Discovery, characterization and applications of natural DNA transformation in *Streptococcus suis*

Edoardo Zaccaria

Thesis committee

Promotors
Prof. Dr Jerry M. Wells
Professor of Host-Microbe Interactomics
Wageningen University

Co-promotor
Dr Peter van Baarlen
Assistant professor, Host-Microbe Interactomics Group
Wageningen University

Other members
Prof. Marco R. Oggioni , University of Leicester, United Kingdom
Prof. Jan-Willem Veening, University of Groningen
Prof. John van der Oost, Wageningen University
Prof. Hanne Ingmer, University of Copenhagen, Denmark

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A Mamma e Papá
Andrea e Chiara
Giorgio e Giotto
e a quelli che mi vogliono bene

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Chapter 1

General Introduction

Edoardo Zaccaria

Streptococcus suis

Streptococcus suis is a Gram-positive, facultative anaerobe belonging to the Firmicutes phylum. It is catalase negative, α- or β- haemolytic and has a low genomic GC content. *S. suis* is a major global porcine pathogen causing high economic losses in the pig production industry [1]. Its natural habitat is considered to be the upper respiratory tract of pigs, and in particular the tonsils and nasal cavity although it can be isolated from the small intestine and faeces of pigs [2]. Although pigs are considered the natural host of *S. suis*, it has been isolated from a wide range of animals including cats, dogs, ruminants and birds [3-5]. *S. suis* is normally encapsulated and more than 35 capsular polysaccharide (CPS) serotypes have been identified using antibody agglutination tests [6]. However, recent analysis of the sequences of 16S rRNA and *cpn60* genes of the 35 characterized serotypes of *S. suis* revealed that serotypes 32 and 34, were in fact phylogenetically distinct from *S. suis* and cluster more closely with the corresponding sequences of the species *Streptococcus orisratti* [7]. In addition, a multilocus sequence typing (MLST) scheme has been described for *S. suis* based on sequencing of seven housekeeping gene fragments from each of 294 *S. suis* isolates [8]. The MLST isolates collection comprises various *S. suis* clinical isolates as well as asymptomatic carriage isolates, representing 28 serotypes and nine different countries of origin. In total 92 sequence types (STs) were distinguished, with complexes ST1, ST27, and ST87 comprising most of the analysed population. Some STs contain multiple serotypes and several serotypes were found in disparate genetic backgrounds suggesting lateral transfer of CPS loci occurs among the *S. suis* population [8]. Most highly virulent porcine *S. suis* isolates belong to ST1.

Epidemiology of pig and human infections

S. suis is an important porcine pathogen and cause of morbidity and mortality in all countries where pigs are reared on a large scale. It can cause different types of invasive disease in pigs, occurs throughout the year, and is most prevalent in pigs reared indoors. Transmission of *S. suis* among animals is believed to occur through ingestion, inhalation or nose-to-nose contact. In adult pigs the rates of asymptomatic carriage of *S. suis* in the intestine or upper respiratory tract may be as high as 80% of the herd. Transmission of *S. suis* from the sow to piglets occurs at, or shortly after birth but may also occur via faeces or contaminated environmental sources. Flies and rodents may also play a role in transmission of *S. suis* to pigs. Young piglets are most susceptible to *S. suis*, and invasive disease most often develops around the time of weaning (4-6 weeks), although infection can occur at any age [9].

Different, and multiple serotypes of *S. suis* can be isolated from symptomless pigs. Among the 35 different serotypes identified, serotype 2 is the most frequently isolated from infected pigs and humans. In addition, the serotypes 1, 9, and 14 have often been associated with porcine disease [1]. The serotype distribution of

invasive *S. suis* isolates appears to be different in different geographic regions, but is inconclusive due to the paucity of epidemiological data from some countries [1]. *S. suis* causes a wide variety of diseases in pigs including septicemia, arthritis, and endocarditis and the serotype per se does not appear to influence the clinical features. Meningitis is the most striking symptom of invasive *S. suis* infections and is characterized by loss of coordination, instability, convulsions and, often, rapid death within 1-2 days. Respiratory symptoms associated with pneumonia may also occur [10,11]. Pig morbidity due to *S. suis* infection has been estimated to be between 1% and 50% [9]. There is recent concern about the emergence of hyper-virulent strains able to cause more severe and rapid infections of pigs and humans [22]. For example, in the recent Chinese outbreak of *S. suis* infections (see below), disease onset was rapid and meningitis was often associated with streptococcal toxic shock-like syndrome and high mortality [22,23].

As mentioned above, *S. suis* is also considered an emerging zoonotic agent of increasing relevance, with the first zoonotic infections recorded in 1968 [12]. To date, 1642 official cases of invasive *S. suis* infection have been reported in humans, of which approx. 90% occurred in Asia, Vietnam, Thailand and China. In these countries, and all of Southeast Asia, *S. suis* zoonosis is considered endemic, due to the high density of pigs, pig production practices and the human consumption of uncooked pork meat and blood. In Vietnam *S. suis* is the main cause of adult bacterial meningitis [13], and is the second most common cause of meningitis in Thailand. Two outbreaks with high mortality rates have been reported in China; the first in 1998, resulted in 24 reported human cases and 14 deaths and the second and largest human outbreak in 2005 was reported to have 215 cases and 38 mortalities.

In Europe, nearly all the reported cases of *S. suis* human infection were associated with close occupational contact with pork or pigs prior to the infection [14]. In the Netherlands 44 cases of *S. suis* meningitis have been described since 1988. In 41 of 44 (93%) cases the source of infection was established: 16 cases involved pig farmers, 11 involved abattoir workers, 10 involved butchers, and 4 involved persons that were occasionally in contact with pigs or pork [15-17]. In order to assess the potential risk of workers with jobs that carry a relatively high risk of exposure to *S. suis* such butchers, slaughterhouse workers, etc., the occurrence of *S. suis* in pharyngeal swabs was assessed and compared with an age and sex matched control group. Of the 132 persons in the case group, 5.3% were colonized by *S. suis* and in good health, but potentially at risk. In contrast, *S. suis* could not be isolated in a single case within the control group [18]. Cases of human-to-human transmission have not yet been reported.

The clinical manifestations of invasive infection with *S. suis* are similar in pigs and humans with infective endocarditis, meningitis and sepsis being the most common [19-21]. Hearing loss is also a common after-effect of meningitis caused by *S. suis*. Among the Dutch cases, hearing loss has been developed in 28 out of 43

survivors (65%) [17].

Most human cases of *S. suis* infection are caused by serotype 2 (97%), followed by serotype 14 (2.8%) and sporadically by other serotypes (4, 5, 16, 21, 24). Recently, the National Institute of Public Health of The Netherlands placed *S. suis* in the top ten priority zoonotic pathogens, highlighting an increased awareness and the necessity of disease surveillance.

Genomics

A typical *S. suis* genome size contains roughly 2 million base pairs [22-24], with a low GC content (median GC%: 41.15). To date, 472 genomes have been sequenced from clinical and non-clinical isolates, sampled worldwide. In 2015, the population structure and evolution of *S. suis* was investigated by whole-genome sequencing of 459 isolates with clinical and sampling data [22]. A striking genetic difference was observed between the systemic, respiratory disease isolates and non-clinical isolates. The average genome size of the systemic disease isolates was smaller and contained more (putative) virulence factors compared with the respiratory disease and non-clinical isolates. Carriage strains had on average the largest genomes and fewer genes associated with virulence and pathogenicity. The genome of the respiratory disease isolates had an average genome size and a virulence factor repertoire intermediate to the non-clinical and systemic disease isolates [22]. These results are consistent with an evolutionary reduction of genome size in disease-causing isolates as described for some other pathogens [25]. As mentioned above, the *S. suis* serotypes 2 and 14 were most commonly associated with human invasive disease, suggesting that these serotypes might represent a host-adapted population. Human disease isolates appeared to originate from a single virulent population in the 1920's, concomitant with the introduction of selective pig breeding, large-scale pig husbandry and indoor rearing in the pig industries worldwide. So far, little geographical clustering of different *S. suis* subpopulations has been reported [22]. Pig and human isolates from Vietnam could not be distinguished on the basis of genomic markers or shared polymorphisms [22] although in some strains, the presence of a large 89kb pathogenicity island, comprising a mobile genetic element and multiple virulence factors, was associated with high virulence and mortality [26,27].

Pathogenicity and virulence factors

As mentioned above, the main natural habitats of *S. suis* in the pig are the pharynx and the gastrointestinal tract. To cause invasive diseases, the bacteria must pass through the mucosal epithelial barrier, enter and survive in the blood stream, and invade and colonize different organs. In human cases, however, it is thought that the most common routes of infection are via lesions in the skin or consumption of contaminated, uncooked pork products. In both pigs and humans the first stage of mucosal infection with *S. suis* involves crossing of an epithelial barrier. Several

studies have reported the capacity of *S. suis* to adhere to human and pig epithelial cell lines [28-30]. Reduced production of the capsule during colonization is thought increase contact between the bacterial surface-exposed adhesins and the host epithelium. Several *S. suis* adhesins have been proposed including: 1. An enolase, highly expressed *in vivo* that binds both the plasminogen and fibronectin [31,32]. 2. A fibronectin binding protein, Fbps, that binds to human fibronectin [30]. 3. ApuA, an amylopullulanase, shown to contribute to adherence of *S. suis* to epithelial cells [33]. Following colonization, *S. suis* can damage the epithelial barrier through the pore-forming activity of the secreted toxin suilysin and gain entry to underlying tissues and vasculature [34,35]. However, suilysin negative strains have been isolated from pigs with invasive infections [36-39] suggesting that suilysin is not essential for *S. suis* to persist in invasive infections. *In vitro*, *S. suis* can form biofilms, a mode of growth that may also be relevant for colonization of the nasopharyngeal mucosa *in vivo* [40]. Currently there are no models that permit reproducible experimental infection via the nasopharyngeal cavity without pre-existing injury to the epithelium. Thus the virulence mechanisms involved in crossing the epithelia, spreading in connective tissues and lymphatic system and gaining entry into the bloodstream have not been fully elucidated, although a model featuring a metabolic switch that regulates virulence factor production, including the capsule, has been proposed [41]. It has been suggested that *S. suis* can be carried on the surface of leukocytes to reach the brain and joints, (the Trojan horse theory) while other studies using choroid plexus epithelial cells and brain endothelial cell lines suggest cell adherence, and invasion mechanisms are instrumental to the development of meningitis [35,36,42,43].

At mucosal sites of infection and in the bloodstream, *S. suis* avoids efficient phagocytosis by neutrophils or tissue macrophages through the capsular polysaccharide (CPS) [42,44-46]. Unencapsulated mutants of *S. suis* are more easily phagocytosed and killed by phagocytes *in vitro* and cleared more rapidly from the bloodstream than the wild-type (WT) encapsulated strains [44,46,47]. A second mechanism *S. suis* may use to evade the immune cells is sialylation of the capsule in serotype 2 and 14 strains, the two most common serotypes isolated from infected hosts [48,49]. The sialic acid linkage to the galactose present on *S. suis* CPS resembles epitopes present on the surface of many mammalian cells [50], suggesting a possible camouflage strategy (molecular mimicry) to avoid the host immune system. The resistance of *S. suis* to phagocytosis also depends on other factors and several molecules present in the bacterial cell wall seem to play crucial roles. PgdA hydrolase is responsible for the N-deacetylation of the peptidoglycan and is highly expressed *in vivo* and *in vitro*. Peptidoglycan modification by N-deacetylation confers resistance to lysozyme in various Gram-positive bacteria and the PgdA deletion mutant in *S. suis* was attenuated for virulence in both a murine and porcine infection model [51]. D-alanylation of the lipoteichoic acid, mediated by the *dlt* operon, increases the positive charge of *S. suis* cell surface, enhances resistance to

killing by host cationic peptides and contributes to *S. suis* virulence in murine and porcine models of infection [52].

S. suis possesses genes that might help the bacterium to survive reactive oxygen species (ROS) generated by phagocytes or to the low pH in the vacuole e.g. the super oxide dismutase and the arginine deiminase system [53-55]. Additionally, *S. suis* has been shown to be cytotoxic to macrophages *in vitro*, probably due to suilysin secretion and its pore forming activity on host cell membranes [34,56]. Some of these stress challenges including exposure of *S. suis* to professional phagocytes, oxidative burst and pH stress were investigated in this thesis to compare the virulence of specific gene deletion mutants to wild-type and unencapsulated mutants.

One of the most common clinical manifestations of *S. suis* infection of pigs and humans is meningitis, which requires bacteria to cross the blood-brain barrier (BBB) or the blood-cerebrospinal fluid (CSF) barrier. The BBB is formed by highly selective endothelium of the blood capillaries in brain and the central nervous system (CNS). Tight junctions between adjacent endothelial cells allow the selective passage of small molecules, water, gases, and lipid-soluble molecules. *In vitro S. suis* has been shown to adhere to and invade porcine brain endothelial cells suggesting a mechanism for dissemination into the brain and CNS [57,58]. Suilysin production by adherent *S. suis* may play a role in crossing the BBB by damaging the integrity of the endothelium [59,60], although a suilysin deletion mutant was still able to cross the BBB in pigs [43]. Thus, suilysin may aid penetration of the BBB, but it is not essential for this process

Another entry to the CNS is via the blood-CSF barrier that separates the peripheral and cerebral blood flow from the CSF. Translocation of *S. suis* across the blood-CSF barrier has been shown *in vitro* using an inverted Transwell system and addition of *S. suis* to the basolateral cell surface [61]. The mechanisms are not well understood but it has been hypothesised that soluble virulence factors, other than suilysin, secreted by *S. suis* may disrupt the blood-CSF barrier [61,62].

S. suis possesses a large number of virulence factors that might play a crucial role in specific stages of infection (Table 1) [22,36,63-67]. Despite our knowledge of *S. suis* virulence mechanisms, it is not possible to predict the virulence of a strain isolated from an healthy carrier based on the presence/absence of virulence factors. Unfortunately, there is no simple, cost-effective animal model to assay virulence of porcine or human isolates. Part of this thesis was therefore focused on the rational choice and evaluation of simple animal models that have in recent years been successfully used to assay virulence of other pathogenic bacteria (see also below).

Table 1. Selection of the established and putative virulence factors of *S. suis*.

Virulence factor	Function	Ref
CpsE/F/2C	CPS biosynthesis	[45,47,68]
NeuB	*N*-acetylneuraminic acid synthetase	[68]
PgdA	Peptidoglycan *N*-deacetylase	[51]
DltA	lipoteichoic acid D-alanylation	[52]
Suilysin	Hemolysin	[34,43,69,70]
Fbps	Adhesin binding to fibronectin	[71]
Enolase	Adhesin binding to fibronectin and plaminogen	[31]
Glyceraldehyde-3-phosphate dehydrogenase	Adhesin binding to plaminogen, and porcine tracheal rings. Plasmin acquisition. Upregulated *in vivo*	[72-74]
Dipeptidylpeptidase IV	Adhesin binding to fibronectin	[75]
6-phosphogluconate-dehydrogenase	Adhesion toHEp-2 and HeLa cells	[76]
Amylopullulanase	Adhesinon to porcine epithelium and mucus	[33]
Adhesin P	Hemagglutinin	[77]
Dpr	Resistance to iron-mediated toxicity	[78,79]
AdcR and Fur	Zinc and iron uptake regulator	[80]
Superoxide dismutase	Resistance to oxidative stress	[55]
Arginine deiminase system	Resistance to acidity	[54,81]
SspA	Subtilisin-like protease	[82-84]
IgA1 protease	IgA1 protease	[85,86]
Serum opacity-like factor	Serum opacification	[87]
Hyaluronate lyase	Degradation of hyaluronic acid	[88]
Collagenase	Degradation of collagen	[68]
SalK/SalR	Two-component signal transduction system	[89]
CiaRH	Two-component signal transduction system	[90]
RevSC21	Orphan response regulator	[91]
CovR	Orphan response regulator	[92]
RevS	Orphan response regulator	[93]
CcpA	Sugar catabolism regulator	[94]
s*crR* gene	Metabolism, repressor of the sucrose operon	[68]
Glutamate dehydrogenase	Glutamate dehydrogenase	[95]
manN gene	Mannose-specific transport PTS IID	[68]
Permease	ABC-type multidrug transporter	[96]
Permease	ABC-type amino acid transporter	[68]

Two-component systems

The ability to sense and respond to environmental changes is necessary for adaptation and survival under adverse and fluctuating conditions. A bacterial pathogen must adjust its physiology and regulate gene expression to adapt the different microenvironments encountered during the infection of the host, e.g. when crossing the epithelia and in contact with the underlying connective tissue of the lamina propria and its immune cells. One of the main mechanisms used by bacteria for rapidly regulating gene expression are the two-component regulatory systems (TCS) [97,98]. TCS are present in most bacteria and the number correlates with the genome size and the range of environments in which the organism can grow and survive [99]. The prototype of a TCS consists of a membrane-bound histidine kinase (HK) and a cognate response regulator (RR). Generally the HK influences the phosphorylation state of the RR, which modulates the expression of specific genes by DNA binding. The RR is usually a transcription factor and its phosphorylation alters its affinity to bind specific motifs in DNA. RR may also act as an enzyme or ATPase [100,101]. TCS may sense and respond to numerous physical and chemical signals such as pH, redox status, oxygen concentration, temperature, and ionic strength. Thus in bacteria, TCS regulate, directly or indirectly, regulons involved in stress responses, metabolism, virulence and resistance to antibiotics [102-104]. TCS have frequently been implicated in the regulation of the expression of important virulence factors, for example, in many streptococcal species, capsule, haemolysis and exotoxin production are under control of TCS [105-107].

The number of TCS encoded by *S. suis* genome varies depending on strain differences, a minimum of 12 to a maximum of 15 have been annotated so far [23,26]. However, to date, predicted orthologues of SalK/SalR, CiaRH, Ihk/Irr, VirR/VirS, NisK/NisR and two orphan response regulators, RevS and CovR, have been characterized in detail. The contribution of these TCS to *S. suis* virulence is briefly summarised in the next section.

The SalKR and NisKR TCS were identified by comparative genomics in two virulent *S. suis* serotype 2 strains (98HAH12 and 05ZYH33) isolated from Chinese patients in the outbreak of 2005 [89,108]. In both isolates *salKR* and *nisKR* TCS are located in a pathogenicity island. *salKR* was shown to be essential for the high virulence observed in experimental infection of piglets and this TCS may protect *S. suis* from neutrophil phagocytosis and killing [89]. However the precise role of SalKR in virulence of *S. suis* is still unknown, as microarray expression analysis of the *salKR* mutant revealed no changes in known virulence genes [89]. A *S. suis* mutant lacking the TCS *ciaRH* was impaired in adherence to human and pig intestinal epithelial cells *in vitro* and in survival after encountering macrophages [90]. Similar results were obtained in *revS* and *covR* deletion mutants [91,92]. The proposed role of TCS Irr/Ihk in virulence of *S. suis* is based on reduced lethality following intraperitoneal injection of mutant and WT strains in mice [109]. Furthermore the

ihk/irr mutant showed increased killing by a mouse macrophage cell line as well as reduced survival after exposure to low pH and hydrogen peroxide, probably due to a decreased expression of manganese-dependent superoxide dismutase in the mutant strain [109]. Deletion of the v*irR/virS* TCS in *S. suis* resulted in morphological changes characterized by short chains and thin capsular walls. The mutant was more easily cleared in whole blood assays, displayed a decreased oxidative stress tolerance and attenuated virulence in a mouse model [110].

The studies mentioned above highlight the critical role TCS may have as mediators of *S. suis* virulence and survival in the host, however, the regulons controlled by these TCS have not been identified, and it is unknown which genes and proteins mediate the virulence functions that enhance *S. suis* survival under these stress conditions. Only a few TCS mutants have been investigated using infection models *in vivo*, most likely due to the fact that pig infection models are challenging and require substantial investments in facilities and expertise and mouse models are considered difficult to translate to the natural host. In this thesis we describe an investigation of two previously uncharacterized TCS in *S. suis.* These TCS were chosen on the basis of their high DNA and protein sequence conservation among *S. suis* isolates (72.3% and 98.7% of 451 genomes analysed contain SSU1930/1931 and SSU0827/0828). In addition to *in vitro* assays with the TCS mutants and wild-type strain, we developed a simple experimental animal model to compare their virulence *in vivo.*

Experimental models of *S. suis* infection

An animal model is a non-human species that is more amenable to study complex biological phenomena under ethical laboratory conditions, the most common example being rodent models. The expectation is that discoveries made in the model organism will provide information on the target organism. Studying model organisms can reveal important information that would not be possible to obtain using the target host, and thus increase our knowledge of, for instance, host-pathogen interactions, but caution is needed in translating data from one organism to another. In *S. suis* several host species have been used to evaluate, assess and determine the role of genetic determinants on pathogenicity and virulence. Pigs and mice have been the most common experimental animals used for studies of *S. suis* virulence [111,112]. Although a standardized pig intravenous infection model for *S. suis* has been described [113], it is not universally accepted and the experimental conditions adopted by research groups varies, e.g. different groups use pigs with different health status or age, and infect pigs with inocula containing different numbers of bacteria [114]. Moreover the use of pigs, or mice as infection models has economical, logistic and ethical disadvantages over non-mammalian models, such as zebrafish, fruit flies and nematodes.

Zebrafish (*Danio rerio*) larvae recently arose as a non-mammalian vertebrate

model for immune and infection studies. Zebrafish (up to 6 days post fertilization) are exempt from ethical legislation and cheap to rear in large numbers. Moreover they are inexpensive and easily standardized conditions allow high-throughput screens to be performed. Adult zebrafish have been used as infection model to study *S. suis* with some success [115,116]. However these studies compared only a few strains and the injected dose of *S. suis* required to cause 50% mortality was high and not consistent between laboratories [117,118]. In addition, adult zebrafish are not exempt from ethical legislation.

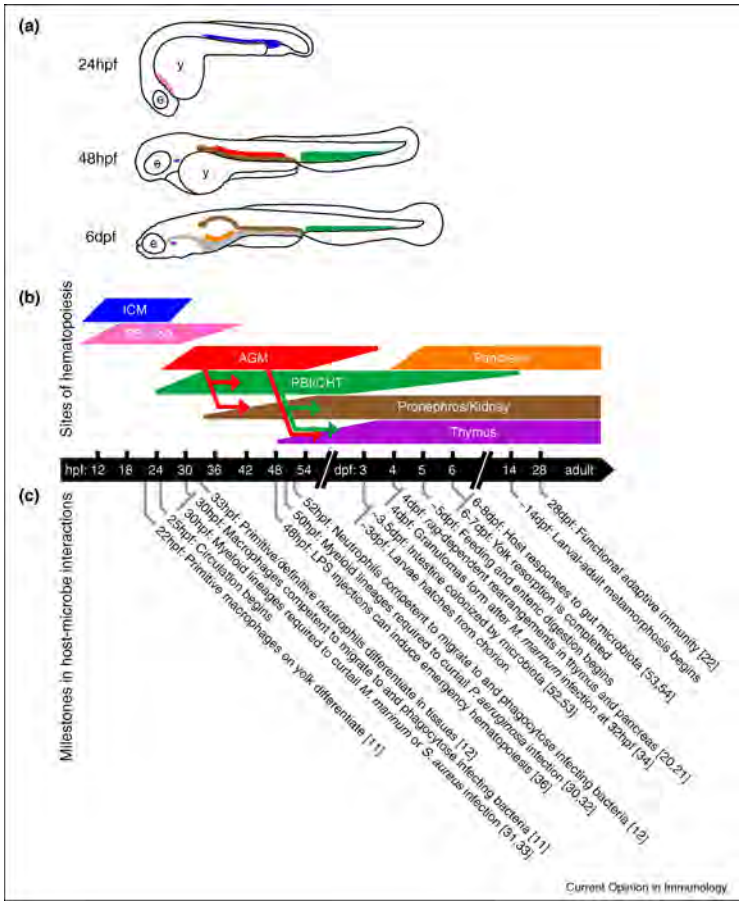

Figure 1. Developmental highlights in zebrafish. (a) Schematic representation of zebrafish larvae anatomy, (b) approximate durations, (c) important milestones relevant to zebrafish immunity. The eye (e), yolk (y) and gastrointestinal tract (grey) are indicated in (a). Reprinted from Kanther M, Rawls JF (2010) Host-microbe interactions in the developing zebrafish. Curr Opin Immunol 22: 10-19 [119], with permission from Elsevier.

Zebrafish at the embryonal and larval stage undergo a rapid development of the innate immune system (Fig. 1): functional phagocytes, granuloma-like structures representing macrophage aggregation, complement factors and antimicrobial enzymes are present in the embryo before or soon after hatching [120-124]. Several Gram-negative and Gram-positive bacterial pathogens have been successfully studied in zebrafish larval models, for example *Salmonella*, *Mycobacterium marinum*, and Gram-positive pathogens including *Listeria monocytogenes* and several streptococcal species [124-128]. To date, use of zebrafish larvae as an experimental infection model for *S. suis* has not been described in the scientific literature. In this thesis we developed a zebrafish larvae infection model by assessing larvae mortality after injection of several porcine *S. suis* isolates and genetic mutants of known virulence in pigs (Chapter 5).

Challenges in prevention and treatment of *S. suis* infections

Despite the importance of *S. suis* as a swine pathogen and zoonotic agent of human disease, little is known about *S. suis* pathogenicity and its mechanisms of infection and adaptation to the host. Our lack of knowledge about the biology and virulence mechanisms in *S. suis* is highlighted by the lack of cross-protective effective control measures and reliable diagnostic markers of strain virulence.

The efficacy of many commercially available *S. suis* vaccines, usually based on formalin killed whole-cell preparations known as bacterins, is low due to the fact that protection is only limited to the serotype present in the vaccine [129]. Also autogenous vaccines, generated using cultures of *S. suis* isolated from infected pigs at specific farms have similar limitations. For instance, in specific-pathogen-free weaning piglets, serotype 2 based bacterins induced production of opsonizing antibodies and evoked protection against infection by *S. suis* but only if those *S. suis* belonged to serotype 2; no protection was provided against serotype 9 strains[130,131]. Interestingly, pigs vaccinated with formalin-killed encapsulated *S. suis* gave a full protection against strains of the same serotype, whereas killed unencapsulated strains conferred only a partial protection [132]. This result suggests that cell wall antigens may contribute to the induction of an immune response, making cell wall proteins possible vaccine candidates. However their cross-protection must be further investigated. Unfortunately, the methods used to quantify the protective efficacy of a vaccine against *S. suis* are not standardized, thus conflicting data have been reported for the same vaccine candidates [133,134].

Antimicrobial therapy is an important tool to treat and control streptococcal infections however, its use should be monitored to minimize spread of antimicrobial resistance genes. Penicillin is commonly used to treat *S. suis* infection but resistant strains have been isolated from pig carriers [135]. Moreover, resistance of porcine *S. suis* isolates to tetracyclines and macrolide antibiotics have been reported worldwide [136-138]. The growing increase in antibiotic resistance among *S. suis*

clinical isolates is a strong motivation to increase our understanding virulence and immunity for the development of new preventative and therapeutic strategies. The use of standardized infection models and the development of more efficient methods and tools for genetic manipulation are a priority target for the advancement of *S. suis* research. The discovery of natural competence in streptococcal species (reviewed below) raised the possibility that a functional competence system might also be found in *S. suis* and exploited for its genetic manipulation.

Natural Competence

Natural competence in the bacterial kingdom

The ability to transfer genetic information between organisms other than the ("vertically") passing on of genes from parents to offspring is called horizontal (or lateral) gene transfer (HGT). In the bacterial kingdom there are three main mechanisms for HGT: (I) transformation, which involves the uptake of short fragments of naked, exogenous DNA by naturally transformable bacteria; (II) transduction, which is the process by which DNA is transferred from one bacterium to another via bacteriophages; and (III) conjugation, which involves the transfer of DNA via cell-to-cell contact, by a cell surface-located needle or pilus. HGT has been proposed to be one of the major evolutionary forces driving bacterial gene flow between bacteria that are not associated by descent and their adaptation to new ecological niches [139,140].

As mentioned above, natural genetic transformation, or competence, is the capacity for uptake, processing and recombination of DNA present in the extracellular environment. The first experiment suggesting that bacteria are capable of transferring genetic information between different strains was performed with *S. pneumoniae* by the British bacteriologist Frederick Griffith in 1928 [141]. It was not until the late 1930s and early 40s that scientists showed isolated bacterial DNA was the "transforming principle" identified by Griffith.

To date, natural genetic transformation has been reported in at least 82 species, uniformly spread throughout the Kingdome of (Eu)Bacteria with an equal representation between Gram-positive and Gram-negative bacteria [142]; these numbers are likely to increase as competence systems are being found in taxa that had not been previously studied [143]. All transformable bacteria, with the exception of *Helicobacter pylori*, seem to share a common mechanism of DNA uptake and recombination involving the aptly named 'transformasome'. This multiprotein complex is well conserved in all naturally transformable Gram-positive bacteria including *S. pneumoniae* and ***B. subtilis*** and relies mainly on a set of genes co-expressed during competence [142].

Natural genetic transformation is believed to play a major role in bacterial evolution [144,145] and its impact on bacterial evolution has been extensively studied in *S. pneumoniae*. Chewapreecha and collaborators [146] have shown the huge contribution of genetic recombination via natural transformation, to the evolution of a pneumococcal population under antibiotic selective pressure [146]. Furthermore a retrospective comparative genomic analysis on the Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* (*Act*) revealed that in this species, competence is evolutionarily linked to genomic diversity and speciation [147]. Interestingly loss of competence has occurred frequently during evolution in *Act*, and has been followed by the loss of clustered regularly interspaced short

palindromic repeats (CRISPRs) involved in bacterial protection against foreign genetic elements such as plasmids and bacteriophages [148,149]. Jorth and Whiteley [147] have shown that competent *Act* bacteria have larger genomes containing multiple rearrangements while the genomes of non-competent strains are more stable but also more susceptible to infective DNA elements. Thus, the genetic diversity of competent *Act* strains is mainly driven by natural genetic transformation and in non-competent strains by transduction and/or DNA mobile elements [147].

Competence in the genus *Streptococcus*

The regulation and expression of the competence regulon relies, in several species, on a single master regulator, which controls transcription of a substantial part of all the genes involved in DNA uptake, process and recombination. In *Haemophilus influenzae*, and possibly in the entire Pasteurellaceae family to which this species belongs, competence regulon expression is regulated by Sxy, which mediates a response to nutritional deprivation [150,151]. In *Bacillus subtilis*, the competence master regulator is ComK, a transcription factor that controls expression of target operons in response to nutritional signals and peptide pheromones [152,153]. In the streptococci, the master regulator is the alternative sigma factor SigX (also known as ComX), of the Sigma-70 family. Two main types of pheromone regulatory systems have been identified that control *comX* expression in streptococci (Fig. 2a, b). The first is found in the *mitis* and *salivarius* groups, where *comX* expression is regulated by the ComCDE system, comprising the TCS ComDE and a peptide pheromone, ComC, also known as the competence inducing peptide (CSP). An exogenous processed form of ComC binds to the histidine kinase (ComD) promoting phosphotransfer to the cognate response regulator ComE (Fig. 2b). The phosphorylated form of ComE (ComE~P), forms a dimer altering its affinity for the operator site upstream of *comx*. Additionally, ComE~P autoregulates expression of the *comCDE* operon via a positive feedback mechanism to amplify induction of the competence state.

The second type of pheromone regulatory system for control of competence induction in streptococci is the ComRS-dependent induction of *comX*. In *S. mutans, S. thermophilus,* and *S. salivarius,* ComR, a transcriptional activator of the Rgg family, positively regulates expression of *comX* and *comS*, through allosteric interaction with the mature (processed) form of the pheromone encoded by *comS* [154-156] (Fig 2a). Both regulatory systems activate the transcription of *comX*-induced genes via interaction of ComX with the CIN-box (or Com-box) and RNA polymerase. The competence regulon includes operons encoding genes for assembly of a Type-4 pilus (T4P) like structure that is required for DNA uptake, and DNA recombination and repair enzymes [157-160].

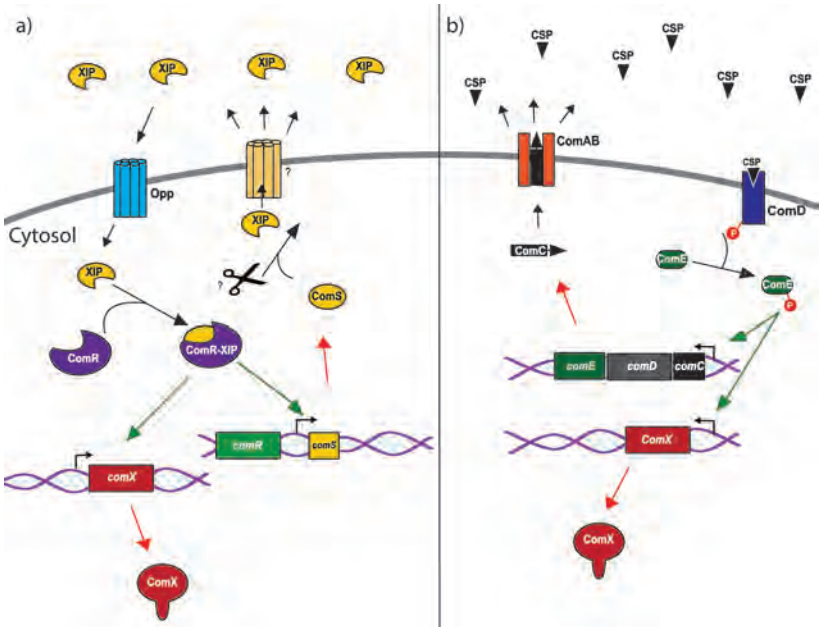

Figure 2. Schematic representation of the two different signalling mechanisms regulating competence in members of the genus *Streptococcus*. a) Regulation of natural transformation via ComRS signalling, present in the *salivarius*, *mutans* and *bovis* groups. The competence-inducing pheromone ComS is secreted by an unidentified transporter and is presumably processed into the mature pheromone SigX-inducing peptide (XIP) during its translocation across the cytoplasmic membrane. Extracellular XIP is imported into the cells by the oligopeptide Opp transporter. Inside the cell, it binds to and activates the transcriptional regulator ComR. Activated ComR binds to an inverted repeat motif, the CIN-box, in the promoter regions of the *comS* and *comX* genes, resulting in amplification of the XIP signal and expression of the late competence genes. b) Regulation of natural transformation via ComCDE signalling, present in the streptococcal *mitis* and *anginosus* groups. ComC is processed and secreted by ComAB into the competence stimulating peptide (CSP). Accumulation of CSP in the extracellular environment triggers the phosphorylation (P) of ComE by ComD, resulting in increased expression of *comCDE* and of *comX*, thereby promoting the amplification of the CSP signal and the expression of the late competence genes.

DNA transformation is an energy-consuming process with potentially adverse effect on bacterial genome integrity and cell division, therefore its induction is usually tightly regulated at different levels [161]. Competence induction is commonly dependent on bacterial density, environmental stress and composition of the medium [161-165].

Although the regulation of competence induction is well understood in different species, we still know relatively little about how competence is shut down. In *S. pneumoniae*, exit from competence seems to be regulated by multiple mechanisms including (I) degradation of ComX and ComW, a ComX-stabilizing protein, due to the protease activity of ClpE-ClpP and ClpC-ClpP [166,167]; (II) repression of *comCDE* by accumulation at high level of the unphosphorylated form of ComE [168]; and (III) interaction of DprA, a protein that helps the RecA-driven

DNA recombination, with ComE~ P, to block ComE-driven transcription [169]. In *S. mutans* and *S. thermophilus* the only mechanism known to facilitate competence exit is mediated by the adaptor protein MecA and relies on the degradation of ComX via the protease complex ClpC-ClpP [166,170,171].

In addition to the apparatus required for the homologous recombination, competence regulates secondary processes in some streptococci. One such process involves the production of bacteriolytic hydrolases also known as fratricins which cause lysis of non-competent strains. It is thought that lysis of non-competent strains functions to release DNA for uptake and transformation of competent strains. Fratricins are most active against bacteria that are closely related to the producer strains, thereby increasing the likelihood of acquiring DNA that is compatible with the genome of the acceptor strain [172-174]. Although the genes encoding fratricin are directly regulated by ComX, they are not required for DNA transformation in *S. pneumoniae* [175]. Conservation of this mechanism has been proposed in other streptococci based on gene homologies and the presence of CIN-boxes in promoter regions [176]. Recently, a bacteriocin-like molecule was identified that is induced by the competence-inducing peptide in *S. mutans*, causing autolysis in only a fraction of the population [177,178]. The existence of these complex competence-associated mechanisms for induction and control of competence and its secondary processes in many bacteria highlights the importance of competence for horizontal gene transfer and bacterial evolution. Moreover, the ability to induce competence in bacteria greatly accelerates generation of gene deletion mutants and enables high-throughput transposon-based methods to be used. Such approaches would be a major break-through in *S. suis* research. Moreover, knowledge on natural competence in *S. suis* would be relevant to appreciate potential for gene flow and the spread of antibiotic resistance genes. In this thesis research *in silico* methods were used to identify *com* regulons in *S. suis* genome sequences and experimentally investigate natural competence and its regulation.

Aims of the thesis

Inefficient DNA transformation and lack of convenient, standardized infection models have hindered genetic approaches and the investigation of *Streptococcus suis* pathogenesis and virulence.

The first aim was to explore the possibility of experimentally inducing natural competence in *S. suis* and to investigate its similarities and differences to other streptococcal species. Our second aim was to study the temporal regulation of competence and identify possible mechanisms of competence shut-down as well as potential secondary processes. We anticipated this knowledge would be useful to optimize experimentally induced competence and provide insights into the biological relevance of competence for *S. suis* in its natural habitats. The third aim was to develop a convenient, standardized infection model for *S. suis* using larval-stage

zebrafish. To demonstrate the usefulness of such a model for predicting virulence in the natural host we decided to test several strains and mutants with different virulence in pigs. The fourth aim was to use experimentally induced competence to construct deletion mutants in two previously uncharacterised TCS loci and investigate their role in virulence *in vitro* and *in vivo* using the new zebrafish larvae infection model.

Chapter 2

Control of competence for DNA transformation in *Streptococcus suis* by genetically transferable pherotypes

Edoardo Zaccaria[1], Peter van Baarlen[1], Astrid de Greeff[2], Donald A. Morrison[3], Hilde Smith[2], Jerry M. Wells[1*]

[1]Host-Microbe Interactomics, Animal Sciences, Wageningen University, Wageningen, The Netherlands, [2]Central Veterinary Institute, Animal Sciences, Wageningen University, Lelystad, The Netherlands, [3]Biological Sciences, University of Illinois at Chicago, Chicago, Illinois, United States of America

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Abstract

Here we show that *S. suis*, a major bacterial pathogen of pigs and emerging pathogen in humans responds to a peptide pheromone by developing competence for DNA transformation. This species does not fall within any of the phylogenetic clusters of streptococci previously shown to regulate competence via peptide pheromones suggesting that more species of streptococci may be naturally competent. Induction of competence was dependent on ComX, a sigma factor that controls the streptococcal late competence regulon, extracellular addition of a SigX-inducing peptide (XIP), and ComR, a regulator of *comX*. XIP was identified as an N-terminally truncated variant of ComS. Different *comS* alleles are present among strains of *S. suis*. These *comS* alleles are not functionally equivalent and appear to operate in conjunction with a cognate ComR to regulate *comX* through a conserved *comR*-box promoter. We demonstrate that these 'pherotypes' can be genetically transferred between strains, suggesting that similar approaches might be used to control competence induction in other lactic acid bacteria that lack ComR/ComS homologues but possess *comX* and the late competence regulon. The approaches described in this paper to identify and optimize peptide-induced competence may also assist other researchers wishing to identify natural competence in other bacteria. Harnessing natural competence is expected to accelerate genetic research on this and other important streptococcal pathogens and to allow high-throughput mutation approaches to be implemented, opening up new avenues for research.

Introduction

In recent years the acceleration in sequencing of bacterial genomes has made genome sequences available for multiple strains in many species. Comparative analysis of genomes has revealed an important role of horizontal gene transfer (HGT) in the evolution of bacterial genomes [142,179,180]. The transfer of genetic information between strains and even species of bacteria can give rise to quantum evolutionary leaps that increase bacterial fitness and their capacity to colonize new ecological niches. Most bacteria appear to be able to transfer genetic information via mobile genetic elements such as bacteriophages, plasmids, or transposons, while to date only about 82 bacterial species have been documented to be naturally transformable [142,181]. Nonetheless, natural competence is conserved in members of at least six different bacterial phyla suggesting that it is very old in evolutionary terms [143,181]. This includes the genus *Streptococcus*, one of the most abundant genera in the human small intestine, oral cavity and pharynx [182,183]. In the gut HGT and natural competence are likely to have played a major role in the evolution and ecology of the microbiota as well as strain diversity [184]. The competent state of ''DNA receptivity'' is controlled by a large, dispersed set of genes encoding proteins responsible for DNA uptake and recombination, as well as other processes related to competence induced cell stress [151,185-189]. Homologues of competence genes are widespread among bacteria that have not yet been demonstrated to be naturally transformable, suggesting that the mechanism may in fact be more common, especially within the phyla that contain members that are already known to be naturally competent. In some bacteria, the competence genes may have become non-functional or evolved such that they are now involved in different processes. An alternative explanation for the presence of competence genes in apparently non-competent bacteria is that they are indeed transformable but only when the right growth conditions and/or specific environmental cues are present. *Streptococcus thermophilus* possesses functional homologues of competence genes found in *Streptococcus pneumoniae*, with the exception of *comCDE*, encoding a two-component regulator and competence pheromone of the pneumococcal competence regulon [189]. However, spontaneous competence development in this species was only recently observed, during early exponential phase growth in a synthetic medium, suggesting that competence in this species is regulated by an alternative mechanism to *comCDE* [190]. Indeed, a novel oligopeptide competence pheromone (XIP) characterized by a double-tryptophan motif near the C-terminus [154], as well as its intracellular target protein (ComR) were subsequently identified in this species. The current model predicts that the oligopeptide pheromone is presumably internalized by an Opp transporter enabling it to bind ComR, thereby altering the affinity of this transcription regulator for an inverted repeat motif upstream of the *comX*, encoding an alternative sigma factor. Binding of ComR to the *comX* promoter is then predicted to initiate expression of

the ComX sigma factor that associates with the RNA polymerase core and binds to the promoters regulating the late competence genes. Mashburn-Warren et al. [154] demonstrated that a system closely resembling the one operating in *S. thermophilus* is present in *S. mutans* and that homologues of *comS*, *comR* and *comX* are present in streptococcal species of the *pyogenic* and *bovis* groups [155].

In the streptococcus phylogenetic tree, 36 species are grouped into six main clusters with the exception of *S. suis* and *S. acidominimus* [191]. *S. suis*, like its more distant relatives in the genera *Lactococcus* and *Lactobacillus*, possesses homologues of all the streptococcal competence genes involved in DNA uptake and recombination including *comX*, the master regulator of competence [181,192]. Yet, natural competence development has not been demonstrated in *S. suis* despite a growing body of research on this important zoonotic pathogen [50]. To investigate the possibility that a natural competence system exists within *S. suis* we mined its genome for sequence motifs conserved in the *comX* promoter regions. Here we report results of genomic analysis of predicted promoter sequence patterns of *comX*, and identification of genes encoding an Rgg-like transcriptional regulator ComR, and a cognate pheromone, ComS, in *S. suis*. Moreover, we identified conditions suitable for efficient pheromone activation of natural competence in 5 different serotypes and around 60% of *S. suis* isolates that were otherwise poorly or not at all transformable. This discovery will open up new avenues for genetic analysis of this important pathogen and should assist in discovery of natural competence systems in other bacteria.

Materials and Methods

Bacterial strains, plasmid and growth conditions

The bacterial strains and plasmids used in the present study are listed in Table S3. *S. suis* strains were grown in Todd-Hewitt broth (THB) (Difco) at 37°C under 5% CO_2. When required, chloramphenicol (5 mg/ml) or spectinomycin (100 mg/ml) was added to the media. Solid agar plates were prepared by adding 12 g/L agar to the medium.

Genome analysis

BLAST searches with *S. suis* genome sequences were performed using non-redundant sequences accessible at the National Centre for Biotechnology Information internet site (http://www.ncbi.nlm. nih.gov). Specific promoter patterns in nucleotide sequences were identified using the Fuzznuc program in the EMBO Open Software suite [193]ÿÿÿÿ□?. Comparative genome analysis among *S. suis* strains was performed using MicrobesOnline (http://www. microbesonline.org) [194] . Sequence alignments were made using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index. html). Peptide alignments were annotated using CHROMA [195].

Preparation of synthetic peptides

Peptides were purchased from JPT Peptide Technologies (Berlin, Germany) at purity grades of 62–89%. Transformation efficiencies might have been higher with highly pure peptides as the preparations used in this study were between 62 to 89% pure. Stock solutions were dissolved in Milli-Q water at a final concentration of 5 mM, taking in consideration their specific purity. Stock solutions were stored in 50 ml aliquots at 280°C.

Natural transformation experiments

S. suis strains were grown overnight in THB broth at 37°C under 5% CO_2. The overnight culture was diluted 1:40 into similar pre-warmed media, and grown at 37°C without shaking. 100 ml samples were removed from the main culture after 1 hour (with an $O.D._{600\ nm}$ between 0.035 and 0.058).

Donor DNA (1.2 mg of pNZ8048) in EB buffer (10 mM Tris-Cl, pH 8.5) was added to the bacteria along with 5 ml of stock peptide at a final concentration of 250 mM. After 2 hours of incubation at 37°C under 5% CO_2 in 1.5 ml Eppendorf Safe Lock TubesTM, the samples were diluted and plated in THB agar plates with the required antibiotics.

For the study of the kinetics of competence induction, plasmid DNA was added at the indicated times after ComS13-21 followed 5 min later by addition of DNase at a concentration of 100 U/ml (Qiagen Ltd,. Crawley, UK) to limit exposure to transforming DNA to 5 min.

Insertional inactivation of apuA

Primers used for mutagenesis are listed in Table S3. The plasmid pG9-*apuA*::*spc* [33] was used as a template for PCR to obtain a linear DNA fragment carrying the *apuA* gene interrupted by the spectinomycin cassette. 1 mg of the purified fragment was used to transform *S. suis* strain 10 with selection on agar containing 100 mg/ml of spectinomycin at 37°C; the double crossover events were verified by PCR using the primer pairs CtrlMutA1/CtrlR1 and CrtlMutB1/CtrlR2.

To study the effect of length of the homologous arms to the transformation efficiency four different PCR fragments with increasing length were synthesized using the primer pairs described in Table S3.

Construction of mutant strains

Mutants in *comX* and *comR* were constructed by transformation of *S. suis* S10 with linear DNA fragments comprising the spectinomycin resistance flanked by about 1 kb of DNA with homology to sequences adjacent to the target gene. The linear DNA fragments were generated by SOE-PCR using the primers listed in Table S4.

Results

Identification of an alternative sigma factor gene (*comX*) and a conserved *comX* promoter in *S. suis*

Inspection of the *S. suis* strain P1/7 genome revealed a single homologue of *comX* (SSU0016) (Table S1) with highest similarity to *comX* of *S. pneumoniae* (46.36%) and *comX* in *S. mutans* (43.23%), both of which mediate expression of the late competence regulon [196]. The *S. suis comX* locus bears strong similarity to the *comX* loci in *S. sanguinis*, *S. pneumoniae*, and *S. gordonii*, with conservation of upstream genes encoding MesJ, hypoxanthine-guanine phosphor-ibosyltransferase (Hpt) and the cell division protease FtsH, and of downstream genes encoding 16 S ribosomal RNA (Fig. S1). In contrast, sequence analysis of the *comX* promoter in *S. suis* revealed a high conservation with *comX* promoters in the *bovis* and *pyogenic* groups (Fig. 1). A predicted Pribnow box (-10 element) of *S. suis comX* lies 31 nt upstream of the initiation codon but, as with the other *comX* promoters, the canonical -35 hexamer is missing. The direct-repeat binding site of the competence regulator *comE* is also missing, replaced by the sequence TGTC C/A TGT T/A, characteristic of the ComR box binding site in the *mutans*, *bovis*, and *pyogenic* groups [197,198]. In *S. suis* the consensus ComR box at the *comX* promoter differs in the most distal inverted repeat sequence upstream of *comX*, which in the *bovis* and *pyogenic* streptococcal species forms part of the 'stem' of the transcription terminator for an upstream tRNA gene.

