Treponema spp. in necrotic skin ulcers in pigs

Phenotypic and genetic features

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Abstract

Treponema belongs to the phylum *Spirochaetes* consisting of bacteria distinguishable by a unique cell architecture that enables corkscrew-like motility. In two case studies, treponemes were isolated from ear necrosis and shoulder ulcers, two types of skin lesions that have impact on animal welfare and may cause economic losses.

This doctoral thesis focuses on a hypothesis that *Treponema* spp. have a pathogenic role in the progression of ear necrosis and shoulder ulcers in pigs and, as *Treponema* spp. are present in gingiva of pigs, that transmission to skin is mediated by biting or licking. The thesis describes their occurrence, phenotypic and genetic features.

We sampled 109 pigs with lesions and 60 apparently healthy piglets. Spirochetes were present in 73% of the shoulder ulcers, in 53% of the ear necroses and in 9.7% of the gingivae.

Many identified phylotypes were similar to *Treponema* spp. considered as pathogens in bovine digital dermatitis, a claw disease in cattle. Similar phylotypes were present both in gingiva and ulcers. There were indications of transmission between gingiva and ulcers.

Twelve isolates were acquired and identified as *T. pedis*, *T. parvum* and *T.* sp. OMZ840-like. Metabolic patterns were similar to those of other treponemes and were not discriminatory between isolates. All except two gingival isolates showed unique DNA fingerprints. *Treponema* sp. OMZ840-like and *T. pedis* isolates were hemolytic, *T. parvum* was not. The isolates were generally susceptible to the tested antimicrobials.

The genome sequence of *T. pedis* strain T A4 from ear necrosis was *de novo* assembled and analyzed. The genome was most similar to that of *T. denticola,* a species strongly associated with human periodontitis. Several of the predicted genes in *T. pedis* were homologous to virulence related genes in *T. denticola*, including those encoding the major surface sheath protein, dentilisin and dentipain.

In *T. denticola*, IdeT is a protein that includes the oligopeptidase domain dentipain. An IdeT-homologue in *T. pedis* strain T A4, TPE0673, was recombinantly expressed in *Escherichia coli* and purified for *in vitro* testing. TPE0673 did not display protease activity towards immunoglobulin G (IgG) or insulin as its homologues. However, western blot analysis showed that IgG in serum from sows with shoulder ulcers bound to purified TPE0673, suggesting an immunogenic property of this gene.

In conclusion, this thesis describes an association between *Treponema* spp. with ear necrosis and shoulder ulcers. The treponemes present in pig ulcers were phylogenetically similar to those of oral origin and transmission between gingiva and ulcer was indicated. Finally, analysis of the genome of *T. pedis* revealed the presence of several putative virulence genes indicating a pathogenic potential for this species.

Keywords: Treponema pedis, Treponema parvum, Treponema sp. OMZ840, Pigs, Ear necrosis, Shoulder ulcers, Spirochetes

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Treponema-arter i nekrotiska hudsår hos grisar - Populärvetenskaplig sammanfattning

Spiroketer är en typ av bakterier med en utmärkande form. Många bakteriesläkten beskrivs till utseendet som stavar eller kocker, spiroketer däremot liknar en korkskruv. Arvsmassan hos spiroketer är så särskiljande att de bildar en egen gren i det evolutionära släktträdet. Man tror också att alla arter inom spiroketerna har sitt ursprung från samma spiroket-förfader.

Spiroketer kan förflytta sig i fasta miljöer, såsom i agar och i vävnad, genom en skruvande rörelse skapad av roterande proteinstrukturer i cellens hölje.

Redan i början av 1900-talet fann man bakterier med form och rörlighet som spiroketer i sår hos grisar. Vid den tiden hade man inte tillgång till dagens moderna teknik för att kunna karaktärisera bakterierna. Sedan dess har endast ett fåtal studier undersökt sambandet mellan spiroketer och hudsår hos grisar. Spiroketer kan orsaka en mängd sjukdomar hos människa och djur, till exempel syfilis, borrelia, tandköttsinflammation och smittsamt klövexem hos nötkreatur.

Vetskapen om spiroketers betydelse vid andra sjukdomar ledde till att vår forskargrupp genomförde pilotstudier på två typer av nekrotiska sår hos grisar – öronnekros och bogbladssår. Pilotstudierna visade att spiroketer av arten *Treponema pedis* kunde isoleras från öronnekros, bogbladssår samt munslemhinna hos gris. *Treponema pedis* är en av de arter som är associerad till smittsamt klövexem hos nöt. Man började misstänka att *T. pedis* och möjligtvis andra *Treponema*-arter förekommer i munnen hos grisar och (genom bitning) kunde spridas till hud där de får betydelse för sjukdomsutvecklingen av öronnekros och bogbladssår vilket också är avhandlingens hypotes.

Öronnekroser uppkommer hos grisar nyligen avvanda från suggan. Såren är vanligtvis lokaliserade på nederkanten av örat men kan också förekomma på toppen. De kan variera mellan små sår, ibland täckta av en skorpa, till stora som omfattar hela örat. Bogbladssår kan uppkomma hos suggor under diperioden. Såren bildas på grisens bog där huden blir utsatt av trycket från den liggande suggan. Det börjar med en rodnad och hårlossning som kan övergå i ett bogbladssår på flera centimeter i diameter. Under våra studier provtogs ett flertal sår som hade en diameter större än 5 centimeter. Både öronnekros och bogbladssår påverkar djurvälfärden negativt och kan leda till ekonomiska förluster hos grisproducenter.

Öronnekros har ansetts bero på bitskador men kan uppkomma plötsligt och spridas likt en smittsam sjukdom. Den specifika lokaliseringen talar också emot att det endast är en bitskada av icke infektiös natur. För bogbladssår är hull, liggtid och vissa typer av golv

riskfaktorer men när såren väl bildas ger de ett intryck av att vara infekterade med död vävnad i kanterna. Det finns dock inte någon bestämd mikrobiologisk orsak till att öronnekros och bogbladssår uppstår eller förvärras.

Avhandlingen bygger på forskargruppens studier av spiroketer som tillhör släktet *Treponema* från öronnekros och bogbladssår hos grisar. Totalt har 18 svenska besättningar besökts där 109 grisar med sår provtagits samt ytterligare munprover tagits från 60 griskultingar. I 73 % av bogbladssåren, 53 % av öronnekroserna och 10 % av munslemhinnorna kunde förekomst av spiroketer påvisas. Förutom *T. pedis* förekom minst tre andra typer av *Treponema* som kan vara betydelsefulla.

Bland de olika typer av *Treponema* som identifierades förekom likadana såväl i mun som i sår. Det fanns även indikationer på spridning mellan mun och sår vilket stödjer hypotesen i avhandlingen. Tolv isolat erhölls av arterna *T. pedis, T. parvum* och en annan som liknar en icke beskriven *Treponema*-art isolerad från plack på tänderna hos hund. Isolaten karaktäriserades genom att enzymatiska aktiviteter testades. *Treponema pedis* och isolat av den icke beskrivna arten visade hemolys på blodagar vilket *T. parvum* inte gjorde. Alla isolat var känsliga för antibiotikum. Den kompletta arvsmassan för *T. pedis* sattes samman, kartlades och analyserades. Vi kunde fastslå att *T. pedis* bär på flera gener som har hög likhet med sjukdomsframkallande gener i *T. denticola* som är en art som orsakar tandköttsinflammationer hos människa. Även om den exakta funktionen för dessa gener i *T. pedis* behöver testas experimentellt, tyder det på att en sjukdomsframkallande potential finns. En av generna studerades vidare genom att proteinet den kodar för framställdes och renades fram. Det visade sig att serum från suggor med bogbladssår innehöll antikroppar mot proteinet som den kodar för vilket tyder på att de blivit infekterade av *T. pedis*.

