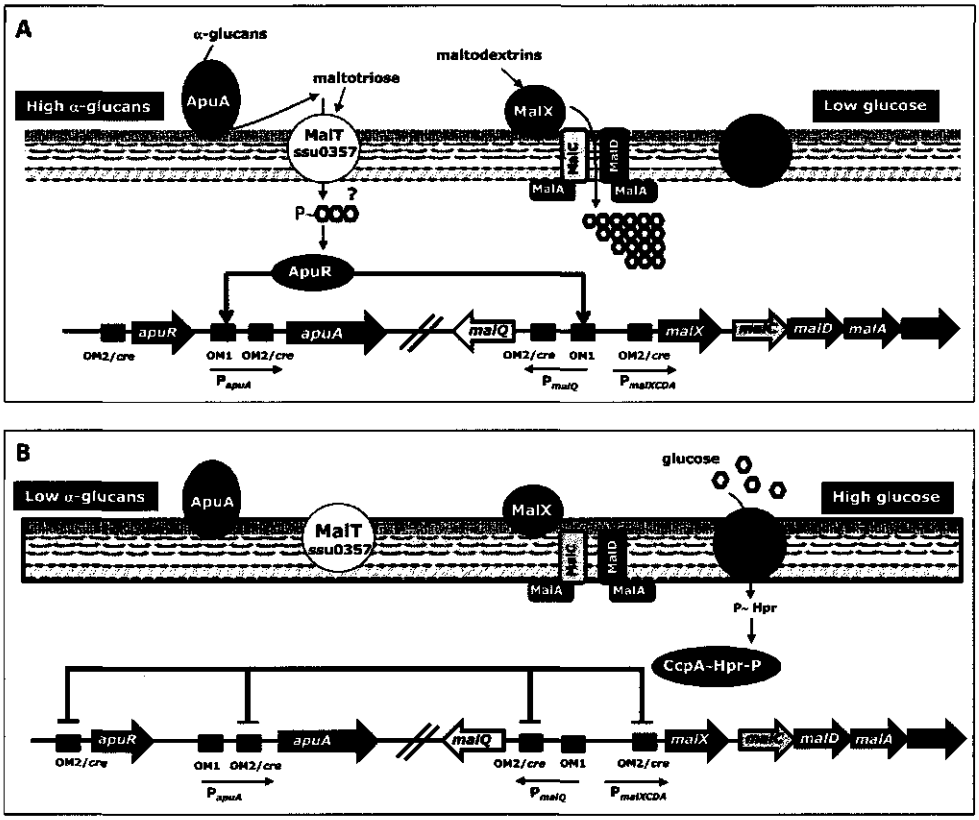


Fig. 4.10. Hypothetical model of α-glucan catabolism regulation in *S. suis*. The proteins/genes indicated in this model are the most likely candidates for the predicted functions based on their homology and genome organization in related organisms. The extracellular surface anchored ApuA (SSU1849) is able to degrade α-glucans. MalX (SSU1914) is a maltodextrin binding protein anchored in the cell wall that is specialized in the binding and uptake of maltodextrins. MalP (SSU0357) is part of a PTS system specific for uptake of maltotriose and/or maltose. The presence of OM1 and OM2/cre are indicated in the promoters of P_{apuA} and the predicted promoters of P_{apuR}, P_{malQ} and P_{malX} (Table 1). **A.** Regulation in the presence of relatively high amounts of α-glucans and low amounts of glucose. In the absence of CCR, the *apuA* gene will be expressed and the ApuA protein will degrade glucans leading to the generation of maltodextrin and maltose or maltotriose. The transport systems, maltose- and maltotriose-specific PTS (MalP) and the ABC permease system (MalX), are than expressed and import maltose and maltodextrins. The intracellular maltotriose is proposed to interact with ApuR to promote binding and transcriptional activation of P_{apuA} (continuous green line) and potentially P_{malX} (broken green line).
B. Regulation in the presence of relatively low amounts of α-glucans and high amounts of glucose. The Hpr protein is activated by the phosphotransfer system for glucose. In its phosphorylated form, Hpr(Ser-P) acts as a cofactor for CcpA to increase the affinity of binding to *cre* operator motifs in P_{apuA} (continuous red line). CcpA is also predicted to bind to *cre* sites in P_{apuR} and the P_{Mal} operon (broken red line). Binding of CcpA is predicted to repress transcription of expression of the *apuR*, *apuA* and *mal* operons.

Materials and Methods

Bacterial strains, plasmids and culture conditions

In this study, the virulent *S. suis* strain S10 (virulent serotype 2 strain) [59] has been used. The genome of *S. suis* S10 is more than 99% identical to the genome of *S. suis* P1/7, a sequenced reference strain of which the genome had been annotated previously (**Chapter 1**). *S. suis* was grown in Todd-Hewitt broth (THB) (Difco) or on Columbia agar plates with 6% sheep blood (Oxoid) at 37°C under 5% CO_2 for 18 hr. A complex medium (CM) comprising of 10 g l^{-1} proteose peptone, 5 g l^{-1} trypticase peptone, 5 g l^{-1} yeast extract, 2.5 g l^{-1} KCl, 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 final concentrations of 1% (w/v) as previously described [44]. The carbohydrates were added separately and sterilized by autoclaving at 100°C for 10 min (pullulan) or filtration with 0.45 µM pore size filter (glucose, lactose and maltotriose). We previously demonstrated that *S. suis* only grows to high density in CM when exogenous carbohydrates are added [18]. Growth in complex medium was determined by measurement of turbidity at OD_{600} using a SpectraMax M5 reader.

Expression and purification of recombinant ApuR and CcpA

To purify the recombinant His-ApuR and His-CcpA proteins we used a similar protocol as described below with some small modifications according the proteins. Briefly, the entire genomic regions encoding the mature regulators ApuR (amino acids 2 to 312) and CcpA (amino acids 2 to 333) were amplified by PCR from *S. suis* S10 genomic DNA using GoTaq (Promega) with primers ApuR_F/R and CcpA_F/R (Table 2).

The purified PCR products were ligated to the pTrcHis TOPO2 TA expression vector (Invitrogen) such that the expressed recombinant regulators would be fused in-frame to a C-terminal polypeptide containing six histidine residues for affinity purification. After transformation of *E. coli* TOPO 10, clones containing the recombinant *apuR* and *ccpA* were selected on LB agar containing 50 µg ml^{-1} of ampicillin; several colonies were checked for the correct insertion of the *apuR* and *ccpA* gene fragments and verified by DNA sequencing. Expression of both regulators was induced by addition of 1 mM isopropyl-β-D-1-tiogalattopiranoside (IPTG, Invitrogen) to an exponentially growing culture (OD_{600} of 0.6) for 4 hrs at 18°C for ApuR and overnight at 18°C for CcpA under shaking (250 rpm). The cells were harvested by centrifugation (8 000 g x 10 min at 4°C) and the pellet was resuspended in lysis buffer (50 mM Tris-Cl; 0.5 M NaCl, pH 7.4) containing a cocktail of protease inhibitors (Roche), and then disrupted using a high pressure cell disrupter (Constant Systems, U.K.). The soluble protein extract was recovered after high speed centrifugation (20 000 x g 40 min at 4°C) and loaded onto a HisTrap affinity chromatography column (Amersham Pharmacia Biotech, Freiburg, Germany). Proteins were eluted in a phosphate buffer containing 500 mM NaCl and increasing concentrations of imidazole (0-500 mM) and checked by SDS-PAGE gel electrophoresis. Fractions containing purified fusion proteins of the expected size (approx. 38 kDa for ApuR and 40 kDa for

CcpA) were collected and dialyzed against buffer (500 mM NaCl, 50 mM Tris-HCl pH 7.4) and stored at −80°C with 10% of glycerol. Protein concentrations were measured using a BCA Protein Assay (Thermo Scientific), according to the supplier's instructions.

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence[a] (5′ to 3′)	Purpose
ApuR_F	ACGACATTAGCCGATGTGG	To produce rApuR
ApuR_R	CTGAGGTGTAGTCTCCCTTTCAA	
CcpA_F	TTAAACACTGACGATACGGTAACG	To produce rCcpA
CcpA_R	CTTAGTTGATTTACGTACTTTGATTCC	
Pr1F	AGGGAGACTACACCTCAGTA	EMSA fragment Pr1
Pr1R	TTAACGGTAACAAGTTTTGA	
Pr2F	AAAGAAGGGGGAGCTATTTAT	EMSA fragment Pr2
Pr2R	CTACCAGTATATACAATTCCAAGG	
Pr3F	CTTGTTACCGTTAACATTTAA	EMSA fragment Pr3
Pr3R	TATTCTGAACGGATTTCTT	
A-CompF	GTTTATCAAGGTGACTTCAGA	Aspecific competitor
A-CompR	GTACCAATTCATCAAAGGA	
ΔOM1	AAAGAAGGGGGAGCTATTTATTCTATTTTTGTTAGATAGTAGCTCAAAACT TGTTACCGTTAACATTTAAACTCCTTGGAATTGTATATACTGGTAG	Synthetic fragment 1 with OM1 deletion
ΔOM2/*cre*	AAAGAAGGGGGAGCTATTTATTCTATTTTTGTTAGATAGTAGCTCAAAA TAAGAAAACGTTTGCAAAGACTCCTTGGAATTGTATATACTGGTAG	Synthetic fragment 2 with OM2 deletion
ASP1	CTCCCCAAGTCCAAA	5′-RACE cDNA
ASP2	CTGTTACCGCTGTCGCTTCA	5′-RACE nested PCR
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	5′-RACE nested PCR
proS1	TTACGTGCGGGCTATGT	qPCR *proS* reference gene
proS2	GCTGTAGCCGTCTTTCATG	
apuR1	CTGAGGTGTAGTCTCCCTTTC	qPCR *apuR* target gene
apuR2	GGGAGAGAAGAAGGCTACAA	
apuA1	CACCACTTGTCGCTTGTC	qPCR *apuA* target gene
apuA2	ACCTTTGACTGCAACAGTG	
sgaT1	GGGTTTATCAAAGCGACAG	qPCR *sgaT* target gene
sgaT2	ACTGCCCAGTAGATACCACAG	
ptsN1	GCAGGAGGCTGTTCACA	qPCR *ptsN* target gene
ptsN2	CTAGGTAAGGACTGTTTTTGG	

5′-Rapid Amplification of cDNA Ends (5′-RACE)

The 5′-rapid amplification of cDNA ends (RACE) system (Invitrogen) was used to determine the transcription start site of the *apuA* gene. Briefly, the first strand cDNA was reversed transcribed from RNA from 1 µg of total RNA *S. suis* grown in CM plus pullulan using the specific primer ASP1 (Table 2). The obtained cDNA was treated with RNase Mix and purified with a S.N.A.P. Column (Invitrogen). A homopolymeric tail was then added to the 3′-end of the cDNA using terminal deoxynucleotidyl transferase (TdT) and the deoxynucleotide dCTP. The tailed cDNA was amplified in nested-PCR with Abridged Anchor Primer (AAP) and a second *apuA* specific primer ASP2 primers upstream ASP1. The resulting 5′-RACE product of ~ 380 bp was sequenced and analyzed by Vector NTI software (Invitrogen).

DNA Binding Reactions and Electrophoretic Mobility Shift Assay

Gel-shift assays were performed utilizing three pairs of fluorescent IRdye 800 labelled primer on the 5' ends (Biolegio, the Netherlands). Primers used to PCR-amplify three DNA fragments (Pr1-Pr3, ~120 bp) containing overlapping regions to cover the full length of P_{apuA} promoter from *S. suis* genomic DNA are listed in Table 2. The fluorescents PCR fragments were purified with QIAquick PCR Purification Kit (Qiagen) and used for the binding reaction at a concentration of around 4-6 ng. DNA binding reactions were performed in 20 µl of binding buffer containing 10 mM Tris-HCl pH 8.5, 50mM NaCl, 10 mM EDTA, 0.5% Tween-20, 10 mM DTT, and 1 mg of poly(dI-dC); labelled PCR fragments at room temperature for 30 min. For the specific and aspecific competition assays, D(+)-glucose 6-phosphate (30 mM) (Sigma) was added to the binding buffer in the EMSA as CcpA cofactor [60]. Purified ApuR and CcpA proteins (200–800 ng) were incubated, in separate experiments, with the fragment Pr2 and a non-specific competitor fragment that was obtained by PCR amplification of the gene (SSU0879) (4-14 ng). Two 95 bp oligonucleotides complementary to Pr2 fragment without the two predicted binding sites (*ΔOM1 and ΔOM2/cre*) were synthesized (Eurogentec, the Netherlands), PCR- amplified with labelled Pr2F/R primers and incubated with the proteins. Free and bound DNAs were separated on 5% Tris-Borate-EDTA (TBE) native gels for 30 min at 10 cm/v. The gels were visualized using a LI-COR Odyssey Imager and scanned at 800 nm wavelengths.

Bioinformatic tools

In silico searches and comparisons of genomic regions containing *apuR* and homologous genes were conducted using the MicrobesOnline web server (http://microbesonline.org).

The reference set of predicted regulons was present within the *Streptococcus* regulons collection, provided by the RegPrecise database (http://regprecise.lbl.gov) [35].

The MEME (http://meme.sdsc.edu/meme/meme.html) software suite (version 4.1.0) was used for the identification of motifs OM1 and OM2. MEME can be used to identify likely motifs within a given input set of aligned sequences [61]. It produces a consensus sequence and a position specific probability matrix, which has probabilities associated with each base at each position. A range of motif widths (15 nt in length) and zero or one motif per sequence were specified in our queries.

We then applied the FIMO (Find Individual Motif Occurrences) module (part of the MEME suite), using the motif weight matrix from the MEME program, to search for the occurrence of operator motifs (OMs) in all *S. suis* operons. Motif hits with a position-specific goodness-of-fit *P* value of less than 10^{-4} [62] were considered to identify putative ApuR-CcpA binding sites across the *S. suis* genome.

Growth of S. suis in CM media supplemented with different sugars and RNA extraction

All growth experiments were performed at least in duplicate. *S. suis* S10 bacteria grown overnight in THB were harvested, washed in 0.1 M phosphate buffer and added to complex media supplemented with 1% of sugars to achieve a uniform starting OD_{600} of 0.05.

To determine the activity of putative inducers, the growth curves for *S. suis* were carried out in CM plus 1% of glucose and CM plus 1% of lactose. During the exponential growth phase (~ OD_{600} 0.24), a putative inducer 0.25% (w/v) was added to the media. Spectrophotometric density readings were then taken after 30 min until completion of the experiment.

For analysis of gene expression under diverse growth conditions, *S. suis* S10 was grown to exponential-early stationary phase in the indicated media. Ten ml of culture was collected and centrifuged for each analyzed point. The pellet was immediately frozen in nitrogen and stored at 4°C overnight with 1.5 ml RNA*later* (Ambion). To isolate total RNA, 1.5 ml RLT lysis buffer (Qiagen) was added to the RNA*later* to dissolve the pellet and after incubation at 15 min at room temperature, centrifuged at high speed (13 000 g for 30 min). The cells were dissolved in 600 µl RA1 buffer (Macherey-Nagel) plus 6 µl β-mercaptoethanol and destroyed using a FastPrep-24 (MP. Biomedicals, Solon, OH) for 20 sec at 6.0 m/sec. Total RNA was purified using NucleoSpin RNA II columns (Macherey-Nagel). Residual DNA was removed on a column with RNase-free DNaseI (Ambion). The quality and the concentration of RNA samples were assessed with the Experion system (Bio-Rad) and by analysis of the A260/A280 ratio (Nanodrop). The absence of DNA from RNA samples was verified by PCR prior to reverse transcription, using prolyl-tRNA synthetase housekeeping gene (*proS*-SSU1753)-specific primers.

cDNA synthesis and quantitative reverse transcription-PCR

cDNA was synthesized from 1 µg of total purified bacterial RNA and random oligonucleotide hexamers using SuperScript® VILO (Invitrogen) according to the manufacturer's recommendations. Primers were designed using Oligo Program version 6 (MedProbe, Oslo, Norway) to have melting temperatures above 83°C and an amplicon size of approximately 400 bp (Table 2). Quantitative PCR was performed with LightCycler 4.0 V and using LightCycler FastStart DNA Master SYBR Green I Kit (Roche). The identities of the resulting amplicons were checked by melting curve analysis using the LightCycler 4.1 software and 1.5% agarose gel electrophoresis. Reaction mixtures containing no template were included in each real-time PCR experiment to control for contamination. Constitutive gene expression in complex media was determined as a ratio of target gene *vs* reference gene *proS* and was calculated according to the following equation: ratio = $(E_{reference})^{Ct\ reference}/(E_{target})^{Ct\ target}$ where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value. Expression following the addition of the possible inducer was determined relative to the expression at the non-induced time point according to the following

equation: Ratio=$(E^{target})Ct^{target\ (control\text{-}sample)}/(E_{reference})Ct^{\ reference(control\text{-}sample)}$ [63]. The level of *proS* expression was constant at all the time points analysed (data not shown). Two replicates of all samples and primer pairs were included and the experiment was performed in triplicate. Non-template controls were included for each gene in each run. Primers for targets and the internal control gene *proS* are listed in Table 2.

Statistical analysis

All qPCR experiments were reproduced at least two times in triplicates and, where indicated, representative experiments are shown. Two-way ANOVA tests were carried out using Bonferroni's post hoc test. Statistical analyses were performed using GraphPad Prism 5 software (La Jolla, CA, USA). Statistical significance was indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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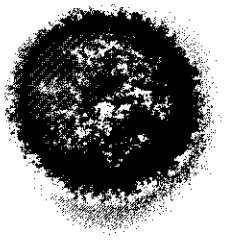
Chapter 5



Mucosal Carbohydrates Serve as Environmental Cues to Regulate Virulence Gene Expression in *Streptococcus suis*

M. Laura Ferrando, Peter van Baarlen, Rosaria Piga, Roger Bongers, Michiel Wels, Astrid De Greeff, Hilde E. Smith and Jerry M. Wells

Submitted in part

Summary

Streptococcus suis is a major pig pathogen as well as an emerging zoonotic pathogen. Previous work has demonstrated that the *S. suis* extracellular amylopullulanase enzyme that degrades complex carbohydrates was also an important adhesin that increased bacterial binding to porcine epithelial cells. This finding suggested a link between carbohydrate metabolism and virulence. To explore this link, *S. suis* whole-genome gene expression microarrays were used to measure *S. suis* transcriptome alterations when bacteria were grown in minimal media supplemented with glucose or pullulan as carbon sources. We found that the relative expression of seventeen virulence genes was increased during growth in presence of pullulan, compared to growth in glucose. To test the hypothesis that *S. suis* grown in pullulan is more virulent, adhesion and invasion experiments were performed using porcine epithelial cells and bacteria grown in the two different media. We show that growth of *S. suis* using complex carbohydrates as carbon source significantly improves bacterial adhesion to host epithelia and significantly increased bacterial invasion of epithelia.