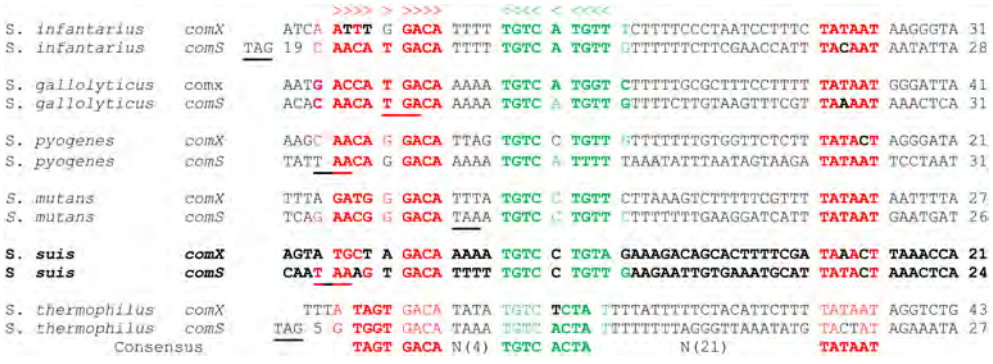


Figure 1. Comparison of S. suis comS and comX (sigX) promoter regions with those of bovis, pyogenic, mutans, and salivarius groups. The Stop triplet of ComR is underlined. Green and red formatted sequences in the comR-box highlight RNA base-pairing in a potential stem-loop structure. The conserved Pribnow box (TATAAT) at −10 is highlighted in red. Note that the core 12 bases of the ComR box are shared by all species. The distance to the start codon of comS or comX is indicated by the numbers at the far right.

Identification of a comR/comS locus in *S. suis*

As the ComR box (Fig. 1) is instrumental to the regulation of early competence genes *comX* and *comS* in *S. mutans* [154] and *S. thermophilus* [189], we searched the *S. suis* genome for ComR box- containing promoters, using the consensus sequence AAAG N(1, 4) GACA N(4) TGTCCTG N(20) TA N(3) in the Fuzznuc program in the EMBO Open Software suite [193] . This pattern was found in a 405-nt intergenic region between a putative transcriptional regulator (SSU0049) and a transposase gene (SSU0051), in an organization resembling the *comRS* locus of the early competence regulatory systems in *S. pyogenes* and *S. mutans* [154,155]. This led us to search for *comS*-like open-reading frames downstream of the apparent ComR box. We identified a 63-nt ORF predicted to encode a 21-residue peptide (SSU0050) that was not annotated in GenBank. The conserved genomic context of *comR* and *comS* in *S. mutans* and *S. suis* suggested that SSU0050 could be a *comS* gene. Furthermore the putative *comS* ORF in *S. suis* shared similar amino acid sequence properties to the *S. mutans* ComS [154] (Fig. 2). All *comS* products have a net positive charge (except in the case of *Streptococcus gallolyticus*) and all the competence inducing peptides (XIPs) identified in *bovis* and *pyogenic* streptococci and *S. mutans* contain a double-tryptophan (WW) motif near the C-terminus (Fig. 2). The C-terminus of the predicted ComS in *S. suis* however, contains two tryptophan residues separated by 2 other amino acids: W-G-T-W. The ComS sequence is identical in genomes of six of the seven *S. suis* serotype 2 strains known to date. In the serotype 7 strain D9, *comX*, *comR* and the *comX*-like promoters are conserved, but the putative competence pheromone contains a double-tryptophan at the C-terminus, as in the *pyogenic*, *mutans*, and *bovis* groups.

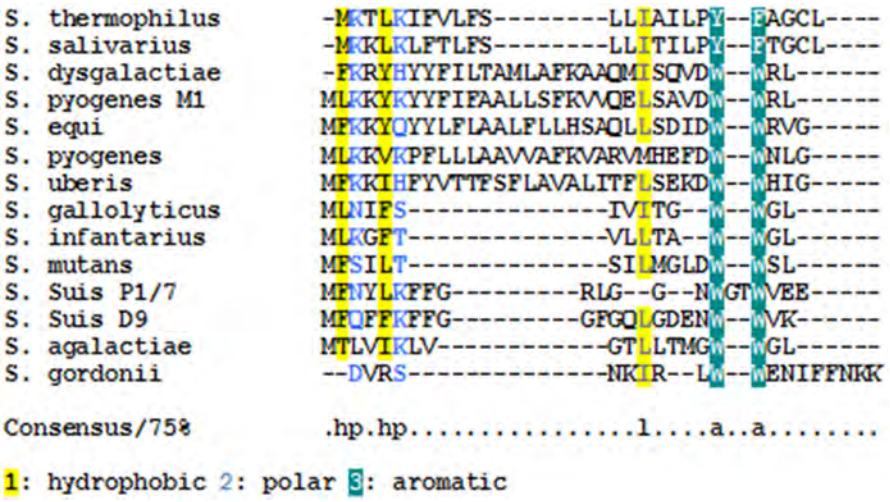
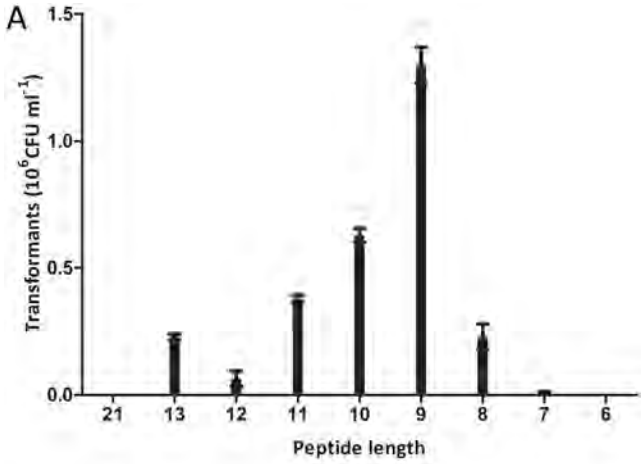


Figure 2. Protein sequences and chemical properties of the known or putative competence pheromones.

Competence development in *S. suis* is induced by extracellular ComS derivatives and depends on ComR and ComX

To test whether *S. suis* strain S10 possesses a ComRS-regulated competence system, we added a synthetic 21-amino acid (aa) peptide based on the predicted open reading frame of *comS* (SSU0050) to low density logarithmic phase cultures of *S. suis* in THB for a 2-hour incubation with 10 ng/μl of the broad host range plasmid pNZ8048. We also tested N-terminal truncated variants of the predicted ComS, as many streptococcal peptide pheromones undergo N-terminal processing to generate the active form of the peptide [152,193,199,200]. As shown in Fig. 3A and B, competence for transformation was maximal with ComS13-21 (GNWGTWVEE), suggesting that ComS13-21 is, or closely resembles, the active form of this pheromone. Neither full-length ComS nor the ComS16-21 variant gave rise to transformants. Transformation was abolished in a *comR* KO mutant under all conditions tested, supporting the hypothesis that the competence- inducing properties of ComS peptides depend on ComR. Furthermore, no transformants were obtained using the same procedure with a mutant deficient in *comX*, which in other naturally transformable streptococci regulates expression of genes involved in DNA uptake and recombination [196]. Together, these experiments demonstrate that the 9-amino-acid ComS13-21 stimulated development of a high level of competence in *S. suis* and that the *S. suis* homologues of ComR and ComX are necessary for transformation.



B

Length	Peptide sequence
21	MFNYLKFFGRLGGNWGTWVEE
13	GRLGGNWGTWVEE
12	RLGGNWGTWVEE
11	LGGNWGTWVEE
10	GGNWGTWVEE
9	GNWGTWVE
8	NWGTWVEE
7	WGTWVEE
6	GTWVEE

Figure 3. Truncated variants of the predicted ComS peptide and their effect on transformation efficiency. A) Induction of competence by the truncated variants (from 13 to 6 amino acids) of the predicted ComS at 250 µM peptide and 10 ng/µl of the plasmid pNZ8048. Error bars show the standard deviation of 4 different experiments. B) Sequences of full length and truncated variants of the competence inducing peptide.

The effects of varying concentrations of peptide and transforming DNA were investigated using ComS13-21 and low-density logarithmic cultures. Transformation efficiency increased with DNA concentration, giving 1.4 million CFU transformants per ml with 10 mg DNA of pNZ8048, as replication mode plasmid [201] (Fig. 4A). The number of transformants also increased with peptide concentration, approaching saturation above 0.2 mM peptide (Fig. 4B). The optimal peptide concentration was higher than previously described for other streptococci [155,202]. Possible factors affecting efficiency of peptide-induced competence have been documented in previously [203].

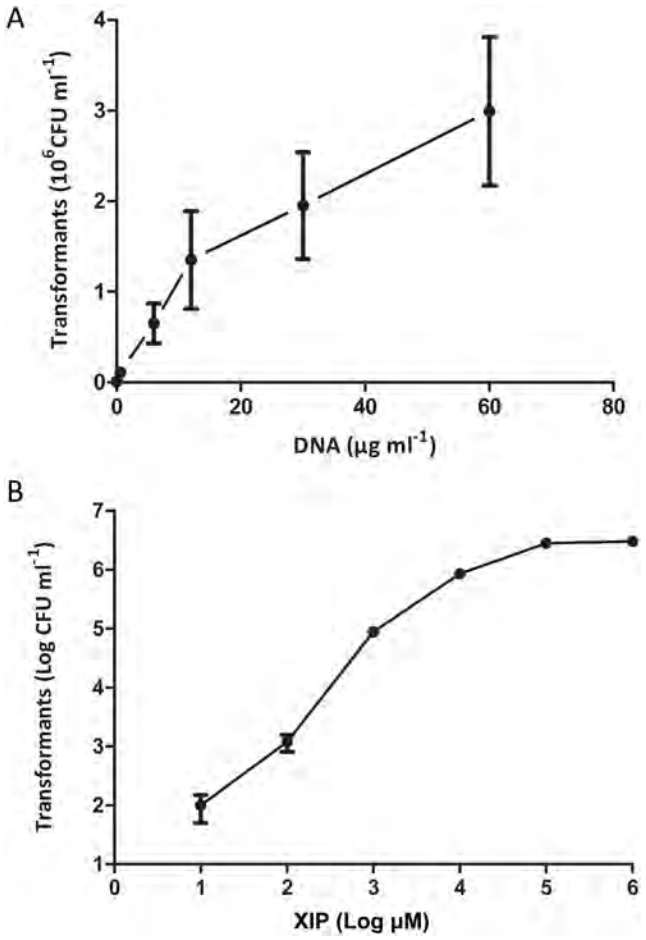


Figure 4. Effect of DNA concentration (A) and competence pheromone concentration (B) on competence development. The experiments were performed using Com13-21 and low-density logarithmic cultures. Error bars show the standard deviation of 4 different experiments.

The susceptibility to peptide pheromone is transient and optimal at low bacterial densities

In other naturally transformable bacteria the state of competence may be transient, depending on the growth conditions and bacterial density in logarithmic growth phase. Commonly, however, this density dependence largely disappears when synthetic peptide is used to obviate the cell-to-cell communication. We determined the transformation efficiency in the presence of the synthetic peptide at different optical densities (O.D. 600 nm) between 0.01 and 0.30 (Fig. 5A) during outgrowth of an overnight culture inoculum. Competence development occurred only within a

narrow window of bacterial densities (O.D. 0.03 to 0.06), with maximal transformation efficiency at O.D. 0.042 (Fig. 5A). To understand better the kinetics of competence development, we determined transformation frequency of bacteria (O.D. 0.04) at different time-points after addition of ComS13-21 by using 5-min exposure to donor DNA followed by DNase treatment (100 U/ ml). The transformation efficiency was low after 5 min incubation with peptide, maximal after 15 min, and then declined to zero by 45 min, suggesting that feedback mechanisms and/or changes in the bacterial density lead to a rapid loss of the capacity for DNA uptake or recombination (Fig. 5B). The large yield of transformants obtained after such brief exposure to DNA indicates that *S. suis* possesses the capacity for a high level of natural competence, with an efficient DNA uptake mechanism.

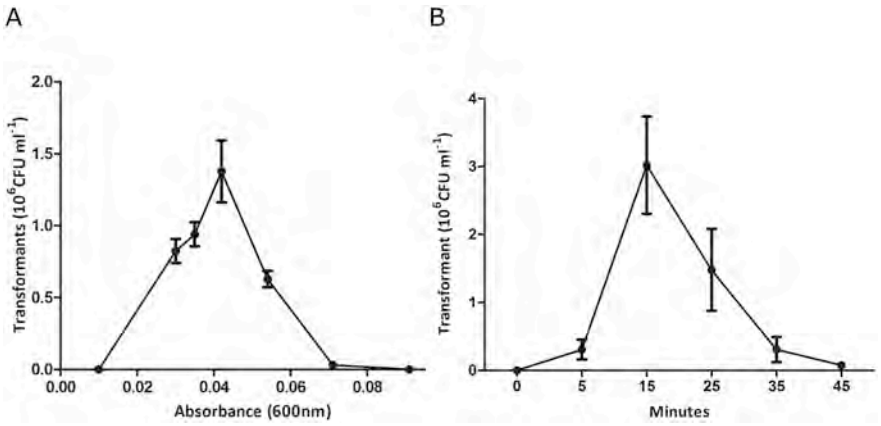


Figure 5. The susceptibility to peptide pheromone is transient and optimal at low bacterial densities. A). Effect of bacterial optical density (O.D. 600 nm) on competence development using a concentration of 10 µg/ml of plasmid DNA (pNZ8048). Competence was not induced over a range of OD values up to 0.3, suggesting that waves of competence do not occur. B). Kinetics of competence induction with plasmid DNA added at the indicated times after ComS13-21 followed 5 min later by addition of DNase (100 U/ml) to degrade any remaining extracellular donor DNA. Error bars show the standard deviation of 4 different experiments.

Exogenous linear and plasmid DNA transform *S. suis* in the competence state

Having established conditions that favor natural competence of *S. suis* by optimizing a transformation protocol using plasmid DNA, we next investigated the efficiency of transformation with linear DNA. For this purpose, we used linear PCR-amplified DNA fragments containing a spectinomycin resistance cassette flanked by sequences identical to 59 and 39 coding regions of the *S. suis apuA* gene [33]. To investigate the influence of the length of the homologous flanking DNA on transformation efficiency, the homologous flanking DNA region was varied in length from 250 to 1500 nucleotides. *S. suis* was transformed with the purified PCR products at the concentration of 10 mg/ml in triplicate experiments (Fig. 6). The strong positive

influence of the length of the flanking homologous segments on integration efficiency, similar to that reported previously for *S. pneumoniae* [204], suggests that the mechanism of recombination resembles that in *S. pneumoniae*, and provides a practical guide for design of gene-replacement donors in *S. suis*.

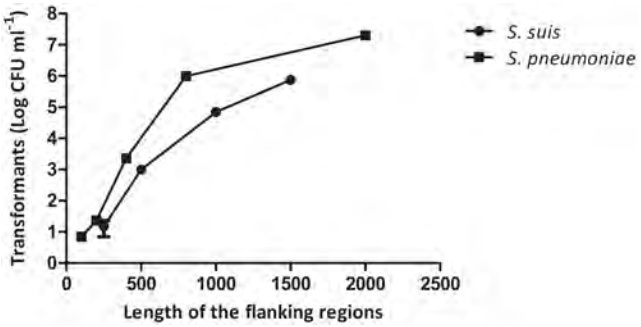


Figure 6. Influence of the length of the homologous flanking DNA on transformation efficiency with linear DNA. The S. pneumoniae data was obtained from Lau, at al 2001 [204]. Error bars show the standard deviation of 4 different experiments.

Early competence regulation is pherotype-specific and genetically transferable

We tested the possibility of inducing competence for DNA transformation in different strains and serotypes of *S. suis* using the ComS (aa13-21) pheromone from strain 10. The ability for competence induction was not serotype specific and 10 of the 15 strains could be transformed by the peptide pheromone of strain S10 (Table S2). The genomes of strain S10 and one of the untransformable serotype 7 strains (isolate 7 in Table S2) revealed differences in the sequence of *comS* and *comR*, suggesting that competence is regulated by different peptide pheromones (or XIPs) in some strains that we refer to here as 'pherotypes'. The coding sequence of the two ComR DNA-binding domains from serotype 2 and serotype 7 strains were identical; all the sequence variation was present in the C-terminal half of the gene, which is hypothesized to interact with XIP. DNA transformation was not induced in serotype 2 strain using the XIP from serotype 7 but became possible after allelic replacement with the serotype 7 *comR*/*comS* (Fig. S2). As a consequence of this allelic exchange the serotype 2 strain also lost its ability to become competent for DNA transformation with serotype 2 XIP (Fig. S2). These results demonstrate that the *comR*/*comS* early competence switch can be used to genetically transfer pheromone-specific induction of competence to heterologous strains possessing *comX* coupled to a canonical *comR* promoter, *comX* and the downstream genes necessary for natural competence.

Discussion

Representative species of the *mitis*, *salivarius*, *mutans*, *pyogenic*, and *bovis* phylogenetic clusters of streptococci have all been shown to control activity of ComX, a master regulator of bacterial competence, via small peptide pheromones [154,202,205-208]. Here we show that *S. suis*, a streptococcal species which does not appear to fall within any of these phylogenetic clusters, also responds to a peptide pheromone by developing competence for DNA transformation. This finding does suggest that additional streptococcal species might also regulate competence via peptide pheromones (Fig. 7). The competence system in *S. suis* was discovered by searching the genome for the conserved promoter elements found upstream of *comX* and *comS* in *S. thermophilus* and *S. mutans* [154,155]. Two such promoter regions were identified in the *S. suis* genome (Fig. 1). Downstream of one promoter we identified a homologue of *comX*, the alternative sigma factor that plays a fundamental role in the competence system in *S. thermophilus* and *S. mutans*. Downstream of a second promoter was a small ORF encoding a potential competence pheromone propeptide that we later designated ComS to highlight its homology with the ComS of *S. thermophilus* and *S. mutans*. In the *bovis*, *pyogenes* and *mutans* species of streptococci the competence propeptide sequence is highly variable except for the presence of two adjacent tryptophan residues near the C-terminus which appear to be essential for competence induction in *S. mutans* [154] (Fig. 3B). In *S. suis* serotype 2 there are also two tryptophan residues near the C-terminus of the competence peptide but they are separated by two different amino acid residues. As the full length 21-aa ComS propeptide of *S. suis* did not induce competence for DNA transformation under our standard conditions, we hypothesized that the propeptide needed to be processed into a biologically active form, and thus tested several N- terminal deletion variants. Competence induction was optimal using the C-terminal 9 residues of ComS, suggesting that the propeptide contains specific N-terminal sequences that determine processing and secretion steps.

In *S. mutans*, ComS is processed to form a 7-aa *comX* inducing peptide [209], which has been detected extracellularly in chemically defined medium. Poor activity of the propeptide ComS itself indicates that it is processed before or during transport across the cytoplasmic membrane [203], via unknown mechanisms. It is likely that a similar mechanism occurs in *S. suis* because truncated variants of ComS but not the full length peptide induce competence for transformation when added exogenously. Moreover *S. suis* contains a predicted orthologue of *S. thermophilus* Eep, data not shown, which is involved in processing of the competence peptide [203]. The putative ComS propeptides of *S. pyogenes*, *S. uberis*, *S. dysgalactiae* and *S. equi* possess a basic N-terminus and hydrophobic central core, which are characteristic of type 2 signal secretion leaders, although the polar C-terminus is absent [210]. In contrast the ComS propeptides encoded by *S. suis* and *S. mutans*

are hydrophobic shorter versions of those found in streptococci in the *pyogenic* and *bovis* groups (17–22 residues) and do not contain N- or C-terminal signatures associated with signal secretion leaders. The existence of propeptides with different chemical properties suggests that these propeptides might be secreted and processed by different mechanisms.

The competence-inducing peptides of *S. mutans* and *S. thermophilus* are imported into the cytoplasm by the ATP- dependent multi-subunit transporters known as Opp or Ami [154,190,197,201]. As the competence-inducing peptide of *S. suis* functions when added exogenously, we predict that the homologues of the *S. suis* Opp multi-subunit transporter (Table S1) are also involved in import of XIP into the cytoplasm for access to ComR.

This study builds on the discovery of a pheromone-regulated natural competence system in *S. mutans* [154] and *S. thermophilus* [155,203] and exemplifies the approaches that can be taken to identify competence regulatory circuits in bacteria possessing homologues of *comX*. Many streptococcal competence systems similar to those of *S. mutans* and *S. thermophilus* possess a conserved ComR box promoter upstream of both *comX* and *comS*. The cognate *comS* ORFs are frequently not annotated due to their small size and can be difficult to identify in bacterial genomes. However homologues of *comX* are commonly annotated when present and, as we show here, genome homology searches with the promoter regions upstream of *comX* can help to identify ORFs encoding ComS peptides. As N-terminal truncations may be necessary for ComS to induce competence, a series of truncated variants of ComS should be tested at concentrations up to 250 mM. Roadblocks may well remain in determining the optimal environment for competence development. For example, the response to XIP requires expression of peptide transporters and of *comR*, as well as freedom from interfering activities such as proteases or nucleases. Therefore ComS peptides should be tested with bacteria grown in a variety of culture media and in multiple phases of growth [209].

ComR has been shown to be necessary for ComS to induce competence and on the basis of its sequence similarity to the PlcR transcriptional regulator we assume that it also forms a dimer or tetramer by directly binding ComS13-21, to form an active transcriptional activator. In *S. suis*, *S. mutans* and *S. thermophilus* ComR is annotated as an Rgg–like transcriptional regulator of which there may be several paralogues in a bacterial genome [207,208]. In the *comRS* regulatory circuits identified to date *comR* was upstream of *comS* but in other organisms it may be at a distant locus. It is also possible that *comR* itself may be regulated by environmental factors highlighting again the need to test for competence induction using a range of growth conditions including multiple carbon sources. An alternative strategy is to increase copy number of *comR* by transfer to a plasmid, which in *S. mutans* increased transformability in the presence of *comS* [154].

The existence of different pherotypes divides competent *S. suis* into

bacterial populations that allow pheromone communication to take place only within, not between, populations. Nevertheless, pherotype switching could occur by uptake of DNA and gene replacement within the species or even between species that utilize the *comS*, *comR* regulatory switch. Thus pherotype switching may be a biologically important mechanism, that enables different species to be induced to competence by the same XIP. Our finding that genetic transfer of *comS*/*comR* from an XIP-responsive strain to a non-responsive strain confers XIP-specific induction of competence suggests an approach to regulate competence development in the species of lactic acid bacteria that appear to possess homologues of *comX* and the late competence genes found in naturally competent streptococci but no homologues of the *comR*/*comS* early competence switch (Table S1). This would require coupling of the canonical *comR* promoter to the *comX* of the host species and introduction of *comR*/*comS* from bacteria with demonstrated natural competence such as *S. suis*. Nevertheless, additional factors may be required for activation as previously shown for *S. pneumoniae* where ComW is required for stabilization and activation of the alternative sigma factor ComX [211]. The current low-efficiency genetic approach to manipulate *S. suis* requires use of *E. coli* shuttle vectors and suicide vectors. Thus there was an urgent need for novel methodology that would allow more rapid genetic manipulation in this important zoonotic pathogen, which is the major cause of bacterial meningitis in adults in Vietnam [13]. *S. suis* provokes more than 300 million dollars of economic losses in the USA pork industry alone [50]. The competence system identified in this paper allows high frequency of transformation of *S. suis* and the possibility to use linear DNA fragments assembled using common PCR-based approaches for rapid targeted gene modification. This will overcome existing problems with low transformation efficiency. Routine genetic manipulation and gene deletion in *S. suis* would allow high throughput mutation approaches to be implemented, opening up new avenues for research on this important pathogen.

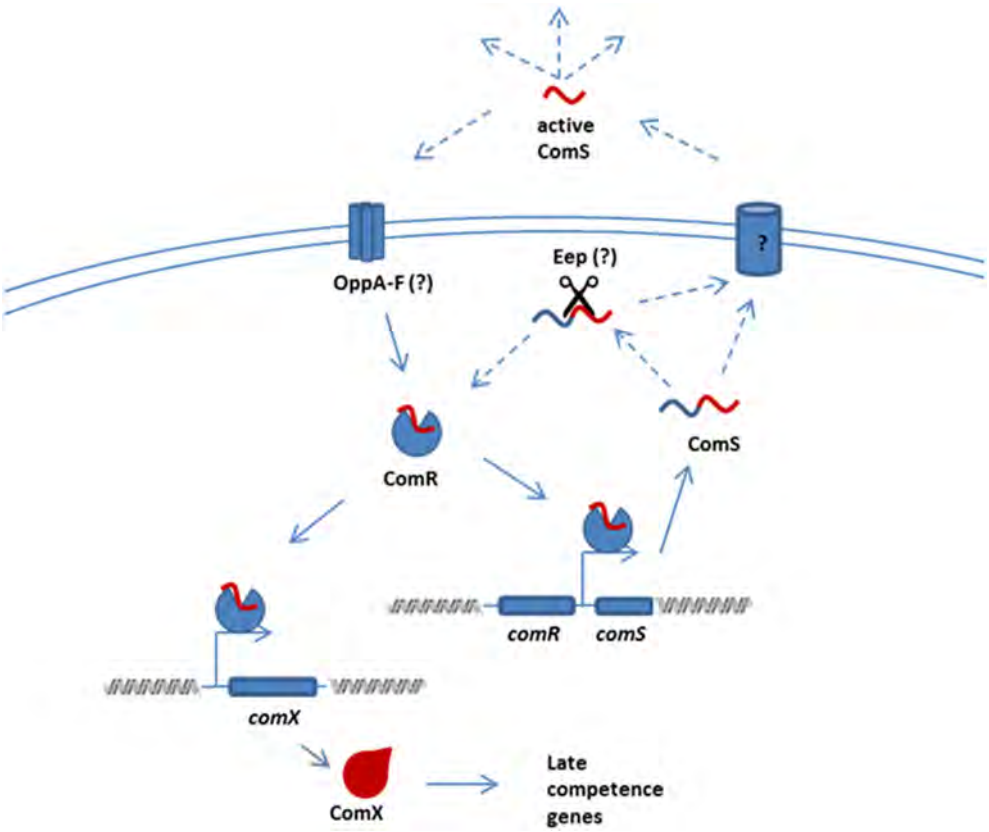
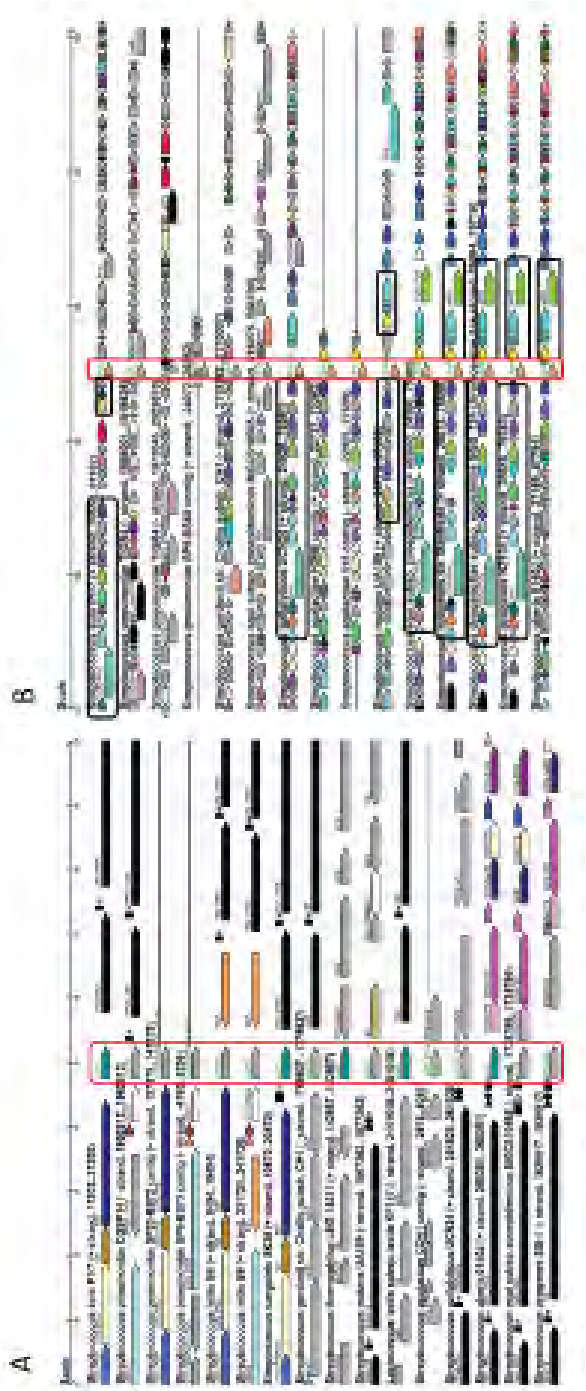


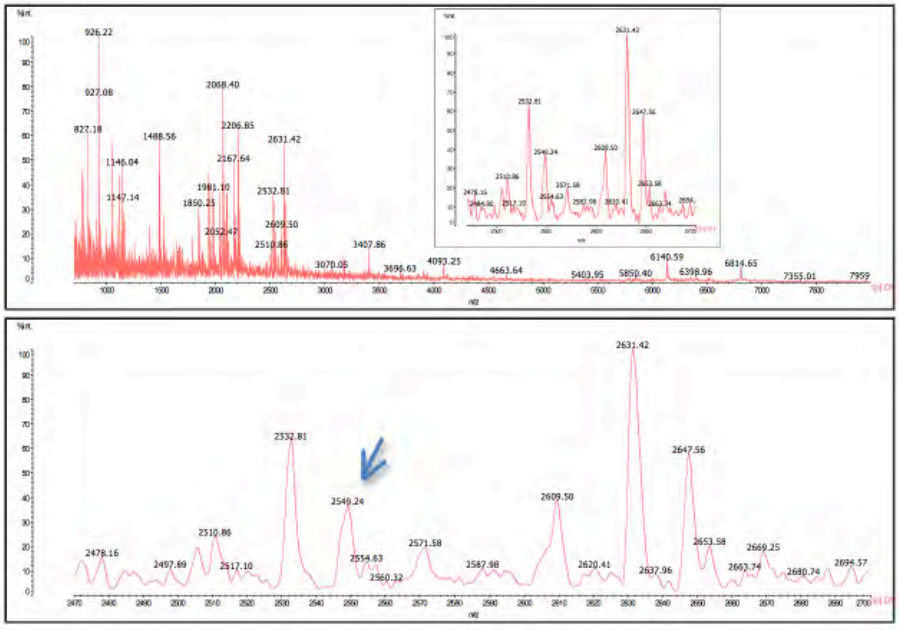
Figure 7. Hypothetical model of competence induction. Given appropriate growth conditions, the transcription of comS is activated and the propeptide is produced. ComS is processed before or during transport and its mature form accumulates in the extracellular environment. Uptake of the competence inducing peptide is mediated via an Opp transporter. Intracellular competence inducing peptide activates ComR by binding to its peptide-binding domain leading to transcription of comS and of comX, the master regulator of the late competence genes involved in the uptake, processing and integration of the extracellular DNA. This hypothetical model of competence induction in S. suis based on published data and data presented in this paper.

Supporting Information



Figure_S1. Schematic view of *comX* and *comRS* regions in *S. suis* and other streptococcal species.
A) Overview of the *comX* region in the genome of different streptococcal species. The *comX* sequences have a sequence identity greater than 37%. Orthologous genes are represented with the same colors if amino acid identity was greater than 50%. B). Overview of the *comRS* locus in *S. suis* and in other representative streptococcal species. The *comR* sequences have a sequence identity greater 37%. Orthologous genes are represented with the same colors if amino acid identity was greater than 50%. (Red box): *comR* genes, (black boxes): conserved genes surrounding *comR*.

A.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927.08
827.18
1488.56
2068.40
2206.85
2631.42
1146.04
2167.64
1147.14
1981.10
1850.25
2332.81
2609.50
2052.47
2510.86
3407.86
3070.05
3696.63
4093.25
4663.64
5403.95
5850.40
6140.59
6398.96
6814.65
7355.01
7969
2631.42
2332.81
2647.56
2549.24
2609.50
2510.86
2478.16
2497.89
2517.10
2554.63
2571.58
2560.32
2587.98
2620.41
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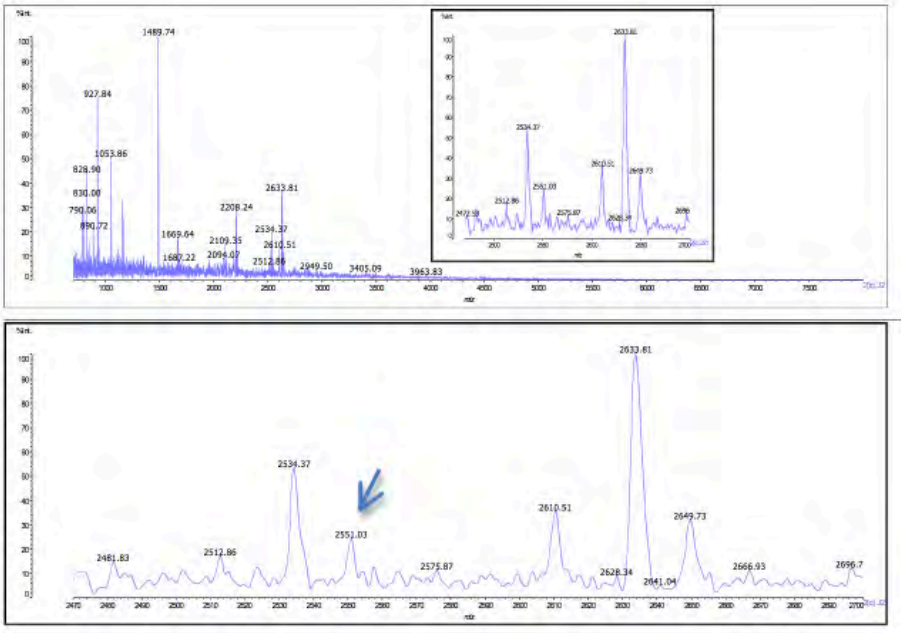
2653.58
2669.25
2663.74
2680.74
2694.57

B.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927.84
1053.86
828.90
830.00
790.06
890.72
2633.81
2208.24
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2109.35
2610.51
1687.22
2094.07
2512.86
2949.50
3405.09
3963.83
2633.81
2534.37
2610.51
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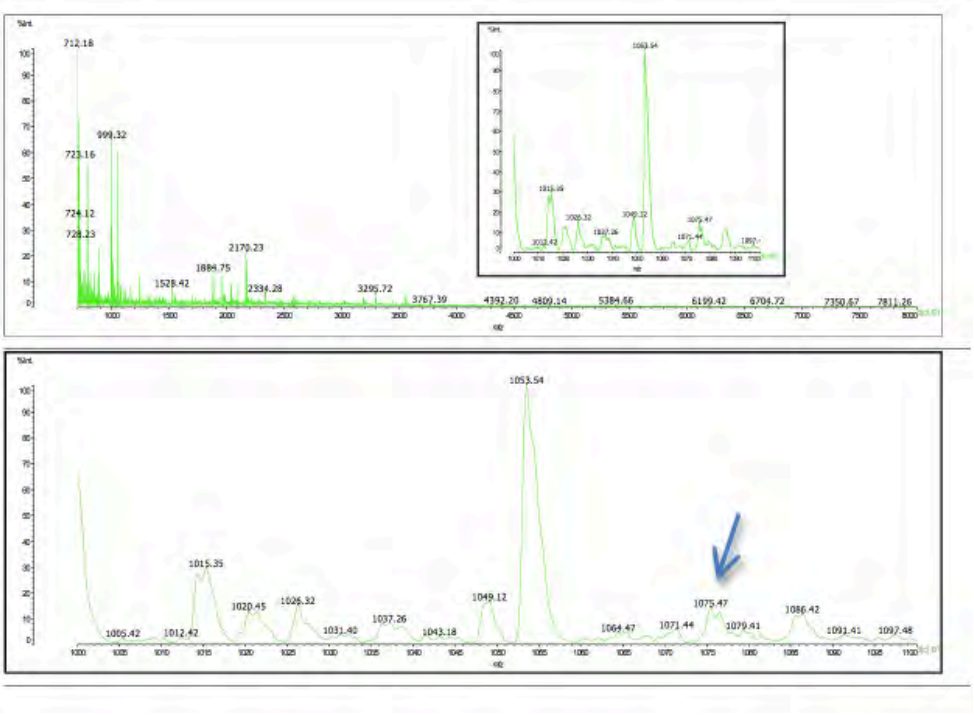
2481.83
2512.86
2575.87
2628.34
2641.04
2666.93
2696.7



Figure_S2. MALDI TOF Mass Spectra of peptides from bacterial cell pellet (A) and *S. suis* culture supernatant (B and C). In all panels the top mass spectrum shows sample complexity in the range 700 Da to 8000 Da. The inserts show an expanded spectrum from 2470 Da to 2700 Da (A and B) and 1000 to 1100 (C). The bottom mass spectrum is expanded to show the putative full length ComS (arrowed in A and B) and truncated form of ComS inducing competence for transformation (arrowed in C). Samples for mass spectrometry were passed through a C18 Solid Phase Extraction (SPE) column and the resulting eluents applied to an analytical Reversed Phase HPLC column running an 25-46% acetonitrile 0.1% TFA gradient over 45 minutes. Fractions were collected at 1 minute intervals and assessed for the presence of peptides of interest using MALDI TOF Mass Spectrometry. Synthetic peptides were used as reference standards.

Table S1. Conservation of competence genes in streptococci, *Lactococcus lactis*, *Lactobacillus plantarum* and *Bacillus subtilis*.

Putative Function	Gene in suis	*S. pneumoniae*	*S. pyogenes*	*S. mutans*	*S. thermophilus*	*L. lactis*	*L. plantarum*	*B. subtilis*
Transcrptional control								
First trascriptional regulator	*ComR*	37.46%	35.93%	43.81%	34.43%	-	-	-
Master regulator	*ComX*	46.36%	40.00%	43.23%	40.52%	32.71%	30.86%	-
DNA uptake-recombination machinery								
helicase required for DNA uptake	*ComFA*	66.36%	50.00%	57.87%	52.79%	51.20%	38.95%	37.35%
membrane DNA-binding receptor	*ComEA*	47.95%	47.59%	53.41%	59.26%	37.56%	46.34%	37.56%
competence protein	*CoiA*	51.60%	45.19%	50.48%	46.01%	42.50%	39.11%	31.91%
component of the DNA transport	*ComGC*	70.37%	55.68%	60.23%	60.23%	56.82%	43.37%	49.33%
competence associated endonuclease	*endA*	69.49%	65.47%	65.38%	61.56%	-	35.29%	-
DNA transporter	*comFC*	49.08%	40.64%	50.91%	44.09%	45.25%	35.24%	31.28%
Signaling function								
Sensing	-	*ComD-E*	-	-	-	-	-	*comA-comP*
peptide export	-	*ComA-B*	-	-	-	-	-	*ComQ*
peptide import								
Oligopeptide binding protein	*OppA*	-	-	22.69%	-	32.26%	-	24.14%
	//////	66.82%	49.92%	-	AmiA1	26.97%	28.43%	-
Oligopepeptide transporter								
	OppB	-	33.44%	35.74%	-	52.66%	-	39.69%
	OppC	32.51%	31.67%	32.16%	32.39%	51.45%	32.89%	33.78%
	OppD	45.03%	46.03%	47.63%	44.27%	55.11%	46.84%	48.28%
	OppF	47.73%	47.53%	47.55%	49.79%	60.13%	47.91%	50.78%

Table S2. Overview of *S. suis* isolates and their ability for competence for DNA transformation to be induced using the synthetic ComS peptide. *serotype 1 reference strain.

S. suis isolate	Serotype	Virulent	Competence induced with S10 pherotype
6388	1	ND	yes
6112	1	ND	yes
6555*	1	ND	no
S10	2	yes	yes
P1/7	2	yes	yes
05ZYH33	2	yes	yes
7917	7	ND	yes
15009	7	ND	yes
7711	7	ND	no
8074	7	ND	no
7	7	ND	no
7997	9	ND	yes
8067	9	ND	yes
22083	9	ND	no
13730	14	ND	yes

Table S3 Bacterial strains and plasmids used in this study.

Plasmid or strain	Description	Reference
pNZ8048	E. coli- L. lactis shuttle vector containing PnisA promoter, CmR (chloramphenicol resistant)	[212]
S. suis S10	Virulent serotype 2 strain S3881	[213]
S. suis mut1	Isogenic apuA::spc mutant of strain S10	[33]
S. suis mut2	Isogenic comX::spc mutant of strain S10	This work
S. suis mut3	Isogenic comR::spc mutant of strain S10	This work
S. suis pNZ	S. suis strain 10 harboring pNZ8048 plasmid	This work
S. suis 7	Serotype 7 strain S8039	[214]
S. suis S10 (XIP 7)	Isogenic *comR/S S10::comR/S 7 spc* mutant of strain S10	This work

Table S4. Oligonucleotide primers used in this study.

Primers	Sequences	Restriction sites	Location
ApuA250F	CTGAGAATACCGCAACCG		1875397-414
ApuA250R	GAGTGAGTACGCGTCAAG		1875936-953
ApuA500F	AGGATGGCGGAACTGTTTG		1875047-065
ApuA500R	ATTGTGGACCCAGCAGAAG		1876207-225
ApuA1000F	CCAGCACTTGACCCTTAAGC		1874599-618
ApuA1000R	AGCAACAAGGTGCGAGTCC		1876675-693
ApuAR	TACCGATGGCAATTATGAT		1877138-156
ApuAF	CTTGTCCAGACGCTTGAG		1874137-154
ComXAF	TTCTGAACGAAGCAGCCCTTGTAG		14857-880
ComXBR	CACCAGACTTCAGCGTTCTACTTG		17027-50
ComXEF	TGAGAGCTCCAGGACATTTTTGTCTAGCATAC	SacI	15889-909
ComXFR	GGCCTCGAGTAGTTACTAACATCACGTTAATCGAAT	XhoI	16441-467
SpecIAF	TCCGAGCTCCAGCTTGATGCCTGCA	SacI	3-18
SpecIAR	CGCCTCGAGATCTGATTACCAATTAGAATG	XhoI	1166-187
CtrlMutA1	TGGGTGTGATTTTGGATGTG		1875779-798
CtrlMutB1	TAAAGGCCAGCTCAATTGCT		1875595-554
CtrlF1	GGTTGGTCGTCCGGATGTTAAAGG		14720-743
CtrlR1	GCGTTACTCACCCGTTCGCAACTC		17067-090
Ctrl2F	GGAGGATGATTCCACGGTACCATTTC		615-631
Ctrl2R	TATTGCGGGAAATGCAGTGG		938-357

Chapter 3

The dynamic competence transcriptome of *Streptococcus suis*

Edoardo Zaccaria[1], Michiel Wels[2], Peter van Baarlen[1], Jerry M. Wells[1]

[1] Host-Microbe Interactomics, Animal Sciences, Wageningen University, Wageningen, The Netherlands [2] NIZO Food Research, Kernhemseweg 2, 6718 ZB Ede

Submitted for publication

Abstract

Temporal studies of genome-wide gene expression were used to identify the pheromone-induced competence regulon of *Streptococcus suis*, where competence development can be induced by a Sigma X (SigX)-inducing peptide (XIP). In streptococci, the XIP pheromone regulates transcriptional activation of *comX* (or *sigX*) probably through allosteric interactions with the C-terminus of ComR and binding to a Competence-damage INducible regulator (CIN)-box motif. A conserved CIN-box (YTACGAAYW), was found in all promoters controlling the late competence transformasome, including gene homologues encoding the type 4-like pilus DNA uptake apparatus and the genes involved in DNA processing, repair and recombination. Furthermore we verified the role of the *S. suis* major pilin, and the recombination enzyme CinA in competence development suggesting that the transformasome performs the same function in *S. suis* as in other streptococci. Competence was a transient state with the *comX* regulon shut down after approximately 15 min even when transcription of *comX* had not returned to basal levels. Finally we identified a putative homologue of fratricin, and a putative bacteriocin gene cluster, that were expressed during competence and thus may play a role in DNA release from non-competent cells, enabling gene transfer between *S. suis* pherotypes or *S. suis* and other species.

Introduction

The process of natural competence for DNA transformation in specific habits or "natural competence" has been established as an important mechanism impacting on bacterial evolution and speciation. It has been proposed that in addition to horizontal gene transfer, natural competence provides a nutritional benefit by providing a nucleotide source from the degraded strand during transport into the cytoplasm. The genes associated with natural competence are widely distributed throughout the bacterial kingdom, although experimental evidence for natural competence is limited to only a few genera.

Most streptococcal species belonging to the *Streptococcus mutans*, *Streptococcus thermophilus*, and *Streptococcus gordonii* phylogenetic groups possess conserved genetic components of the competence machinery [142], and natural competence in the genus *Streptococcus* has been experimentally demonstrated in around sixteen species [154,202,206,215]. In streptococci competence is induced by an alternative sigma factor, ComX or SigX, which regulates expression of the late competence genes encoding functions in DNA uptake and recombination. Two main types of pheromone regulatory systems control the proximal switch for *comX* expression. The first is exemplified by *Streptococcus pneumoniae*, which uses a two-component system (TCS) to sense and respond to a competence stimulating peptide (CSP). In the presence of the peptide pheromone, *S. pneumoniae* histidine kinase ComD phosphorylates the cognate response regulator ComE, thereby altering its affinity for binding to an operator site upstream of *comX*. Phosphorylated ComE also auto-induces expression of the *comCDE* operon to increase production of the pheromone and the TCS, as a positive feedback mechanism to amplify induction of the competence state.

Recently, a second pheromone regulatory system for induction of competence was discovered in *S. mutans*, *S. thermophilus* and *S. pyogenes*. In these species, ComR, an Rgg family transcriptional activator, positively regulates expression of *comX* and *comS*, through allosteric interaction with a processed form of the pheromone encoded by *comS* [154,156,203,205]. The mature pheromone peptide induces competence from outside the bacteria but its mechanism of export is unknown. In *S. mutans* and *S. thermophilus*, the import of the mature pheromone is dependent on Opp, a peptide transporter not dedicated to competence regulation [165,190].

In streptococci ComX controls the late competence regulon via interaction with the Com box, also known as the CIN-Box, and RNA polymerase. The late competence regulon includes operons encoding genes for assembly of the type 4-like pilus, a DNA uptake system [157], and DNA recombination and repair enzymes [158,159,187]. In *S. pneumoniae* it is thought that once the pilus is polymerized, a channel is formed that passes through the cell wall and the capsule, allowing

the exogenous DNA to be internalised into the cytoplasm [216,217]. Before or concomitant with DNA translocation, the activity of EndA generates a single stranded DNA (ssDNA) molecule. In *S. pneumoniae* ComFA and ComFC appear to be required to internalize ssDNA while SsbB/A, DprA, RadC and RecA protect the ssDNA [218-220]. Additionally CoiA, DprA and RecA, a DNA-dependent ATPase, promote formation of the recombination synapse, heteroduplex formation and strand exchange between homologous DNAs [220,221]. Homologues of these genes are conserved in other streptococcal species shown to possess functional competence systems.

In several bacteria, *in vitro* competence induction is dependent on bacterial density, environmental stress and composition of the growth medium, indicating a precise control of natural transformability [163-165,209,222]. In streptococci, competence is a transient physiological bacterial state and the mechanisms mediating shut-down have only been partially elucidated in some species [170,223-226]. In *S. pneumoniae*, the exit from competence is regulated by multiple ComX dependent- and independent mechanisms. When accumulated at high levels, unphosphorylated ComE will lead to a rapid repression of *comCDE* transcription antagonizing the binding of phosphorylated ComE dimer to the ComE-box [168]. In *S. pneumoniae* a second mechanism of competence shut-down involves degradation of ComX and ComW due to the protease activity of ClpE-ClpP on ComX, and ClpC-ClpP activity on ComW and is probably mediated by the adaptor protein MecA [166,167]. Recently the late competence protein DprA was also proposed to be involved in shutting down competence in *S. pneumoniae* by binding to phosphorylated ComE, the transcriptional activator of *comX* [169,226]. The DprA protein is also involved in binding and protecting the incoming ssDNA and probably, presenting it to RecA, thus facilitating homologous recombination [219,227].

In *S. mutans* and *S. thermophilus*, ComX abundance is controlled by the MecA-dependent ClpC-ClpP protease activity and ClpE-ClpP probably has no role in competence exit. Thus *mecA* deletion leads to a stabilization of ComX and to expression of the late competence genes [170,225]. Moreover, *in vitro* degradation of ComX by ClpC-ClpP was shown to be strictly dependent on MecA [224]. To date it has not been possible to identify a gene(s) controlled by the ComRS system encoding protein(s) involved in the degradation of ComX and thus exit from competence.

In some streptococci ComX regulates secondary processes including expression of stress response pathways and fratricin, a cell wall hydrolase. In *S. pneumoniae* competence-induced expression of fratricin and the associated immunity gene are not required for DNA transformation but are considered to be a mechanism to acquire DNA from non-competent pneumococci [172,175,228]. Conservation of this mechanism has been proposed in other streptococci based on gene homologies and the presence of CIN-boxes in promoter regions [176]. Recently, in *S. mutans* a bacteriocin-like molecule was identified that is induced by the competence-

inducing peptide in *S. mutans*, causing autolysis in part of the population [177,178]. The existence of these complex mechanisms for induction, and control of natural competence and their involvement in secondary processes regulating cell fate in many bacteria highlights their importance in horizontal gene transfer and bacterial evolution.