Treponemors roll i nekrotiska sår hos grisar är ännu inte fullständigt kartlagd. De resultat som läggs fram i avhandlingen ger stöd för hypotesen om att *Treponema*-arter i grisens mun sprids via bitning till hud där de har betydelse för utvecklingen av nekrotiska sår.

Dedication

To bacon and ham, my favourite animals.

Homer J. Simpson

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Karlsson F, Svartström O, Belak K, Fellström C, Pringle M (2013). Occurrence of *Treponema* spp. in porcine skin ulcers and gingiva. *Vet Microbiol* 165(3-4), 402-409.

II Svartström O, Karlsson F, Fellström C, Pringle M (2013). Characterization of *Treponema* spp. isolates from pigs with ear necrosis and shoulder ulcers. *Vet Microbiol* 166(3-4), 617-623.

III Svartström O, Mushtaq M, Pringle M, Segerman B (2013). Genome-Wide Relatedness of *Treponema pedis*, from Gingiva and Necrotic Skin Lesions of Pigs, with the Human Oral Pathogen *Treponema denticola*. *PLoS One* 8(8), e71281.

IV Svartström O, Johnzon C-F, Zuerner R, Rosander A. Characterization of TPE0673, a *Treponema denticola* IdeT homologue, in *Treponema pedis*. Manuscript in preparation.

The contributions to the papers included in this thesis was as follows:

I Frida Karlsson and Olov Svartström performed the experiments, analyzed the results and wrote the manuscript under supervision by Märit Pringle and Claes Fellström. Katinka Belák performed the histopathological analyses.

II Olov Svartström and Frida Karlsson performed the experiments, analyzed the results and wrote the manuscript under supervision by Claes Fellström and Märit Pringle.

III Olov Svartström and Memoona Mushtaq performed the experiments and analyzed the results under supervision by Bo Segerman. Olov Svartström and Bo Segerman wrote the manuscript. Märit Pringle and Memoona Mushtaq revised the manuscript critically and contributed with intellectual input.

IV Olov Svartström and Anna Rosander wrote the manuscript. Carl-Fredrik Johnzon, Olov Svartström and Anna Rosander performed the experiments and analyzed the results. Richard Zuerner and Anna Rosander planned the study.

Abbreviations

BANA	N-α-benzoyl-DL-arginine-2-naphthylamide
BDD	Bovine digital dermatitis
bp	Base pairs
FISH	Fluorescent *in situ* hybridization
IgG	Immunoglobulin G
ISR2	intergenic spacer region 2
Msp	Major surface sheath protein
NGS	Next generation sequencing technology
PFGE	Pulsed-field gel-electrophoresis
RAPD	Randomly amplified polymorphic DNA
rRNA	Ribosomal RNA
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

1 Introduction

1.1 Pig production and pig welfare in Sweden and the European Union

Meat consumption is increasing globally. The average person in most countries in the European Union consumes more than 80 kg of meat-products intended as food per year. In Sweden, pork constitutes approximately 45% of the meat consumed (Jordbruksverket, 2013). Intensification of meat production implies an increased stress on the animals that may enhance disease. To ensure animal welfare and health, pig management is regulated by legislation (Jordbruksverket, 2011). In addition to compliance with these regulations, promoting animal health may also have an impact on productivity for pig-farmers. Minimization of diseases, e.g. skin lesions, that have negative impacts on profit, animal welfare and consumers' attitude towards the pig industry is therefore of great importance.

1.2 Infectious skin diseases of pigs

The specific functions of the skin involve temperature control and protection against the environment. Infectious bacteria will meet several obstacles when invading the skin. The first is the outer layer of epidermis consisting of degenerated cells stuffed with the protein keratin. As long as this layer remains intact it prevents passing of bacteria. It even prevents passing of water. Proliferating cells from deeper layers in the epidermis continuously replace the cells of the outer layer. This prevents excessive colonization of bacteria on the skin surface. The epidermis is connected to the underlying dermis by a dermo-epidermal junction layer. The dermis is connective tissue made of fibroblasts and the molecules that these cells produce, including collagen fibers and hyaluronic acid. Hyaluronic acid and other molecules form

a gel that prevents invading microorganisms from passing further. In addition, immune cells, e.g. neutrophils, macrophages and lymphocytes, are present in the skin ready to fight invading microorganisms (Dyce *et al.*, 1987). An ulcer is defined as an area where complete interruption of the epidermal layer has occurred. The protecting features of intact skin are disturbed in an ulcer, thereby making it more susceptible for infections.

In pig herds, various skin lesions and ulcers can be seen. Superficial lesions caused from fights or self-inflicted traumas are examples of non-infectious skin lesions. However, the damage of the skin may lead to infections caused by microorganisms such as bacteria. For example, certain *Staphylococcus hyicus* strains may infect the skin and cause a disease characterized by greasy exudates named exudative epidermitis (Zimmerman *et al.*, 2012).

Ear necrosis (Figure 1) is a name commonly used for skin lesions appearing at the lower margin or at the tip of the ear in pigs. The lesions may spread in a contagious manner causing outbreaks in a pen or herd. Ear necrosis usually occurs after weaning where affected pigs are of age 5 to 10 weeks (Zimmerman *et al.*, 2012; Harcourt, 1973). Although the general condition of the pig rarely is affected, necrosis may spread and, in rare cases, aggravate to loss of the entire ear (Pringle *et al.*, 2009). In addition, ear necrosis limits the possibility of selling affected pigs.

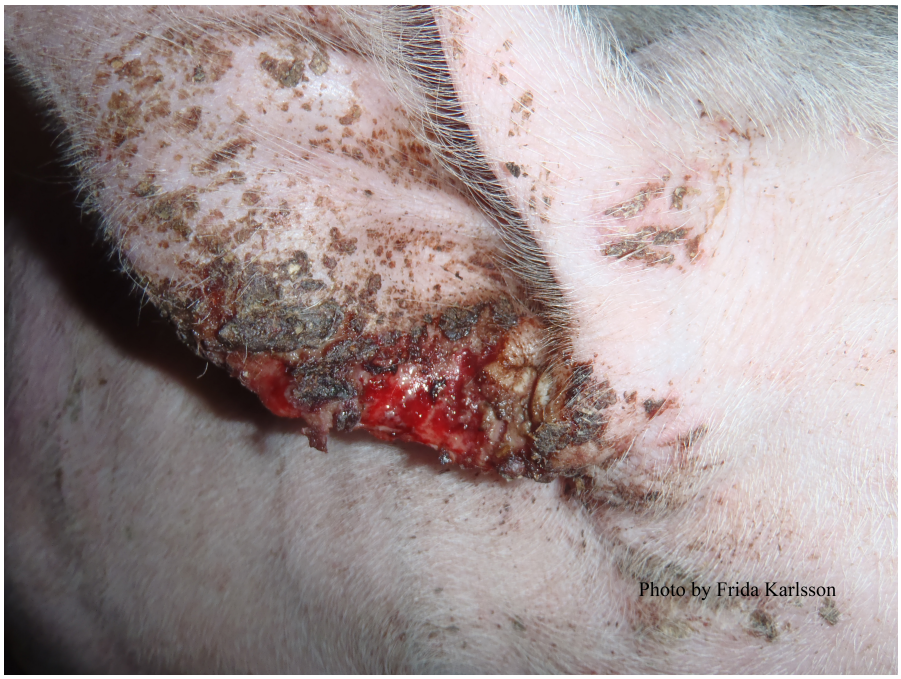

Figure 1: Ear necrosis.