Introduction

Streptococcus suis is a major swine pathogen and an emerging zoonotic pathogen of humans [1,2]. Young pigs typically acquire *S. suis* by vertical or horizontal transmission, resulting in colonization of the tonsils and the nasal cavity (oropharynx). Some carrier piglets may develop invasive disease leading to bacteremia, meningitis, septicemia and arthritis, whereas other piglets also carrying *S. suis* may never develop disease [3,4]. Humans are thought to become infected via skin lesions or via the oral route upon contact with, or consumption of, contaminated pork [2,5].

There are only few studies on the interactions between *S. suis* and mucosal epithelial cells but according to these, the ability of *S. suis* to invade epithelial cells or pass the epithelial barrier remains somewhat controversial [6,7,8,9]. Some studies reported that only unencapsulated strains can invade epithelial cells [8] and it has been hypothesized that capsule production might be suppressed in response to environmental signals to promote adhesion and invasion, but this remains to be proven *in vivo* [7,8].

Passage of the epithelial barrier depends on several bacterial molecules, bacterial effector molecules or virulence factors, that interfere with host defense responses and promote disease. Suilysin (Sly) produced by some but not all *S. suis* isolates is an extracellular thiol-activated haemolysin which belongs to the cholesterol-binding toxin family. Suilysin forms pores in host cells upon oligomerization [10] and has been shown to be cytotoxic for epithelial [7,11], endothelial [12,13,14] and immune cells *in vitro* [15,16]. Production of suilysin has been proposed to play a role in epithelial damage and translocation, enabling *S. suis* to invade the mucosal tissues and thereafter disseminate via the blood [11]. Suilysin may not be essential for host invasion since strains that do not produce suilysin have also been associated with invasive disease [14].

Host colonisation also depends on the efficiency by which bacteria can utilize host tissues as nutrient source. Starch α-glucans (large polymers of glucose) are used as an energy storage by plants and are present in large amounts in the staple diet of food production animals [17]. Consequently starch α-glucans are found in high concentrations in the saliva and oropharyngeal cavity of domestic animals including pigs [18,19,20,21]. Presence of high amounts of complex carbohydrates is of relevance to microbial ecology since the utilization of different carbohydrates by bacterial pathogens is known to influence their metabolism and as a consequence, pathogen virulence [22,23,24,25].

Glucose, a common component of most diets, may also be present in the oral cavity but concentrations diminish rapidly (within 30 min) after ingestion [26] since glucose is readily metabolised by microbes. In human the concentration of available glucose in the human oropharynx is too low (0.02–0.4 mM) to support *S. suis* growth, which means that other carbon sources are needed for bacterial proliferation [27]. Generally, host-associated bacteria do not synthesize degradative (catabolic) enzymes unless the substrates for these enzymes are present in their environment, and host-associated bacteria have evolved elaborate control mechanisms for selective uptake and utilization of the carbon source that allows fastest growth [28,29]. In the presence of glucose, a carbon catabolite control (CCC) mechanism controls (usually suppresses) the expression of genes encoding degradative enzymes, transporters and metabolic pathways for other sugars [30,31,32,33]. The catabolite control protein A (CcpA) is well-conserved among Firmicute species and can repress (the most common activity) or activate transcription by binding to *cis*-acting catabolite response element (*cre*) sites that are present in promoter regions of genes controlled by CcpA [33]. Consensus *cre* sequences have been determined in several species [32]. Studies in *Bacillus* species have shown that the binding of CcpA to *cre* DNA sites is enhanced by interaction of CcpA with the histidine-containing phosphocarrier protein HPr-Ser-46-P [34,35]. The HPr phosphorylation status is determined by the action of HPr kinase/phosphorylase (HPrK/P), which, in turn, is affected by intracellular concentrations of fructose 1,6-bisphosphate (FBP) that is produced during glycolysis. Orthologues of *Bacillus* genes regulating CcpA-mediated carbon catabolite control are present in *S. suis* isolate P1/7 (serotype 2), the pathogenic European reference strain of which a fully sequenced genome is available [36]. These genes comprise (i) the transcriptional regulator CcpA (SSU1202), (ii) *ptsG*-IIBCA^{Glc} (including enzyme I-HPr; SSU1309), (iii) the glycolytic enzyme fructose-bisphosphate aldolase (*fba*; SSU0312), (iv) the HPr kinase/phosphorylase (*hprK*; SSU1419). In addition to CcpA, other transcriptional repressors or activators play a role in the regulation of carbon metabolism in Gram-positive bacteria [23,37] (**Chapter 4**).

CcpA is important for virulence of streptococcal species, influencing growth, haemolysin production, biofilm formation and capsule expression [22,38,39,40]. In streptococci, several virulence factors are known to be controlled by CcpA, including genes that regulate and mediate capsule formation, the virulence M protein positive transcriptional regulator Mga, and several cytotoxins [38,40,41,42,43]. CcpA loss-of-function mutants in

Streptococcus pneumoniae were attenuated for virulence in mouse infection models [38,41] whereas CcpA depletion in *Streptococcus pyogenes* led to both hypervirulence and hypovirulence, again in experimental mouse infection models [22,43].

Recently, the role of CcpA was investigated by microarray analysis of wild-type *S. suis* and an isogenic Δ*ccpA* mutant grown in THB. Deletion of *ccpA* altered the expression of the surface-associated virulence factors *arcB*, *sao* and *eno* as well as *ofs* and *cps2A* in the capsule locus [44]. In electron micrographs of the Δ*ccpA* mutant, the thickness of the capsule was shown to be markedly reduced. Reduced capsule was proposed to be the reason for enhanced binding of the deletion mutant to porcine plasma proteins and a reduced resistance of *S. suis* to killing by porcine neutrophils [44].

The recently obtained genome sequences of 13 *S. suis* strains [36,45] led to the identification of a large number of predicted surface proteins and secreted proteins that might play a role in virulence, including a number of putative complex carbohydrates degrading enzymes. Many streptococcal pathogens possess the ability to metabolize α-glucans from the environment [46,47,48,49,50]. Here we took an experimental approach to study the role of *S. suis* carbohydrate metabolism on global *S. suis* metabolism and virulence, by comparing the transcriptomes of *S. suis* P1/7 grown in complex medium plus pullulan (an α-glucan) or glucose in both exponential and early stationary growth phases. Pullulan is a polymer of α-glucose linked by glycosidic bonds that is degraded by bacterial enzymes such as pullulanase or amylopullulanase into maltodextrins that can be metabolised further [9,48,51,52,53]. A metabolic pathway map was generated from the genome sequence of *S. suis* P1/7 and the transcriptome data. We also quantified production of suilysin and the expression of known virulence genes and performed adhesion and invasion assays of *S. suis* P1/7 grown in pullulan or glucose, to study the effect of different carbon sources on *in vitro* virulence. The results provide new insights into carbohydrate metabolism in *S. suis* and demonstrate links between carbohydrate metabolism and virulence gene expression concurrent with adhesion and invasion of epithelial cells.

Results

The metabolic map of S. suis

The metabolic map of *S. suis* includes most of the metabolic pathways that have been found in other *Streptococcus* species; these include all standard metabolic pathways for fatty acid biosynthesis, nucleotide, amino acid and carbohydrate metabolism (Fig. 5.1). *Streptococcus* species do not encode all enzymes necessary for completion of the tricarboxylic acid (TCA) cycle, the series of chemical reactions whereby aerobic organisms generate energy through the oxidization of acetate derived from carbohydrates, possibly because streptococcal species tend to be facultative anaerobes. *S. suis* appears to produce energy (ATP) through homolactic and/or mixed-acid fermentation of carbohydrates. Under conditions of excess glucose and in presence of oxygen, glucose is oxidized via the glycolysis (or Embden-Meyerhof pathway) to pyruvate. In several streptococcal pathogens, the oxidative respiratory enzymes of the TCA cycle then metabolize pyruvate via an incomplete TCA cycle [54,55] (Fig. 5.1). Alternatively, under limited oxygen conditions, *S. suis* can reduce pyruvate to lactic acid and products such as formate, ethanol and acetate. In addition, *S. suis* possesses the genes encoding proteins participating in the Leloir pathway, where UDP-glucose (UDP-Glc) and UDP-galactose (UDP-Gal) can be generated from glucose-1-phosphate or α-galactose. To date two ABC transporters and 17 phosphotransferase systems (PTSs) for the uptake of carbohydrates have been predicted for *S. suis* in the KEGG database highlighting the potential for *S. suis* to ferment a variety of sugars and carbohydrates (Fig. 5.1). Indeed, metabolic pathways for metabolism of fructose, mannose, glucans, galactose and sucrose appear to be present in *S. suis* and the capacity to ferment ribose, L-arabinose, mannose, sorbitol, lactose, raffinose, maltotriose, and α-glucans such as glycogen and pullulan has been described for *S. suis* [9,56]. For the metabolism of α-glucans, the extracellular amylopullulanase (ApuA) is required to degrade α-D-glucose polymers into smaller units for transport across the cytoplasmic membrane [9]. ApuA is the only extracellular α-glucan catabolic enzyme found in the sequenced genomes of *S. suis* except in the pathogenic *S. suis* ST3 serotype 3 [57] which possess an additional secreted amylase gene (SSUST3_0537). The broad fermentative capabilities of *S. suis* enables this organism to utilize dietary and host sources of carbohydrates for colonization of host mucosal tissues.

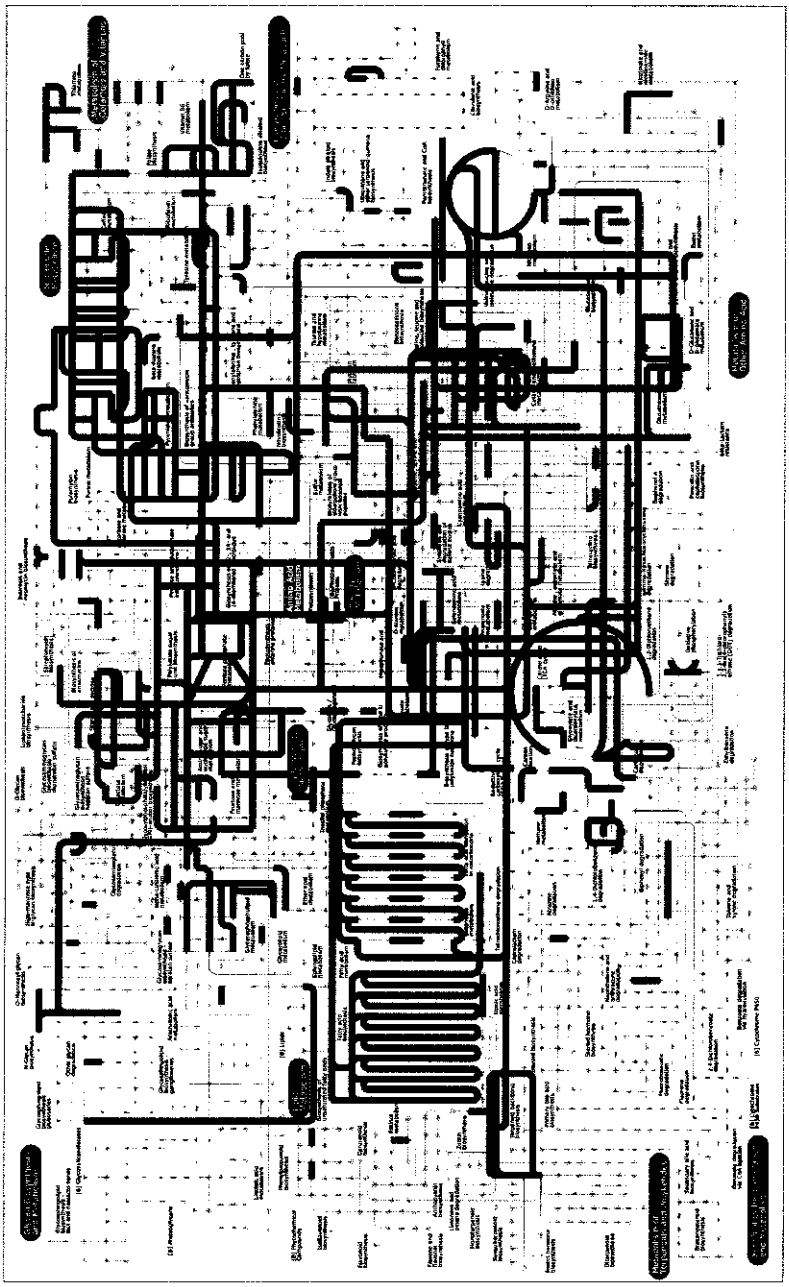


Fig. 5.1. Metabolic pathways chart, generated using iPath [58]. (Fragments of) pathways that include genes previously annotated in *S. suis* P1/7 are indicated in red.

Fermentation of pullulan has pleiotropic effects on gene expression

To compare the effects of pullulan (Pul) and glucose (Glc) on bacterial growth and transcriptional activity, we performed a gene expression profiling experiment, hybridizing RNA prepared from bacteria in exponentially growth phase (e) and in early stationary phase (s) (Fig. 5.2) to whole-genome microarrays.

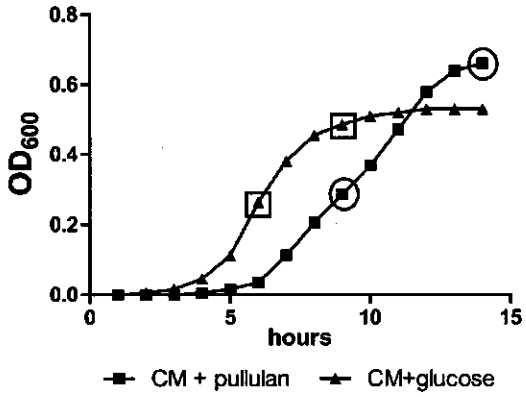


Fig. 5.2. *S. suis* S10 growth curve at 37°C in presence of different carbon sources. OD_{600} measured in complex media (CM) supplemented with 1% (w/v) glucose or pullulan.

At stationary phase, *S. suis* populations grown in CM + pullulan consistently yielded a higher biomass compared to *S. suis* grown in CM + glucose. The numbers of genes differentially expressed during growth in pullulan versus glucose in exponential (e) or early stationary (s) phase were 1028 (52% of annotated genes) and 1015 (51% of annotated genes), respectively (Fig. 5.3). In total 738 (37% of annotated genes) genes were differentially regulated in pullulan compared to glucose, irrespective of the growth phase. In pullulan, 209 genes were differentially regulated between the exponential and early stationary phases of growth; in glucose, 432 genes were differentially regulated for the same comparison.

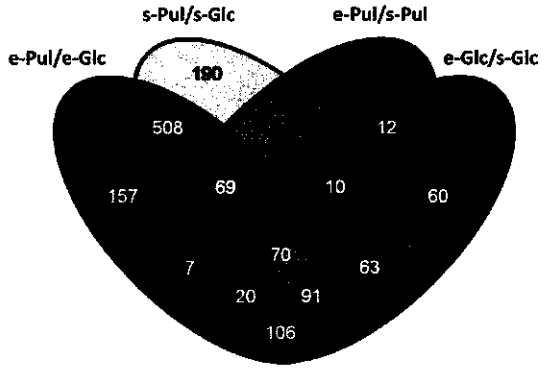


Fig. 5.3. Venn diagram of the genes differentially regulated growing *S. suis* in pullulan (Pul) vs glucose (Glc) in exponential (e) or stationary (s) phase. This diagram was created using VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html). For all intersections the numbers of differentially expressed genes are indicated.

For all genes and proteins identified in the *S. suis* P1/7 genome, Gene Ontology (GO, http://www.geneontology.org) and KEGG pathway annotations were obtained using the BLAST2GO software (www.blast2go.org) [59] including annotations based on terms obtained from EBI using the InterPROScan feature [60] that is part of BLAST2GO. The GO enrichment analysis feature of BLAST2GO was used to identify the functional categories that were statically over- or underrepresented in the set of genes differentially expressed in pullulan vs glucose in exponential and early stationary phases of growth. GO enrichment was calculated using a Fisher's Exact Test and P value cut-offs of 0.05 and lower. We determined over- and under-represented GO categories of genes differentially regulated in pullulan versus glucose in early exponential and early stationary growth phases (Fig. 5.4A and 5.4B) . Similar analyses were performed for a subset of differentials, namely all upregulated genes with the GO annotation "carbohydrate metabolism" (Fig. 5.5A and 5.5B).

The functional categorization of genes revealed that the highest number of genes differentially expressed in both growth phases participated in carbohydrate transport into the cell (11.7% of annotated sequences were annotated with this GO term). Related to carbohydrate transport, genes annotated with GO term "carbohydrate metabolism" (18.6%) (Fig 5.4A) and its sub-categories galactose metabolism (4.1%), starch-sucrose

metabolism (3.4%), pyruvate metabolism (7.1%) and fructose-mannose metabolism (6.0%) were also important components of the transcriptomes (Fig. 5.5A). The starch metabolic pathways comprise the genes encoding ApuA, transport systems for the maltose/maltodextrin breakdown products, and genes encoding enzymes that convert maltose and maltodextrin into Glc1P or Glc6P for other metabolic pathways (see below). The category "galactose metabolism" was possibly over-represented due to increased expression of the Leloir pathway enzymes that convert galactose into Glc1P (Fig. 5.5). Interestingly, genes in the GO category "energy reserve metabolism" (2.1%) were also enriched when bacteria were grown in pullulan versus glucose. Polysaccharide biosynthesis (4.1%) was over-represented in pullulan (Fig. 5.5), presumably due to the increased expression of the α-glucan branching enzymes (SSU0870, SSU0874 and SSU0873) that are required for the synthesis of glycogen from glucose-1-phosphate (Glc1P) (Fig. 5.5).

Another GO category that was enriched during growth in pullulan was lipid metabolism (11.7%) which is the metabolic pathway during which precursors of lipoteichoic acids and membrane phospholipids are generated. Linked to this GO category is glycolipid metabolism (2.7%), which includes glycosphingolipids and globosides (Fig. 5.5), molecules which are involved in membrane biosynthesis and biofilm formation [61]. Other enriched GO categories included amino acid metabolic pathways for arginine and proline, which in *S. suis* and other streptococcal pathogens are repressed in the presence of glucose by carbon catabolite repression (CCR) [44,62]. The under-represented categories in pullulan included ribosome biogenesis, trans-membrane ATPase activity, and regulation of gene expression by transcriptional regulatory proteins including two-component systems. Under-represented categories suggest possible cross-regulation (pathway repression) which could be due to activity of CcpA and other transcriptional regulatory systems.

In early stationary phase (Fig. 5.4B), "rRNA binding" is the first over–represented category that includes genes encoding stress proteins binding to 5S RNA, possibly due to the depletion of the carbon sources that were metabolised to generate energy [63]. Carbohydrate transport systems were more enriched at early stationary than in early exponential phase (17.9%) while cellular carbohydrate metabolism was under-represented (6.4%), possibly also due to the diminished availability of carbohydrate sources.