The bacterial oligopeptide transport system Opp (or Ami) belongs to a family of ATP-dependent transporters that transport peptides across the cytoplasmic membrane. The Opp transporters consist of two transmembrane hydrophobic pore-forming domains (OppB and OppC) and two ATP-binding proteins (OppD and OppF), that hydrolyse ATP to provide the energy required for peptide transport [229]. In addition to these conserved proteins, the Opp operon encodes a ligand-binding protein (OppA) that is responsible for recognizing and binding extracellular peptides, thus conferring specificity to the transport system (20).

We recently identified a pheromone-induced mechanism of competence in *Streptococcus suis*, an important pig pathogen and zoonotic agent of human meningitis [156]. The competence system of *S. suis* appears to be similar to the ComRS-driven mechanism that has been discovered in *S. mutans*, *S. thermophilus* and *S. pyogenes*, although *S. suis* belongs to a different phylogenetic group [156]. Important gaps remain in our understanding of how competence is turned off in species like *S. suis* that regulate competence by a ComRS mechanism. Thus the aim of this study was to unravel the dynamic mechanisms of competence development in *S. suis* and identify the pheromone-induced secondary processes using transcriptomics. At three biologically relevant times after pheromone induction competence [156], *S. suis* RNA was extracted and hybridized to commercially available whole-genome microarrays. To verify predicted functions based on orthology of major *S. suis* competence-regulated genes, knock-out mutants were constructed for these genes. We found that induction and repression of major DNA repair and RNA metabolic genes occurred within 5 and 15 minutes, indicating that uptake, processing and incorporation of exogenous DNA into the *S. suis* genome occurs effectively within 15 to 30 min. Our data can be used to predict the *S. suis* transformasome by orthology and pinpoint processes that are both crucial to genomic integrity and gene transfer. These processes are therefore not only relevant from a fundamental biological viewpoint but could also be targets of future antimicrobials.

Materials and Methods

Bacterial strains and culture conditions

The *S. suis* strains used in the present study are listed in Table 1. S. *suis* S10 genome is 99% identical to the genome of *S. suis* 2 strain P1/7 [230], a sequenced reference strain of which the genome had been annotated previously [23]. *S. suis* was grown at 37°C at 5% atmospheric CO_2 in Todd Hewitt Broth (THB, Thermo Scientific, Oxoid) or on THB plates containing 1.2 % of agar (BD). When required, spectinomycin (Invitrogen) at a concentration of 100 µg/ml was supplemented in the medium for the selection and growth of mutant strains. Insertional deletion mutants of the genes *cinA, oppA* and *comYC* were constructed in *S. suis* strain S10 by Gene Splicing Overlap Extension PCR (SOE-PCR) and allelic replacement as previously described [156]. The primers used for gene splicing are shown in Table 1. Successful deletion of the genes was verified by colony PCR using primer combinations based on DNA sequences of the inserted DNA and proximal chromosomal DNA (Table 1) and verified by sequencing of the resulting fragments. Growth phase was determined by measuring optical density at 600 nm (OD_{600nm}) using a SpectraMax M5 reader (Molecular Devices LLC).

RNA extraction

RNA extraction was preceded by induction of competence as described in [156]. Briefly *S. suis* S10 was grown to OD_{600nm} 0.04. Thirty five ml of culture was collected and donor DNA (pNZ8048, 350 µg) in EB buffer (10 mM Tris-Cl, pH 8.5) was added to the bacteria along with synthetic XIP (GNWGTWVEE) at a final concentration of 250 µM [6]. Control extracts were obtained using same quantities of culture with donor DNA added but without XIP stimulation. Ten ml of the induced culture were collected after 5, 15 and 45 minutes following XIP exposure. Ten ml of uninduced cultures were collected at 15 and 45 minutes, spun down and the pellets were resuspended in 2.5 mL PBS plus 5 mL RNAprotect buffer (Promega). After 5 minutes of incubation the bacteria were collected via centrifugation and immediately frozen in liquid nitrogen until further handling. The frozen pellet was dissolved in 110 µl of TE containing proteinase K and lysozymes (1.25 µg/mL and 15 ug/ml respectively) and incubated for 10 minutes. The bacterial pellet was dissolved in 700 µL RLT buffer (Promega) containing 7 µl of freshly added β-mercaptoethanol and the bacteria were disrupted using a FastPrep-24 (MP. Biomedicals, Solon, OH) for 20 sec at 6.0 m/sec. Total RNA was purified using the RNeasy Mini Kit (Qiagen). The quality and the concentration of RNA were assessed with an Experion System (Bio-Rad) and by analysis of the A260/A280 ratio (NanoDrop 8000 UV-Vis Spectrophotometer). cDNA was synthesized with a SuperScript III Reverse Transcriptase kit (Invitrogen) using Aminoallyl-dUTP as dUTPs source and purified with the Illustra CyScribe GFX Purification Kit (GE Healthcare). The cDNA was labelled with CyDye Post-Labeling Reactive Dye Pack (GE Healthcare).

Table1: *S. suis* strains and primers used

Strain	**Relevant characteristics**	**Source of reference**
S. suis S10	Wild-type; reference strain	[213]
S. suis ΔcinA	S10 ΔcinA	Smith HE, 1999
S. suis ΔoppA	S10 ΔoppA	This study
S. suis ΔcomYC	S10 ΔcomYC	This study
Primers	**Nucleotide sequence**	**Purpose**
CinA 1F	TGCGGCCATGACAGATAGCG	Creation of *cinA* deletion fragment
CinA 1R	CTTGCCAGTCACGTTACGTTTCCGTCCCAACGGCGATTAG	Creation of *cinA* deletion fragment
CinA 2F	CTAATCGCCGTTGGGACGGAAACGTAACGTGACTGGCAAG	Creation of *cinA* deletion fragment
CinA 2R	GTCCTCTGTTGATTCCGGTTTCGGTACCCTATGCAAGGGTTT	Creation of *cinA* deletion fragment
CinA 3F	CCTTGCATAGGGTACCGAAACCGGAATCAACAGAGGACAAC	Creation of *cinA* deletion fragment
CinA 3R	TCTTTCTGGGCTTGAGCTACTG	Creation of *cinA* deletion fragment
CinA ctrl F	GGAGTTTCTATGTCCCGTTGTG	Control of Δ*cinA*
CinA ctrl R	GTACAAGGGCTGCAACCGAGTC	Control of Δ*cinA*
OppA 1F	CGGAAACCGACGTGTAAATC	Creation of *oppA* deletion fragment
OppA 1R	CTTGCCAGTCACGTTACGTTTCGGGTACAGGTCTTGCTTATG	Creation of *oppA* deletion fragment
OppA 2F	TAAGCAAGACCTGTACCCGAAACGTAACGTGACTGGCAAGAG	Creation of *oppA* deletion fragment
OppA 2R	GTTCTTGCAGCATGTGGTTCTCGGTACCCTATGCAAGGGTTTA	Creation of *oppA* deletion fragment
OppA 3F	AACCCTTGCATAGGGTACCGAGAACCACATGCTGCAAGAACAGA	Creation of *oppA* deletion fragment
OppA 3R	CACGAATGCAGCTTCGCTACC	Creation of *oppA* deletion fragment
OppA ctrl F	GTTATGCAAGCCCATGATGGTC	Control of Δ*oppA*
OppA ctrl R	AGGCGTTTAGCGAGGTAATGTC	Control of Δ*oppA*
ComYC 1F	ACCTACCTGACGGCCTATTACG	Creation of *comYC* deletion fragment
ComYC 1R	TCTCTTGCCAGTCACGTTACGTTCCACCAGAGTGAACCCTTT	Creation of *comYC* deletion fragment
ComYC 2F	AAGGGTTCACTCTGGTGGAACGTAACGTGACTGGCAAGAGAT	Creation of *comYC* deletion fragment
ComYC 2R	CCTTTCGCCTGCAAATCTGCTCGGTACCCTATGCAAGGGTTTA	Creation of *comYC* deletion fragment
ComYC 3F	ACCCTTGCATAGGGTACCGAGCAGATTTGCAGGCGAAAGGTT	Creation of *comYC* deletion fragment
ComYC 3R	CTGGACAGCCATCTGTGCTAAG	Creation of *comYC* deletion fragment
ComYC ctrl F	GATTGAGGTGGCGACCTATCCG	Control of Δ*comYC*
ComYC ctrl R	AGAGGCTACCTGACAGAATGAC	Control of Δ*comYC*
Spec F	ACCGTGGAATCATCCTCCCAAAC	Control of all mutants
Spec R	CCACTGCATTTCCCGCAATATC	Control of all mutants

Microarray transcriptome analysis

An *S. suis* oligoarray (8×15 K) containing *in situ* synthesized 60-mers was produced by Agilent Technologies (Santa Clara, USA), based on the genome sequence of S. *suis* P1/7 [23]. A total of 7651 unique 60-mers having a theoretical melting temperature of approximately 81°C and representing 1960 ORFs were selected as described [231]. Genes were represented by 4 (91%), 3 (4%), 2 (2%), or 1 probe(s) (3%). Twenty-five putative genes were not represented on the array because no unique probe satisfying the selection criteria could be selected. Co-hybridization with labelled cDNA probes was performed on these oligonucleotide arrays at 42°C for 16 h in Slidehyb#1 (Ambion, Austin, USA). The data were normalized using Lowess normalization [232] as available in MicroPrep [233] and corrected for inter-slide differences on the basis of total signal intensity per slide using Postprep [233]. Significance of differential gene expression was based on false discovery rate (FDR) values lower than 0.05. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE74507 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wrwfquyqfhijfeh&acc=GSE74507).

Transcriptome data mining

Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used to filter the data for the most differently expressed genes using the following parameters: standard deviation: 250; at least 4 observations with absolute value higher than 20; subtraction between maximum and minus minimum: 200.

Heatmaps were generated by the MultiExperimental Viewer (MeV) program (http://www.tm4.org/mev.html) [234].

Transformation experiments

S. suis strains were grown overnight in THB broth at 37°C under 5% CO_2. The overnight culture was diluted 1:40 into pre-warmed THB broth, and grown at 37°C without shaking. Aliquots of 100 µl were removed from the main culture after 1 hour, at OD_{600nm} approx. 0.04 and combined with transforming DNA (1.2 µg of pNZ8048) in EB buffer (10 mM Tris-Cl, pH 8.5) and 5 µl of XIP at a final concentration of 250 µM. After 2 hours of incubation at 37°C under 5% CO_2 in 1.5 ml Eppendorf Safe Lock Tubes™, the samples were diluted and plated onto THB agar plates containing antibiotics when required.

Results

The competence pheromone induces distinct clusters of differentially regulated genes at specific time-points

Competence pheromone-induced transcriptional changes were identified by microarray analysis of RNA isolated from bacteria at 5, 15 and 45 min in the presence and absence of the competence-peptide and in the presence of exogenous DNA as previously described [156]. Additionally the peptide pheromone was added without adding DNA, to identify possible effects of DNA addition. As competence induction is only possible in early lag phase we also investigated possible effects of changes in growth rate at 15 and 45 min by not adding peptide to some of the bacterial cultures. Five minutes after addition of the competence pheromone, 556 differentially expressed genes were up-regulated more than 2-fold and 215 genes were down-regulated more than 2-fold. At 15 min 148 and 185 differentially expressed genes were respectively up-regulated or down-regulated. At 45 min 140 and 48 were up- and downregulated, respectively. Genes that were not expressed or did not change expression at the multiple time points and controls were removed by filtering the data as described in materials and methods. Genes with altered expression were clustered according to their relative expression values at the different time points (Fig. 1). Four major clusters were observed (Fig. 1), the first of which (cluster 1) contains genes that were down-regulated upon induction of competence until 45 min post-addition of peptide (Fig. 1b). Cluster 1 contains 13 of the 14 fatty acid biosynthetic pathway genes and 3 genes involved in cell envelope metabolism. Cluster 2 contains genes of diverse functions including a cation-transporting ATPase, a putative peptidase and a predicted transcriptional regulator that were up-regulated only at 15 min. Cluster 3 genes were all highly (> 4 fold) up-regulated at 5 min, after which their expression decreased until 45 min, when expression reached the same level as measured for the uninduced control samples. Cluster 3 contained 37 genes including *comX*. Of these 37 genes, 8 genes were annotated to be involved in DNA repair and recombination, and 4 genes were annotated as homologues of the multi-protein Type 4 pilus-like DNA uptake and transport apparatus, part of the transformasome recently described in *S. pneumoniae* [157]. Cluster 4 contains 28 genes that were highly expressed at 5 and 15 min and downregulated at 45 min. This cluster contained genes involved in mechanisms directly related to competence for DNA transformation, as well as the chaperones *groES and groEL*, a putative fratricin gene, 3 enzymes in the mevalonate pathway and an operon (SSU0038-SSU0045) of unknown function.

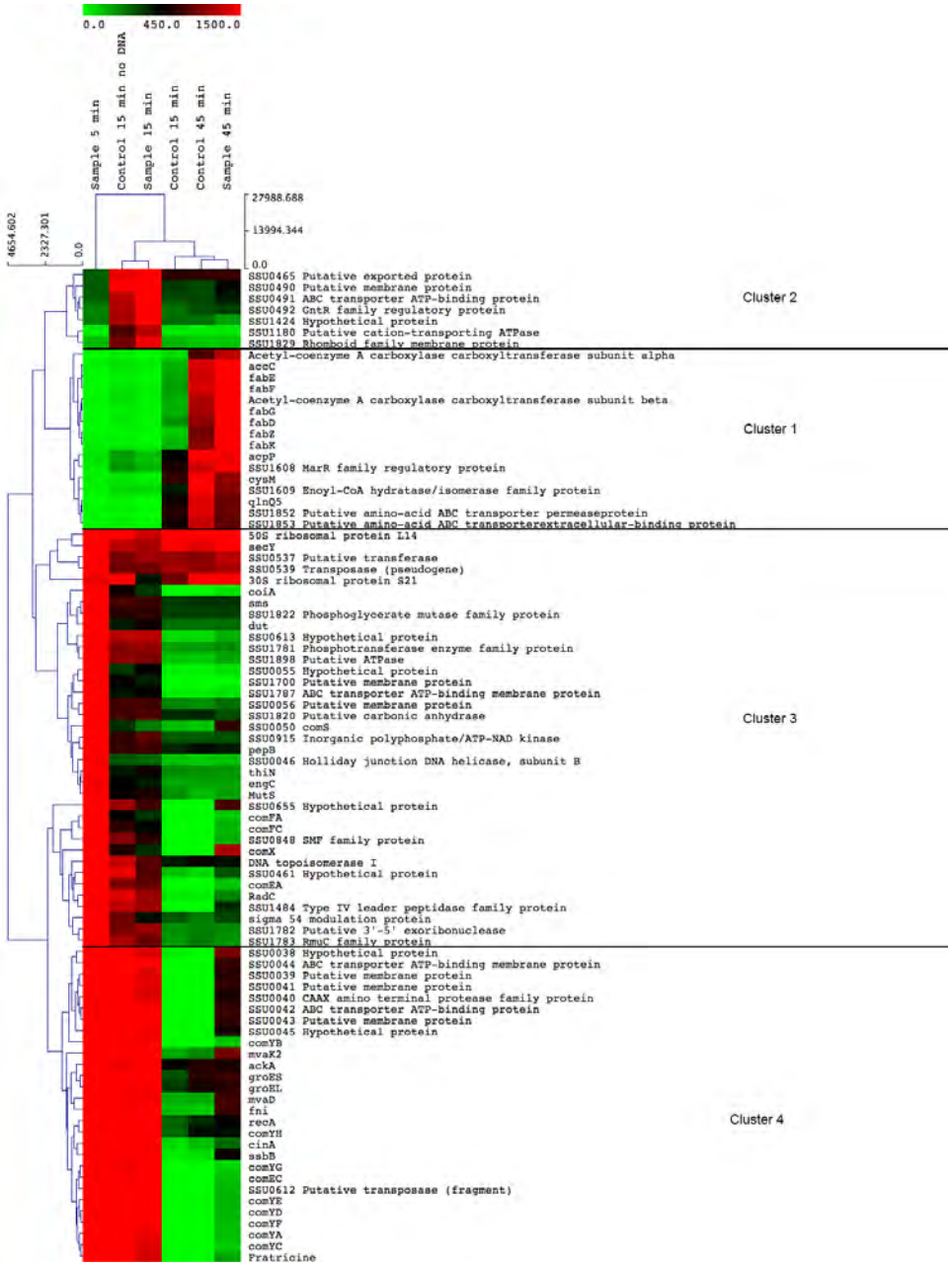


Figure 1: Heatmap displaying the most differentially expressed genes in *S. suis* during competence. Genes were filtered using Cluster 3.0 (see Methods) and were clustered using average linkage and Euclidian distance using MultiExperiment Viewer (MeV, see Methods).

The *S. suis* transformasome is regulated by ComX via a conserved CIN-box

In both *S. pneumoniae* and *Bacillus subtilis* competence induction leads to expression of a similar set of genes encoding membrane and cytosolic proteins involved in DNA internalization, collectively producing the transformasome. In *S. pneumoniae* the promoters of the genes encoding the transformasome contain the conserved CIN-box, a DNA motif where the alternative sigma factor ComX (or SigX) interacts with the RNA polymerase to initiate transcription. To identify the consensus CIN-box regulon in *S. suis*, promoters of competence regulated genes or operons were searched for consensus motifs using MEME (see Methods). A conserved consensus 9 nt motif was identified with similarity to the CIN-box of *S. pneumoniae* (Fig. 2). In *S. suis* the CIN-box genes were present in 9 operons, 4 of which were identified using the FIMO module of the MEME software suite (see Methods). The *S. suis* CIN-box genes encode homologues of all the known transformasome proteins in *S. pneumoniae*, *S. mutans* and *S. thermophilus* (Table 2), showing its conservation across streptococcal species. *S. suis* CIN-box regulon contains a dedicated set of genes, the *comYA-YH* operon (*comGA-GH* in *S. pneumoniae*), with homology to the Type 4 pili (T4P) of Gram-positive bacteria, predicted to encode a putative ATPase (ComYA), a membrane protein (ComYB) and five other proteins named ComYC, ComYD, ComYE, ComYF, ComYG and ComYH [157]. With the exception of *recA*, that has its own promoter and is expressed constitutively, the expression levels of all 9 CIN-box-containing operons are similar. Of note, the ComX regulated 'late competence' genes of *S. pneumoniae* and *S. gordonii* are induced approximately 15 min after induction [235,236] but the same genes were expressed at 5 min after competence induction in *S. suis* (see clusters 3 and 4, Fig. 1b).

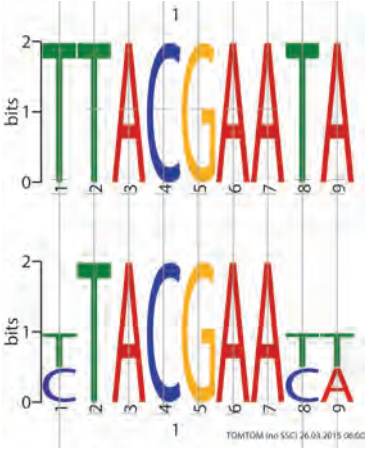


Figure 2: Consensus CIN-box of *S. pneumoniae* (top) and *S. suis* (bottom).

Homologues of all the CIN-box-containing late competence genes involved in the transformasome described for *S. pneumoniae* and *S. gordonii* [187,235,236] are conserved in *S. suis* and possess a CIN-box in their promoter regions, except

endA. EndA is a DNA specific nuclease that converts the dsDNA bound by ComEA and ComEC [169,237] into ssDNA before or concomitant with delivery into the cytoplasm through ComEC [238]. To further investigate the role of EndA in *S. suis* competence we attempted to create a *endA* deletion mutant but were unable to obtain transformants.

Streptococcal genes previously shown to be involved in the formation of the recombination synapsis, heteroduplex formation and strand exchange between homologous DNAs are also members of the *S. suis* ComX regulon (Table 2). In *S. suis* the single stranded binding protein A (SsbA), which protects the transforming ssDNA from degradation in the cytoplasm is not regulated during competence induction whereas this gene is a member of the ComX regulon in *S. pneumoniae* and *S. mutans* [218-220].

Table 2: Transformasome genes under ComX regulation with their homologs in *S. mutans, S. pneumoniae and S. thermophilus* and their (putative) function.

ComX regulated genes	*S. mutans* UA159	*S. pneumoniae* TIGR4	*S. thermophilus* LMD-9	Name	Function
SSU0061	67%	66%	72%	CinA	DNA binding and homologous recombination
SSU0062	86%	84%	87%	RecA	
SSU0126	66%	63%	58%	ComYA	Pilus assembly
SSU0127	58%	63%	53%	ComYB	
SSU0128	59%	65%	60%	ComYC	
SSU0129	48%	45%	44%	ComYD	
SSU0130	49%	42%	48%	ComYE	
SSU0131	47%	52%	47%	ComYF	
SSU0132	36%	37%	35%	ComYG	
SSU0133	60%	62%	61%	ComYH	
SSU0144	76%	75%	72%	SsbB	SSDNA binding and protection
SSU0610	46%	46%	60%	ComEA	dsDNA receptor and channel
SSU0611	57%	53%	50%	ComEC	
SSU1083	50%	52%	46%	CoiA	Implicated in DNA homologous recombination
SSU0924	66%	64%	61%	RadC	DNA binding and protection
SSU0393	58%	66%	53%	ComFA	ssDNA binding and intracellular translocation
SSU0394	51%	49%	44%	ComFC	

Deletion of the major pilin gene *comYC* prevents DNA transformation

The ComYA-YH operon of *S. suis* contains all homologues of the known membrane-associated proteins and the pilus required for exogenous DNA binding [157] (Table 2)**.** To verify if the conserved ComY operon was necessary for competence in *S. suis* we generated a *S. suis* deletion mutant of the pilin *comYC* and verified the correct mutation by PCR and sequencing (Table 1). The *comYC* deletion in *S. suis* prevented subsequent attempts to obtain DNA transformation after competence induction with the SigX-inducing-peptide (XIP) providing further evidence for the conserved role of this protein in competence for DNA transformation.

Deletion of *cinA* reduces natural transformation efficiency in *S. suis*

CinA is a DNA repair enzyme that is important for efficient competence development in *S. pneumoniae* and *S. mutans* [239,240]. As in *S. mutans* and *S. pneumoniae*, *recA* is immediately downstream of *cinA* and expression of both genes was strongly enhanced at 5 and 15 minutes after XIP exposure. *recA* was also constitutively expressed at a lower basal level in the absence of XIP, reflecting its 'housekeeping' role in DNA recombination. To determine whether the predicted *S. suis* orthologue of CinA may have a role in competence, a *cinA* deletion mutant (Δ*cinA*) was generated using previously described methods [156] and verified by PCR and sequencing. The ΔcinA mutant resulted in substantially reduced DNA transformation efficiency compared to the parent wild-type (WT) strain S10 (about 8% of WT efficiency) suggesting that CinA has an important but not essential role in DNA transformation (Fig. 3).

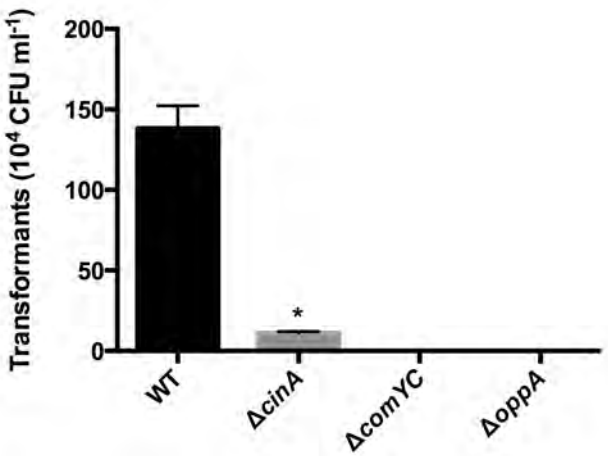


Figure 3: Number of transformants obtained with Δ*cinA*, Δ*comYC* and Δ*oppA* (see below) deletion mutants in *S. suis* strain S10. * P-value < 0.05 using Student's t test. Results were obtained in 3 independent experiments"

Expression profiles of the transcriptional regulators of competence, *comR* and *comX*

In all the streptococcal species in which natural competence has been demonstrated, its activation leads to expression of the alternative sigma factor X (*comX* or *sigX*). We have previously shown that *comX* is essential for natural transformation in *S. suis* S10 [156]. In our transcriptome data *comX* expression was strongly up-regulated at 5 minutes (1300 fold compared with the control), relatively mildly up-regulated at 15 minutes (105 fold) and up-regulated again at 45 minutes (327 fold). Despite the high amount of *comX* expression at the 45 min time point, the CIN-box genes under its direct regulation were not increased in expression at 45 min compared to 15 min in the XIP-induced samples with DNA provided exogenously, although they were expressed significantly higher compared to uninduced control samples.

We have shown that ComR is required for competence induction in *S. suis* [156] and postulated that ComR interacts with the mature ComS pheromone to induce *comX* and *comS*, in a similar way as described for other streptococci possessing the ComRS regulatory system [154,203]. In support of this hypothesis, the expression of *comS* is similar to *comX* expression, characterized by a strong induction at 5 minutes, a decline at 15 minutes and an induction at 45 minutes. Importantly, the basal (uninduced) expression of *comX* and *comS* differs: *comX* expression values are close to zero, preventing entering the competence state in absence of exogenous XIP. During competence, *comR* expression reaches its peak value at 5 minutes (2.3 fold compared with the control), rapidly decreasing (at 15 min) to expression values measured prior to competence induction.

Where is the exit? - regulation of competence shut down

In *S. suis* expression of MecA, the adapter protein regulating ClpCP degradation of ComX in *S. thermophilus*, was not substantially different over the time course of competence induction. *mecA* expression was down-regulated at 5 min (0.42 fold) and 45 min (0.88 fold) and up-regulated at 15 min (1.28 fold). Similar fold changes in the expression were measured for ClpC at the same respective time points.

The *dprA* gene is regulated by ComX and has a dual role in the natural transformation system of *S. pneumoniae*. DprA promotes the homologous recombination facilitating RecA binding to the ssDNA but can also bind to ComE~P, preventing its interaction with the ComX promoter [169,226]. As *S. suis* utilises ComRS rather than a TCS for regulation of *comX* it is not clear that DprA has a role in shutting down *comX* expression. Nevertheless *dprA* was highly induced 5 min after competence induction despite the absence of a CIN-box in its promoter.

Conserved function of the oligopeptide permease gene cluster of *S. suis*

The Opp transport system is essential for competence development of *S. mutans* and *S. thermophilus* [154,190,203,207], and it appears to be responsible for the

internalization of the XIP. In *S. suis* the five genes encoding the Opp transporter are organized in two transcriptional units, an operon of 4 genes and OppA which encodes the subunit A of the Opp transporter complex. The expression of the full transporter system had not significantly changed during competence induction. To understand whether the XIP transporter role of Opp is conserved in *S. suis*, we deleted *oppA* and found it prevented XIP-induced DNA transformation. We also observed a reduced capacity for growth in rich medium by the *oppA* deletion mutant, which could have negatively influenced the competence development.

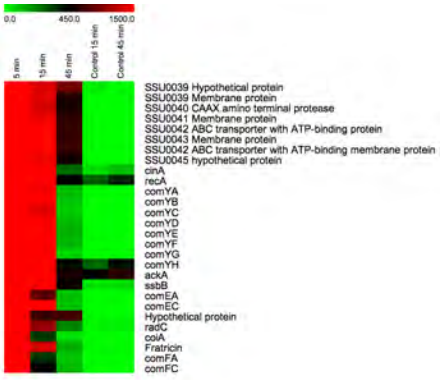


Figure 4. Temporal expression of the genes under direct regulation of ComX.

S. suis contains a CIN-Box-regulated homologue of fratricin and a putative bacteriocin-producing operon

In addition to the set of genes conferring the ability for competence sensing and DNA uptake and recombination, naturally transformable streptococci appear to express a murein hydrolase during competence, usually called CbpD or fratricin, under the control of ComX [173,174,176,241,242]. We identified a fratricin-like gene in *S. suis* (SSU1911) downstream of a CIN-box promoter that contains an N-terminal CHAP (Cysteine, Histidine-dependent Amidohydrolases/Peptidases) domain and two SH3b (central Src homology 3b) domains. These domains are also present in the pneumococcal fratricin protein [174]. Expression of the gene encoding the putative *S. suis* fratricin was up-regulated at 5, 15 and 45 min by 722, 208 and 22 fold respectively; the expression profile of this gene was similar to the expression profile of the ComX-regulated genes of *S. suis* (Fig. 4). These findings strongly suggest that *S. suis* also produces a fratricin-like protein during competence development, possibly also functioning to lyse non-competent bacteria and release their DNA such that this becomes available for uptake by the competent part of the local *S. suis* population [174].

The immunity protein ComM of *S. pneumoniae* is expressed prior to the fratricin synthesis, which protects the competent bacteria from the muralytic activity

by an unknown mechanism [228,243]. We could not identify a candidate immunity gene among the set of genes collectively upregulated during competence development nor a likely homologue of ComM in the *S. suis* genome based on protein homology.

Interestingly, among the genes under direct regulation by ComX, we identified an operon consisting of 8 genes that do not show significant homology with other competence genes. The operon SSU0038-45 comprises 3 putative membrane proteins, one CAAX amino terminal protease and two ABC transporters with ATPase activity. In addition we measured high expression of two relatively small putative ORFs (SSU0038 and SSU0045) represented on the microarray but not annotated in *S. suis* genome. These ORFs are predicted to encode two small (42 and 57 amino acids) proteins with unknown function but their size and association with a CAAX peptidase and two ABC transporters suggests a possible role as bacteriocins. This is also supported by the peptide leader sequence of SSU0045 that features a double-glycine motif which is a characteristic of bacteriocins secretion by ABC transporters [244].

Discussion

Temporal studies of genome-wide transcription during pheromone-induced competence in *S. suis* identified a regulon comprising 9 operons containing conserved CIN-box like motifs (YTACGAAYW) in the promoter regions that were originally identified and characterised in *S. pneumoniae*, *S. mutans* and *S. thermophilus* [158,245]. In streptococci CIN-box promoters are regulated by the alternative sigma factor ComX or SigX, which is induced by competence-inducing pheromones and one of two different competence-regulatory systems. The *S. suis* CIN-box regulated genes were all highly expressed at 5 min and thereafter showed decreased expression, eventually returning to basal levels after 45 min. This pattern of temporal expression has also been described in *S. pneumoniae* with the difference that the ComX regulated genes peak at 15 min after induction of competence. In *S. suis* expression of *comX* is regulated by the ComRS regulatory system that is conserved in *S. mutans*, *S. thermophilus* and *S. pyogenes* and that is predicted to be present in the *salivarius*, *pyogenes*, *mutans* and *bovis* groups of *Streptococcus* species [246].

In representative species of these phylogenetic groups, the competence-inducing pheromone ComS is processed into the mature form, the XIP, by proteolytic cleavage and exported by an unknown mechanism. XIP mediates the quorum sensing mechanism of competence induction by transport back into the cell via the Opp oligopeptide ABC type transporter [154,190]. We generated a knockout of *oppA*, the component of the transporter that recognises XIP, and were unable to transform this mutant. Although this is consistent with similar findings in other streptococcal species, we cannot completely rule out that the slower growth of the OppA mutant impacted on competence development. It has been shown in all investigated ComRS systems that efficient binding of ComR to its operator motif is strictly dependent on the presence of the XIP pheromone [197,207,208,247].

In streptococci, competence is a short-lived, transient physiological bacterial state of which the exit mechanisms have only been partially elucidated in some streptococcal species. Expression of MecA and ClpC, which tightly control amounts of ComX in the uninduced state through targeted proteolysis were not substantially different over the time course making it less likely that only these two proteins are responsible for exiting the competence state after induction. Expression of DprA, which has a role in DNA processing and competence regulation in *S. pneumoniae*, was upregulated after 5 min despite the absence of a CIN-box. Thus DprA may have a role in regulation of the ComRS system in *S. suis* but the potential target of DprA binding remains to be identified.

In *S. suis* the ComX- regulated operons (the late competence genes) include all homologues of the multiprotein Type IV pilus (T4P) required for exogenous DNA binding, and processing to ssDNA before or concomitantly with delivery into the

cytoplasm [157,238] (Fig. 3a). To confirm the role of the DNA binding pilus in competence development we constructed a deletion mutant of the pilin *comYC* and showed that ComYC was required to obtain DNA transformation after competence induction with XIP.

In pneumococcus, ComX regulates production of endonuclease A (EndA), an integral component of the transformasome [248]. EndA converts DNA taken up by the pilus complex into single-stranded form before or concomitant with its transport to the cytoplasm. Moreover *endA* deletion mutants in pneumococcus were found to accumulate DNA at the cell surface, and EndA is required for natural transformation of *S. pneumoniae* [237]. There is probably a similar role for EndA in *S. suis* but we were unable to verify this as we were not able to obtain *endA* gene deletion mutants suggesting EndA has an additional essential role in *S. suis*. This is consistent with an absence of a CIN-box in the *endA* promoter and the constitutive expression of EndA in *S. suis*.

As previously demonstrated in *S. pneumoniae* and *S. mutans*, the *cinA* promoter of *S. suis* contains a CIN-box and is regulated by ComX. Additionally we confirmed a role for this gene in natural competence by constructing a *cinA* deletion mutant in *S. suis*, which led to reduced efficiency of transformation. In *S. suis* the *cinA* open reading frame is followed by the *recA* gene, as also observed in *S. pneumoniae*, *S. mutans* and *B. subtilis* [249]. The *recA* gene encodes a protein with a central role in homologous recombination during competence and in DNA repair. In *S. suis recA* is constitutively expressed from a promoter that lacks a CIN-box but is upregulated during competence development in a manner resembling that of CIN-box regulon. This is most likely due to the fact that the CIN-box-regulated *cinA* lies directly upstream of r*ecA* making it likely that read-through transcription into *recA* during competence induction occurs, as described in *S. mutans* [239]. Previously Mair et al. [239] showed that *cinA*-complementation of a *cinA* deletion only partly restored transformability of the strain by natural competence, suggesting polar effects of *cinA* deletion on *recA* expression.

Pheromone-induced competence in streptococci leads to production of bacteriolytic cell wall hydrolases, so-called fratricins, exemplified by CbpD in pneumococcus [174-176,250]. The proposed biological function of fratricin is to promote DNA release from non-competent bacteria by hydrolysis of peptidoglycan and weakening of the cell wall [173,241,242]. Fratricins appear to be most active against bacteria that are relatively closely related to the producer strain, which may favour beneficial DNA recombination over detrimental genetic events [172]. Fratricins might theoretically also damage producing strains, suggesting that hypothetical immunity proteins might be produced by fratricin-positive strains. Immunity proteins have so far been identified only in *S. pneumoniae* and they are expressed early in competence, indeed to protect the producers against their own fratricins [228,243]. In *S. suis* we identified a fratricin homologue possessing

the conserved N-terminal CHAP domains and two SH3b domains found in other streptococci. The putative fratricin had the same expression profile as the other CIN-box regulated genes during competence development supporting the notion that this gene is having the same function in *S. suis*. We were unable to predict any candidate immunity proteins encoded by the genes upregulated during competence or in *S. suis* genome by protein homology.

In addition to fratricin, a second operon containing 8 genes potentially associated with bacteriocin secretion via an ABC transporter (SSU0038-45) was induced during competence development. The operon consists of 8 genes that do not show significant homology with competence genes from other streptococci and comprises 3 putative membrane proteins, one CAAX amino terminal protease and two ABC transporters with ATPase activity. In addition, the operon contains two relatively small putative ORFs (SSU0038 and SSU0045) that were highly expressed according to the microarray data (Fig. 4). These ORFs have not been annotated in the *S. suis* P1/7 genome and are predicted to encode two small (42 and 57 amino acids) proteins with unknown function. Their small size and association with a CAAX peptidase and two ABC transporters suggests that these ORFs might encode bacteriocins. This idea is also supported by the amino acid sequence of the peptide leader of SSU0045 that features a double-glycine motif, characteristic of bacteriocins secreted by ABC transporters [244]. In *S. mutans*, competence induction indirectly controls bacteriocin production [198,251]. In *S. gordonii*, a locus with a CIN-box in the promoter region that encodes a bacteriocin has also been reported. The encoded bacteriocin peptide contains a double-glycine motif, prerequisite for processing and secretion by peptide-exporting ABC-type transport systems [252]. This bacteriocin was shown to be active against *S. gordonii* and *S. mitis* [253]. Further studies are needed to show that the bacteriocin is produced during competence and identify the spectrum of target species killed by the bacteriocin.

In conclusion we predicted the conserved CIN-box/ComX regulon in *S. suis* that mediates pheromone-induced expression of the transformasome and associated DNA processing and recombination genes. As our description of the *S. suis* competence system is a model, we verified the essential role of the *S. suis* major pilin, and CinA for efficient competence development, supporting the notion that our predicted multi-protein transformasome indeed appears to function as described for other streptococci. Moreover, induction of competence was not possible in an *oppA* deletion mutant, consistent with its previously described role in XIP transport in *S. mutans* and *S. thermophilus*. We cannot rule out an effect of the growth rate of this mutant on competence development. Competence was a transient state, in that expression of the genes making up the ComX regulon was suppressed after 15min, when transcription of *comX* had not returned to basal levels. One possible candidate for mediating exit from competence was DprA, which in pneumococcus inhibits binding of the ComE-Phos regulator to its DNA motif. However, further studies are

required to determine whether DprA may be involved in competence exit in *S. suis* and if so, to identify the mechanism.

Finally we identified a homologue of fratricin, and a putative bacteriocin gene cluster, that were expressed during competence and thus may a role in DNA release from non-competent cells. We propose a time-resolved model of competence in *S. suis* (Fig. 5) that illustrates the production and proteolytic break-down of pilus and main regulators at 5, 15 and 45 minutes. This model can be used for further investigation into pheromone-induced DNA uptake and recombination and repair, processes that are relevant to genome integrity, and highlights proteins such as RecA and EndA that appear to perform crucial functions in *S. suis* bacteria. The results from this study may therefore also provide data that can be translated into applied studies including antibiotic resistance.

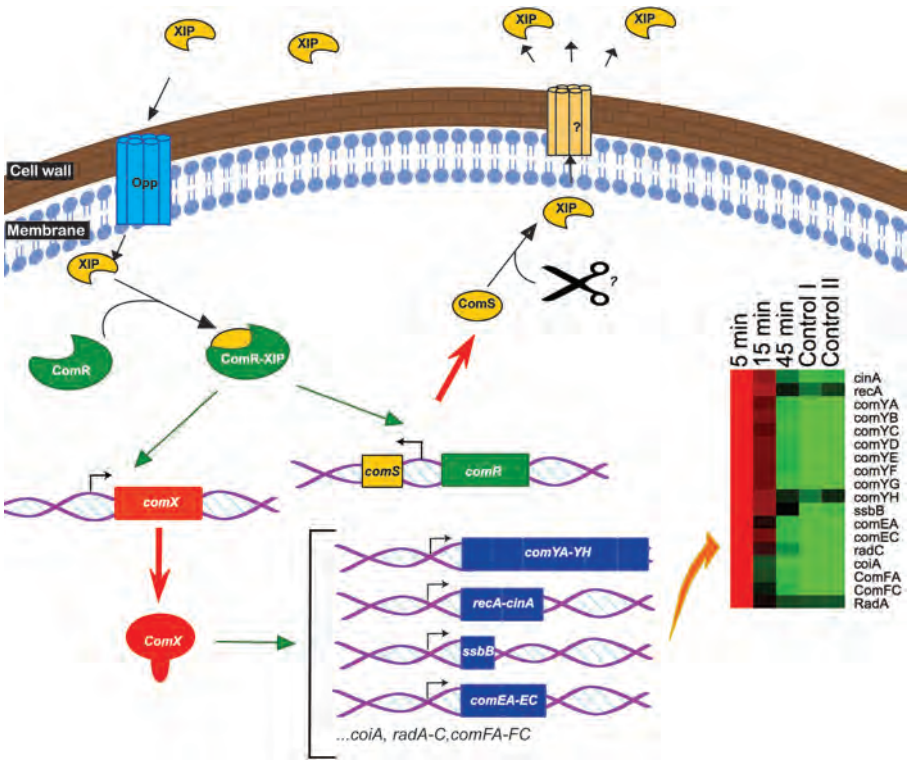


Figure 5. Simplified model of competence induction in *S. suis*. Extracellular SigX-inducting-peptide (XIP) enters the bacteria via the Opp transporter system. Intracellularly, the transcriptional regulator ComR binds to XIP and the complex ComR-XIP promotes the expression of *comS*, encoding the full-length form of the pheromone, and of *comX* (green arrow). ComX activates the expression (green arrow) of the late-competence genes involved in the transformasome having a CIN-box in their promoter (heatmap). ComS is processed and secreted by an unknown mechanism inducing a positive feedback loop.

Chapter 4

Metabolic context of the competence-induced checkpoint for cell replication in *Streptococcus suis*

Edoardo Zaccaria, Jerry M. Wells and Peter van Baarlen

Host-Microbe Interactomics, Animal Sciences, Wageningen University, Wageningen, The Netherlands

Manuscript in preparation

Abstract

Natural genetic transformation is a transient, rapidly progressing energy-consuming process characterized by expression of the transformasome and competence-associated regulatory genes. This transient state is tightly controlled and potentially may have adverse effect on bacterial genome integrity and cell division. We investigated the global response of *Streptococcus suis* to exposure to the SigX-inducing peptide (XIP), and thus to the activation of the competence machinery, using time series analysis together with PCA analysis, gene clustering followed by heatmap visualisation, and GO enrichment analysis. We explored the possible regulatory link between metabolism and competence, and predicted the physiological adaptation of *S. suis* during the competence induction, progression and exit using transcriptome analysis. We showed that competence development is associated with a suppression of basal metabolism, which may have consequences for the microbe's resilience to fluctuations in the environment, as competence is costly in terms of use of energy and protein translation. Furthermore our data suggest that several basal metabolic pathways are incompatible with activation of competence in *S. suis*. Finally this study also showed that, upon competence induction, targeting specific pathways might render *S. suis* more vulnerable toward novel antibiotic therapies.

Introduction

In the previous chapter, the *S. suis* pheromone-induced competence regulon was characterised using a temporal transcriptomics approach, revealing that competence in *S. suis* is indeed transient as shown in previous studies (Chapter 2, [156]) and characterised by tightly controlled expression of the transformasome and associated regulatory genes (Chapter 3). Natural genetic transformation is associated with internalization and chromosomal integration of exogenous DNA into the bacterial genome, enabling bacteria to obtain new genetic traits that may improve fitness in changing environmental conditions, including those involving survival of host immune defences. Competence-inducing conditions in Gram-positive bacteria are generally related to stress challenges, circumstances, which in nature would select for adaptation and resilience and eventually, may improve fitness [254]. The *comCDE* operon of *S. pneumoniae* is located close to the chromosomal replication origin and as a consequence antibiotics that target DNA replication cause stalling of the replication fork, leading to increased copy number of *comCDE* and competence induction [255]. Under laboratory conditions, competence is influenced, among others, by temperature, growth medium, pH and the concentrations of magnesium and calcium [256]. Integrity of the plasma membrane or of the bacterial cell wall, perception of external stress, nitrogen concentration and cell density are some of the stimuli regulating, positively or negatively, the activation of competence [142,161]. Interestingly, pheromone induction of competence in proliferating *S. mutans* leads to the competence state in only a proportion of the bacteria, the remaining population undergoing cell death [257]. However, the interaction of these processes with ComCDE or ComRS and how they may participate in the modulation of the physiological state of the cells and with decisions to commit to competence or to cell death are not well understood.

Despite the evolutionary advantages of natural competence, uptake and incorporation of “foreign” DNA into the recipient genome is not without potential risks. Recombination of DNA with different genetic content may cause a gain or loss of functionalities. Additionally, DNA recombination during the process of elongation and chromosome replication is potentially dangerous to genome integrity. Furthermore competence development may negatively affect the organism’s basal metabolism and fitness as the process is costly in terms of use of energy and protein translation. The biological relevance of the possible physiological changes that might co-occur with competence development prompted us to investigate the possible regulatory link between metabolism and competence development, and to find out more about the physiological adaptation of bacteria upon induction of the competence state.

One molecular link between competence and metabolism is the small peptide pheromone SigX-inducing-peptide (XIP), that, in a number of streptococcal

species, is transported into the intracellular environment by the general peptide transporter system designated Opp [155,190]. Opp transporters are located in the plasma membrane, and their main function is to take peptides from the extracellular environment to serve as sources of plasma carbon and nitrogen that are necessary for bacterial proliferation [258], thus providing some contextual information on extracellular availability of nutrients. The use of a general peptide transporter for uptake of the pheromone may not be coincidental as it could be an indirect sensor of available oligopeptides and thus competitors occupying the same niche. For example, the addition of a high concentration of tryptone extract (but not casamino acids), to complex media can inhibit XIP induction of competence in *S. mutans* [209] and in *S. thermophilus* [203].

Temporal transcriptome data obtained during competence development (Chapter 3) were analyzed under the hypothesis that it would reveal the metabolic context of bacteria entering, executing and exiting the competence state. We investigated which basal processes appeared to be induced during competence, and which processes were incompatible with bacterial proliferation. Such analyses would, in addition to unravelling the genetic regulation of competence in *S. suis*, hint at why competence induction can be potentially unfavourable for the bacteria. This is not only of fundamental interest but might also contribute to the development of future therapeutic applications, for instance induction of competence might render *S. suis* bacteria more vulnerable to antibiotics and the host immune response.

Materials and Methods

Bacterial strains and culture conditions

The virulent *S. suis* serotype 2 strain S10 was used in this study. *S. suis* S10 genome is 99% identical to the genome of *S. suis* 2 strain P1/7 [230], a sequenced reference strain of which the genome had been annotated previously [23]. *S. suis* was grown at 37°C at 5% atmospheric CO_2 in Todd Hewitt Broth (THB, Thermo Scientific, Oxoid) with 1.2% of agar (BD) if solid medium. Growth phase was determined by measurement the optical density (OD_{600nm}) using SpectraMax M5 reader (Molecular Devices LLC).

RNA extraction

Induction of competence was performed as previously described [156]. Briefly *S. suis* S10 was grown until OD_{600nm} 0.04. Thirty five ml of culture was collected and donor DNA (pNZ8048, 350 µg) in EB buffer (10 mM Tris-Cl, pH 8.5) was added to the bacteria along with synthetic XIP (GNWGTWVEE) at a final concentration of 250 µM. For the control same quantity of culture was collected and donor DNA added. Ten ml of the induced cultures were collected after 5, 15 and 45 minutes of XIP addition. Ten ml of the uninduced cultures were collected at 15 and 45 minutes. The samples were centrifuged for 2 min at 8000 g and the pellet resuspended in 2.5 mL PBS plus 5 mL RNAprotect buffer (Promega). After 5 minutes of incubation the bacteria were again collected by centrifugation and immediately frozen in liquid nitrogen until further use. The frozen pellet was dissolved in 110 µl of TE containing 1.25 µg/mL proteinase K and 15 ug/ml lysozyme and incubated for 10 minutes. Then 700 µL RLT buffer (Promega) containing 7 µl of freshly added β-mercaptoethanol was added to the dissolved pellet and the bacteria were lysed using a FastPrep-24 (MP Biomedicals, Solon, OH) for 6.0 m/sec at 20 sec. Total RNA was purified using RNeasy Mini Kit (Quiagen). The quality and the concentration of RNA were assessed with an Experion System (Bio-Rad) and by analysis of the A260/A280 ratio (NanoDrop 8000 UV-Vis Spectrophotometer). cDNA was synthesized with SuperScript III Reverse Transcriptase kit (Invitrogen) using Aminoallyl-dUTP as a source dUTPs and purified with Illustra CyScribe GFX Purification Kit (GE Healthcare). The cDNA was labelled with CyDye Post-Labeling Reactive Dye Pack (GE Healthcare).

Microarray transcriptome analysis

An *S. suis* oligoarray (8×15 K) containing *in situ* synthesized 60-mers was produced by Agilent Technologies (Santa Clara, USA), based on the genome sequence of *S. suis* P1/7 [23]. A total of 7651 unique 60-mers having a theoretical melting temperature of approximately 81°C and representing 1960 ORFs were selected as described [231]. The majority (91%) of genes were represented by 4 probes with the remaining probe

distribution as follows: 3 (4%), 2 (2%), or 1 probe (3%). Twenty-five putative genes were not represented on the array because no unique probe satisfying the selection criteria could be selected. Co-hybridization with labelled cDNA probes was performed on the oligonucleotide arrays at 42°C for 16 h in Slidehyb#1 (Ambion, Austin, USA). The data were normalized using Lowess normalization [232] as available in MicroPrep [233] and corrected for inter-slide differences on the basis of total signal intensity per slide using Postprep [233]. Significance of differential gene expression was based on false discovery rate (FDR) values lower than 0.05. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE74507 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wrwfquyqfhijfeh&acc=GSE74507).

