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Chapter 8

General Discussion
Discussion

1 Identification of virulence factors

Streptococcus suis is a major cause of infections in piglets, leading to meningitis, synovitis and septicemia (Staats et al., 1997). Due to costs of treatment, prevention, and loss of animals S. suis infections lead to economical losses in the pig industry. Control of the disease is hampered by lack of protective vaccines and sufficient, and sensitive diagnostic tools. To overcome these shortages, insight into the pathogenesis of S. suis infections is required. Until now, little is known of the pathogenesis of these infections and only a limited number of virulence factors of S. suis have been identified (see Figure 1 of Chapter 1). In addition, although several studies indicate that the pathogenicity of S. suis is host dependent (Vecht et al., 1997), host-associated factors important for the pathogenic process are unknown. We focused on the identification and characterization of potential new virulence factors of S. suis and their role in the pathogenesis. In order to study the role of virulence factors in the pathogenesis of S. suis infections, several experimental infection models in pigs as well as in mice have been described (Vecht et al., 1997). The data obtained from the various models clearly showed that strains pathogenic in piglets appeared less pathogenic in mice, and vice versa. This indicates, that virulence of S. suis strains is host dependent and that experimental infections have to be performed in the natural host to study the virulence of S. suis strains. In order to detect differences in virulence between the many S. suis strains, large numbers of pigs are required. For ethical reasons this is unacceptable. To circumvent this problem, we used a competitive challenge infection model, in which piglets were infected with a mixture of wild-type and mutant strain in a 1:1 ratio. An upshift in the ratio between wild-type and mutant bacteria reisolated from affected organs of piglets, indicates that the mutant is less virulent. A pre-requisite for the use of this infection model is that both strains used can be easily discriminated, e.g. by differences in their antibiotic resistance. Competitive challenge experiments have been successfully used to determine the virulence of Streptococcus agalactiae (Jones et al., 2000), Actinobacillus pleuropneumoniae (Fuller et al., 2000) and Salmonella typhimurium mutants (Shea et al., 1999). Therefore this model is also suitable to study the interaction between pathogen and host, and to study the role of virulence factors in the pathogenesis of S. suis infections.

To identify new virulence factors, several strategies were used. In Chapter 2, a subtractive phage display technique was described, while in Chapters 4, 5, 6, and 7 environmentally regulated promoters of S. suis were studied. Since the subtractive phage
display failed due to technical problems as described in Chapter 3, only the outcome of the second strategy will be discussed in more detail. Assuming that any function that is up- or downregulated in the host contributes to the fitness of a pathogen within a host and thus is a virulence factor, putative virulence factors were identified by selecting environmentally regulated promoters of *S. suis* (Smith *et al.*, 2001). With this “*in vivo* expression technology”, IVET-strategy, we successfully selected a number of known and unknown virulence factors. Previously, we showed that expression ofextracellular factor (EF) is associated with virulence of *S. suis* serotype 2 strains (Vecht *et al.*, 1991), and EF was selected with the IVET-system. In Chapter 5, we described the characterization of a fibronectin- and fibrinogen-binding protein of *S. suis* (FBPS), a new virulence factor of *S. suis* that was identified by IVET. FBPS was clearly shown to be involved in virulence since the isogenic knock-out mutant of the *fbps*-gene was attenuated in colonizing the organs specific for an *S. suis* infection. The isogenic mutant of the regulator of virulence of *S. suis* (*RevS*) was also attenuated in colonizing the *S. suis*-specific organs (Chapter 7). Therefore, *RevS* is a new virulence factor of *S. suis*. Besides the work described in this thesis, two other proteins selected with the IVET were described to be virulence factors for other streptococci: NADH-oxidase of *Streptococcus pneumoniae* (Yu *et al.*, 2001), and an ABC-transporter of *S. pneumoniae* (Brown *et al.*, 2001).