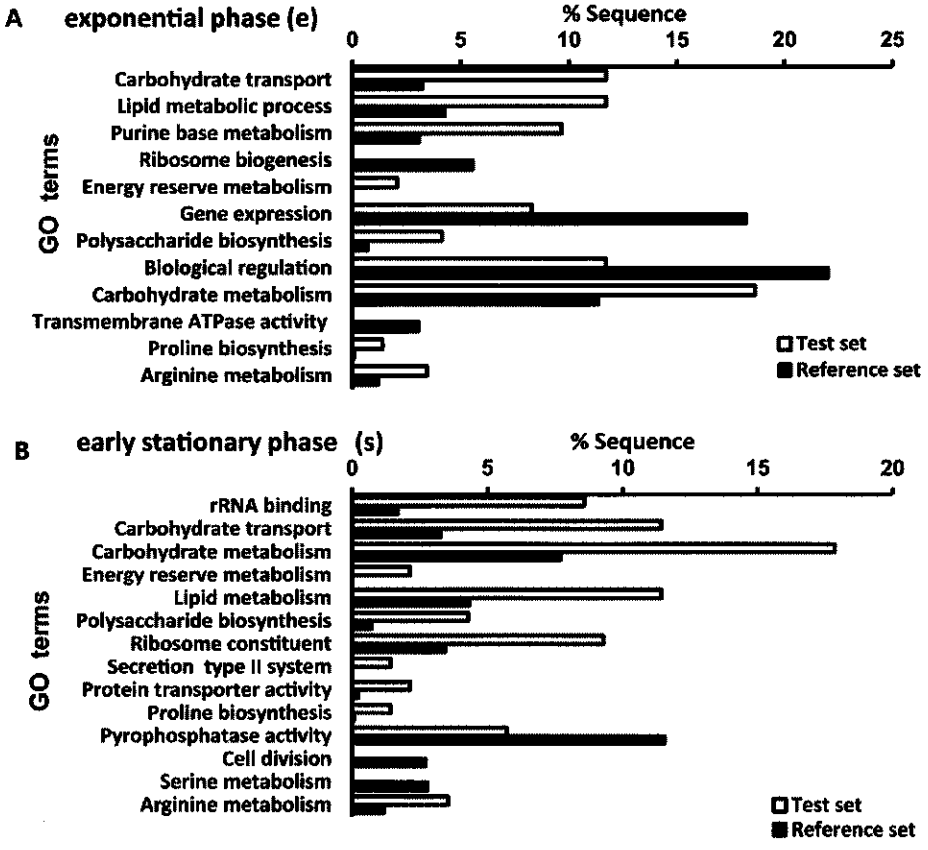


Fig. 5.4. GO term distribution of *S. suis* genes upregulated in pullulan vs glucose. A. early exponential (e) and **B.** early stationary phase (s) GO Enrichment analysis performed using BLAST2GO (P= 0.05, two-tailed Fisher's Exact test).

with their predicted function as a repressor and activator of the maltodextrin utilization system, respectively (**Chapter 4**).

Glucose-1-P (Glc1P), released upon pullulan degradation, can be metabolized in different pathways. Phosphoglucomutase (*pgm*-SSU0826), which is highly upregulated in pullulan compared to glucose, can isomerise Glc1P to Glc6P which can enter the glycolytic pathway. However, the glycolytic pathway enzymes were either not modulated or slightly down-regulated in pullulan compared to glucose under all conditions (Fig. 5.6). In agreement with this, genes participating in the pentose-glucuronate pathway were upregulated in pullulan. The pentose-glucuronate pathway is an alternative to glycolysis for the metabolism for Glc6P.

Glc1P is a direct precursor substrate for synthesis of glycogen (glucose polymers) as an energy reserve. The three genes encoding the ADP glucose pyrophosphorylase, glycogen synthase, and branching enzyme (*glgC-glgA-glgB*; SSU0870 to SSU0874) were all strongly induced (Fig. 5.6). The glycogen utilization genes encoding glycogen phosphorylase (*glgP2*-SSU0354) and *malQ2* (SSU0353), which convert glycogen or maltodextrins to Glc1P, were not differentially regulated (Fig 5.6). Enzymes for maltodextrin utilization and glycogen synthesis are classified in starch-sucrose pathway (Kegg ssi00500).

The genome of *S. suis* encodes also predicted enzymes belong both starch-sucrose and galactose metabolism that participate to the interconversion of different sugars [69,70]; their activities have not been biochemically tested yet. These genes, sucrose phosphorylase (*gtfA*-SSU1369), β-fructofuranosidase/interconvertase [71] (*invrtsC*-SSU1169) and raffinose galactohydrolase [72] (*rafgH*-SSU0167) were strongly induced in pullulan compared to glucose (Fig 5.6) and may be associated with the downstream induction of genes associated with galactose metabolism (Fig. 5.6).

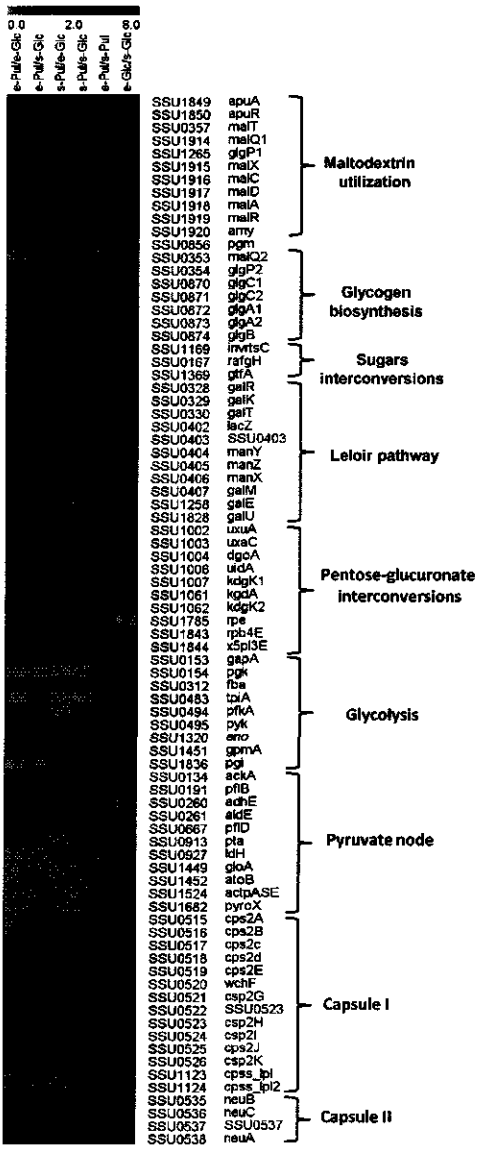


Fig. 5.6. Heatmap showing the effect of pullulan on the transcription of genes involved in key basic metabolic processes. Expression (ratios) of genes participating in 9 different metabolic processes (indicated at the right of the heatmap) are shown for 6 different comparisons (indicated at the top of the heatmap). At the top of the figure, a color scale depicts the ratio of expression during growth in pullulan vs. glucose. Red colour indicate induction (upregulation of the respective genes) and blue indicates repression (downregulation of the respective genes) for each comparison. For each gene, the *S. suis* P1/7 locus tag and the gene name is given on the right.

Interestingly, the *galT* (galactose-1-phosphate uridylyltransferase) and *galK* (galactokinase) genes that participate in the Leloir pathway were strongly induced. The GalT enzyme (EC 2.7.7.9) interconverts galactose-1-phosphate (Gal1-P) and UDP-Glucose (UDP-Glc) to UDP-galactose (UDP-Gal) and Glc1P. Activated UDP-sugars are important intermediates in polysaccharide biosynthesis, e.g. as capsule components or exopolysaccharides. Expression ratios of capsule biosynthesis genes were slightly lower (*cps2C*-SSU0517, *cps2D*-SSU0518, *cps2E*-SSU0519, *cpss_lpl*-SSU1123) for bacteria grown in pullulan compared to glucose, suggesting capsule production would not be increased in pullulan. Indeed, similar levels of capsule production were observed in transmission electron micrographs of *S. suis* grown in pullulan or glucose (Fig. 5.7).

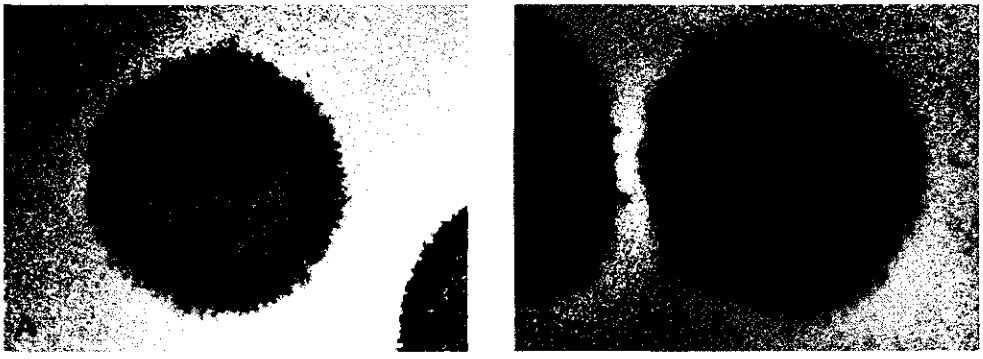


Fig. 5.7. EM picture of *S. suis* grown in CM supplemented with A. pullulan or B. glucose.

In lactic acid bacteria including streptococci, pyruvate, the end product of glycolysis, is converted to lactate during homolactic fermentation. Mixed-acid fermentation of pyruvate may also occur in lactic acid bacteria, resulting in end products such as acetate, ethanol, formate or lactate, the growth conditions. The fermentation of pullulan may result in the formation of formate and ethanol, predicted from the induction of genes encoding pyruvate formate lyase (*pflB*-SSU0191) and alcohol dehydrogenase (*adhE*-SSU0260) (Fig. 5.6; **Chapter 6**).

The prediction of cre-sites in the genome of S. suis

The microarray analysis did show that carbohydrate metabolism is substantially altered when *S. suis* bacteria are grown in presence of pullulan or glucose. The CcpA transcription factor that mediates carbon catabolite repression (CCR) or activation (CCA) is a major

Table 5.1: *cre*-site prediction in the genome of *S. suis* P1/7.

Sequence	P/Glc[1]	Locus	Operon *S. suis* P1/7	Pos[3]	COG	ΔccpA[2]
GAAAACGTTTGC	U	SSU1849	*apuA*	P	Carbohydrate metab/virul	
GAAAACGTTTGC	U	SSU1850	*apuR*	P	Carbohydrate regulator	
GAAAACGTTTGC	U	SSU1915	*malX-malC-malD-malA-malR*	P	ABC Carbohydrate transport	U
GAAAACGTTTGC	U	SSU0844	SSU0844	P	Unknown function	U
GAAAACGTTTGC	U	SSU1914	*malQ1*	P	Carbohydrate metabolism	
GCAAACGTTTGC	U	SSU0357	*ptsG-elsH*	P	PTS Carbohydrate transport	
GAATACGTTCGC	U	SSU0766	*glpR-fruB-fruA*	G	PTS Carbohydrate transport	U
GATAATGTTTGC	U	SSU1583	*manL-manM-manN*	G	PTS Carbohydrate transport	U
GAACGCGTTTGC	D	SSU1320	*eno*	G	Carbohydrate metabolism	D
GAAAACGTCGGC	D	SSU1467	*xseA-xseB-fps-SSU1464-argR-recN*	P	DNA replication	
GAAAACGTTTAC	D	SSU0249	*gla*	P	Energy production	D
GAAAACGTTTAC	D	SSU1256	*dnaE-rpoD*	P	Transcription	
GAAAACGGTTGC	U	SSU0395	SSU0395	P	Unknown	U
GTAAACGTTTGC	D	SSU0355	*gntR*	P	Carbohydrate regulator	
GTAAACGTTTGC	U	SSU1726	*murQ-ptsG*-SSU1724	P	Carbohydrate transp/metab	
GTAAGCGGTTGC	U	SSU0402	*lacZ*-SSU0403-*manY-manZ-manX*	P	Carbohydrate metabolism	
GGAAGCGGTTGC	U	SSU1050	*hyl-hepII/III*	P	Virulence	U
TAAAACGTTTAC		SSU0245	SSU0245-SSU0246-SSU0247	P	Regulation multidrug efflux	
GAAAACGTTTTG	D	SSU0284	*his-hisM*	P	Amino acid transporter	D
AAAAACGTTTTC	D	SSU0353	*malQ2-glgP2*	P	Carbohydrate metabolism	D
GAAAACATTTAC	D	SSU0421	*typA*-SSU0422	P	Energy production	D
AAAAACGTTTGG		SSU0449	*SSU0449-SSU0450-SSU0451*	P	Unknown	
AAAAACGTTTGG		SSU0524	*csp2H*	P	Envelope/virulence	D
AAAAACGTTTTC	U	SSU0938	*rsmC-deoA-deoC-cdd*	P	Energy production	U
AAAAACGTTTTC		SSU0939	*coaA*	P	Energy production	U
GAAAACATTTGG	U	SSU1688	*paaD*	P	Unknown	
GAAAACTTTTGT	D	SSU1969	*HtrA-SpoOJ*	P	DNA replication	
GAAAACGATTAC	U	SSU0328	*galR-galK-galT*	P	Carbohydrate regulator	U
GAAAACGATTAC	U	SSU0329	*galK-galT*	P	Carbohydrate metabolism	U
GAAGAAGTTTGC	D	SSU0375	*SSU0375-SSU0374*	P	Unknown	
GAAAACGGTTAC	D	SSU0377	*SSU0377*	P	Hydrolase	
GATAACGTTTTC	D	SSU0515	*cps2A*	P	Envelope/virulence	D
GATAAGGTTTGC		SSU0573	*norM*	P	Multidrug transporters	
GAAATCGTTTTC		SSU0613	SSU0613	P	Carbohydrate regulator	U
GTAAATGTTTGC	U	SSU1033	SSU1033	P	Energy production	
GAAAACGGTTAC	U	SSU1172	SSU1172-SSU1171-SSU1170-*invrtsC*	P	Carbohydrate transp/metab.	U
GAAAACGGTTAC	U	SSU1175	SSU1175	P	Unknown	U
GTAAACGTTTTC	U	SSU1265	*glgP1*	P	Carbohydrate metabolism	U
GTAAACGTTTTC		SSU1618	*scrA-ScrK*	P	PTS Carbohydrate transport	
GTAAACGTTTTC		SSU1619	*scrB-scrR*	P	Carbohydrate metabolism	
GAAAAGGTTAGC	D	SSU1794	*stdB*-SSU1795-SSU1794-*ksgA*	P	Energy production	
GATAACGTTTGA		SSU1883	*srtB-srtC-srtD*	P	Hydrolase	
GAAAAAGCTTGC	D	SSU0006	SSU0006-*pth-trcF*-SSU0009-SSU0010-SSU0011-*penP-mesJ-hpt-ftsH*	P	Energy production	D
GCAAACGTTTTC	U	SSU0240	*SSU0240-SSU0241-SSU0242*	P	Unknown	
AAAAACGCTTGC	D	SSU0287	*SSU0287-SSU0288*	P	Unknown	D
GCAAACGTTTAC	U	SSU0356	*elsH-ptsG*	P	Unknown	
GAAAATGCTTGC	U	SSU0482	*tufA*	P	DNA replication	
GAAAAGGCTTGC	U	SSU0673	*hcaD-SSU0674-glpK-glpO-glpF1*	P	Carbohydrate metabolism	U
GCAAAGGTTTGC	U	SSU0775	*glkA*	P	Carbohydrate transport	
GACAACGTTTAC	D	SSu0789	SSU0789-*tdK*	P	Unknown	D
GAAAATGTTCGC	D	SSU1960	*guaA*	P	Energy production	
GAAAGCGTTTCC	U	SSU0870	*glgC-glgC-glgA-glgB*	P	Carbohydrate metabolism	U
GAAAACGCTTAC	U	SSU1008	*ptsMelB-pfkB-uidA-fadR-dgoA-uxaC-kduD-Had-bglX-bglX*	P	Carbohydrate transport	U
GCAAAGGTTTGC	D	SSU1183	SSU1183	P	Stress response	

ACAAACGTTTGC	U	SSU1231	*sly*	P	Virulence	
GAAAACGCTTTC	U	SSU1713	SSU1713-*ams-nag-gcnA*	P	Carbohydrate metabolism	U
AAAAACGTCTGC	U	SSU1760	*ssnA*	P	Virulence	
TAAAGCGTTTTCA	U	SSU1373	*aga-msmE-msmF*	P	Carbohydrate metabolism	
GAAAACGCTTCC	U	SSU0260	*adhE-adhP*	P	Energy production	U
GAAAACGCTTCC	U	SSU0675	*glpK-glpO-glpF1*	P	Energy production	U
GAAATGCTTGTCG	D	SSU1308	*bglB-ptsG*-SSU1310	P	Carbohydrate metabolism	D
GAGAACGTATGC		SSU0558	*aroK-pheA-LytR*	P	Amino acid transport	
CAAAACTTTTGC		SSU0134	*ackA*	G	Carbohydrate metabolism	U
GAAACCGCTTCGA	U	SSU0856	*pgm*	P	Carbohydrate metabolism	U
GTAAACGGTTGC	U	SSU0766	*glpR-fruB-fruA*	P	Carbohydrate regulator	U
TAAAGCGTTTTCA	U	SSU1701	*msmK*	P	ABC Carbohydrate transport	U
GAAAGGTTTTTCA	U	SSU0191	*pflB*	P	Energy production	U
CAAAGGAGTTTT	U	SSU1749	*dak1-dak1-SSU1751-glpF2*	P	Energy production	D
GAAAACGATGGC	U	SSU0580	*arcA-arcB-arcC*	G	Energy produc/virulence	U
GAAAACGATGGC	U	SSU0347	SSU0347-SSU0348-SSU0349-SSU0350-*fms*	G	Energy production	U
GAAAACGACTTC	U	SSU1855	*ptsCelB*	P	PTS Carbohydrate transport	U

P/Glc[1] upregulated (U) or downregulated (D) expression when *S. suis* was grown in pullulan compared to glucose
ΔccpA[2] upregulated (U) or downregulated (D) expression in wild type *S. suis* 10 compared to its isogenic *ΔccpA* mutant (data from Willenborg *et al.* [44])
Pos[3] tag locus located upstream of the start codon of predicted proteins (P) or in proximity of the gene transcription start (G)

regulator of carbohydrate metabolism. Catabolite control, activated in presence of glucose, leads to high-affinity binding of CcpA to a catabolite responsive element (*cre*). Inactivation of *ccpA* in *S. suis* was reported to alter expression of 259 genes (13.2% of the genome) [44]. For this chapter, it was of interest to investigate if (part of) these genes were differentially expressed during growth in pullulan (compared to glucose) and thus, might be regulated by CcpA. Therefore, using MEME [73] and MAST-algorithms [74], the genome sequence of *S. suis* strain P1/7 was mined for presence of a *cre*-site motif (tGAAAACGTTTGCat); this motif is similar to a *B. subtilis* consensus *cre*-site (TGWAARCGYTWNCW) [75,76]. A total of 472 putative *cre*-sites were identified within 71 sites in the tag locus located upstream of the start codon of predicted proteins (P) or in proximity of the gene transcription start (G) (Table 5.1). A total of 171 genes, including other genes within the same operon, were predicted to be under control of CcpA. In Table 5.1 we listed 38 *cre* sites and their downstream genes or operons, and indicated if those genes were differentially expressed in pullulan compared to glucose (our study), and if genes were differentially expressed in a Δ*ccpA* mutant [44].