Shoulder ulcers (Figure 2) have a startling appearance that may lead to penalty for producers for misconduct regarding animal welfare. The ulcers usually develop during the lactating period and are often bilateral. The prevalence in herds with affected sows is often above 10%. In 12,502 examined sows in sweden, an average prevalence of 18% has been reported (Gunnar Johansson, Swedish Animal Health Service). When the sow is laying down the pressure of the body weight on the shoulder leads to a traumatic challenge of the skin. The progress goes from redness and hair-loss to deep necrotic ulcers. In severe cases, the diameter of the ulcer passes 5 cm. Rough bedding and underfeeding are considered as risk factors (Zurbrigg, 2006; Bonde *et al.*, 2004). The ulcers usually heal without treatment after weaning of the piglets. However, they often return at the next lactating period (Herskin *et al.*, 2011). Sows with shoulder ulcers are likely in pain. A recent report associates pain with behavior and with results from histological examinations of affected tissue from sows with shoulder ulcers. It was suggested that sows with a healed or unhealed shoulder ulcer experience pain (Dahl-Pedersen *et al.*, 2013). Sows with severe shoulder ulcers may be taken out of the production chain thereby affecting the economy for the producers.

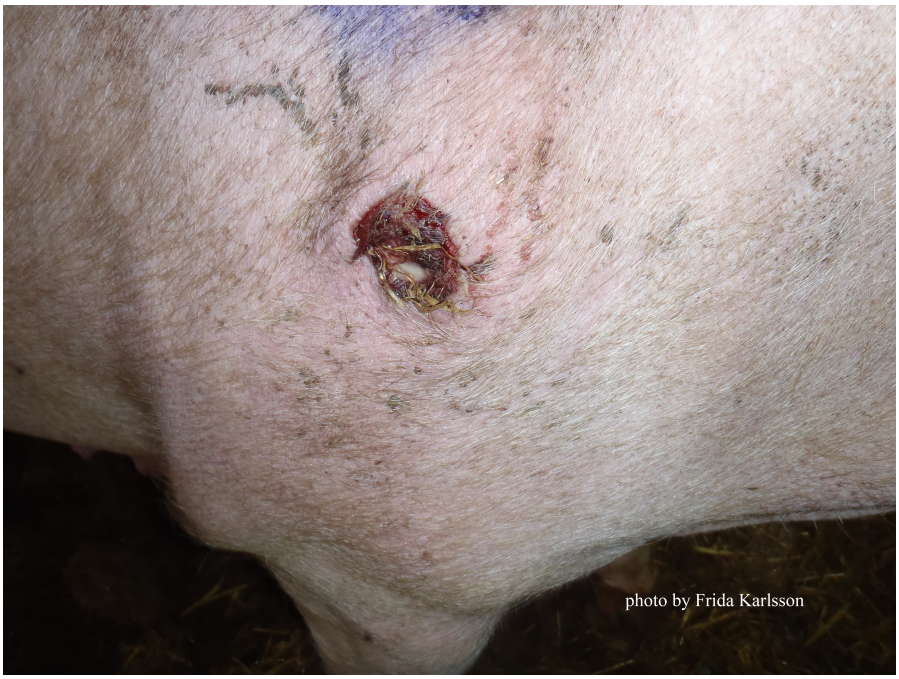

Figure 2: Shoulder ulcer.

Ear necrosis and shoulder ulcers have not been associated to infection by a specific bacterial species. In ear necrosis, mixed infections with staphylococci and streptococci have been suggested as possible causes in combination with wave-formed bacteria, i.e. spirochetes (Zimmerman *et al.*, 2012).

1.3 The bacterial phylum *Spirochaetes*

Spirochaetes is a phylum within the domain bacteria that forms a monophyletic lineage that divides first at the taxonomic rank family; hence all spirochetes are believed to have evolved from a common spirochetal ancestor. Spirochetes are characterized by their cell architecture and genetic properties.

When studying evolutionary relationships among prokaryotes, phylogenetic distances are commonly estimated by sequence comparisons of the 16S ribosomal RNA (rRNA) gene. The 16S rRNA genes of spirochetes have unique signatures that separate spirochetes from all other bacterial species (Paster & Dewhirst, 2000; Woese, 1987). The same conclusion is attained by comparisons of multiple genes from whole-genome sequences (Gupta *et al.*, 2013; Wu *et al.*, 2009).

Spirochetes are long and thin with a characteristic waveform. Some species have a helical shape whereas others are flat. The diameter of the cell varies between 0.1 – 3 μm and the length between 4 – 250 μm (Krieg *et al.*, 2011).

Only spirochetes have their flagella located in the periplasmic space, i.e. between the inner membrane that surrounds the cytoplasm and their outer sheath. This causes the waveform and enables corkscrew-like motility. Flagella are protein structures crucial for motility of bacteria. Simplified, the flagellum consists of three parts: motor, hook and filament. The motor is anchored in the inner membrane and utilizes force from protons. The cell creates an electric potential by pumping protons from the cytoplasm and across the inner membrane. The protons flow through the motor, back into the cytoplasm, which makes the motor produce a rotational energy around an imaginary axis between the inside and outside of the membrane. Outside the cytoplasm, the hook links the motor to the long and flexible filament. The hook transfers the rotational energy from the motor to the filament. The filament forms a rotating helix when the motor runs. In non-spirochete, motile bacteria, the filament is protruding into the surrounding. The flagellum then works as a propeller that gives the cell thrust. In spirochetes, motility is created differently. The filament of the spirochetal flagella is located in the periplasmic space and is not in contact with the surrounding of the cell. The motor part is attached sub terminally at the ends of the cell. A spirochete may have up to 100 flagella

attached at each end. The filament is aligned with the cell body towards the opposite terminal. As periplasmic flagella results in a waveform of the cell, rotation of these flagella causes a movement of the waves. If the cell is in a viscous environment, translation occurs. Mutants lacking genes coding for flagella loose both motility and waveform. When spirochetes translate, flagella at either terminal turn in opposite directions. If the turns are switched, translation occurs in the opposite direction. Equal turning results in a non-translational mode of conformal change of the cell that can be used to change its course. The motility of spirochetes is considered to contribute to virulence for pathogenic species (Charon & Goldstein, 2002).

The outer sheath of spirochetes is similar to the outer membrane of gram-negative bacteria. However, Gram staining of spirochetes is not reliable. Silver impregnation is commonly used to visualize these bacteria in tissue (Krieg *et al.*, 2011) as stained spirochetes can be identified due to their characteristic morphology.

The spirochetes are naturally resistant to the antibiotic substance rifampicin. The resistance is likely due to low affinity of the rifampicin molecule to a specific region of the RNA polymerase in spirochetes (Stamm *et al.*, 2001).

