RESEARCH REPORT
SWINE HEALTH

Title: Development of an Avirulent Vaccine Strain of *Streptococcus suis*
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I. Abstract

The gene encoding the hemolysin of *Streptococcus suis*, suilysin, has been cloned and a deletion derivative of the gene created which lacks hemolytic activity. The mutant form of the gene was then tagged with an spectinomycin resistance marker. The inactivated gene was cloned into a Staphylococcus-based shuttle plasmid and introduced into *Staphylococcus hyicus*. For introduction of the deleted allele of the suilysin gene into *S. suis*, a co-mobilization system was set up. The *S. hyicus* donor strain was mixed with a virulent type 2 strain of *S. suis*. The bacterial mixture was collected on 0.45□m nitrocellulose filters which were then overlaid onto blood agar plates. Following incubation to allow the mating to occur between the donor and recipient strains, the cells were harvested and plated on media containing spectinomycin. Antibiotic resistant and non-hemolytic colonies have been obtained. These strains will be examined to ensure that the inactivation of the suilysin gene is correct. Now that this mutation system has been shown to work, we will repeat the process with a heavy metal resistance marker in place of the spectinomycin resistance cassette. The resulting suilysin-negative mutant will then be evaluated for virulence properties and, if attenuated, will be tested as a possible vaccine strain.

II. Introduction

Streptococcus suis is an important pathogen of swine as the causal agent of arthritis, meningitis, pneumonia, septicemia, endocarditis, encephalitis, polyserositis, abortions, and abscesses (reviewed in 1). *S. suis* has been shown to cause disease outbreaks in pigs of all ages, but it primarily affects nursery pigs. In the acute form of the

disease, it is not unusual for death to occur without apparent clinical signs. Without treatment, mortality from *S. suis* infections approaches 20% (2). There are substantial economic losses associated with infections with this pathogen annually in the United States. Thirty-five capsular serotypes of *S. suis* have been identified. Type 2 is the serotype most commonly associated with disease and also the one which is most frequently isolated (3). *S. suis* type 2 is usually transmitted nasally or orally and colonizes the palatine tonsils of both clinically ill and apparently healthy pigs. Carriers of *S. suis* type 2 constitute a source of infection of other pigs. Control of *S. suis* disease by vaccines and antimicrobials has been generally ineffective. Clinical isolates of this bacterium are frequently resistant to multiple antibiotics including tetracyclines, penicillin, clindamycin, tilmicosin, erythromycin, and the aminoglycosides (1). Animals which are treated with antibiotics to which the organism is susceptible *in vitro* rarely recover fully (1,4). Furthermore, antibiotics used for medicated early weaning techniques are ineffective in eliminating the tonsillar carrier state of *S. suis* in pigs. Commercial vaccines against *S. suis* are available, but their efficacy is poor. This is due in large part to a paucity of information regarding the protective antigens and virulence factors of this bacterium. Therefore, current measures for control of this bacterial pathogen are inadequate. As additional antibiotic resistance is acquired by this organism, prevention of infections and treatment of infected animals will become increasingly more difficult.

Virulence of *S. suis* for pigs varies markedly both between serotypes and also among serotype 2 isolates (5-7). The features of *S. suis* which define its virulence are just now becoming recognized. We have developed a system which allows isolates of *S. suis* to be classified on the basis of virulence (7). Characterization of the highly virulent strains relative to the isolates exhibiting reduced virulence has revealed a requirement for the production of the hemolytic toxin (suilysin) for complete expression of virulence. Other virulence factors have been identified by other investigators, including extracellular protein factor (EF) and muramidase-released protein (MRP) (8,9). Our studies demonstrated that there is an absolute dependence on suilysin production for the bacteria to be highly virulent in pigs (10). The highly virulent strains also produce EF and MRP, although these proteins are also present in strains of reduced virulence. Thus suilysin is the first essential virulence factor described for this bacterium.

III. **Objectives.**

The gene for suilysin has been cloned and its DNA sequence determined (11). Thus it is now possible to apply recombinant DNA techniques to create a strain of *S. suis* which is reduced in virulence but which allows for the production of an immune response to the virulence-enhancing hemolysin protein. The nature of *S. suis* infections dictates that mucosal immunity is necessary for full immunity to be achieved. This necessitates the development of a live, avirulent vaccine rather than parenteral administration of a bacterin or purified protein vaccine which would generate a good humoral, but not mucosal, immune response. The goal of this research is to create a serotype 2 *S. suis* strain which is derived from a fully virulent isolate but with an inactivated suilysin gene (*sly*). Recombinant DNA procedures would be utilized to inactivate the suilysin gene to reduce the virulence properties of the strain, but to retain production of the inactivated protein so as to allow for

the development of antibodies to this important virulence protein. Thus an antigenically intact, but functionally inactive suilysin protein will be produced by the constructed *S. suis* strain. Following creation of this Sly^- strain, it will be introduced intranasally into pigs to determine its 1) relative virulence, 2) its effectiveness in eliciting mucosal and humoral antibodies in treated animals, and 3) its relative protection against subsequent challenge by a highly virulent *S. suis* isolate.