In table 5.1, we observed that genes belonging to all main functional classes were represented in the *ccpA*-regulon prediction, but with a relative enrichment of *cre*-sites in the upstream regions of genes predicted to encode carbohydrate PTS and ABC transporters (17%), genes (operons) with regulatory functions (10%), and genes encoding enzymes involved in carbohydrate metabolism (25%) and, noteworthy, in virulence (8%).

A total of 171 genes, including other genes within the same operon, were predicted to be under control of CcpA. In Table 5.1 we listed 38 *cre* sites and their downstream genes or operons, and indicated if those genes were differentially expressed in pullulan compared to glucose (our study), and if genes were differentially expressed in a Δ*ccpA* mutant [44]. In table 5.1, we observed that genes belonging to all main functional classes were represented in the *ccpA*-regulon prediction, but with a relative enrichment of *cre*-sites in the upstream regions of genes predicted to encode carbohydrate PTS and ABC transporters (17%), genes (operons) with regulatory functions (10%), and genes encoding enzymes involved in carbohydrate metabolism (25%) and, noteworthy, in virulence (8%).

Links between carbohydrate metabolism and virulence gene regulation

To investigate if growth of *S. suis* in α-glucans could affect expression of known or predicted virulence genes, the expression of these genes were compared during bacterial growth in pullulan or glucose (Fig 5.8).

Seven genes involved in the invasion of mucosal tissues or avoidance of host defenses by streptococcal pathogens were upregulated in pullulan compared to glucose. These genes included *sly* (suilysin-SSU1231) [11,77,78,79,80], *hepII/III* (putative oligohyaluronate lyase-SSU1048) [81], *hyl* (hyaluronidase precursor-SSU1050) [82], *arcB* (arginine deiminase-SSU0580) [83,84], *igaP* (surface metallo serine protease-SSU1773) [85] *dppIV* (Xaa-Pro dipeptidyl peptidase-SSU0187) [86,87], and *pepD* (putative surface dipeptidase-SSU1215) [88].

Regarding the envelope structures which contribute to *S. suis* virulence, only two capsule biosynthesis genes (*cpss_lpl*-SSU1124 and *cpss_lpl2*-SSU1123) were slightly downregulated, but not the gene encoding the cps operon regulator. These transcriptional changes did not appear to have a visible effect on capsule production (Fig. 5.7). The *neuACB* operon, containing genes involved in sialylation of the capsule polysaccharides [89] was slightly induced. The only adhesin highly upregulated during bacterial growth in pullulan was ApuA.

Remarkably, the genes with highest (*sly*, *apuA*, *arcB*, *hyl-hepII/III*) or moderate (*ssnA*, *cps2A*) differential induction in pullulan all have a predicted *cre* element site in their gene or operon promoter region (Table 5.1) indicating they might be subject to CcpA-mediated repression when concentrations of glucose are high enough. However, these genes can be co-regulated by other transcriptional regulators, for instance suilysin may be co-regulated by the two-component system CovS/CovR (SSU1190-SSU1191)[90].

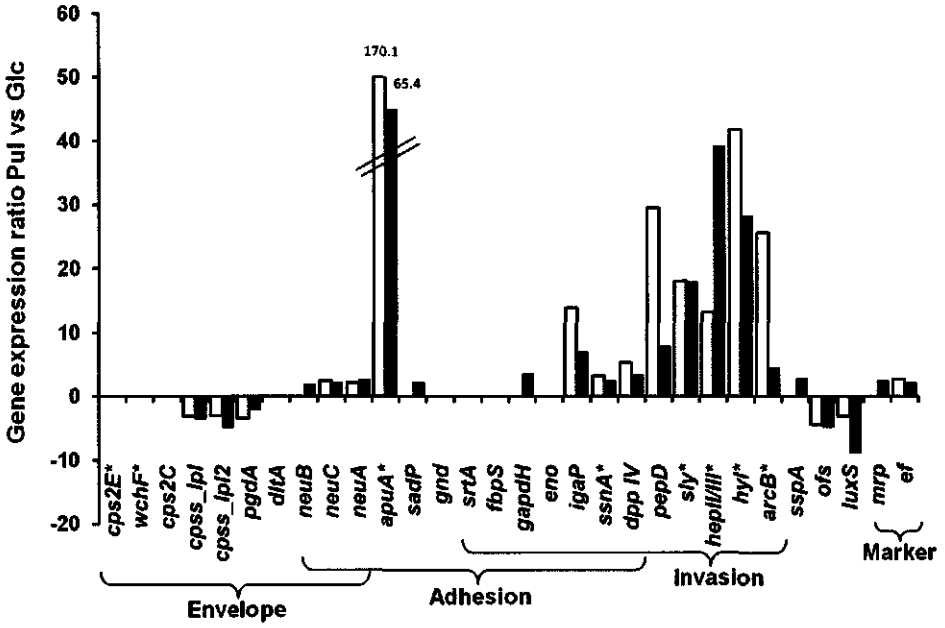


Fig. 5.8. Gene expression ratio of putative and characterized virulence factors of *S. suis* grown in pullulan or glucose in exponential (white bar) and early stationary (black bar) phases. The genes are grouped together according to their predicted or described function in *S. suis* pathogenesis. Envelope: *cps2E**-SS0519 putative galactosyl transferase; *wchF**-SSU0520 putative rhamnosyl transferase; *cps2C*-SSU0517 tyrosine-protein kinase; *cpss_lpl*-SSU1123 putative glycosyltransferase; *cpss_lpl2*-SSU1124 putative rhamnosyl transferase *pgdA*-SSU1448 peptidoglycan GlcNAc deacetylase, *dltA*-SSU0596 D-alanine-poly(phosphoribitol) ligase subunit1; Envelope/Adhesion *neuB*-SSU0535 putative N-acetylneuraminic acid synthase; *neuC*-SSU0536 putative UDP-N acetylglucosamine 2-epimerase; *neuA*-SSU0538 N-acylneuraminate cytidylyltransferase; Adhesion: *apuA**-SSU1849 amylopullulanase; *sadP*-SSU0253 putative surface-anchored protein receptor; *gnd*-SSU1541 6-phosphogluconate dehydrogenase; Adhesion/Invasion: *srtA*-SSU0925 sortase; *fbpS*-SSU1311 fibronectin-fibrinogen binding protein; *gapdH*-SSU0153 glyceraldehyde-3-phosphate dehydrogenase; *eno*-SSU1320 enolase; *pepD*-SSU1215 putative surface-anchored dipeptidase; *dpp IV*-SSU0187 Xaa-Pro dipeptidyl-peptidase; Invasion: *sly** SSU1231 suilysin (haemolysin); *hepII/III**-SSU1048 heparinase II/III-like protein; *hyl**-SSU1050 hyaluronidase precursor; *ssnA**-SSU1760 surface-anchored DNA nuclease; *arcB**-SSU0580 arginine deaminase; *igaP*-SSU1773 putative surface-anchored serine protease; *sspA*-SSU0757 cell envelope proteinase; *ofs*-SSU1474 serum opacity factor; *luxS*-SSU0376 S-ribosyl homocysteinase; Marker: *mrp*-SSU0706 muramidase-released protein precursor; *ef*-SSU0171 putative surface-anchored protein. * Indicates the presence of predicted *cre*-site in the promoter region of the gene virulence factor.

Effect of carbohydrates on haemolytic activity of S. suis

According to our microarray analysis, the expression of suilysin, an important *S. suis* virulence factor, was strongly induced in pullulan compared to glucose, both at exponential and stationary growth stages (+18.1/+17.9 ratio pullulan vs glucose). To test if secretion of suilysin was indeed increased during bacterial growth in pullulan, we measured erythrocyte haemolytic activity (HA) of culture supernatants of *S. suis* grown in glucose or pullulan. Haemolytic activity was higher in CM supplemented with pullulan than in CM plus glucose, over a wide range (ca. 0.5) of OD values (Fig 5.9). In early stationary

phase, HA for pullulan and glucose cultures was approx. 91% and 18%, respectively (Fig. 5.9). We assume that suilysin (SSU1231) is the only active haemolysin that is produced by *S. suis* P1/7. A haemolysin-like protein (SSU1464) has been annotated in the genome of *S. suis*, but this gene was not differentially expressed under these conditions.

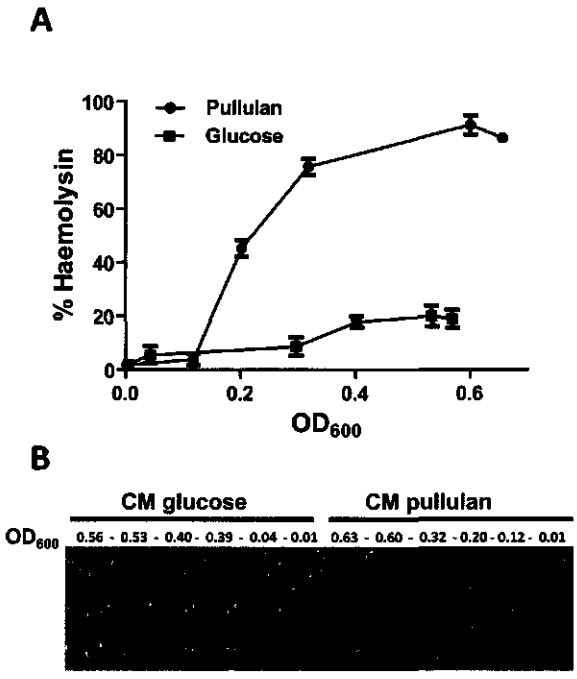


Fig. 5.9. Hemolysis assay of *S. suis* growing in two different carbon sources. A. The hemolysis production was measured by analyzing the supernatant of *S suis* grown in CM plus 1% of glucose or pullulan in the lag, exponential and stationary phase (OD_{600} values 0 to 0.56). **B.** Deep-well titer plate showing hemolytic activity of supernatants collected from *S. suis* grown in CM supplemented with glucose or pullulan.

Effect of carbohydrate source on S. suis adhesion and invasion

From our microarray data, and the expression of (putative) virulence factors, we hypothesized that the adhesion and invasion capacity of *S. suis* might be enhanced upon growth in pullulan compared to glucose. Because of its porcine epithelial origin, the newborn pig tracheal cell line (NPTr) [9,91] was chosen to investigate the adhesion and invasion of *S. suis* S10 grown in pullulan or in glucose. Exponentially growing *S. suis* grown in CM plus 1% of either carbohydrate were harvested and incubated for 2 h at a multiplicity of infection (MOI) of approximately 50 (number of bacteria per NPTr cell). To

maintain similar growth conditions, the cell culture medium was replaced with glucose-free DMEM and supplemented with 1% glucose or pullulan during the incubation with *S. suis*. In agreement with previous adhesion studies that used the NPTr cell line [9], we found that adherence of *S. suis* bacteria grown in CM plus glucose was 19.4 ± 1.0 % of original inoculum (averaged over 3 independent replicates). *S. suis* had a significantly higher adhesion capacity (24.9 ± 1.7 %; P <0.05; 3 replicates) after bacteria had been grown in CM plus 1% pullulan (Fig. 5.10A). In accordance with previous studies using a human Hep-2 cell line [8], *S. suis* S10 showed low invasion capacity (0.05% of original inoculum) in glucose. Strikingly, invasiveness of *S. suis* was nearly 9 times higher when grown in pullulan as a unique carbon source (0.43% ± 0.01) (*** P <0.01) (Fig. 5.10B). In conclusion, *S. suis* bacteria grown in pullulan showed a slightly but significantly increased adherence to, and a strongly increased invasiveness of NPTr cells, always compared to glucose.

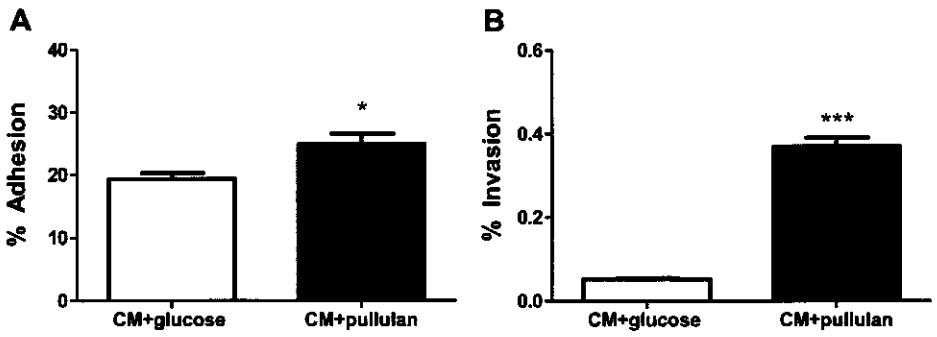


Fig. 5.10. Comparison of NPTr adherence A. and invasion B. of *S. suis* after growth in CM+1% pullulan (black columns) vs. CM+1% glucose (white columns).
NPTr confluent monolayers were co-cultivated for 2 h with *S. suis* S10 bacteria grown in CM medium complemented with pullulan or glucose. NPTr cells were washed and lysed and lysates plated on Columbia agar to determine number of adherent bacteria as calculated from CFU. To determine real invasion, antibiotics were added for 1 additional hours to kill extracellular bacteria before plating. Results were expressed as % adherence or % invasion (% recovered CFU of the inoculum) as described in Experimental procedures. Plotted values indicate means ± SD averaged across three independent experiments.

Discussion

S. suis is an important pathogen of swine; some isolates have also shown to be able to infect humans. *S. suis* is among the more ubiquitous bacteria that colonise the oropharynx and oral cavity of asymptomatic carrier pigs [92,93]. It is unclear how, and under what external conditions *S. suis* bacteria can switch from an asymptomatic to a pathogenic association with their host. Host susceptibility to *S. suis* infection may occur due to inadequate host immune responses but may also be due to reduced antagonism by commensal bacteria, concurrent infections or environmental factors such as the availability of nutrients. As shown in this study, carbohydrate availability may be an important environmental factor as carbohydrates can dramatically alter gene expression and may promote an increased production of toxins and other virulence factors. In the mammalian oropharynx and saliva, dietary α-glucans are present at high concentrations [18,19,27]. The importance of these substrates to the ecology of bacteria in this niche is evident from the high number of α-glucans degrading enzymes and starch metabolic pathway genes present in genome sequences of commensals [9,48,51,52,53].

Previously, we showed that the ability of *S. suis* to utilize α-glucans is dependent on a bifunctional α-glucan degrading enzyme (amylopullulanase, ApuA) that also mediates adhesion to porcine epithelial cells [9] (**Chapter 3**). Growth in pullulan led to an induction of *S. suis* starch metabolic pathway genes, including homologues of the maltodextrin ABC transport operon and a predicted PTS for maltose/maltotriose (*ptsG*-SSU0357), as described in other *Streptococcus* species [94,95] (Fig. 5.11). PTS systems were important to utilize the two different carbon sources: one of the GO categories with the highest number of genes over-represented during exponential growth in pullulan was the phosphotransferase system (PTS) category (Fig. 5.4). 70% (12 out of 17) of the annotated PTS were upregulated in pullulan and 40% of the genes encoding these PTSs contained a *cre* binding site in the promoter region. As several PTS genes are transcribed at higher levels in pullulan, we cannot exclude the possibility that one of these is the major *S. suis* transporter for maltose or maltotriose.

Intracellular *glg1* and *malQ1* are predicted to convert maltodextrins to Glc1P which can be converted by phosphoglucomutase (*pgm*-SSU0856) to Glc6P, a substrate for glycolysis (Fig. 5.11). We observed that transcription of glycolytic pathway genes were not substantially altered by growth in pullulan compared to glucose, suggesting that this

pathway is similarly expressed under both conditions. Strikingly, the pentose and glucuronate interconversion pathway for metabolism of Glc6P were stronger induced during growth in pullulan compared to glucose, suggesting an accumulation of intracellular α-glucose. This suggestion is supported by the finding that glycogen biosynthesis genes including three α-glucan branching enzymes (SSU0870, SSU0874 and SSU0873) that synthesize glycogen from Glc1P were stronger induced in pullulan than in glucose (Fig. 5.11). Thus growth in pullulan may promote glycogen energy storage. We noted that in early stationary phase, when glucose is depleted, the expression of glycogen biosynthesis genes was suppressed, whereas the expression of two genes encoding glycogen-degradation enzymes (*glg2* and *malQ2*, SSU0353-SSU0354) was induced, presumably to enable the intracellular glycogen reserves to be converted to glucose for fermentation. This could explain why *S. suis* bacteria grow to higher OD_{600} in pullulan than in glucose (Fig. 5.2). Glycogen concentrations can increase up to 50% of the cell dry weight without having detrimental effects on internal osmotic pressure in the bacterial cell [96,97]. *S. suis* appears to be able to metabolize pyruvate to different end-products including lactate, acetate, ethanol and formic acid (but not acetoin). This assumption is supported by the finding that the genes involved in metabolism of pyruvate to formate and ethanol were more strongly induced during growth in pullulan than in glucose.

Expression of the two Leloir pathway genes *galTK* were highly upregulated in pullulan compared to glucose. The enzymes GalT and GalK catalyze the interconversion of UDP-galactose (UDP-Gal) to UDP-glucose (UDP-Glc) [98,99] (Fig. 5.11). Although galactose was not a standard component of the bacterial growth medium, it can presumably be derived through enzymatic interconversion of other sugars. Activated sugars such as UDP-Glc and UDP-Gal are key components in the biosynthetic pathway of extracellular polysaccharides (EPS) [100,101]. Exopolysaccharides produced by *Lactococcus* and *Streptococcus* species have a range of functions including adhesion and biofilm formation [102]. In *Streptococcus thermophilus*, the overexpression of both phosphoglucomutase (PGM) and GalE increases EPS synthesis more than 2-fold; increased production of GalK, GalT, and GalU led to further increases of EPS production [103]. During our experiments, no difference in production of capsule was evident from comparing electron micrographs of *S. suis* grown in glucose or pullulan (Fig. 5.7). Recently, Willenborg *et al.* reported that the capsule of *S. suis* S10 Δ*ccpA* mutant strain was downregulated compared to the wild type. Moreover, the glycosyl transferases present in the capsule operon (*cpss_lpl* and *cpss_lpl2*) and the

capsule operon regulator (*cps2A/wzg*) were slightly downregulated in exponential phase. *S. suis* cps and sialic acid transfer gene clusters have been proposed to be activated by CcpA [44]. However, in other *Streptococcus* species, production of capsule is influenced by carbon source but in a CcpA-independent manner [38,64] suggesting that the influence of CcpA control of capsule biosynthesis is not a straightforward consequence of glucose (or another monosaccharide) availability.