There are both free-living and host-associated spirochetes, of which the genera *Treponema, Brachyspira, Borrelia,* and *Leptospira* involve species with considerable impact on human and animal health. Skin diseases of pigs caused by spirochetes have been suggested in studies conducted as early as 1906 (Dodd, 1906).

1.4 The bacterial genus *Treponema*

The genus *Treponema* belongs to the family *Spirochaetaceae* under the phylum *Spirochaetes*. The word *Treponema* means to turn and thread.

A typical treponemal cell is helical, 10 µm long, and 0.2 µm thick with 6-10 waves.

Treponemes are fastidious, slow growing, motile and require an anaerobic environment for continuous *in vitro* culturing. Many *Treponema* spp. are not yet cultivable. *Treponema pallidum* is a microaerofilic, obligate pathogen that can only be propagated by inoculation to testicles of live rabbits. A 100-fold increase of cells may be achieved in culturing medium; however this culture fails to grow as subcultures. Carbohydrates and amino acids serve as energy and carbon sources for treponemes. There are few reports of successful cultivation on sugars other than glucose. Treponemes display inactivity for many metabolic bacterial enzymes, although most have tested positive for acid phosphatase, naphthol phosphohydrolase, alkaline phosphatase, C4 esterase,

and C8 esterase lipase. Treponemes are susceptible to many antibiotics, e.g. penicillin, ampicillin, tetracycline, and erythromycin. As other spirochetes, *Treponema* spp. are not inhibited by rifampicin (Krieg *et al.*, 2011).

Presence of *Treponema* spp. has been demonstrated at various locations, e.g. termite gut, tonsils and gut of pigs, in the human oral microbiome and as free-living organisms (Lowe *et al.*, 2012; Lowe *et al.*, 2011; Chen *et al.*, 2010; Graber *et al.*, 2004; Leser *et al.*, 2002; Cwyk & Canale-Parola, 1979; Veldkamp, 1960). The free-living treponeme was described in a study from the 60s and may today be classified differently. Intact, healthy skin of humans does not appear to be inhabited by *Treponema* spp., nor any other spirochete (Segata *et al.*, 2012; Grice *et al.*, 2008; Gao *et al.*, 2007; Dekio *et al.*, 2005).

Several species of *Treponema* are considered as pathogens or are associated with disease. *Treponema pallidum* ssp. *pallidum* is a frank pathogen that causes human syphilis. Initial symptoms are superficial wounds located at the genitals that usually disappear after 2-3 weeks. These wounds contain high numbers of treponemes. If not treated with antibiotics (e.g. penicillin), the disease may become systemic and undergo a secondary and tertiary phase. The secondary phase occurs within 8 weeks with influenza-like symptoms. The symptoms of the tertiary phase are due to which organ that is mostly affected. Organ failure may lead to death (Radolf & Lukehart, 2006).

T. pallidum ssp. *pertenue*, causes yaws, a disease with symptoms different from those of syphilis. Yaws is recognized by skin nodules, skin ulcerations, joint and soft tissue destruction, bone affections, and is transmitted by skin contact (Radolf & Lukehart, 2006).

Treponema denticola is a member of the human oral microbiome (Chen *et al.*, 2010). *Treponema denticola* is associated with periodontitis, a disease affecting gum and underlying bone. Periodontitis is a polymicrobial disease involving *T. denticola,* other treponemes and species from other phyla, e.g. *Tannerella forsythia* and *Porphyromonas gingivalis* (Dashper *et al.*, 2011; Ishihara, 2010; Sela, 2001). *Treponema denticola* was first described from an isolate originating from gingival debris (Socransky *et al.*, 1969). During the 70s and 80s, a large number of studies assessing the role of spirochetes in periodontitis were conducted. A quantitative relationship between *T. denticola* and periodontitis was demonstrated (Simonson *et al.*, 1988) and *T. denticola* became considered as one of the most important treponemes for periodontitis. Today, there are several review articles summarizing studies demonstrating roles and features of *T. denticola* in periodontitis (Dashper *et al.*, 2011; Ishihara, 2010; Sela, 2001).

Bovine digital dermatitis (BDD) is a contagious disease that affects the foot of cattle and causes lameness. The response to antibiotic treatment indicates a

bacterial cause of BDD. Multiple research groups worldwide, including ours, have performed studies that altogether strongly associates BDD with treponemes (Yano *et al.*, 2009; Klitgaard *et al.*, 2008; Nordhoff *et al.*, 2008; Pringle *et al.*, 2008; Trott *et al.*, 2003; Walker *et al.*, 1995). Treponemal isolates from BDD have been shown to produce skin lesions comparable with other pathogenic *Treponema* spp. in a murine model (Elliott *et al.*, 2007). In addition, a *Treponema vincentii*-like isolate and homogenates from clinical BDD samples can induce BDD lesions in cattle (Gomez *et al.*, 2012). Several *Treponema* spp. have been proposed as important etiological agents of BDD, e.g. the cultivable *T. pedis* and phylotypes similar to *T. phagedenis* and *T. medium* (Evans *et al.*, 2009; Evans *et al.*, 2008).

The type strain of *T. pedis* T3552B^{T} was originally isolated from a BDD lesion (Walker *et al.*, 1995) and characterized by Evans *et al.* (Evans *et al.*, 2009). *Treponema pedis* is one of the predominating treponemes in BDD that have been observed at high densities within diseased tissue (Klitgaard *et al.*, 2013). Exclusively *T. pedis* was isolated during the case studies of skin lesions in pigs' prior this thesis work (Pringle & Fellström, 2010; Pringle *et al.*, 2009).

Sheep may be affected by severe virulent foot rot, a disease with similar characteristics as BDD. Infection with *Dichelobacter nodosus* and *Fusobacterium necrophorum* are mainly discussed as causes but involvement of spirochetes have also been shown (Demirkan *et al.*, 2001).

1.5 Genomes of treponemes

The first treponemal genome to become sequenced and assembled was that of *T. pallidum* subsp. *pallidum* strain Nichols (Fraser *et al.*, 1998). This was performed using the same technique as for the first bacterial genome sequenced. Copies of the chromosome were sheared into fragments that were randomly inserted into cloning vectors. The clones were amplified in bacterial hosts. The plasmids were extracted and sequenced by the Sanger method. This was repeated until the combined lengths of the reads covered the entire genome multiple times. Computer software and manual editing was used to combine the reads until a consensus sequence emerged. This sequencing technique is now replaced by the next generation sequencing technology (NGS) that generates millions of sequencing reads within hours at costs affordable for most laboratories. At the time this thesis work was conducted, two techniques were commonly used, the 454 sequencing technology from Roche Diagnostics Corporation and the Illumina Technology. Template DNA is fragmentized and clonally amplified in an emulsion PCR (454) or on a solid surface by bridge amplification (Illumina). In 454, the four types of nucleotides are added

separately in cycles and will be incorporated if they agree with the template. Each incorporated nucleotide involves release of one inorganic pyrophosphate molecule. The chemistry of 454, known as pyrosequencing, converts this molecule to a photon. The photons are detected and since the type of added nucleotide is known, incorporation of that certain base is registered (Margulies *et al.*, 2005). Illumina's instrument adds reversible terminator nucleotides that carry a fluorophore with a base-specific color. Before the elongation resumes, the terminator is removed and the fluorophore of that incorporated nucleotide is registered. This technique enhances the accuracy (Bentley *et al.*, 2008). Generally, the 454 technique is considered to yield longer reads whilst Illumina's platform holds higher accuracy and cost effectiveness. Sanger sequencing, that requires primers specific for the target, is still appropriate and cost effective for limited sequencing, e.g. PCR products. This technique may give sequencing reads up to 1,000 base pairs (bp).