Summarizing, the IVET-strategy has been a successful approach for the identification of virulence factors of *S. suis*. However, additional virulence factors may be identified with other mechanisms, since virulence factors that are constitutively expressed are not selected with the IVET-system. Now that 99.9% of the genome sequence of a pathogenic *S. suis* serotype 2 strain has become available in the database, this information can be used to identify these constitutively expressed new virulence factors. The genome sequence makes genomic and proteomic approaches for the identification of virulence factors feasible. Since it is generally accepted that nearly all bacterial virulence factors are located on the bacterial surface or are secreted (Finlay and Falkow, 1997), the selection of surface located proteins from the genome sequence can yield many virulence factors. Besides, surface localized proteins are very attractive vaccine candidates. Therefore, the availability of new tools such as genomics and proteomics may lead to the identification of more virulence factors of *S. suis* and potential vaccine candidates. Similar strategies has lead to the identification of new vaccine candidates for *Neisseria meningitidis* (Pizza *et al.*, 2000).
2 Pathogenesis of *Streptococcus suis*

Five major events can be recognized in the pathogenesis of *S. suis*: (1) colonization of epithelia, (2) invasion of the epithelia, (3) systemic migration, (4) invasion and colonization of the organs specifically involved in an *S. suis* infection (synovia, meninges, pericardium, and to a lesser extent pleura and peritoneum), and (5) replication inside specific organs (see also Figure 1 in Chapter 1). The isogenic knock-out mutants of FBPS, RevS and LspS colonized the tonsils of piglets as efficiently as the wild-type strain, and were all capable of reaching the deeper organs (Chapters 5, 6, and 7). Therefore, these factors are obviously not involved in colonization and invasion of the epithelia during an *S. suis* infection. The role of FBPS, LspS, and proteins regulated by RevS in the systemic migration in blood was studied in an *in vitro* model by determining the survival of the isogenic mutants in whole blood of piglets. No differences in survival were found for any of the mutant strains compared to the wild-type strain (Chapter 7, and unpublished data). Although *in vitro* the host defense mechanisms present in blood cannot be mimicked completely, these findings indicate that FBPS, LspS, and proteins regulated by RevS are not involved in systemic migration. The FBPS-mutant strain and the RevS mutant strain were clearly attenuated in colonizing the organs specific for an *S. suis* infection (meninges, synovia, pericardium and serosae). Since FBPS and RevS play a role in the colonization of the target organs or the replication inside the target organs, the genes may affect adhesion and/or invasion or the growth inside the organs. A role for FBPS in the adhesion to and the invasion of endothelial cells can easily be envisaged. Fibronectin- and fibrinogen-binding proteins of several streptococci are involved in adhesion and invasion to epithelial and endothelial cells (reviewed by Joh *et al.*, 1999). The attenuation of RevS is most likely an effect of one or more of the proteins regulated by RevS. These proteins, very likely to be novel virulence factors of *S. suis*, are involved in the entry into the target organs or in the replication inside the target organ.

In the competitive challenge experiments described in chapter 5, 6, and 7, one strain is often dominantly present in each organ. This was clearly observed for the LspS-mutant, which is not attenuated. Only a limited number of affected organs is colonized by both wild-type and mutant strains (Chapter 6). Therefore, we conclude that the colonization of the target organs is a limiting and critical step in the pathogenesis of *S. suis* infections. Once the bacteria passed this barrier they multiply extensively. Similar organ barriers were observed during *Haemophilus influenzae* infections by Moxon and Murphy (1978). This organ barrier was also found in the *in vivo* selection of environmentally regulated genes with the IVET-system.
Eighty percent of the affected organs was colonized by only one specific clone, and therefore only a limited number of clones was selected, independent of the inocula ranging from $5 \times 10^5$ - $5 \times 10^8$.

Summarizing the data from the literature (Chapter 1) and from this thesis, we conclude that we identified virulence factors and the site of infections involved, but that much is to be learned. The mechanisms by which \textit{S. suis} colonizes the epithelia, invades these epithelia and migrates systemically, and the virulence factors involved in these processes are largely unknown. Additional strategies for the identification of virulence factors are required to fill in these gaps in our knowledge of the pathogenesis of \textit{S. suis} infections.

