MASTER THESIS IN MEDICAL MICROBIOLOGY (BIO-3930)

A molecular study of putative pili structures in *Enterococcus faecium*.

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Abbreviations

Ace	Adhesion to collagen from *E. faecalis*
AP1	Activator protein 1
AP2	Activator protein 2
AS	Aggregation substance
ATG	Start codon
*att*X	Attachment site X, were x denotes L, R, 1 or 2
Bp	Base pairs
CACC	Overhang in cloning
CC17	Clonal complex 17
Cfu	Colony forming unit
CWSS	Cell wall sorting signal
CWAPs	Cell wall anchored proteins
ddNTP	dideoxynucleotide triphosphate
df	degree of freedom, a statically term
DMSO	dimethyl sulfoxide
dNDT	deoxynucleotide diphosphate
dNMP	deoxynucleotide monophosphate
dNTP	deoxynucleotide triphosphate
dsDNA	Double stranded DNA
DNA	Deoxyribonucleic acid
E	In χ^2: Expected number
Ebp	Endocarditis and biofilm-associated pili
ECM	Extra cellular matrix
Esp	Enterococci surface protein
EtBr	Etidium Bromide
EXO	Exonuclease
G	Glycine in peptides
	Gravity in centrifugation
G/C	Guanine/cytosine content

GI	Gastrointestinal tract
His	Histidine
IgX	Immunoglobulin, x denotes M, D, A, G or E
kDa	Kilo Dalton
LB	Luria-Bertani media
LPXTG	Leucine- Proline- any amino acid- Threonine- Glycine
µg	Micro gram
µl	Micro litre
MRSA	Meticillin resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface cell recognition adhesion matrix molecules
N	In DNA sequence: Any nucleotide
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
ng	Nano gram
Ni	Nickel
O	In χ^2: Observed number
OD	Optic density
PBP	Penicillin binding protein
PCR	Polymerase Chain Reaction
PFGE	Pulsfield gel electrophoresis
PGC	Pili gene cluster
RBS	Ribosome binding site
RNase	Ribonuclease
rpm	Rotations pr minute
SAP	Shrimp Alkaline Phosphatase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ssDNA	Single stranded DNA
T	Threonine in peptides
TBE	Tris/Borate/EDTA
TBS	Tris buffered saline
TBST	TBS with triton

TE	Tris EDTA
UV	Ultra violet
V	Volt
VBNC	Viable but not culturable
VRE	Vancomycin resistant enterococci
VREfm	Vancomycin resistant *Enterococcus faecium*

Introduction

Enterococci

Enterococci were first described as a group in 1984 when it was discovered that *Streptococcus faecium* and *Streptococcus faecalis* did not belong to the streptococci. Today over 20 species of enterococci are described [Franz et al. 1999, Klein 2003). The enterococci are gram-positive, facultative anaerobic, lactic acid bacteria. They are catalase negative, and belong to the low G/C content of the genra *Firmicutes*. The normal habitat for enterococci is the gastro intestinal tract (GI) of mammals and humans, and soil [Franz et al. 1999, Foulquie Moreno et al. 2006]. The enterococci occur as single cells, in pairs or as short chains, the optimum growth temperature is 35˚C, but they can grow at temperatures between 10˚C and 45˚C. Enterococci can even survive at 60˚C for 30 minutes [Franz et al. 1999, Foulquie-Moreno et al. 2006, Top et al. 2008]. They grow in broth containing 6,5% NaCl, and hydrolyse esculin in the presence of 40% bile salts [Foulquie Moreno et al. 2006]. The enterococci are also able to survive extreme temperature and chemical disfectants like chlorine, glutaraldehyde and alcohol [Kearns et al. 1995, Bradley & Fraise 1996]. In addition to these harsh conditions enterococci can survive in environments with little nutrients by entering a viable but non-culturable (VBNC) state, where the cells are metabolically active and can resume a normal state when the environment are back to normal [Fischetti et al 2006], an example of such an environment are the treated root canals in teeth which are treated with harsh chemicals and are a poor nutritional environment [Pinheiro et al. 2003], and tap water were the enterococci are exposed to a environment which is low on nutrients and thereby causes nutritional stress on the cells [Byrd et al. 1991, Hartke et al.1998].

28 enterococci species are known [Foulquie-Moreno et al. 2006]. There has been an attempt of dividing these species into enterococci species groups based on 16S rRNA data [Franz et al. 1999, Klein 2003, Foulquie-Moreno]. In 2003 Günter Klein divided the then 22 described species into 7 different groups:

1) *E. faecalis* group: *E. faecalis, E. haemoperoxidus* and *E. moravinensis*.
2) *E. faecium* group: *E. faecium, E. durans, E. hirae, E. mundtii, E. porcinus* and *E. villorum*.
3) *E. avium* group: *E. avium, E. pseudoavium, E. maoldoratus* and *E. raffinosus*.
4) *E. casseliflavus* group: *E. casseliflavus, E. gallinarum* and *E. flavescens*.
5) *E. cecrum* group: *E. cecrum* and *E. columbae*.
6) *E. dispar* group: *E. dispar* and *E. asini*.
7) *E. saccharolyticus* group: *E. saccharolyticus* and *E. sulfureus*.

According to molecular data *E. solitarius* belongs to the Tetragenococci [Klein 2003], *E. solitarius* is mentioned as enterococci in 2006 by the Foulquie-Morenos group [Foulquie-Moreno et al. 2006]. Other enterococci species that does not belong to any group: *E. gilvus, E. pallens, E. ratti* [Klein 2003, Foulquie-Moreno et al. 2006], *E. canis, E. phoenoculicola* and *E. saccharominimus* [Foulquie-Moreno 2006]. *E. faecium* and *E. faecalis* are the enterococci that are most often associated with infections in humans.

Enterococci are opportunistic bacteria, and when found in niches they do not normally live in, such as in the urinary tract, heart giardi cells, vagina, blood and mouth, they can cause serious infections like bacteraemia, endocarditis and urinary tract infections [Donelli et al. 2004]. There are two ways enterococci can cause infections. In one way infections are caused by the enterococci that originate from the patients native flora, these enterococci are unlikely to possess acquired antibiotic resistance, but possess intrinsic resistance that is normal for the species. The other type of infections is caused by bacteria that often possess acquired resistance to antibiotics, in addition to the intrinsic, and is capable of nosocomial transmission. Spread of enterococci between patients probably goes via hands of health-care providers and medical devices, while spread between hospitals is caused by patients with prolonged intestinal colonization [Huycke et al. 1998]

Clinical infections can involve almost any anatomic site, and may be life-threatening during bacteriemia and endocarditis, the features of infections are variable [Murray 1990, Lu et al. 2002]. Resistance to vancomycin in enterococci more than doubles the odds for

dying during bloodstream infections by VRE [DiazGrandos et al. 2005]. In one large study, enterococci were the only Gram-positive pathogen independently associated with high risk of death in bloodstream infections [Weinstein et al 1983]. Enterococcal bacteriemia leads to death in 12%-68% of the cases, with death due to enterococcal sepsis in 4%-50% of these cases [Jett et al. 1994].

Use of Enterococci

Enterococci are usually considered heavy fermentors due to their lack of Krebs cycle and respirator chain [Huycke et al 1998], and because of this enterococci have been used as fermentors in the food industry.

Enterococci are present in many sorts of food, especially food that origin from animals, due to the presence of enterococci in the GI of animals. Historically isolation of *E. faecium* and *E. faecalis* from food has been an indication of faecal contamination in the production line of the food [Klein 2003]. Today enterococci are considered a natural part of the normal microbial flora of food [Klein 2003].

Because of their ability to produce bacteriocins, and their organoleptic[1] properties, enterococci are used in the food industry as e.g. in the cheese industry were they might contribute to the ripening and aroma of the cheese [Foulquié Moreno et al. 2006]. The levels of enterococci in cheese curds lies between 10^4 and 10^6 colony forming units (cfu)/g, while in the ripened cheese the cfu/g is between 10^5 to 10^7, *E. faecium* and *E. faecalis* are the dominant species [Foulquié Moreno et al. 2006]. In some cheeses, like Mozzarella, the enterococci are the predominant microorganism used [Foulquié Moreno et al. 2006].

The levels of enterococci varies from cheese to cheese as a result from the production season, the extent of contamination of the milk and water used, the survival in the dairy

[1] Sensory properties of a product including: taste, color, smell and consistency/ how it feels. Organoleptic testing is done by visually inspection, feeling and smelling the product.

environment, and survival and growth under the particular conditions of the cheese production and ripening [Foulquié-Moreno et al. 2006].

When a bacterial strain is to be used as a probiotic, live microbial feed supplements that benefit and improve the host intestinal microbial balance, they have to be able to adhere to cells and exclude or reduce the adherence of pathogenic adherence, they have to persist and multiply, and produce acids, hydrogen peroxide and bacteriocins antagonistic[2] to pathogen growth. The strains have to be safe, in the meaning of being non-invasive, non-carcinogenic and non-pathogenic, and it has to contribute to the formation of a balanced normal flora [Salminen et al. 1996]. Both *E. faecalis* and *E. faecium* have been used as probiotics. *E. faecium* SF68 has been clinically effective in the prevention of antibiotic associated diarrhea in children, and has made the duration of diarrhea in adults, it has also been used as feed probiotic, in dry dog food, and in milk in Denmark [Foulquié-Moreno et al. 2006], which shows the broad use of probiotics in society.

The uses of enterococci as probiotics are controversial. While some strains benefit the host, the emergence of antibiotic resistant strains, and the increase infections in humans caused by antibiotic resistant enterococci, especially VRE, has lead to concern regarding the use of enterococci as probiotic [Folquié-Moreno et al. 2006].

Virulence of Enterococci

Enterococci have failed to cause chronic or sever infections after inoculation into subcutaneous tissue or peritoneal cavity in laboratory animals, which has been used as the main argument of enterococci being non-pathogen organisms [Jett et al. 1994], despite this the enterococci have emerged as a major nosocomial pathogen over the last decades.

Known and putative virulence factors in enterococci are: Cytolysin, pheromones, aggregation substance (AS), protease, lipoteiochoic acid, hylauronidase, AS 48, hemolysin, gelatinase [Jett et al. 1994], enterococci surface proteins (esp), adhesion to

[2] Chemical substance that interfere with the physiological action of another chemical

collagen from *E. faecalis* (Ace) (Budzik & Schneewind 2006), biofilm production, ebp [Singh et al. 2006], MSCRAMMs [Sillanpää et al. 2004], resistance to phagocytosis by neutrophils [Arduino et al. 1994, Rakita et al. 1999], secreted antigens SagA [Teng et al. 2003] and Acm [Nallapareddy et al. 2003], quorum sensing [Nakayama et al 2001], and production of hydrogen peroxide [Moy et al. 2004].