The genome of *T. pallidum* ssp. *pallidum* strain Nichols was determined to 1,138,006 bp with 1,041 predicted coding sequences. This is one of the smallest known bacterial genomes. The hopes that the genome information would result in knowledge for continuous culturing of *T. pallidum* have not been fulfilled. The genome of *T. pallidum* ssp. *pallidum* is nearly identical to that of *T. paraluiscuniculi* that causes rabbit syphilis (Smajs *et al.*, 2011). Despite the similarities between the genomes of these organisms, *T. paraluiscuniculi* does not cause a systemic infection and is not infectious to humans. In addition, *T. pallidum* ssp. *pertenue* exhibits minimal genomic difference to the syphilis-causing agent (Cejkova *et al.*, 2012). Still, the clinical symptoms from *T. pallidum* ssp. *pertenue* differ from those of syphilis.

The genome of *T. denticola* ATCC 35405 has been determined to 2,843,201 bp and comparative genomics showed that *T. denticola* and *T. pallidum* differed significantly in several parameters such as size, homology, gene synteny, and G+C content. These results rejected a hypothesis that *T. pallidum* originates from a *T. denticola* ancestor that has reduced its genome (Seshadri *et al.*, 2004). *Treponema denticola* is cultivable and genetically manipulatable. Thus, the genome sequence provides an excellent source of information when studying the genes of *T. denticola* or those from other non-cultivable treponemes using *T. denticola* as a host.

The prosperity of NGS allows sequencing of multiple strains when characterizing a genome of a bacterial species. Comparisons of genomes from *Streptococcus agalactiae* strains showed that the species have a core-genome, shared by all strains, and a dispensable accessory part that is variably present among strains (Tettelin *et al.*, 2005). This emphasizes importance of

sequencing multiple strains to give a more complete genomic picture of a species.

1.6 Virulence factors of pathogenic treponemes

Virulence factors are molecules expressed by a microorganism that enable or enhance the ability to cause disease. Examples are the M protein of *Staphylococcus,* that helps the cell to evade the immune system, or neuro-toxins produced by the botulism causing species *Clostridium botulinum*.

Current research suggests that *Treponema pallidum,* that causes serious infections, lack the classical types of virulence factors. Still, *T. pallidum* ssp. *pallidum* can infect virtually any organ in the human body and persist for a long time. *Treponema pallidum* is thought to express a low number of surface-exposed proteins and undergo antigenic variations thereby evading the immune system of the host (Centurion-Lara *et al.*, 2004). Due to difficulties in cultivation, genes that may be involved in pathogenesis cannot be studied by gene knockout. This has hampered syphilis research (Radolf & Lukehart, 2006).

Virulence of the cultivable periodontal pathogen *T. denticola* has been associated to several specific genes and gene-products. Two that have received much attention is the major surface sheath protein (Msp) and the dentilisin operon.

The Msp constitutes a large portion of the outer-membrane proteins of *T. denticola*, hence the name. It was first identified from a complex with other proteins and appeared as a 53 kDa band when a sample of purified outer membrane proteins was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and a 1629 bp DNA sequence encoding the Msp gene was subsequently determined (Fenno *et al.*, 1996; Haapasalo *et al.*, 1992). Msp adheres to extracellular matrix proteins, is cytotoxic and form pores in cultured human cells (Fenno *et al.*, 1998; Mathers *et al.*, 1996).

Dentilisin is a protein complex from an operon of three open reading frames named *prcB*, *prcA* and *prtP* (Godovikova *et al.*, 2010). Dentilisin is a cell-surface located prolyl-phenylalanine-specific protease with cytopathic effects (Fenno *et al.*, 1998; Ishihara *et al.*, 1996). PrtP-deficient mutants of *T. denticola* have a reduced ability to penetrate epithelial cell-layers (Chi *et al.*, 2003) and show a significant reduction of both protease activity and abscess formation in subcutaneously challenged mice (Ishihara *et al.*, 1998).

Treponema denticola also contain proteases with N-α-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolysis. This feature is associated with periodontal pathogens and linked to the gene paralogues TDE1195 and

TDE2140 in the genome of *T. denticola* ATCC 35405 (GenBank accession: NC_002967.9) (Dashper *et al.*, 2011; Fenno *et al.*, 2001).

The *lrrA* gene in *T. denticola* has been proposed important for adherence to epithelial cells and for the synergistic interactions that occur in the periodontal biofilm (Ikegami *et al.*, 2004).

Dentipain is the designation of a domain recently characterized from the open reading frame *ideT* harboring the locus tag TDE0362 in the genome of *T. denticola* ATCC 35405. The gene is required for full virulence of *T. denticola* when inoculated to mice. The mutant strain lacking dentipain produced smaller sized lesions compared to wild type *T. denticola*. Dentipain was characterized as an oligopeptidase (Ishihara *et al.*, 2010).

1.7 Hypothesis

The hypothesis of this thesis is that *Treponema* spp. have a pathogenic role in the progression of ear necrosis and shoulder ulcers in pigs. Moreover, that the gingiva of pigs is the reservoir for these bacteria, thus biting may mediate transmission from mouth to susceptible skin.

2 Specific aims

- To determine occurrence and diversity of *Treponema* spp. in samples from ear necrosis, shoulder ulcers and gingiva of pigs by culture-independent techniques.
- To isolate and characterize *Treponema* spp. from the material used to investigate occurrence.
- To sequence and *de novo* assemble the genome of *T. pedis* strain T A4, isolated from pig ear necrosis, and search for virulence factors.
- To characterize potentially virulence-related gene-products identified in the genome of *T. pedis* strain T A4.

3 Comments on materials and methods

3.1 Pig herds and sampling

Obtaining pigs with ulcers for sampling was crucial for studying occurrence of treponemes. Pig herds, where affected animals could be sampled, were identified by personal contact with pig farmers and local veterinarians. We sampled 169 pigs, 52 with shoulder ulcers, 57 with ear necrosis and additionally 60 apparently healthy piglets (gingiva only). A detailed description of the sampled animals can be found in supplementary file 1 in paper **I**. We recorded the number of sampled animals in each herd, the geographical location, date of sampling, and the type of herd visited. The age, breed and if the pig was a sow, grower or weaner was also registered. The sampling procedure is described in paper **I**. During the sampling procedure, the pigs were restrained with a firm tension applied around the snout (Figure 3). This technique is traditionally used to immobilize the animal and has a calming effect thus making the situation less stressful. The samples were processed for culture-independent assessment and culturing as described in paper **I** and **II** respectively.