Transcriptome data mining

Short Time-series Expression Minor (STEM) [259] was used to cluster and compare gene expression intensities to identify genes of which the expression was significantly changed across all the time-points.

Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) was used to filter the data for the most differentially expressed genes using the following parameters: standard deviation: 250; at least 4 observations with absolute value higher than 20; subtraction between maximum and minus minimum: 200.

The heatmaps were generated by MultiExperimental Viewer (MeV) program (http://www.tm4.org/mev.html) [234]. For all genes and proteins identified in the *S. suis* P1/7 genome, and KEGG pathway annotations were obtained using the BLAST2GO software (www.blast2go.org) [260] including annotations based on terms obtained from EBI using the InterPROScan feature [261] that is part of BLAST2GO while GO terms over-represented were calculated via the integrated FatiGO package [262].

Results

Half the *S. suis* transcriptome is differentially expressed in response to competence induction

Based on our initial competence kinetics study (Chapter 2, [156]), we examined the transcriptome of *S. suis* in response to competence induction after 5, 15 and 45 minutes exposure to XIP. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE74507 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=wrwfquyqfhijfeh&acc=GSE74507). After 5 minutes, almost half the *S. suis* transcriptome (918 of the 1969 known ORFs, 46.6%) showed a statistically differential expression compared to the control; 82% of the differentials were downregulated. At 15 minutes, 333 (16.9%) genes were differentially expressed; at 45 min 115 (5.8%) were differentially expressed (Table 1). These data show that competence induction has a major impact on *S. suis* gene expression, with more than 80% of the differentials being suppressed after 5 min of competence induction. This differential regulation emphasises that competence has a major impact on *S. suis* regulatory processes, likely including processes involved in physiology and metabolism. The finding that more than 80% of the differentials were down regulated prompted us to identify suppressed cellular processes and evaluate what bearing these may have on bacterial function and persistence.

Table 1: Number of genes with expression significantly changed at 5, 15 and 45 minutes compared with the control.

	5 Min	15 Min	45 Min
Up regulated	163	162	78
Down regulated	755	171	37

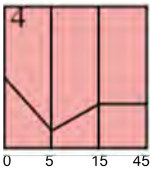
STEM analysis

In the next section, we outline the processes that were differentially regulated during competence (exemplified by DNA repair) with special attention for regulation of bacterial metabolism. Because competence involves DNA repair, a process that is largely incompatible with whole-genome replication, we hypothesised that competence is incompatible with most cellular processes apart from pathways involved in DNA uptake, DNA processing, recombination and repair. Thus, we expected to identify expression profiles of genes that were downregulated at the 5-min time-point, when competence was activated. We reported (Chapter 2, [156]) that competence development in *S. suis* peaks at 15 minutes after bacterial exposure to the XIP, and exit from competence is established at 45 min. As competence is a dynamic process that is tightly orchestrated by specific transcriptional regulators that may be differentially expressed throughout competence development and exit, we

first identified genes that altered their expression across all time-points using STEM software (see Methods) [259].

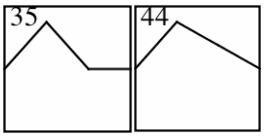
STEM analysis first identified genes of which the expression had significantly changed at 5 min, when bacteria were entering competence state, at 15 minutes (peak competence) and 45 minutes (exit from competence), and retrieved their respective gene ontology (GO) (geneontology.org) terms. These GO terms helped us to identify the biological processes that had changed across the three time-points, from the early stage of competence at 5 min after induction to the shutdown of competence at 45 min. The possible link between competence and metabolism led us to hypothesise that in our transcriptome data, we should be able to find clusters of co-expressed genes with GO terms involved in metabolism, energy state and cellular fate. Moreover, we reasoned that consistently downregulated genes would be annotated with GO terms pointing at cellular processes that were repressed during competence. We set out to investigate clusters of co-expressed genes, expected to together carry out specific tasks, in a hypothesis-driven manner, by postulating cellular functions that can be expected to be induced or repressed before, around and after the induction of natural competence. In the upcoming section, we list expression profiles representing significantly altered genes and their enriched GO terms resulting from STEM analysis (Table 2).

Table 2: overview of the STEM profiles. The coloured profiles had a statistically significant number of genes assigned. Non-white profiles of the same colour represent profiles that could be grouped into a single cluster. The enlarged profile shows the time-point locations; for all profiles, the time-points can be schematically displayed like this.



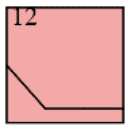
STEM profile	Number of genes assigned	p-value	GO terms, general description
	30	1	Competence related functions (DNA binding and repair, response to external stimuli)
	41	0.7	Competence related functions (ATP binding domain, DNA binding and repair)
	145	$6*10^{-76}$	Cell wall and cell membrane (organization and biogenesis). RNA metabolism
	125	$3*10^{-34}$	Redox balance and transfer of acetyl groups
	53	$2*10^{-2}$	Protein synthesis (ribosome and ribosome structural components, translation, peptide synthesis)
	204	$2*10^{-75}$	Nucleotide and nucleoside transmembrane transport. ATP synthesis and ATPase activity
	41	$2*10^{-5}$	RNA metabolism and gene expression
	176	$3*10^{-75}$	Protein folding, cell cycle and cell division
	11	0.4	Carbohydrate and unsaturated fatty acids metabolic process, cellular metabolism
	175	$3*10^{-50}$	Carbohydrate uptake and transport, hydrolase activity and general transport of substances

We first searched for profiles including the known competence genes.



Profile 35 and 44 include the competence genes, including those under direct control of ComX. As predicted, the associated GO terms: “DNA binding”, “response to external stimuli” and “DNA metabolic process” reflect common functions of natural genetic transformation. The associated GO term “ATP binding” also shows that energy metabolism is significantly altered during competence. White profiles do not contain a statistically significant number of genes showing that trend [259]B. Note that absence of statistical significance does not imply that the trend bears no biological relevance.

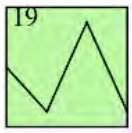
Profile 4 contained genes that were downregulated during competence, with the lowest expression at 5 min. The genes belonging to this profile participated in cell wall, cell division and RNA metabolism, suggesting that gene expression and bacterial proliferation were suppressed when competence was induced. Interestingly genes associated with the GO term “oxidoreductase activity”, representing the redox processes that are at the basis of biochemical reactions, were included in this cluster. Note that this should be interpreted as a significantly lower expression compared to the non-competent state, rather than no gene expression during competence induction at all.



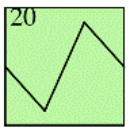
Profile 12 represents a second cluster of co-expressed genes with significantly reduced expression throughout the competence period. Note that 4 and 12 display the same colour; this indicates that these profiles include a statistically significant number of genes that could be grouped into a single larger cluster characterised by an expression profile encompassing the two separate profiles. The genes included in profile 12 were involved in redox balance and transfer of acetyl groups, a common event in biological metabolic synthetic - and catabolic reactions. The expression profiles of the genes included in profiles 4 and 12 support the notion that the competence state is characterised by a suppression of general metabolism, and these

profiles represent part of the 80% of the significantly differentially expressed genes that were downregulated at 5 minutes.

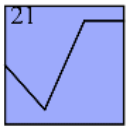
Profile 33 included genes that were upregulated at 5 and 45 minutes and downregulated at 15 min, at the peak of transformability. The genes showing this expression pattern were encoding structural components of the ribosome and involved in rRNA binding, gene expression and protein translation. Suppression of ribosome gene expression is a straightforward way to suppress translation and *de novo* cellular protein production, thereby suppressing the activation of any pathway that relies on *de novo* protein production.



We were interested which genes were necessary for competence to proceed; we reasoned that at the peak of competence induction, defined as the highest number of colonies after competence-induced transformation, a specific set of genes would be induced. Profile 19 was of interest to us since it included genes that were downregulated at all time-points except at 15 min. This profile included genes involved in transmembrane transport and (purine) nucleoside and nucleotide transport and six transcriptional regulators of which SSU0388 encodes an orthologue of a protein induced by cell wall stress conditions and SSU1826 encodes an orthologue of a protein involved in competence in other streptococci [162].

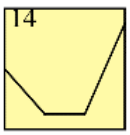


We also observed a cluster of genes that were induced at peak of competence induction but that were not downregulated at 45 min as in profile 19, but of which the expression was restored to the uninduced state. This cluster included genes participating in RNA metabolism and gene expression. This profile, together with the previously reported profiles, suggests that expression of at least some genes is induced between peak and shutdown of competence. Interestingly this profile includes a PadR-like transcriptional regulator, a protein that is usually part of the response to phenolic acids, facilitating the bacteria to adapt to stressful environments [263,264]. We searched for other clusters of genes induced at peak competence.



Profile 21 included genes that were suppressed at 5 minutes, induced between 5 and 15 min and remained induced between 15 and 45 min. The genes in this profile participated in protein folding and the cell cycle. This cluster together with the previous two clusters suggested that at 15 and 45 minutes, genes involved in specific metabolic pathways including protein folding and involved in the cell cycle had been induced. This was of interest since this hinted at induction of cell proliferation at the later time-points, whereas these processes had been suppressed at the onset of competence.

Next, we searched for a gene profile that included genes downregulated at the earlier time-points and upregulated at later time-points. The small cluster 7 contained genes continuously induced after 5 min that were involved in metabolism of carbohydrates and unsaturated fatty acids, processes that play roles in basal metabolism and bacterial proliferation, more specifically, during the synthesis of novel membranes.



Profile 14 contained genes that had been downregulated until 15 min (peak of competence) but were strongly induced between 15 and 45 minutes. These genes were involved in carbohydrate uptake and transport across the membrane, and amide metabolic processes that commonly involve the modification of carboxylic acids including amino acids and organic acids via biochemical reactions that include formation of amides. Profile 14, together with the previous two profiles represent clusters of genes with distinct metabolic functions.

The STEM analysis results suggested that competence is characterised by two main phases: a first phase when competence is induced and the competence state develops (peaks) that is characterised by a general repression of gene expression, protein production, cell cycle and metabolism (note that 80% of the differentials had been downregulated from the onset of competence); and a second phase when competence is exited, characterised by an induction of genes involved in protein

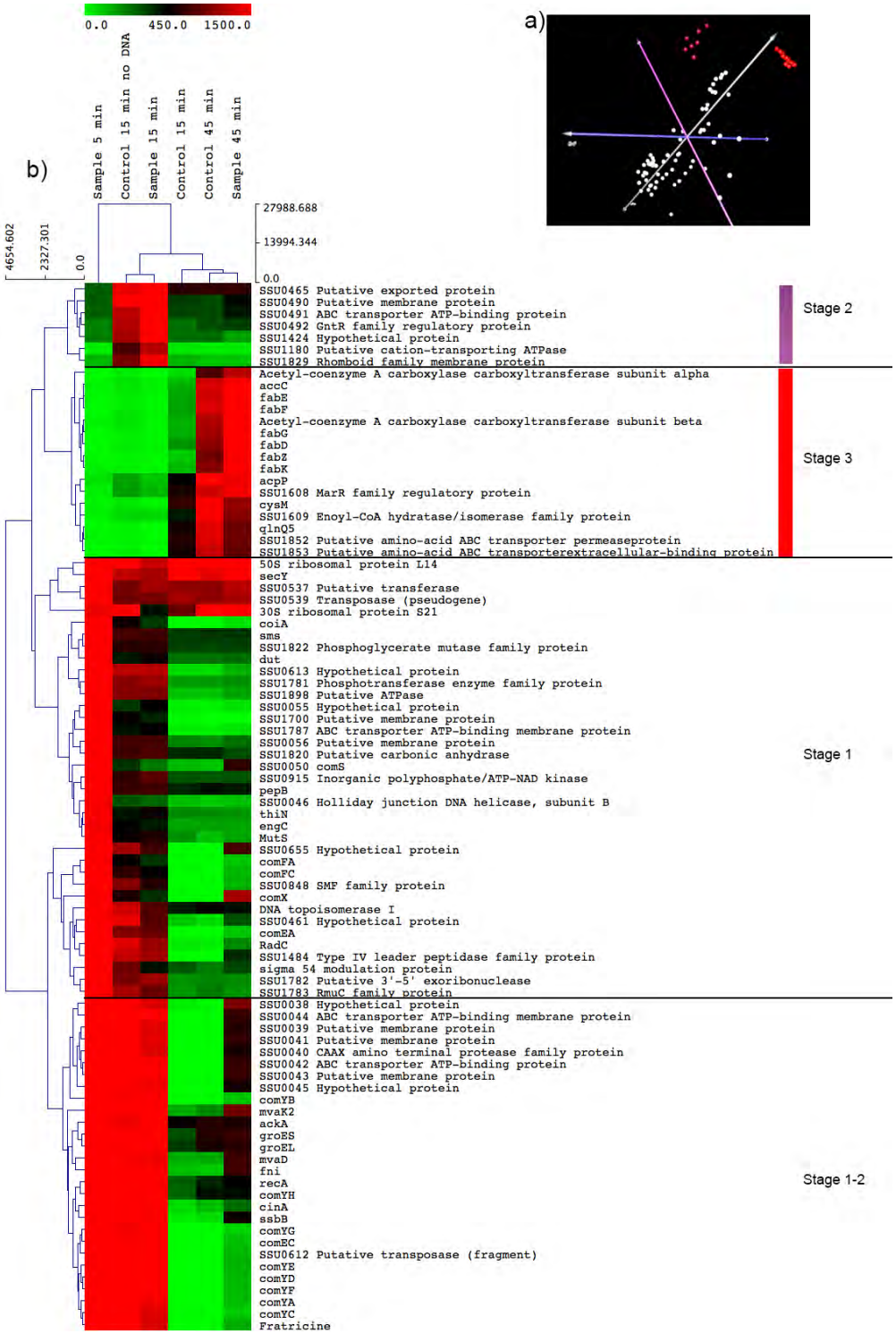
folding, cell cycle and fatty acid metabolism. To explore this further, we filtered the transcriptomes for those genes that showed the highest differences in expression between the three time-points, using the raw gene expression values, performed principal component analysis (PCA) and plotted the PCA output on a heatmap representing raw gene expression of the filtered genes.

PCA and cluster analysis shows that initiation, peak and exit of competence are regulated by distinct gene clusters

STEM analysis, in particular profile 14, 7, 4 and 12, suggested that competence induction might be incompatible with bacterial growth at 5 minutes but not or less so at later time-points. To investigate this notion, we expected to be able to find clusters of genes involved in cell proliferation strongly induced at especially 45 minutes. To investigate this, we generated heatmaps to display gene expression as normalised values, not as ratio data, to identify genes that were highly expressed. Pre-filtering genes (see Methods) resulted in a set of 88 genes, 4.5% of the *S. suis* genome, including genes encoding proteins with diverse metabolic functions, transcriptional regulators, heat-shock proteins and chaperones, proteases, and genes involved in competence including the gene SSU0050 encoding the competence peptide pheromone (Figure 1,b). We inferred that the presence of these key competence (and competence-related) genes in the 88-gene set indicated that the filtering of genes had resulted in a set that was still biologically relevant to competence. The 88 genes were clustered using average linkage and Euclidian distance (see Methods) and displayed in a heatmap. Visual inspection of the raw gene expression across the heatmap showed that these 88 genes had clustered into distinct time-dependent stages, I-III (Figure 1,b). PCA analysis successfully separated the dataset into six components, with the first 2 components explaining 90,9% of the total variation and the first 3 components explaining 97,8% (Figure 1,a). 2D and 3D analysis of the PCA plot showed that at least three major clusters could be identified, coloured white, red and purple in Figure 1A. Plotting the three major PCA clusters onto the heatmap showed that the red and purple clusters corresponded with distinct clusters in the heatmap (Figure 1.b). The combined PCA and heatmap clusters showed that the initiation and peak competence gene clusters contain the white and purple PCA clusters and span phases I, I-II and II, and that the red PCA cluster corresponds to a separate heatmap cluster, phase III. Phase III includes the fatty acid metabolism operon, and all genes in this cluster were only strongly expressed upon competence exit. This PCA and heatmap analysis of the most differentially expressed genes again shows that the earlier competence time-points are characterised by gene expression profiles that are very different from the later time-point, when the competence state is shut down. The PCA and heatmap analysis also shows that genes involved in cell proliferation, the fatty acid metabolism genes of the red PCA cluster and the stage III cluster of the heatmap, are only strongly expressed when the bacteria have exited the

competence state (Chapter 2, [156]). The STEM and PCA analyses therefore support the hypothesis that induction and execution of competence is incompatible with bacterial proliferation. We decided to investigate this prediction more extensively using a GO term enrichment approach.

Figure 1: Most differentially expressed genes. a) Principal Component Analysis (PCA) plot showing three major clusters (in white, red and purple). Red and purple clusters correspond with distinct clusters in the heatmap indicated by the same colours. **b)** Heatmap displaying the 88 most differentially expressed genes clustered using average linkage and Euclidian distance into 3 distinct time-dependent stages.


a)
b)
0.0
450.0
1500.0
Sample 5 min
Control 15 min no DNA
Sample 15 min
Control 15 min
Control 45 min
Sample 45 min
27988.688
13994.344
0.0
4654.602
2327.301
0.0
SSU0465 Putative exported protein
SSU0490 Putative membrane protein
SSU0491 ABC transporter ATP-binding protein
SSU0492 GntR family regulatory protein
SSU1424 Hypothetical protein
SSU1180 Putative cation-transporting ATPase
SSU1829 Rhomboid family membrane protein
Acetyl-coenzyme A carboxylase carboxyltransferase subunit alpha
accC
fabE
fabF
Acetyl-coenzyme A carboxylase carboxyltransferase subunit beta
fabG
fabD
fabZ
fabK
acpP
SSU1608 MarR family regulatory protein
cysM
SSU1609 Enoyl-CoA hydratase/isomerase family protein
qlnQ5
SSU1852 Putative amino-acid ABC transporter permeaseprotein
SSU1853 Putative amino-acid ABC transporterextracellular-binding protein
50S ribosomal protein L14
secY
SSU0537 Putative transferase
SSU0539 Transposase (pseudogene)
30S ribosomal protein S21
coiA
sms
SSU1822 Phosphoglycerate mutase family protein
dut
SSU0613 Hypothetical protein
SSU1781 Phosphotransferase enzyme family protein
SSU1898 Putative ATPase
SSU0055 Hypothetical protein
SSU1700 Putative membrane protein
SSU1787 ABC transporter ATP-binding membrane protein
SSU0056 Putative membrane protein
SSU1820 Putative carbonic anhydrase
SSU0050 comS
SSU0915 Inorganic polyphosphate/ATP-NAD kinase
pepB
SSU0046 Holliday junction DNA helicase, subunit B
thiN
engC
MutS
SSU0655 Hypothetical protein
comFA
comFC
SSU0848 SMF family protein
comX
DNA topoisomerase I
SSU0461 Hypothetical protein
comEA
RadC
SSU1484 Type IV leader peptidase family protein
sigma 54 modulation protein
SSU1782 Putative 3'-5' exoribonuclease
SSU1783 RmuC family protein
SSU0038 Hypothetical protein
SSU0044 ABC transporter ATP-binding membrane protein
SSU0039 Putative membrane protein
SSU0041 Putative membrane protein
SSU0040 CAAX amino terminal protease family protein
SSU0042 ABC transporter ATP-binding protein
SSU0043 Putative membrane protein
SSU0045 Hypothetical protein
comYB
mvaK2
ackA
groES
groEL
mvaD
fni
recA
comYH
cinA
ssbB
comYG
comEC
SSU0612 Putative transposase (fragment)
comYE
comYD
comYF
comYA
comYC
Fratricine
Stage 2
Stage 3
Stage 1
Stage 1-2

BLAST2GO and FatiGO

To further examine our hypothesis of a cell proliferation blockade as a consequence of competence activation, we analysed which GO terms were over-represented for each time-point using Blast2GO and its integrated FatiGO package [260,265] using a Fisher's Exact Test (Table 3). For each time-point, we compared significantly up- or downregulated genes and calculated which GO terms, associated with the up- or downregulated gene sets, were significantly enriched or over-represented. For convenience, we report a selection of the GO terms that were statistically over-represented. At 5 and 15 minutes, the down-regulated genes carried GO terms associated with cell proliferation and basal metabolism whereas GO terms associated with DNA binding were over-represented in the up-regulated gene sets. Interestingly, genes carrying the GO term "ATP binding" were up-regulated, highlighting that the competence state is an energy-demanding process, probably linked with transformasome formation, active DNA uptake, processing, recombination and repair.

At 45 minutes, the over-represented GO-terms were associated with activation of metabolic processes, cell growth and proliferation, whereas these processes had been suppressed at the earlier time-points. The low number of genes down-regulated at 45 minutes: 37, representing 1.9% of the *S. suis* genome, exemplifies how similar bacterial gene expression was at 45 minutes compared to pre-competence induction conditions. In contrast, 38% of the transcriptome had been downregulated at the 5-min time-point. The combined STEM, PCA and GO enrichment results are in agreement and show that the competence state of *S. suis* is characterised by an initial suppression of processes associated with bacterial proliferation and activation of the natural transformation machinery, followed by induction of fatty acid metabolism, protein translation and cell division at 45 minutes, effectively re-establishing bacterial growth.

Table 3: selection of over-represented GO terms for each time-point

Time-point	Down-regulated		Up-regulated	
	Term	P-Value	Term	P-Value
5 minutes	Cellular catabolic process	7.56E-06	Structure-specific DNA binding	2.35E-04
	Lipid biosynthetic process	8.31E-05	DNA binding	2.49E-04
	Cellular biosynthetic process	1.13E-04	ATP binding	7.65E-04
	Cell wall organization or biogenesis	1.33E-04	Hormone biosynthetic process	4.00E-03
	Regulation of primary metabolic process	6.47E-04		
15 minutes	Fatty acid biosynthetic process	1.78E-10	ATP binding	3.53E-04
	Carboxylic acid biosynthetic process	7.29E-09	Hormone biosynthetic process	3.55E-04
	Organic acid metabolic process	1.40E-06	Carbohydrate derivative binding	6.37E-04
	Single-organism biosynthetic process	2.41E-05	DNA binding	2.75E-03
	Acetyl-CoA carboxylase complex	5.74E-05	DNA mediated transformation	5.37E-03
45 minutes	Membrane	1.84E-02	Fatty acid biosynthetic process	5.81E-16
	Regulation of transcription, DNA-templated	2.67E-02	Monocarboxylic acid biosynthetic process	1.62E-15
	Regulation of RNA metabolic process	4.73E-02	Lipid biosynthetic process	5.85E-14
			Ligase activity, forming carbon-carbon bonds	1.38E-06
			Single-organism biosynthetic process	7.01E-03

Discussion

Natural genetic transformation is the ability of bacteria to uptake DNA present in the extracellular environment and to stably integrate this "foreign" DNA in their genome. The physiological state during which this mechanism takes place is called competence state. Chromosomal replication during the competence state, when DNA is being actively taken up and recombined may result in an accumulation of mutations in daughter cells or double-strand breaks that are not efficiently repaired during competence. In *Bacillus subtilis* natural competence state is linked to inhibition of both cell elongation and cell division which resumes 90 min after exit from competence [266,267]. This delay or checkpoint prevents chromosomal replication and thus, bacterial proliferation until the transforming DNA recombination event has been carried out.

In *S. suis*, *in vitro* competence induction is possible by exposing a *S. suis* culture to XIP at a very short and specific window of the bacterial lag phase. We previously showed (Chapter 2, [156]) that *S. suis* cells need around 5 minutes of exposure to XIP in order to produce the necessary machinery able to take up and recombine exogenous DNA [156]. Transformation efficiency reaches its peak at 15 minutes from competence induction and competence is lost after 45 minutes. In this chapter we described the transcriptional changes during 45 minutes following competence induction via the analysis of 3 time-points: at 5, 15 and 45 minutes. Our data show substantial modulation of gene expression with nearly half (918 or 47% of the 1969 known *S. suis* ORFs) being significantly differentially expressed (See Method) at 5 min. At 15 and 45 minutes only 333 and 115 genes respectively, were differentially expressed. Our data differ from transcriptomic analyses performed in other streptococcal species [187,235,236]. After competence induction in *S. gordonii* only 35 and 127 differentially expressed genes were identified at 5 and 15 minutes respectively [235]; similar results were obtained in *S. pneumoniae* [187,236]. *S. gordonii* and *S. pneumoniae* use a different proximal switch for induction of *comx* expression and might also differently regulate the competence-induced checkpoint for cell replication. Moreover, the growth phase of competence induction differs, being lag phase for *S. suis* but exponential phase for *S. gordonii* and *S. pneumoniae* [142,161]. In this chapter we wanted to analyse the physiological impact that competence induction may have on bacterial metabolism to come up with hypotheses that can be verified in metabolic and other studies. The global response of *S. suis* to exposure to XIP, and thus the activation of the competence machinery, was analysed using STEM time series analysis together with PCA-heatmap analysis and GO enrichment analysis. These analyses enabled us to identify sets of co-regulated genes of which the expression significantly changed during competence induction, development and termination and to identify the biological processes that were differentially regulated at the onset of, and exit from, competence. Transcriptome

analysis by STEM is a useful way to achieve this since STEM analysis clusters genes based on similar gene expression values across all the time-points, comparing expression profiles to pre-modelled trends, and calculating GO enrichment for each cluster.

We initiated our analysis from profile 4 and 12, characterized by repressed expression during the whole experimental set up. Together these profiles encompass 270 genes carrying strongly overrepresented GO terms, corresponding with a significantly lower expression of genes involved in bacterial basal metabolism compared with the uninduced samples. For instance, we observed a down-regulation of genes involved in transfer of acetyl groups, a common event in biological metabolic and breakdown reactions and a usual feature of metabolically active cells. We reasoned that competence, a state where bacteria were mainly carrying out processes involving DNA uptake and genome recombination and repair, would be incompatible with activation of biochemical or other cellular pathways that required extensive genome-wide expression and protein translation. By inspecting all STEM profiles, we obtained an excellent overview of which processes were induced or repressed at each of the time-points representing induction, the competence state, and exit from competence. We found that the earlier two time-points were mainly characterised by suppression of rRNA binding, gene expression, protein translation, protein folding, cell proliferation and the cell cycle. In contrast, we found induction of transformasome formation, DNA uptake and recombination, and genes involved in transmembrane transport, nucleoside and nucleotide transport, and RNA metabolism at the two later time-points. Moreover six transcriptional regulators, and the enzymes acetyl-coA acetyltransferase and diacylglycerol kinase were also induced at these later time-points. Acetyl-coA acetyltransferase is necessary for microbial carbon fixation, fatty acid elongation, pyruvate fermentation and fatty acid degradation while the diacylglycerol kinase encoded by SSU1226 is involved in lipid metabolism and signalling. These observations, shown in profile 19, 20 and 21, suggest that some metabolic genes are induced between the peak and shutdown of competence. The profiles also suggest that the expression of a specific metabolic gene cluster involved in carbohydrate and fatty acid metabolism had been induced at the later time-points. We indeed found clusters of genes that started to be induced at 15 minutes and remained induced up to 45 minutes involved in carbohydrate uptake and metabolism, hydrolase activity and the cell cycle (Fig. 2).

Importantly, the genes involved in the initiation and elongation of short fatty acids were present among the most differentially expressed genes across the three time-points and the controls. The same genes were also represented as a separate cluster in PCA analysis of the most differentially expressed genes. They were not expressed at the earlier time-points but it showed a strong expression at 45 minutes after competence induction, when cells have exited the competence state (Fig. 1). To further mine the competence transcriptomes, we calculated which GO terms were

over-represented at the three time-points compared with the uninduced control. Table 3 shows that at 5 and 15 minutes, the enriched or over-represented GO terms belong to downregulated genes associated with cell proliferation and basal metabolism. At 45 minutes, the over-represented GO-terms belong to induced genes associated with activation of the metabolic processes and are compatible with an induction of cell growth and proliferation. It is worth mentioning that genes carrying the GO term "ATP binding" were strongly induced during competence, exemplifying the high energetic costs that are commonly ascribed to transformasome formation and DNA uptake and recombination. We hypothesise that, in the competence state, energy molecules are partially depleted and responses to environmental changes will be slower.

Taken together these results show the massive impact competence activation has on *S. suis* physiology and metabolism. At 5 and 15 min after competence induction ribosome function and basal metabolism were suppressed, compatible with a delay or checkpoint in DNA replication and cell division. It is of interest to consider that in *S. suis*, competence induction reduces cell proliferation and bacterial growth despite the bacteria growing in a very rich medium and at a bacterial growth phase usually characterized by fast replicating bacteria. At 15 minutes, at the peak of competence, we observed an upregulation of the genes involved in DNA uptake, processing and recombination, which is in complete agreement with peak competence. At 45 minutes after competence induction, *S. suis* gene expression is compatible with the physiology that is typical for bacteria growing *in vitro* under good growth conditions, including activation of the fatty acid and carbohydrates metabolism, enabling cell proliferation and bacterial growth. The delay or checkpoint in DNA replication and cell division during competence is not surprising, as homologous recombination of the transforming DNA during chromosomal replication may lead to replication errors [266,267]. In *S. pneumoniae*, it has been demonstrated that ParBS regulated chromosome segregation machinery negatively influences competence development [268] showing how, in species in which the competence activation machinery differs, cell growth and competence might act antagonistically. In *B. subtilis* the checkpoint is controlled at two levels: (I) Inhibition of cell elongation through competence induced expression of ComGA [266] and (II) cell inhibition of cell division by ComGA and the highly conserved protein Maf [266]. Recently, In *B. subtilis* ComCG was shown to sequester MreB preventing cell elongation and cell division before exit from competence [269]. Interestingly, a homologue of ComGC, ComYA, is also induced by the competence pheromone in *S. suis*, although it is not known if ComYA is required for checkpoint control of cell division.

Our data suggest that basal metabolic pathways are incompatible with activation of competence in *S. suis*. The 5-min time-point is characterised among others by cellular activities involving ATP binding and energy-costly processes such as transformasome formation, depleting the bacteria's energy pool. Thus we

can hypothesise that induction of competence and energy depletion might render bacteria more vulnerable to antimicrobial therapy and immune defences in the host. Moreover, our study proposes that *S. suis* is characterised by an unfavourable energy balance and not well suited to control its redox balance since key antioxidant genes as thioredoxin and superoxide dismutase are hardly expressed during the competence state, making bacteria in the competence state more susceptible to reactive oxygen species which are produced by neutrophils and macrophages after contact with bacteria. We consider that it might be of interest to investigate if induction of competence of *S. suis* could render the bacteria less virulent during infection. Oggioni and collaborators' studies [270,271] provide an indication that this may be a realistic scenario. They evaluated the effect of competence induction on virulence in a mouse model of pneumococcal sepsis and demonstrated a significant increase in mouse survival and a reduction in blood *S. pneumoniae* counts. However, induction of the competence system increased virulence if the bacteria were in a biofilm-like state, e.g. as described in pneumonia. Our transcriptome analyses have provided us with pathways that may be more vulnerable to small molecule inhibitors when targeted during the competence state, and that interfering with these pathways may contribute to suppress proliferation of *S. suis*. Blocking essential pathways, rather than blocking individual genes, might decrease the incidence of resistance, since chances of developing resistance in all the pathway genes, at the same time keeping the pathway functional, are very low.

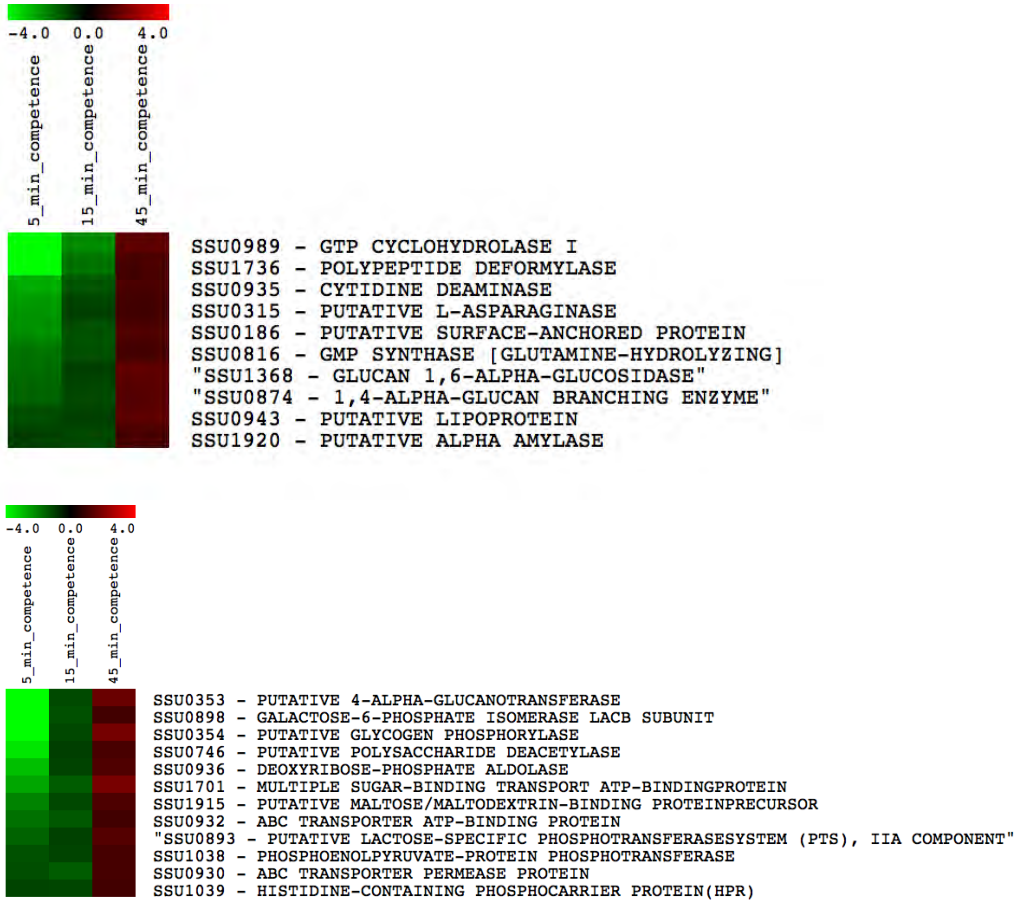


Figure 2: Heatmaps of the genes involved in carbohydrates metabolism (a), and in hydrolase activity (b).

Chapter 5

A multi-site infection model for *Streptococcus suis* using zebrafish larvae

Edoardo Zaccaria, Rui Cao, Jerry M. Wells and Peter van Baarlen

Host-Microbe Interactomics, Animal Sciences, Wageningen University, Wageningen, The Netherlands

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Abstract

Streptococcus suis is an encapsulated Gram-positive bacterium, and the leading cause of sepsis and meningitis in young pigs resulting in considerable economic losses in the porcine industry. Moreover, it is considered an emerging zoonotic agent with increasing numbers of human cases over the last years. In the environment, both avirulent and virulent strains occur in pigs, and virulent strains appear to cause severe disease in both humans and pigs. There is a need for a convenient, reliable and standardized animal model to assess *S. suis* virulence. The zebrafish larvae infection model has some advantages over the use of adult zebrafish (*Danio rerio*), including transparency of larvae, low cost, ease of use and exemption from ethical legislation up to 6 days post fertilization, but has not been previously established as a model for *S. suis*. Infection by microinjection into the yolk sac or Ducts of Cuvier is leads to similar rates of mortality for different strains and was highly reproducible. Microinjection of several different strains of *S. suis* in zebrafish larvae resulted in dose- and strain-dependent larval death, strongly correlating with presence of the *S. suis* capsule and to the original virulence of the strain in pigs. Additionally we compared the virulence of the two-component system mutant of *ciaRH*, which is attenuated for virulence in both mice and pigs *in vivo.* Infection of larvae with the Δ*ciaRH* strain resulted in significantly higher survival rate compared to infection with the S10 wild-type strain. Our data demonstrate that zebrafish larvae are a rapid and reliable model to assess the virulence of clinical porcine *S. suis* isolates.

Introduction

Streptococcus suis is a zoonotic Gram-positive bacterial pathogen and the leading cause of sepsis and meningitis in young pigs. Infections with *S. suis* occur worldwide throughout pig production industry, resulting in considerable economic losses [1,9]. In recent years *S. suis* isolates causing more rapid and severe infections in both humans and pigs have been identified leading to concerns about the emergence of more virulent strains [65,272]. At least 35 different serotypes of the capsular polysaccharide have been identified of which serotype 2 is the most frequently isolated from infected pigs and humans [65,273]. In addition, serotypes 1, 9, and 14 have often been associated with porcine disease [274,275]. Its natural habitat is the upper respiratory tract of pigs, the tonsil and nasal cavity in particular [2] and the intestine [276]. Healthy pigs that carry *S. suis* are a source for its transmission to the herd [2]. At present, *S. suis* is the most common cause of adult bacterial meningitis in Vietnam [13] and the second most common in Thailand [277]. Moreover, two human outbreaks with high mortality rates have been reported in China [27,273,278] underlining its importance as a zoonotic agent in Asia. In Europe, the largest number of zoonotic infections due to *S. suis*, have been recorded in the Netherlands (Figure 1 in [273]). In pigs and humans, meningitis, endocarditis and streptococcal toxic shock-like syndrome are the most common symptoms caused by virulent *S. suis* strains [15,19,279]. Currently it is impossible to assess the virulence of an *S. suis* strain using molecular markers [36] highlighting the need for better genomic markers and animal models to establish the genetic determinants of virulence in different isolates.

Notwithstanding its prominence as a zoonotic agent, little is known about *S. suis* pathogenicity, virulence and mechanism of infection. Genetic analysis of virulence and pathogenicity is challenging because *S. suis* produces multifactorial virulence factors [36] and because natural populations are characterized by high rates of recombination [22,280], creating many different genotypes of which only few have been well characterized for virulence [1]. One generally accepted virulence factor of *S. suis* is the capsular polysaccharide, and its critical role in virulence and pathogenicity has been demonstrated in multiple independent studies [45,46,281]. However, unencapsulated strains have been isolated from pigs with invasive disease [282].

A simple and reliable animal model would be a valuable tool to assess virulence of natural isolates of *S. suis* and establish the role of genetic determinants in virulence. Pigs and mice have been successfully employed for virulence studies [111,112], but have economical, logistic and ethical disadvantages over non-mammalian models, like zebrafish, fruit flies and nematodes. Zebrafish (*Danio rerio*) larvae (up to 6 days post fertilization) are exempt from ethical legislation and cheap to rear in large numbers, allowing high-throughput screens to be performed that would be much less feasible in experimental mammalian models of infection. Adult

zebrafish have been used as an infection model to study *S. suis* with some success [115,116], however these studies used only one or two strains and did not assess the usefulness of the model to predict strain virulence in pigs or humans. In addition, adult zebrafish are not exempt from ethical legislation. Here we evaluated the use of pre-feeding-stage zebrafish larvae, which are exempt from legislation, as an animal model to assess virulence of natural porcine *S. suis* isolates. A first step towards this goal is to test clinical porcine strains that differ in virulence as benchmarks and controls.

Zebrafish is a teleost fish of the Cyprinidae family. The zebrafish embryonal and larval innate immune system develops rapidly and functional phagocytes, complement factors, and antimicrobial enzymes are present in the embryo before or soon after hatching [120-122,283]. Also granuloma-like structures, the result of macrophage aggregation, do occur during infection in zebrafish larvae [124,284] showing that the larval immune system is competent to provide resistance against bacterial infection. Zebrafish larvae have been used extensively as an animal infection model for a wide range of Gram-negative and Gram-positive bacterial pathogens [120,125]. *Salmonella*, *Mycobacterium marinum* and *Listeria monocytogenes* infection studies, as well as several streptococcal studies, have been successfully accomplished using this model organism [126,127,285].

To date, *S. suis* infection of zebrafish larvae has not been explored. Theoretically, using larvae has several advantages over using adult fish. Short-term (1-3 days) maintenance and handling of larvae in the laboratory environment is low-cost and easy, and their transparency allows various optical microscopy and imaging technologies at high resolution. As mentioned above, larvae 24-48 hours post-fertilization (hpf), already carry a mature innate immune system [119].

Here we examined *S. suis* infection in zebrafish larvae at 72 hpf. We found that zebrafish larvae are susceptible to infection by different virulent porcine *S. suis* strains belonging to different serotypes and determined the median lethal dose (LD_{50}). Importantly, bacterial isolates reported as weakly virulent, and unencapsulated mutants were attenuated in the larval model. These findings propose a novel system to assess *S. suis* virulence using a convenient, cost-effective and reproducible zebrafish larval infection model.

Materials and methods

Bacterial strains, plasmids and culture conditions

The *S. suis* strains described in this manuscript are listed in Table 1. *S. suis* strains were grown in Todd-Hewitt Broth (THB) (Difco, USA) at 37°C, in the presence of 5% CO_2. When required, spectinomycin (100 µg/ml) was added to the media to select for genetically modified strains. Solid agar plates were prepared by adding 12 g/L agar to the medium. Overnight cultures were diluted 1:100 in fresh THB and grown until exponential phase (optical density at 600nm ca 0.4) and stored at -80°C in 15% of glycerol. After thawing bacterial colony forming units (CFU) were determined by plating on THB agar.

Table 1: *S. suis* strains used in this study and their virulence. V, virulent; WV, weakly virulence; AV, avirulent.

S. suis **strain**	**Serotype**	**Virulence in pigs**	**Reference**
S10	2	V	[45]
S10-J28	2	WV	[45]
p1/7	2	V	[23]
6555	1	WV	[286]
6388	1	V	[230]
5218	9	AV	[286]
8067	9	AV	[131]
S735	2	WV	[213]

Zebrafish stock and larvae

Zebrafish embryos were obtained from breeders of the Zod2F8 line and were bred at the animal facility of Wageningen University. In the animal facility, a female zebrafish can lay up to 200 eggs a week. Ca. 60 eggs/dish were kept in oxygenated sterile egg water (60 µg/mL Sea salts) at 28˚C. Debris and unfertilized eggs were removed twice a day until hatching.

Larvae were sacrificed by an overdose of the anesthetic 3-aminobenzoic acid (Tricaine, Sigma-Aldrich, USA) buffered with 1.5% $NaHCO_3$ at 50 hours post infection (hpi).

All zebrafish were raised, maintained, and handled in compliance with the local animal welfare regulations, and according to standard protocols (zfin.org) under the guidelines of Wageningen University and Research Centre Institutional Animal Care and Use Committee.

Bacterial staining

Overnight cultures of *S. suis* S10 were diluted 1:100 in fresh THB, grown until

exponential phase and collected by centrifugation (5000 g, 10 min). The pellet was resuspended in 5µm CFSE in PBS buffer and incubated in the dark for 30 minutes. Bacteria were washed in PBS three times, diluted and use for microinjection. For each condition (microinjection into the Duct of Couvier, yolk sac and controls group), at 1 hpi, the viability of 10 anesthetized larvae was visually checked using a fluorescence stereo-microscope (M205 FA, Leica).

Zebrafish microinjection

Zebrafish larvae were anesthetized with 200 µg/mL 3-aminobenzoic acid approximately 10 min prior to injection. The larvae were kept in a Petri dish filled with egg water and with a layer of 1% agarose on the bottom. The larvae were checked for blood circulation consistency under a stereo-microscope and vital larvae were injected with 1 nl of bacterial suspension into the yolk sac or into the Duct of Cuvier (DoC). Prior to injection, bacteria were obtained by centrifugation (5000 rpm, 5 minutes) and resuspended in 2% polyvinylpyrrolidone (PVP). To determine the bacterial count, 1 nl of bacterial suspension was added to 100 µl of sterile PBS, diluted and plated in THB agar plates with the required antibiotics. To obtain an optimal number of bacteria per inoculum, total numbers of 100, 500, 1000, 2500 and 5000 CFU were initially tested. At 48 hpf, 25 larvae per condition, per strain were collected using filtered water and sterile Petri dishes under aseptic conditions in a flow cabinet. Experiments were performed in triplicate.

Statistical analysis

GraphPad Prism version 6.0c was used for statistical analysis. Paired T-test was used determine the significant difference between *S. suis* growth curves at 28°C and at 37°C. Significant differences between mortality rates were determined by the Kruskal-Wallis test, and the Mantel-Cox test was used to determine significant differences between survival curves. Statistical significance was accepted at $p < 0.05$.

Histological Examination

Zebrafish larvae selected for histological examination were immersed in 4% paraformaldehyde at 4˚C overnight and embedded in 1.5% agarose. The agarose-embedded zebrafish are then paraffinated overnight. Paraffin sections (5 mm) were attached to poly-L-lysine–coated glass slides (Thermo Fisher Scientific, Dreieich, Germany). After overnight incubation at 37°C, slides were dewaxed and hydrated stepwise using 100% xylene followed by several solutions of distilled water containing decreasing amounts of ethanol. Sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy.

Results

S. suis grows efficiently at 28°C

To determine the ability of *S. suis* to efficiently grow at 28°C, the rearing temperature of zebrafish larvae, an overnight culture was diluted and incubated at 28°C and at 37°C, both at 5% of CO_2. We sampled 1 ml of both cultures every 2 hours and measured their absorbance at 600 nm. At 28°C *S. suis* grows to almost the same final OD_{600nm} as cultures incubated at 37°C but the lag phase is longer and the growth rate slightly slower than at 37°C (Fig. 1).

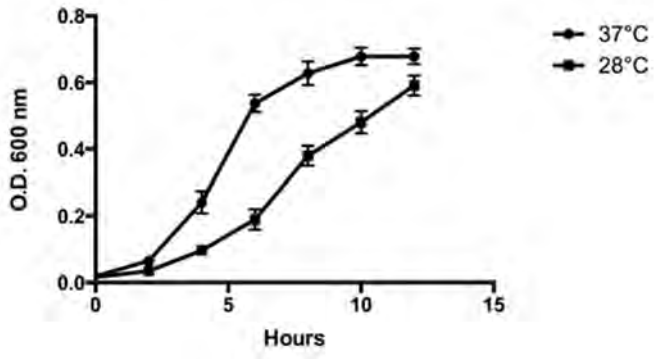

Figure 1: *S. suis* growth curves at 28°C and at 37°C. 1 ml of culture was sampled and its absorbance at 600 nm measured every 2 hours.

Localization of fluorescent *S. suis* in injected zebrafish larvae

In order to ensure the successful injection of *S. suis* into the zebrafish larvae, visual tracking of injected bacteria was performed. Carboxyfluorescein succinimidyl ester (CFSE), a dye that readily crosses intact cell membranes, was used to stain bacterial cells. Once inside the cells, intracellular esterases cleave the acetate groups to yield the fluorescent carboxyfluorescein molecule. The succinimidyl ester group reacts with primary amines, crosslinking the dye to intracellular proteins. CFSE-stained *S. suis* S10 was injected in the yolk sac and in the Duct of Cuvier (DoC) and the zebrafish larvae were observed via a fluorescence stereo microscope (M205 FA, Leica) at 1 hpi. Bacteria were visible in the yolk sac and in the bloodstream (green fluorescent signals indicated by red arrows in Fig. 2a, b) confirming successful injection and showing that bacteria injected in the DoC injection become efficiently dispersed via the bloodstream to blood vessels.

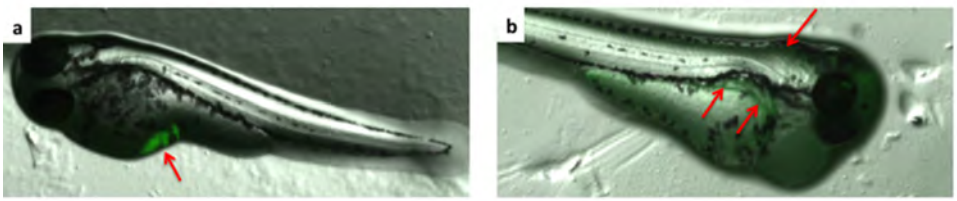

Figure 2: Fluorescent *S. suis* in injected zebrafish larvae. Zebrafish larvae were injected with 1000 colony forming units in the yolk sac and in the DoC and observed under a Leica M205 FA fluorescent stereo-microscope. a) Localization of fluorescent *S. suis* S10 injected in the yolk sac, 1 hpi, b) Localization of fluorescent *S. suis* S10 injected in the DoC, 1 hpi.

Microinjection of zebrafish larvae with *S. suis* S10 is fatal and dose-dependent

To determine the susceptibility of zebrafish larvae to *S. suis* infection and establish lethal dosages, the virulent serotype 2 isolate S10 was microinjected into the yolk sac of the larvae at 72 hpf. Zebrafish larvae were injected with 100, 500, 1000, 2500 and 5000 colony forming units (CFU) resuspended in polyvinylpyrrolidone (PVP). The control group was injected with PVP only. Each group contained 25 larvae per condition, and each experiment was performed in triplicate. The LD_{50} for larval death after injection of strain S10 was determined 24 and 48 hpi and shown to be dose-dependent. At 24 hpi the LD_{50} was $1.6*10^3$ CFU and at 48 hpi $2.7*10^3$ CFU per fish (Fig. 3a, b).