In addition enterococci are known to transfer mobile genetic elements that often contain genes for antibiotic resistance and/or other virulence factors to other enterococci species, Gram-positive and Gram-negative species [Donelli 2004]. Antibiotic resistance is considered as an important pathogenicity property of enterococci [Foulquié Moreno et al. 2006].

Antibiotic Resistance

Antibiotic resistance is often caused by antibiotic pressure in the environment of the bacteria, and the occurrence of antibiotic resistance is more frequent in hospital environment than in the community. A study from Germany showed that antibiotic resistant *E. faecalis* in the community was highly associated with recent intake of antibiotics, but resistance to quinolones in *E. faecium* was more common and not associated with recent use of the antibiotic [Lietzau et al. 2006]

Main mechanisms for antibiotic resistance are inactivation of the drug, prevention of the drug to reach its target site, reduction of the target susceptibility, and acquisition of a new less sensitive target [Berger-Bächi 2002]. Enterococci harbour both intrinsic and acquired drug resistance. A new drug, tigecycline, with anti-enterococci activity can be used to treat infections caused by enterococci [Pankey 2005].

Intrinsic drug resistance is due to mechanisms that are natural for the organism, and the drugs enterococci have intrinsic resistance to vary among the species. Enterococci can harbour intrinsic resistance against new drugs like *E. faecalis* resistance to quinopristin-dalfopristin, community reservoir in US and Europe have emerged due to the widespread use of the analogue virginiamycin as growth promoter [Acar et al. 2000].

Acquired drug resistance is often due to mutations, and exchange or acquisition of genetic mobile elements such as transposones and plasmids. The genetic mobile elements often contains the genes for other virulence factors instead of resistance to antibiotics, or in addition to, and enterococci can transfer these elements to both Gram negative and Gram positive bacteria, which make them even more potent. *E. faecium, E. hirae* and *E. faecalis* are known to acquire high level resistance to ampicillin by overproduction of PBP5 or in some cases production of β-lactamase [Top et al. 2008].

Biofilm

Enterococci are known to produce slime, an amorphous extracellular substance made of polysaccharides, and is one of the major components of bacterial biofilm [Donelli et al. 2004], which is the term used about microbial colonies encased in adhesive material, i.e. polysaccharide, attached to a surface [Madigan et al. 2003]. Biofilm protects the microorganisms from host defence and some antimicrobial drugs [Merode et al. 2006] and is an important factor in the attachment of enterococci to surfaces and other cells; it is one of the virulence factors in many pathogenic enterococci strains [Hufnagel et al. 2004]. The composition of the growth media has an influence on the production of biofilm by the enterococci, the presence of additional carbohydrates or de depletion of iron favour the biofilm production [Baldassarri et al 2001]. Other factors that favour the production of biofilm are the transcriptional regulator BopD [Hufnagel et al. 2004], the gene products of the quorum sensing locus *fsr* and gelatinase (GelE) [Hancock & Perego 2004] and ebp [Singh et al 2006].

In clinical strains of *E. faecium* slime production is more frequent than in environmental strains and isolates from healthy individuals, and because of this biofilm is thought to be one of the virulence factors of enterococci [Donelli et al. 2004]. Compared to Streptococci, enterococci are poor producers of biofilm.

Aggregation Substance

”Aggregation substance” (AS) is regarded as one of the main virulence factors in enterococci [IsenMann et al. 2002], and is described in both *E. faecalis* and *E. faecium* [Donelli et al. 2004]. AS is a plasmid encoded surface protein that is expressed as a response to specific pheromones, and this lead to the promotion the formation of mating pair and conjugative transfer via the sex pheromone system [Donelli et al 2004].

Enterococci expressing AS are frequently found among clinical isolates, and expression of AS promotes enterococci adhesion to cultured human cells, this is either directly or indirectly by increasing the number of organisms taken up as a clump [Donelli et al. 2004, IsenMann et al. 2002], and it significantly enhance *E. faecalis* adherence to immobilized ECM [IsenMann et al. 2002]. Expression of AS also promotes the invasion of enterococci into different cell types, including macrophages [IsenMann et al. 2002].

Cell Wall Associated Proteins

Cell wall anchored proteins (CWAPs) exposed on the surface are used by bacterial pathogens to interact with other cells, and potentially mount a successful infection of the host [Dramsi et al. 2008]. Cell wall sorting signals (CWSS) is needed for an efficient sorting to the cell wall; the CWSS are N-terminus leader peptides. CWSS in Gram-positive bacteria are generally longer, more hydrophobic and more charged at their N-terminus than their counterparts in Gram-negative bacteria [Dramsi et al. 2008]. The C-terminus of the CWAPs consists of an LPXTG motif, and hydrophobic domain and a positively charged tail; these characteristics are also needed for an efficient sorting to the cell wall [Dramsi et al. 2008]. The LPXTG motif of CWAPs are typically considered LPXTG sortase substrate, after the translocation over the plasma membrane CWAPs are anchored to the cell wall by a the transpeptidase activity in the sortases [Hendrickx et al. 2007].

Proteins exposed on the cell surface are frequently considered as potential virulence factors [Desvaux et al. 2006], esp and acm are examples of CWAPs found in *E. faecium*

and associated with the formation of biofilm and better adherence to ECM resulting in a possible selective advantage of *E. faecium* in the hospital environment [Hendrickx et al. 2007].

Enterococcus faecium

Together with *Enterococcus faecalis*, *Enterococcus faecium* is the most common enterococci in clinical isolates from human infections. While the numbers of infections caused by *E. faecalis* and other human pathogen enterococci are stable, the infection rate of *E. faecium* is increasing [Iwen et al. 1997, Top et al. 2007].

E. faecium is often responsible for urinary tract infections, surgical site infections, bacteriemia and endocarditis. *E. faecium* is the enterococci most often associated with increasing resistance to different classes of antibiotics such as penicillins, aminoglycosides and glycopeptides [Hendrickx et al. 2007].

Geographic Distribution

Ampicillin resistance in enterococci is due to penicillin binding proteins (PBP) with low affinity for β-lactam, high level of resistance is often associated with *E. faecium*. In the US the first reports of infections caused by ampicillin resistant *E. faecium* was reported in the early 1980s. The same was the case for Europe- 10 years later [Top et al. 2007].

In 1986 the first cases of glycopetide resistant enterococci were reported in Europe, and it spread all over the world. Infection caused by vancomycin resistant enterococci (VRE) in the US increased from 0% in 1989 to 28,5% in 2003, and in the 1990s VRE had become the second most common nosocomial pathogen, and were endemic in many hospitals around the US. [Top 2007]

The differences of occurrence of enterococci the US and Europe are severe. In the US colonization of VRE in hospitalised patients increased rapidly in the 1990s to reach the

present levels. In Europe the prevalence rates in hospitals remained much lower until 2000, when it started to increase. The suggested reason for this is the much higher use of vancomycin in US hospitals than in European hospitals, a comparison made between US and European countries with similar numbers of inhabitants [Top 2007]

In Europe there is a large reservoir of VRE in healthy people and animals in the community. This is not the case for the US. The reason for this is the massive use of avoparcin, a glycopetide with the same function as vancomycin, as a growth promoter in animal husbandry in most European countries since the 1970s. After the ban of avoparcin use in the food industry the number of healthy animal and humans with VRE decreased. In the US vancomycin has been strictly restricted to hospitals, and was not used in the agricultural industry, and the reservoir in healthy humans and animals have therefore not been a problem in the US [Top 2007].

Population Structure

Isolates from humans, pigs and poultry have shown specific genetic lineages, poultry and pigs tend to cluster in distinctive branches [Willems et al. 2000, Leavis et al. 2006]. Vancomycin resistant *E. faecium* from human volunteers cluster together with pig isolates, while vancomycin resistant *E. faecium* from documented nosocomial outbreaks and infections clustered in another distinct branch, showing the existence of host specific lineages and distinct genetic a distinct genetic subpopulation of enterococci representing clinical and hospital outbreak isolates [Willems et al. 2000, Leavis et al. 2006]. Figure 1 shows the distinct branch made up of the clinical isolates.

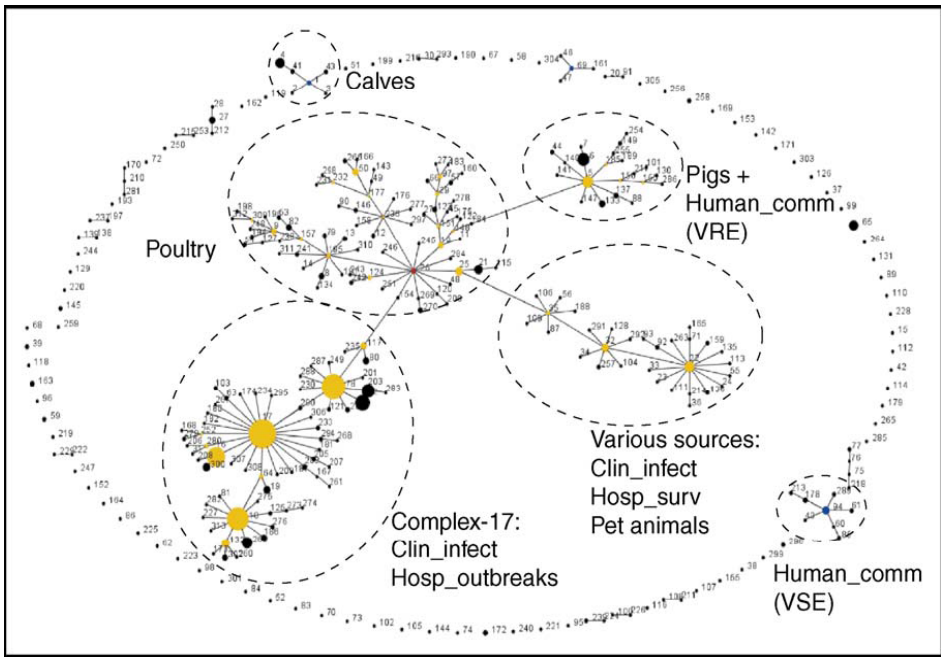

Figure 1- The figure shows the population structure of enterococci. [Leavis et al. 2006]

Clonal Complex 17

Successful clonal complexes have adapted to the environment through a series of mutations and acquisition of genetic elements giving the bacteria a selective advantage in the selective pressure it is exposed to. Such acquired properties can be resistance to antibiotics, increased virulence e.g. due to new MSCRAMMs, increased production of biofilm and increased ability to use alternative metabolic pathways [Hendrickx et al. 2007, Leavis et al. 2007, Top et al. 2007].