IV. **Procedures**

Creation of suilysin nonhemolytic mutations.

The suilysin gene has been amplified from *S. suis* chromosomal DNA by polymerase chain reaction (PCR) and cloned into a plasmid vector. A deletion derivative of the *sly* gene was created by restriction endonuclease digestion. The protein product of the deletion lacks 136 of the 497 amino acids of the suilysin protein. However, the protein sequence on either side of the site of the deletion remains the normal sequence. Deletion mutants are advantageous because they are stable mutations, which cannot genetically revert back to produce an active protein.

Introduction of the mutant suilysin determinant into *S. suis*.

To create an *S. suis* strain with an inactivated suilysin gene, we began with a highly virulent, suilysin-producing strain. We then introduced our inactivated suilysin gene. To accomplish this, we genetically tagged the inactivated gene with a spectinomycin resistance marker. As a back-up, the intact suilysin determinant was digested with HpaI, which is a unique site 660 bp into the suilysin open reading frame. The spectinomycin resistance cassette was inserted into this site, physically disrupting the suilysin open reading frame. The resistance tagged, inactivated suilysin gene was subcloned onto a shuttle plasmid capable of replication in both *E. coli* and staphylococci. The recombinant plasmid was then isolated from the *E. coli* host strain and introduced into a temperature-sensitive mutant of *Staphylococcus hyicus*. This strain harbors a conjugation-proficient plasmid which is capable of mobilizing the vector plasmid bearing the inactivated suilysin determinant. The *S. hyicus* strain harboring both the conjugation plasmid as well as the suilysin containing plasmid was used as a donor strain in filter mating experiments to transfer the inactivated suilysin allele into *S. suis*. Following incubation to allow the mating to occur, the bacteria were harvested from the filters and plated on yeast-extract supplemented Todd Hewitt broth containing 100 mg/ml of spectinomycin. The plating was carried out at 39□C, to inhibit growth of the *S. hyicus* temperature-sensitive donor strain. Spectinomycin-resistant *S. suis* were then patched onto sheep blood agar plates to check their hemolytic phenotype.

V. **Results**

1. Creation of nonhemolytic mutant forms of the suilysin gene.

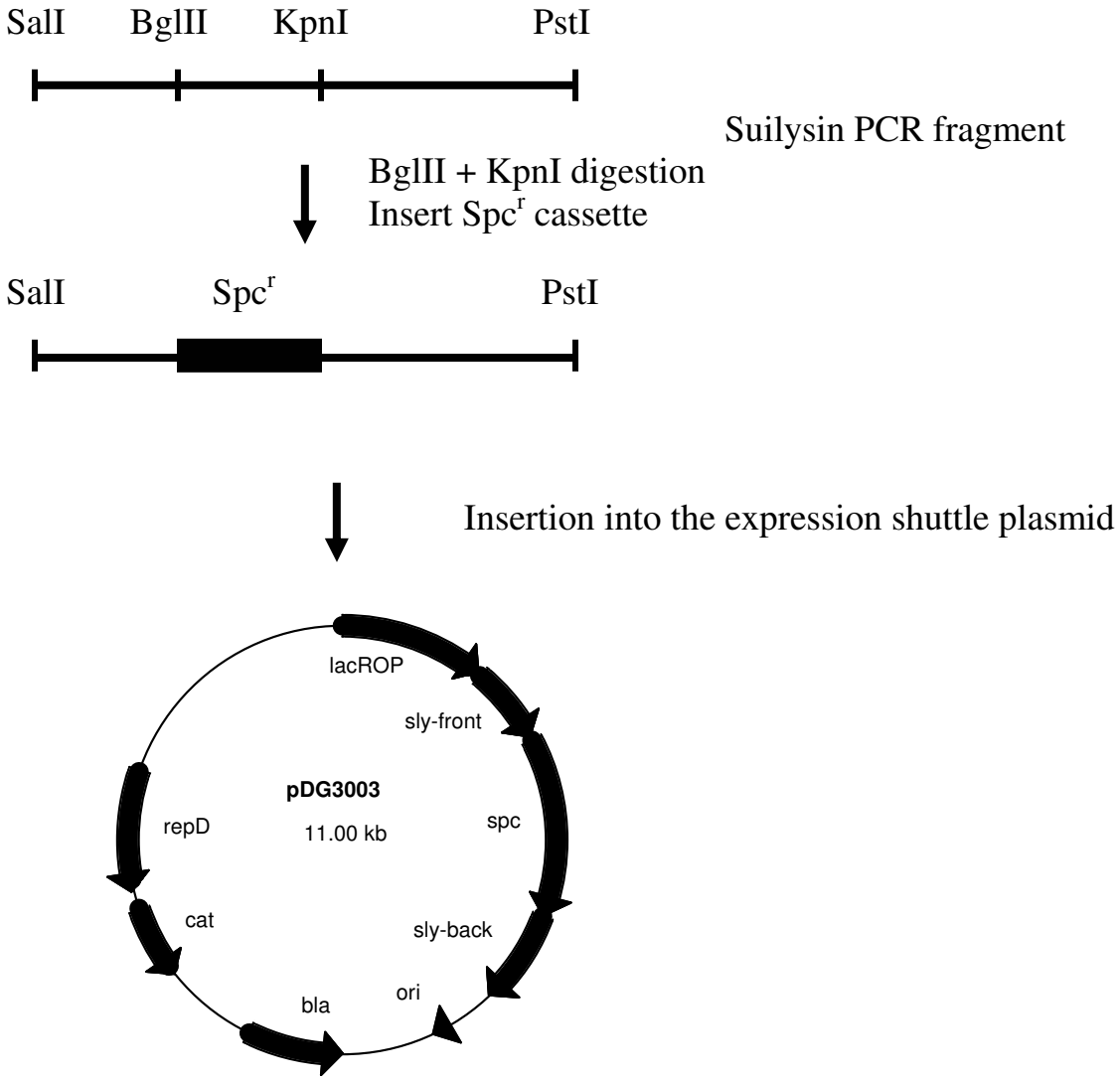
The cloned suilysin gene was digested with a pair of restriction endonucleases, BglII and KpnI, which cleave at single sites within the suilysin gene. As a result, a 408 bp sequence from within the gene was deleted, producing a truncated version of suilysin which is 136 amino acids shorter than the 497 amino acid wild-type protein. The amino acid sequences at the beginning of the toxin are normal. This shortened version of the toxin no longer has hemolytic activity, but should retain some of the antigenicity of the original toxin.