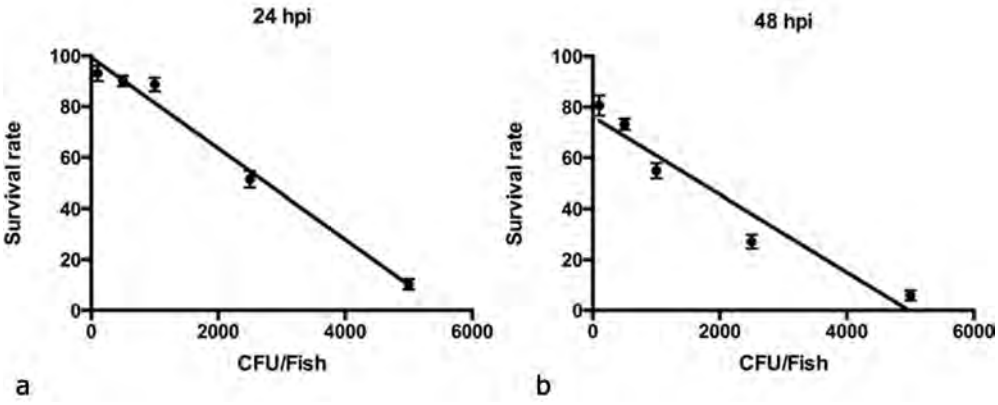

Figure 3: LD_{50} determination. Zebrafish larvae were injected with 100, 500, 1000, 2500 and 5000 colony forming units. a) Survival rate of infected larvae at 24 hpi, b) Survival rate of infected larvae at 48 hpi. At both time points, results were obtained in 3 independent experiments, and 25 larvae were injected per group.

Zebrafish larval mortality depends on the virulence of the injected strain

To assess the accuracy of the zebrafish larvae infection model for predicting the virulence of *S. suis* strains in pigs, we tested strains with known differential virulence in pigs. Yolk injection with strain S735, reported as weakly virulent [213,230],

showed a mortality of 10% at 48 hpi. In contrast, the virulent strain S10 caused nearly 50% of larval death after 48 hours. The zebrafish larvae infected with the virulent strain p1/7 [23] also resulted in about 50% larval mortality. No significant difference was found between mortality of larvae infected with virulent strains p1/7 or S10 (Fig. 4a).

Infection with serotype 9 strains 8067 and 5218, reported as avirulent and weakly virulent porcine strains respectively [287], resulted in very low mortality rates of 7.5% for 8067 and 11.6% for 5218. Serotype 1 strain 6388, which has been described as a hypervirulent porcine strain [230], caused nearly 50% of larval death at 48 hpi (Fig. 4b). In comparison, serotype 1 strain 6555, reported as weakly virulent [286] showed no significant difference in larval killing compared to the PVP control group (Fig. 4b).

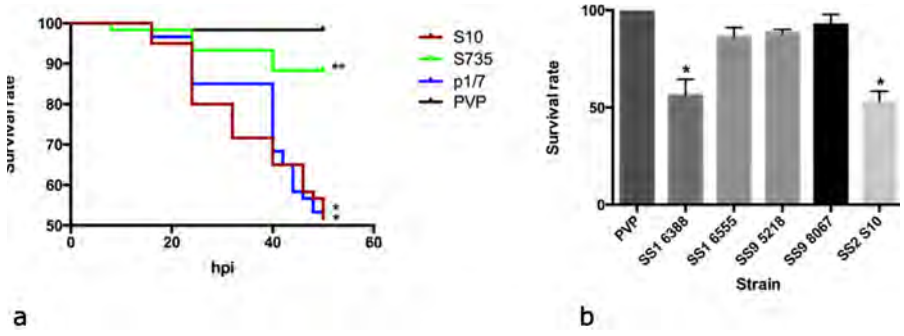

Figure 4: Zebrafish larval mortality correlates with virulence of the injected *S. suis* strain in pigs. a) Survival curve of yolk-injected larvae with three different *S. suis* serotype 2 strains. Larval viability was checked every 8h for the first 48h, then every 2h. * P-value < 0.05 compared with S735 and PVP curves. ** P-value < 0.05 compared with S10, P1/7 and PVP. b) Survival rate of yolk-injected larvae with different *S. suis* strains; larval survival was determined at 48 hpi. * P-value < 0.05. Results were obtained in 3 independent experiments, and 25 larvae were injected per group.

Microinjection of *S. suis* in the zebrafish yolk sac or the Ducts of Cuvier leads to similar rates of mortality

In pigs, the largest problems upon *S. suis* infections derive from bloodstream infections. In zebrafish larvae, injections into the bloodstream are technically more challenging than injection into the yolk sac. To evaluate if injections into the bloodstream or yolk sac resulted in differential killing of zebrafish larvae, the same dose of four *S. suis* serotype 2 strains were injected in the yolk sac and in the Ducts of Cuvier (DoC), the common cardinal vein that directs the blood that returns from the tail regions back to the heart. Percentage of survival was determined at 24 hpi and 48 hpi. Virulent strains caused 50% of larval death at 48 hpi, with a minor increase in

mortality when the bacteria were inoculated directly into the bloodstream. As in the previous infection assays, infection with weak or avirulent strains resulted in very low mortality (Fig. 5), with no significant difference with the control group. These data show that injection into the readily accessible yolk sac leads to comparable larval death rates as compared to injection into the DoC, which is technically more difficult to access.

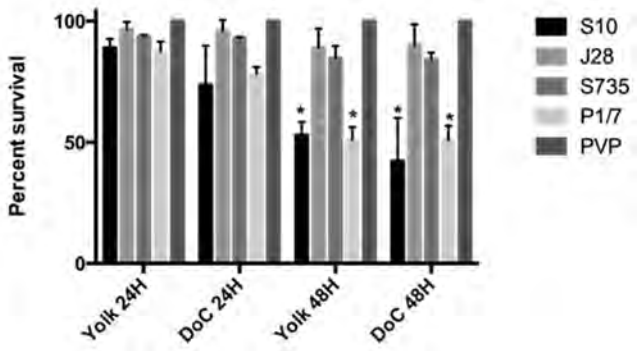

Figure 5: Microinjection in the yolk sac or the ducts of Cuvier leads to similar mortality rates. Survival rate of injected larvae with four different *S. suis* strains in two different infection sites, the yolk sac and the Ducts of Cuvier. The survivors were counted 48 hpi. * P-value < 0.05. Results were obtained in 3 independent experiments, and 25 larvae were injected per group.

Capsular polysaccharide is an important virulence factor for *S. suis* infection of zebrafish larvae

In a previous study, an unencapsulated mutant was reported to be less infectious in pigs [288] and to be phagocytized by human and porcine dendritic cells at higher rates than the corresponding wild-type strain S10 [46,281]. To investigate if the same difference in virulence could be observed during infection of zebrafish larvae, *S. suis* strain S10 and the unencapsulated mutant J28 were injected into the yolk sac and the infection was monitored for 48h. Infection of zebrafish larvae by the unencapsulated J28 mutant resulted in a significant decrease in mortality compared with the S10 wild-type strain, thus showing the same differential infection capacity as previously found in controlled pig infections (Fig. 6).

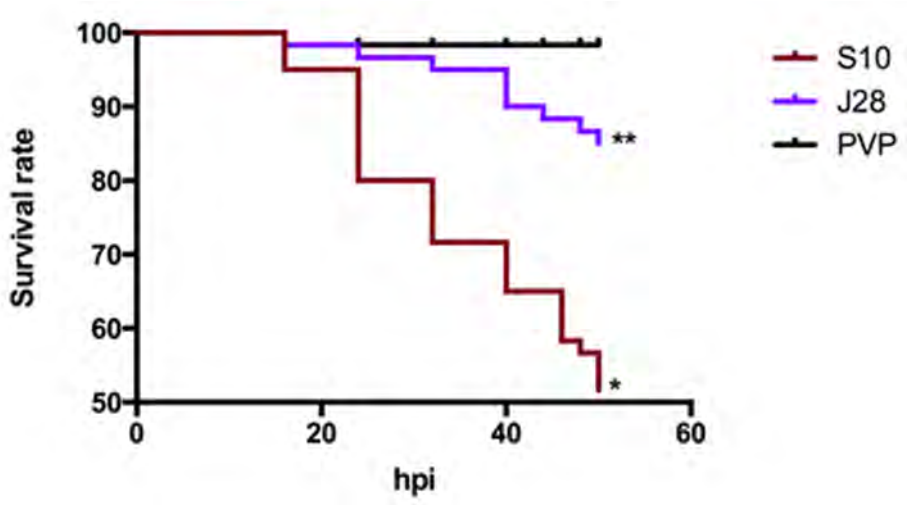

Figure 6: Relevance of the capsular polysaccharide during infection of zebrafish larvae. Survival curve of injected larvae with *S. suis* S10, its unencapsulated mutant strain J28 and PVP control. Larval viability was checked every 8h for the first 40h, then every 2h. *P-value < 0.05 compared with J28 and PVP curves. **P-value < 0.05 compared with S10 and control curves. Results were obtained in 3 independent experiments, and 25 larvae were injected per group.

Deletion of the two-component system *ciaRH* reduced *S. suis* virulence in zebrafish larvae.

In a previous study Li *et al* [90] showed the relevant contribution to the virulence of *S. suis* serotype 2 of the two component system *ciaRH*. The deletion of this operon resulted in a lower mutant survival rate in a bactericidal assay compared to the wild-type. Moreover, the mutant was attenuated for virulence in both mice and pigs *in vivo*, as indicated by lower mortality and morbidity. In order to assess whether the same reduced virulence of *S. suis* strain S10 Δ*ciaRH* would be observed in zebrafish larvae, the mutant and corresponding wild-type strain were separately injected into the yolk sacs of groups of zebrafish larvae and mortality monitored for 48h. Infection of larvae by the Δ*ciaRH* strain resulted in significantly higher survival rate compared to infection with the S10 wild-type strain (Fig. 7), thus reproducing previous results obtained with the same two isolates in mouse and pig infection studies. Taken together these results show that the virulence of *S. suis* strains in experimental pig infections is reflected in the zebrafish larvae infection model, inferring that zebrafish larvae can be used to reproducibly assess virulence of porcine *S. suis* strains, in a biologically meaningful way.

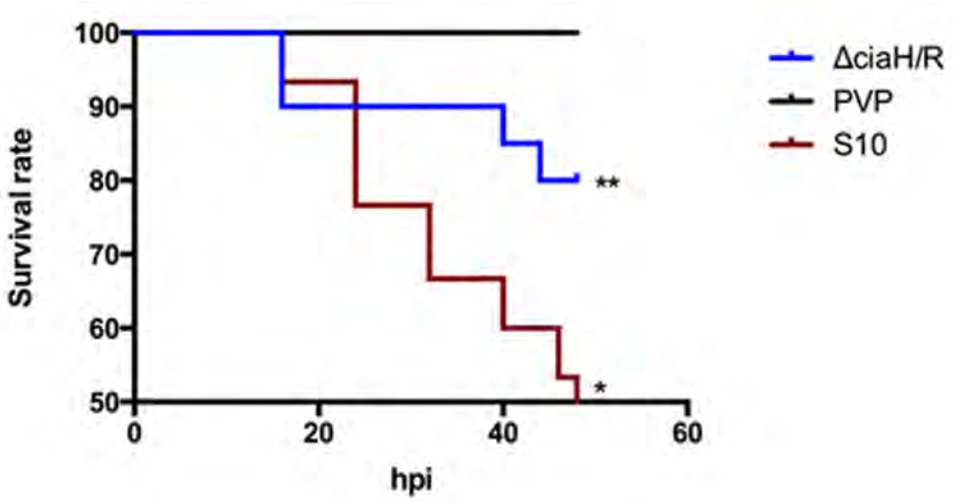

Figure 7: Increased survival rate of zebrafish larvae injected with S10 Δ*ciaRH* compared with the wild-type strain. Survival curve of injected larvae with *S. suis* S10, its mutant strain Δ*ciaRH* and control. Larval viability was checked every 8h for the first 40h, then every 2h. * P-value < 0.05 compared with Δ*ciaRH* and PVP curves. ** P-value < 0.05 compared with S10 and control curves. Results were obtained in 3 independent experiments, and 25 larvae were injected per group.

Discussion

Given the societal importance of *Streptococcus suis* infections in pigs and humans, and the need for epidemiological markers of strain virulence and the notion that virulent isolates can infect both pigs and humans, there is an urgent need for a standardized and cost-effective measure of relative virulence. *S. suis* infection studies using adult zebrafish have been performed successfully in the past to assess virulence of specific strains and mutants [116-118,289,290] but this has some disadvantages compared to the zebrafish larval model. Adult zebrafish are more expensive to rear than larvae due to the need for long-term maintenance and aquaria for experimentation. Unlike larvae in the first few days of their life, adult zebrafish are not transparent, precluding the use of high-resolution optical microscopy and imaging technologies. Published studies indicate that number of CFU of *S. suis* that need to be injected to cause 50% mortality in adult zebrafish is relatively high and not consistent between laboratories. For example, in one study the median LD_{50} for *S. suis* strain HA9801 was $3.8*10^4$ [117] and in a second study it is was reported to be more than 10 fold higher [118].

As zebrafish larvae models have been used to study the pathogenesis and virulence mechanisms of several different pathogens [125-127,285] we evaluated their potential to assess virulence of porcine *S. suis* isolates. Complement components are transferred from the mother to the eggs and effective innate immune responses against microbial challenges, including functional macrophages, can be seen at 24 hours post fertilization (hpf) [121,291,292]. At 48 hpf the first granulocytes are visible [293] and at 72 hpf the innate immune system is fully functional [119]. Therefore, we chose the developmental stage at 72 hpf to conduct our experiments. To ensure reproducibility and standardization, we found that it is crucial to infect zebrafish larvae at the same developmental stage. Zebrafish embryos develop very rapidly, and larvae that differ 4-8 hours of age can differ in development [119].
In nature, zebrafish pathogens may enter and infect their hosts in numerous ways, via the gastrointestinal tract, the gills or open wounds. Accordingly, in preliminary experiments, we wanted to evaluate the ability of *S. suis* to successfully infect zebrafish larvae in immersion assays where bacteria were added to the water containing the larvae. Larvae were immersed in Petri dishes in 2 ml of water inoculated with different numbers of *S. suis*, from $5*10^6$ to $5*10^7$ CFU/well (Fig. 8), incubated at 28°C for 24 h after which larval viability was checked. We found that larval death was dose dependent and that death rates were approaching 100% at the highest doses (Fig. 8).

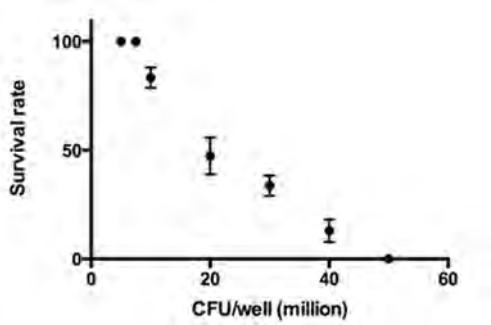

Figure 8: Survival rate of zebrafish larvae in immersion assay with different doses of *S. suis*. For each infective dose, 25 larvae were tested with 5 larvae per well and larval death determined at 24 hpi.

To investigate if larval death could be independent of the bacterial species used, zebrafish larvae were incubated with nonpathogenic *Lactobacillus plantarum* bacteria, using the same experimental conditions. Incubation with *L. plantarum* did not lead to zebrafish death at the same concentration used for *S. suis* infections (Fig. 9). However, high doses ($5*10^8$ CFU/well) of *L. plantarum* did result in about 20% larval death. This may be caused by *L. plantarum* over-growth due to the presence of growth medium in the inoculum (final concentration 1% v/v), with consequential high concentration of lactate in the water and a decrease in pH.

These data show the natural ability of *S. suis* to successfully infect and cause death of zebrafish larvae. However, we found that immersion assays are less reproducible and less consistent than microinjection as reported by others [294]. Thus microinjection was selected as the preferred mode of infection in subsequent experiments.

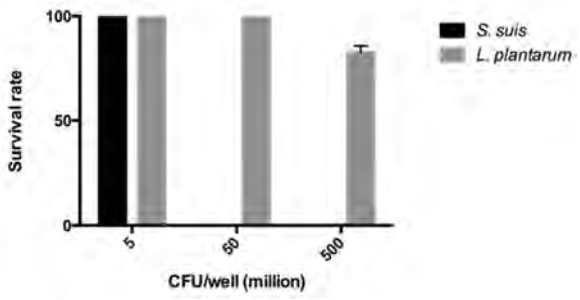

Figure 9: Survival rate of zebrafish larvae immersed in water containing different doses of *S. suis* or *L. plantarum*. For each sample, 25 larvae were tested with 5 larvae per well and larval death determined at 24 hpi.

We did not observe tissue damage or other morphological anomalies in histological sections of the brain and intestine of zebrafish larvae infected with *S. suis* (Fig. 10 a, b). Moreover, in immersion assays, no death was observed after 24 hpi. These

observations, in addition to the rapid mortality, led us to hypothesize that larval death may have been due to sepsis induced inflammation causing tiny blood clots to form, which can block oxygen and nutrients from reaching vital organs [295,296]. This hypothesis is in agreement with the reports stating that a septic shock syndrome is among the most common symptoms caused by virulent *S. suis* strains [15,19,279].

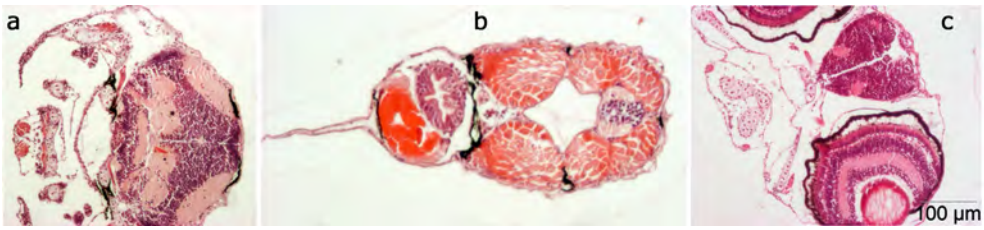

Figure 10: Histology sections of zebrafish larvae infected via immersion assay with *S. suis* (a, b) and control (c). The larvae were kept in 5 wells per group, 5 larvae per wells, 25 fishes per condition. Larvae were fixated with paraformaldehyde 4% at 4 ℃ overnight, then embedded in agarose and paraffinated. To allowed examination haematoxylin and eosin staining were used.

To evaluate suitability of the zebrafish larval infection model to assess virulence of *S. suis* strains, we selected *S. suis* serotype 2 strain S10, a porcine highly virulent strain [286], at doses of 100, 500, 1000, 2500 and 5000 CFU by injection into the yolk sac. We noted that the larval mortality rates were dose dependent, as observed in the immersion assays, ranging from 6.9% of larval death at the lower dose at 24 hpi to 95% at the higher dose at 48 hpi. The LD_{50} values for larvae at 24 and 48 hpi were calculated as $1.6*10^3$ and $2.7*10^3$ CFU per larvae, respectively. These values were comparable to those obtained for other pathogens in the zebrafish larvae model [125,297,298]. Furthermore, the results were highly reproducible: we injected more than 500 larvae in independent experiments using the same bacterial load and always obtained the same virulence ranking of strains.

To evaluate use of zebrafish larvae to assess *S. suis* strain virulence, we compared larval death rates after infection via two injection sites: the yolk sac and the Duct of Cuvier (DoC), a major blood vessel connecting the heart to the trunk blood circulation system that can be used as an infection site [299]. The yolk sac is a convenient and easy injection site; it has previously been used to achieve systemic infection of slow growing bacteria [300]. However when injected with fast growing bacteria it can lead to rapid bacterial proliferation and rapid death of the larvae. In pigs, dissemination of *S. suis* via the blood to peripheral organs and the brain is a relevant virulence trait [36]. We were therefore interested to compare death rates of zebrafish after injection of two different *S. suis* strains into the zebrafish bloodstream or yolk sac. No significant differences were obtained for the two infection routes using zebrafish larvae from the same egg batch. Thus for convenience, most virulence assays were performed by microinjection into the yolk sac.

To further evaluate of the usefulness of a zebrafish larvae infection model for assessing the virulence of *S. suis* strains in their natural host, we tested the virulence of seven clinically relevant *S. suis* strains comprising three serotypes that had been previously studied in porcine infections. Initially, we infected zebrafish larvae with two serotype 2 strains reported as virulent (p1/7) and weakly virulent (S735) [213]. Both strains induced increased mortality rates of zebrafish larvae compared to PVP-injected larvae. At the same dose, the virulent strain p1/7 caused larvae death at a similar rate to S10, while S735 infection resulted in a very low larval mortality. Next, we sought to determine death rates induced by other *S. suis* serotypes in zebrafish larvae. Two strains of serotype 1, and two strains of serotype 9 with different pathogenicity in pigs were selected for injection. As predicted the virulent pig strain 6388 caused a high mortality rate in zebrafish, whereas mortality rates induced by the two avirulent (strains 5218 and 8067) and a weakly virulent pig strain 6555 were not significantly different to the control group. The virulence of seven natural *S. suis* strains and one weakly virulent mutant, S10-J28, in the zebrafish larvae infection model showed consistency with the reported pig virulence studies (table 2) apart from the isolate 5218. This strain, the reference isolate for serotype 9, is of unknown origin and reported to be avirulent, based on a single piglet infection study [287]. This strain contains a prophage that can be activated *in vitro* [301] and it is possible that this prophage differentially modulates virulence depending on changes in environmental conditions, a common feature of prophages that use bacterial pathogens as host [302].

Table 2: *S. suis* isolates and their relative virulence in zebrafish larvae and in pig infection studies. [R], reference strain for the corresponding serotype

***S. suis* strain**	**Serotype**	**Origin**	**Virulence in pigs**	**Virulence in zebrafish larvae**	**Reference**	**Suilysin gene***
S10	2	Tonsil	V	V	[286]	+
S10-J28	2	Laboratory	WV	WV	[286]	+
p1/7	2	Organs	V	V	[23]	+
6555 [R]	1	Unknown	WV	WV	[287]	ND
6388	1	Organs	V	V	[230]	+
5218 [R]	9	Unknown	AV	WV	[287]	ND
8067	9	CNS	AV	AV	[131]	+
S735	2	Lungs	WV	WV	[213]	+

*+, present in the genome; ND, not determined.

Next, we tested whether the capsule polysaccharide, a well-characterized *S. suis* virulence factor that contributes to disease pathogenesis in porcine infection models, also plays the same important role in infection of zebrafish larvae. We used the

unencapsulated mutant J28 that is less virulent [45,287] and phagocytized more efficiently than the virulent parent strain S10 by human and porcine dendritic cells [46,281]. In agreement with previous studies, infection of zebrafish larvae with the J28 strain resulted in significantly lower mortality rates compared to infection by the wild-type strain. This result shows that capsule, a confirmed *S. suis* virulence factor in pigs, is also required for full virulence in zebrafish larvae, lending further support to the reliability of the zebrafish larval model to assess virulence of porcine *S. suis* strains.

We further investigated the impact of the deletion of *ciaRH* on *S. suis* serotype 2 virulence. The *ciaHR* operon encodes a two-component system (TCS) consisting of a histidine kinase receptor and a cognate response regulator. TCSs play fundamental roles in regulating bacterial gene expression in response to environmental stimuli. The *ciaHR* TCS contributes to *in vivo S. suis* virulence [90]. In a bactericidal assay, using a macrophage cell line, deletion of this TCS resulted in a lower survival rate compared to the wild-type. Moreover, survival rates of mice and pigs infected with the *ciaHR* -deletion mutant strain were higher than for the wild-type bacteria. The *ciaHR* mutant was also significantly attenuated in the zebrafish larvae model causing larval mortality of 20% at 48 hpi, compared to 50% mortality with the parent strain. This finding is of interest as it suggests that this TCS is necessary for full virulence in three diverse hosts, and that this TCS may therefore also be necessary for virulence in humans. The *ciaHR* TCS may be an antimicrobial target to treat animal and human infections.

In conclusion we show for the first time that it is possible to use a zebrafish larvae model to assess the relative virulence of *S. suis* strains in porcine infections. By exploiting the favorable features of the breeding and maintenance of zebrafish larvae, the number of experiments can be easily scaled-up and results can be obtained within days. Because of its convenience and cost-effectiveness, this model may be used to assay virulence of environmental *S. suis* strains that are ubiquitous in pig husbandry and may infect a variety of animals, with most clinical relevance to infection of pigs and humans. Furthermore, a large number of bacterial mutants and strains can be screened for their virulence and *in vivo* pathogenicity, opening up new avenues to investigate the so far undiscovered pathways mediating successful host infection by *S. suis*.

Chapter 6

Genetic, *in vitro* and *in vivo* analysis of *S. suis* two component systems involved in virulence and in acid and oxidative stress survival

Edoardo Zaccaria, Jery Baan, Ellen Kranenbarg-Stolte, Peter van Baarlen, Jerry M. Wells

Host-Microbe Interactomics, Animal Sciences, Wageningen University, Wageningen, The Netherlands

Submitted for publication

Abstract

Streptococcus suis is an important swine pathogen and an emerging zoonotic agent of human infectious diseases, causing large economic losses in the porcine industry worldwide. *S. suis* can cause severe infections in pigs and humans resulting in meningitis, endocarditis and septicaemia, among others. Despite its importance, relatively little is known about the mechanisms employed by *S. suis* to infect its host. In many pathogenic bacteria, two component systems (TCS) are involved in the regulation of virulence factors responsible for the pathology and bacterial adaptation in the host. More than 500 two-component systems have been identified in all kingdoms of life with the exception of mammals, suggesting that TCS may be good targets for novel antibacterial drugs. In this study, we analysed the effect of the deletion of two TCS (SSU1930/31 and SSU0827/28) on *S. suis* virulence and its capacity to respond to adverse conditions. We determined that the two TCS mutants are characterized by a reduced ability to survive in acidic or oxidative environments. Additionally, the mutants showed a lower survival rate when compared with the wild-type in *in vitro* neutrophil assays and reduced pathogenicity *in vivo*, in zebrafish. Collectively, our data show that the two TCS that we studied here contribute to the virulence of *S. suis* 2 strain S10 in and may have a primary role on the survival and persistence of the bacteria in the bloodstream and host tissues.

Introduction

Streptococcus suis is one of the major bacterial pathogens of swine, causing meningitis, pneumonia, septicemia and arthritis [275]. The same clinical features are observed in zoonotic *S. suis* infections of humans, resulting in high mortality [64]. Among the 35 known serotypes *S. suis* serotype 2 is most frequently associated with severe disease in humans and animals [7]. However, it is unknown why the *S. suis* serotype is a determinant of virulence, and the molecular details of *S. suis* pathogenesis remain relatively poorly understood compared to other pathogens.

To ensure survival, bacterial pathogens need to rapidly modulate their gene expression profiles in response to environmental cues perceived during infection of their hosts. The main environmental cues include acidity, osmotic and redox changes [303]. The ability to regulate gene expression also avoids energy expenditure on the expression of unnecessary pathways and of factors required for one particular condition that have adverse effects on growth or virulence under other conditions [304-306].

Two-component systems (TCS) are important regulatory circuits of gene expression in bacteria [98]. TCS are present in most bacteria and the number of TCS for a given species is correlated to genome size and the range of environments in which the organism can grow and survive [99]. Typically TCS signaling involves autophosphorylation of a membrane-bound histidine kinase (HK), and phosphotransfer of the phosphoryl group to a cognate response regulator (RR). The RR is usually a transcription factor that, upon phosphorylation, binds differentially to operator sites in gene promoters. RRs may also act as enzymes or ATPase [100,101]. TCS systems may also involve intracellular kinase sensors and auxiliary proteins [99,307] involved in phosphorelay of the transduced signal. Bacterial TCS regulate virulence, metabolic pathways and antibiotic resistance mechanisms in some bacteria. Furthermore, some conserved TCS have been shown to be essential or conditionally essential in different groups of bacteria, making them interesting targets for novel antibacterials and anti-infective drugs [308,309].

Among the 15 predicted TCS in *S. suis* [26] only predicted orthologues of CiaRH, Ihk/Irr, and Salk/Salr have been characterized in any detail. The SalKR TCS was identified by comparative genomics in two chinese virulent *S. suis* serotype 2 outbreak strains (98HAH12 and 05ZYH33) [89]. In both isolates, the *salKR* TCS was located in a genomic region showing characteristics of a pathogenicity island, and in experimental piglets infection studies, *salKR* was shown to be essential for the high virulence of these two isolates [89]. However the precise role of SalKR in virulence of *S. suis* is still unknown, as microarray expression analysis of the SalKR mutant revealed no changes in known virulence genes or metabolic pathways compared to the WT [89].

Several studies have been carried out to elucidate how the CiaRH TCS

regulates virulence of *S. suis* [90,310]. One study showed that CiaRH was necessary for efficient adherence of *S. suis* bacteria to human and pig intestinal epithelial cells *in vitro* and for survival upon phagocytosis by macrophages [90]. Furthermore, the *ciaRH* mutant was attenuated for virulence in experimentally infected mice and pigs, as indicated by increased survival of the infected animals and lower blood counts compared to the WT strain [90]. The regulon for this TCS, has also not yet been elucidated in *S. suis*.

S. suis mutants of the Irr/Ihk TCS were attenuated for virulence in mice, evidenced by lower mortality after intraperitoneal injection compared to WT strain [109]. Additionally the *irr/ihk* mutant was killed more effectively than the WT by mouse macrophages, consistent with *in vitro* data showing reduced survival of the mutant strain after exposure to low pH and hydrogen peroxide [109]. The reduced survival of the *irr/ihk* mutant to oxidative stress was attributed to decreased expression of manganese-dependent superoxide dismutase, Sod. The bacterial Sod protein is essential to counteract the effects of the macrophage oxidative burst and thus, lower production of Sod in the *irr/ihk* deletion mutants may account for the observed phenotypes seem *in vitro* and *in vivo*.

To date the role of the other twelve TCS in *S. suis* remain unexplored. Here we describe the phenotypic characterization of deletion mutants of the highly conserved *S. suis* TCS SSU0827/0828 and SSU1930/1931 *in vitro* using survival assays after acid, oxidative or neutrophil challenge. The contribution of these TCS to virulence was also assessed *in vivo* using a zebrafish larvae infection model.

Materials and methods

Bacterial strains and growth conditions

The *S. suis* WT and mutant strains used in this study (Table 1) were grown at 37°C at 5% atmospheric CO_2 in Todd Hewitt Broth (THB, Thermo Scientific, Oxoid) or on solid THB containing 1.2% of agar (BD). For the selection of mutant strains, the medium was supplemented with spectinomycin (Invitrogen) at a concentration of 100 µg/ml. Insertional deletion mutants of the TCS genes SSU0827/0828 and SSU1930/1930 were constructed in *S. suis* strain S10 by Gene Splicing Overlap Extension PCR (SOE-PCR) and allelic replacement as previously described [156]. The primers used for gene splicing are shown in Table 1. Successful deletion of the TCS operons in *S. suis* was verified by colony-PCR using primer combinations based on DNA sequences of the inserted DNA and proximal chromosomal DNA (Table 1).

Growth curves

S. suis S10, *S. suis* Δ0827/28 and *S. suis* Δ1930/31 were grown overnight in liquid THB containing spectinomycin as described above. Erlenmeyer flasks containing 200 ml of pre-warmed (37°C) THB plus spectinomycin (100 µg/ml), were inoculated with 2 ml of the overnight cultures and incubated at 37°C in the presence of 5% CO_2. Measurements at OD_{600nm} were recorded every 30 minutes using a SpectraMax M Series (Molecular Devices) until the cultures reached stationary phase. All *S. suis* growth experiments were performed in triplicate.

Electron microscopy

Samples of exponential phase bacteria were fixed according to the lysine-acetate-based formaldehyde/glutaraldehyde ruthenium red-osmium (LRR) fixation procedure, as described previously [29]. Transmission electron microscopy of bacteria was performed using a JEOL JEM 2100 microscope and images were obtained at 200000x magnification.

Antibiotic exposure assays

To investigate antibiotic sensitivity of *S. suis* S10 and TCS mutants *S. suis* Δ0827/28 and *S. suis* Δ1930/31, 100 µl of overnight cultures of each strain were spread evenly on THB-agar plates using sterile swabs. Antimicrobial susceptibility disks (Thermo Scientific, Oxoid) for ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (30 µg), gentamicin (10 µg) and penicillin G (10 units) were then placed on the agar plates using a dedicated dispenser (Thermo Scientific Oxoid). The plates containing a total of 4 replicate disks for each antibiotic were incubated overnight at 37°C in the presence of 5% CO_2. The radius of growth inhibition was measured as the distance in millimetres from the centre of the disc to the outer edge of growth inhibition.

Table 1 Bacterial strains and oligonucleotide primer sequences used in this study

Strain	**Relevant characteristics**	**Source or reference**
S. suis S10	Wild-type; reference strain	[45]
S. suis J28	S10 ΔcpsEF	[45]
S. suis Δ0827/28	S10 ΔSSU0827/0828	This study
S. suis Δ1930/31	S10 ΔSSU1930/1931	This study
Primers	**Nucleotide sequence**	**Purpose**
1930 F1/31	AGCCAAGAGGCCAAAGCCCAAC	Creation of 1930/31 deletion fragment
1930/31 R1	CTCTTGCCAGTCACGTTACGTAGCTGATTTCCACTGCAATCC	Creation of 1930/31 deletion fragment
1930/31 F2	GGATTGCAGTGGAAATCAGCTACGTAACGTGACTGGCAAGAG	Creation of 1930/31 deletion fragment
1930/31 R2	GCTAAGTGTGAGACTGCCACCCCCTATGCAAGGGTTTATTGTTT	Creation of 1930/31 deletion fragment
1930/31 F3	ACAATAAACCCTTGCATAGGGGGTGGCAGTCTCACACTTAGC	Creation of 1930/31 deletion fragment
1930/31 R3	TGCAACAGCATGCCATGCTCTC	Creation of 1930/31 deletion fragment
1930/31 CtrlF	CTCATCTGCCGCACCTGAAC	Control of Δ1930/31
1930/31 CtrlR	TATGCCGAGGCTGAGTTAAG	Control of Δ1930/31
0827/28 F1	CTTGGGCATAAGGCGTAATCAG	Creation of 0827/28 deletion fragment
0827/28 R1	CTCTTGCCAGTCACGTTACGTTTCCGTGGAAAGTCAACTGG	Creation of 0827/28 deletion fragment
0827/28 F2	TCTAAATCAATCGCGCATGGCACGTAACGTGACTGGCAAGAG	Creation of 0827/28 deletion fragment
0827/28 R2	GATTGCTGGTAAAGAACGCCCCTATGCAAGGGTTTATTGT	Creation of 0827/28 deletion fragment
0827/28 F3	CAATAAACCCTTGCATAGGGAAAGCCAGGACACAGGTCTTG	Creation of 0827/28 deletion fragment
0827/28 R3	TCGCCTTGTGCGAGATTTCTTG	Creation of 0827/28 deletion fragment
0827/28 CtrlF	TGTTTGCCTCCCAGACTTTC	Control of Δ0827/28
0827/28 CtrlR	CTGGGTGCAGCCTTGATTAT	Control of Δ0827/28

Acid stress survival assay

To investigate whether the TCS gene deletions affected bacterial survival at low pH, the WT strain and mutants were exposed to acid stress for four different periods of time. In preliminary experiments the most differentiating pH range and lengths of exposure time had been determined. Briefly, $1*10^8$ CFU of mutant and WT strains of *S. suis* growing exponentially in THB (approx. OD_{600nm} 0.4) were resuspended in a solution containing 20 mM Na_2PO_4, 1 mM $MgCl_2$ and 25 mM arginine HCl at

different pH values, ranging from pH 1.5 to pH 5.0. Bacteria were then recovered at different times by centrifugation (0, 60, 120 and 240 minutes), serially diluted, and plated on THB agar medium. The percentage of surviving cells was calculated based on CFU at time zero. Based on the initial results survival assays were performed at pH 4.0 and 5.0 on three independent cultures and plated in duplicate.

H_2O_2 stress survival assay

To determine whether the TCS mutants showed an altered response to oxidative stress, $2.5*10^9$ CFU of WT and mutant strains were harvested from cultures growing exponentially in THB (approx. OD_{600nm} 0.4) and resuspended in PBS solutions containing either 1, 2.5, 5 or 0 mM H_2O_2 for 120 min. Surviving bacteria were enumerated by plating of serial dilutions on THB agar plates. The assays were performed in triplicate on different occasions using three independent bacterial cultures.

Growth and differentiation of HL-60 cells

Cell line HL-60 (human promyelocytic leukemia cells) were grown under standard conditions [311]. Briefly, a frozen stock was diluted 1:20 and maintained in tissue culture flasks (T-75; Corning, Corning) in RPMI 1640 medium containing 1% L-glutamine (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (penicillin-streptomycin solution; Life Technologies). Cell suspensions were grown at 37°C in the presence of 5% CO_2. Undifferentiated cells were sub-cultured twice a week (1:20 dilution in RPMI tissue culture medium). Undifferentiated cells were grown to a cell density of $5*10^4$ cells/ml and then differentiated into neutrophils in RPMI 1640 medium without antibiotics containing 1% L-glutamine, 10% FBS and 1.25% dimethylsulfoxide (DMSO) by further incubation for 3-4 days without replacement of the medium. The viability of the differentiated cells, determined by trypan blue exclusion, was 80–85%. Neutrophil differentiation was verified by visual examination of the tissue culture flasks with an inverted microscope (Axiovert 10, Zeiss).

Neutrophil survival assay

Differentiated HL60 cells were plated in 96 wells/plate (Corning, Corning N.Y., USA) at a concentration of $2*10^5$ cells/well in 100 µl. The plate was incubated at 37°C in presence of 5% CO_2 for 2 hours to promote cell adherence to the bottom of the well. Approximately $2*10^6$ CFU of each *S. suis* strain was incubated in human serum for 1 hour at 37°C prior to incubation with the differentiated neutrophils. The human serum had not been heat-inactivated in order to preserve the functionality of complement proteins. The bacteria were incubated for three different periods of time (30, 60, and 90 minutes) and three multiplicities of infection (MOI 10, 1, and 0.1) to optimize the conditions for the assay. Phagocytosis was stopped by cooling the

sample on ice for 5 minutes, after which the extracellular bacteria were harvested from the cell-free supernatant by centrifugation and the CFU determined by serial dilution and plating on THB agar. To enumerate viable intracellular bacteria, HL-60 cells were lysed by incubation for 10 minutes in sterile ice-cold Milli-Q water and bacterial CFU determined as above.

Zebrafish maintenance

Zebrafish (*Danio rerio*) embryos were obtained from breeders of the Zod2F8 line and were bred at the animal facility of Wageningen University and Research. Eggs were kept in oxygenated sterile egg water (60 µg/mL Sea salts) ca. 60 eggs/dish at 28˚C. At 50 hours post infection (hpi) larvae were sacrificed by an overdose of the anesthetic 3-aminobenzoic acid (Tricaine, Sigma-Aldrich, USA) buffered with 1.5% $NaHCO_3$. Zebrafish were raised, maintained, and handled according to standard protocols (zfin.org) and in compliance with the guidelines of Wageningen University and Research Centre Institutional Animal Care and User Committee.

Microinjection of two-day-old zebrafish embryos

Groups of 25 zebrafish were used per condition. Three days post fertilization (dpf) larvae were anesthetized with 200 µg/mL 3-aminobenzoic acid (MS-222, Sigma) approximately 10 min before the injection. The larvae were kept in a Petri dish filled with egg water and with a layer of 1% agarose on the bottom. Each *S. suis* strain was grown until exponential phase (approx. OD_{600nm} 0.4), spun down, resuspended in 2% polyvinylpyrrolidone (PVP) and injected into zebrafish yolk sacs in a volume of 1 nl using a microinjector device (FemtoJet®, Eppendorf, Germany). Inocula contained 3% phenol red (Sigma, M.O., USA) to visually verify accurate injection by binoculars. Once injected the zebrafish embryos were placed in clean fish water and examined every 8 h for presence of a heartbeat until 40 hours and then every 2 hours until 50 hours. The precise bacterial inoculum was determined for each strain by serial dilution and plating on THB agar. The experiment was repeated three times.

Statistical analysis

A Student t-test was used to analyse significance. Significant differences between survival curves were calculated using the Mantel-Cox test. P-values < 0.05 were considered significant. All statistical tests were carried out using GraphPad Prism (version 6.0c)

Results

S. suis deletion mutants of SSU0827/0828 and SSU1930/1931 TCS do not display altered *in vitro* growth rate but are attenuated for virulence in a zebrafish larvae infection model

Using the web-based genome database browser microbesonline.org [194] and BLAST [312] both SSU0827/0828 and SSU1930/1931 TCS were predicted to be in bicistronic operons and encode a membrane sensor kinase and a cytoplasmic response regulator. BLAST search revealed high conservation of these TCS among *S. suis* isolates; 72.3% and 98.7% of 451 sequenced isolates (NCBI) contain SSU1930/1931 and SSU0827/0828 respectively. For the membrane sensor kinase SSU0827 we found less than 50% identity to genes in other bacterial species. Deletion mutants were generated for each TCS in *S. suis* strain S10 using the recently described pheromone-induced competence system [156] and verified by PCR and sequencing (Table 1). There were no apparent visual differences in the colony morphology of mutant and WT strains. Furthermore, no ultrastructural differences were observed in transmission electron micrographs of these strains (Fig. 1b). The growth rates of the mutants and WT in liquid culture were similar (Fig. 1a) except that the final maximum optical density (OD_{600nm}) and CFU was consistently lower in the mutants compared to the WT. On average, a final OD_{600nm} of 0.687, 0.598 and 0.610, corresponding to $2.5*10^9$, $1.7*10^9$, $2.0*10^9$ CFU/ml, was measured for WT, Δ1930/31 and Δ0827/28 respectively. Visual microscopic inspection showed that this growth phenotype was not due to differences in bacterial chain length or aggregation in liquid medium.

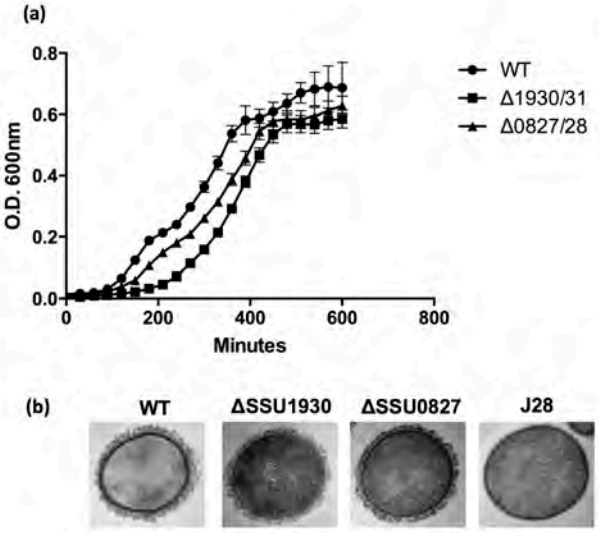

Figure 1. Characterization of two TCS mutant strains in comparison to the WT. (a) Growth curves of the mutants and WT strains in liquid THB culture. Each point represents the mean ± standard deviation. OD_{600nm} value from triplicate independent experiments. **(b)** Transmission electron micrographs of WT and mutant strains showing the presence of a capsule, for comparison J28 an unencapsulated strain of S10 is shown.

As several TCS mutants have been previously associated with changes in cell envelope structures and increased membrane permeability to antibiotics [313-315] the sensitivity of two TCS deletion mutants and the corresponding *S. suis* S10 WT strains were tested against a panel of antibiotics. Disc diffusion antibiotic sensitivity assays with mutant and WT strains revealed no significant differences in sensitivity to a panel of antibiotics (Fig. 2).

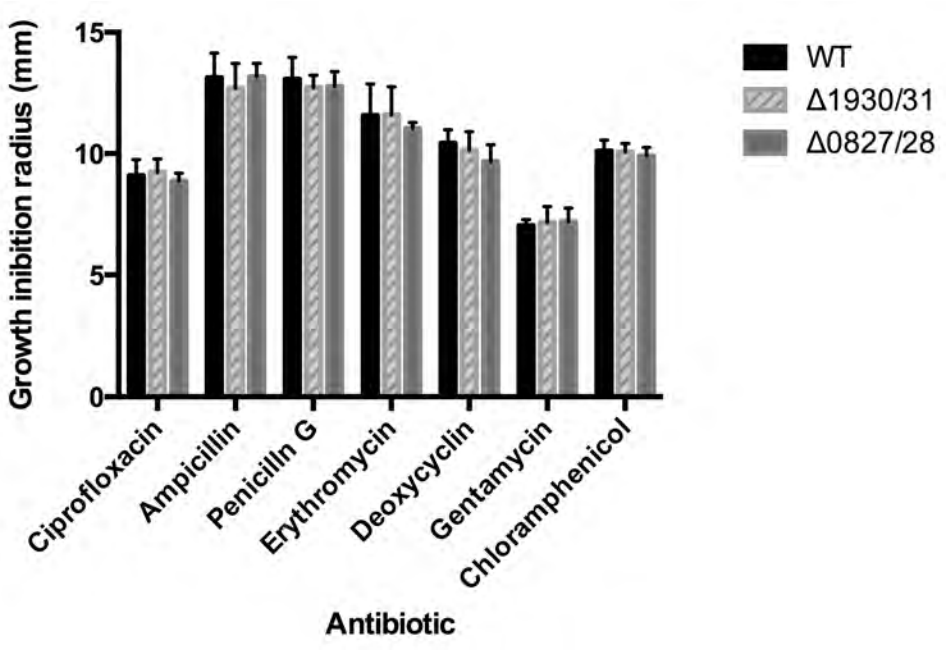

Figure 2. Antibiotic sensitivity assay. Overnight cultures of WT, *S. suis* mutants Δ1930/31 and Δ0827/28 were plated onto THB agar plates and incubated at standard growth conditions. Immediately after plating, disks containing antibiotics were placed onto the plates. After overnight incubation, the radius of growth inhibition (in mm) was measured. Performed in quadruplicate, error bars show standard deviation.

Given the absence of an *in vitro* growth phenotype, the zebrafish larvae infection model was used to determine whether TCS mutants SSU0827/28 and Δ1930/31 were attenuated for virulence. Each *S. suis* strain was microinjected into the yolk sac of 60 zebrafish larvae giving a final inoculum size of $1.3*10^3$ CFU per larvae. The numbers of viable larvae were reduced by almost 50% at 50 hours post injection (hpi) with WT *S. suis* (Fig. 3). TCS mutant Δ1930/31 was less virulent than the WT resulting in approximately 30% larvae mortality at 50 hpi. The TCS mutant Δ0827/28 was more attenuated for virulence than TCS Δ1930/31 with only 10% larvae mortality at 50 hpi.

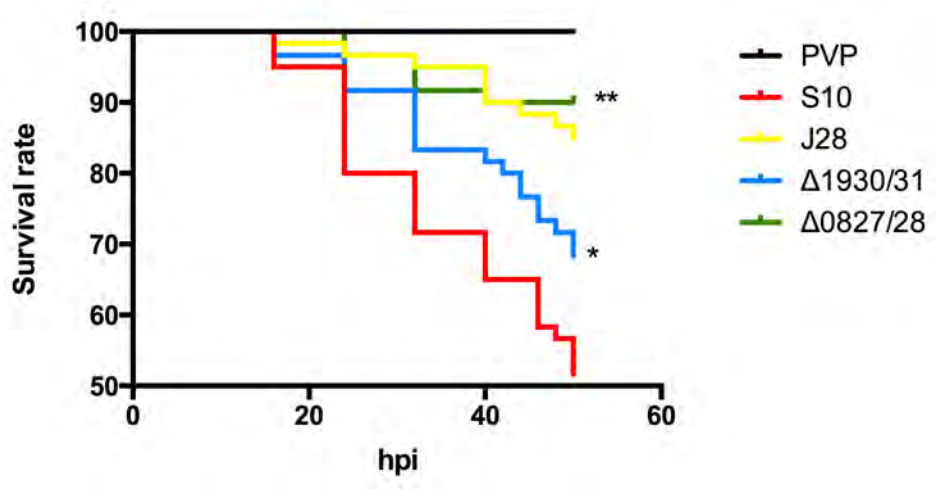

Figure 3. Survival curves for zebrafish larvae challenged with *S. suis* WT and mutant strains. Kaplan–Meier Survival curve comparing killing phenotypes of the mutant Δ0827, Δ1930 and J28 with the WT strain. Larvae were examined every 8 h for presence of a heartbeat until hour 40 and every 2 hours until hour 50. The experiment was repeated three times and groups of 20 zebrafish were used per condition. *, statistically different from the WT as determined by the Mantel-Cox test, p-value: 0.0492. **, statistically different from the WT as determined by the Mantel-Cox test, p-value < 0.0001 .