Many clinical *E. faecium* isolates contains clonal complex 17, these *E. faecium* are called *E. faecium* CC17 isolates, or just CC17 isolates. There are two key features of CC17 isolates; the first is high resistance to antibiotics like ampicillins and ciprofloxacins. The other key feature is the presence of putative pathogenicity islands harboring the esp and acm genes and probably the hyl_{Efm} [Hendrickx et al. 2007]. These features of CC17 isolates suggest that they might be more virulent than non-CC17 isolates, and contribute to selective advantage in hospital settings [Hendrickx et al. 2007]. Other features

associated with CC17 are ampicillin resistance and higher levels of quinolone resistance, which is also the characteristic of globally spread CC17 [Leavis et al. 2006].

The adaptation of CC17 to hospitals is probably a multistep process involving the sequential acquisition of adaptive mechanisms that gives *E. faecium* CC17 a selective advantage over non-CC17 *E. faecium*, and thus leading to the acquisitions of more selective advantages, eventually leading to a specialized genetic subpopulation that have a greater chance of surviving and spreading in the hospital environment [Leavis et al. 2006, Hendrickx et al. 2007].

It has been suggested that *E. faecium* may enter the community during the food chain [Lu et al. 2001], humans and animals in close contact seem to harbor identical strains of *E. faecium*. Transfer between poultry and pigs seem to be less common, but both poultry and pigs can be the strain source of *E. faecium* in humans. Primary transmission is suggested to be from animal to human, not human to animal. [Lu et al. 2001]. A study performed in China after an outbreak of *E. faecium*-related sepsis in both humans and pigs in 1998 were 12 out of 40 infected humans died [Lu et al. 2001]. The PFGE patterns of the isolates obtained from both humans and pigs indicated that the isolates were clonal [Lu et al. 2001].

Pili

Pili in Gram-positive bacteria were first described by Yanagawa in 1968 in Croynebacteria, and a while later in streptococci that inhabits the oral cavity [Scott and Zähner 2006]. Pili-like structures have been indentified in electron microscopy in Croynebacteria, streptococci, enterococci and pneumococci and has been characterized both genetically and biochemically [Ton-That & Schneewind 2003, Lauer et al. 2005]. The structures have been found to be extended polymers formed by a transpeptidase reaction performed by a specific sortase that cross-link the LPXTG motifs in the subunits. The sortases have also been found to be responsible for the covalent attachment to the peptidoglycan in the cell wall [Barocchi et al. 2006]. The typical heterodimeric pili are

composed of one major subunit that forms the backbone of the pili structure, one minor subunit and a subunit forming the tip of the pili [Hendrickx et al. 2008].

One model for pili in Gram positives is the pneumococcal pili. The genes encoding the pili subunits and its sortases are found to be in an rlrA islet containing seven genes [Barocchi et al. 2006]. Three of these genes, rgrA, rgrB and rgrC, are thought to encode LPXTG containing microbial surface components recognizing adhesive matrix molecules, so called MSCRAMMs that bind to components of the ECM of the host. RgrB is thought to be the main pili subunit; this is based on the result of antibody labelling to the pili structure. Antibodies for rgrB protein labelled the whole pili structure, whereas antibodies for rgrA protein labelled the cell surface indicating that this protein associates the pili structure to the surface, and antibodies for the rgrC protein decorated the tip of the structure [Barocchi et al. 2006].

Three other genes, srtB, srtC and srtD, are predicted to encode sortases [Barocchi et al. 2006]. A transcriptional repressor, mgrA, is located external to the rlrA islet, and is involved in the regulation of the pili genes.

Gram positive can use pili in the attachment to the host target cell; one model for such an attachment is [Telford 2006]: The initial contact with the target cell or extracellular matrix (ECM) might be facilitated through an AP2 component on the tip. The properties of main pili (and/or AP1) components will then interact with the host target cell and the bacteria will be drawn closer to the host cell. This would make it possible for AP1 and other non-pili adhesins on the bacterial surface to interact with the receptors on the host cells, the result of this would be intimate attachment to and colonization of the host cells and tissue.

Expression of pili bacteria might lead to the initial bacterial adherence to host cells, and in this way promote colonization of e.g. the nasopharynx [Barocchi et al. 2006]. Colonization by pneumococci expressing pili structures more often cause mucosonal

infection, this might be triggered by the pili structures, and the structures can aid the pneumococci in a potentional invasion of the colonized tissue [Barocchi et al. 2006].

Pili has also shown to promote the aggregation of other bacteria, this might help the bacteria to colonize tissues and other surfaces, make the bacteria involved more resistance against host defences and allow beneficial interactions between the bacteria involved [Telford 2006].

Pili from enterococci, and other Gram positive, are ideal candidates for vaccines due to their exposure on the cell surface. Sera from infected patients have been showed to contained antibodies against various surface proteins, among them enterococci pili proteins, which indicate that infection, lead to natural production of antibody response to enterococci pili. These pili based vaccines cannot be used as general vaccines because of the lack of pili in some pathogen strains [Mandlik et al. 2007].

Pili in *E. faecium* has been suggested to be enriched in hospital-acquired environment, indicating that expression of pili might be a selective advantage in this environment, and may promote pathogenesis for *E. faecium* [Hendrickx et al. 2008].

Types of Pili

In Gram-positives two types of pili have been identified; Fibrils and Pili [Telford et al. 2006]. Pili are recognized as flexible rods with a length of 0,3-3µm and a diameter of 2-10nm. There are 2-3 types of pili proteins and 1-4 assembly components, in some species the assembly is sortase-mediated. The function of the pili is host tissue adhesion, co-aggregation and immunomodulation [Telford et al. 2006, table 1].
The fibrils are 0,07-0,5µm long and has a diameter of 1-2nm, they consists of 2 pili proteins and have the same function as the pili, except for the immunomodulation [Telford et al. 2006, table 1].

In *E. faecium* PilA has been observed in 40% of the cells, the structure appears as relatively short thin rigid appendages highly expressed at the poles of dividing cells [Hendrickx et al.. 2008]. Another pili has been identified in *E. faecium*, the PilB pili, which is longer, more flexible, and thicker than the PilA pili structure, it has been observed at the poles of the cells, and in single cocci, and PilB are less frequent than PilA [Hendrickx et al. 2008].

Assembly of Pili

There are to models for pili assembly in gram-positive, both are distinctively different from how the pili assembly in Gram-negative bacteria, and the first model is supposed to be more likely than the second model due to the energy requirements [Telford et al.. 2006]. Both models explain how the pili are thought to be composed of several proteins that are covalently linked into a chain [Scott and Zähner 2006, Telford et al.. 2006].

Assembly model 1, shown in figure 2:
This model contains four steps that explain the assembly process which is mediated by the sortase catalyzing transpeptidase reactions [Scott & Zähner 2006, Telford et al. 2006] and it suggests that the growth happens from the bottom of the structure:

1. Sec dependent secretion of the three pili proteins, which all contains the LPXTG (Leucine- Proline- any amino acid- Threonine- Glycine) motif and a C-termini membrane spanning domain that anchor the protein to the cell membrane.
2. Cell-anchored proteins are cleaved by a sortase-dependent reaction between the threonine (T) and glycine (G) residue in the LPXTG motif. The result is acyl-enzyme intermediates where covalent thioester bonds are formed between the thiol groups of the cysteine residue located in the catalytic site of the sortase enzyme and the carboxyl group of the threonine residue in the LPXTG motif in the pili protein.
3. and 4. oligomerization of the pili protein subunits and the anchoring of the pili to the cell wall. These two steps happen due to a nucleophilic attack of the thioester

bond that links the threonine residue in the pili protein subunit to the cysteine residue in the sortase.

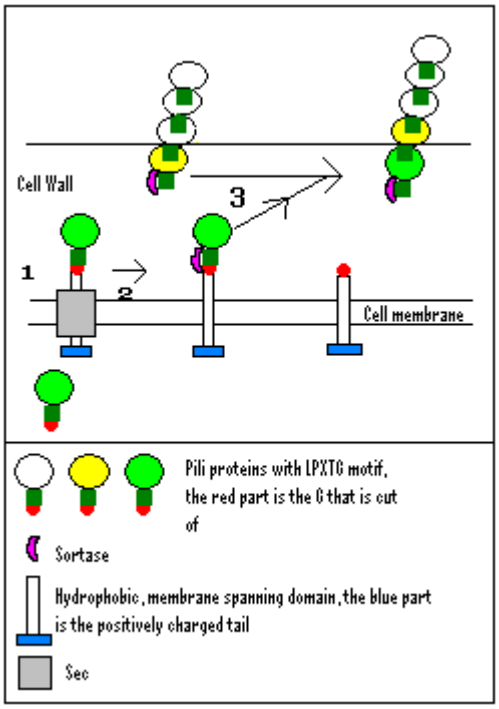

Figure 2- showing the pili assembly in Gram-positives. 1) The sec dependent secretion of the pili protein over the plasma membrane. 2) Sortase mediated cleavage between the T and G in the LPXTG domain, leaving the G, the hydrophobic domain and the positively charged tail behind. 3) Oligomerization and anchoring of the structure to the cell wall.

The second model suggests that new pili proteins are added to the top of the pili, growth in this way will start with the binding of the first pili subunit to the peptidoglycan precursor lipid II. For the addition of a new pili subunit the fiber have to be bent towards the membrane in a process that is thought to require energy to promote an effective bending of the pili and thereby an effective growth of the pili. Even though electron-microscopy analyzes have shown pili that lie on the bacterial surface this model is thought the less likely of the two because, at the present, there are no clues of where the energy for such a bending should come from [Telford et al. 2006].

Sortases and LPXTG motif

LPXTG proteins, derived from the name of the main conserved residues, x denotes any amino acid, is a cell wall sorting motif located at the C-terminal of the surface protein. The LPXTG motif is followed by a hydrophobic domain and a positively charged tail that is thought to allow the recognition of the LPXTG motif by the sortase by keeping the protein from being secreted into the medium [Fischetti et al 1990, Boekhorst et al. 2005].

Sortases are membrane associated transpeptidases that recognize surface protein precursors with a C-terminal cell wall sorting signal (CWSS) containing a pentapeptid followed by a hydrophobic region containing 30-40 amino acids and a charged tail [Scott & Zähner 2006]. The hydrophobic domain and the charged tail anchor the protein to the cell membrane where the sortase is located [Scott & Zähner 2006].

There are four different sortase classes, sortase A, B, C and D, where the main sortases in class A and C recognize and cleave at the LPXTG domain [Dramsi et al.. 2008].

Sortase A class is found in almost all low G/C Gram-positive bacteria, and it is believed to be required for anchoring the majority of all LPXTG-containing proteins. The sortase A class is not clustered with its substrate and is constitutively expressed [Dramsi et al.. 2008].

The sortase C class is found in both high G/C and low G/C Gram-positive bacteria, often represented by several copies in the genome, clustered with the genes for their substrate and associated with mobile genetic elements. Unlike sortase class A, sortase class C is a polymerizing enzymes that are involved in the formation of pili in Gram-positive bacteria, where it catalyzes the formation of the cross-links between two pili subunits [Dramsi et al.. 2008].