Many of the transcriptional changes we observed could be attributed to carbon catabolite control (CCC). To predict which of the differentially expressed *S. suis* genes could have been regulated by CcpA we searched for conserved catabolite-responsive elements (*cre*) motifs in the genome of *S. suis* P1/7. 172 genes (or operons) were predicted to be under control of CcpA (Table 5.1). Of these 172 genes, 145 (84%) were differentially regulated during growth in pullulan compared to glucose. The difference may be due to incorrect *cre* presence predictions, or to the fact that genes and operons may have dual regulation involving not only CcpA but also other transcription factors (**Chapter 4**).

The 145 genes that appeared to be controlled by CCC were also compared to a published microarray data set generated by comparison of a Δ*ccpA* mutant with the corresponding wild-type strain [44]. In total, 99 genes comprising 38 operons were differentially regulated in both datasets (Table 5.1), lending further support to the accuracy of *cre* site predictions for at least a subset (70%) of genes.

The CCC-regulated gene set included genes encoding PTS and ABC carbohydrate transporters (17%), genes encoding proteins with regulatory functions (10%), and genes encoding enzymes involved in carbohydrate metabolism (25%) and in virulence (8%) (Table 5.1). These genes participate in metabolic pathways including Leloir pathway, pyruvate metabolism, starch metabolism, glycogen biosynthesis and pentose glucuronate interconversion pathway (Fig. 5.11). In *S. pneumoniae*, one of the genes activated by CcpA is lactate dehydrogenase (*ldh*). Under anaerobic conditions, CcpA CCR leads to increased production of lactate and repression of two mixed-acid fermentation genes, *pfl* and *adhE* [64]. A similar regulation mechanism may also occur in *S. suis*, where decreased expression of *ldh* (containing a predicted *cre* site) and induction of genes participating in mixed acid fermentation was observed during growth in pullulan compared to glucose.

Most striking was the induction of nearly 30 genes encoding virulence factors associated with mucosal invasion and the avoidance of host defenses in pullulan compared to glucose. One of these was the gene encoding a secreted suilysin, a pore-forming toxin

which we showed to be highly induced during growth in pullulan, using a haemolytic *in vitro* assay (Fig. 5.9). Suilysin has been shown to play a role in damaging host epithelial [7,11], endothelial [12,14] and immune cells [16], suggesting that suilysin could faciltate the entry of *S. suis* into tissues and avoidance of host defences [5,104]. In contrast to our own results, *sly* expression was not increased in a recent study which compared gene expression of a wild-type strain with a Δ*ccpA* mutant grown to early exponential phase [44]. The reason for these different findings is unknown but may be related to the use of THB medium [44] instead of the supplemented CM medium. Also upregulated in pullulan (compared to glucose) was a gene encoding hyaluronidase, the enzyme which can breakdown hyaluronan, an anionic, non-sulphated glycosaminoglycan distributed widely throughout the extracellular matrix of eukaryote connective tissue cells. Its role in *S. suis* infection remains to be demonstrated but in *Streptococcus intermedius*, hyaluronidase may be important for detachment from a biofilm [105]. Additionally, two peptidyl peptidases (*dppIV* and *pepD*) were transcribed at higher levels when *S. suis* was grown in pullulan. In *S. suis*, the function of *pepD* is unknown but the *S. suis* di-peptidyl peptidase IV (DppIV) has been shown to interact with human fibronectin, and a *dppIV*-deficient mutant was greatly attenuated in a mouse infection model [86]. A putative heparinase II/II enzyme was also highly upregulated but its function and potential role in virulence is unknown. In our experiments, the *sspA* gene which encodes a secreted serine protease was also induced. This protease can degrade eukaryote pro-inflammatory interleukin-8 (IL-8) molecules and, by degrading IL-8, SspA is predicted to inhibit chemotaxis and infiltration of neutrophils and other leukocytes *in vivo* [106,107]. The *ssnA* gene, encoding a recently described DNase which is secreted by *S. suis* and is potentially involved in the breakdown of neutrophil entrapments (NETS) [108], was slightly upregulated in pullulan compared to glucose.

Several adhesion factors, namely ApuA [9], FbpS [109], Eno [110], GAPDH [111,112], Gnd [113] and SadP [114]) have been characterized in *S. suis*. Transcription of *apuA* was strongly induced, whereas *sadP* and *gapdH* were only slightly induced during growth in pullulan compared to glucose. Adhesion and invasion of porcine epithelial cells was significantly increased when *S. suis* was grown in pullulan rather than glucose which may be due to increased expression of adhesins (*apuA*, *sadP* and *gapd*H) and possibly other genes including genes that modify the cell envelope by transfer of sialic acids (*neu* operon, capsule II; Figs. 5.6 and 5.8). In other streptococcal pathogens, it was shown that the

external part of the capsule was needed for binding alveolar cells by sialic acids contained in the envelope [115].

In blood, glucose levels are relatively high (6–9 mM) [116] compared to the oropharyngeal cavity where dietary sources of α-glucans will be the predominant carbon source that will be available for bacterial fermentation. Here we show that fermentation of α-glucans in the absence of extracellular glucose leads to differential expression of approximately 1000 genes, of which about 145 contained predicted *cre* sites in their promoter regions. We propose that the altered expression of these 145 genes is most likely due to relief from CcpA-dependent carbon catabolite repression; most of the genes that we predicted to include a *cre* site in their promoter regions are predicted to participate in diverse metabolic pathways, including biosynthesis of glycogen (as energy reserves). In addition, nearly 30 genes encoding putative or confirmed virulence factors were transcribed at higher levels during growth in pullulan including the genes encoding the toxin suilysin and ApuA, an adhesion and extracellular enzyme required for growth on α-glucans. These findings demonstrate a clear link between carbohydrate utilisation and regulation of virulence, and suggests that fermentation of α-glucans favours colonisation and early stages of invasion at the mucosal epithelium. These findings are in agreement with studies in other streptococcal pathogens that have shown a role for CcpA and CCC in colonization and infectivity in animal models [22,42].

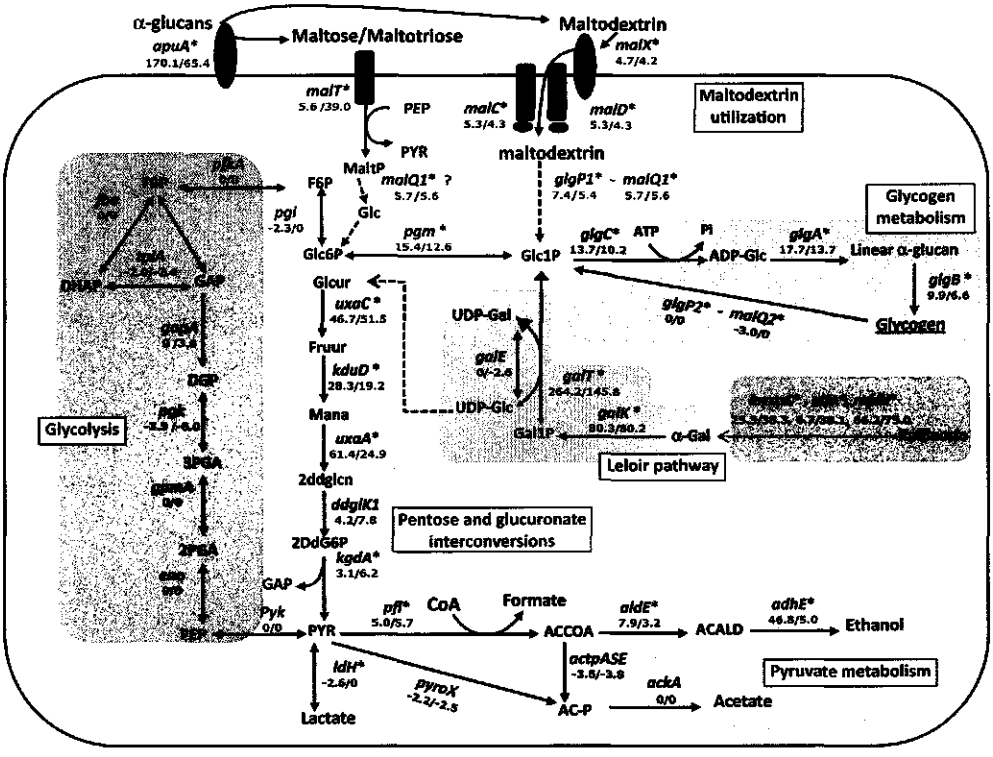


Fig. 5.11. Schematic representation of *S. suis* metabolic pathways differentially regulated in pullulan vs glucose α-glucans (i.e. pullulan). α-glucans are degraded by extracellular amylopullulanase (ApuA) and the end degradation products, maltose/maltotriose and maltodextrins, are transported by PTS for maltose/maltotriose (*malT*) and maltodextrin ABC transport inside the bacteria (*malX*, *malC* and *malD*). Maltodextrins and maltose are presumably converted to glucose-1-phosphate (Glc1P) or α-glucose by 4-α-glucanotransferase and maltodextrin phosphorylase (*malQ*1 and *glgP*1 respectively). Glc1P can be metabolized in different pathways: phosphoglucomutase (*pgm*) isomerize Glc1P to glucose-6-phosphate (Glc6P) which may enter glycolysis (violet box) where it is consequently oxidated to pyruvate (pyr). Homolactic fermentation reduces pyruvate into lactate, whereas mixed-acid fermentation leads to other products, such as formate, acetate and ethanol (pyruvate metabolism, yellow box). The excess of Glc1P that cannot enter in glycolysis may be used for synthesis of glycogen as an energy reserve (light blue box). The genome of *S. suis* is predicted to encode the enzymes sucrose phosphorylase *gtfA*, α-fructofuranosidase (interconvertase) *invrtsC*, and raffinose galactohydrolase, *rafgH* for the interconversion of sugars like raffinose. These enzymes participate in the starch and galactose-Leloir pathway. Part of Leloir pathway genes (e.g. galactose-1-phosphate uridylyltransferase *galT* and galactokinase *galK*) was induced more strongly in pullulan. GalT interconverts galactose-1-phosphate (Gal1-P) and UDP-Glucose (UDP-Glc) to UDP-galactose (UDP-Gal) and Glc1P. Alternatively, UDP-Glc may be converted into glucuronic acid (Glcur) by UDP-D-glucuronate (UDP-Glcur) to enter in an alternative (to glycolysis) pathway for pyruvate (pyr) production. Pathway predictions were reconstructed based on genome information (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi), literature and database surveys (KEGG, MetaCyc). The following gene annotation was downloaded from NCBI: *galM*, aldose 1-epimerase; *galK*, galactokinase; *galE*, UDP-glucose 4-epimerase; *galT*, galactose 1-phosphate uridylyltransferase; *pgm*, phosphoglucomutase/phosphomannomutase; *pfkA*, 6- phosphofructokinase; *fba*, fructose bisphosphate aldolase; *tpiA*, triosephosphate isomerase; *gap*, glyceraldehyde-3-phosphate dehydrogenase; *pgk*, phosphoglycerate kinase; *gpmA*, phosphoglyceromutase; *eno*, phosphopyruvate hydratase; *pyk*, pyruvate kinase; *ldh*, L-lactate dehydrogenase; *pyrox*, pyruvate oxidase; *ackA*, acetate kinase; *pfl*, pyruvate formate-lyase; *adlE* acetaldehyde-CoA dehydrogenase; *adhE* alcohol dehydrogenase; *glgB*, *glgA* glycogen synthase; *glgC* glucose-1-phosphate adenylyltransferase; *glgP* glycogen phosphorylase

Materials and methods

Bacterial strains, plasmids and culture conditions

S. suis S10 strain was grown in Todd-Hewitt broth (THB) (Difco) or on Columbia agar plates with 6% sheep blood (Oxoid) at 37°C under 5% CO_2 for 18 h. An optical density (OD) of 1.2 at 600 nm with a 1 cm path length corresponds to approximately 10^9 bacterial colony forming units per milliliter (c.f.u. ml^{-1}). The genome of *S. suis* S10 is more than 99% identical to the genome of *S. suis* P1/7, a sequenced reference strain of which the genome had been annotated previously (**Chapter 1**).

A complex medium (CM) comprising of 10 g l^{-1} proteose peptone, 5 g l^{-1} trypticase peptone, 5 g l^{-1} yeast extract, 2.5 g l^{-1} KCl, 1 mM urea, and 1 mM arginine, pH 7.0 was used to assess growth on different carbon sources (glucose-Glc, pullulan-Pul) by supplementation with different carbohydrates at a final concentration of 1% (w/v) as previously described [53]. Growth in complex medium was determined by measurement of turbidity at OD_{600} using a SpectraMax M5 (Molecular Devices LLC) reader.

RNA extraction

For RNA extraction, *S. suis* S10 was grown to exponential and early stationary phase in indicated in Fig. 5.2. 10 ml of culture was collected and centrifuged for each analyzed point. The pellet was immediately frozen in liquid nitrogen and stored over night at 4°C in 1.5 ml RNA*later* (Ambion). To isolate RNA, 1.5 ml RLT lysis buffer (Qiagen) was added to the pellet dissolved in RNA*later*, incubated at 15 min at r.t. and centrifuged (13000 g for 30 min). The cells were dissolved in 600 µl RA1 reagent (Macherey-Nagel) plus β-mercaptoethanol and lysed using a FastPrep-24 (MP. Biomedicals, Solon, OH) for 6.0 m/sec at 20 sec. Total RNA was purified using NucleoSpin RNA II (Macherey-Nagel). The quality and the concentration of RNA were assessed with an Experion™ System (Bio-Rad) and by analysis of the A_{260}/A_{280} ratio (NanoDrop 8000 UV-Vis Spectrophotometer).

Transcriptome analysis, bioinformatics tools and data mining

A two-color microarray-based gene expression analysis was performed on a custom-made 60-mer oligonucleotide array (Agilent Biotechnologies, submitted in GEO under platform GPL9359) to determine the global gene transcription levels of WCFS1 and the lp_2991 deletion mutant. Cy3- and Cy5-labeled cDNAs were prepared using a Cyscribe post labeling kit (GE Healthcare, United Kingdom). Slides were pre-hybridized for 45 min at 42°C in 20 ml prehybridization solution (1% bovine serum albumin, 5SSC, 0.1% sodium dodecyl sulfate; filtered), washed in filtered deionized water, and dried. Co-hybridization with Cy5-and Cy3-labeled cDNA probes was performed overnight at 42 °C for 16 h in Slidehyb#1 (Ambion, Austin, TX). The slides were then washed twice in 1SSC - 0.1% sodium dodecyl sulfate (1SSC is 0.15 NaCl plus 0.015 M sodium citrate) and twice in 1SSC before they were scanned. Slides were scanned with a ScanArray Express 4000 scanner (Perkin Elmer, Wellesley, MA), and image analysis and processing were performed using the ImaGene

Version 7.5 software (BioDiscovery Inc., Marina Del Rey, CA, USA). The microarrays were scanned at different intensities. For each of the individual microarrays the best scan was selected on the basis of signal distribution (combination of a low number of saturated spots and a low number of low signal spots). The data were normalized using lowess normalization as available in MicroPrep [117]. The data were corrected for inter-slide differences on the basis of total signal intensity per slide using Postprep [117]. The median intensity of the different probes per gene was selected as the gene expression intensity. CyberT was used to compare the different transcriptomes, taking into account the duplicates (dye swaps) of each of the conditions [118]. This analysis resulted in a gene expression ratio and false discovery rate (FDR) for each gene. Genes with FDR values under 0.05 and with expression ratios greater than 2 or lower than -2 were considered to be statistically significant and biologically relevant. All microarray data are MIAME compliant and are available in GEO.

Venn diagrams

Differential gene expression of *S. suis* bacteria grown in CM supplemented with pullulan (Pul) or glucose (Glc) and harvested at early exponential (E) or early stationary (S) phase was cross-compared in different combinations (Pul_E vs Glc_E, Pul_S vs Glc_S, Pul_E vs Pul_S and Glc_E vs Glc_S). Overlapping and unique differentials were visualised using Venn diagrams generated by the on-line software program Venny, available at http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Enrichment analysis using BLAST2GO

BLAST2GO was used to annotate all known *S. suis* genes and proteins according to standard Gene Ontology (www.geneontology.org) nomenclature. BLAST2GO uses the integrated Gossip package [59] for statistical assessment of differences in GO term abundance between two sets of sequences. This package employs the Fisher's Exact Test and corrects for multiple testing. For our analysis we GO term enrichment using standard and more specific settings, employing similarity term filter settings ranging from P or FDR<0.05 to P or FDR<0.01. The similarity term takes into account the sequence similarity to the homologue sequence (max.sim), modulated by the individual evidence code (EC) of its corresponding annotations.

Searching cre motifs in the S. suis genome using MEME

The MEME (http://meme.sdsc.edu/meme/meme.html) software suite (version 4.1.0) was used for the identification of motifs OM1 and OM2. MEME represents motifs as position-dependent letter-probability matrices which describe the probability of each possible letter at each position in the pattern and uses statistical modeling techniques to identify likely motifs within the input set of sequences [119]. After alignment of sequences that are thought to contain conserved motifs, MEME produces a consensus sequence and a position- specific probability matrix, which has probabilities associated with each base at each position. As input, we used the promoter sequences of the *MdxE*

and *malE* genes, with well-characterised *cre* sites, from *Bacillus* and *Listeria*. A range of motif widths (15 nt in length) and zero or one motif per sequence were specified in our queries.

We then applied the FIMO (Find Individual Motif Occurences) program, using the motif weight matrix from the MEME program, to search for the operator motifs (OMs) in the operon as well as for a random sequence model based on the letter composition of the target sequence. The algorithm in MAST calculates position scores for the motif at each possible position within a sequence [74]. Only the motif hits with a position-specific goodness-of-fit *P* value of less than 10^{-4} were considered to identify putative CcpA binding sites. *In silico* searches and comparisons of predicted *cre* sites within the *S. suis* genome sequence and reconstruction of *cre* locations in the predicted operons was conducted using the corresponding databases provided by the MicrobesOnline web server (http://microbesonline.org). As a further check for our predictions, we performed a cross-database comparison using predicted reference sets of *cre*-containing regulons that had been annotated for *Streptococcus* species and deposited within the RegPrecise database (http://regprecise.lbl.gov) [120].

Electron microscopy

For morphological analysis of the capsule structure, samples of exponential phase (~ 0.5 OD_{600}) bacteria were fixed according to the lysine-acetate-based formaldehyde/glutaraldehyde ruthenium red-osmium (LRR) fixation procedure, as described previously [8] and studied by JEOL JEM 2100 transmission electron microscope at magnifications of 25.000 X.

Titration of hemolytic activity

The hemolytic activity was assayed as previously described [79]. Two different independent assays using triplicate were carried out. Briefly, *S. suis* bacteria were grown in CM+Glc and CM+Pul at three different growth stages: lag (OD_{600} 0.1-0.2) exponential (OD_{600} 0.2-0.5) and stationary (OD_{600} 0.5-0.7). The supernatant was collected from 1 ml for each culture by centrifugation at max speed (12000 g x 1 min).