3 Prevention and control of \textit{S. suis}

A promising prospect for prevention of disease due to \textit{S. suis} infections in pigs is vaccination. However, until now, only very limited vaccines are available, based on formalin-killed whole cells. Commercially available vaccines are all derived from one serotype of \textit{S. suis}. No cross-protective activity against other serotypes is expected from these vaccines. Consequently, their use will be limited. Since the burden of disease is by many different serotypes, a vaccine that protects against multiple serotypes is required.

In this thesis a new, very attractive vaccine candidate has been identified. FBPS is immunogenic in piglets, and is present in all but three of 35 serotypes of \textit{S. suis} (Chapter 4). These three serotypes are very distinct from the other serotypes, and are hardly ever isolated from diseased animals in the field. In addition, we showed that FBPS is a virulence factor, since knock-out mutants of FBPS are attenuated in colonizing the specific organs during an \textit{S. suis} infection. The protection by vaccination with FBPS against \textit{S. suis} infections remains to be determined, but FBP54, a protein of \textit{S. pyogenes} that is very homologous to FBPS confers protection against \textit{S. pyogenes} infections in mice (Kawabata \textit{et al.}, 2001). Besides FBPS, one or more of the proteins regulated by RevS can be interesting vaccine candidates as well. We argued RevS is a virulence factor involved in the colonization of the specific organs, and therefore the proteins regulated by RevS are likely to be virulence factors as well.

Protein based vaccines offer several advantages. Since proteins are T-cell dependent antigens, they are likely to be highly immunogenic in piglets, and elicit immunologically memory (Paton, 1998). The costs of protein based vaccines can be relatively low, since the production is easy nowadays with all the cloning and expression systems and advanced purification systems. Alternatively, \textit{S. suis} vaccines can be based on capsular polysaccharide structures, since capsule is a virulence factor of \textit{S. suis} (Smith \textit{et al.}, 1999). Capsule
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Polysaccharide vaccines have been developed for other encapsulated bacteria. Unfortunately, at low age the response to polysaccharide antigens is limited due to immaturity of the immune system. In addition, polysaccharides are T-cell independent antigens not inducing immunological memory. Similar problems can be expected for *S. suis* capsular vaccines in piglets. By conjugation of polysaccharides to proteins, the polysaccharides become T-cell dependent antigens, eliciting high antibodies at low age and immunological memory (reviewed by Rappuoli, 2001). However, introduction of a conjugated pneumococcal vaccine in the population increased the carriage of serotypes not present in the vaccine. Such an effect was not seen with the *Haemophilus influenzae* type b vaccine (reviewed by Lipsitch, 1999). Besides, the conjugated vaccine is very expensive, which makes the approach of conjugation unsuitable for usage in the veterinary industry.

It was suggested that the risk of escape mutants after introduction of a vaccine based on (conjugated) polysaccharides in the population, is not negligible. Coffey *et al.* (1998) have shown that recombinational exchanges at the capsular polysaccharide locus of *S. pneumoniae* lead to frequent serotype changes among natural isolates of *S. pneumoniae*. It is not known yet whether *S. suis* has a natural competence to take up DNA. However, among the environmental regulated clones several genes have been identified that show homology to genes involved in competence of streptococci (Chapter 4), suggesting that *S. suis* possesses genes necessary for natural competence. Based on these considerations, a *S. suis* vaccine is preferably a protein-based vaccine. To limit selection of vaccine induced escape mutants, and to protect against as many serotypes as possible, it is desirable to include more than one protein in a vaccine.

FBPS is a good vaccine candidate since it is immunogenic in young pigs, is present in all but three serotypes of *S. suis*, and is a virulence factor of *S. suis*. Besides, FBP54, a protein of *S. pyogenes* that is very homologous to FBPS confers protection against *S. pyogenes* infections in mice (Kawabata *et al.*, 2001). MRP and EF can be added as vaccine candidates since these proteins confer protection against *S. suis* serotype 2 strains in piglets (Wisselink *et al.*, 2001). A high percentage of European serotype 1, 1/2, and 14 strains expresses MRP and EF, and more than 80 per cent of the serotype 9 strains produce an enlarged form of MRP (Wisselink *et al.*, 2000).