Sortase class C cleave the LPXTG domain between the T and the G to form an acyl-enzyme intermediate, the enzyme is released by a nucleophilic attack by an amino group provided by another peptidoglycan precursor for the cell wall anchored proteins, resulting

in cleavage and removal of the C-terminal segment from the cell wall anchored protein and covalent linkage between the protein and peptidoglycan [Dramsi et al. 2008].

Little is known about the Sec pathway in Gram-positive bacteria. The sec pathway, in general, is an exocytic pathway, which means that the cell secrete molecules using this pathway, the pathway coordinates the biosynthesis and secretion of bacterial molecules [Pollard and Earnshaw 2004].

Nomenclature of the Putative Main Pili Genes

Pili in *E. faecium* have been identified *in silico* in four gene clusters, PGC1, PGC2, PGC3 and PGC4 [Hendrickx 2008].

There has been an attempt for establishing names for the presumed main pili genes. Hendrickx et al. [2008] uses *pil*A about the putative main pili in PGC1, and *pil*B about the putative main pili in PGC3.

In this thesis the work has been concentrated on PGC2 and PGC3, the main pili are, from now on, in this thesis called C3:44, which are located in PGC2, and C3:48, which are located in PGC3, and is the same as *pil*B. The screenings have been done on the gene encoding pili1:44 in PGC2, and it will be referred to as pili 1:44. For the screening for PGC3 pili 1:48 was used, and it will be referred to as pili 1:48.

Aims of this Study

The overall objectives with this study were to detect pili structures in *E. faecium* by electron microscopy, and the second aim was to screen for putative pili genes in given invasive and non-invasive strains of *E. faecium*.

The work was defined into five steps:

1) To design PCR tools for amplification of putative pili coding sequences from *E. faecium*.
2) To perform TOPO-cloning of amplicons into pENTR-vector by directional insertion.
3) To purify the putative pili proteins by affinity chromatography and inject rabbits for production of polyclonal antibodies.
4) To detect pili by electron microscopy with immunogold labeling.
5) To design PCR tools for screening of pili genes in a collection of *E. faecium* isolates.

Materials

The kits used are in table 1

Table 1- Kits used in this thesis.

Provider	Catalogue nr	Kit
E.Z.N.A	D6945-02	Plasmid Mini Kit II
E.Z.N.A	D6943-02	Plasmid Mini Kit I
Invitrogen	K2420-20	pENTR™ Directional TOPO® Cloning Kits
Invitrogen	11824-026[1)]	*E. coli* Expression System with Gateway® Technology
Invitrogen	LC6033	InVision™ His-Tag In-gel Stain

1) There are several kits covered in the protocols, catalogue number is registered for the kit used in this thesis.

Chemicals used in the experiments are in table 2; this table also includes enzymes, buffers and antibodies ordered from an external provider.

Table 2- Chemicals, enzymes and buffers used in the work for this thesis.

Provider	Catalogue nr[1)]	Enzyme/ Chemicals
Invitrogen	11791-020	Gateway LR Clonase Enzyme Mix
USB	-	Exonuclease I (Exo)
USB	-	Shrimp Alkaline phophatase (SAP)
Invitrogen	NP0007	NuPAGE LDS Sample Buffer
Invitrogen	NP0005	NuPAGE Antioxidant
Invitrogen	NP0005	Antioxidant
Roche	13279400	25mM MgCl2
Thermo Scientific	AB0575/DC/LD/B	ReadyMix PCR mastermix
Sigma	092K8930	EtBr
Applied biosystems	-	BigDye 3.1
Applied biosystems	-	BigDye Sequencing Buffer
Qiagen		Penta-His HRP Conjugate
Sigma Aldrich	EC200-664-3	DMSO
Arcus Kjemi	600068	Absolute Alkohol Prime
BDH laboratory supplies	663684B	Tween 20
BDH laboratory supplies	306324N	Triton x-100
Invitrogen	NP0002	NuPAGE Running buffer 20x

1) The lab has replaced Exo and SAP with a ready to use ExoSAP, catalogue number for the products depends on the volume of the enzymes, and this also applies on the BigDye 3.1 and BigDye Sequencing buffer.

Molecular Weight Standards

Table 3 gives an overview over the used molecular weight standards, figure 3 and 4 shows the band sizes of 1kb+ and MagicMarker molecular standards, and these were the molecular standards used to check the size of the PCR product and protein, respectively.

Table 3- molecular weight standards used.

Provider	Catalogue nr	Marker
Invitrogen	LC5677	Mark12 Unstained Standard
Invitrogen	LC5602	MagicMarker XP Western Standard
Invitrogen	LC5925	SeeBlue 2 Marker
Invitrogen	10787.026	1Kb+ molecular weight standard

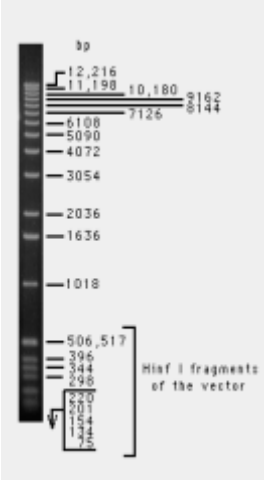

Figure 3 - 1kb+ molecular weight standard, figure from invitrogen.com.

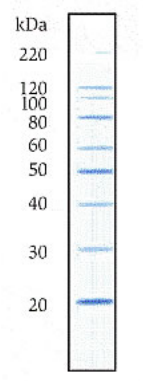

Figure 4- MagicMarker Molecular weight standard from Invitrogen. Picture from invitrogen.com. The bands are visualized with comassie blue.

Vectors and competent cells

The competent cells and vectors used in this thesis are in table 4.

Table 4- The competent cells and vectors used.

Provider	Catalogue nr	Cells/ vectors
Invitrogen	C6070-03	OneShot BL21-AI Chemically competent cells
Invitrogen	Provided by kit K2420-20	OneShot TOP10 Chemically competent cells
Made in the department	-	DH5α Chemically Competent cells
Invitrogen	Provided by kit K2420-20	pENTR entry vector
Purified from cells	-	pDEST17 expression vector

DH5α chemically competent cells are suitable for maintaining plasmids over time due to their *rec*A1 and *end*A1 mutations which increase insert stability and quality of plasmids purified by minipreps. In addition to this DH5α cells can be used in blue white screening due to the presence of lacZΔM15 promoter [invitrogen.com].

OneShot TOP10 chemically competent cells allow stable replication and maintaining of plasmids, they take up unmetylated DNA, like PCR products, effectively during transformation. They can also be used in blue-white screening due to the presence of lacZΔM15 promoter [invitrogen.com].

OneShot BL21-AI™ Strain is designed for expression of recombinant proteins from the T7 promoter, the expression from this promoter can be tightly regulated by L-arabinose and glucose [*E. coli* Expression with Gateway® Technology protocol].

The pENTR™/SD/D-TOPO® vector contains a gene 10 translational enhancer, a ribosome binding site (RBS), a T7 promoter/priming site, M13 forward (-20) and reverse priming sites, attL 1 and 2 and NotI [pENTR™ Directional TOPO® Cloning Kits], as indicated in figure 5. The figure also shows the location of selective gene, kanamycin resistance, origin of replication and T2 and T1.

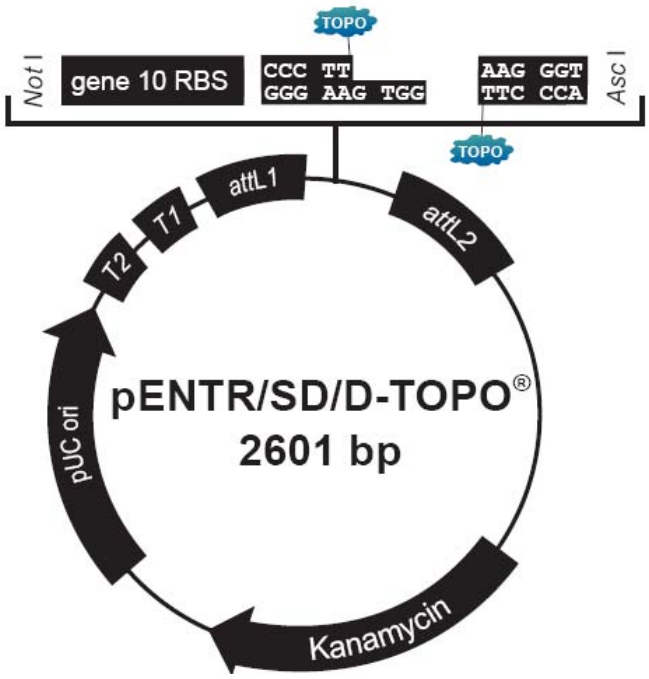

Figure 5- The pENTR/SD/D- TOPO® vector [http://www.emblhamburg.de/~geerlof/webPP /genetoprotein/cloning_strategy/gateway/ENTR_vectors/pentrsd_dtopo_map.pdf]

The sequence in figure 6 was used to make primers for the cloning into the vector. The restriction sites are marked, and the darker region will be transferred to pDEST via LR recombination between the vectors.

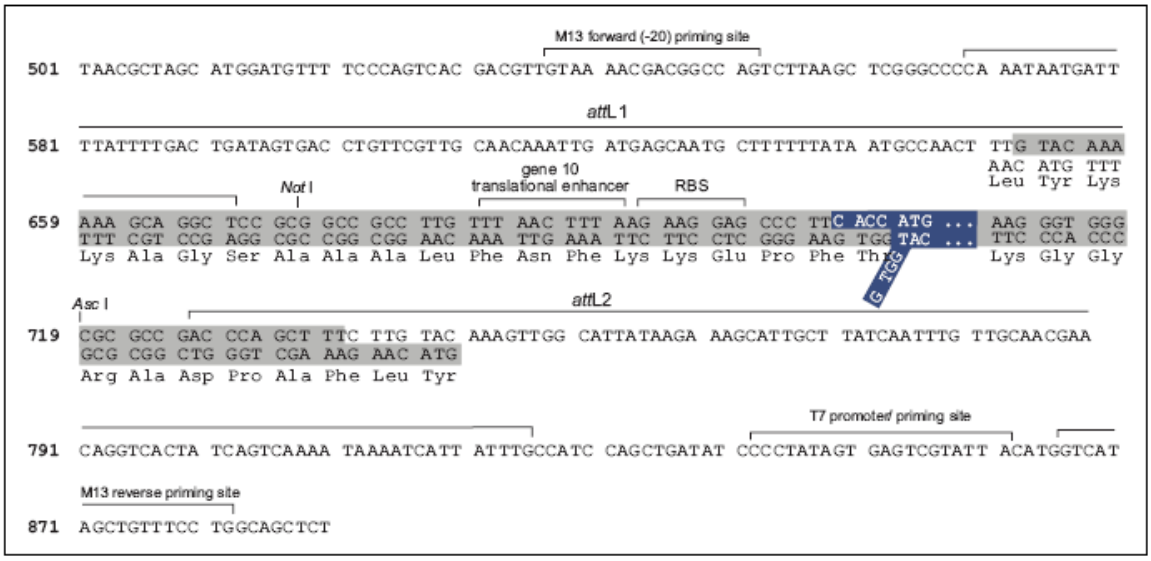

Figure 6- The sequence of pENTR/SD/D-TOPO vector [invitrogen.com].