Δ1930/31 and Δ0827/28 mutants are more susceptible to hydrogen peroxide stress

Professional phagocytes play a key role in the innate defence against bacterial infection in larvae and adult zebrafish as in mammals [119]. We investigated the bacterial resistance to hydrogen peroxide (H_2O_2), which is formed in neutrophils during the oxidative burst. Mutant and WT strains were exposed to different doses (0-5 mM) of H_2O_2 for 120 minutes. Both mutants showed an increased sensitivity to H_2O_2 compared to WT strain over a range of doses (Fig. 4) and this was similar for both TCS. For example, after 120 min exposure of the WT to 5 mM H_2O_2, survival was reduced from $3*10^8$ at time zero to $5*10^4$, whereas the CFU of Δ0827/28 mutant and Δ1930/31 mutant were reduced from $2*10^8$ to $3*10^2$ and from $3*10^8$ to $9*10^2$ respectively.

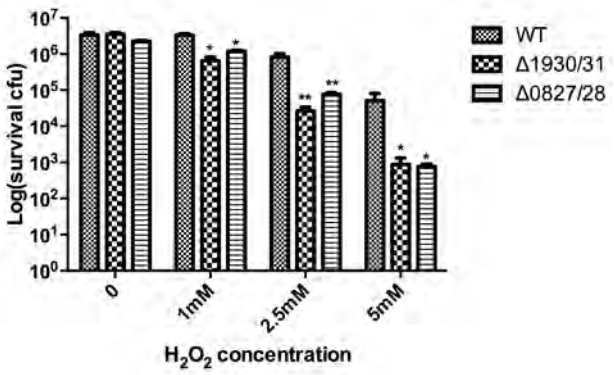

Figure 4. Bacterial survival under oxidative stress. The bacterial resistance to hydrogen peroxide was investigated by incubating the mutants and the WT strains with different concentrations of H_2O_2 in sodium phosphate buffer. Surviving bacteria were plated on THB agar plates after 2 h incubation with H_2O_2. *, statistically different from the WT at $P < 0.05$ using Student's *t* test; **, statistically different from the WT and from the other mutant at $P < 0.05$ using Student's *t* test.

Survival in neutrophil killing assays

The capacity of *S. suis* to resist oxidative killing mechanisms of neutrophils is likely to be critical for dissemination and establishment of invasive disease. To test the hypothesis that the TCS deletion mutants of *S. suis* would be more sensitive to killing by neutrophils we quantified bacterial survival after phagocytosis by HL60-derived human neutrophils (see Methods). To optimize the conditions for the assay, mutant and WT strains of *S. suis* were incubated with neutrophils at different MOI for 30 to 90 min. Based on these results an MOI of 0.1 was chosen to ensure a high rate of bacterial phagocytosis and to avoid lysis and formation of neutrophil-extracellular traps (NETs) due to excessive neutrophil activation and subsequent induction of apoptosis or NETtosis. After 60 min incubation with the WT strain at MOI 0.1, only 3.4% of the initial bacterial inoculum remained extracellular (Fig. 5). After removal of the supernatant, the neutrophils attached to the culture plate were lysed, diluted and plated to determine the CFU of surviving intracellular bacteria. For the WT strain, 1.1% of the original inoculum, amounting to ca. 200 bacteria was recovered after neutrophil lysis (Fig. 5). Both TCS mutants have significantly reduced intracellular survival in neutrophils, with numbers of surviving bacteria being reduced to less than 100 and 50 bacteria, respectively (Fig. 5) showing that the TCS play a role in *S. suis* resistance to the oxidative and non-oxidative killing mechanisms of professional phagocytes.

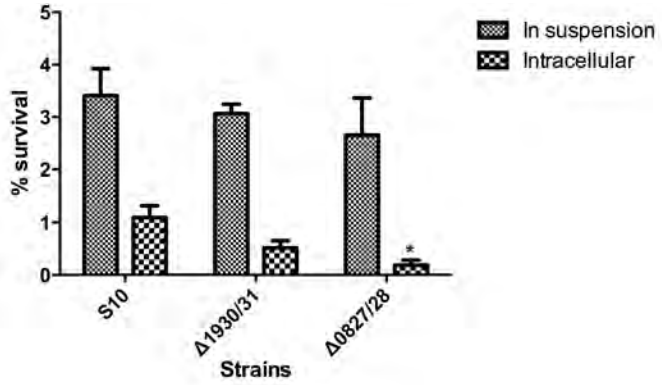

Figure 5. Neutrophil killing assay. The bacterial resistance to phagocytosis and killing by neutrophils was investigated incubating the mutants and the WT strains with HL60-derived human neutrophils at MOI 0.1. After 60 min live bacteria in supernatant and inside neutrophils were enumerated by plating on THB agar. *, statistically different from the WT at $P < 0.05$ using Student's *t* test.

TCS mutants Δ1930/31 and Δ0827/28 mutants are more sensitive to low pH than WT

After uptake of pathogens the neutrophil or macrophage phagosome undergoes a series of transformations prior to lysosome fusion including a continuous reduction in pH from around 7 to 4.5 [316]. To determine whether the two investigated *S. suis* TCS also play a role in sensitivity to pH we determined the survival of the WT strain and TCS mutants after 1, 2 and 4 h incubation at a range of pH values (1.5 to 5.0). At pH 4 and 5, the survival of both TCS mutants was reduced significantly compared to the WT strain. For example, after one hour incubation at pH 5, 36% of the initial WT inoculum survived, whereas this was 17.7% for *S. suis* Δ0827/28 and 21.1% of *S. suis* Δ1930/31 (Fig. 6). A statistically significant difference between survival of the mutant and WT strains was only found at pH values below 6 (not shown).

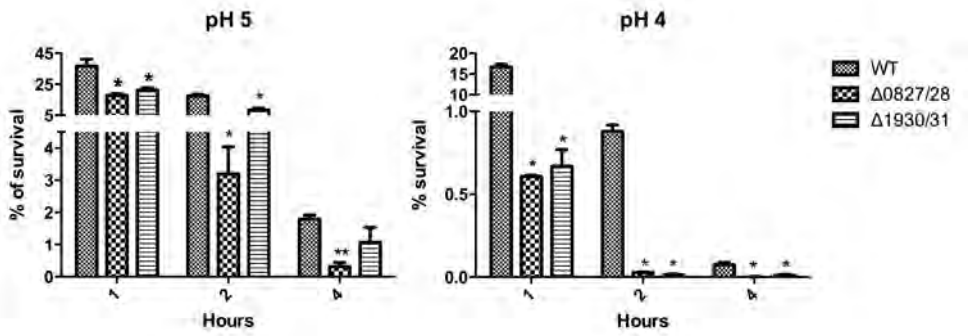

Figure 6. Bacterial survival following acid stress. To investigate whether the TCS also play a role in sensitivity to pH we determined the survival of the WT strain and TCS mutants after 1, 2 and 4 h incubation at pH 5.0 and 4.0. Surviving bacteria were plated on THB agar plates. *, statistically significantly different from the WT at $P < 0.05$ using Student's *t* test, **, statistically significantly different from the WT and from the 1930/1 mutant at $P < 0.05$ using Student's *t* test.

Discussion

Two bicistronic operons, SSU0827/0828 and SSU1930/1931 in the genomes of *S. suis* WT strain S10 were predicted to encode two TCS comprising proteins of the histidine kinase and DNA-binding response regulator families. TCS play important roles in the regulation of bacterial adaptation to changing environmental conditions, including those encountered in the host during infection. Here we investigated the potential role of these two uncharacterized TCS in *S. suis* virulence. *In vitro* growth of TCS mutant strains was not significantly different to the WT strain except for a small reduction in final density, as expected from the common definition of a virulence factor. No apparent differences were observed between the WT and TCS mutants in their morphology or capsule using transmission electron microscopy and Gram-staining. As several TCS mutants have been previously associated with changes in cell envelope structures and increased membrane permeability to antibiotics [313-315] the sensitivity of two TCS deletion mutants and the corresponding *S. suis* S10 WT strains were tested against a panel of antibiotics. Disc diffusion antibiotic sensitivity assays with mutant and WT strains revealed no significant differences in sensitivity to a panel of antibiotics (Fig. 2).

To screen for potential effects of the TCS deletion on virulence *in vivo*, we used a zebra fish larvae infection model which permits us to differentiate between the virulence of porcine *S. suis* isolates and their isogenic mutants, enabling us to assay large (hundreds) numbers of larvae in a highly standardised virulence assay (Chapter 4). As in mammals, professional phagocytes play a key role in the innate defence of adult and larval stages of the zebrafish against bacterial infection [121,317], providing one reason why zebrafish larvae are a useful model to measure differential virulence of porcine *S. suis* isolates (Chapter 4). Microinjection of $1.3x10^3$ CFU of WT *S. suis* S10 resulted in killing of 50% of the injected larvae after 50 hours. TCS mutant Δ0827/28 showed the highest decrease in virulence, only killing 10% of the injected larvae after 50 hours. This low virulence was comparable to the virulence of J28, an unencapsulated mutant of the WT strain S10. In *S. suis* and capsulated pathogenic streptococci in general, the capsule is a major virulence determinant [318,319]. Additionally, unencapsulated *S. suis* J28 was more efficiently phagocytosed than the S10 WT strain [46]. The finding that the TCS mutant Δ0827/28 displays a similar low *in vivo* virulence as an unencapsulated *S. suis* strain suggests that the corresponding TCS is necessary for efficient host colonisation, and might be a useful antibiotics target. TCS mutant Δ1930/31 also displayed a significantly lower virulence *in vivo* but to a lesser degree than the Δ0827/28 mutant.

Neutrophil killing of bacteria is an important innate defence mechanism in zebrafish larvae and in mammals, and neutrophils are among the first immune cells that migrate towards sites of inflammation. Zebrafish neutrophils share morphological, biochemical, and functional features with their mammalian counterparts including

production of primary and secondary granules, myeloperoxidase (MPO) enzyme that generates antimicrobial products using H_2O_2 as substrate, and a functional NADPH oxidase [292,320] that is involved in the oxidative killing of bacteria through the production of the reactive oxygen species, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\cdot$). We hypothesized that the attenuated virulence phenotype of the TCS mutants might be due to a reduced capacity to evade neutrophil killing. To test this hypothesis, the survival of the TCS mutants and WT *S. suis* strains was determined after incubation with neutrophils *in vitro*.

Previously published results [321,322] showed that a relatively low amount of bacteria per cell must be used to prevent the formation of neutrophil extracellular traps (NETs), usually followed by neutrophil apoptosis and bacterial overgrowth. Moreover it has been shown *in vitro* that incubation with serum and/or with specific antibodies, or opsonisation, enhanced *S. suis* phagocytosis and killing by pig neutrophils [42]. To test whether opsonisation of *S. suis* with serum or specific IgG antibodies would also enhance phagocytosis by HL60-derived human neutrophils, the bacteria were first incubated in complete human serum, heat-inactivated human or complete human serum with addiction of IgG, derived from crude bacterial lysates, and phosphate buffer prior to incubation with neutrophils. The presence of human serum resulted in an enhanced phagocytosis, suggesting an important opsonizing effect of complement (data not shown). Phagocytic killing of the serum opsonised TCS mutant Δ0827/28 by neutrophils was significantly higher than killing of the corresponding WT. Also TCS mutant Δ1930/31 showed a lower survival rate than the WT in neutrophil killing assays. Overall these results underline the importance of these TCS in response to adverse environmental conditions such as those encountered during phagocytosis by leukocytes.

After phagocytosis, lysosomes fuse with the phagosome, loading hydrolytic enzymes and antimicrobial peptides into the newly formed phagolysosome. Additionally the respiratory burst increases uptake of oxygen to generate reactive oxygen species that promote killing of internalised bacteria. The respiratory burst is catalysed by the enzyme NADPH oxidase, which pumps superoxide (O_2^-) into the phagocytic vacuole [323,324]. Due to the very rapid dismutation of the superoxide into hydrogen peroxide (H_2O_2) inside the phagosome, a H_2O_2stress assay was performed to assay H_2O_2 tolerance of both WT and TCS mutants. Both mutants showed an increased sensitivity to H_2O_2 compared to WT strain over a range of doses (Fig. 4). This increased sensitivity of TCS mutants to oxidative stress would account for their lower survival compared to WT after incubation with neutrophils and may indeed explain the significantly lower virulence of the TCS mutants in the zebrafish larvae infection model, as hypothesised.

Prior to lysosome fusion, the phagosome pH decreases from 7 to 4.5. The acidification of the phagosome is due to the hydrolysis of ATP and the translocation of H^+ across the membrane by the vacuolar-type H^+-ATPase (V-ATPases). Phagosomal

acidification produces a harsh environment that negatively impacts microbial metabolism, cell wall and membrane. Moreover low pH enhances the activity of hydrolytic enzymes in the phagolysosome, further hampering survival and growth of bacteria. *S. suis* can be isolated from the small intestine of pigs after weaning, showing that it can survive passage through the stomach which has a pH lower than 2 [325]. *S. suis* was unable to survive exposure to pH lower than 4 even for a short period of time. However, various factors such as food consumption, liquid intake and a rapid change of diet, can increase stomach pH, favouring microbial survival and passage to the intestine. Bacteria have developed several mechanisms to survive low pH including alteration of cell membrane composition, proton export, production of protective macromolecules, and production of basic molecules to increase pH; these survival mechanisms are at least partially regulated by TCS [326]. A common bacterial defence mechanism against acid stress is the arginine deiminase system which has also been characterised in *S. suis* [54,327,328]. This system comprises three main enzymes, ArcA, ArcB and ArcC, which catalyse the conversion of arginine into ornithine in acid environments. To investigate whether the TCS SSU0827/0828 and SSU1930/1931 play a role in pH stress responses we incubated WT strain and the two TCS deletion mutants at pH ranging from 1.5 to 5.0 for 1, 2 and 4 h. At pH 4 and 5, the survival of both TCS mutants was reduced significantly compared to the WT strain (Fig. 6). This striking decrease in survival of mutants after acid challenge highlights the central role of these two TCS in promoting bacterial survival in acidic conditions.

In agreement with the common definition of "virulence factor", the two TCS mutants generated in this study did not show any significant change in growth compared with the WT. At present, we do not know the mechanisms by which TCS SSU1930/1931 and SSU827/0828 contribute to virulence, but it is likely that these TCS contribute to the capacity of *S. suis* to survive oxidative and pH stress conditions. We observed substantial differences between the two mutants; deletion of the TCS SSU0827/28 caused a lower survival rate and a strongly compromised infection capacity compared with Δ1930/31. Further research is necessary to identify the regulatory targets of each TCS. We propose that SSU0827/28 may provide a possible novel antibacterial drug target for ongoing efforts to develop inhibitors of bacterial TCS based on protein structures of the catalytic domains [308,309]. The TCS encoded by SSU0827/28 is well conserved across all *S. suis* isolates that have been sequenced to date. Our data suggests that interfering with proper functioning of this TCS might lead to a reduced survival in the porcine stomach and a more efficient clearance of *S. suis* by the innate immune system.

Chapter 7

General Discussion

Edoardo Zaccaria

Natural competence in streptococci and its discovery in *S. suis*

Streptococci, are oxygen-tolerating lactic acid bacteria that ferment carbohydrates to produce mainly lactate and, to a lesser extent, acetate. The genus has been extensively studied and includes species of pathogens, commensals and food bacteria with relevance to the food industry or animal and human health. Streptococcal species can be phylogenetically grouped into six main clusters with the exception of *Streptococcus acidominimus* and *Streptococcus suis* [191].

S. suis is a normal member of the adult pig microbiome but can cause serious diseases in weaned piglets and humans. Most human infections are considered the consequence of occupational exposure, where bacteria enter the bloodstream via breaks in the skin due to contact with *S. suis* contaminated porcine carcasses, meat or blood. Experimental studies of *S. suis* virulence and pathology have thus far been hampered by (I) the lack of efficient methods for genetic transformation, allowing high-throughput studies, and (II) the lack of a simple, cost-effective model to investigate *S. suis* virulence. In some streptococcal species, notably *Streptococcus thermophilus, S. pneumoniae* and *S. mutans,* genetic transformation can be carried out very efficiently as these species can be experimentally induced to take up and recombine homologous extracellular, "naked" linear DNA in the genome. The previous discovery of natural competence in some streptococci and potential to open up new avenues for genetic analysis of *S. suis* was the motivation for investigating natural competence in this important pathogen [66,67].

Among the streptococci, the regulation of pheromone-induced DNA transformation, termed (natural) competence, has been best characterised in *S. thermophilus, S. pneumoniae* and *S. mutans*. In all these species the master regulator of competence development is an alternative sigma factor, ComX (or SigX) [142,192] that controls the genes involved in DNA uptake, processing and recombination. The proximal control of *comX* is mediated through secreted peptide pheromones interacting with two main types of regulatory systems. In *S. pneumoniae* the ComC pheromone and a two-component histidine kinase and response regulator (ComD and ComE) control expression of *comX*. The *comC*, *comD*, and *comE* reside in a single operon, which is conserved in some other streptococcal species. The polypeptide encoded by *comC* contains a double glycine leader, which is processed concomitant with export via a dedicated ABC transporter [252,329]. At a threshold concentration the secreted pheromone, or competence stimulating peptide (CSP), is sensed by the membrane associated histidine kinase ComD. This results in autophosphorylation of ComD and phosphotransfer to ComE, thereby altering its affinity of binding to promoters of the early competence regulon, including *comX*. In *S. mutans, S. thermophilus, S. infantarius, S. macedonicus* and *S. salivarius* the proximal control of *comX* is regulated by the ComRS system [154,155,206]. The Sigma X-inducing peptide (XIP) pheromone is produced from a precursor peptide ComS and exported by an unknown mechanism. The XIP is matured by protease(s) that have not yet been

identified in many species. In the best-studied species extracellular XIP was shown to be reimported by the oligopeptide transporter Opp/Ami and interact with the transcriptional regulator ComR [197]. The resulting ComR-XIP complex activates the transcription of early genes including *comX* and *comS* as a positive feedback.

To investigate the possibility that a natural competence system exists within *S. suis* we, (I) mined its genome for the competence genes known to be required for competence development in other streptococci, and (II) reconstructed-by-orthology the complete streptococcal apparatus needed for DNA uptake, process and recombination from the available *S. suis* genome sequences. The promoter of the *S. suis comX* was eventually found, by manual "sliding-window" scanning of the *S. suis* P1/7 genome sequence, upstream of a small ORF encoding a potential competence pheromone propeptide ComS. The sliding-window scan was based on genomic features described for other streptococci utilising the ComRS type regulatory system for induction of competence [154]. As competence-inducing pheromones in *S. mutans* and *S. pneumoniae* were previously shown to be N-terminally cleaved variants of ComS [154,202], we tested different truncated variants of the predicted *S. suis* ComS, of which one was optimally active in inducing natural genetic transformation in laboratory conditions (Chapter 2, [156]).

The proximal competence switch

In *S. suis* pheromone induction of competence was strongly dependent on the number of amino acids truncated at the N-terminus with the full length ComS peptide giving no transformants. In *S. mutans* and *S. thermophilus* mature extracellular XIPs detected in the medium or on the cell surface differ in size [203,330]. In *S. mutans* XIP is a heptamer [330], while in *S. thermophilus* several forms were identified, the major one being 11 amino acids in length [203].

Fontaine and collaborators [161] classified putative or known XIPs in three classes: class I, are specific for streptococci of the *salivarius* group and are characterized by the presence of a P(F/Y)F motif. Class II are XIPs are found in *pyogenic*, *mutans* and *bovis* group and around 30% of *S. suis* isolates (25 of the 81 total *S. suis* genomes examined). They contain two conserved tryptophan residues (WW) 2-3 residues away from the C-terminal end together with basic or acidic amino acids [154,156]. The third class is specific for certain *S. suis* groups and contains a WG(T/K)W motif [156]. The shortest XIPs are found in mutans and bovis streptococci (12–17 aa) and the longest in pyogenic species (16–32 aa). We were unable to demonstrate production of the processed pheromone in normal laboratory medium using HPLC and mass spectrometry suggesting that natural competence develops under different growth conditions. In *salivarius* streptococci Eep, a zinc metaloprotease, is required for maturation of the peptide pheromone [330] but not in *S. mutans* [203]. In *S. mutans* and *S. thermophilus*, the import of the mature pheromone is dependent on OppA, a peptide transporter not dedicated to

competence regulation [165,190]. This was also demonstrated for *S. suis* (Chapter 3) with the caveat that deletion of OppA caused slower growth of *S. suis* which may also influence competence induction. The use of a general peptide transporter for uptake of the pheromone may not be coincidental as it could be an indirect sensor of available oligopeptides and thus competitors occupying the same niche. For example, the addition of a high concentration of tryptone extract (but not casamino acids), to complex media can inhibit XIP induction of competence in *S. mutans* [209] and in *S. thermophilus* [203]. This may be an explanation for the requirement of a relatively high XIP concentration to induce competence in *S. suis* in rich medium (Chapter 2, [156]).

We have shown the existence of distinct pherotypes in *S. suis* (Chapter 2, [156]) and we observed that, under laboratory conditions, competence induction was pherotype specific. Thus the XIPs examined were not functionally equivalent and appeared to operate only in conjuction with their cognate ComR. *S. suis* producing an XIP of the same pherotype would trigger development of the competent state at a critical concentration. This concentration of XIP is dependent on rate of loss of XIP through uptake by other Opp transporters present in other strains and species and the concentration of producers for that pherotype.

In different streptococci XIP has been shown *in vitro* to interact with ComR altering binding to promoters of the early competence genes including *comX*. The closest homologues of ComR are PlcR of *Bacillus thuringiensis* and PrgX of *Enterococcus faecalis* [154,215] both of which are known to be regulators, activated by interactions of pheromones with tetratricopeptide repeats (TPR) in their C-terminal alpha-helical domain. Phylogenetic analyses have suggested coevolution of the C-terminal domain of XIP, and the putative peptide-binding domain, of ComR [161]. This suggests that ComR is also a determinant of the pherotype, ensuring induction of competence to a specific pheromone. From our preliminary study of sequence diversity of *comR* alleles, it appears that the C-terminal domain of ComR is less conserved than the N-terminal DNA-binding domain which is consistent with a possible coevolution of the C-terminal peptide-binding domain and *comS*.

A nucleotide BLAST search on all the *S. suis* isolates sequenced [22] revealed that the 418 isolates having a *comR* homolog can be clustered in 5 groups containing in total 31 classes, based on the degree of sequence conservation of the *comR* alleles (Table 1) (Zaccaria et al., unpublished). The *comR*-groupings of *S. suis* isolates presented in Table 1 can be categorised reasonably well, according to the main groups identified by Weinert and collaborators using whole genome sequences and Bayesian Analysis of Populations [22] (Fig. 1).

Table 1. *S. suis* isolates grouped based on *comR* identity

comR group	*comR* classes*	Alignment length (nt)	Number of isolates	Identity**
I	1-4	900	266	100-99.44%
II	5-12	900	34	98.44-95.00%
III	13-18	900	32	91.11-87.33%
IV	19-20	358	55	100-99.16%
V	21-31	685-353	33	86.28-83.99%

*Based on the degree of sequence conservation of the *comR* alleles, a class differs by at least 1 nt from a different class
**Relative sequence identity to the *comR* sequence of *S. suis* P1/7

This result suggests that *S. suis* isolates can be typed using DNA sequence polymorphisms in *comR*, and that clustering *S. suis* isolates using *comR* sequence variation yields groups that resemble groupings based on whole-genome sequences. If the use of *comR* sequence variation is further explored and validated, it may show that *S. suis* population structure follows DNA variation in one of the main competence genes. If this holds true, *comR* alleles may provide a powerful marker to analyse *S. suis* evolution and subpopulation distribution.

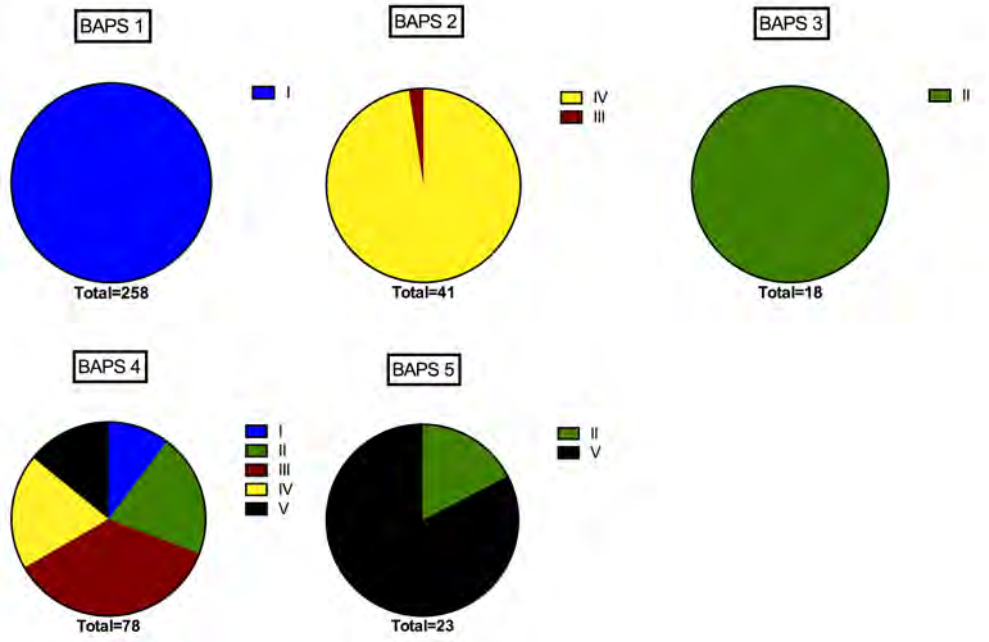

Figure 1. *comR*-groupings of *S. suis* isolates aligned with the main groups reported by Weinert and collaborators [22]. Five distinct populations (BAPS1-5) identified with Bayesian Analysis of Population, were compared with the *comR* groups I to V shown in Table 1. The number of isolates contained in each BAPS group is indicated below the graphs.

As we showed in Chapter 3 the induction of competence in *S. suis* promotes the expression of several genes not directly involved in DNA binding, processing and recombination. Maybe these secondary metabolic and energy status-related processes may give us a clue with respect to the effect that competence induction has on a *S. suis* population in an ecological niche. In the following section the possible reason for existence of multiple pherotypes in *S. suis* and their impact on evolution and natural diversity is discussed.

The role of competence in nature: on pherotypes, fratricins, bacteriocins and competition

As described in chapter 3, the ComX-regulon of *S. suis* consists mainly of genes encoding proteins involved in DNA uptake, processing and recombination. However, ComX also triggers the expression of genes not required for natural transformation. Some of these genes encode proteins with unknown function, for example, SSU0655 or proteins involved in diverse metabolic processes. One interesting process is the fratricide mechanism, observed first and studied mostly in *S. pneumoniae*. During competence, *S. pneumoniae* expresses and secretes a murein hydrolase, called CbpD or fratricin, under the control of ComX [241]. This protein binds to the septum of proliferating cells [243] and provokes their lysis, thereby releasing DNA that can be taken up by competent cells. The importance of this lytic mechanism has been studied both in liquid cultures and biofilms [331,332]. In liquid cultures CbpD strongly increases the acquisition of an antibiotic marker from non-competent pneumococcal cells compared to the wild-type [331] and in multispecies biofilms, which are the natural habitat of *S. pneumoniae* in the human oral cavity and nasopharynx [332]. Similar results were obtained when the targets cells were not *S. pneumoniae* but other species that were sensitive to the lytic activity of the S. *pneumoniae* fratricin. Pneumococcal fratricins have a limited target range due to the specificity of the choline-binding domain present in CbpD; this enzyme can only bind pneumococcal cell walls or of species very closely related to *S. pneumoniae* including the species *Streptococcus pseudopneumoniae*, *S. mitis* and *S. peroris* [174,176]. This limited range of activity could be a means to avoid potential hazards of recombining distantly related DNA, thereby compromising genome integrity or introducing non-functional or potentially deleterious genes into the genome.

Homologs of the pneumococcal fratricin have been identified in a number of streptococcal species. All fratricins seem to be regulated by ComX, and have a conserved catalytic domain, and a variable region. Presumably the variable domain binds to the cell wall of target cells, providing specificity that limits the range of action of the fratricin, although this has not been proven [174]. Our transcriptome data revealed that expression of a homolog of *cbpD* is induced by *S. suis* during competence development and is regulated by ComX (Chapter 3). The *S. suis* fratricin is characterized by a CHAP (Cysteine, Histidine-dependent Amidohydrolases/

Peptidases) domain (Fig. 2) which is found in a family of cysteine- and histidine-dependent amidohydrolases/peptidases whose members function either as N-acetylmuramoyl-L-Ala amidases or as endopeptidases that cleave within the peptide part of peptidoglycan [333,334]. As in pneumococcus the *S. suis* fratricin contains two SH3b (central Src homology 3b) domains, that probably bind to peptidoglycan (Fig. 2) giving specificity to the fratricin [335].

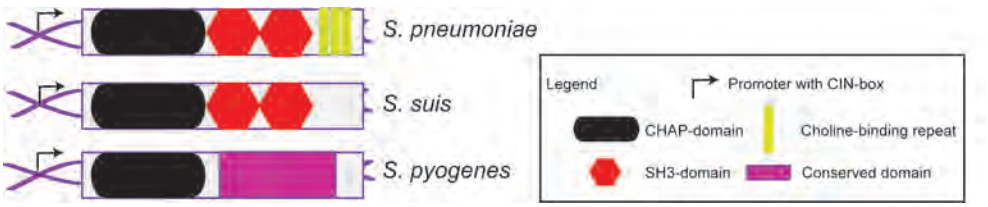

Figure 2. Domain organization of murein hydrolases of *S. pneumoniae*, *S. suis* and *S. pyogenes*. CHAP, cysteine, histidine-dependent amidohydrolases/peptidases; SH3, binds peptidoglycan (22); choline-binding repeats, binds choline residues linked to teichoic acid; Conserved domain, uncharacterized domain that probably mediates binding to the cell wall of target cells [174].

Nucleotide BLAST search using all the available *S. suis* genome sequences revealed that at least 26 alleles of fratricin are distributed among the sequenced isolates (data not shown). Although these alleles show good degree of conservation among *S. suis* isolates (sequence identity > 75%) the most variable region encodes the SH3 domains, suggesting they might target different species. In *S. pneumoniae* ComM, an immunity protein, confers protection against lytic activity of fratricin although the mechanism is unknown [228]. We were unable to identify a homologue of the fratricin immunity protein in *S. suis* or in other streptococcal species possessing a putative fratricin, with the exception of *S. pseudopneumoniae*, *S. mitis*, *S. oralis*, *S. infantis*, and *S. peroris* [174]. However preliminary experiments we conducted on the viability of *S. suis* during competence development suggest that the XIP producer strain is protected from lysis (Zaccaria et al., unpublished). More work is needed to unravel the specificity of *S. suis* fratricin and the mechanism of immunity but it is likely that it serves to lyse non-competent bacteria to release DNA for uptake. In *S. suis* the putative fratricin protein was variable in the SH3 domain, suggesting that the variants might have different strain or species specificities. Thus the presence of different fratricins and XIP pherotypes would mean competence induction in one population of *S. suis* could induce fratricin-mediated lysis in another. The existence of pherotypes very likely has a positive contribution to *S. suis* population fitness by favouring survival of subpopulations at the expense of others under particular stressful circumstances or during adverse fluctuations in the environment. Such a mechanism would also ensure that the DNA released is very similar to the DNA of the acceptor, decreasing the possibility of harmful recombination events while on the

other hand reducing the chance to obtain entirely new genetic traits.

In the oral, pharyngeal and intestinal habits biofilms are the most common mode of growth. *S. suis* can form biofilms *in vitro* [40,336] and most likely exists within such natural communities, although this has not been investigated *in vivo*. It is known that other naturally transformable streptococci such as *Streptococcus gordonii*, S. *oralis*, and *S. sanguinis* coaggregate with distantly related genera. Natural biofilms most often contain mixed communities and DNA is present in the extracellular matrix [337]. Thus competent *S. suis* inhabiting a biofilm would be exposed to DNA in the biofilm matrix but it is not known to what extent it is serves as a source of DNA for recombination. Thus fratricins may play a role in releasing DNA from more closely related strains and species of streptococci. In *S. pneumoniae*, fratricins are indeed mainly active against non-competent con-specific bacteria [172,228,241].

A future goal would be to investigate condition(s) during which competence is induced in nature, including *in vivo* in the host, and which impact it can have on the selection of particular genetic traits, e.g. antibiotic resistance or virulence factors. This could be investigated using mixed biofilms *in vitro* based on the composition of *S. suis c*ontaining biofilms *in vivo.*

Apart from fratricins some species also produce bacteriocins to compete with other bacteria in the same niche or to regulate cell fate. Interestingly our transcriptomic data revealed ComX-dependent expression of a putative bacteriocin-like operon (Chapter 3). The operon SSU0038-45 consists of 8 genes that do not show significant homology with competence genes from other streptococci and comprises 3 putative membrane proteins, one CAAX amino terminal protease and two ABC transporters with ATPase activity. In addition, the operon contains two relatively small putative ORFs (SSU0038 and SSU0045) that were highly expressed according to the microarray data (Chapter 3). These ORFs have not been annotated in the *S. suis* P1/7 genome and are predicted to encode two small (42 and 57 amino acids) proteins with unknown function (Chapter 3). Their small size and association with a CAAX peptidase and two ABC transporters suggests that these ORFs might encode bacteriocins. This idea is also supported by the amino acid sequence of the peptide leader of SSU0045 that features a double-glycine motif, characteristic of bacteriocins secreted by ABC transporters [244]. In *S. mutans*, competence induction indirectly controls bacteriocin production [198,251]. In *S. gordonii*, a locus with a CIN-box in the promoter region that encodes a bacteriocin has also been reported. The encoded bacteriocin peptide contains a double-glycine motif, prerequisite for procession and secretion by peptide-exporting ABC-type transport systems [252]. This bacteriocin was shown to be active against *S. gordonii* and *S. mitis* [253]. Further studies are needed to characterize the putative bacteriocin produced by *S. suis*, to show that the bacteriocin is produced during competence, and to identify the bacteriocin targets. Understanding the bacterial species targeted by *S. suis* fratricin and bacteriocin may

provide insights into the ecological niche of *S. suis*, its bacterial competitors and the available genetic reservoir.

If the putative fratricins and bacteriocins are indeed functional in nature, a picture emerges where *S. suis* pherotypes release fratricins and bacteriocins into the environment, which can ultimately impact on genetic exchange, genome heterogeneity, and population diversity in mixed biofilms. The latter property also has practical implications, one major topic being possible spreading of antibiotic resistance genes in and between *S. suis* populations. Transfer of *S. suis* genes is at present not characterised, and can probably proceed along different routes since spread can occur within each porcine host but can also include transfer of bacteria (maybe different pherotypes) between different pigs that are co-housed. Transfer can be by direct pig-to-pig contact, via released aerosols or body fluids including aerosols producing during sneezing and via the faeces; theoretically transfer can also occur between different pens via farmers, exemplifying that natural spread and transfer of *S. suis* bacteria will be difficult to trace and quantify.

Conservation of the transformasome

The competence state is characterized by the coordinated expression of a set of genes encoding for a peculiar multiprotein complex known as the transformasome. The transformasome comprises a Type-4 pilus (T4P) like structure and a set of proteins required for uptake, processing and recombining exogenous DNA (Fig. 3). In *S. suis*, all the genes that are involved in producing the transformasome (with the exception of EndA) contain a CIN-box motif in their promoter region indicating that their expression can be regulated by ComX (Chapter 3).

In Gram-positive bacteria the competence pilus is conserved [338,339] and formed by a major pilin, ComGC (or ComYC), and other structural proteins encoded by the *comG* (*comY*) operon. A T4P directly binding to extracellular DNA has been identified and visualized by electron microscopy in *S. pneumoniae* [157,217]. The mechanism of DNA entry is controversial. One hypothesis proposes that the pilus extrusion and secretion provides an opening in the bacterial cell wall, allowing the entrance of extracellular DNA. According to this hypothetical scenario, the DNA must enter the “pilus hole” while avoiding the interference by the secreted pilus [157,340]. The other hypothesis is that the pilus transfers extracellular DNA to the DNA receptor ComEA via pilus retraction or a charge-based sliding mechanism. For both hypotheses, experimental evidence is lacking [157,217]. In *S. pneumoniae* ComEA delivers the dsDNA to the endonuclease EndA, allowing the passage of a ssDNA through the pore protein ComEC [341,342]. ComFA and ComFC are also probably required to internalize the ssDNA while SsbB/A, DprA, RadC and RecA protect it [218-220]. Additionally CoiA, DprA and RecA, a DNA-dependent ATPase, promote formation of the recombination synapse, heteroduplex formation and strand exchange between homologous DNAs [220,221] (Fig. 3). With the exception of *ssbA*

and *endA*, *S. suis* encodes conserved homologs of all transformasome genes, with a range of sequence identity from 36% to 87%, and our transcriptome analysis shows that all of these genes are regulated by pheromone-induced competence (Chapter 3). We have also shown that the major pilin ComYC is required for transformation in *S. suis*, and that *cinA* is necessary for optimal transformation efficiency (Chapter 3).

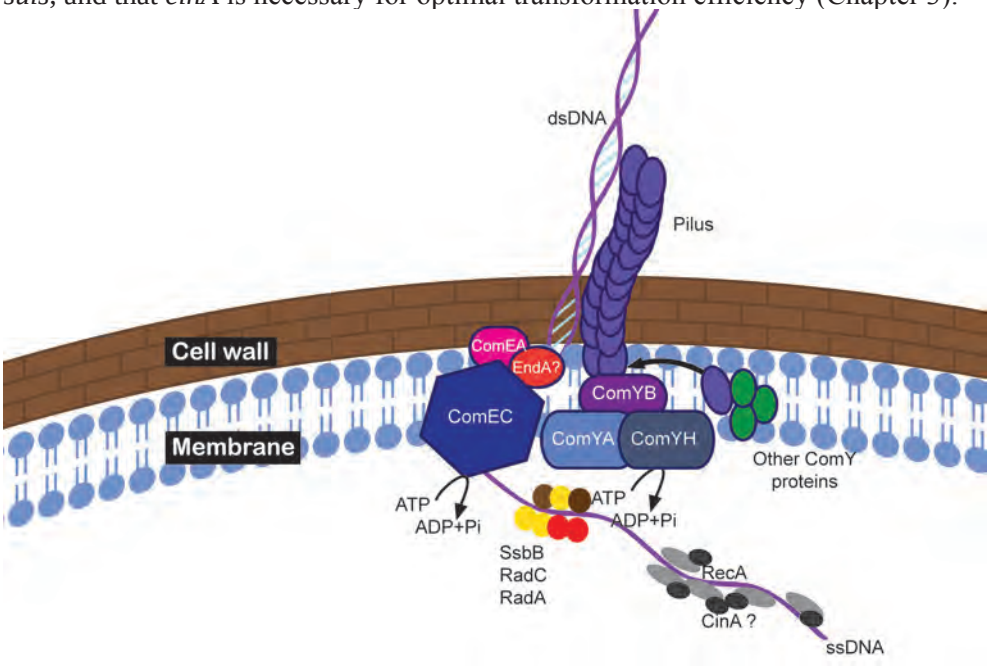

Figure 3. The transformasome. Transforming dsDNA is captured by the pilus, mainly formed by ComYC. After capture, dsDNA is passed to the DNA receptor ComEA, before transfer to the EndA nuclease. EndA degrades one strand of the captured dsDNA, and the resulting ssDNA is transferred across the membrane via the ComEC pore. After internalization, ssDNA is coated with SsbB, which protects it from nucleases and creates a reservoir of ssDNA for homologous recombination. The transformation-dedicated recombinase loader RadC and RadA and CinA. This promotes binding of the recombinase RecA onto the ssDNA. RecA can then polymerize on the ssDNA and promotes homology search and strand exchange.

Competence exit and dual role of competence proteins

In contrast to the wealth of information on the conditions of competence induction and the transformasome, little is known about the mechanism(s) mediating exit from competence. In several streptococcal species, post-translational control of ComX degradation plays a major role [167,171,225,226]. In *S. pneumoniae*, degradation of ComX is performed by the protease complex ClpE-ClpP (Fig. 4); the ClpC-ClpP complex is responsible for the degradation of ComW [167], a small protein required for stabilization and activation of ComX [211,343]. In *S. thermophilus* and *S. mutans* ComX degradation is performed by the protease complex ClpC-ClpP, and requires MecA binding to ComX and ATP hydrolysis (Fig. 4) [170,224,225]. *S. suis* possesses

homologues of *mecA* and *clpCP* but these were not differentially expressed during competence (Chapter 3). However MecA and ClpCP could play a role in ensuring that production of ComX is tightly regulated and dependent on the proximal regulatory switch.

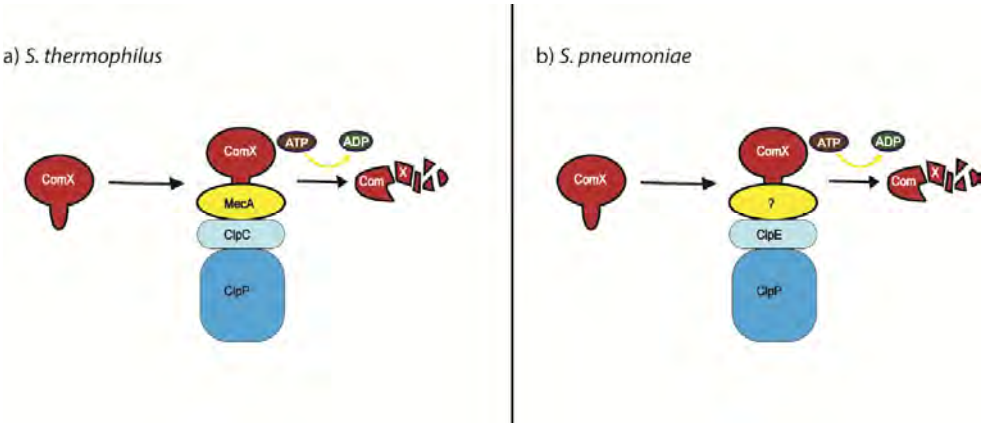

Figure 4. Post-translational regulation of ComX (σ^x). a) In *S. thermophilus* and in *S. mutans*, ComX degradation is performed by the protease complex ClpC-ClpP, with additional requirement of MecA and ATP hydrolysis. b) In *S. pneumoniae* ClpE-ClpP complex is responsible for degradation of ComX and may involve an unknown adaptor protein (?).

Several transcriptional regulators were differentially expressed early after competence induction (5 minutes), but the mechanism(s) that these regulators participate in are not well characterised. In *S. pneumoniae*, high expression of *comE* represses its auto-activation via a feedback mechanism, thus favouring competence exit [168]. Based on overall comparisons of streptococcal competence, we hypothesize that a mechanism involving ComR degradation regulates exit from competence in *S. suis* but further research is needed to confirm or falsify this hypothesis.

Interestingly it has been observed that many proteins that together produce the transformasome have a dual role. In *S. pneumoniae*, DprA is involved in facilitating the binding of RecA to ssDNA but it also inhibits binding of the phosphorylated form of ComE to the DNA, thus favouring competence exit [226] (Fig. 5). In *Bacillus subtilis* it has been demonstrated that ComGA, one of the minor pilin proteins, plays a major role in blocking cell division and DNA replication during competence [266]60; ComGA also sequesters MreB to prevent cell elongation [269]. Our transcriptome data suggest that when *S. suis* enters the competence state, basal metabolism is suppressed to delay DNA replication and division (Chapter 4). The purpose of suppression of basal metabolism would be to avoid genome-wide gene expression while chromosome replication is in progress, which could negatively affect genome

integrity. *S. suis* does not produce ComE and MreB, thus DprA and ComYA (the *S. suis* homolog of ComGA) may only have competence-related functions, or may carry out altogether different functions. In the streptococcal species that rely on ComE for *comX* regulation, the sequence of the N-terminal domain of DprA (which interacts with ComE) differs to the N-terminal domain encoded by the *dprA* allele that is present in the genomes of streptococci that signal via ComRS (Fig. 5).

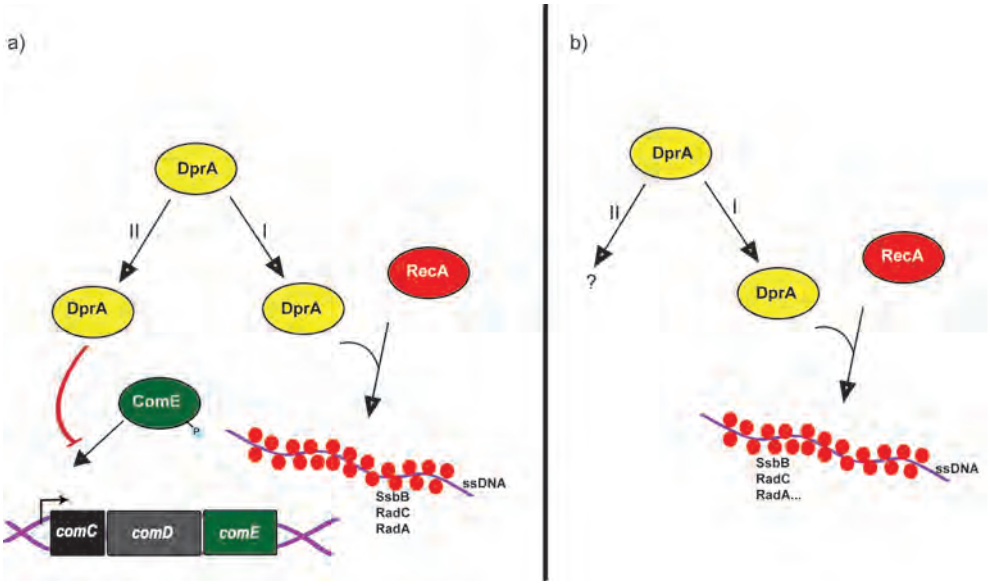

Figure 5. **Dual role of DprA.** a) In *S. pneumoniae*, DprA is involved in (I) facilitating the binding of RecA to ssDNA; (II) in inhibiting binding of the phosphorylated form of ComE (ComE~P) to the promoter region of the *comCDE* operon, thus favouring competence exit. This mechanism is probably conserved in all streptococci regulating competence via ComCDE. b) In *S. suis* and in streptococci that signal via ComRS, the putative role of DprA seems to be confined to facilitating RecA binding to ssDNA (I) [227] but an additional role is not excluded (II).

This suggests that DprA has a conserved role in competence exit in the species that signal competence via ComCDE but not in the species that signal via ComRS including *S. suis*. Further studies must be performed to investigate a possible dual role of the competence proteins such as DprA and ComYA in preventing DNA replication and cell division in *S. suis* or in facilitating competence exit [142]. Since DNA replication and competence are basal to bacterial proliferation, deeper knowledge on these processes in *S. suis* may have practical relevance as well, e.g. translation into pathways that could be rationally targeted by next-generation antimicrobials.

Competence in a natural context

Competence is one of the mechanisms of horizontal gene transfer, a crucial factor for evolution and speciation of microorganisms that reproduce clonally. Several

studies [146,147,344,345] have investigated the role that competence has on the capacity of a bacterial population to adapt and increase fitness under changing, potentially disadvantageous, environmental conditions. In *S. pneumoniae*, it has been shown that pneumococcal genome evolution is driven by vaccine and antibiotic stress and mediated by competence [146,345]. It is possible that competence has similar role in natural populations of clonally reproducing *S. suis* as suggested by the extensive genome variation and recombination in *S. suis* populations, based on whole-genome sequences of hundreds of porcine and human isolates from the United Kingdom and Vietnam [22]. We were unable to identify growth conditions that promote spontaneous competence development in *S. suis* but this knowledge would give insights into where it occurs in nature. It would be of much interest to study the microenvironment in which *S. suis* grows in nature, thus where the uptake of "foreign" DNA takes place, and to characterise the gene pool that is available locally. Biofilms are likely to be important habitats for gene transfer as was shown for other organisms in the oral cavity [346-348]. Very little is known about biofilm mode of growth for *S. suis* except that they can form biofilms *in vitro* [40,349]. We hypothesise that the surface and deep crypts in the porcine tonsilar epithelium may be one *in vivo* location that favours formation of mixed-organism biofilms that could include *S. suis*. Such biofilms would be of practical interest since colonization of pig tonsils by *S. suis* is considered a risk factor for invasive disease [112].