Serial twofold dilutions (150 µl) of test samples were prepared in polystyrene deep-well titer plates (Beckman) with 10 mM Tris-buffered saline (PBS, pH 7.4). Subsequently, 150 µl of a 2% (washed) horse erythrocyte suspension in 10 mM Tris-buffered saline containing 0.5% BSA was added to each well. After the wells were sealed, the plates were incubated on a Coulter mixer for 2 h at 37°C. After unlysed erythrocytes were sedimented by centrifugation (1500 g for 10 min), 150 µl portions of the supernatant were transferred to a polystyrene flat-bottom microtiter plate and measured at 540 nm with a microELISA (enzyme-linked immunosorbent assay) reader (SpectraMax M5, Molecular Devices LLC). A 100% lysis reference sample was obtained by lysing bacteria with 1% Triton-X and background lysis was subtracted before calculation of hemolytic activity.

Cell line and culture conditions

Newborn pig tracheal cells (NPTr) [91] were maintained in Dulbecco's Modified Eagle 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 of cells from flasks with 0.25% w/v trypsin, 1 mM Na-EDTA (trypsin-EDTA, Gibco-Invitrogen) and replacement of the medium [121]. For the adherence assay, approximately 2.3 x 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.

Adhesion and invasion 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 in fresh cell culture medium without antibiotics. Bacterial suspensions (around 1.15 x 10^7 c.f.u.) diluted in cell culture medium (glucose-free DMEM) were added to wells containing a monolayer (2.3 x 10^5 cell/well) of epithelial cells in 1 ml of medium (multiplicities of infection (m.o.i.) were approximately 50 bacteria per cell). Plates were incubated for 2 h at 37°C with 5% CO_2. The cell monolayers were washed three times with PBS and detached and lysed by scraping in 800 µl of ice-cold Milli-Q water. To enumerate the viable bacteria, serial dilutions of each 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. For the invasion assay, the co-coture bacteria-cells (m.o.i 50) were incubated for 2 h at 37 °C with 5% CO_2, to allow cellular invasion by the bacteria. The monolayers were then washed three times with PBS, 1 ml of cell culture medium containing 100 µl/ml gentamicin and 5 µg/ml penicillin G was added to each well, and the plates were incubated for 2 h at 37 °C, 5% CO_2 to kill extracellular and surface-adherent bacteria. The monolayers were washed three times with PBS, and cells were disrupted by the addition of 800 µl of ice-cold Milli-Q water and repeated pipetting to liberate intracellular bacteria. To enumerate the viable bacteria, serial dilutions of each cell lysate were plated in triplicate on Columbia sheep blood agar plates and incubated at 37°C for 24 h. The rate of invasion was expressed as the total number of c.f.u. recovered per well. Assays were performed in triplicate and repeated two times.

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Chapter 6

General discussion

M. Laura Ferrando

1. Ecological niches of *Streptococcus suis*

Infections caused by *Streptococcus suis* are considered a worldwide economical problem for the pig industry [1]. Moreover, *S. suis* is emerging as a zoonotic pathogen of humans due increased awareness of its association with meningitis and septicaemia [2,3]. Although *S. suis* is considered a major swine pathogen, it has been isolated from a wide range of different hosts including domestic animals and birds [3] and has frequently been found to be a normal inhabitant of the intestine of a variety of ruminants [4,5]. These findings indicate that *S. suis* can colonize and proliferate in different hosts leading to a complex epidemiology [6,7,8,9].

S. suis can colonize the buccal gingiva, tonsils, nasal conchae [10], intestine and genitals of weaning pigs [11,12]. Disease occurs primarily in young pigs 2-3 weeks of age whereas asymptomatic carriage appears to be the norm in older pigs [13,14,15]. However, also in young pigs colonization by *S. suis* does not always lead to disease [5].

In the study of Baele *et al.* (2011) [10], *S. suis* was found to be the most abundant bacterial group isolated from the 40 study pigs; but none showed any symptoms of disease. In this study, twelve *S. suis* serotypes were found; none of the *S. suis* strains was of serotype 2, and none of the strains expressed the virulence markers MRP or EF (**Chapter 1**). This does not contradict the notion that *S. suis* serotype 2 is more strongly associated with disease. However, in an earlier study on 38 pigs *S. suis* serotype 2 strains were isolated from diseased as well as healthy pigs [16]. In this study 3 out of the 4 (11%) of the *S. suis* isolates came from healthy pigs and produced bacteriocin-like compounds that inhibited growth of other *S. suis* strains, as well as several streptococcal pathogens and other few Gram+ and - bacteria often associated with microflora of pig tonsils and nasal cavities. Thus it is clear that *S. suis* can colonize pigs asymptomatically, and as such be a member of the pig nasal and oral commensal microbiota. *S. suis* is among the most abundant bacteria in pigs, and it frequently co-occurs with *Rothia* sp. (*R. nasimurium*) [10,17]. *Rothia* species have been associated with disease, at least in human [18,19].

In this respect *S. suis* might be considered a "pathobiont" which is the term given to a symbiont that does not normally elicit any pathology in the host except under special circumstances (e.g. in susceptible hosts and environmental conditions including altered microbiota). We showed in **Chapter 3** and **5** that a change in diet (a diet high in carbohydrates and low in glucose, or vice versa) alters virulence gene expression. Thus

changes in carbohydrate composition and availability could have a major influence on the ability of *S. suis* to cause disease.

Changes in commensal microbiota may also influence *S. suis* pathogenicity, e.g. via synergistic or competitive interactions. Cooperation between bacterial pathogens in order to optimize metabolism via quorum sensing [20] and iron uptake [21] has also been described. It is therefore of interest to consider that bacteria co-occurring with *S. suis* might complement its metabolism, such as has been shown for short carbohydrate conversions in populations of different bacteria co-occurring in the small intestine [22].

1.1 Transmission of *S. suis*

Young pigs typically acquire *S. suis* by vertical or horizontal transmission, resulting in colonization of the tonsils, nasal cavity (oropharynx) and occasionally, invasive disease leading to bacteremia, meningitis, septicemia and arthritis. Infections of humans have been considered for a long time to be sporadic and to have low prevalence and only affect people working with pigs or pig-derived products [12]. Two recent large outbreaks that occurred in China [23,24,25] changed that perception of the potential threat posed by *S. suis* bacteria to human health. The current view is that humans become infected via skin lesions or via an oral route, due to contact with contaminated pork [3,26]. However, in some cases patients did not recall any contact with pigs or pork products [27,28,29,30] suggesting other potential routes of transmission. For instance, clinical case studies have reported the presence of *S. suis* in the nasopharynx of workers in the pig slaughter industry [31]. In one study, colonisation of the mucosal epithelia with *S. suis* was estimated to be around 5.3% of the case group [32], highlighting this as a potential route of invasion.

Colonization of the nasopharyngeal cavity by *S. suis* was shown to be an important risk factor for the infection of young pigs [33,34], and *in vivo* distributions of bacteria have pointed toward the pharyngeal and palatine tonsils as principal portals of entry [35]. Other circumstantial evidence for invasion via the tonsils comes from the finding that *S. suis* serotype 2 bacteria were frequently identified in the lymph nodes draining the upper respiratory tract [35].

Adhesion of *S. suis* to mucosal epithelia of the nasal and oral cavities is likely to be important for colonization, as demonstrated for other opportunistic pathogens that colonize the oropharynx [36,37,38]. It is still unclear what triggers the switch from an

asymptomatic to pathogenic association of *S. suis* with the host. Vulnerability to *S. suis* infection may occur due to weaknesses in host defences including reduced antagonism by commensals [10,15], concurrent or sequential infections by other pathogens, or environmental factors such as pH and the type of nutrients that are available.

The purpose of this thesis research was to increase our knowledge of the interactions of *S. suis* with the host in the mucosal environment. The specific aims were investigate the following:

1. The interaction of *S. suis* with human dendritic cells which are abundant in the mucosa.
2. The functions of a surface amylopullulanase ApuA that was predicted to be involved in the catalytic breakdown of complex carbohydrates found in the oropharyngeal cavity and intestinal tract.
3. The mechanisms regulating expression of ApuA.
4. How carbohydrate sources alter global regulation of metabolism and importantly, expression of virulence factors involved in invasion and dissemination of bacteria.

2. Alpha-glucans degrading enzymes are key factors for colonization of the host oropharynx

To colonize host mucosal surfaces, bacteria typically must i) survive in critical niches with limited nutrient resources, ii) compete with other commensal microbes, iii) bind residues of host cell receptors, and iv) avoid or tolerate the host immune defences. Typically, such interactions are dynamic and multifactorial and are mediated by a diversity of bacterial proteins including surface-exposed, cell wall-anchored and secreted proteins and metabolites.

In the mammalian oropharynx and saliva, different α-glucans (e.g. starch and dextrans) are present at high concentrations [39,40,41,42]. Apart from high abundance of starch in the animal diet [40,43], eukaryotic glycogen stores, released from damaged or lysed cells may be an important substrate for pathogen growth during the early stages of infection [44]. In contrast to α-glucans, glucose, the most readily metabolized polysaccharide, is present in too low an amount to support *S. suis* growth, highlighting the need for alternative carbon sources to support bacterial proliferation at the mucosal surface [45,46]. The importance of α-glucans as substrates to the ecology of bacteria in the oral and oro-/nasopharynx niche is evident from the high number of α-glucans-degrading

enzymes, maltodextrin transport systems, and the diversity of starch metabolic pathway genes that are typically present in genome sequences of commensals or pathogens of the host mucosal epithelia [47,48,49,50,51,52,53].

Genes encoding carbohydrate-degrading enzymes are common in the genomes of streptococcal pathogens and play key roles in nutrient acquisition for colonization of mucosal surfaces and proliferation. Transcriptome studies of *Streptococcus pyogenes* growing in saliva and in a mouse infection model [42,54,55,56] have identified genes that were highly expressed during the adaptive metabolic responses of bacteria that colonized, or dispersed into, different host organs. Among the highly expressed genes were genes involved in utilization of α-glucans. The metabolism of these complex carbohydrates requires the presence of specific transport systems and catabolic enzymes such as amylase, pullulanase or amylopullulanase that permit the utilization of sugars other than glucose in the host mucosal environment [52].

This led us to consider that *S. suis* might be able to survive and proliferate in host mucosal epithelia via the degradation of α-glucans that are present in high amounts compared to glucose. In **Chapter 3** we characterized a large *S. suis* surface amylopullulanase (we designated as ApuA), which is the only extracellular α-glucan-degrading catabolic enzyme found in the sequenced genomes of *S. suis*, except for the pathogenic *S. suis* isolate ST3 (serotype 3) [57] which possesses an additional gene (SSUST3_0537) that is predicted to encode a secreted amylase. ApuA was shown to possess both α-amylase and pullulanase catalytic activities that can breakdown α-glucans to maltodextrins. The evolution of this type of bifunctional enzyme could have resulted from recombination events (fusions) between genes encoding an α-amylase and type I pullulanase. Similar events are thought to be responsible for the origin of genes encoding bifunctional endo- and exoglucanase enzymes in *Caldocellum saccharolyticum* [58]. Indeed, as shown in **Fig. 3.2 of Chapter 3**, the presence of a bifunctional ApuA gene seems to be peculiarity of *S. suis* and is not found in the sequenced genomes of other streptococcal species. The fusion of both catalytic functions in one protein may have provided an evolutionary advantage for *S. suis*. We hypothesized that ApuA would play an important ecological role by allowing *S. suis* to remain competitive in the environment of the host where environment where food sources change. As expected, our phenotypic characterization of *apuA* mutant demonstrated that ApuA was required for fermentative growth on food-derived starch and glycogen. Glycogen released from damaged host cells may also be degraded by ApuA

and used for fermentative growth. This agrees with the findings that both PulA from *S. pyogenes* and the related pneumococcal pullulanase SpuA bind with high affinity to alveolar type II cell glycogen in the mouse lung tissue [44].

Apart from its role in carbohydrate utilization, the extracellular pullulanase from *S. pyogenes* has been shown to bind to several carbohydrates including submaxillar mucin [49]. Furthermore, recombinant SAP, a type I pullulanase from *Streptococcus agalactiae*, was shown to bind human epithelial cells *in vitro* [59,60]. In **Chapter 3** we investigated the possible role of *S. suis* ApuA in adhesion to a tracheal newborn pig cell line (NPTr) as *in vitro* model. In adhesion assays, our *S. suis apuA* mutant showed significantly less adherence to NPTr cells compared to the wild type *S. suis*. The host molecule or receptor bound by ApuA *in vivo* has not been identified so far. In *S. pneumonia* a library of 300 ($PhoA^+$) fusion mutants were screened against multiple eukaryotic sugar receptors [61]. A mutant pullulanase (PulA-$PhoA^+$) was shown to have lost the capacity for adhesion to β(1-3)Gal glycoconjugate receptor [62]. Similar studies involving eukaryotic sugar receptors need to be performed with *S. suis* to identify which receptors they bind to *in vivo* in pigs and humans. ApuA is one of four adhesins identified *in vitro* for *S. suis*, together with streptococcal adhesin P (SadP) [63], 6-phosphogluconate-dehydrogenase (6PGD-SSU1541) [64] and glutamine synthetase (GlnA-SSU0157) [65]. The latter 6PGD and GlnA are cytoplasmic glycolytic enzymes that are present on the surface of other pathogens and reported to be involved in adhesion to epithelia.

3. Carbohydrate metabolism in *S. suis*

Before the completion of this thesis, there was little published information about carbohydrate metabolismin *S. suis* and its implications for colonization of host tissues, virulence factors production and tissue invasion. Given that we had established a role for ApuA in adhesion and as a potential virulence factor involved in mucosal colonization, we sought to gain a better understanding of its regulation with the aim of finding correlations between the regulation of carbon metabolism and virulence.

We hypothesized that, in order to most efficiently use the entire spectrum of bacterial factors involved in adhesion and nutrient acquisition during the colonization of host mucosa, enzymes such as ApuA would need to be tightly regulated, depending on substrate availability. Genetic regulatory systems are fundamental to optimize (in terms of energy production) expression of adhesins and other colonization factors in response to

changes in the environment. The regulation of ApuA was studied using two different approaches, one involving transcriptome and qPCR transcript quantification of *S. suis* grown in defined complex media (CM) containing different carbohydrates, and one focusing on transcription factor binding to the apuA promoter region.

At the start of our study, little was known about the global metabolic potential of *S. suis* except for annotation of the genome and proteome sequence of *S. suis* P1/7. The latter is a sequenced strain that is more than 99% identical to the often used S10 strain. We functionally annotated the P1/7 strain using Gene Ontology (GO) terms and EBI protein domain annotations. We found that 1447 ORFs (73.5% of the total number of P1/7 ORFs) matched known proteins [66] and we constructed a metabolic map, using the Cluster of Orthologous Group (COG) annotations of all known *S. suis* proteins. The metabolic map of *S. suis* is described first time in this thesis and includes more than 80% of the metabolic maps known for other *Streptococcus* species (**Chapter 5**). The metabolic map indicated that like other lactic *Streptococcus* species, *S. suis* is able to ferment a wide diversity of sugars, including mono- and di-saccharides but also α-glucans such as glycogen, starch and pullulan, to glucose [67]. To date, two ABC transporters and 17 phosphotransferase systems (PTSs) for the uptake of carbohydrates have been predicted for *S. suis* in the KEGG database, highlighting the potential for *S. suis* to ferment a variety of carbohydrates. Like other *Streptococcus* species, *S. suis* mainly produces energy through degradation of glucose via two different metabolic routes: homolactic or mixed-acid fermentation, depending on the environmental growth conditions.

Comparative transcriptome analysis of *S. suis* grown in pullulan or glucose revealed that growth in pullulan induced expression of the maltodextrin utilization gene cluster that includes ApuA and the corresponding PTS and ABC transporters. Also induced were two intracellular phosphatase enzymes, *glg1* and *malQ1,* predicted to convert maltodextrins to glucose-1P (Glc1P). Glc1P is then transformed, by intracellular phosphoglucomutase (*pgm*), into glucose-6P (Glc6P) that may enter glycolysis. However, expression of the glycolysis pathway genes did not change significantly during growth in pullulan compared to glucose, suggesting that activity of this pathway was not altered during growth in both carbon sources. Rather, it appeared that the excess of intracellular glucose was converted in glycogen as energy reserves or had entered into hexose-pentose carbohydrate pathways as an alternative to glycolysis. We noted that the genes that participate in the Leloir pathway were highly induced in pullulan compared to glucose. Leloir pathway

enzymes catalyze the interconversion of glucose or galactose to activated sugars (UDP-Glc and UDP-Gal) which are key components in the biosynthetic pathway of extracellular polysaccharides (EPS) production. *S. suis*, as other lactic acid bacteria, appears to be able to metabolize pyruvate, the end product of fermentation, to different end-products including lactate, acetate ethanol and formic acid. This pathway appeared to be upregulatedduring growth in pullulan, as the genes involved in pyruvate conversion to ethanol and lactate were transcribed at higher levels in pullulan compared to glucose. *S. suis* has an incomplete tricarboxylic acid cycle (TCA cycle) (Fig. 6.1) as reported for other *Streptococcus* pathogens [68,69] and is unable to generate ATP from oxidative phosphorylation.

Other metabolic pathways induced during growth in pullulan were lipid metabolism, important for generating lipoteichoic acid (LTA) precursors and membrane phospholipids important during adhesion/invasion [70,71], and metabolic pathways for arginine and proline, which in *S. suis* and other Firmicutes are used as reducing agents to maintain redox balance [72] during metabolism of pyruvate.

In order to investigate the effects of α-glucans on global gene expression, we performed a transcriptome analysis of *S. suis* grown in defined complex media (CM) supplemented with an α-glucan (i.e. pullulan) or glucose. Fermentation of α-glucans enhanced the metabolic activity of *S. suis*, resulting in a higher biomass production (Fig. 5.1) and production of intracellular energy reserves (Fig. 6.1).

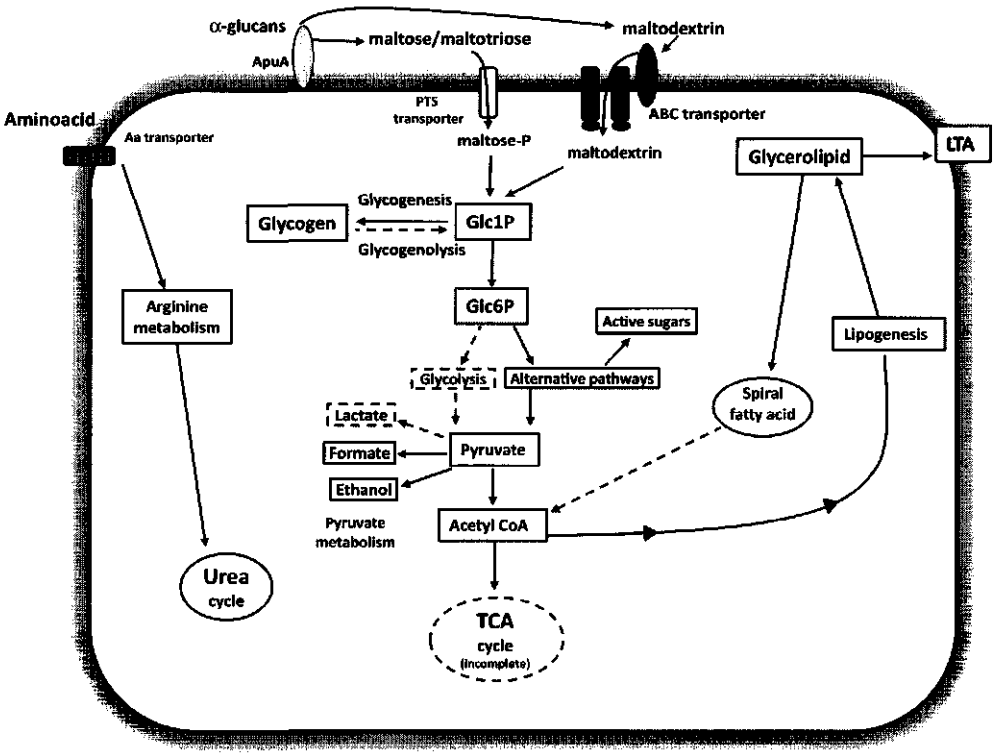

Fig. 6.1: Diagram depicting the major changes in metabolism of *S. suis* based on transcriptome analysis of bacteria grown in CM (see Methods, **Chapter 5**) supplemented with pullulan and compared to growth in CM with glucose. Continuous and dotting lines indicate induction and repression respectively.