The pDEST^{TM}17 are N-terminal fusion vectors that contain an ATG initiation codon upstream of the GST and 6xHis tags. Shine-Dalgarno is located upstream for the ATG, and the T7 promoter and transcriptional start is located upstream for the Shine-Dalgarno. The attachment sites, *att*B1 and *att*B2, are located downstream for the 6xHis tags, as indicated in figure 7. The gene of interest is recombined in between the two attachment sites. Figure 7 also shows the location of the selective gene ampicillin resistance, origin of replication (ori) and rop. [*E. coli* Expression System with Gateway® Technology]

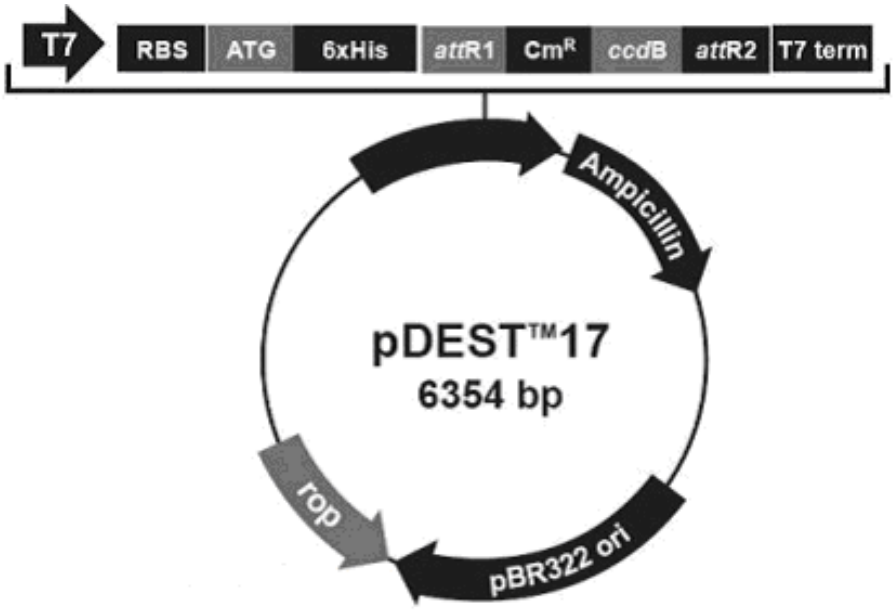

Figure 7- The pDEST^{TM} vector [http://ecoliwiki.net/colipedia/images/thumb/5/5f/Pdest_17.jpg/440px-Pdest_17.jpg]

The gene of interest is inserted in the darker area; figure 8 shows the sequence of pDEST17 vector.

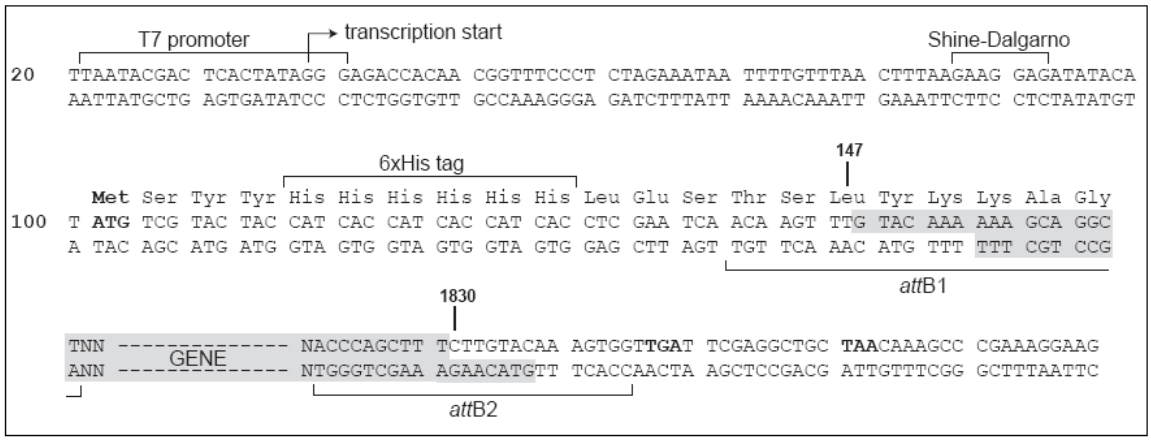

Figure 8- The sequence of the pDEST17 vector [invitrogen.com]

Buffers

Blocking buffer (20 ml)[3]:

Blocking reagent	0,1 g
Blocking reagent buffer	20 ml
Heat to 70ºC	
10% Tween 20	200 µl

Blotting buffer (10X)

Glycine	144 g
Tris base	30,3 g
Distilled water to 1 L	

Blotting buffer (1X)

Blotting buffer 10X	50 ml
Deionised water	450 ml

[3] See QIAexpress Detection and Assay handbook 10/2002 protocol 7, Immunodetection with Anti-his Antibodies or Anti-His HRP conjugate (Chemiluminicent method)

Fixing solution for NuPAGE Novex gels

Ethanol	40ml
Acetic acid	10ml
Distilled water to	100ml

Lysis Buffer

Potassium phosphate, pH7,8	50 mM
NaCl	400 mM
Glycerol	10%
Triton X-100	0,5%
Imidazole	10 mM

NuPAGE MES running buffer 1X

NuPAGE MES Running buffer (20X) [Invitrogen catalogue number NP0002]	40 ml
Distilled water	760 ml

Phophate buffer, 20mM

Sodium phosphate (monobasic)	2,6g

Adjust pH to 7,8 with 3M NaOH

Distilled water to 1000ml

TBS (10X)

Trizama HCl	24,3 g
NaCl	80,06 g
Distilled water	800 ml

pH adjusted to 7,6

Distilled water to 1 L

1X TBST

TBS 10X	100ml
Deionised water	899ml
Tween 20	1ml

TBST + Triton (0,5 L)[4]

TBST	0,5 ml
Triton x-100	1 ml

TE buffer

1 M HCl pH8	20 ml
0,5 M EDTA	4ml
Distilled water up to 2 L	

[4] See QIAexpress Detection and Assay handbook 10/2002 protocol 7, Immunodetection with Anti-his Antibodies or Anti-His HRP conjugate (Chemiluminicent method)

Media

Blood Agar

Blood Agar Base Oxoid CM 271	40 g
Distilled water	1 L

LB medium and plates

Tryptone	1,0%
Yeast extract	0,5%
NaCl	1,0%
pH	7,0

For LB medium and plates with antibiotics:

Kanamycin	50 µg/ml
Ampicillin	100 µg/ml

S.O.C.

Deionised water	950ml
Tryptone	20g
Yeast extract	5g
NaCl	0,5g
Glucose	20mM

Primers

The primers used are in table 5, the reverse primers are showed as the primer sequence and as reverse complement.

Table 5 - Primers used in the experiments.

Primer	Sequence	Reverse complement of reverse primers
Pili 1:44 F	CGGGAGCTCAAGACGGAA	-
Pili 1:44 R	TAACTGATAACGTGGTGGTCCA	TGGACCACCACGTTATCAGTTA
Pili 2:44 F	AACCGAGGAACTGCAAGAGA	-
Pili 2:44 R	GCGGATTCATAGGCAGAGAC	GTCTCTGCCTATGAATCCGC
Pili 3:44 F	ATGAAGCTGGAGAAGCGGTA	-
Pili 3:44 R	CGCTTCCGTTTTCAAGGACA	TGTCCTTGAAAACGGAAGCG
Pili C3:44 F	CACCATGAGTGTACTGTTACTCGTTACTG	-
Pili C3:44 R1	TTATACTTCAGACTTCATTTTAGATTTAC	GTAAATCTAAAATGAAGTCTGAAGTATAA
Pili C3:44 R2	TACTTCAGACTTCATTTTAGATTTACG	CGTAAATCTAAAATGAAGTCTGAAGTA
Pili 3:44 FR	GAAAGTCACAACAGGTGGA	-
Pili 3:44 FM	CCAGAATCGCGTCTCCA	-
Pili 3:44 RL	CCATGTAGAACCATCCCACA	TGTGGGATGGTTCTACATGG
Pili 3:44 RM	GGTCTTCATTCCACAATTCGA	TCGAATTGTGGAATGAAGACC
Pili 1:48 F	GAAAATGAGGACTTCCATCCAA	-
Pili 1:48 R	ATCCTTTTCACCTGTAGAGGAA	TTCCTCTACAGGTGAAAAGGAT
Pili 2:48 F	GCACAAGCAGAGATCGCAA	-
Pili 2:48 R	CCGCACTATCTGACAATTCGA	TCGAATTGTCAGATAGTGCGG
Pili 3:48 F	TTTGAACGAAAAAGCAGATCCA	-
Pili 3:48 R	GCCATATTTTAGACCTGTCACA	TGTGACAGGTCTAAAATATGGC
Pili C3:48 F	CACCATGTTAGGAGTCCTTTTCCTTATTT	-
Pili C3:48 R1	CTAAATCTGACTGTGCTTGCG	CGCAAGCACAGCTAGATTTAG
Pili C3:48 R2	AATCTGACTGTGCTTGCGTC	GACGCAAGCACAGTCAGATT
Pili 3:48 RL	TCTAGTTCTTCTGTCCCGTA	TACGGGACAGAAGAACTAGA
Pili 3:48 FM	ACCAAAAGACGGAGTGTCA	-
Pili 3:48 RM	GCAGCGTCATGAGTATCGA	TCGATACTCATGACGCTGC
Pili 3:48 FR	GATCCTGGCAAATAGCCAA	-
M13 F	GTAAAACGACGGCCAG	-
M13 R	CAGGAAACAGCTATGAC	GTCATAGCTGTTTCCTG

Figure 9 gives an overview of the pili operon, the figure is based on the pili operon from the pathogenicity islet in pneumococci

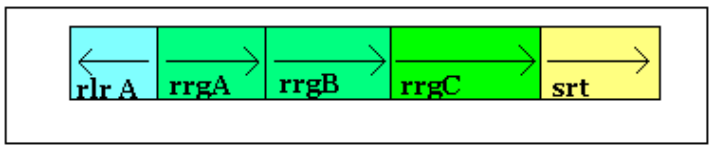

Figure 9- The pili operon in the rlr islet.

