RESEARCH REPORT
SWINE HEALTH

Title: Bacteriocins as potential alternative therapeutic agents for the control and prevention of *Streptococcus suis* infections in pigs - **NPB #04-005**

Investigator: Daniel Grenier, Ph.D.

Institution: Université Laval, Québec, Canada

II. Abstract: The swine industry is of significant importance in the North American economy. *Streptococcus suis* serotype 2, a Gram positive bacterium colonizing the upper respiratory tract of the pig, is responsible for many cases of septicaemias, meningitis and endocarditis in pig herds. Treatments by antibiotics can be effective to control *S. suis* infections if they are administrated early. However, the literature indicates the frequent isolation of *S. suis* strains resistant to penicillin and to other antibiotics often used. Therefore, studies aiming to develop alternative methods for the prevention and control of *S. suis* infections are essential. In this project, we investigated the ability of *S. suis* to produce bacteriocins, which are antibacterial molecules of proteinaceous nature. The non-pathogenic strain *S. suis* 94-623 was found to produce an antibacterial substance having the characteristics of a classical bacteriocin, including low molecular mass, resistance to heat, and susceptibility to proteolytic enzymes. In addition to be active on pathogenic isolates of *S. suis*, the bacteriocin also showed the capacity to inhibit growth of other Gram positive and Gram negative bacterial species isolated from swine. The addition of yeast extract to the culture medium significantly increased the production of the bacteriocin by *S. suis* 94-623. A purification protocol was designed and allowed to recover a fraction enriched in bacteriocin activity. Analysis of this fraction by electrophoresis on polyacrylamide gel suggested that the bacteriocin 94-623 possesses a molecular mass of 4-5 kDa. The logical extension of the project will be to evaluate the potential of a theraby based on bacterial interference, in which piglets would be inoculated with the bacteriocin-producing *S. suis* strain. This bacteria, once established, would thus confer protection against colonization by pathogenic isolates of *S. suis*.

III. Introduction: The swine industry is of significant importance in the North American economy. Only in Canada, it was estimated that the economic losses caused by bacterial infections represent approximately 80 million dollars per annum. *Streptococcus suis*, a Gram positive bacterium colonizing the higher respiratory tract of pigs, is regarded as an important pathogen being responsible for numerous cases of septicaemias, meningitis and endocarditis. Among the 35 known serotypes, *S. suis* serotype 2 is most often associated with infections. Antibiotics, more particularly penicillin, can be effective for treating *S. suis* infections if they are given early. However, the recent literature reports the frequent isolation of *S. suis* strains resistant to penicillin and to other antibiotics such as tetracycline and clindamycin. Since the use of antibiotics in the swine industry raises more and more fears in regard to the apparition of bacterial resistances, which can ultimately affect humans, studies aiming to develop alternative methods for the prevention and control of *S. suis* infections are essential. Vaccination is well known as an efficient procedure to protect animals and humans against a number of viral and bacterial infections. Until now, most vaccines tested to protect pigs against *S. suis* infections have used inactivated bacteria and the results have been inconsistent. Possible explanations for vaccine failure may be related to loss of antigenicity of the bacteria caused by the heat or formalin processing, weak immunogenicity of the capsulated bacteria, existence of a large number of capsular types, or production of antibodies to antigens not associated with virulence factors.

Bacterial interference, which refers to the capacity of a microorganism (probiotic agent) to protect the host against certain pathogens, is considered by several groups of investigators as a reliable alternative treatment and preventive regimen to antibiotics in the future. The production of antibacterial substances, called bacteriocins, is a mechanism often used by the protective species to eliminate pathogens. Bacteriocins are bacteriocidal substances of proteinaceous nature produced by certain bacteria and being able to show a spectrum of inhibition either narrow (active against the same species) or broad (active against various species). The producer strain itself exhibits a specific immunity to its bacteriocin. The potential of a human therapy based on bacterial interference using streptococci or lactobacilli species has been previously reported in the case of recurrent acute otitis media as well as infections of the intestinal and urogenital tracts. A review of the literature indicated that there was no data available concerning the production of bacteriocins by *S. suis*. Considering the high therapeutic potential of bacteriocins and bacteriocin-producing bacteria for preventing and treating infections in pigs, we proposed to the National Pork Board a research project on this aspect.

IV. Objectives: The long term objective of our research program is to develop alternative methods of prevention and control of *S. suis* infections. The proposed research was aimed to characterize the bacteriocin produced by *S. suis* 94-623 and to obtain evidences supporting the potential for using the purified bacteriocin or the bacteriocin-producing strain *S. suis*.

V. Materials and Methods: A variety of well-recognized microbiological and biochemical methods were used. The methods for detection and quantification of bacteriocin activity were adapted from previously published methodologies. In regard to the purification of the bacteriocin, various methods were used including hydrophobic, anionic, and cationic chromatographies, ultrafiltration, acidic precipitation, and preparative electrophoresis.