4. Transcriptional regulation of carbohydrate metabolism

The expression of *apuA* was highly induced in presence of pullulan or maltotriose (which is the most abundant degradation product of pullulan) and repressed in presence of glucose. An analysis of the promoter region of ApuA revealed the presence of two transcription binding motifs (**Chapter 4**). Studies conducted on the molecular mechanisms of regulation of *apuA* (**Chapter 4**) showed that *apuA* expression was co-regulated by catabolite control protein A (CcpA) and a dedicated regulator (ApuR) that we suggest to be an activator that induces *apuA* transcription in the presence of (phosphorylated) maltotriose or maltose. Additionally, CcpA, in the presence of rapidly metabolized sugars such as glucose, mediates a repression of *apuA* transcription upon binding to catabolite response elements (*cre*) sites that we predicted to overlap with the *apuA* -35 promoter region. Similar binding sites for ApuR and CcpA were also found in the promoters of the maltodextrin-specific

ABC transporter operon and 4-α-glucanotransferase (*malQ1*) (**Chapter 5**). We proposed that *in vivo*, dual regulation may ensure that *apuA* is expressed in the mucosa where glucose levels are low and when the substrates that can be degraded by ApuA are present (Fig. 5.10).

Recently, the role of CcpA was investigated by microarray analysis of *S. suis* strain S10 and its isogenic Δ*ccpA* mutant growth in standard THB culture media. Inactivation of *ccpA* in *S. suis* altered expression of 259 genes (13.2% of the genome) including cell surface-associated virulence factors and capsule genes [73]. In **Chapter 5** we searched for *cre* motifs in *S. suis* P1/7 and predicted for 172 genes (8.7% of the genome; this number included genes belonging to the same operon) that these were under direct control of CcpA (Table 5.1). The number of genes is lower than that affected in the Δ*ccpA* deletion mutant possibly due to the inaccuracy of informatics predictions or indirect effects in the mutant background. Genes belonging to all main functional classes were represented in the *ccpA* regulon prediction; a relative enrichment of *cre*-sites was found in the upstream regions of genes predicted to encode PTS and ABC carbohydrate transporters (17%), proteins with regulatory functions (10%), and enzymes involved in carbohydrate metabolism (25%) and in virulence (8%).

5. Carbon catabolite control and virulence gene regulation in *S. suis*

As mentioned above, bacteria alter transcription of carbohydrate utilization genes and virulence factor production in response to changes in environmental conditions encountered during host colonization and infection [56,74,75,76]. Therefore, pathogenic bacteria have developed molecular strategies to directly link regulation of carbohydrate utilization and virulence factor production. CcpA has been shown to be important for virulence of several streptococcal species, influencing growth, haemolysin production, biofilm formation and capsule expression [56,77,78,79]; several virulence factors including capsule genes and cytotoxins are directly controlled by CcpA [77,79,80,81,82]. CcpA mutants in *Streptococcus pneumoniae* are often attenuated for virulence in mouse infection models [77,80]. However, in *Streptococcus pyogenes*, CcpA depletion did lead to both hypervirulence and hypovirulence, in the same set-up of experimental mouse infection [56,82].

Table 6.1: *S. suis* virulence genes differentially expressed in pullulan (P) compared to glucose (Glc)

Annotation	Protein	Function	Virulence	P/Glc[1]
Galactosyl / rhamnosyl transferase	CpsE/F	CPS biosynthesis	Attenuated-pig	
Tyrosine-protein kinase Wze	Cps2C	CPS biosynthesis	Attenuated-pig	
N-acetylneuraminic acid synthase	NeuB	Sialic acid synthesis	Attenuated-pig	U
Peptidoglycan GlcNAc deacetylase	PgdA	Peptidoglycan	Attenuated-pig	D
D-alanine-poly(phosphoribitol) ligase	DltA	LTA D-alanylation	Attenuated-pig	
Fibronectin-fibrinogen binding	FbpS	Adhesion ECM	Attenuated-pig	
Enolase	eno	Adhesion ECM	no Mutant	
Glyceraldehyde-3-P-dehydrogenase	GAPDH	Adhesion ECM	no Mutant	U
Di-peptidyl peptidase IV	DppIV	Adhesion ECM	Attenuated-mouse	U
6-phosphogluconate-dehydrogenase	6-PGD	Adhesion epithelium	No Mutant	
Amylopullulanase	ApuA	Adhesion epithelium	Not tested	U
Glutamine synthetase	GlnA	Adhesion epithelium	Attenuated-mouse	D
Streptococcal adhesin P	SadP	Adhesion epithelium	no Mutant	U
Arginine deaminase	ArcB	Resistance to acidity	Not tested	U
Anchored DNA nuclease	SsnA	Host DNA degradation	Not tested	U
Cell envelope proteinase	SspA	Subtilisin-like protease	Attenuated-mouse	U
Metallo-serine protease	IgA1	IgA1 protease	Attenuated-pig	U
Suilysin	Sly	Haemolysin	Unaffected-pig	U
Hyaluronate lyase	Hyl	Hyaluronan degradation	Not tested	U
Sortase A	SrtA	Protein sorting	Attenuated-pig	
Serum opacity-like factor	OFS	Serum opacification	Attenuated-pig	D
S-ribosyl homocysteinase	LuxS	Quorum sensing	Attenuated zebrafish	D
Muramidase released protein	MRP	Unknown	Unaffected-pig	U
Extracellular protein factor	EF	Unknown	Unaffected-pig	U

P/Glc[1] upregulated (U) or downregulated (D) expression when *S. suis* was grown in pullulan (P) compared to glucose (Glc)

These results could be explained by considering that CcpA may have a pleiotropic effect in host colonization and virulence since it can mediate the repression or activation of different virulence factors, depending on the location in the body where *S. suis* is actually dispersed during the progression of the infection.

Our transcriptome and *cre* site analyses predicted that CcpA regulated multiple virulence genes in *S. suis* and that carbohydrate utilization would play a key role in virulence regulation *in vivo*. In **Chapter 4 and 5** we reported that the expression of 17 virulence genes associated with mucosal adhesion and invasion and avoidance of host defences were significantly differentially expressed when bacteria were grown in CM supplemented with pullulan (Table 6.1).

One of these genes encodes a secreted suilysin, a pore-forming toxin which has been shown to play an important role in damaging host epithelial [83,84], endothelial [70,85] and immune cells [86]. Suilysin is cytotoxic to different cell and tissue types including

leukocytes [1,3] thereby facilitating bacterial tissue invasion. Increased expression of the suilysin gene by *S. suis* bacteria grown in pullulan (compared to glucose) was concomitant with a higher suilysin production *in vitro* and an increased hemolytic capacity of bacteria *in vitro* assays using horse red blood cells (**Chapter 5**).

Additionally, a hyaluronidase (Hyl) gene, encoding an enzyme which is predicted to break down hyaluronan, a component from the extracellular matrix of eukaryote connective tissue cells, was highly induced in pullulan. Hyaluronidase, in combination with suilysin, could contribute to destruction of epithelial tissue, thereby promoting the release of glycogen from host cells. Glycogens can be degraded by amylopullulanase, and the degradation products (maltotriose/maltodextrin) can be imported by the invading bacteria via specific transport systems (MalT, part of the corresponding PTS and MalX part of a specific ABC transporter) and then metabolized to sustain bacterial proliferation in an infection site (Fig. 6.2).

Bacterial-induced damage to host epithelia (e.g. brought about by suilysin) may expose ECM and may contribute to microbial colonization and infection. Some virulence genes could encode factors that contribute to bacterial binding to extracellular matrix (ECM) components such as collagen and fibronectin (Table 6.1). *S. suis* encodes several additional secreted surface proteins that may bind ECM, two of which, GAPDH and peptidyl peptidase DppIV, were also induced in presence of α-glucans (Fig. 6.2).

Bacteria that have translocated across epithelia and have invaded the connective tissue are very likely to be perceived by the mucosal immune system including dendritic cells (DCs). In **Chapter 2**, we investigated the fundamental role of capsule during interactions of *S. suis* bacteria with DCs. In addition to capsule production, *S. suis* may use additional virulence factors that modulate DC functions and escape immune surveillance, mainly by modulating cytokine release and avoiding phagocytosis. Invading *S. suis* bacteria may also interact with neutrophils at infection sites. We observed that the *ssnA* gene that encodes a secreted DNase and is potentially involved in the breakdown of neutrophil entrapments (NETS) [87], was upregulated in bacteria grown in presence of pullulan (compared to glucose). Human and pig plasma cells in the lamina propria secrete large amounts of immunoglobulin A (sIgA) which is transported to the lumen and secreted via epithelial cells into the lumen. Secretory IgA can effectively limits translocation of bacteria and several bacterial pathogens encode IgA proteases. In S. suis a metallo-serine protease with homology to IgA proteases was shown at hogh concentrations to cleave human IgA [88].

In our experiments, the SspA gene which encodes a secreted serine protease was also induced in pullulan compared to glucose. This protease can degrade eukaryote pro-inflammatory interleukin-8 (IL-8) molecules thereby inhibiting chemotaxis and infiltration of neutrophils and other leukocytes to infection sites *in vivo* [89,90]. Note that these predicted or validated virulence genes were all induced when *S. suis* bacteria were grown in CM supplemented with pullulan (compared to glucose) and that induction of these genes correlated with a significantly increased adherence and cell invasion of bacteria *in vitro* assays using a porcine tracheal epithelial cell line (**Chapter 5**).

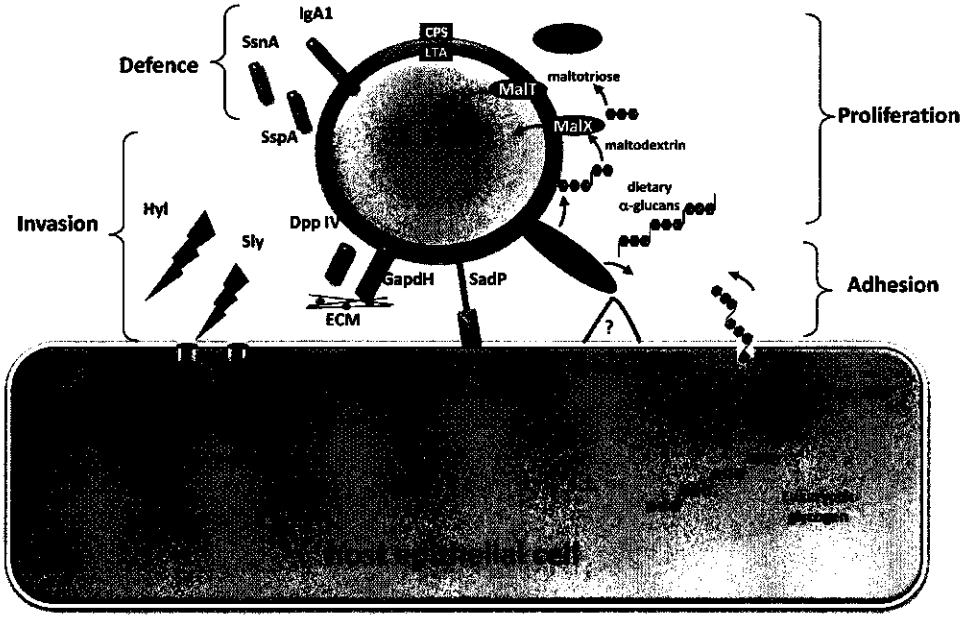

Fig. 6.2 Virulence factors upregulated in pullulan and their (confirmed or proposed) role during adhesion to and invasion of host mucosal epithelia.

Secreted suilysin promotes *S. suis* invasion, possibly by damaging host epithelial barriers exposing basolateral membrane receptors or extracellular matrix components (ECM) for interactions with *S. suis*. Surface-expressed proteins DppIV and GAPDH promote binding to ECM components, such as fibrinogen and fibronectin, and degradation of host cells and tissues once the epithelial barrier has been breached. Several *S. suis* adhesins (ApuA and SadP) and LTA have been reported to contribute to cellular invasion [70]. The bacteria can proliferate upon degradation by ApuA of dietary α-glucans or eukaryotic glycogen coming from damaged cells in the infection site. Other secreted or cell wall-anchored proteins (IgA1, SsnA and SspA) protect the bacteria against the mucosal immune response.

Abbreviations: ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Sly, suilysin; Hyl, hyaluronidase; Dpp IV, peptidyl peptidases; ArcB, arginine deaminase; ApuA, amylopullulanase; SadP, streptococcal adhesin P; SspA, cell envelope proteinase; SsnA, (extracellular) DNA nuclease; Iga1, metallo-serine protease; CPS, capsule; LTA, lipoteichoic acid.

6. The role of *S. suis* capsule during infection

In a previous study, higher levels of adhesion and invasion were obtained with unencapsulated mutants compared to wild type strains [91]. Unencapsulated *S. suis* strains, isolated from pigs that had developed endocarditis, also showed increased adherence to HEp-2 cell lines and porcine and human platelets compared to capsulated strains [91,92]. These findings suggest that the presence or composition of the capsule or its charge, may hinder the binding of surface adhesins to receptors on epithelial cells. A recent genetic analysis of the capsular polysaccharide synthesis locus of fifteen *S. suis* serotypes predicted that capsules of serotypes 1, 2, and 14 may contain sialic acid [93]. In *Streptococcus agalactiae*, capsule sialic acid has been shown to increase the hydrophilic surface properties of the bacteria and to have an inhibitory effect on phagocytosis [94]. Based on these findings, it has been hypothesized that during infection, *S. suis* could down regulate capsule expression for increased adhesion to epithelial cells and, after passing the epithelial barrier, in response to external stimuli and after entering the bloodstream, up regulate capsule expression for protection against phagocytosis [1]. In a recent study, a Δ*ccpA* deletion mutant of *S. suis* showed reduced transcription of the capsule gene *cps2A* and reduced thickness of the capsule in electron micrographs [73]. However, reduced capsule thickness was not observed in our study when *S. suis* was cultured in CM plus pullulan but no glucose (**Chapter 4**). The reason for these different findings is not clear but may be related to the use of a different serotype 2 strain or differences in the growth media (THB vs. CM supplemented by glucose or pullulan). Furthermore, reduced expression of the capsule during colonization remains to be proven *in vivo*.

The capsule was previously shown to be essential for full virulence of *S. suis* in a porcine infection model [95]. However, not all capsulated isolates of *S. suis* (including serotype 2) are virulent, highlighting the (existence and) relevance of additional virulence factors in disease pathogenesis [1]. The capsular polysaccharide was shown to interfere with phagocytosis by porcine dendritic cells (DCs) and consequently, the level of DC maturation and cytokines production was reduced compared to an unencapsulated strain [96]. DCs are important sentinels in the host mucosa that play a key role in immune homeostasis and tolerance [97]. Phagocytosis of invading microbes typically results in DC activation and antigen presentation to T cells in the Peyer's patches or mesenteric lymph nodes.

Cytokines secreted by activated DCs have a major influence on T cell polarization, differentiation and clonal expansion [98].

Considering the emergence of *S. suis* as a significant cause of meningitis in humans, we investigated the effect of different European serotypes that have been associated with human infections on the maturation and expression of cytokines in human monocyte-derived DCs. As references, we also included the capsulated *S. suis* serotype 2 (SS2) and its own unencapsulated mutant (SS2J28) in these DC assays. Additionally, we compared the efficiency by which the different isolates were phagocytosed by DCs and studied the intracellular survival of internalized *S. suis* serotype 2 isolates. We measured a high variation in the induction of cytokine profiles and resistance to phagocytosis between the tested strains; the unencapsulated mutant SS2J28 was the most effective in stimulating and activating DCs. Interestingly, the capsulated SS2, which is the serotype most commonly associated with invasive disease in pigs and humans [99], was least effective in activating DCs. This might be due to presence of sialic acid in the capsule. Sialylated capsules and sialylated lipo-oligosaccharides are known to render bacteria more resistant to complement killing, and sialylation can affect bacterial interactions with neutrophils and epithelial cells [94].

The contribution of capsule to resistance to phagocytosis became clearly apparent when comparing the significantly higher phagocytosis of the unencapsulated SS2J28 with the much lower phagocytosis of SS2 capsule-bearing bacteria. It appears that the presence of capsule during colonization is needed to shield the bacteria from phagocytosis by DCs. To rule out the possibility that the quantified differences in phagocytosis of SS2 and the unencapsulated SS2J28 by DCs might be due to strain variation in intracellular survival, we measured kinetics survival over a period of 4 hours after phagocytosis. We found that once internalized, both the wild-type strain and its unencapsulated SS2J28 were killed at similar rates.

Surprisingly, after 24 hours of incubation, we were still able to recover 10^3 CFU/ml from both strains. This has important consequences for pathogenesis because activated DCs eventually undergo apoptosis and may release viable *S. suis*. As DCs traffic from the mucosa via the blood to lymphoid tissues [35], intracellular survival may enable phagocytosed *S. suis* to disseminate in the body and contribute to invasive disease **(Chapter 2)**.

7. A revised model of *S. suis* colonization and pathogenesis

Based on the literature and novel results obtained in the course of this thesis we propose a revised model for colonization and pathogenesis taking into account the role of carbohydrate metabolism (this thesis; Fig. 6.3).

S. suis bacteria, upon entering the host by a nasal or oral route, will colonize the mucosal epithelia. The composition of carbohydrates within this niche can be highly variable because of differences in parameters such as diet and resident microbiota. When the local carbohydrate composition is low in glucose and high in α-glucans, *S. suis* bacteria may behave as described in **Chapter 3** and we propose that ApuA may contribute to adhesion of bacteria to host mucosa and acquisition of mucosal carbohydrates (Fig. 6.3 step A). During this colonization phase, the capsule may be thinner than when the bacteria are growing inside the body to promote adhesion of bacterial surface structures to host epithelia, possibly to specific epithelial receptors (Fig. 6.3 step A).