Figure 10 shows an overview of the suspected orf of the main pili, it is based on the operon in figure 9 and is the rrgC gene in that figure. The figure includes the primers used for sequencing of the orfs, and each primer pair has the same color. M13 primers were located in the vector, outside the gene.

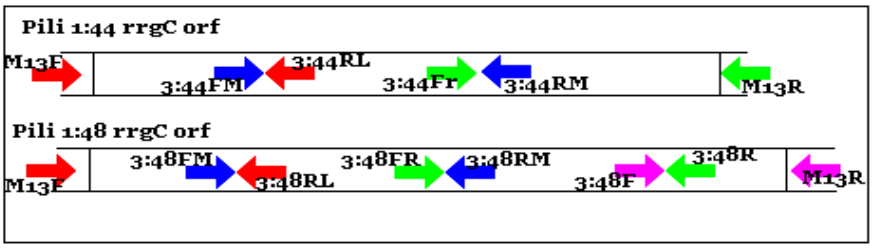

Figure 10- The suspected pili 1:44 rrgC orf with primer localization and the suspected pili 1:48 rrgC orf with primer localization.

Table 6 shows the position of the primer and their function in the experiments, for the Pili 44 primers the position is in operon 44, and for the Pili 48 primers the position is in operon 48 [*Enterococcus faecium* DO JGI 4/6/04 Assembly]

Table 6- The position and function of the primers

Primer Pair Forward/Reverse	Position Forward	Position Reverse	Function
Pili 1:44F/ Pili 1:44R	3038-3055	3387-3408	Screening
Pili 1:48F/ Pili 1:48R	4205-4226	4705-4726	Screening
M13F/ Pili3:44RL	in the vector	7022-7041	Sequencing
Pili 3:44FM/ Pili 3:44RM	6959-6975	7698-7718	Sequencing
Pili 3:44FR/ M13R	7624-7642	in the vector	Sequencing
M13F/ Pili3:48RL	in the vector	7709-7728	Sequencing
Pili 3:48FM/ Pili 3:48RM	7619-7638	8301-8319	Sequencing
Pili 3:48FR/ Pili 3:48R	8104-8122	8803-8824	Sequencing
Pili 3:48F/ M13R	8503-8524	in the vector	Sequencing
Pili C3:44F/ Pili C3:44 R1	6468-6499	8121-8149	Cloning
Pili C3:48F/ Pili C3:48R1	7220-7248	9056-9076	Cloning

Isolates

The isolates used for screening for the pili 1:44 and pili 1:48 genes are noted in table 7. Other strains used were *E. faecium* DO for positive control, *Staphylococcus epidermis* as negative control. *E. faecium* BM 4105RF and *E. faecium* 64/3 were used in the designing of PCR tools.

Table 7- Isolates used for screening, showing the 96 well setup.

Well	Reference number	Other names	Geographic Origin	Sample	Sample Source	Origin	Van Type	Reference
1a	TUH 44-39	399/F99/A9 (248)	Norway/Østfold	Animal	Faeces	Community survey	VanA	Johansen et al. 2003
1b	TUH 44-29	399/F99/H8 (106)	Norway/ Østfold	Human	Faeces	Community survey	VanA	(Johensen et al. 2003)/AMV
1c	TUH 44-34	399/F98/A4 (225)	Norway/ Østfold	Animal	Faeces	Community survey	VanA	Johensen et al. 2003
1d	TUH 7-57	BM4147	France	Human	Faeces	Clinical isolate	VanA	Leclercq et al. 1988
1e	TUH 7-58	BM4147-1	France	Human	Faeces	Curing of BM4147	-	Leclercq et al. 1988
1f	TUH 41-64	U37 (pRUM)	USA/ Ohio	Human		Clinical isolate	-	Grady et al. 2003
1g	TUH 12-1	C 68	USA / Ohio	Human	Faeces	Clinical isolate	VanB	(Carias et al. 2003, Dhal et al. 2000)
1h	TUH 12-16	64/3	Germany/IN	Human	Faeces	IN	-	Wolfgang Witte/AMV
2a	TUH 12-4	3332	USA/ Ohio	Human	DN	Outbreak	VanB	(Carias et al. 2003, Dhal et al. 2000)
2b	TUH 2-18	25942/96	Norway/ Bergen	Human	Urine	Outbreak	VanB	Mikalsen, M. R./AMV
2c	TUH 2-19	28865/96	Norway/ Bergen	Human	Wound	Outbreak	VanB	Mikalsen, M. R./AMV
2d	TUH 2-21	17112/96	Norway/ Bergen	Human	IN	Outbreak	-	Mikalsen, M. R./AMV
2e	TUH 2-8	95 T0070	Sweden/ Ørebro	Human	Faeces	Clinical isolate	VanA	Mikalsen, M. R./AMV
2f	TUH 32-56	DO (TX0016) (TEX16)	USA/ Houston	Human	Blood	Clinical isolate	-	Arduino et al. 1994
2g	TUH 32-61	E1293 (10A243)	Italy/ Genua	Human	Blood	Clinical isolate	-	Willems, R. J. L.
2h	TUH 32-62	E0510 (9801894)	Australia/ Melbourne	Human	Blood	Hospital outbreak	VanB	Willems, R. J. L.
3a	TUH 32-63	E1626 (85/65)	Netherlands/IN	Human	Peritoneal fluid	Clinical isolate	-	Willems, R. J. L.
3b	TUH 32-64	E0013 (58155)	Great Britain/ Oxford	Human	Urine	Hospital outbreak	VanA	Willems, R. J. L.
3c	TUH 32-65	E1272 (05A433)	France/ Lyon	Human	Blood	Clinical isolate	-	Willems, R. J. L.
3d	TUH 32-66	E1279 (07A217)	Denmark/ Freiburg	Human	Blood	Clinical isolate	-	Willems, R. J. L.
3e	TUH 32-67	E0403 (SKH26)	Netherlands/IN	Human	Faeces	Community survey	VanA	Willems, R. J. L.
3f	TUH 32-68	E0996 (9800265E)	Netherlands/IN	Human	Faeces	Community survey	-	Willems, R. J. L.
3g	TUH 32-69	E0470 (TY286)	Netherlands/ Amsterdam	Human	Blood/ Faeces	Hospital outbreak	VanA	Willems, R. J. L.
3h	TUH 32-70	E0734 (A135)	Netherlands/ Amersfoort	Hospital Environment	Faeces /Sink	Hospital outbreak	VanA	Willems, R. J. L.
4a	TUH 32-71	E0745 (UMC015820G)	Netherlands/ Utrecht	Human	Faeces	Hospital outbreak	VanA	Willems, R. J. L.
4b	TUH 32-72	E0155 (VS2)	USA/ Chicago	Human	Faeces	Hospital outbreak	VanA	Willems, R. J. L.
4c	TUH 32-73	E1652 (02126628)	Netherlands/ Amersfoort	Human	Faeces	Hospital outbreak	VanA	Willems, R. J. L.
4d	TUH 32-74	E0300 (Detroit 12)	USA/ Detroit	Human	Urine	Hospital outbreak	VanA	Willems, R. J. L.
4e	TUH 32-75	E0073 (10-D) (7260931)	Netherlands/ Rotterdam	Human	Faeces	Clinical isolate	VanA	Willems, R. J. L.
4f	TUH 32-76	E1304 (15A159)	Portugal/ Coimbra	Human	Blood	Clinical isolate	VanA	Willems, R. J. L.
4g	TUH 32-77	E1173 (15D169)	Portugal/ Coimbra	Human	Wound/soft tissue	Clinical isolate	VanA	Willems, R. J. L.
4h	TUH 32-78	E0125 (10-G) (0244208)	Netherlands/ Rotterdam	Human	Bile	Clinical isolate	VanA	Willems, R. J. L.

Table 7- Continued

Well	Reference number	Other names	Geographic Origin	Sample	Sample Source	Origin	Van Type	Reference
5a	TUH 32-79	E0729 (A155)	Netherlands/ Amersfoort	Human	Faeces	Hospital survey	VanA	Willems, R. J. L.
5b	TUH 32-80	E0092 (9700228)	Netherlands/IN	Human	Faeces	Community survey	VanA	Willems, R. J. L.
5c	TUH 340		Norway/ Østfold	Animal	Faeces	Community survey	-	AMV
5d	TUH 4-15	ATCC 19434 (NCTC 7171)	IN/IN*	IN	IN	IN	-	ATCC
5e	TUH 4-21	94 I	Norway/ Øsfold	Animal	Chicken meat	Community survey	VanA	Mikalsen, M. R./AMV
5f	TUH 4-22	111 III	Norway/ Øsfold	Animal ?	Chicken floor	Community survey	VanA	Mikalsen, M. R./AMV
5g	TUH 4-41	Bm 4105- RF	France/IN	Animal	Faeces	Wild strain	-	(3)/AMV
5h	TUH 4-65	E57-1	USA/IN	Human	IN	IN	VanB	Dahl et al. 1999
6a	TUH 44-23	399/F98/H2 (95)	Norway/ Østfold	Human	Faeces	Community survey	VanA	[20] Aasnæs, B
6b	TUH 44-31	399/F98/A1 (222)	Norway/ Østfold	Animal	Faeces	Community survey	VanA	Johnsen et al. 2005
6c	TUH 44-40	399/F99/A10 (241)	Norway/ Østfold	Animal	Faeces	Community survey	VanA	Johnsen et al. 2005
6d	TUH 45-12	S399/S99/A4	Norway/ Østfold	Animal	Faeces	Community survey	-	Johnsen et al. 2005
6e	TUH 45-22	S399/F99/A14	Norway/ Østfold	Animal	Faeces	Community survey	-	Johnsen et al. 2005
6f	TUH 45-3	S399/F98/H3	Norway/ Østfold	Human	Faeces	Community survey	-	Johnsen et al. 2005
6g	TUH 45-5	S399/S99/H5	Norway/ Østfold	Human	Faeces	Community survey	-	(Johnsen et al. 2005)/AMV
6h	TUH 44-52	64/S99/A5	Norway/ Østfold	Animal	Faeces	Community survey	VanA	Johnsen et al. 2005
7a	TUH 44-50	64/F98/A2	Norway/ Østfold	Animal	Faeces	Community survey	VanA	(Johnsen et al. 2005)/AMV
7b	TUH 44-42	64/F98/H1	Norway/ Østfold	Human	Faeces	Community survey	VanA	Johnsen et al. 2005
7c	TUH 44-47	64/F99/H6	Norway/ Østfold	Human	Faeces	Community survey	VanA	Johnsen et al. 2005
7d	TUH 45-35	S64/S99/A5	Norway/ Østfold	Animal	Faeces	Community survey	-	Johnsen et al. 2005
7e	TUH 45-25	S64/F98/H1	Norway/ Østfold	Human	Faeces	Community survey	-	(Johnsen et al. 2005)/AMV
7f	TUH 45-30	S64/F99/H6	Norway/ Østfold	Human	Faeces	Community survey	-	(Johnsen et al. 2005)/AMV
7g	TUH 7-15	110-1309-1	USA/IN	Human	Blood	Outbreak	VanB	Dahl et al. 1995
7h	TUH 7-55	UW 1551	Germany/IN	Human	Urine	Clinical isolate	VanB	Dahl et al. 1995
8a	TUH D2-34	D34	Denmark/ Aarhus	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
8b	TUH D2-6	D6	Denmark/ Aarhus	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
8c	TUH D2-69	D69	Denmark/ Aarhus	Human	Urine	Clinical isolate	-	Simonsen et al. 2003
8d	TUH D2-91	D91	Denmark/ Aarhus	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
8e	TUH I1-82		Iceland/ Reykjavik	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
8f	TUH N1-42		Norway/ Bergen	Human	Urine	Clinical isolate	-	Simonsen et al. 2003
8g	TUH N1-59		Norway/ Bergen	Human	Urine	Clinical isolate	-	Simonsen et al. 2003
8h	TUH N1-80		Norway/ Bergen	Human	Blood	Clinical isolate	-	Simonsen et al. 2003