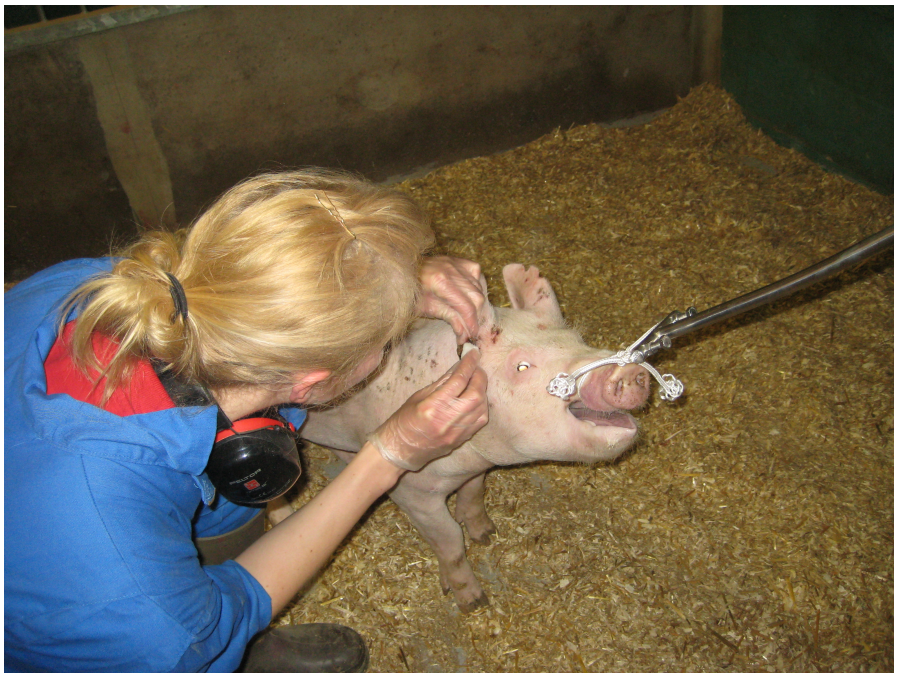

Figure 3: Sampling of a pig with ear necrosis.

3.2 Detection, phylogenic analysis and phenotyping of *Treponema* spp.

Occurrence of *Treponema* was investigated by a combination of culture-independent techniques described in paper **I**. The number of isolates acquired for culturing, as described in paper **II**, was much lower than the presence demonstrated in paper **I**. No isolate was retrieved from an ulcer that did not show occurrence of *Treponema* by the methods used in paper **I**.

Isolates, acquired as described in paper **II**, were used for phylogenetic analyses and phenotypic studies. DNA sequences from 16S rRNA and a short variable intergenic spacer region (ISR2) suitable for discrimination between *Treponema* phylotypes (Stamm *et al.*, 2002) were used for phylogenetic analyses. DNA sequences were retrieved from lysates of tissue, gingival samples and from isolates.

DNA fingerprinting-methods are used for showing strain variations thereby making it suitable for epidemiology. According to our hypothesis, we wanted to test if there were gingival isolates identical to those isolated from ulcers.

Pulsed-field gel-electrophoresis (PFGE) is commonly used in epidemiological studies. Genomic DNA is extracted in agar and digested with rare-cutting restriction enzymes. Electric current with alternating directions separates large-sized fragments and clone-specific banding-patterns appear (Schwartz & Cantor, 1984). This method was repeatedly tested but failed due to weak appearance of the bands. The randomly amplified polymorphic DNA (RAPD) technique is also attended to produce clone specific patterns (Williams *et al.*, 1990). The technique is based on PCR with an oligonucleotide primer short enough to bind randomly in chromosomal DNA. In theory, primers annealed in proximity will amplify the surrounded region producing a fragment detectable by gel electrophoresis. Since the primers are unspecific, the banding patterns produced are dependent on the template DNA hence clone-specific patterns can be achieved. The purchased RAPD kit (illustra, GE Healthcare Life Sciences) comes delivered with six primers. All primers were tested, however only primer four yielded comparable banding patterns.

3.3 Whole genome sequencing of *Treponema pedis*

The features and functions of an organism are encoded in its genome. Access to the genomes of *Treponema* spp. present in pig gingiva and ulcers may help discover genes responsible for infection and ulcer development, i.e. virulence factors. *Treponema pedis* strain T A4, isolated from pig ear necrosis, was chosen for *de novo* sequencing since basic information of this strain was already published (Pringle *et al.*, 2009).

An initial assembly of the genome was constructed by sequencing genomic DNA on Roche's FLX 454 platform. This technique has commonly been used for *de novo* sequencing as the sequencing reads holds a relatively long length (~400 bp) compared to other NGS available at the time of this study. The lengths of the sequencing reads influence the number of obtained contigs. Contigs are continuous DNA sequences that together represent the genome and are made of aligned sequencing reads. Longer reads will presumably yield a lower number of contigs making completion of the chromosome(s) more feasible. At present, there are manufacturers (Pacific Biosciences) of new sequencing platforms that claim read length longer than 20 kbp, which in many cases give contigs representing entire chromosome(s) without manual processing. The assembly of the genome of *T. pedis* strain T A4 required use of several sequencing technologies, manual processing and PCR. The 454 reads were assembled by computer software into ~50 contigs. After manual inspection of the aligned reads, it became evident that the computer software produced many misassemblies. There were reads incorrectly joined within

contigs. Moreover, at certain positions there were accumulations of reads not corresponding exactly to the consensus. Contigs were broken at the positions of incorrectly joining and mismatched reads were removed and used to produce new contigs. Joining of the manually inspected and processed contigs resulted in a reduction of the number of contigs. There were now two remaining problems, low quality regions within contigs and low quality regions at the end of contigs making joining impossible. For this, PCR with primers designed to amplify low quality regions or to anneal at the end of a contig were used. A PCR product over an internal low quality area, with expected size, indicated a correct assembly. Most PCR-products could also be Sanger-sequenced and resulting reads were added to the assembly. Primers annealing to the end of a contig, directed outwards, were used in a pair-wise, all-against-all PCR. This generated sequencing reads that filled gaps and further lowered the number of contigs. One reaction generated a long-range PCR product that was sequenced using several primers annealing at various locations within the fragment. This is called primer walking and resulted in addition of a ~3,5 kbp contig that was used to redirect the consensus. There were now few remaining gaps. PCR results had indicated joining, however the PCR product failed Sanger-sequencing. Most likely, these regions contained hairpin structures or long homopolymers. A decision was taken that sequencing on Illumina's HiSeq2000 would hopefully give sequences from the desired regions and also improve the overall quality due to the massive output. Alignment of the Illumina reads was used to identify and correct dozens of small errors. The 30,012,760 additional reads did also cover the remaining gaps. A complete circular chromosome of *T. pedis* was assembled. A brief summary of the assembling process and information on software used are described in paper **III**.

To investigate variability within *T. pedis*, six additional isolates were sequenced on an Illumina MiSeq instrument. These isolates originated from ear necrosis, shoulder ulcer and gingiva. The genomes were only assembled as drafts (no manual processing) together with 12 additional genomes of the closely related species *T. denticola.* The sequencing reads for the *T. denticola* strains were downloaded from experiments deposited in NCBI's sequence read archive (http://www.ncbi.nlm.nih.gov/sra). The draft genomes were used to compare gene content from gingival isolates and those from ulcers and assess intra-strain variability.

Genetic comparisons were made using the stand-alone BLAST program from NCBI (Altschul *et al.*, 1990). These tools generated large lists of information difficult to sort manually. For this, PERL scripts were written that could perform desired analyzes from BLAST results.