Invasion of host mucosa may be a consequence of bacterial translocation across epithelia or after internalization and transport of bacteria by mucosal DCs (**Chapter 2**) (Fig. 6.3 step B). Eukaryotic glycogen, released by (suilysin) damaged host cells, can be used as substrate by ApuA and fermented during growth (Fig. 6.3 step C). Once bacteria have breached the epithelium and reach the loose connective tissues of the lamina propria, adhesins and proteases such as hyaluronidase are thought to bind and digest components of the ECM, promoting spreading of the infection (Fig. 6.3 step C). In the tissues and bloodstream, glucose concentrations are substantially higher compared to oral and nasal mucosa or saliva. The higher (around 5 mM) glucose concentrations will induce a change in metabolic gene expression in order to optimize bacterial metabolism and survival. In presence of high concentrations of glucose capsule genes will be induced and capsule thickness will increase to protect *S. suis* from phagocytosis by leukocytes recruited to the site of infection (Fig. 6.3 step D).

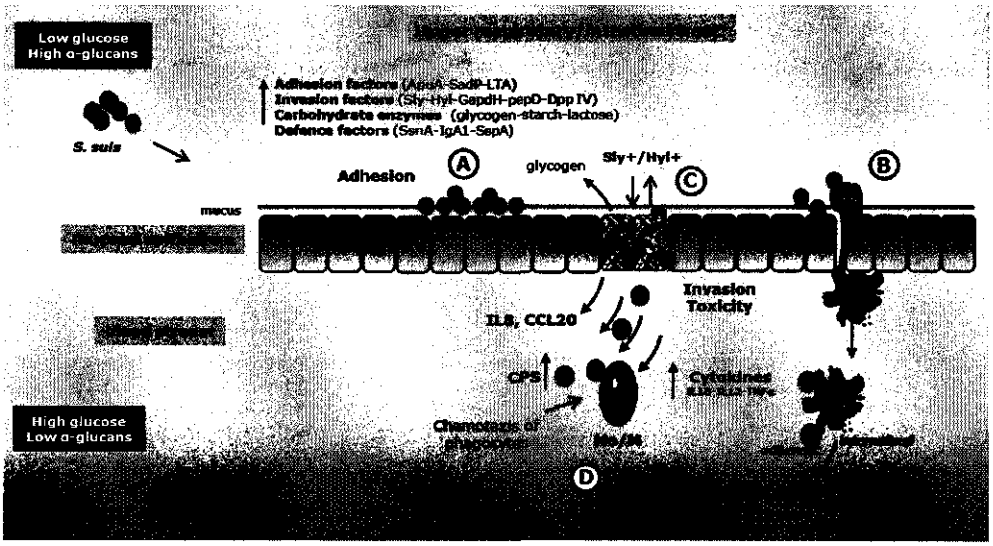

Fig. 6.3: ***S. suis*** **colonization model of host mucosal epithelia. Sly,** suilysin; **Hyl,** hyaluronidase; **CPS,** capsular polysaccharides; **DC** dendritic cells, **Mo/M** Monocytes/Macrophages, **IL8–IL10-IL12-TNFα** interleukins. The description of pathogenesis steps are found in the text.

8. The future of *S. suis* research in the veterinary and medical field.

S. suis continues to be an unresolved problem for the pig industry due to the lack of new preventive measures. New insights from our work suggest a few possible strategies to "combat" this important swine and human pathogen.

1. Is ApuA a vaccine target? Possibly not since *apuA* may not be expressed in blood. However it could be useful to immunize sows with ApuA and test if anti-ApuA antibodies in the colostrum protect pigs against pathogenic colonization by *S. suis*. In order to do so, we should validate that ApuA (or other adhesins mentioned in this thesis) is (are) expresssed and immunogenic *in vivo*. Adhesins with demonstrated *in vivo* functionality could be combined in a single vaccine and tested.
2. In pigs, *S. suis* is frequently present in the oral cavity. It may be worthwhile determining which members of the microbiota could influence *S. suis* colonization and invasion of the host mucosa.
3. What is the contribution of resident microbiota in suppression or induction of pathogenic factors in *S. suis*? If *S. suis* could be suppressed by certain bacterial groups, this could indicate novel approaches for *S. suis* control.
4. In addition, could the diet be a major factor in promoting *S. suis* pathogenicity while at the same time, reducing competition by other bacteria? *S. suis* infections of piglets are most common around weaning, when piglets no longer receive the sow's milk but instead, receive solid pig feed. Interestingly, starch is among the most abundant carbohydrate present in the feed of weaned piglets. Since the extracellular ApuA enzyme is a unique feature of *S. suis*, we propose that the unique (compared to other bacteria that do not possess extracellular ApuA) capacity of *S. suis* to degrade starch provides it a major advantage over other resident bacteria. We propose that the starch from the piglet's feed provides a rich source of nutrients for *S. suis* but not for other bacteria that lack extracellular ApuA enzymes. Extracellular ApuA therefore provides *S. suis* a huge temporal competitive advantage. This could explain the frequent infections of weaned piglets, whereas infections of piglets receiving milk or of adult pigs occur far less often. We propose that it could be of interest to modify dietary composition to promote growth of competitive bacteria.

The last decade has clearly highlighted the clinical relevance of human infections by *S. suis* and the increasing numbers of cases of human infections reported in the literature.

1. How does *S. suis* colonize the human mucosa cause and which route(s) is used to invade the host? Contaminated pork products have been demonstrated as a source for *S. suis* infections. A possible route of human entry leading to infection could be via colonization of the oral cavity, and then enter a bloodstream or other route to the brain.
2. What determines differences in human or pig dendritic cell responses to *S. suis*? it appears that sialic acid decorations could play a role here [100] although capsule sialic acids do not appear to function as virulence factor for serotype 2 isolates [101].
3. What (if any) are the correlations between *S. suis* serotype and an isolate's capacity to disperse and cause disease? It is clear that especially serotype 2 is associated with human disease, and that distribution of highly virulent isolates in humans may change considerably within a decade [102,103].

There are many more research questions to be addressed, all relating to pathogenicity, ecology and infection of different hosts by *S. suis*. Based on the global distribution of *S. suis*, and increased awareness of *S. suis* as a cause of human meningitis we anticipate that clinical relevance of *S. suis* as a human pathogen will increase during the coming years.

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Summary

Summary

Streptococcus suis (*Firmicutes, Lactobacillales*) is a major pathogen of swine and an emerging zoonotic pathogen that induces diverse serious diseases including septicaemia and meningitis. *S. suis* bacteria show clear characteristics of pathobionts, which are symbionts that do not normally elicit any damage in the host except under specific circumstances (e.g. host hypersusceptibility or major changes environmental conditions including changes in microbiota composition and carbon sources). The purpose of this thesis research was to increase our knowledge of the interactions of *S. suis* with the host mucosa and to investigate conditions that could alter virulence in *S. suis*.

The interaction of *S. suis* with human dendritic cells was investigated by measuring phagocytosis and intracellular survival of different strain serotypes. Pathogen adhesins play a major role in colonization of the host which is typically a re-requisite to cause disease. Therefore, the role of an extracellular surface amylopullulanase (ApuA) in bacterial adhesion to porcine tracheal epithelial cells was investigated. ApuA indeed contributed significantly to bacterial adhesion to host cells and was also required for the utilization of α-glucans for fermentative growth. The expression of *apuA* gene was shown to be regulated by the presence of α-glucans (specifically pullulan) and maltotriose a breakdown product generated by the catalytic activity of ApuA. To understand the mechanisms regulating expression of *apuA* the roles of an upstream transcriptional regulator (ApuR) and the carbon catabolite control protein A (CcpA) were investigated. From these studies, it became clear that growth of bacteria in glucose or α-glucans greatly impacted on bacterial growth and possibly, on virulence. To determine whether carbohydrate sources could alter the global regulation of metabolism and the expression of virulence factors, *S. suis* whole-genome gene expression microarray experiments were performed. Based on the recent literature and novel results obtained in the course of this thesis, a revised model for colonization and pathogenesis of *S. suis* was proposed, taking into account the role of carbohydrate metabolism. According to the model, *S. suis* bacteria, upon entering the host by a nasal or oral route, colonize mucosal epithelia. The composition of carbohydrates within this niche can be highly variable due to differences in parameters such as diet and resident microbiota. When the local carbohydrate composition is low in glucose but high in complex α-glucans, the expression of several

virulence factors is upregulated. This thesis exemplifies how carbon sources determine genetic regulation of *S. suis* metabolism and virulence. The results described in this thesis suggest how dietary carbohydrates could influence *S. suis* virulence and provide information that may contribute to the rational design of novel strategies to control this emerging zoonotic pathogen.

Samenvatting

Samenvatting

Streptococcus suis (*Firmicutes, Lactobacillales*) is een belangrijk pathogeen van varken en een opkomend zoonotisch pathogeen van de mens dat een aantal uiteenlopende ernstige ziektes kan veroorzaken inclusief septicemia en hersenvliesontsteking. *S. suis* bacteria vertonen duidelijke kenmerken van pathobionten, oftewel symbionten die onder normale omstandigheden geen schade in de gastheer veroorzaken behalve onder specifieke omstandigheden (bv. hypervatbaarheid van de gastheer of drastische veranderingen in de omgeving waartoe veranderingen in microbiota samenstelling en koolstofbronnen behoren). Het doel van dit onderzoek was het vergroten van onze kennis omtrent de interacties van *S. suis* met de gastheer mucosa en te onderzoeken welke omstandigheden de virulentie van *S. suis* zouden kunnen veranderen.

De interactie van *S. suis* met humane dendritische cellen werd onderzocht door fagocytose en intracellulaire overleving te meten van een aantal isolaten met verschillende serotypes. Pathogeen adhesines spelen een belangrijke rol in kolonisatie van de gastheer, een algemene voorwaarde om ziekte te veroorzaken. Om die reden werd de rol van een extracellulair oppervlakte amylopullulanase (ApuA) enzym in adhesie van bacteriën aan varkens tracheale epitheel cellen onderzocht. ApuA droeg inderdaad significant bij aan bacterie adhesie aan gastheercellen en was ook noodzakelijk om α-glucanen voor fermentatieve groei te kunnen gebruiken. Het kon aangetoond worden dat de expressie van het *apuA* gen gereguleerd werd door de aanwezigheid van α-glucanen (vooral pullulan) en maltotriose, een afbraakprodukt dat gevormd werd door de katalytische activiteit van het ApuA enzym. Om de mechanismen te begrijpen welke bijdragen aan regulatie van *apuA* werden de functies van een upstream transcriptie regulator (ApuR) en het carbon catabolite control protein A (CcpA) onderzocht. Uit deze studies werd duidelijk dat groei van bacteriën in glucose of α-glucanen in belangrijke mate van invloed was op bacteriegroei en mogelijk ook op virulentie. Om vast te stellen of carbohydrate bronnen de globale regulatie van metabolisme en expressie van virulentiefactoren konden veranderen werden *S. suis* compleet-genomische genexpressie microarray experimenten uitgevoerd. Uitgaande van de recente vakliteratuur en de nieuwe resultaten verkregen tijdens de uitvoering van dit promotie onderzoek wordt hier een aangepast model voor kolonisatie en pathogenese van *S. suis* voorgesteld, met

inachtneming van de rol van het carbohydraat metabolisme. Volgens dit model koloniseren *S. suis* bacteriën het mucosale epitheel na binnendringen van de gastheer via de neus of mondholte. De samenstelling van de carbohydraten in deze niche kan erg verschillend zijn vanwege verschillen in de samenstelling van de lokale microbiota. Wanneer de plaatselijke carbohydraat samenstelling van glucose laag is maar α-glucanen algemeen zijn neemt de expressie van een aantal *S. suis* virulentie genen toe, waaronder expressie van suilysin, een bekend cytotoxine.

In dit proefschrift wordt aangetoond hoe koolstofbronnen de genetische regulatie van *S. suis* metabolisme en virulentie bepalen. De resultaten die in dit proefschrift beschreven worden stellen voor hoe carbohydraten, onderdeel van het dieet, virulentie van *S. suis* zouden kunnen beïnvloeden, en verschaffen informatie die kan bijdragen tot het rationeel ontwikkelen van nieuwe strategieën om dit belangrijke, opkomende zoönotische pathogeen.

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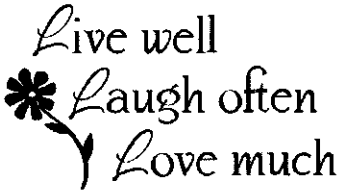
Vi voglio bene! Un abbraccio forte a tutti!

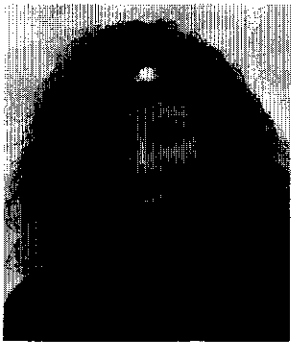
Laura


Live well
Laugh often
Love much

Personalia

If the doors of perception were cleansed
everything would appear to man as it is: ***Infinite.***
W. Blake

Curriculum vitae



M. Laura Ferrando was born in Cagliari (Italy) on 10th September 1972. In 1992, after graduating at Classic Gymnasium "Siotto Pintor", she started her bachelor and masters in Biological Sciences education at the University of Cagliari. She had been working for almost eight years as team-researcher in several Italian research institutes (University of Parma - Division of Genetics and Environmental Biotechnologies; Istituto zooprofilattico di Brescia - Centro substrati celluari; Università di Cagliari- Public Health Institute) mainly focussing on molecular diagnostics of animal and human pathogens. During these years, she acquired good work experience in the development of molecular methods for detection of pathogens in food, veterinary and environmental research areas.

In the meantime, she also obtained the title of "Clinical microbiology and virology specialist" after successful completion of a 4-year specialization course at the Faculty of Medicine and Surgery of University of Cagliari. She completed this course with a thesis titled "Role of the Two-Component Systems (TCS) of *Streptococcus pneumoniae* in virulence", performed during a long-term fellowship at Swammerdam Institute (SILS) in Amsterdam under the supervision of Prof. J. M. Wells. After this, the author started as PhD student on the project entitled "Role of cell envelope proteins in virulence and pathogenicity of *Streptococcus suis* infections in pigs" funded by "Programma binnen KB-thema 8 Diergezondheid", I.POP and CVI "kennisbasis". The project was carried out at the Host-Microbe Interactomics group, under the supervision of Prof. Dr, J. M. Wells, Dr Hilde E. Smith and Dr Ir Peter van Baarlen.

Currently the author is employed as PostDoc at the Medical Microbiology Department of the Academic Medical Center of Amsterdam in the group of Dr Constance Schultsz.

List of publications

Meijerink M., Ferrando M.L., Lammers G., Taverne N., H.E. Smith, Wells J.M. (2012). "Immunomodulatory effects of *Streptococcus suis* type 2 capsule on human dendritic cell responses and intracellular survival". PLoS ONE, in press.

Ferrando M.L., S. Fuentes, de Greeff A., Smith H. E. &. Wells J. M. (2010). ApuA, a multifunctional alpha-glucan-degrading enzyme of *Streptococcus suis*, mediates adhesion to porcine epithelium and mucus. *Microbiology* 156: 2818-2828.

Orru G., Ferrando M. L., Meloni M., Liciardi M., Savini G. & De Santis P. (2006). Rapid detection and quantitation of Bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay. *J Virol Methods* 137: 34-42.

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Bertasi B., Bignotti E., Ferrando L., D'Abrosca F., Scaratti L. & Pomati F. (2003). The standardization of a molecular biology method to verify the presence of *Microcystis aeruginosa*. *Vet Res Commun* 27 Suppl 1: 277-279.

Training and Supervision Plan

The Basic Package	**Year**
WIAS Introduction Course, Wageningen, The Netherlands	2008
Course on Ethics and Philosophy in Life Sciences, Wageningen, The Netherlands	2009
Subtotal Basic Package	**3.0 credits**
Scientific Exposure	
International conference	
CSHL, Microbial Pathogenesis & Host Response, New York, USA	2009
Scientific Spring Meeting NVMM&NVvM Papendal, The Netherlands	2010
Scientific Spring Meeting NVMM&NVvM Papendal, The Netherlands	2011
XVIII Lancefield International Symposium, Palermo, Italy	2011
Seminars and workshops	
WIAS Science day, Wageningen, The Netherlands	2008
HMI Workshop, Cagliari, Italy	2008
WIAS Science day, Wageningen, The Netherlands	2011
Presentations	
"Surface virulence determinants of *S. suis*" WIAS Science day (Poster)	2008
"Surface virulence factors of *S. suis*" (Italy) HMI Workshop (Oral)	2008
"Role of a multifunctional carbohydrate utilization gene *apuA*, in the virulence of *S. suis*" CSHL (Poster)	2009
"ApuA a multifunctional α-glucan-degrading enzyme of *S. suis* mediates adhesion to porcine epithelium" NVMM&NVvM (poster)	2010
"A transcriptional control mechanism linking carbohydrate metabolism and virulence in *Streptococcus suis*" WIAS Science day (Oral)	2011
"Catabolite regulation of a bifunctional adhesin and α-glucan utilization enzyme in *Streptococcus suis*" NVMM&NVvM (Poster)	2011
"Transcriptional control mechanisms linking carbohydrate metabolism, colonization and virulence in *S. suis.*" XVIII Lancefield (Oral)	2011
Subtotal Scientific Exposure	**11.8 credits**
In-Depth Studies	
Disciplinary and interdisciplinary courses	
Workshop on phylogeny & genetics in microbiology and virology, Rotterdam, The Netherlands	2008
2nd Workshops molecular microbiology of Infectious diseases, Rotterdam, The Netherlands (Oral presentation)	2008
WIAS Adavnced statistics course design of experiment, Wageningen, The Netherlands	2009
Advance in genomics 2010, Ghent, Belgium	2010
Stars network and training event Siena, Italy (Oral presentation)	2011
Advanced visualisation, integration and biological interpretation of -omics data, Wageningen, The Netherlands	2011
Statistics for the Life Sciences, Wageningen, The Netherlands	2011
Subtotal In-Depth Studies	**6.8 credits**
Statutory Courses	
International course on laboratory animal science, Utrecht, The Netherlands	2011
Subtotal Statutory Courses	**3 credits**
Professional Skills Support Courses	
Techniques for writing and presenting a scientific paper	2011
Carrier and sperpective	2011
Effective behavour surround	2010
Subtotal Professional Skills Support Courses	**3.5 credits**
Research Skills Training	
Preparing own PhD research proposal	2008
Subtotal Research Skills Training	**6.0 credits**
Didactic Skills Training	
Supervising practicals and excursions	
HMI practical course	2008
HMI practical course	2009
HMI practical course	2010
HMI practical course	2010
Supervising theses	
Supervising 1 MSc minor	
Tutorship	
Tutor of 1 WIAS student	2008

Tutor of 1 MBO student		2009
Preparing course material		
HMI practical course 2009		2009
	Subtotal Didactic Skills Training	**6.5 credits**
Management Skills Training		
Organization of seminars and courses		
Host Microbe Interactomics Workshop at University of Cagliari (Italy) on September 2008		2008
	Subtotal Management Skills Training	**1.0 credits**

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