Table 7 Continued

Well	Reference number	Other names	Geographic Origin	Sample	Sample Source	Origin	Van Type	Reference
9a	TUH N1-90		Norway/ Bergen	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
9b	TUH N2-1		Norway/ Tromsø	Human	Urine	Clinical isolate	-	Simonsen et al. 2003
9c	TUH N2-26		Norway/ Tromsø	Human	Urine	Clinical isolate	-	Simonsen et al. 2003
9d	TUH N2-78		Norway/ Tromsø	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
9e	TUH N2-85		Norway/ Tromsø	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
9f	TUH S1-102		Sweden/ Uppsala	Human	Urine	Clinical isolate	-	Simonsen et al. 2003
9g	TUH S1-28		Sweden/ Uppsala	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
9h	TUH S1-7		Sweden/ Uppsala	Human	Blood	Clinical isolate	-	Simonsen et al. 2003
10a	TUH S1-95		Sweden/ Uppsala	Human	Urine	Clinical isolate	-	Simonsen et al. 2003
10b	K8-42	V1-59	Norway/ Oslo	Human	Faeces	Clinical isolate	VanA	K-res
10c	K8-43	V1-60	Norway/ Oslo	Human	Faeces	Clinical isolate	VanA	K-res
10d	K8-45		Norway/ Kristiansand	Human	Urine	Clinical isolate	VanA	K-res
10e	K9-26		Norway/ Kristiansand	Human	Faeces	Clinical isolate	VanA	K-res
10f	K9-27		Norway/ Kristiansand	Human	Faeces	Clinical isolate	VanA	K-res
10g	K9-28		Norway/ Kristiansand	Human	Faeces	Clinical isolate	VanA	K-res
10h	K9-72		Norway/ Ålesund	Human	Faeces	Clinical isolate	VanA	K-res
11a	K8-50	14-4, 25509/99	Norway/ Ålesund	Human	Urine	Clinical isolate	VanA	Simonsen, G
11b	TUH 2-40	14-5, 39487covIII	Norway/ Oslo	Human	Faeces	Clinical isolate	VanA	Simonsen, G
11c	TUH 2-13	14-7, 273-78	Norway/ SIR	Human	UVI	Clinical isolate	VanA	Simonsen, G
11d	TUH 7-56	14-13	Norway/ DNR	Human	Faeces	Clinical isolate	VanA	Simonsen, G
11e	K8-61	14-16, 1049698-1/98	Norway/ Bergen	Human	?	Clinical isolate	VanA	Simonsen, G
11f	K8-62	14-17, 1105035-3/99	Norway/ Bergen	Human	?	Clinical isolate	VanA	Simonsen, G
11g	K8-64	Prøvenr UNN 80728/01	Norway/ Akershus	Human	Faeces	Hospital survey	VanB2	Halvorsen, D
11h	K8-74	Prøvenr UNN 24024/02	Norway/ Akertshus	Human	Faeces	Hospital survey	VanA	Halvorsen, D
12a	K8-69	Prøvenr UNN 134568/01	Norway/ Akershus	Human	Faeces	Hospital survey	VanA	Halvorsen, D
12b	K8-68	Prøvenr UNN 109480/01	Norway/ Akershus	Human	Faeces	Hospital survey	VanA	Halvorsen, D
12c	K9-10	Prøvenr UNN 121311/02	Norway/ Akershus	Human	Faeces	Hospital survey	VanA	Halvorsen, D
12d	K18-79	Prøvenr St.Olavs 50144	Norway/ St.Olavs	Human	Faeces	Hospital survey	VanA	Halvorsen, D
12e	41-73	EA-AN26	Italy	Animal	Faeces	?	VanA	AMV
12f	Control	Negative control 1:44, Positive Control 1:48						
12g	Control	Positive control 1:44, Mastermix control 1:48						
12h	Control	Mastermix control 1:44, Negative control 1:48						

Other materials used

Table 8 notes the machines used for the work, it includes analytical machines like the spectrophotometer.

Table 8 - Machines

Provider	Machine
M7 Research	PTC-200
Heraeus	Biofuge pico
New Brunswitch scientific	Incubator shaker
Not known	E-base
Not known	Electrophoresis
Pharmacia Biotech	Spectrophotometer, ultrospec 2000
Not known	Western Blot

For analyzing the lab results some computer work had to be done, table 9 gives an overview of the software used.

Table 9 - software

Copyright	Software
Bioedit®	BioEdit
www.flu.org.cn	Chromas
Synoptics Ltd	SynGene
Thermo Fisher Scientific	NanoDrop

Gels, tubes and other basic materials used in the work for this thesis is shown in table 10

Table 10- Other stuff used.

Provider	Catalogue nr	Other
Applied BioSystems	4306737	MicroAmp
AB gene	AB 0337	0,2ml eppendorf tube
		1,5ml eppendorf tube
Invitrogen	G7008-02	E-Gel 96 2% agarose (GP)
Invitrogen	NP0322BOX	NuPAGE 4-12% Bis-Tris Gel, 1,0mm, 12 well
-		dry ice
		Agarose
Thermo Scientific	AB-0266	0,2ml thermo-strip
Whatman	708111	Protran BA 85 Nitrocellulose
Roche	11666916001	Chemiluminicent Detection film
-	-	PCR Film
Whatman	3030917	3mm Chromatography paper (filter paper)
-		Blotting Pads
Amershman biosciences		HyperCassette

Methods

Figure 11 shows the flow in the experiments

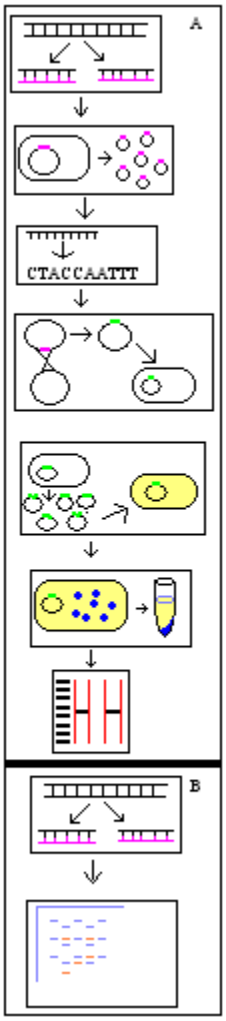

Figure 11 A
Designing of PCR tools for cloning.

Cloning into pENTR vector, check for inserts and purification of plasmid with insert.

Sequencing to check ends and orientation of the region to be cloned.

Recombination between the pENTR and pDEST vectors to move insert into the destination vector. Transformation into DH5α competent cells for maintenance.

Plasmids purified from DH5α cells for transformation into BL21-AI competent cells for expression.

Expression of protein in BL21-AI cells, segregation of water insoluble and soluble proteins by centrifugation at 4ºC. Water insoluble proteins in the pellet.

Western blot and detection to see which fraction the protein is located in.

Figure 11 B
Designing PCR tools for screening for pili genes.

Screening for pili genes in 93 isolates.

Cell Cultures

Overnight Cultures

Over night cultures for crude DNA extraction was made by plating out bacteria from the freeze storage. Cells were kept from thawing by incubation on ice and minimum hand contact. By scraping the ice surface with a needle it was possible to transfer a few bacteria from the tube to the plate with non-selective media. Selective media was used when necessary for maintaining vector and constructs.

Each bacterial isolate has an In House identification number, and this number was used to find the bacteria.

Over night cultures were inoculated from single colonies on plates. One single colony was picked up by an inoculation needle and transferred to a tube containing selective medium. The cells were incubated at 37˚C over night, shaking at 200 rpm in an incubation shaker.

Freezing of Cell Cultures

Procedure

930µl overnight culture containing selective antibiotic was mixed with 70µl DMSO and stored at –80ºC.

DNA Isolation

Crude Extracts of Total DNA

Boiling overnight cultures in a solution like TE buffer causes the cell wall to break and the DNA will be released in sufficient quantity and purity for PCR amplification. The

DNA is separated from the rest of the cell by centrifugation; the DNA will be in the supernatant and the pellet contains the rest of the cell.

Procedure

1) 8-10 single colonies from one plate was collected and dissolved in 1000µl TE buffer in 1,5 ml eppendorf tubes. The tubes were labelled with the strain name.
2) The suspension was centrifuged for 5 min at 2012xg.
3) The supernatant was removed, and the pellet was resuspended in 100µl TE buffer.
4) The suspension was then boiled for 10 min at 100ºC, before the boiling the lids were pierced.
5) The suspension was centrifuged for 5 min at 2012xg, and put on ice.
6) The supernatant, containing the DNA, was transferred to a new, clean 1,5ml eppendorf tube and stored at -20˚C.

Plasmid Extraction

Plasmids are relatively small DNA molecules and does not precipitate at the same salt concentrations as chromosomal DNA, this quality can be used to purify plasmids from chromosomal DNA. By adding a salt solution to the cells, the cells will lyse, and after centrifugation chromosomal DNA will be in the pellet while plasmids will be in the supernatant. [Snyder & Champness 2007]. E.Z.N.A uses this combined with a column binding the plasmids, centrifugation causes the chromosomal DNA to go through the column with the flow through. The plasmids can be eluted from the column by using an elution buffer with a low salt concentration, or using water.