VI. Results: While screening *S. suis* strains for their capacity to produce antibacterial substances, five out of 40 strains were found to be positive. An interesting finding was the fact that strains producing antibacterial substances were those categorized as non-pathogenic. The strain *S. suis* 90-1330 was found to be highly productive and was selected for pursuing the project. When a

culture supernatant of *S. suis* 94-623 was added to an active culture of the pathogenic strain *S. suis* S735, the growth stopped immediately (Figure 1).

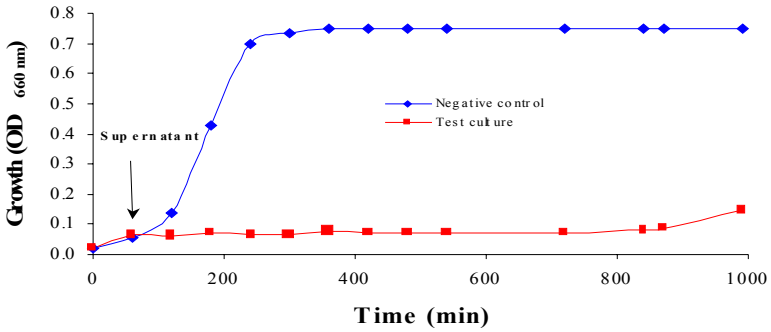

Figure 1. Growth inhibition of *S. suis* S735 following the addition of a culture supernatant of *S. suis* 94-623, which contains the antibacterial substance.

All pathogenic isolates of *S. suis* tested were inhibited by *S. suis* 94-623. Analysis of the inhibitory spectra revealed that the antibacterial substance produced by *S. suis* 94-623 also inhibited the growth of *Actinobacillus minor, Actinobacillus porcinus, Enterococcus durans, Streptococcus agalactiae, Streptococcus dysgalactiae* subsp. *dysgalactiae, Streptococcus equi* subsp. *zooepidemicus,* and *S. dysgalactiae* subsp. *equisimilis*.

The antibacterial substance was found to be dialyzable, heat resistant, and protease sensitive. Based on those characterictics, the antibacterial substance produced by *S. suis* 94-623 represents a classical bacteriocin. We tested whether the susceptible bacterial strain *S. suis* 24 could become resistant to the bacteriocin produced by *S. suis* 94-623. This was done by cultivating *S. suis* 24 in the presence of increasing concentrations of the bacteriocin-containing culture supernatant of *S. suis* 94-623. It appears that the sensitive strain could not acquire a capacity to resist to the bacteriocin.

We evaluated the influence of the nature and concentration of the carbon and nitrogen sources on bacteriocin production by *S. suis* 94-623. Over 60 different conditions were tested. Our results indicated that bacteriocin production was optimal in a minimal culture medium supplemented with 1% glucose, 2% proteose-peptone, and 2% yeast extract. This medium will be used for preparing a culture supernatant that will be used for the purification.

We also improved significantly the sensitivity of our method for detection of bacteriocin activity. Briefly, culture plates are inoculated with a standardized inoculum (10^8 cells) of the pathogenic strain *S. suis* 24 serotype 2 used as the susceptible bacteria. Penicylinders are placed on the surface of the culture media and 25 µl of two-fold serial dilutions of the fractions are applied in the penicylinders. Following incubation of 24 h to allow bacterial growth of the target bacteria, bacteriocin activity is expressed in arbitrary units (AU), which correspond to the reciprocal of the highest dilution giving a clear inhibitory zone against *S. suis* 24. Using potassium biphthalate-HCl at pH 3 as the dilution solution, we are able to detect 640 AU per ml of *S. suis* 94-623 culture supernatant. This is a significant improvement in sensitivity compared to our previous method which allowed to detect 20 AU/ml. This method will offer an interesting advantage for screening fractions during the purification process.

The next step was to design a purification protocol for bacteriocin 94-623. The protocol so far developed allowed to obtain a fraction significantly enriched in bacteriocin activity. Proteins present in a culture supernatant of *S. suis* 94-623 are first precipitated with ammonium sulfate at 20% saturation. Following centrifugation, ammonium sulfate is further added to the resulting supernatant to obtain a 45% saturation. The solution is centrifuged and proteins in the pellet are solubilized in water and dialyzed using a membrane with a molecular weight cut-off of 6-8 kDa. As summarized in table 1, these steps yielded a purification factor of 11.1 with a recovery of 22%. When this fraction was assayed in our diffusion assay, a strong bacteriocin activity toward *S. suis* 24 was demonstrated (Figure 2). Analysis of this fraction by electrophoresis on polyacrylamide gel showed that it contains few protein bands, including one with bacteriocin activity and having a molecular mass in the range of 4-5 kDa (Figure 3).