In some streptococcal species including *S. pneumoniae* [350], competence can be induced by DNA damaging agents that act, directly or indirectly, on DNA replication [351]. Slager and collaborators [255] showed that antibiotics inducing replication stress cause an increase in the copy number of genes proximal to the origin of replication (*oriC*). In *S. pneumoniae* and in other streptococci of the *mitis* group, these proximal genes include the early-competence operons *comCDE* and *comAB* [255]. The increased copy number of *comCDE* induces competence and, thereby, natural genetic transformation. Interestingly, the SOS response regulator LexA (HdiR) is not present in the species where the operon *comCDE* is proximal to *oriC*, suggesting that competence may substitute for the SOS stress responses in the bacterial species lacking it [255,350]. The proximal location of *comCDE* to the origin of replication may have positively selected, to promote competence and genetic recombination when bacteria encounter fluctuating and adverse (stress) conditions.

Several streptococcal species harbouring the ComRS system produce the SOS response regulator, however in these species *oriC* is located at a distance from the *comRS* locus. Moreover, in *S. thermophilus* DNA damaging agents inhibit competence development exemplifying that it is not possible to generalise what we know about competence based on one or a few bacterial species.

S. suis comRS is about 50 kb from *oriC*, like the species present in the *bovis* and the *mutans* groups, suggesting that competence may not function as

part of a stress response pathway that induces DNA repair. This exemplifies that related bacteria can respond to the same stressor in different ways, presumably in accordance with their adaptation to different ecological niches and the available nutrients. Nutrient availability also partially dictates which metabolic and energy-generating pathways the microbes in that niche will carry out. Natural transformation is energy consuming, and uptake and incorporation of non-self or foreign DNA from the environment into a genome is not without potential danger to genome integrity and metabolic functionality. Thus competence development is tightly regulated by a complex genetic and metabolic network that integrates internal and external parameters including fluctuations in nutrient availability and diverse stressors. Several studies reported that environmental changes that negatively influence the bacterial cell wall and/or plasma membrane, cell division and growth, modulate the activation of *comX*. Genome replication and competence must be coordinated to ensure a correct chromosome rearrangement and homologous recombination. Several studies, indeed, suggested that competence imposes a temporary stress on cells and triggers growth arrest [186,266] and cell fate. In *S. mutans*, induction of competence on an actively proliferating population of bacteria leads to a proportion of the bacteria entering the competent stage, and the remaining bacteria undergoing cell death [257], highlighting the association between competence and cell fate.

In this thesis, we show that, in *S. suis*, competence suppresses the organism's basal metabolism (Chapter 4) and that there are at least two molecular links between competence and metabolism: the small peptide pheromone XIP (SigX-inducing peptide); and the general peptide transporter system Opp that transports external peptides, including the XIP, into the bacterial intracellular environment. Opp transporters are located in the plasma membrane, and their main function is to import peptides from the extracellular environment to serve as sources of carbon and nitrogen necessary for bacterial proliferation [258], thus providing information on extracellular availability of nutrients. However, these transporters can also carry out conserved competence-related functions as shown for a number of streptococcal species [155,190], suggesting that Opp transporters have two conserved roles, as transporters in competence and metabolism. This is consistent with the finding that a high concentration of peptides in the medium reduces competence activation, probably due to saturation of the Opp transporter system. Interestingly, in *S. pneumoniae*, in which the Opp transporter system does not function in competence, oligopeptide-binding proteins repress *comCDE* basal transcription and repress competence induction [352]. It is not possible to generalize the finding that a negative regulation of competence via small peptides is a common feature of competence, because casein peptides from the medium may function as competence triggers in *S. thermophilus* [197], and in *S. pneumoniae*, competence can be induced by peptide fragments produced by competitor species present in the same ecological niche [353]. Thus, our working hypothesis is that environmental oligopeptides may be

used by bacteria as indicator of the nutritional status of the ecological niche, and may enable bacteria to optimize their metabolism and cell fate decisions.

New avenues to investigate *S. suis* biology

S. suis is a one of the major swine pathogens, responsible for great economic losses in the pig industry worldwide. Moreover it is considered an important zoonotic agent, gaining more attention in the scientific community after two large human outbreaks and the frequent occurrence of human meningitis cases caused by *S. suis* in South-East Asia [66,67]. Despite the impact and the increased effort and interest in *S. suis* research, the mechanisms of pathogenesis are still not well understood. Moreover, a commercially available cross-protective vaccine against multiple serotypes of *S. suis* bacteria is still not available. This highlights the imperative need for a better understanding of the protective immune responses against this important human and pig pathogen.

A major bottle-neck in *S. suis* research has been the lack of genetic tools allowing easy and rapid genetic manipulation. Before our discovery of the *S. suis* competence system and XIP amino acid sequence, the available low-efficiency genetic transformation approach required use of *E. coli* shuttle vectors, such as temperature sensitive replication vectors to introduce double cross over recombination events in the chromosome. The competence system identified and characterized in this thesis provided a simple method to obtain high frequency of transformation of *S. suis* and the possibility to use linear DNA fragments obtained via PCR for rapid targeted gene modification. Discovery of the competence system has not only overcome the problem of low transformation efficiency, but also allows routine genetic manipulation and gene deletion. Performing high throughput genome-wide mutation approaches using transposon insertions coupled to RNA-Seq methods has now been possible, opening up new avenues for research on this important pathogen. Together with collaborators, we were able to generate 4 different *S. suis* saturated transposon insertion libraries, allowing us to identify all essential genes of *S. suis* (Bem et al., unpublished) and providing us with libraries that can be used to quantify gene fitness under different environmental conditions. Moreover, we identified conditions suitable for efficient pheromone activation of natural competence in 5 different serotypes, enabling us to induce competence in 60% of those *S. suis* isolates that were otherwise poorly or not at all transformable (Chapter 2).

This study exemplified the approaches that can be taken to identify competence regulatory circuits in other bacteria possessing homologues of *comX*. Many streptococcal competence systems similar to those of *S. mutans*, *S. thermophilus* and *S. suis* possess a conserved ComR box promoter upstream of *comX* and *comS*. Our study suggests that a series of truncated variants of ComS should be tested at concentrations up to 250 μM. More challenging may be determining the optimal environmental conditions for competence induction, for example using defined

medium and different growth phases. Such an approach may be useful in order to identify the conditions to induce competence in model bacteria such as *Lactobacillus plantarum* and *Lactococcus lactis*, but also in bacteria with other industrial relevance, e.g. to generate probiotics that produce antimicrobial compounds.

In addition, our study suggests that understanding the microenvironment, the competitors and the ecological niche in which *S. suis* is present will offer us an insight into how we might be able to avoid excessive *S. suis* proliferation and how to manage antibiotic resistance in *S. suis*. Genomic rearrangements and acquisition of foreign genes from bacteria occupying the exact same niche as *S. suis* can be considered crucial factors that contribute for fast evolution of *S. suis* virulence and for acquisition of antibiotic resistance genes. The discovery of a large pathogenic island in the Chinese epidemic strain suggests that the aforementioned processes do indeed occur in nature, to a measurable degree [354,355]. It would be stimulating to uncover the favourable conditions for a spontaneous competence induction in *S. suis*, and to observe what influence peptides present in the medium may have on natural transformation and efficiency of transformation. Chemically defined medium, tryptone or casamino acids may be tested to have an better insight of the possible natural conditions in which competence may occur.

Animal models that can be used to investigate *S. suis* virulence

Another limiting factor for a better understanding of the biology of *S. suis* has been the lack of suitable, well-standardised experimental animal models [114,356]. Pigs and mice are the most common animals used to perform controlled *S. suis* infections and some research groups have successfully employed these two animals to investigate *S. suis* virulence [111,112]. A standardized swine infection model has been described [113], but the research community has not universally adopted this model. Up to now, the research community has used different animal species, pigs with different health status and at different ages, variable bacterial doses, different routes of infection and different pig stressors prior to infection [114]. This has made it extremely challenging to obtain common insights from these different studies. Moreover the use of pigs or mice has economical, logistic and ethical disadvantages over non-mammalian models, like fruit flies, nematodes and zebrafish. In this thesis we demonstrated, for the first time, that it is possible to use a zebrafish larvae model to assess the virulence of porcine *S. suis* strains. By exploiting the favourable features of the breeding and maintenance of zebrafish larvae, the number of experiments can be easily scaled-up under well-established standardized conditions. Moreover a large number of bacterial mutants and strains can be screened for their virulence and *in vivo* pathogenicity in relatively short time, and results can be obtained within days. This opens up new avenues to investigate the so far undiscovered pathways mediating successful host infection by *S. suis*.

Two-component system study, an application of competence and zebrafish larvae infection model

As we were the first to identify how the competence system of *S. suis* can be used to obtain mutants, and that differential virulence of different *S. suis* isolates can be reliably measured using a zebrafish larvae infection model, these two methods have not been previously employed by a single research group. To test the practical feasibility of these methods, we applied the competence system and the zebrafish larval model to characterize two different two-component regulatory systems (TCS) of *S. suis*, SSU1930/31 and SSU0827/28. TCS are important players in the regulation of bacterial adaptation to changing environmental conditions, including those encountered in the host during infection. Moreover, some conserved TCS have been shown to be essential or conditionally essential in different groups of bacteria, suggesting that TCS may be interesting targets for novel antibacterial and anti-infective drugs [308,309].

Our results showed that it was relatively straightforward to obtain mutants for the two TCS and that the zebrafish larvae model showed differential virulence of the two TCS deletion mutants compared to a WT strain, but also between the two TCS mutants (Chapter 6). The larvae infection model that we present here does not require specialised lab facilities apart from a microinjector. In fact, it is possible for skilled persons to micro-inject zebrafish larval yolk sacs by hand, without requiring a micromanipulator device.

We showed that both TCS but especially, the TCS encoded by SSU0827/28 are necessary for full *in vivo* virulence of *S. suis*. Additional *in vitro* pH and redox stress tolerance assays suggested which pathways may have been inappropriately regulated in the TCS deletion mutants, hampering their virulence. Further investigation, however, is necessary to identify the direct targets of both TCS. In addition, we proposed SSU0827/28 as a possible novel antibacterial drug target; it is well conserved across all *S. suis* isolated sequenced so far whereas homologs with sufficient sequence identity have so far not been found in other bacteria. Our data suggest that its inhibition might lead to a reduced survival in the stomach and to a more efficient bacterial clearance by the host immune system, which is especially relevant to bloodstream infections.

Summarising, this thesis research has resulted in development of novel methodology (Chapter 2) that will enable diverse research groups to accelerate discovery of novel features of *S. suis* ecology and pathology, especially with respect to virulence. This methodology does not require gross investments in terms of devices or personnel. Application of this methodology during the thesis research, in combination with *S. suis* transcriptomics approaches and data mining strategies developed in our lab [41] (http://edepot.wur.nl/213812), has provided a detailed model of genetic regulation of competence in *S. suis* and a hypothetical model of the *S. suis* transformasome (Chapter 3). We have also characterised the differential

metabolic states that enable competence, and the metabolic state that is incompatible with competence but rather, associated with competence exit and (Chapter 4). These detailed studies have also provided practical insights: novel antimicrobial targets have been proposed such as the TCS SSU0827/28 (Chapter 6), and pathways that, once targeted, would render *S. suis* more sensitive to antimicrobials and less resilient to a mammalian immune response, making resistance development less likely.

We have also discussed how competence may contribute to gene flow between different *S. suis* strains and maybe even between *S. suis* and other bacteria, potentially accelerating the evolution of novel traits including antibiotic resistance in *S. suis*.

The competence system has been applied during the thesis research period to perform high-throughput transposon-based genome-wide gene knockout studies involving transcriptome shotgun sequencing or RNA-Seq by colleagues from Radboud University (RU), Nijmegen in a collaboration with CVI, Lelystad. Application of the competence method has resulted in four mutant libraries that are expected to include mutants for each *S. suis* gene; similar studies by RU using *S. pneumoniae* has resulted in a wealth of knowledge on *S. pneumoniae* genetics, essential genes, genes conditionally essential for stress survival and in host persistence. We are sure that the research presented in this thesis will substantially contribute to the ability of the *S. suis* research community to unravel important aspects of *S. suis* genetics and population biology, and to translate novel findings to practical applications, with an eye to developing antimicrobials to control *S. suis* proliferation and avoid disease.

References

1. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M (2014) Streptococcus suis, an important pig pathogen and emerging zoonotic agent-an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect 3: e45.
2. Marois C, Bougeard S, Gottschalk M, Kobisch M (2004) Multiplex PCR assay for detection of Streptococcus suis species and serotypes 2 and 1/2 in tonsils of live and dead pigs. J Clin Microbiol 42: 3169-3175.
3. Devriese LA, Haesebrouck F (1992) Streptococcus suis infections in horses and cats. Vet Rec 130: 380.
4. Devriese LA, Desmidt M, Roels S, Hoorens J, Haesebrouck F (1993) Streptococcus suis infection in fallow deer. Vet Rec 132: 283.
5. Devriese LA, Haesebrouck F, de Herdt P, Dom P, Ducatelle R, et al. (1994) Streptococcus suis infections in birds. Avian Pathol 23: 721-724.
6. Palmieri C, Varaldo PE, Facinelli B (2011) Streptococcus suis, an Emerging Drug-Resistant Animal and Human Pathogen. Front Microbiol 2: 235.
7. Hill JE, Gottschalk M, Brousseau R, Harel J, Hemmingsen SM, et al. (2005) Biochemical analysis, cpn60 and 16S rDNA sequence data indicate that Streptococcus suis serotypes 32 and 34, isolated from pigs, are Streptococcus orisratti. Vet Microbiol 107: 63-69.
8. King SJ, Leigh JA, Heath PJ, Luque I, Tarradas C, et al. (2002) Development of a multilocus sequence typing scheme for the pig pathogen Streptococcus suis: identification of virulent clones and potential capsular serotype exchange. J Clin Microbiol 40: 3671-3680.
9. Staats JJ, Feder I, Okwumabua O, Chengappa MM (1997) Streptococcus suis: past and present. Vet Res Commun 21: 381-407.
10. Windsor RS (1977) Meningitis in pigs caused by Streptococcus suis type II. Vet Rec 101: 378-379.
11. Power SB (1978) Streptococcus suis type 2 infection in pigs. Vet Rec 102: 215-216.
12. Perch B, Kristjansen P, Skadhauge K (1968) Group R streptococci pathogenic for man. Two cases of meningitis and one fatal case of sepsis. Acta Pathol Microbiol Scand 74: 69-76.
13. Mai NT, Hoa NT, Nga TV, Linh le D, Chau TT, et al. (2008) Streptococcus suis meningitis in adults in Vietnam. Clin Infect Dis 46: 659-667.
14. de Ceuster LM, van Dillen JJ, Wever PC, Rozemeijer W, Louwerse ES (2012) [Streptococcus suis meningitis in a meat factory employee]. Ned Tijdschr Geneeskd 156: A5080.
15. Arends JP, Zanen HC (1988) Meningitis caused by Streptococcus suis in humans. Rev Infect Dis 10: 131-137.
16. van de Beek D, Spanjaard L, de Gans J (2008) Streptococcus suis meningitis in the Netherlands. J Infect 57: 158-161.
17 van Samkar A, Brouwer MC, Schultsz C, van der Ende A, van de Beek D (2015) Streptococcus suis meningitis in the Netherlands. J Infect.
18. Strangmann E, Froleke H, Kohse KP (2002) Septic shock caused by Streptococcus suis: case report and investigation of a risk group. Int J Hyg Environ Health 205: 385-392.
19. Wangkaew S, Chaiwarith R, Tharavichitkul P, Supparatpinyo K (2006) Streptococcus suis infection: a series of 41 cases from Chiang Mai University Hospital. J Infect 52: 455-460.
20. Wongsawan K, Gottschalk M, Tharavichitkul P (2015) Serotype- and virulence-associated gene profile of Streptococcus suis isolates from pig carcasses in Chiang Mai Province, Northern Thailand. J Vet Med Sci 77: 233-236.
21. Kay R, Cheng AF, Tse CY (1995) Streptococcus suis infection in Hong Kong. QJM 88: 39-47.
22. Weinert LA, Chaudhuri RR, Wang J, Peters SE, Corander J, et al. (2015) Genomic signatures of human and animal disease in the zoonotic pathogen Streptococcus suis. Nat Commun 6: 6740.
23. Holden MT, Hauser H, Sanders M, Ngo TH, Cherevach I, et al. (2009) Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen Streptococcus suis. PLoS One 4: e6072.
24. Zhang A, Yang M, Hu P, Wu J, Chen B, et al. (2011) Comparative genomic analysis of Streptococcus suis reveals significant genomic diversity among different serotypes. BMC Genomics 12: 523.
25. Merhej V, Georgiades K, Raoult D (2013) Postgenomic analysis of bacterial pathogens repertoire reveals genome reduction rather than virulence factors. Brief Funct Genomics 12: 291-304.
26. Chen C, Tang J, Dong W, Wang C, Feng Y, et al. (2007) A glimpse of streptococcal toxic shock syndrome from comparative genomics of S. suis 2 Chinese isolates. PLoS One 2: e315.
27. Tang J, Wang C, Feng Y, Yang W, Song H, et al. (2006) Streptococcal toxic shock syndrome caused by Streptococcus suis serotype 2. PLoS Med 3: e151.

28. Lalonde M, Segura M, Lacouture S, Gottschalk M (2000) Interactions between Streptococcus suis serotype 2 and different epithelial cell lines. Microbiology 146 (Pt 8): 1913-1921.
29. Benga L, Goethe R, Rohde M, Valentin-Weigand P (2004) Non-encapsulated strains reveal novel insights in invasion and survival of Streptococcus suis in epithelial cells. Cell Microbiol 6: 867-881.
30. Esgleas M, Lacouture S, Gottschalk M (2005) Streptococcus suis serotype 2 binding to extracellular matrix proteins. FEMS Microbiol Lett 244: 33-40.
31. Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD, et al. (2008) Isolation and characterization of alpha-enolase, a novel fibronectin-binding protein from Streptococcus suis. Microbiology 154: 2668-2679.
32. Zhang A, Chen B, Mu X, Li R, Zheng P, et al. (2009) Identification and characterization of a novel protective antigen, Enolase of Streptococcus suis serotype 2. Vaccine 27: 1348-1353.
33. Ferrando ML, Fuentes S, de Greeff A, Smith H, Wells JM (2010) ApuA, a multifunctional alpha-glucan-degrading enzyme of Streptococcus suis, mediates adhesion to porcine epithelium and mucus. Microbiology 156: 2818-2828.
34. Gottschalk MG, Lacouture S, Dubreuil JD (1995) Characterization of Streptococcus suis capsular type 2 haemolysin. Microbiology 141 (Pt 1): 189-195.
35. Gottschalk M, Segura M (2000) The pathogenesis of the meningitis caused by Streptococcus suis: the unresolved questions. Vet Microbiol 76: 259-272.
36. Fittipaldi N, Segura M, Grenier D, Gottschalk M (2012) Virulence factors involved in the pathogenesis of the infection caused by the swine pathogen and zoonotic agent Streptococcus suis. Future Microbiol 7: 259-279.
37. Wei Z, Li R, Zhang A, He H, Hua Y, et al. (2009) Characterization of Streptococcus suis isolates from the diseased pigs in China between 2003 and 2007. Veterinary Microbiology 137: 196-201.
38. Segers RP, Kenter T, de Haan LA, Jacobs AA (1998) Characterisation of the gene encoding suilysin from Streptococcus suis and expression in field strains. FEMS Microbiol Lett 167: 255-261.
39. King SJ, Heath PJ, Luque I, Tarradas C, Dowson CG, et al. (2001) Distribution and genetic diversity of suilysin in Streptococcus suis isolated from different diseases of pigs and characterization of the genetic basis of suilysin absence. Infect Immun 69: 7572-7582.
40. Grenier D, Grignon L, Gottschalk M (2009) Characterisation of biofilm formation by a Streptococcus suis meningitis isolate. Vet J 179: 292-295.
41. Ferrando ML, van Baarlen P, Orru G, Piga R, Bongers RS, et al. (2014) Carbohydrate availability regulates virulence gene expression in Streptococcus suis. PLoS One 9: e89334.
42. Chabot-Roy G, Willson P, Segura M, Lacouture S, Gottschalk M (2006) Phagocytosis and killing of Streptococcus suis by porcine neutrophils. Microb Pathog 41: 21-32.
43. Allen AG, Bolitho S, Lindsay H, Khan S, Bryant C, et al. (2001) Generation and characterization of a defined mutant of Streptococcus suis lacking suilysin. Infect Immun 69: 2732-2735.
44. Segura M, Gottschalk M, Olivier M (2004) Encapsulated Streptococcus suis inhibits activation of signaling pathways involved in phagocytosis. Infect Immun 72: 5322-5330.
45. Smith HE, Damman M, van der Velde J, Wagenaar F, Wisselink HJ, et al. (1999) Identification and characterization of the cps locus of Streptococcus suis serotype 2: the capsule protects against phagocytosis and is an important virulence factor. Infect Immun 67: 1750-1756.
46. Meijerink M, Ferrando ML, Lammers G, Taverne N, Smith HE, et al. (2012) Immunomodulatory effects of Streptococcus suis capsule type on human dendritic cell responses, phagocytosis and intracellular survival. PLoS One 7: e35849.
47. Charland N, Harel J, Kobisch M, Lacasse S, Gottschalk M (1998) Streptococcus suis serotype 2 mutants deficient in capsular expression. Microbiology 144 (Pt 2): 325-332.
48. Van Calsteren MR, Gagnon F, Lacouture S, Fittipaldi N, Gottschalk M (2010) Structure determination of Streptococcus suis serotype 2 capsular polysaccharide. Biochem Cell Biol 88: 513-525.
49. Van Calsteren MR, Gagnon F, Calzas C, Goyette-Desjardins G, Okura M, et al. (2013) Structure determination of Streptococcus suis serotype 14 capsular polysaccharide. Biochem Cell Biol 91: 49-58.
50. Gottschalk M, Xu J, Calzas C, Segura M (2010) Streptococcus suis: a new emerging or an old neglected zoonotic pathogen? Future Microbiol 5: 371-391.
51. Fittipaldi N, Sekizaki T, Takamatsu D, de la Cruz Dominguez-Punaro M, Harel J, et al. (2008) Significant contribution of the pgdA gene to the virulence of Streptococcus suis. Mol Microbiol 70: 1120-1135.
52. Fittipaldi N, Sekizaki T, Takamatsu D, Harel J, Dominguez-Punaro Mde L, et al. (2008) D-alanylation of lipoteichoic acid contributes to the virulence of Streptococcus suis. Infect Immun 76: 3587-3594.
53. Fulde M, Willenborg J, Huber C, Hitzmann A, Willms D, et al. (2014) The arginine-ornithine antiporter ArcD contributes to biological fitness of Streptococcus suis. Front Cell Infect Microbiol 4: 107.

54. Gruening P, Fulde M, Valentin-Weigand P, Goethe R (2006) Structure, regulation, and putative function of the arginine deiminase system of Streptococcus suis. J Bacteriol 188: 361-369.
55. Langford P, Williams AE, Kroll JS (1991) Superoxide dismutases of pathogenic and non-pathogenic Streptococcus suis type 2 isolates. FEMS Microbiol Lett 61: 347-350.
56. Segura M, Gottschalk M (2002) Streptococcus suis interactions with the murine macrophage cell line J774: adhesion and cytotoxicity. Infect Immun 70: 4312-4322.
57. Vanier G, Segura M, Gottschalk M (2007) Characterization of the invasion of porcine endothelial cells by Streptococcus suis serotype 2. Can J Vet Res 71: 81-89.
58. Vanier G, Segura M, Friedl P, Lacouture S, Gottschalk M (2004) Invasion of porcine brain microvascular endothelial cells by Streptococcus suis serotype 2. Infect Immun 72: 1441-1449.
59. Charland N, Nizet V, Rubens CE, Kim KS, Lacouture S, et al. (2000) Streptococcus suis serotype 2 interactions with human brain microvascular endothelial cells. Infect Immun 68: 637-643.
60. Lv Q, Hao H, Bi L, Zheng Y, Zhou X, et al. (2014) Suilysin remodels the cytoskeletons of human brain microvascular endothelial cells by activating RhoA and Rac1 GTPase. Protein Cell 5: 261-264.
61. Tenenbaum T, Papandreou T, Gellrich D, Friedrichs U, Seibt A, et al. (2009) Polar bacterial invasion and translocation of Streptococcus suis across the blood-cerebrospinal fluid barrier in vitro. Cell Microbiol 11: 323-336.
62. Tenenbaum T, Adam R, Eggelnpohler I, Matalon D, Seibt A, et al. (2005) Strain-dependent disruption of blood-cerebrospinal fluid barrier by Streptoccocus suis in vitro. FEMS Immunol Med Microbiol 44: 25-34.
63. Jiang H, Fan HJ, Lu CP (2009) Identification and distribution of putative virulent genes in strains of Streptococcus suis serotype 2. Vet Microbiol 133: 309-316.
64. Feng Y, Zhang H, Ma Y, Gao GF (2010) Uncovering newly emerging variants of Streptococcus suis, an important zoonotic agent. Trends Microbiol 18: 124-131.
65. Feng Y, Zhang H, Wu Z, Wang S, Cao M, et al. (2014) Streptococcus suis infection: an emerging/reemerging challenge of bacterial infectious diseases? Virulence 5: 477-497.
66. Segura M, Zheng H, de Greeff A, Gao GF, Grenier D, et al. (2014) Latest developments on Streptococcus suis: an emerging zoonotic pathogen: part 2. Future Microbiol 9: 587-591.
67. Segura M, Zheng H, de Greeff A, Gao GF, Grenier D, et al. (2014) Latest developments on Streptococcus suis: an emerging zoonotic pathogen: part 1. Future Microbiol 9: 441-444.
68. Wilson TL, Jeffers J, Rapp-Gabrielson VJ, Martin S, Klein LK, et al. (2007) A novel signature-tagged mutagenesis system for Streptococcus suis serotype 2. Vet Microbiol 122: 135-145.
69. Lun S, Perez-Casal J, Connor W, Willson PJ (2003) Role of suilysin in pathogenesis of Streptococcus suis capsular serotype 2. Microb Pathog 34: 27-37.
70. Jacobs AA, Loeffen PL, van den Berg AJ, Storm PK (1994) Identification, purification, and characterization of a thiol-activated hemolysin (suilysin) of Streptococcus suis. Infect Immun 62: 1742-1748.
71. de Greeff A, Buys H, Verhaar R, Dijkstra J, van Alphen L, et al. (2002) Contribution of fibronectin-binding protein to pathogenesis of Streptococcus suis serotype 2. Infect Immun 70: 1319-1325.
72. Jobin MC, Brassard J, Quessy S, Gottschalk M, Grenier D (2004) Acquisition of host plasmin activity by the Swine pathogen Streptococcus suis serotype 2. Infect Immun 72: 606-610.
73. Brassard J, Gottschalk M, Quessy S (2004) Cloning and purification of the Streptococcus suis serotype 2 glyceraldehyde-3-phosphate dehydrogenase and its involvement as an adhesin. Vet Microbiol 102: 87-94.
74. Wang K, Lu C (2007) Adhesion activity of glyceraldehyde-3-phosphate dehydrogenase in a Chinese Streptococcus suis type 2 strain. Berl Munch Tierarztl Wochenschr 120: 207-209.
75. Jobin MC, Martinez G, Motard J, Gottschalk M, Grenier D (2005) Cloning, purification, and enzymatic properties of dipeptidyl peptidase IV from the swine pathogen Streptococcus suis. J Bacteriol 187: 795-799.
76. Tan C, Fu S, Liu M, Jin M, Liu J, et al. (2008) Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconate-dehydrogenase, of Streptococcus suis serotype 2. Vet Microbiol 130: 363-370.
77. Tikkanen K, Haataja S, Finne J (1996) The galactosyl-(alpha 1-4)-galactose-binding adhesin of Streptococcus suis: occurrence in strains of different hemagglutination activities and induction of opsonic antibodies. Infect Immun 64: 3659-3665.
78. Niven DF, Ekins A (2001) Iron content of Streptococcus suis and evidence for a dpr homologue. Can J Microbiol 47: 412-416.
79. Haataja S, Penttinen A, Pulliainen AT, Tikkanen K, Finne J, et al. (2002) Expression, purification and crystallization of Dpr, a ferritin-like protein from the Gram-positive meningitis-associated bacterium Streptococcus suis. Acta Crystallogr D Biol Crystallogr 58: 1851-1853.
80. Aranda J, Garrido ME, Fittipaldi N, Cortes P, Llagostera M, et al. (2010) The cation-uptake regulators AdcR

and Fur are necessary for full virulence of Streptococcus suis. Vet Microbiol 144: 246-249.
81. Winterhoff N, Goethe R, Gruening P, Rohde M, Kalisz H, et al. (2002) Identification and characterization of two temperature-induced surface-associated proteins of Streptococcus suis with high homologies to members of the Arginine Deiminase system of Streptococcus pyogenes. J Bacteriol 184: 6768-6776.
82. Bonifait L, Grenier D (2011) The SspA subtilisin-like protease of Streptococcus suis triggers a pro-inflammatory response in macrophages through a non-proteolytic mechanism. BMC Microbiol 11: 47.
83. Bonifait L, Vaillancourt K, Gottschalk M, Frenette M, Grenier D (2011) Purification and characterization of the subtilisin-like protease of Streptococcus suis that contributes to its virulence. Vet Microbiol 148: 333-340.
84. Slater JD, Allen AG, May JP, Bolitho S, Lindsay H, et al. (2003) Mutagenesis of Streptococcus equi and Streptococcus suis by transposon Tn917. Vet Microbiol 93: 197-206.
85. Zhang A, Mu X, Chen B, Han L, Chen H, et al. (2011) IgA1 protease contributes to the virulence of Streptococcus suis. Vet Microbiol 148: 436-439.
86. Zhang A, Mu X, Chen B, Liu C, Han L, et al. (2010) Identification and characterization of IgA1 protease from Streptococcus suis. Vet Microbiol 140: 171-175.
87. Baums CG, Kaim U, Fulde M, Ramachandran G, Goethe R, et al. (2006) Identification of a novel virulence determinant with serum opacification activity in Streptococcus suis. Infect Immun 74: 6154-6162.
88. Allen AG, Lindsay H, Seilly D, Bolitho S, Peters SE, et al. (2004) Identification and characterisation of hyaluronate lyase from Streptococcus suis. Microb Pathog 36: 327-335.
89. Li M, Wang C, Feng Y, Pan X, Cheng G, et al. (2008) SalK/SalR, a two-component signal transduction system, is essential for full virulence of highly invasive Streptococcus suis serotype 2. PLoS One 3: e2080.
90. Li J, Tan C, Zhou Y, Fu S, Hu L, et al. (2011) The two-component regulatory system CiaRH contributes to the virulence of Streptococcus suis 2. Vet Microbiol 148: 99-104.
91. Wu T, Chang H, Tan C, Bei W, Chen H (2009) The orphan response regulator RevSC21 controls the attachment of Streptococcus suis serotype-2 to human laryngeal epithelial cells and the expression of virulence genes. FEMS Microbiol Lett 292: 170-181.
92. Pan X, Ge J, Li M, Wu B, Wang C, et al. (2009) The orphan response regulator CovR: a globally negative modulator of virulence in Streptococcus suis serotype 2. J Bacteriol 191: 2601-2612.
93. de Greeff A, Buys H, van Alphen L, Smith HE (2002) Response regulator important in pathogenesis of Streptococcus suis serotype 2. Microb Pathog 33: 185-192.
94. Willenborg J, Fulde M, de Greeff A, Rohde M, Smith HE, et al. (2011) Role of glucose and CcpA in capsule expression and virulence of Streptococcus suis. Microbiology 157: 1823-1833.
95. Okwumabua O, Persaud JS, Reddy PG (2001) Cloning and characterization of the gene encoding the glutamate dehydrogenase of Streptococcus suis serotype 2. Clin Diagn Lab Immunol 8: 251-257.
96. Vanier G, Fittipaldi N, Slater JD, de la Cruz Dominguez-Punaro M, Rycroft AN, et al. (2009) New putative virulence factors of Streptococcus suis involved in invasion of porcine brain microvascular endothelial cells. Microb Pathog 46: 13-20.
97. Beier D, Gross R (2006) Regulation of bacterial virulence by two-component systems. Curr Opin Microbiol 9: 143-152.
98. Yamada S, Shiro Y (2008) Structural basis of the signal transduction in the two-component system. Adv Exp Med Biol 631: 22-39.
99. Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. Annu Rev Biochem 69: 183-215.
100. Lee SY, De La Torre A, Yan D, Kustu S, Nixon BT, et al. (2003) Regulation of the transcriptional activator NtrC1: structural studies of the regulatory and AAA+ ATPase domains. Genes Dev 17: 2552-2563.
101. Djordjevic S, Goudreau PN, Xu Q, Stock AM, West AH (1998) Structural basis for methylesterase CheB regulation by a phosphorylation-activated domain. Proc Natl Acad Sci U S A 95: 1381-1386.
102. Dubrac S, Msadek T (2004) Identification of genes controlled by the essential YycG/YycF two-component system of Staphylococcus aureus. J Bacteriol 186: 1175-1181.
103. Paterson GK, Blue CE, Mitchell TJ (2006) Role of two-component systems in the virulence of Streptococcus pneumoniae. J Med Microbiol 55: 355-363.
104. Mohedano ML, Overweg K, de la Fuente A, Reuter M, Altabe S, et al. (2005) Evidence that the essential response regulator YycF in Streptococcus pneumoniae modulates expression of fatty acid biosynthesis genes and alters membrane composition. J Bacteriol 187: 2357-2367.
105. Kadioglu A, Weiser JN, Paton JC, Andrew PW (2008) The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 6: 288-301.
106. Heath A, DiRita VJ, Barg NL, Engleberg NC (1999) A two-component regulatory system, CsrR-CsrS,

represses expression of three Streptococcus pyogenes virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect Immun 67: 5298-5305.
107. Graham MR, Smoot LM, Migliaccio CA, Virtaneva K, Sturdevant DE, et al. (2002) Virulence control in group A Streptococcus by a two-component gene regulatory system: global expression profiling and in vivo infection modeling. Proc Natl Acad Sci U S A 99: 13855-13860.
108. Xu J, Fu S, Liu M, Xu Q, Bei W, et al. (2014) The two-component system NisK/NisR contributes to the virulence of Streptococcus suis serotype 2. Microbiol Res 169: 541-546.
109. Han H, Liu C, Wang Q, Xuan C, Zheng B, et al. (2012) The two-component system Ihk/Irr contributes to the virulence of Streptococcus suis serotype 2 strain 05ZYH33 through alteration of the bacterial cell metabolism. Microbiology 158: 1852-1866.
110. Wang H, Shen X, Zhao Y, Wang M, Zhong Q, et al. (2012) Identification and proteome analysis of the two-component VirR/VirS system in epidemic Streptococcus suis serotype 2. FEMS Microbiol Lett 333: 160-168.
111. Dominguez-Punaro MC, Segura M, Plante MM, Lacouture S, Rivest S, et al. (2007) Streptococcus suis serotype 2, an important swine and human pathogen, induces strong systemic and cerebral inflammatory responses in a mouse model of infection. J Immunol 179: 1842-1854.
112. Madsen LW, Bak H, Nielsen B, Jensen HE, Aalbaek B, et al. (2002) Bacterial colonization and invasion in pigs experimentally exposed to Streptococcus suis serotype 2 in aerosol. J Vet Med B Infect Dis Vet Public Health 49: 211-215.
113. Berthelot-Herault F, Cariolet R, Labbe A, Gottschalk M, Cardinal JY, et al. (2001) Experimental infection of specific pathogen free piglets with French strains of Streptococcus suis capsular type 2. Can J Vet Res 65: 196-200.
114. Gottschalk M, Higgins R, Quessy S (1999) Dilemma of the virulence of Strptococcus suis strains. J Clin Microbiol 37: 4202-4203.
115. Wu Z, Zhang W, Lu Y, Lu C (2010) Transcriptome profiling of zebrafish infected with Streptococcus suis. Microb Pathog 48: 178-187.
116. Tang F, Zhang W, Lu C (2013) Lysogenic Streptococcus suis isolate SS2-4 containing prophage SMP showed increased mortality in zebra fish compared to the wild-type isolate. PLoS One 8: e54227.
117. Zhang H, Fan H, Lu C (2010) Identification of a novel virulence-related gene in Streptococcus suis type 2 strains. Curr Microbiol 61: 494-499.
118. Wu ZF, Wang WX, Tang M, Shao J, Dai C, et al. (2014) Comparative genomic analysis shows that Streptococcus suis meningitis isolate SC070731 contains a unique 105 K genomic island. Gene 535: 156-164.
119. Kanther M, Rawls JF (2010) Host-microbe interactions in the developing zebrafish. Curr Opin Immunol 22: 10-19.
120. Cui C, Benard EL, Kanwal Z, Stockhammer OW, van der Vaart M, et al. (2011) Infectious disease modeling and innate immune function in zebrafish embryos. Methods Cell Biol 105: 273-308.
121. Herbomel P, Thisse B, Thisse C (1999) Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development 126: 3735-3745.
122. Trede NS, Langenau DM, Traver D, Look AT, Zon LI (2004) The use of zebrafish to understand immunity. Immunity 20: 367-379.
123. Novoa B, Figueras A (2012) Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases. Adv Exp Med Biol 946: 253-275.
124. Miller JD, Neely MN (2004) Zebrafish as a model host for streptococcal pathogenesis. Acta Trop 91: 53-68.
125. Kim BJ, Hancock BM, Del Cid N, Bermudez A, Traver D, et al. (2015) Streptococcus agalactiae infection in zebrafish larvae. Microb Pathog 79: 57-60.
126. van der Sar AM, Musters RJ, van Eeden FJ, Appelmelk BJ, Vandenbroucke-Grauls CM, et al. (2003) Zebrafish embryos as a model host for the real time analysis of Salmonella typhimurium infections. Cell Microbiol 5: 601-611.
127. Davis JM, Clay H, Lewis JL, Ghori N, Herbomel P, et al. (2002) Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity 17: 693-702.
128. Harvie EA, Green JM, Neely MN, Huttenlocher A (2013) Innate immune response to Streptococcus iniae infection in zebrafish larvae. Infect Immun 81: 110-121.
129. Pallares FJ, Schmitt CS, Roth JA, Evans RB, Kinyon JM, et al. (2004) Evaluation of a ceftiofur-washed whole cell Streptococcus suis bacterin in pigs. Can J Vet Res 68: 236-240.
130. Baums CG, Kock C, Beineke A, Bennecke K, Goethe R, et al. (2009) Streptococcus suis bacterin and subunit vaccine immunogenicities and protective efficacies against serotypes 2 and 9. Clin Vaccine Immunol 16:

200-208.
131. Wisselink HJ, Vecht U, Stockhofe-Zurwieden N, Smith HE (2001) Protection of pigs against challenge with virulent Streptococcus suis serotype 2 strains by a muramidase-released protein and extracellular factor vaccine. Vet Rec 148: 473-477.
132. Wisselink HJ, Stockhofe-Zurwieden N, Hilgers LA, Smith HE (2002) Assessment of protective efficacy of live and killed vaccines based on a non-encapsulated mutant of Streptococcus suis serotype 2. Vet Microbiol 84: 155-168.
133. Feng Y, Pan X, Sun W, Wang C, Zhang H, et al. (2009) Streptococcus suis enolase functions as a protective antigen displayed on the bacterial cell surface. J Infect Dis 200: 1583-1592.
134. Esgleas M, Dominguez-Punaro Mde L, Li Y, Harel J, Dubreuil JD, et al. (2009) Immunization with SsEno fails to protect mice against challenge with Streptococcus suis serotype 2. FEMS Microbiol Lett 294: 82-88.
135. Cain D, Malouin F, Dargis M, Harel J, Gottschalk M (1995) Alterations in penicillin-binding proteins in strains of Streptococcus suis possessing moderate and high levels of resistance to penicillin. FEMS Microbiol Lett 130: 121-127.
136. Wisselink HJ, Veldman KT, Van den Eede C, Salmon SA, Mevius DJ (2006) Quantitative susceptibility of Streptococcus suis strains isolated from diseased pigs in seven European countries to antimicrobial agents licensed in veterinary medicine. Vet Microbiol 113: 73-82.
137. Hendriksen RS, Mevius DJ, Schroeter A, Teale C, Jouy E, et al. (2008) Occurrence of antimicrobial resistance among bacterial pathogens and indicator bacteria in pigs in different European countries from year 2002 - 2004: the ARBAO-II study. Acta Vet Scand 50: 19.
138. Zhang C, Ning Y, Zhang Z, Song L, Qiu H, et al. (2008) In vitro antimicrobial susceptibility of Streptococcus suis strains isolated from clinically healthy sows in China. Vet Microbiol 131: 386-392.
139. Gyles C, Boerlin P (2014) Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. Vet Pathol 51: 328-340.
140. Koonin EV, Makarova KS, Aravind L (2001) Horizontal gene transfer in prokaryotes: quantification and classification. Annu Rev Microbiol 55: 709-742.
141. Griffith F (1928) The Significance of Pneumococcal Types. J Hyg (Lond) 27: 113-159.
142. Johnston C, Martin B, Fichant G, Polard P, Claverys JP (2014) Bacterial transformation: distribution, shared mechanisms and divergent control. Nat Rev Microbiol 12: 181-196.
143. Lorenz MG, Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev 58: 563-602.
144. Levin BR, Cornejo OE (2009) The population and evolutionary dynamics of homologous gene recombination in bacterial populations. PLoS Genet 5: e1000601.
145. Wylie CS, Trout AD, Kessler DA, Levine H (2010) Optimal strategy for competence differentiation in bacteria. PLoS Genet 6: e1001108.
146. Chewapreecha C, Harris SR, Croucher NJ, Turner C, Marttinen P, et al. (2014) Dense genomic sampling identifies highways of pneumococcal recombination. Nat Genet 46: 305-309.
147. Jorth P, Whiteley M (2012) An evolutionary link between natural transformation and CRISPR adaptive immunity. MBio 3.
148. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, et al. (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315: 1709-1712.
149. Marraffini LA, Sontheimer EJ (2008) CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322: 1843-1845.
150. Redfield RJ, Findlay WA, Bosse J, Kroll JS, Cameron AD, et al. (2006) Evolution of competence and DNA uptake specificity in the Pasteurellaceae. BMC Evol Biol 6: 82.
151. Redfield RJ, Cameron AD, Qian Q, Hinds J, Ali TR, et al. (2005) A novel CRP-dependent regulon controls expression of competence genes in Haemophilus influenzae. J Mol Biol 347: 735-747.
152. Solomon JM, Lazazzera BA, Grossman AD (1996) Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in Bacillus subtilis. Genes Dev 10: 2014-2024.
153. Hamoen LW, Venema G, Kuipers OP (2003) Controlling competence in Bacillus subtilis: shared use of regulators. Microbiology 149: 9-17.
154. Mashburn-Warren L, Morrison DA, Federle MJ (2010) A novel double-tryptophan peptide pheromone controls competence in Streptococcus spp. via an Rgg regulator. Mol Microbiol 78: 589-606.
155. Fontaine L, Boutry C, de Frahan MH, Delplace B, Fremaux C, et al. (2010) A novel pheromone quorum-sensing system controls the development of natural competence in Streptococcus thermophilus and Streptococcus salivarius. J Bacteriol 192: 1444-1454.

156. Zaccaria E, van Baarlen P, de Greeff A, Morrison DA, Smith H, et al. (2014) Control of competence for DNA transformation in streptococcus suis by genetically transferable pherotypes. PLoS One 9: e99394.
157. Laurenceau R, Pehau-Arnaudet G, Baconnais S, Gault J, Malosse C, et al. (2013) A type IV pilus mediates DNA binding during natural transformation in Streptococcus pneumoniae. PLoS Pathog 9: e1003473.
158. Campbell EA, Choi SY, Masure HR (1998) A competence regulon in Streptococcus pneumoniae revealed by genomic analysis. Mol Microbiol 27: 929-939.
159. Luo P, Morrison DA (2003) Transient association of an alternative sigma factor, ComX, with RNA polymerase during the period of competence for genetic transformation in Streptococcus pneumoniae. J Bacteriol 185: 349-358.
160. Muschiol S, Balaban M, Normark S, Henriques-Normark B (2015) Uptake of extracellular DNA: competence induced pili in natural transformation of Streptococcus pneumoniae. Bioessays 37: 426-435.
161. Fontaine L, Wahl A, Flechard M, Mignolet J, Hols P (2015) Regulation of competence for natural transformation in streptococci. Infect Genet Evol 33: 343-360.
162. Ahn SJ, Kaspar J, Kim JN, Seaton K, Burne RA (2014) Discovery of novel peptides regulating competence development in Streptococcus mutans. J Bacteriol 196: 3735-3745.
163. Seaton K, Ahn SJ, Burne RA (2015) Regulation of competence and gene expression in Streptococcus mutans by the RcrR transcriptional regulator. Mol Oral Microbiol 30: 147-159.
164. Federle MJ, Morrison DA (2012) One if by land, two if by sea: signalling to the ranks with CSP and XIP. Mol Microbiol 86: 241-245.
165. Guo Q, Ahn SJ, Kaspar J, Zhou X, Burne RA (2014) Growth phase and pH influence peptide signaling for competence development in Streptococcus mutans. J Bacteriol 196: 227-236.
166. Chastanet A, Prudhomme M, Claverys JP, Msadek T (2001) Regulation of Streptococcus pneumoniae clp genes and their role in competence development and stress survival. J Bacteriol 183: 7295-7307.
167. Piotrowski A, Burghout P, Morrison DA (2009) spr1630 is responsible for the lethality of clpX mutations in Streptococcus pneumoniae. J Bacteriol 191: 4888-4895.
168. Martin B, Soulet AL, Mirouze N, Prudhomme M, Mortier-Barriere I, et al. (2013) ComE/ComE~P interplay dictates activation or extinction status of pneumococcal X-state (competence). Mol Microbiol 87: 394-411.
169. Mirouze N, Berge MA, Soulet AL, Mortier-Barriere I, Quentin Y, et al. (2013) Direct involvement of DprA, the transformation-dedicated RecA loader, in the shut-off of pneumococcal competence. Proc Natl Acad Sci U S A 110: E1035-1044.
170. Tian XL, Dong G, Liu T, Gomez ZA, Wahl A, et al. (2013) MecA protein acts as a negative regulator of genetic competence in Streptococcus mutans. J Bacteriol 195: 5196-5206.
171. Biornstad TJ, Havarstein LS (2011) ClpC acts as a negative regulator of competence in Streptococcus thermophilus. Microbiology 157: 1676-1684.
172. Claverys JP, Martin B, Havarstein LS (2007) Competence-induced fratricide in streptococci. Mol Microbiol 64: 1423-1433.
173. Eldholm V, Johnsborg O, Haugen K, Ohnstad HS, Havarstein LS (2009) Fratricide in Streptococcus pneumoniae: contributions and role of the cell wall hydrolases CbpD, LytA and LytC. Microbiology 155: 2223-2234.
174. Berg KH, Biornstad TJ, Johnsborg O, Havarstein LS (2012) Properties and biological role of streptococcal fratricins. Appl Environ Microbiol 78: 3515-3522.
175. Kausmally L, Johnsborg O, Lunde M, Knutsen E, Havarstein LS (2005) Choline-binding protein D (CbpD) in Streptococcus pneumoniae is essential for competence-induced cell lysis. J Bacteriol 187: 4338-4345.
176. Berg KH, Ohnstad HS, Havarstein LS (2012) LytF, a novel competence-regulated murein hydrolase in the genus Streptococcus. J Bacteriol 194: 627-635.
177. Perry JA, Jones MB, Peterson SN, Cvitkovitch DG, Levesque CM (2009) Peptide alarmone signalling triggers an auto-active bacteriocin necessary for genetic competence. Mol Microbiol 72: 905-917.
178. Lemme A, Grobe L, Reck M, Tomasch J, Wagner-Dobler I (2011) Subpopulation-specific transcriptome analysis of competence-stimulating-peptide-induced Streptococcus mutans. J Bacteriol 193: 1863-1877.
179. Takeuchi N, Kaneko K, Koonin EV (2014) Horizontal gene transfer can rescue prokaryotes from Muller's ratchet: benefit of DNA from dead cells and population subdivision. G3 (Bethesda) 4: 325-339.
180. Stanhope MJ, Walsh SL, Becker JA, Italia MJ, Ingraham KA, et al. (2005) Molecular evolution perspectives on intraspecific lateral DNA transfer of topoisomerase and gyrase loci in Streptococcus pneumoniae, with implications for fluoroquinolone resistance development and spread. Antimicrob Agents Chemother 49: 4315-4326.
181. Johnsborg O, Eldholm V, Havarstein LS (2007) Natural genetic transformation: prevalence, mechanisms and function. Res Microbiol 158: 767-778.