Another potential vaccine candidate is suilysin. It has recently been shown that suilysin is a virulence factor for *S. suis*, since isogenic knock-out mutants of suilysin are attenuated in piglets (Allen *et al.*, 2001). Suilysin is very homologous to the hemolysin of *S. pneumoniae*, named pneumolysin (AlonsoDeValasco *et al.*, 1995). Pneumolysin as a vaccine
elicits some protection against *S. pneumoniae* (AlonsoDeValasaco *et al.*, 1995). When pneumolysin is combined with another protective protein, PspA, these proteins together elicit better protection against *S. pneumoniae* than either one alone (Briles *et al.*, 2001). Jacobs *et al.* (1995) showed that purified suilysin protects against *S. suis* serotype 2 challenge. However, a substantial number of isolates recovered from diseased pigs lack suilysin (Segers *et al.*, 1998). Whether suilysin in combination with other *S. suis* proteins also displays a synergistic effect on protection against *S. suis* infections remains to be determined.

In *S. pneumoniae*, components of ABC-transporters involved in iron-uptake are reported to protect mice against systemic *S. pneumoniae* infection (Brown *et al.*, 2001). Several *S. suis* ABC-transporters were selected with the IVET approach (see Tables 1A and 1B of chapter 1). As described in Chapter 3, these ABC-transporters are present in all *S. suis* strains, and therefore these transporters are of interest to study as subunits for a vaccine.

The mentioned vaccine candidates are all virulence factors. Such a mixture containing several virulence factors can target more than one of the sequential events that occur in the pathogenesis of *S. suis* infections. FBPS and the target proteins of RevS are good candidates to target the last two stages of the pathogenic process. Suilysin is a vaccine candidate that might target one of the first steps, invasion of the epithelium. However, the colonization of *S. suis* on the tonsil, and the systemic migration of *S. suis* cannot be targeted with the available candidates. Additional vaccine candidates are required to target all sequential steps of the pathogenesis.

In addition to vaccines, sensitive diagnostics are required to control the problems of *S. suis* in the swine industry. In Chapters 4 and 7, we describe that RevS is only present in virulent phenotypes of serotype 1 and 2 strains. RevS can be detected in virulent and weakly virulent serotype 2 strains, and in virulent and highly virulent serotype 1 strains, but not in avirulent serotype 2 strains. Therefore, RevS can be used to improve the available diagnostic methods that are unable to specifically detect virulent serotype 1 strains. This could easily be done by developing a sensitive and specific PCR assay based on the presence of *revS*.

### 4 Concluding remarks

Two novel virulence factors of *S. suis* have been identified, RevS and FBPS. Both proteins can contribute to control and prevention of *S. suis* infections in the field. RevS can be used to improve the current diagnostic tools. A PCR-assay based on the *revs*-gene, can be used to detect virulent phenotypes among serotype 1 and 2 strains. FBPS is very attractive as a vaccine candidate to include in a subunit vaccine, although FBPS alone will probably not
The ideal subunit vaccine contains more than one virulence factor, to target as many steps of the pathogenesis as possible. To identify other virulence factors that can be used as vaccine candidates, the remaining environmentally regulated genes can be studied. However, now that 99.9% of the genome of *S. suis* is available from the database, we propose to apply a genomics approach for the identification of additional virulence factors. An approach similar to that described for *N. meningitidis*, where all surface located and excreted proteins were tested for their potential as vaccine candidates, will most likely lead to the identification of novel virulence factors for *S. suis*. Besides, it is possible to search the proteome of *S. suis* for proteins that are homologous to virulence factors of other organisms. These virulence factors can help to elucidate the "black holes" that still exist in the pathogenesis of *S. suis*, like the colonization on the tonsil and the systemic migration.

References