3.4 *In vitro* assessment of putative virulence genes of *T. pedis*

The function of a gene derived from a DNA sequence is a prediction based on homology to genes in other organisms. To achieve more credibility to the proposed function, *in vitro* testing is essential. The manuscript designated as paper **IV** describes isolation and *in vitro* testing of the putative virulence-related gene product of TPE0673 in *T. pedis* strain T A4.

Using the technique described in paper **IV**, attempts were also made to isolate the proteins Msp (TPE2758), PrtP (TPE1950), PtrB (TPE0822) and PtrB2 (TPE0746). Transformed cells of *Escherichia coli* carrying an expression vector with TPE2758, coding for Msp, were retrieved, whereas transformation of the other three did not succeed. When expression was induced, the gene-product of TPE2758 was not soluble and purification therefore failed.

Attempts were also made to isolate native dentilisin of *T. pedis* using the same strategy as for *T. denticola* (Fenno *et al.*, 1998). One liter of *T. pedis* isolate B 683 was grown for 7 days and subjected to Triton X-114 extraction and phase partitioning. The detergent fraction was size separated on a high capacity Mini Protean II electrophoresis apparatus (Bio-Rad). Presence of proteins in the fractions were then analyzed by SDS PAGE and silver staining (Fenno *et al.*, 1996). Despite several attempts, no protein of expected size appeared in the size-separated fractions of outer-membrane proteins from *T. pedis*.

4 Results and Discussion

4.1 Occurrence and diversity of *Treponema* spp. in ear necrosis, shoulder ulcers and gingiva in pigs.

Prior to the studies performed in this thesis work, pilot studies, on a limited number of animals, had demonstrated presence of *T. pedis* in ear necrosis, shoulder ulcers and gingiva of pigs (Pringle & Fellström, 2010; Pringle *et al.*, 2009). To test the hypothesis that *T. pedis* and possibly other treponemes are involved in the pathogenesis of these ulcers, occurrence studies of a larger data-set was undertaken on samples collected from 109 pigs with lesions and the gingiva of additionally 60 piglets.

Occurrence of spirochetes was demonstrated in 73% of the shoulder ulcers, 53% of the ear necroses and 9.7% of the gingival samples (paper **I**). The occurrences in ulcers are based on detection using a combination of techniques of which two rely on identification of the unique morphology of spirochetes. Therefore, we could not be certain that all detected spirochetes were of genus *Treponema*. However, the results from the PCR-based detection system, with *Treponema*-specific primers, in shoulder ulcers and ear necroses were only slightly lower, 52% and 46% respectively. In addition, all spirochetes isolated (paper **II**) were of genus *Treponema*. Our conclusion is that the spirochetes occurring in necrotic ulcers in pigs are solely of genus *Treponema*.

In a recent study, prevalence of *Treponema,* using fluorescent *in situ* hybridization (FISH), was shown in 100% of 85 biopsies from BDD lesions (Rasmussen *et al.*, 2012). Even though different detection techniques were used, there seems to be a lower prevalence of treponemes in necrotic ulcers in pigs compared with BDD. Our group has speculated that the treponemes may be localized to specific regions of the ulcers which did not constitute the sampling material or that the bacteria were of significance prior or post to the sampling moment.

Phylotypes similar to *Treponema phagedenis* are abundant among the different treponemes present in BDD. A *Treponema pedis*-like phylotype is also present but at a lower frequency than *T. phagedenis*. *Treponema pedis* however, can be seen in high densities in the affected tissue (Klitgaard *et al.*, 2013). In pigs, *T. pedis* was frequently detected but no presence of *T. phagedenis* was seen (paper **I** and **II**). These findings suggest a difference in the diversity of treponemes between BDD and lesions in pigs and that *Treponema pedis* has a broad animal host range. Generally, many phylotypes within the diverse population of treponemes found in skin ulcers and gingiva of pigs are similar to those associated with ulcerative skin diseases of cattle and with periodontitis (paper **I** and **II**).

Treponemes are found habitating skin, gingiva and gut. There are, to our knowledge, no studies demonstrating treponemes on healthy skin of humans or animals. Therefore, the probability that the treponemes occurring in pig lesions are present on healthy skin should be considered low. Our hypothesis suggests a relationship between treponemes present in ulcers and gingiva. Based on phylogenetic analysis (paper **I** and **II**), metabolic activity patterns (paper **II**) and whole genome comparisons (paper **III**), no distinction between the treponemes in ulcers and those of oral origin could be made. On the basis of ISR2 analysis, transmission of treponemes was indicated between animals within the same herd and between piglets and the sow in the same pen (paper **I**).

4.2 Phenotypic traits and DNA fingerprints of *Treponema* spp. isolated from ear necrosis, shoulder ulcers and gingiva in pigs.

Treponemes are difficult to isolate, slow-growing and fastidious compared to other bacteria. Consequently, phenotypic data from treponemes, especially from pigs, are scarce. Moreover, isolates are important to enable studying of the genomes, gene expressions and proteins of *Treponema*.

Isolates of *T. pedis* and *T. parvum* and isolates similar to *T.* sp. OMZ840 were acquired and characterized (paper **II**). The yield of isolates (Table 1, paper **II**) was low considering the occurrence determined by culture-independent techniques (Table 1, paper **I**) even though material from the same sample was used. Additional isolates were retrieved but were excluded due to weak growth or difficulties in separating mixed phylotypes. The metabolic activities (Table 2, paper **II**) were similar to those reported from other *Treponema* spp. and were not reliable for differentiation between the species. There were several phenotypic features that can be associated with virulence.

All isolates were motile, a feature that can be used for tissue invasion. All except *T. parvum* showed trypsin and α-chymotrypsin activity, proteolytic features that can be used to destroy tissue (Uitto *et al.*, 1988). In addition, mutant strains of the pathogenic spirochete species *Brachyspira hyodysenteriae* that lacks a gene encoding a hemolysin loses virulence (Hyatt *et al.*, 1994), hence these functions may as well be of importance for infectivity for isolates of *T. pedis* (Figure 4) and isolates similar to *T.* sp. OMZ840.

The isolates were generally susceptible to the tested antimicrobials (Table 3, paper **II**). Similar studies performed on isolates of *Treponema* from BDD also show a generally high susceptibility towards multiple antimicrobial compounds with the exceptions of those of which spirochetes are naturally resistant to (Yano *et al.*, 2010; Pringle *et al.*, 2008).

All except two isolates displayed unique DNA fingerprints from RAPD (Fig. 2. paper **II**). The two *T. pedis* isolates of oral origin that displayed possibly identical RAPD patterns originated from different herds, hence transmission between these animals is not likely.

4.3 Genome sequence of *Treponema pedis*

The complete genome of *T. pedis* strain T A4 was sequenced, *de novo* assembled and analyzed (paper **III**). The *T. pedis* strain T A4 was isolated from pig ear necrosis and chosen as a representative for *T. pedis* from pig ulcers since it had been described previously (Pringle *et al.*, 2009). The main goal was to identify virulence factors but also to characterize the genome of *T. pedis* for the first time.

The genome of *T. pedis* T A4 consisted of a 2,889,325 bp long circular replicon. The G+C content was 37.9% and there were 2,806 predicted protein-coding genes. There were a number of gene predictions containing the YD repeat motif (TIGR01643) that were interpreted as non-functional and were designated as pseudo genes; hence the number of proteins coding genes for *T. pedis* strain T A4 in NCBI (accessions NC_022097.1/CP004120.1) is 2,778.