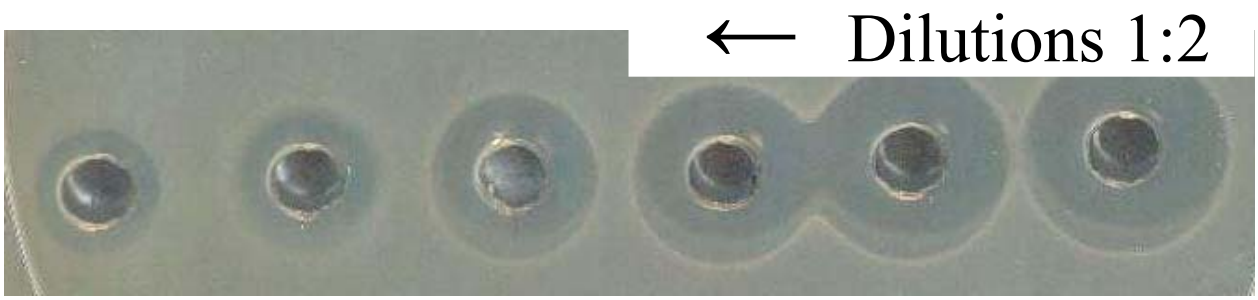

Figure 2. Bacteriocin activity of the enriched fraction prepared from *S. suis* 94-623. Serial dilutions of the final fraction were tested using our method of diffusion in penicylinder and the pathogenic strain *S. suis* 24 as the susceptible bacteria.

Table 1. Stepwise purification of bacteriocin 94-623.

Fractions of *S. suis* 94-623	Volume (ml)	Total proteins (mg)	Total activity (AU)	Specific activity (AU/mg)	Purification factor	Yield (%)
Culture supernatant	100	2103,20	32000	15,21	1,0	100
Supernatant 20% $(NH_4)_2SO_4$	125	1580,00	10000	6,33	0,4	31,25
Pellet 45% $(NH_4)_2SO_4$	10	46,36	6400	138,05	9,1	20
Retentate 6-8 kDa dialysis	11	41,55	7040	169,45	11,1	22

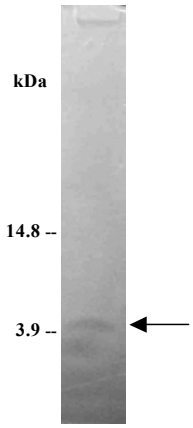

Figure 2. Analysis of the enriched bacteriocin fraction by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A protein band, with a molecular mass in the range of 4-5 kDa, showed bacteriocin activity.

In order to improve our purification yield and the purity of the bacteriocin 94-623, our enriched fraction was subjected to additional steps of purification by chromatography (cationic, anionic, and

hydrophobic), ultrafiltration, precipitation at acidic pH, and preparative electrophoresis. Unfortunately, none of these assays appeared to be applicable to our bacteriocin.

In the eventuality that we obtain additional financial support, the enriched fraction will be used as starting material for purification by FPLC. As soon as a pure fraction will be obtained, it will be sequenced to determine its amino acid sequence and establish its degree of similarity with bacteriocins purified from other bacterial species.

VII. Discussion: Bacterial resistances to antibiotics constitute a growing problem for the porcine industry. Our research project was aimed to explore new avenues for the treatment and the prevention of *S. suis* infections in pigs. The results obtained in this project are entirely original. We clearly showed that the non-pathogenic strain *S. suis* 94-623 was able to inhibit growth of pathogenic isolates of *S. suis*. The antibacterial substance produced by *S. suis* 94-623 possesses the characteristics of a classical bacteriocin. Athough our results clearly support the potential of the bacteriocin or bacteriocin-producing *S. suis* 94-623 for preventing and treating infections in pigs, additional studies are required.

VIII. Lay interpretation: The swine industry is of significant importance in the North American economy. *Streptococcus suis* serotype 2, a bacterium colonizing the upper respiratory tract of the pig, is responsible for many cases of septicaemias, meningitis and endocarditis in pig herds. Treatments by antibiotics can be effective to control *S. suis* infections if they are administrated early. However, the literature indicates the frequent isolation of *S. suis* strains resistant to penicillin. Therefore, studies aiming to develop alternative methods for the prevention and control of *S. suis* infections are essential. In this project, we investigated the ability of *S. suis* to produce antibacterial substances, called bacteriocins. Our results indicated that the non-pathogenic isolate *S. suis* 94-623 produces a bacteriocin having a low molecular and a great stability to heat and pH. The conditions for optimal production of the bacteriocin by *S. suis* 94-623 were determined and a partially purified fraction of the bacteriocin was obtained. The logical extension of the project will be to evaluate the potential of a theraby based on bacterial interference, in which piglets would be inoculated with the bacteriocin-producing non-pathogenic *S. suis* strain. Once established, this bacterium would confer protection against colonization by pathogenic isolates of *S. suis*, thus having a positive impact on the animal and public health.

For additional information please contact: Dr. Daniel Grenier, Groupe de recherche en écologie buccale, Faculté de médecine dentaire, Université Laval, Québec, Canada G1K 7P4. E-mail : daniel.grenier@greb.ulaval.ca