182. Booijink CC, Zoetendal EG, Kleerebezem M, de Vos WM (2007) Microbial communities in the human small intestine: coupling diversity to metagenomics. Future Microbiol 2: 285-295.
183. Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, et al. (2012) The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME J 6: 1415-1426.
184. Booijink CC, El-Aidy S, Rajilic-Stojanovic M, Heilig HG, Troost FJ, et al. (2010) High temporal and inter-individual variation detected in the human ileal microbiota. Environ Microbiol 12: 3213-3227.
185. Chen I, Dubnau D (2004) DNA uptake during bacterial transformation. Nat Rev Microbiol 2: 241-249.
186. Dagkessamanskaia A, Moscoso M, Henard V, Guiral S, Overweg K, et al. (2004) Interconnection of competence, stress and CiaR regulons in Streptococcus pneumoniae: competence triggers stationary phase autolysis of ciaR mutant cells. Mol Microbiol 51: 1071-1086.
187. Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, et al. (2004) Identification of competence pheromone responsive genes in Streptococcus pneumoniae by use of DNA microarrays. Mol Microbiol 51: 1051-1070.
188. Maughan H, Redfield RJ (2009) Tracing the evolution of competence in Haemophilus influenzae. PLoS One 4: e5854.
189. Blomqvist T, Steinmoen H, Havarstein LS (2006) Pheromone-induced expression of recombinant proteins in Streptococcus thermophilus. Arch Microbiol 186: 465-473.
190. Gardan R, Besset C, Guillot A, Gitton C, Monnet V (2009) The oligopeptide transport system is essential for the development of natural competence in Streptococcus thermophilus strain LMD-9. J Bacteriol 191: 4647-4655.
191. Facklam RR (1977) Physiological differentiation of viridans streptococci. J Clin Microbiol 5: 184-201.
192. Martin B, Quentin Y, Fichant G, Claverys JP (2006) Independent evolution of competence regulatory cascades in streptococci? Trends Microbiol 14: 339-345.
193. Rice P, Longden I, Bleasby A (2000) EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet 16: 276-277.
194. Dehal PS, Joachimiak MP, Price MN, Bates JT, Baumohl JK, et al. (2010) MicrobesOnline: an integrated portal for comparative and functional genomics. Nucleic Acids Res 38: D396-400.
195. Goodstadt L, Ponting CP (2001) CHROMA: consensus-based colouring of multiple alignments for publication. Bioinformatics 17: 845-846.
196. Lee MS, Morrison DA (1999) Identification of a new regulator in Streptococcus pneumoniae linking quorum sensing to competence for genetic transformation. J Bacteriol 181: 5004-5016.
197. Fontaine L, Goffin P, Dubout H, Delplace B, Baulard A, et al. (2013) Mechanism of competence activation by the ComRS signalling system in streptococci. Mol Microbiol 87: 1113-1132.
198. Okinaga T, Niu G, Xie Z, Qi F, Merritt J (2010) The hdrRM operon of Streptococcus mutans encodes a novel regulatory system for coordinated competence development and bacteriocin production. J Bacteriol 192: 1844-1852.
199. Slamti L, Lereclus D (2002) A cell-cell signaling peptide activates the PlcR virulence regulon in bacteria of the Bacillus cereus group. EMBO J 21: 4550-4559.
200. Bouillaut L, Perchat S, Arold S, Zorrilla S, Slamti L, et al. (2008) Molecular basis for group-specific activation of the virulence regulator PlcR by PapR heptapeptides. Nucleic Acids Res 36: 3791-3801.
201. Kleerebezem M, Beerthuyzen MM, Vaughan EE, de Vos WM, Kuipers OP (1997) Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for Lactococcus, Leuconostoc, and Lactobacillus spp. Appl Environ Microbiol 63: 4581-4584.
202. Havarstein LS, Coomaraswamy G, Morrison DA (1995) An unmodified heptadecapeptide pheromone induces competence for genetic transformation in Streptococcus pneumoniae. Proc Natl Acad Sci U S A 92: 11140-11144.
203. Gardan R, Besset C, Gitton C, Guillot A, Fontaine L, et al. (2013) Extracellular life cycle of ComS, the competence-stimulating peptide of Streptococcus thermophilus. J Bacteriol 195: 1845-1855.
204. Lau PC, Sung CK, Lee JH, Morrison DA, Cvitkovitch DG (2002) PCR ligation mutagenesis in transformable streptococci: application and efficiency. J Microbiol Methods 49: 193-205.
205. Mashburn-Warren L, Morrison DA, Federle MJ (2012) The cryptic competence pathway in Streptococcus pyogenes is controlled by a peptide pheromone. J Bacteriol 194: 4589-4600.
206. Morrison DA, Guedon E, Renault P (2013) Competence for natural genetic transformation in the Streptococcus bovis group streptococci S. infantarius and S. macedonicus. J Bacteriol 195: 2612-2620.
207. Fleuchot B, Gitton C, Guillot A, Vidic J, Nicolas P, et al. (2011) Rgg proteins associated with internalized small hydrophobic peptides: a new quorum-sensing mechanism in streptococci. Mol Microbiol 80: 1102-1119.
208. Fleuchot B, Guillot A, Mezange C, Besset C, Chambellon E, et al. (2013) Rgg-associated SHP signaling

peptides mediate cross-talk in Streptococci. PLoS One 8: e66042.
209. Desai K, Mashburn-Warren L, Federle MJ, Morrison DA (2012) Development of competence for genetic transformation of Streptococcus mutans in a chemically defined medium. J Bacteriol 194: 3774-3780.
210. von Heijne G (1984) Analysis of the distribution of charged residues in the N-terminal region of signal sequences: implications for protein export in prokaryotic and eukaryotic cells. EMBO J 3: 2315-2318.
211. Sung CK, Morrison DA (2005) Two distinct functions of ComW in stabilization and activation of the alternative sigma factor ComX in Streptococcus pneumoniae. J Bacteriol 187: 3052-3061.
212. Kuipers OP, de Ruyter PG, Kleerebezem M, de Vos WM (1997) Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol 15: 135-140.
213. Vecht U, Wisselink HJ, Stockhofe-Zurwieden N, Smith HE (1996) Characterization of virulence of the Streptococcus suis serotype 2 reference strain Henrichsen S 735 in newborn gnotobiotic pigs. Vet Microbiol 51: 125-136.
214. Vecht U, Arends JP, van der Molen EJ, van Leengoed LA (1989) Differences in virulence between two strains of Streptococcus suis type II after experimentally induced infection of newborn germ-free pigs. Am J Vet Res 50: 1037-1043.
215. Fontaine L, Dandoy D, Boutry C, Delplace B, de Frahan MH, et al. (2010) Development of a versatile procedure based on natural transformation for marker-free targeted genetic modification in Streptococcus thermophilus. Appl Environ Microbiol 76: 7870-7877.
216. Petersen FC, Tao L, Scheie AA (2005) DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. J Bacteriol 187: 4392-4400.
217. Laurenceau R, Krasteva PV, Diallo A, Ouarti S, Duchateau M, et al. (2015) Conserved Streptococcus pneumoniae spirosomes suggest a single type of transformation pilus in competence. PLoS Pathog 11: e1004835.
218. Attaiech L, Olivier A, Mortier-Barriere I, Soulet AL, Granadel C, et al. (2011) Role of the single-stranded DNA-binding protein SsbB in pneumococcal transformation: maintenance of a reservoir for genetic plasticity. PLoS Genet 7: e1002156.
219. Berge M, Mortier-Barriere I, Martin B, Claverys JP (2003) Transformation of Streptococcus pneumoniae relies on DprA- and RecA-dependent protection of incoming DNA single strands. Mol Microbiol 50: 527-536.
220. Morrison DA, Mortier-Barriere I, Attaiech L, Claverys JP (2007) Identification of the major protein component of the pneumococcal eclipse complex. J Bacteriol 189: 6497-6500.
221. Desai BV, Morrison DA (2006) An unstable competence-induced protein, CoiA, promotes processing of donor DNA after uptake during genetic transformation in Streptococcus pneumoniae. J Bacteriol 188: 5177-5186.
222. Seaton K, Ahn SJ, Sagstetter AM, Burne RA (2011) A transcriptional regulator and ABC transporters link stress tolerance, (p)ppGpp, and genetic competence in Streptococcus mutans. J Bacteriol 193: 862-874.
223. Dong G, Tian XL, Gomez ZA, Li YH (2014) Regulated proteolysis of the alternative sigma factor SigX in Streptococcus mutans: implication in the escape from competence. BMC Microbiol 14: 183.
224. Wahl A, Servais F, Drucbert AS, Foulon C, Fontaine L, et al. (2014) Control of natural transformation in salivarius Streptococci through specific degradation of sigmaX by the MecA-ClpCP protease complex. J Bacteriol 196: 2807-2816.
225. Boutry C, Wahl A, Delplace B, Clippe A, Fontaine L, et al. (2012) Adaptor protein MecA is a negative regulator of the expression of late competence genes in Streptococcus thermophilus. J Bacteriol 194: 1777-1788.
226. Weng L, Piotrowski A, Morrison DA (2013) Exit from competence for genetic transformation in Streptococcus pneumoniae is regulated at multiple levels. PLoS One 8: e64197.
227. Mortier-Barriere I, Velten M, Dupaigne P, Mirouze N, Pietrement O, et al. (2007) A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. Cell 130: 824-836.
228. Havarstein LS, Martin B, Johnsborg O, Granadel C, Claverys JP (2006) New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor. Mol Microbiol 59: 1297-1307.
229. Higgins CF (2001) ABC transporters: physiology, structure and mechanism--an overview. Res Microbiol 152: 205-210.
230. de Greeff A, Wisselink HJ, de Bree FM, Schultsz C, Baums CG, et al. (2011) Genetic diversity of Streptococcus suis isolates as determined by comparative genome hybridization. BMC Microbiol 11: 161.
231. Saulnier DM, Santos F, Roos S, Mistretta TA, Spinler JK, et al. (2011) Exploring metabolic pathway

reconstruction and genome-wide expression profiling in Lactobacillus reuteri to define functional probiotic features. PLoS One 6: e18783.
232. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, et al. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res 30: e15.
233. van Hijum SA, Garcia de la Nava J, Trelles O, Kok J, Kuipers OP (2003) MicroPreP: a cDNA microarray data pre-processing framework. Appl Bioinformatics 2: 241-244.
234. Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, et al. (2006) TM4 microarray software suite. Methods Enzymol 411: 134-193.
235. Vickerman MM, Iobst S, Jesionowski AM, Gill SR (2007) Genome-wide transcriptional changes in Streptococcus gordonii in response to competence signaling peptide. J Bacteriol 189: 7799-7807.
236. Peterson S, Cline RT, Tettelin H, Sharov V, Morrison DA (2000) Gene expression analysis of the Streptococcus pneumoniae competence regulons by use of DNA microarrays. J Bacteriol 182: 6192-6202.
237. Lacks S, Greenberg B, Neuberger M (1974) Role of a deoxyribonuclease in the genetic transformation of Diplococcus pneumoniae. Proc Natl Acad Sci U S A 71: 2305-2309.
238. Seitz P, Pezeshgi Modarres H, Borgeaud S, Bulushev RD, Steinbock LJ, et al. (2014) ComEA is essential for the transfer of external DNA into the periplasm in naturally transformable Vibrio cholerae cells. PLoS Genet 10: e1004066.
239. Mair RW, Senadheera DB, Cvitkovitch DG (2012) CinA is regulated via ComX to modulate genetic transformation and cell viability in Streptococcus mutans. FEMS Microbiol Lett 331: 44-52.
240. Masure HR, Pearce BJ, Shio H, Spellerberg B (1998) Membrane targeting of RecA during genetic transformation. Mol Microbiol 27: 845-852.
241. Guiral S, Mitchell TJ, Martin B, Claverys JP (2005) Competence-programmed predation of noncompetent cells in the human pathogen Streptococcus pneumoniae: genetic requirements. Proc Natl Acad Sci U S A 102: 8710-8715.
242. Perez-Dorado I, Gonzalez A, Morales M, Sanles R, Striker W, et al. (2010) Insights into pneumococcal fratricide from the crystal structures of the modular killing factor LytC. Nat Struct Mol Biol 17: 576-581.
243. Eldholm V, Johnsborg O, Straume D, Ohnstad HS, Berg KH, et al. (2010) Pneumococcal CbpD is a murein hydrolase that requires a dual cell envelope binding specificity to kill target cells during fratricide. Mol Microbiol 76: 905-917.
244. van Belkum MJ, Worobo RW, Stiles ME (1997) Double-glycine-type leader peptides direct secretion of bacteriocins by ABC transporters: colicin V secretion in Lactococcus lactis. Mol Microbiol 23: 1293-1301.
245. Dufour D, Levesque CM (2013) Cell death of Streptococcus mutans induced by a quorum-sensing peptide occurs via a conserved streptococcal autolysin. J Bacteriol 195: 105-114.
246. Havarstein LS (2010) Increasing competence in the genus Streptococcus. Mol Microbiol 78: 541-544.
247. Aggarwal C, Jimenez JC, Lee H, Chlipala GE, Ratia K, et al. (2015) Identification of Quorum-Sensing Inhibitors Disrupting Signaling between Rgg and Short Hydrophobic Peptides in Streptococci. MBio 6: e00393-00315.
248. Berge MJ, Kamgoue A, Martin B, Polard P, Campo N, et al. (2013) Midcell recruitment of the DNA uptake and virulence nuclease, EndA, for pneumococcal transformation. PLoS Pathog 9: e1003596.
249. Kaimer C, Graumann PL (2010) Bacillus subtilis CinA is a stationary phase-induced protein that localizes to the nucleoid and plays a minor role in competent cells. Arch Microbiol 192: 549-557.
250. Biornstad TJ, Ohnstad HS, Havarstein LS (2012) Deletion of the murein hydrolase CbpD reduces transformation efficiency in Streptococcus thermophilus. Microbiology 158: 877-885.
251. Reck M, Tomasch J, Wagner-Dobler I (2015) The Alternative Sigma Factor SigX Controls Bacteriocin Synthesis and Competence, the Two Quorum Sensing Regulated Traits in Streptococcus mutans. PLoS Genet 11: e1005353.
252. Havarstein LS, Diep DB, Nes IF (1995) A family of bacteriocin ABC transporters carry out proteolytic processing of their substrates concomitant with export. Mol Microbiol 16: 229-240.
253. Heng NC, Tagg JR, Tompkins GR (2007) Competence-dependent bacteriocin production by Streptococcus gordonii DL1 (Challis). J Bacteriol 189: 1468-1472.
254. LaSarre B, Federle MJ (2013) Exploiting quorum sensing to confuse bacterial pathogens. Microbiol Mol Biol Rev 77: 73-111.
255. Slager J, Kjos M, Attaiech L, Veening JW (2014) Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. Cell 157: 395-406.
256. Morrison DA (1997) Streptococcal competence for genetic transformation: regulation by peptide pheromones. Microb Drug Resist 3: 27-37.
257. Wenderska IB, Lukenda N, Cordova M, Magarvey N, Cvitkovitch DG, et al. (2012) A novel function for the

competence inducing peptide, XIP, as a cell death effector of Streptococcus mutans. FEMS Microbiol Lett 336: 104-112.
258. Monnet V (2003) Bacterial oligopeptide-binding proteins. Cell Mol Life Sci 60: 2100-2114.
259. Ernst J, Bar-Joseph Z (2006) STEM: a tool for the analysis of short time series gene expression data. BMC Bioinformatics 7: 191.
260. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, et al. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21: 3674-3676.
261. Zdobnov EM, Apweiler R (2001) InterProScan--an integration platform for the signature-recognition methods in InterPro. Bioinformatics 17: 847-848.
262. Al-Shahrour F, Minguez P, Tarraga J, Medina I, Alloza E, et al. (2007) FatiGO +: a functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. Nucleic Acids Res 35: W91-96.
263. Gury J, Barthelmebs L, Tran NP, Divies C, Cavin JF (2004) Cloning, deletion, and characterization of PadR, the transcriptional repressor of the phenolic acid decarboxylase-encoding padA gene of Lactobacillus plantarum. Appl Environ Microbiol 70: 2146-2153.
264. Tran NP, Gury J, Dartois V, Nguyen TK, Seraut H, et al. (2008) Phenolic acid-mediated regulation of the padC gene, encoding the phenolic acid decarboxylase of Bacillus subtilis. J Bacteriol 190: 3213-3224.
265. Al-Shahrour F, Diaz-Uriarte R, Dopazo J (2004) FatiGO: a web tool for finding significant associations of Gene Ontology terms with groups of genes. Bioinformatics 20: 578-580.
266. Haijema BJ, Hahn J, Haynes J, Dubnau D (2001) A ComGA-dependent checkpoint limits growth during the escape from competence. Mol Microbiol 40: 52-64.
267. Briley K, Jr., Prepiak P, Dias MJ, Hahn J, Dubnau D (2011) Maf acts downstream of ComGA to arrest cell division in competent cells of B. subtilis. Mol Microbiol 81: 23-39.
268. Attaiech L, Minnen A, Kjos M, Gruber S, Veening JW (2015) The ParB-parS Chromosome Segregation System Modulates Competence Development in Streptococcus pneumoniae. MBio 6.
269. Mirouze N, Ferret C, Yao Z, Chastanet A, Carballido-Lopez R (2015) MreB-Dependent Inhibition of Cell Elongation during the Escape from Competence in Bacillus subtilis. PLoS Genet 11: e1005299.
270. Oggioni MR, Iannelli F, Ricci S, Chiavolini D, Parigi R, et al. (2004) Antibacterial activity of a competence-stimulating peptide in experimental sepsis caused by Streptococcus pneumoniae. Antimicrob Agents Chemother 48: 4725-4732.
271. Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, et al. (2006) Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 61: 1196-1210.
272. Lun ZR, Wang QP, Chen XG, Li AX, Zhu XQ (2007) Streptococcus suis: an emerging zoonotic pathogen. Lancet Infect Dis 7: 201-209.
273. Wertheim HF, Nghia HD, Taylor W, Schultsz C (2009) Streptococcus suis: an emerging human pathogen. Clin Infect Dis 48: 617-625.
274. Allgaier A, Goethe R, Wisselink HJ, Smith HE, Valentin-Weigand P (2001) Relatedness of Streptococcus suis isolates of various serotypes and clinical backgrounds as evaluated by macrorestriction analysis and expression of potential virulence traits. J Clin Microbiol 39: 445-453.
275. Gottschalk M, Segura M, Xu J (2007) Streptococcus suis infections in humans: the Chinese experience and the situation in North America. Anim Health Res Rev 8: 29-45.
276. Ferrando ML, de Greeff A, van Rooijen WJ, Stockhofe-Zurwieden N, Nielsen J, et al. (2015) Host-pathogen Interaction at the Intestinal Mucosa Correlates With Zoonotic Potential of Streptococcus suis. J Infect Dis 212: 95-105.
277. Suankratay C, Intalapaporn P, Nunthapisud P, Arunyingmongkol K, Wilde H (2004) Streptococcus suis meningitis in Thailand. Southeast Asian J Trop Med Public Health 35: 868-876.
278. Yu H, Jing H, Chen Z, Zheng H, Zhu X, et al. (2006) Human Streptococcus suis outbreak, Sichuan, China. Emerg Infect Dis 12: 914-920.
279. Watkins EJ, Brooksby P, Schweiger MS, Enright SM (2001) Septicaemia in a pig-farm worker. Lancet 357: 38.
280. Marini E, Palmieri C, Magi G, Facinelli B (2015) Recombination between Streptococcus suis ICESsu32457 and Streptococcus agalactiae ICESa2603 yields a hybrid ICE transferable to Streptococcus pyogenes. Vet Microbiol 178: 99-104.
281. Lecours MP, Segura M, Lachance C, Mussa T, Surprenant C, et al. (2011) Characterization of porcine dendritic cell response to Streptococcus suis. Vet Res 42: 72.
282. Lakkitjaroen N, Takamatsu D, Okura M, Sato M, Osaki M, et al. (2011) Loss of capsule among Streptococcus

suis isolates from porcine endocarditis and its biological significance. J Med Microbiol 60: 1669-1676.
283. Stockhammer OW, Zakrzewska A, Hegedus Z, Spaink HP, Meijer AH (2009) Transcriptome profiling and functional analyses of the zebrafish embryonic innate immune response to Salmonella infection. J Immunol 182: 5641-5653.
284. Sullivan C, Kim CH (2008) Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol 25: 341-350.
285. Levraud JP, Disson O, Kissa K, Bonne I, Cossart P, et al. (2009) Real-time observation of listeria monocytogenes-phagocyte interactions in living zebrafish larvae. Infect Immun 77: 3651-3660.
286. Smith HE, Vecht U, Wisselink HJ, Stockhofe-Zurwieden N, Biermann Y, et al. (1996) Mutants of Streptococcus suis types 1 and 2 impaired in expression of muramidase-released protein and extracellular protein induce disease in newborn germfree pigs. Infect Immun 64: 4409-4412.
287. Smith HE, Rijnsburger M, Stockhofe-Zurwieden N, Wisselink HJ, Vecht U, et al. (1997) Virulent strains of Streptococcus suis serotype 2 and highly virulent strains of Streptococcus suis serotype 1 can be recognized by a unique ribotype profile. J Clin Microbiol 35: 1049-1053.
288. Vecht U, Wisselink HJ, van Dijk JE, Smith HE (1992) Virulence of Streptococcus suis type 2 strains in newborn germfree pigs depends on phenotype. Infect Immun 60: 550-556.
289. Wu Z, Zhang W, Lu C (2008) Comparative proteome analysis of secreted proteins of Streptococcus suis serotype 9 isolates from diseased and healthy pigs. Microb Pathog 45: 159-166.
290. Ju CX, Gu HW, Lu CP (2012) Characterization and Functional Analysis of atl, a Novel Gene Encoding Autolysin in Streptococcus suis. Journal of Bacteriology 194: 1464-1473.
291. van der Vaart M, van Soest JJ, Spaink HP, Meijer AH (2013) Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. Dis Model Mech 6: 841-854.
292. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE (2001) Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood 98: 3087-3096.
293. van der Sar AM, Appelmelk BJ, Vandenbroucke-Grauls CM, Bitter W (2004) A star with stripes: zebrafish as an infection model. Trends Microbiol 12: 451-457.
294. van Soest JJ, Stockhammer OW, Ordas A, Bloemberg GV, Spaink HP, et al. (2011) Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen Edwardsiella tarda. BMC Immunol 12: 58.
295. Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, et al. (2012) Into the eye of the cytokine storm. Microbiol Mol Biol Rev 76: 16-32.
296. Bisno AL, Brito MO, Collins CM (2003) Molecular basis of group A streptococcal virulence. Lancet Infect Dis 3: 191-200.
297. Li YJ, Hu B (2012) Establishment of multi-site infection model in zebrafish larvae for studying Staphylococcus aureus infectious disease. J Genet Genomics 39: 521-534.
298. van der Sar AM, Stockhammer OW, van der Laan C, Spaink HP, Bitter W, et al. (2006) MyD88 innate immune function in a zebrafish embryo infection model. Infect Immun 74: 2436-2441.
299. Benard EL, van der Sar AM, Ellett F, Lieschke GJ, Spaink HP, et al. (2012) Infection of zebrafish embryos with intracellular bacterial pathogens. J Vis Exp.
300. Carvalho R, de Sonneville J, Stockhammer OW, Savage ND, Veneman WJ, et al. (2011) A high-throughput screen for tuberculosis progression. PLoS One 6: e16779.
301. Tang F, Bossers A, Harders F, Lu C, Smith H (2013) Comparative genomic analysis of twelve Streptococcus suis (pro)phages. Genomics 101: 336-344.
302. Fortier LC, Sekulovic O (2013) Importance of prophages to evolution and virulence of bacterial pathogens. Virulence 4: 354-365.
303. Groisman EA, Mouslim C (2006) Sensing by bacterial regulatory systems in host and non-host environments. Nat Rev Microbiol 4: 705-709.
304. Dominguez-Bernal G, Pucciarelli MG, Ramos-Morales F, Garcia-Quintanilla M, Cano DA, et al. (2004) Repression of the RcsC-YojN-RcsB phosphorelay by the IgaA protein is a requisite for Salmonella virulence. Mol Microbiol 53: 1437-1449.
305. Garcia-Calderon CB, Garcia-Quintanilla M, Casadesus J, Ramos-Morales F (2005) Virulence attenuation in Salmonella enterica rcsC mutants with constitutive activation of the Rcs system. Microbiology 151: 579-588.
306. Humphreys S, Rowley G, Stevenson A, Anjum MF, Woodward MJ, et al. (2004) Role of the two-component regulator CpxAR in the virulence of Salmonella enterica serotype Typhimurium. Infect Immun 72: 4654-4661.
307. Parashar V, Mirouze N, Dubnau DA, Neiditch MB (2011) Structural basis of response regulator

dephosphorylation by Rap phosphatases. PLoS Biol 9: e1000589.
308. Bem AE, Velikova N, Pellicer MT, Baarlen P, Marina A, et al. (2015) Bacterial histidine kinases as novel antibacterial drug targets. ACS Chem Biol 10: 213-224.
309. Velikova N, Bem AE, van Baarlen P, Wells JM, Marina A (2013) WalK, the Path towards New Antibacterials with Low Potential for Resistance Development. ACS Med Chem Lett 4: 891-894.
310. Marx P, Nuhn M, Kovacs M, Hakenbeck R, Bruckner R (2010) Identification of genes for small non-coding RNAs that belong to the regulon of the two-component regulatory system CiaRH in Streptococcus. BMC Genomics 11: 661.
311. Sun H, Wang Y (1995) Apoptosis of human leukemic HL-60 cells induced to differentiate by treatment with RA or DMSO[ast]. 5: 181-186.
312. Coordinators NR (2015) Database resources of the National Center for Biotechnology Information. Nucleic Acids Res 43: D6-17.
313. Bisicchia P, Bui NK, Aldridge C, Vollmer W, Devine KM (2011) Acquisition of VanB-type vancomycin resistance by Bacillus subtilis: the impact on gene expression, cell wall composition and morphology. Mol Microbiol 81: 157-178.
314. Jansen A, Turck M, Szekat C, Nagel M, Clever I, et al. (2007) Role of insertion elements and yycFG in the development of decreased susceptibility to vancomycin in Staphylococcus aureus. Int J Med Microbiol 297: 205-215.
315. Fridman M, Williams GD, Muzamal U, Hunter H, Siu KW, et al. (2013) Two unique phosphorylation-driven signaling pathways crosstalk in Staphylococcus aureus to modulate the cell-wall charge: Stk1/Stp1 meets GraSR. Biochemistry 52: 7975-7986.
316. Flannagan RS, Cosio G, Grinstein S (2009) Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol 7: 355-366.
317. Renshaw SA, Trede NS (2012) A model 450 million years in the making: zebrafish and vertebrate immunity. Dis Model Mech 5: 38-47.
318. Moxon ER, Kroll JS (1990) The role of bacterial polysaccharide capsules as virulence factors. Curr Top Microbiol Immunol 150: 65-85.
319. Iida K, Seki M, Saito M, Kawamura Y, Kajiwara H, et al. (2006) Capsule of Streptococcus pyogenes is essential for delayed death of mice in a model of streptococcal toxic shock syndrome. Microbiol Immunol 50: 127-130.
320. Brothers KM, Newman ZR, Wheeler RT (2011) Live imaging of disseminated candidiasis in zebrafish reveals role of phagocyte oxidase in limiting filamentous growth. Eukaryot Cell 10: 932-944.
321. Clifton-Hadley FA (1984) Studies of Streptococcus suis type 2 infection in pigs. Vet Res Commun 8: 217-227.
322. Zhao J, Pan S, Lin L, Fu L, Yang C, et al. (2015) Streptococcus suis serotype 2 strains can induce the formation of neutrophil extracellular traps and evade trapping. FEMS Microbiol Lett 362.
323. Babior BM, Curnutte JT, Kipnes RS (1975) Biological defense mechanisms. Evidence for the participation of superoxide in bacterial killing by xanthine oxidase. J Lab Clin Med 85: 235-244.
324. Rossi F, Zatti M (1964) Biochemical aspects of phagocytosis in polymorphonuclear leucocytes. NADH and NADPH oxidation by the granules of resting and phagocytizing cells. Experientia 20: 21-23.
325. Su Y, Yao W, Perez-Gutierrez ON, Smidt H, Zhu WY (2008) Changes in abundance of Lactobacillus spp. and Streptococcus suis in the stomach, jejunum and ileum of piglets after weaning. FEMS Microbiol Ecol 66: 546-555.
326. Liu Y, Burne RA (2009) Multiple two-component systems modulate alkali generation in Streptococcus gordonii in response to environmental stresses. J Bacteriol 191: 7353-7362.
327. Casiano-Colon A, Marquis RE (1988) Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl Environ Microbiol 54: 1318-1324.
328. Cunin R, Glansdorff N, Pierard A, Stalon V (1986) Biosynthesis and metabolism of arginine in bacteria. Microbiol Rev 50: 314-352.
329. Hui FM, Zhou L, Morrison DA (1995) Competence for genetic transformation in Streptococcus pneumoniae: organization of a regulatory locus with homology to two lactococcin A secretion genes. Gene 153: 25-31. 330. Khan R, Rukke HV, Ricomini Filho AP, Fimland G, Arntzen MO, et al. (2012) Extracellular identification of a processed type II ComR/ComS pheromone of Streptococcus mutans. J Bacteriol 194: 3781-3788.
331. Johnsborg O, Eldholm V, Bjornstad ML, Havarstein LS (2008) A predatory mechanism dramatically increases the efficiency of lateral gene transfer in Streptococcus pneumoniae and related commensal species. Mol Microbiol 69: 245-253.

332. Wei H, Havarstein LS (2012) Fratricide is essential for efficient gene transfer between pneumococci in biofilms. Appl Environ Microbiol 78: 5897-5905.
333. Layec S, Decaris B, Leblond-Bourget N (2008) Diversity of Firmicutes peptidoglycan hydrolases and specificities of those involved in daughter cell separation. Res Microbiol 159: 507-515.
334. Bateman A, Rawlings ND (2003) The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. Trends Biochem Sci 28: 234-237.
335. Ponting CP, Aravind L, Schultz J, Bork P, Koonin EV (1999) Eukaryotic signalling domain homologues in archaea and bacteria. Ancient ancestry and horizontal gene transfer. J Mol Biol 289: 729-745.
336. Bonifait L, Grignon L, Grenier D (2008) Fibrinogen induces biofilm formation by Streptococcus suis and enhances its antibiotic resistance. Appl Environ Microbiol 74: 4969-4972.
337. Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8: 623-633.
338. Claverys JP, Martin B, Polard P (2009) The genetic transformation machinery: composition, localization, and mechanism. FEMS Microbiol Rev 33: 643-656.
339. Mann JM, Carabetta VJ, Cristea IM, Dubnau D (2013) Complex formation and processing of the minor transformation pilins of Bacillus subtilis. Mol Microbiol 90: 1201-1215.
340. Balaban M, Battig P, Muschiol S, Tirier SM, Wartha F, et al. (2014) Secretion of a pneumococcal type II secretion system pilus correlates with DNA uptake during transformation. Proc Natl Acad Sci U S A 111: E758-765.
341. Puyet A, Greenberg B, Lacks SA (1990) Genetic and structural characterization of endA. A membrane-bound nuclease required for transformation of Streptococcus pneumoniae. J Mol Biol 213: 727-738.
342. Berge M, Moscoso M, Prudhomme M, Martin B, Claverys JP (2002) Uptake of transforming DNA in Gram-positive bacteria: a view from Streptococcus pneumoniae. Mol Microbiol 45: 411-421.
343. Luo P, Li H, Morrison DA (2004) Identification of ComW as a new component in the regulation of genetic transformation in Streptococcus pneumoniae. Mol Microbiol 54: 172-183.
344. Straume D, Stamsas GA, Havarstein LS (2015) Natural transformation and genome evolution in Streptococcus pneumoniae. Infect Genet Evol 33: 371-380.
345. Chewapreecha C, Marttinen P, Croucher NJ, Salter SJ, Harris SR, et al. (2014) Comprehensive identification of single nucleotide polymorphisms associated with beta-lactam resistance within pneumococcal mosaic genes. PLoS Genet 10: e1004547.
346. Tribble GD, Rigney TW, Dao DH, Wong CT, Kerr JE, et al. (2012) Natural competence is a major mechanism for horizontal DNA transfer in the oral pathogen Porphyromonas gingivalis. MBio 3.
347. Molin S, Tolker-Nielsen T (2003) Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr Opin Biotechnol 14: 255-261.
348. Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2001) Natural genetic transformation of Streptococcus mutans growing in biofilms. J Bacteriol 183: 897-908.
349. Dawei G, Liping W, Chengping L (2012) In vitro biofilm forming potential of Streptococcus suis isolated from human and swine in China. Braz J Microbiol 43: 993-1004.
350. Charpentier X, Polard P, Claverys JP (2012) Induction of competence for genetic transformation by antibiotics: convergent evolution of stress responses in distant bacterial species lacking SOS? Curr Opin Microbiol 15: 570-576.
351. Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP (2006) Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. Science 313: 89-92.
352. Alloing G, Martin B, Granadel C, Claverys JP (1998) Development of competence in Streptococcus pneumonaie: pheromone autoinduction and control of quorum sensing by the oligopeptide permease. Mol Microbiol 29: 75-83.
353. Hathaway LJ, Battig P, Reber S, Rotzetter JU, Aebi S, et al. (2014) Streptococcus pneumoniae detects and responds to foreign bacterial peptide fragments in its environment. Open Biol 4: 130224.
354. Li M, Shen X, Yan J, Han H, Zheng B, et al. (2011) GI-type T4SS-mediated horizontal transfer of the 89K pathogenicity island in epidemic Streptococcus suis serotype 2. Mol Microbiol 79: 1670-1683.
355. Zhu SR, Wang ZG, Chen EF, Yang TT, Zhang Z, et al. (2008) [The emergence of candidate pathogenicity island 89K DNA sequence of Streptococcus suis isolated from sporadic patients in Zhejiang province]. Zhonghua Liu Xing Bing Xue Za Zhi 29: 1110-1113.
356. Berthelot-Herault F, Gottschalk M, Morvan H, Kobisch M (2005) Dilemma of virulence of Streptococcus suis: Canadian isolate 89-1591 characterized as a virulent strain using a standardized experimental model in pigs. Can J Vet Res 69: 236-240.

Summary and Acknowledgements

Summary

Streptococcus suis is Gram-positive bacterium and its natural habitat is the upper respiratory tract of pigs, and in particular the tonsils and nasal cavity. *S. suis* is one of the most important swine pathogens world-wide, causing a wide variety of diseases in pigs including septicemia, arthritis, endocarditis, and meningitis that leads often to a rapid death within 1-2 days. Human infections are considered to be the consequence of occupational exposure to infected pigs or pork and are characterized by a similar symptomatology as in pigs. In the last decade the number of human cases has increased and isolates with multi-drug resistance genes have been isolated. Despite the economic losses in pig production caused by *S. suis* and its importance as emerging zoonotic agent, the mechanisms of virulence and pathogenesis remain poorly understood. Research on these topics has been hampered by the lack of efficient methods for genetic transformation and the lack of a simple, cost-effective model to investigate *S. suis* virulence.

In some streptococcal species, genetic transformation can be carried out very efficiently as these species can be experimentally induced to take-up and recombine homologous extracellular DNA. The discovery of natural competence in some streptococci and the potential of opening up new avenues for genetic analysis of *S. suis,* was the motivation for investigating natural competence in this important pathogen.

In **Chapter 2** we showed that a peptide pheromone induces competence in *S. suis*. The induction was dependent on ComX, a sigma factor that controls the streptococcal late competence regulon; the SigX-inducing peptide (XIP); and ComR, a regulator of *comX*. XIP was identified as an N-terminally truncated variant of ComS. This discovery resulted in the development of an efficient and inducible transformation method that will greatly facilitate the application of genetic approaches to study *S. suis*.

In **Chapter 3** we investigated the genetic regulation of competence in *S. suis* and provided a hypothetical model of the *S. suis* transformasome. We verified the essential role of the *S. suis* major pilin, and CinA for efficient competence development, supporting the notion that our predicted multi-protein transformasome indeed appears to function as described for other streptococci. We have also characterised the metabolic states linked to competence development and competence exit (**Chapter 4**).

In **Chapter 5** we investigated for the first time the use a zebrafish larvae model to assess the relative virulence of *S. suis* strains in porcine infections. Because of its convenience and cost-effectiveness, this model may be used to assay virulence of environmental *S. suis* strains, in particularly those of clinical relevance to infection of pigs and humans. Furthermore, a large number of bacterial mutants and strains can be screened for their virulence and *in vivo* pathogenicity, opening up new avenues to

investigate the so far undiscovered pathways mediating successful host infection by *S. suis*.

In **Chapter 6** we applied these two innovative methods (the competence system and the zebrafish larval model) to characterize two different two-component systems (TCS) of *S. suis*. TCS are important players in the regulation of bacterial adaptation to changes in environmental conditions, including those encountered in the host during infection. We showed that two previously uncharacterised TCS of *S. suis* 2 strain S10 play a role in virulence *in vivo* and investigated their role in resistance to stresses encountered in the host during infection.

Chapter 7 summarizes and discusses the key results and the future prospective of the thesis research.

Samenvatting

Streptococcus suis is een Gram-positieve bacterie die de bovenste luchtwegen van varkens, en dan met name de amandelen en neusholte, als natuurlijke habitat gebruikt. Hoewel deze bacterie als een standaard lid van het microbioom van volwassen varkens wordt beschouwd kan deze ernstige ziektes veroorzaken in varkens en de mens. *S. suis* is in feite één van de belangrijkste mondiale pathogenen van het varken en veroorzaakt een divers aantal ziekten in het varken, waaronder bloedvergiftiging, arthritis, endocarditis en hersenvliesontsteking die meestal tot een snelle dood leidt, binnen 1 tot 2 dagen. Alhoewel de meeste infecties van de mens beschouwd worden als het gevolg van blootstelling tijdens werkzaamheden, is het aantal ziektegevallen bij de mens gedurende de laatste jaren toegenomen, en er zijn verschillende isolaten met multi-resistentiegenen geïsoleerd. Humane infecties die door *S. suis* worden veroorzaakt worden gekenmerkt door eenzelfde symptoom-ontwikkeling als bij varkens. Ondanks de financiële verliezen in de varkensindustrie ten gevolge van *S. suis* infecties en het belang van deze bacterie als opkomende zoönotische bacterie, zijn experimentele studies van de virulentie en pathologie van *S. suis* gehinderd door het ontbreken van efficiënte methodes tot genetische transformatie, en het ontbreken van een eenvoudig kostenefficiënt model om de virulentie van *S. suis* vast te stellen.

Genetische transformatie kan in een aantal streptococcus soorten heel efficiënt worden uitgevoerd aangezien het in deze soorten mogelijk is om het opnemen van extracellulair DNA, en het inbouwen hiervan in homologe delen van het genoom, experimenteel te induceren. De ontdekking van natuurlijke competentie in een aantal streptococcen en de mogelijkheid om nieuwe manieren te ontdekken ter genetische analyse van *S. suis* was de drijfveer om natuurlijke competentie in dit belangrijke pathogeen te onderzoeken.

In **Hoofdstuk 2** laten we zien dat een eiwit feromoon competentie induceert in *S. suis*. De inductie was afhankelijk van ComX, een sigma-factor die het streptococcus “late-competentie” regulon beheert; het SigX-inducerende eiwit (XIP); en ComR, een regulator van *comX*. XIP bleek een variant van ComS te zijn welke aan het N-uiteinde afgebroken was. Dit heeft geleid tot het ontwikkelen van een nieuwe methode welke een breed scala aan onderzoeksgroepen in staat zal stellen om sneller nieuwe kenmerken van de ecologie en pathologie van *S. suis* te ontdekken, in het bijzonder op het gebied van virulentie.

In **Hoofdstuk 3** onderzochten we de genetische regeling van competentie in *S. suis* en presenteren we een hypothetisch model van het *S. suis* “transformasoom”. We verifiëerden de essentiële rol van het belangrijkste pilin eiwit van *S. suis*, en de noodzaak van het CinA eiwit om efficiënte competentie ontwikkeling te verkrijgen, ter ondersteuning van de opvatting dat ons hypothetische multi-eiwit transformasoom model, dat eerder werd beschreven voor andere streptococcen, inderdaad functioneel blijkt te zijn. We hebben ook de onderscheidende metabole

toestanden die competentie mogelijk maken beschreven, naast de metabole toestand die betrokken is bij het afsluiten van competentie (**Hoofdstuk 4**).

In **Hoofdstuk 5** hebben we voor de eerste keer onderzocht of een zebravis-larve model gebruikt kon worden om de relatieve virulentie van *S. suis* isolaten voor infectie van varkens vastgesteld kon worden. Vanuit het oogpunt van gemak en kosten efficiëntie is dit model bruikbaar om de virulentie van omgevingsisolaten van *S. suis* vast te stellen, in het bijzonder die isolaten welke clinisch relevant zijn, gelet op infectie van varkens en mensen. Verder kunnen grote aantallen bacterie mutanten en wild-type isolaten gescreend worden op virulentie en *in vivo* ziekteverwekkend vermogen, zodat nieuwe methodes gebruikt kunnen worden om de tot dusver onbekende routes die succesvolle infectie van gastheren door *S. suis* mogelijk maken, te vinden.

In **Hoofdstuk 6** hebben we deze twee vernieuwende methodes, zowel het competentie systeem als het zebravis-larve model, toegepast om het belang van twee verschillende twee-component-systemen (TCS) van *S. suis* in virulentie te omschrijven. TCS zijn belangrijke onderdelen in de regeling van bacteriële aanpassing aan veranderingen in de omgeving, waaronder diegene welke in de gastheer een rol spelen tijdens infectie. In dit onderzoek hebben we de rol van twee TCS van *S. suis* serotype 2 strain S10 in het ziekteverwekkend vermogen en de overleving van de bacteriën in het bloed en gastheerweefsel onderzocht.

Hoofdstuk 7 vat de hoofdresultaten van het promotie-onderzoek samen en bediscussiëert zowel deze resultaten als de toekomstperspectieven voor verder onderzoek in navolging van dit promotie-onderzoek.

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Thanks to all my students, **Jery, Jori, Simon, Rui, Ingrid, Koen** and **Jasper.** I am quite sure we had a good time together and I hope I could transfer you at least part of my curiosity and friendly approach to science. I am also glad that already 3 of them

are Ph.D. candidates, Simon and Jori joined the HMI recently. Take a good care of the group!

I was extremely happy to find out that Wageningen is very international and extremely easy going. I think it is a special place. You might have noticed a lot of people complain dramatically about the city and the city-life. Nothing to say about that, no much is happening here but still, most of them miss Wageningen and, mostly, miss the people living in Wageningen (or Bennekom). I will. I am already missing friends that left, when finally I will leave I will miss you all.
Danilo, Alberto, Domenico, Brian and **Bruno** for the fantastic FIFA nights, the unforgettable nights in the International Club ("look at his leg, he cannot help, last one and we go, che cinghiale di macchia!" will sound in my ear so often). And how can I forget the beautiful beers in De Zaaier with **Dimitri, Joao, Elsa, Simon, Erika** (you still owe me something from there...), **Vale B.B., Vale B.E., Stefano, Sourya, Lavinia, Walter**. The time in Droevendaal, the dinners and the soccer-table tournaments!
I have wonderful memories of the time spent together with one of the sweetest person I have ever met in my life, **Eva**.

Danilo, Gerald...the moving/cleaning we had to do? It was F-A-N-T-A-S-T-I-C!

As I love being home, I must spend special words for the ones who managed to live with me, **Dimitri, Alvaro**, my sista **Inma, Danilo, Alberto**, the tall **Dimitri** (Bambino!), Martin(o), Thibaut, Raffaele and Alexia . I like every one of you! Different reasons, different feelings all positive no worries! I wish I could have enjoyed more special moments we spent together (from "ciccio" to fantastic mandolin songs, from wiping out the vegetation of the garden to Bolognese, from "u Ba'" to Family guy...). **Tibó**, now it is almost 4 years we know each other, among dinners, whiskeys, cakes and games we had quite a good time, eh? Daje **Raffa**, you didn't trust me a bit at the beginning, but you see? Everything turned out to be good. Now I know how to over-use oil and still make everything tasty. For **Alexia** I should spent many words, she managed to convinced me to clean my house and my life. Even if it doesn't look like, I think we are quite similar in some peculiar aspects of our personality. Love you much Hun!

A personal paragraph has to be dedicated to my two Paranimf but I guess I also have to feel in with nice words, eh? Alright...**Costas**! Papi Costas, Costí! With your tsipouro, your Mexican glass, your St. Pauli box and the Dolce and Gabbana glasses (and the Ic! Berlin one!)...Grande, Costas!
Marcela, what can I say? We should speak at the same microphone during our defences, as we have been always confronting, helping and supporting each other. We always end up in screaming and laughing so much, my Ph.D. would have been

millions of times more boring (maybe faster though :p). Keep going, keep working hard and you also know that you will be a great scientist! I am not sure you will be able to get rid of me ;)

Imogen May Morris, I love you!

About the Author

Training Activity

Conferences and meeting

Scientific Spring Meeting NVMM & NVvM, Arnhem, the Netherlands 2011
Scientific Spring Meeting NVMM & NVvM, Arnhem, the Netherlands 2012 (Oral presentation)
Scientific Spring Meeting NVMM & NVvM, Arnhem, the Netherlands 2014 (Oral presentation)
Cross Talk Milano's Workshop, Milan, Italy 2011 (Poster presentation) XVIII Lancefield International Symposium, Palermo, Italy, 2011 XIX Lancefield International Symposium, Buenos Aires, Argentina 2014 (Oral presentation)
Antimicrobial Drug Conference Madrid, Spain, 2013 (Poster presentation)
WIAS Science day, Wageningen, the Netherlands, 2012 (Poster presentation)
WIAS Science day, Wageningen, the Netherlands, 2013 (Oral presentation)
WIAS Science day, Wageningen, the Netherlands, 2014
STARS 1st Network Meeting, Valencia, Spain, 2010 (Oral presentation)
STARS 2nd Network Meeting, Amsterdam, the Netherlands, 2011 (Oral presentation)
STARS 3rd Network Meeting, Siena, Italy, 2011 (Oral presentation)
STARS 4th Network Meeting, Riga, Latvia, 2012 (Oral presentation)
3rd symposium and workshop on microbial pathogen Rotterdam, the Netherlands, 2011(Oral presentation)
Platform Moleculaire Genetica, Lunteren, the Netherlands 2013
Platform Moleculaire Genetica, Lunteren, the Netherlands 2014 (Oral presentation)

Courses

WIAS Course Ethics and Philosophy in Life Sciences, Wageningen, 2011
Advanced visualisation integration and biological interpretation of -omics data, Wageningen 2011
Genetics and physiology of food-associated micro-organism, Wageningen 2013

Spring School 'Host-Microbe Interactomics, Wageningen 2014
Infection meets Immunity, summer school, Utrecht, 2014
Use of Laboratory Animals (FELASA requirements, level C), Utrecht ,2011
Course Techniques for Scientific Writing and presenting scientific paper, Riga, Latvia, 2012
Project and Time Management, Wageningen, the Netherlands, 2011
Information Literacy including EndNote, Wageningen, the Netherlands, 2012

Basic Introduction Course on SPSS Erasmus MC, Rotterdam, the Netherlands, 2013
External training period, LAMMB, University of Siena, Siena, Italy (two months) 2012

Others

Host-Microbes Interactomics practical course 2010/2011/2012
Supervision of 7 thesis (3 MSc major, 1 MSc minor, 3 BSc thesis)

Curriculum Vitae

Edoardo Zaccaria was born on August the 6th 1984, in Rome, Italy. After completing elementary and middle school, he started the Second Level College of Science at the Farnesina High School, where he had his first contact with what he thought was, proper science. After few years of biological studies, he understood how he could free the world from the atrocious full-of-seeds-watermelons and the horrific hard-skin-and-full-of-seeds-grapes using transgenic crops and recombinant DNA technology. After obtaining his diploma, he started a bachelor program in Biotechnology at La Sapienza. Soon he realized GMO technology improved way faster than his knowledge and his fruit-battle was already won by somebody else. He decided to fall back on the development of cellular and molecular methods for the study of pathogenesis of acute secondary otiti. However, this was not enough and he enrolled in the Master program of Industrial Biotechnology. Here he joined the plant and molecular biotechnology laboratory at the Ente Nazionale per l'Energia e l'Ambiente (ENEA, research centre Casaccia), where he studied the use of plants as biofactories for vaccine production. In particular, he focused on the expression and purification of the HIV-1 protein Nef fused to a single chain antibody in *Nicotiana benthamiana*.

From October 2010, Edoardo was appointed as a Ph.D. candidate at the Host-Microbes Interactomics group at Wageningen University under the supervision of Prof Jerry Wells and Dr Peter van Baarlen. Here he helped fighting the very dangerous life-threatening bacteria *Streptococcus suis*. During his Ph.D period he was part of the EU FP7-funded Marie Curie ITN STARS (Scientific Training in Antimicrobial Research Strategy) with immense joy.

He is an avid traveller and a gastronomy passionate (he thinks money should be only spend on meals or train tickets). Since 2011 he is happily living in Bennekom (his second hometown :)), stuffing himself with blueberries and rarely eating grapes and watermelon.

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