Comparative genomics revealed that two thirds of the genes in *T. pedis* were most closely related to those in *T. denticola*. Several genes in *T. pedis* were homologous to virulence related genes in *T. denticola*, of which some have been thoroughly characterized (Table 3, paper **III**).

The additional draft genomes enabled estimation of the pan-genome structures of *T. pedis* and *T. denticola*. The structures were similar between the species. There were 988 core functions in *T. pedis*, i.e. gene functions present in all isolates. Each sequenced *T. pedis* isolate contributed with, on average, 576 unique genes. In this dataset, gene-transfer events with orally related

species from other phyla were indicated for *T. pedis*. No difference was detected between isolates of *T. pedis* originating from gingiva to those from ulcers and the putative virulence factors, identified in *T. pedis* strain T A4, were virtually present in all genomes.

In conclusion, the results from the analysis of the genomes of *T. pedis* suggest multiple pathogenic properties for this species (Figure 4).

4.4 Characterization of potentially virulence-related genes in *T. pedis* strain T A4.

Comparative genomics is used to annotate the functions of genes predicted in a genome sequence. Since the proposed function is based on sequence comparisons, *in vitro* assessment of the gene product is essential to determine the function.

In the genome analysis of *T. pedis* strain T A4 (paper **III**), an open reading frame (TPE1565) coding for a putative Ig-like protein homologous to IdeT (TDE0362) in *T. denticola* was identified. The gene product of TDE0362 has been characterized and proposed as a virulence factor for *T. denticola* (Ishihara *et al.*, 2010). Attempts were made to clone TPE1565 for recombinant expression in *E. coli* but without success. However, at subsequent analysis of the genome, a paralog to TPE1565 designated as TPE0673 was identified and successfully cloned and expressed (paper **IV**).

The gene product of TPE0673 did not display the proteolytic properties as its homologues in *Streptococcus* (von Pawel-Rammingen *et al.*, 2002) or *T. denticola* (Ishihara *et al.*, 2010). However, results from western blot analyzes suggest that sows with shoulder ulcers, where presence of *T. pedis* had been shown (paper **I**), had developed antibodies with affinity towards the product of TPE0673 (paper **IV**). The results suggest an immunogenic property of TPE0673 and it may be useful for serological screening for *T. pedis* infection (Figure 4).

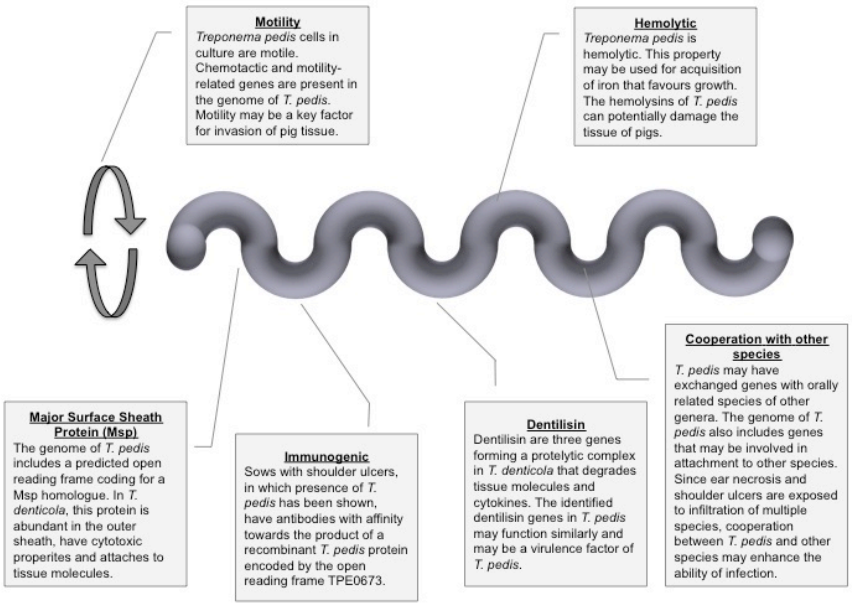

Figure 4: Putative virulence traits of *T. pedis*.

5 Conclusions

Several factors may predispose ear necrosis and shoulder ulcers in pigs, e.g. stress, animal density, nutritional status of the animal and the type of floor.

In this thesis, the tested hypothesis is that *Treponema* spp. present in the gingiva of pigs are transmitted to the skin, mediated by biting or licking, where they play a pathogenic role when these ulcers develop.

The following conclusions from the results of the performed studies supports the hypothesis:

- A diverse population of *Treponema* spp. can be found in a majority of ear necroses and shoulder ulcers in pigs. The identified phylotypes are related to treponemes present in other skin diseases. The treponemes found in ulcers are genetically similar to those present in gingiva, which indicates transmission between gingiva and ulcer.
- Treponemes can be isolated from ear necrosis, shoulder ulcers and gingiva. The same species could be isolated from both ulcer and gingiva. A majority of the isolates were hemolytic, a feature that may promote tissue destruction.
- The genomes of *T. pedis*, isolated from ear necrosis, shoulder ulcer and pig gingiva, include several putative virulence factors suggesting a pathogenic potential for this species.
- Sows with shoulder ulcers, in which presence of *T. pedis* has been demonstrated, display a humoral response towards a recombinantly expressed, putative virulence-related, protein of *T. pedis* strain T A4.

6 Suggested future studies

When comparing the outcome of the culture-dependent and culture-independent studies, it is clear that many *Treponema* phylotypes are difficult or impossible to isolate and cultivate. These species may be as important for ulcer development as those more commonly found, e.g. *T. pedis*. To study this further, development of new culturing techniques or more informative culture-independent techniques would be needed.

The progression over time of the infection in ear necrosis and shoulder ulcers is unexplored. Surveillance of pigs over time is therefore of interest. Continuous sampling and clinical examination can detect the presence of treponemes before, during and after the occurrence of ear necrosis and shoulder ulcers.

The microbiota in a sample can be investigated using high-throughput sequencing. There are two approaches, whole genome shotgun sequencing and 16S rRNA amplicon sequencing. The latter includes a step of PCR with primers designed to amplify the 16S rRNA gene from all existing bacteria. The dataset retrieved is comprehensive and can be compared to a large database of 16S rRNA sequences to determine the genus or species. However, this approach is biased by the use of primers that may favor certain bacteria and give an inaccurate diversity profile. Whole genome shotgun sequencing is independent from primers and sequences all present DNA from all microorganisms in a sample. This technique enables exploration of all present organisms, including archea and fungi, and may also be used to focus on e.g. presence of certain enzymes. It should be noted that much of the acquired data will not give a reliable database match (Carlos *et al.*, 2012). Either of these methods may be used to further explore the diversity of treponemes in ear necrosis and shoulder ulcers. The results may also reveal presence and functions of bacteria from other genera that may be of importance.

Finally, the putative virulence factors identified in the genomes of *T. pedis* need further characterization by *in vitro* assessments. The homologues to Dentilisin and Msp in *T. pedis* are top candidates for investigation as these are proposed as important virulence factors in *T. denticola* (Dashper *et al.*, 2011). Morover, since *T. pedis* may be involved in the pathogenesis of BDD, knowledge of virulence for this species can also contribute to research regarding BDD.

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