2. Creation of a shuttle plasmid vector for introduction of genes into *S. suis*.

We have created an expression plasmid which allows genes to be expressed using regulatory signals from the *Staphylococcus aureus* lactose operon. Using this plasmid, we have expressed the cloned suilysin determinant and confirmed that the cloned gene retains hemolytic activity. Furthermore, this system has been utilized to demonstrate that the deletion derivative of the suilysin gene produces a non-hemolytic product. To create a shuttle vector by which the expression plasmid can be stably introduced into the *Staphylococcus hyicus* donor strain, a shuttle plasmid was created by inserting a staphylococcal plasmid into the expression plasmid. The plasmid, pC221, confers replication functions and a chloramphenicol resistance gene for selection. This shuttle plasmid was utilized to develop a process to introduce DNA into *S. suis*.

3. Validation of the *S. suis* DNA exchange system.

The procedure we utilized to introduce DNA into *S. suis* is a conjugation based plasmid co-mobilization system. The plasmid to be introduced into *S. suis* is first introduced into a temperature-sensitive strain of *S. hyicus* by a highly efficient electroporation procedure. This strain also harbors a conjugation-proficient plasmid. When this staphylococcal strain is mixed with the *S. suis* recipient strain, the conjugation proficient plasmid promotes its own entry into the recipient strain. The pC221-based shuttle plasmid is co-mobilized into the recipient. To determine the feasibility of this genetic system, we incorporated a transposon onto the shuttle plasmid and monitored the introduction of the transposon=s antibiotic resistance into the *S. suis* recipient. Co-mobilization occurred at a frequency of 10^{-3}, which provides adequate numbers for the proposed experiments involving the inactivated suilysin determinant.


SalI
BglII
KpnI
PstI
Suilysin PCR fragment
BglII + KpnI digestion
Insert Spc^r cassette
SalI
Spc^r
PstI
Insertion into the expression shuttle plasmid
lacROP
sly-front
pDG3003
11.00 kb
repD
spc
cat
sly-back
ori
bla

4. Inactivation of the *S. suis* suilysin determinant.

The mating experiments to introduce the inactivated suilysin allele into *S.* suis have been carried out several times, but the frequency of recombination of the inactivated suilysin allele into the streptococcal chromosome appears to be very low. We have carried out a series of experiments varying the donor to recipient cell ratios and changing the nature of the selective medium used to obtain the transconjugant *S. suis* colonies. As a result of the fine tuning of the experimental protocol, we have recently been able to isolate spectinomycin-resistant colonies which appear to be non-hemolytic.

Experiments in progress include southern blot-DNA hybridization studies to verify that the suilysin gene has been inactivated by a genetic exchange between the chromosomal copy of the suilysin determinant and the inactivated allele introduced on the plasmid. When the strain has been verified, we will repeat the process with a suilysin allele which carries a heavy metal resistance cassette, rather than one encoding an antibiotic resistance marker. This will be to avoid introduction of additional antibiotic resistance markers into this pathogen. We will then assay the suilysin-negative mutant for virulence in pigs. If the mutant is attenuated for virulence, we will proceed to evaluate its potential as a vaccine candidate strain.

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