High copy number

This was done by E.Z.N.A.™ Plasmid Miniprep Kit II

Procedure

1) Single overnight colonies from selective plates was picked up and added to 10-15 ml selective medium. The cultures were incubated over night at 37˚C, shaking at 200 rpm in an incubation shaker. Kanamycin was used as selective marker in the TOPO® cloning.
2) 1000µl overnight culture was centrifuged in room temperature for 10 minutes at 2012xg to pellet the cells. The supernatant (medium) was discarded.
3) The pellet (cells) was completely resuspended in 500µl Solution I/RNase A solution by pipetting up and down.
4) The resuspended cells were transferred to two clean 1,5ml eppendorf tubes, 250µl too each.
5) 250µl Solution II was transferred to each eppendorf tube and mixed gently by inverting and rotating the tube several times to get a clear lysate. In some cases it was necessary with 2 - 5 minutes incubation at room temperature, but the lysis reaction should not go on for more than 5 minutes.
6) 350µl Solution III was added and rapidly mixed with the lysate, by rotating and inverting the tube, until a flocculent with precipate formed.
7) The tubes were centrifuged at 13600 xg to pellet the cell debris. The supernatant was transferred to a clean HiBind® Miniprep column (II) that was placed in a 2ml collection tube, one column for each sample. The columns were centrifuged at 13600 xg for 1 minute at room temperature to pass the lysate through the column. The flow-through was discarded and the rest of the lysate was added to the column. The columns were centrifuged at 13600 xg.
8) Flow-through was discarded and 500µl buffer HB was added to wash the HiBind® Miniprep column (II). The columns were centrifuged at 13600 xg for 1 minute to pass the lysate through the column.
9) Flow-through was discarded and 700µl DNA wash buffer diluted with absolute ethanol was added to the columns. The columns were centrifuged at room

temperature for 1 minute at 13600 xg to pass the lysate through the columns. This step was done twice.

10) The empty columns were centrifuged for 2 minutes at 13600 xg at room temperature to dry the column matrix.
11) The columns were placed in clean 1,5ml eppendorf tubes without caps, 100µl elution buffer was added to the column matrix and the columns were centrifuged for 1 minute at 13600 xg in room temperature to elute the DNA.
12) The concentration of DNA was estimated by NanoDrop. Elution buffer was used as blank.
13) DNA was maintained by freezing it at -20˚C.

Low copy number plasmids

This was done by E.Z.N.A.^{TM} Plasmid Miniprep Kit I

Procedure

1) Single colonies from an overnight selective plate with 100µg/ml ampicillin used to inoculate starter cultures if 5 ml LB medium containing 100µg/ml ampicillin. The starter cultures were incubated over night in room temperature, shaking at 200 rpm in an incubation shaker.
2) 1000µl culture were centrifuged at 10000xg for 2 minutes to make a cell pellet, the supernatant were discarded.
3) The cell pellet was resuspended in 250µl SolutionI/ RNase A solution, this was done by pipetting up and down until the whole pellet was dissolved. The suspension was transferred into a clean 1,5µl eppendorf tube.
4) 250µl Solution II was added to the suspension and mixed by inverting the tube gently several times, until a clear lysate was obtained. Vigorously mixing would shear chromosomal DNA and lower plasmid purity. The reaction did not go for more than 5 minutes!
5) 350µl of Solution III was added and the tube was inverted several times until a white flocculent precipitate formed.

6) Centrifuge at 13000xg for 10 minutes at room temperature. Put HiBind Miniprep Columns (I) was put in clean 2ml collection tubes.
7) The clear supernatant was carefully added to a clean HiBind Miniprep Column (I). Centrifuge for 1 minute at 13600 xg at room temperature.
8) Flow through was discarded and 500µl buffer HB was added to wash the HiBind Miniprep Column (I). Centrifuge at 13600 xg for 1 minute at room temperature.
9) Flow- through was discarded and 700µl of DNA wash buffer diluted with absolute ethanol before use, was added to the column. The column was centrifuged at 13600 xg for 1 minute at room temperature.
10) Step 9) was repeated
11) The empty column was centrifuged at 13600 xg for 1 minute at room temperature.
12) The column was placed in a 1.8µl eppendorf tube (the lid was marked and removed, and taken care of for use when the plasmid isolation was finished). 50µl – 100µl[5] elution buffer. The column was centrifuged at 13600xg for 1 minute at room temperature.
13) Yield and quality of isolated plasmid was determined by NanoDrop, elution buffer was used as blank.
14) The isolated plasmids were stored at -20˚C.

Amplification Detection and Determination of DNA Sequences

Amplification of Pili Genes by Polymerase Chain Reaction (PCR)

The polymerase chain reaction copies a section of double stranded DNA, and its power lies in the possibility to produce a large number of copies of the target DNA sequence in a short period of time. Normally the primers flanking the DNA target sequence have a length of 17-30 nucleotides and have approximately the same sequence as the strand they are binding to; one primer will bind to the leading strand, while the other will bind to the lagging strand. [Reece 2004]

[5] Most often 80µl was used, and when possible a gradient with volumes 50µl, 70/80µl and 100µl were used.

The PCR reaction goes through three different steps; denaturation, annealing and elongation, these steps make up a cycle that is repeated 20-35 times to achieve a satisfying amount of amplified DNA [Reece 2004].

Procedure

1) Mastermix was made; table 11 shows the recipe for the mastermix used in this experiment. Primers were the last things added to the mastermix. The reagents was diluted according to manufactures recommendation,

Table 11- PCR reaction mastermix for nine samples with a total volume of 25µl

Reagent	Volume
ReadyMix™	125 µl
Primer F	25µl
Primer R	25µl
25mM $MgCl_2$	10µl
MQH_2O	40µl
Total	225µl

2) The primers were optimized by using a temperature gradient ranging from 53ºC to 66ºC, the lowest temperature with a positive + control, and a negative – control was registered as the optimal temperature for the primers.
3) Mastermix and DNA template was mixed by centrifuging in PCR tubes. 22µl mastermix was added to the tubes, then 3µl template to make a reaction solution with a total volume of 25µl. Table 12 (next page) shows the PCR programs used in this experiment.

Table 12- The table shows the PCR programs that were used.

Product	Primer Pair (forward/ Reverse)	PCR Program[2]				Expected size (bp)
		Denaturation	Annealing	Elongation	Cycles	
Pili 1:44[1)]	Pili 1:44F/ Pili 1:44R	95˚C 30''	64,2˚C 30''	72˚C 1'	35	370
Pili 2:44[1)]	Pili 2:44F/ Pili 2:44R	95˚C 30''	61,2˚C 30''	72˚C 1'	35	497
Pili 3:44[1)]	Pili 3:44F/ Pili 3:44R	95˚C 30''	64,2˚C 30''	72˚C 1'	35	419
Pili C3:44R1[1)]	Pili C3:44F/ Pili C3:44R1	95˚C 30''	57˚C 30''	72˚C 2'	25	1677
Pili C3:44R2[1)]	Pili C3:44F/ Pili C3:44R2	95˚C 30''	61,2˚C 30''	72˚C 2'	30	1674
Pili C3:44 5'	M13F/ Pili 3:44RL	95˚C 30''	55˚C 30''	72˚C 1'	30	720
Pili C3:44M	Pili 3:44FM/ Pili 3:44RM	96˚C 30''	56˚C 30''	72˚C 1'	30	756
Pili C3:44 3'	Pili 3:44 FR/ M13 R	95˚C 30''	55˚C 30''	72˚C 1'	30	699
Pili 1:48[1)]	Pili 1:48F/ Pili 1:48R	95˚C 30''	61,2˚C 30''	72˚C 1'	35	521
Pili 2:48[1)]	Pili 2:48F/ Pili 2:48R	95˚C 30''	64,2˚C 30''	72˚C 1'	35	417
Pili 3:48[1)]	Pili 3:48F/ Pili 3:48R	95˚C 30''	64,2˚C 30''	72˚C 1'	35	321
Pili C3:48R[1)]	Pili C3:48F/ Pili C3:48R1	95˚C 30''	57,4˚C 30''	72˚C 2'	30	1856
PiliC3:48R2[1)]	Pili C3:48F/ Pili C3:48R2	95˚C 30''	65,8˚C 30''	72˚C 2'	35	1851
Pili C3:48 5'	M13F/ Pili 3:48RL	95˚C 30''	56˚C 30''	72˚C 1'	30	650
Pili C3:48M1	Pili 3:48FM/ Pili 3:48RM	96˚C 30''	56˚C 30''	72˚C 1'	30	699
Pili C3:48M2	Pili 3:48FR/ Pili 3:48R	95˚C 30''	55˚C 30''	72˚C 1'	30	722
Pili C3:48 3'	Pili 3:48F/ M13R	95˚C 30''	56˚C 30''	72˚C 1'	30	746

1) PCR programs that were optimized

2) Each program started with a 1 minute denaturation at 95˚C, and after the cycles the programs ended with a 4 minutes elongation at 72˚C followed by a 4˚C unlimited hold. Temperatures in clams show the primer pairs temperature range.

Detection of PCR Products by Agarosegel Electrophoreses

Dry agarose suspended in a buffer, i.e. 0,5X TBE, and boiled to make a clear solution of agar that will form a gel when the temperature gets beneath 50˚C. The pore sizes in agarosegels cannot be accurately controlled, and therefore the bands will be less distinct than the bands in gels were the pore size can be controlled [Reece 2004].

The movement of the fragments is decided by several parameters; size, shape and agarose concentration are the most important parameters. The running time and electrical current are also important and controllable parameters. Agarose gels can separate DNA fragments in the size range of 200-15000 base pairs [Reece 2004].

Visualization of DNA in the agarose gels are done by staining the DNA fragments, this is most commonly done by soaking the gel in Etidium bromide (EtBr) [Reece 2004], but because of the carcinogenic effects of EtBr other substances such as cyber safe should be used. EtBr is a flat planar molecule that can intercalate between stacked pairs of DNA and bind efficiently, this result in a distortion of the DNA double helix and localized unwinding of the structure. EtBr binds less efficiently to single stranded DNA and RNA due to the relative lack of stacking in these molecules [Reece2004]. In UV light EtBr-DNA shows up as light bands.

Procedure

1) Dry agarose was added to in 0,5X TBE buffer (made from a 10X stock solution), for a 40ml 0,8% gel 0,32 g was dissolved in 40ml buffer and for a 60ml 0,8% gel 0,48 g was dissolved in buffer. Because of evaporation when the mixture was boiled 3-4 ml extra buffer was needed.
2) This solution was boiled in a microwave to dissolve all the agarose, the solution was taken out and carefully shaken once in a while, and after boiling. It was allowed to boil 2-3 times to ensure that all agarose was dissolved.

3) The solution was allowed to cool to about 40ºC before EtBr was added to a final concentration of 0,5 µg/ml. The gel was cast, and allowed to set before the samples was loaded.
4) Each well was loaded with 10µl sample, and the first and the last well loaded with 3,5µl 1kb+ molecular weight standard with DNA of known sizes. The samples included PCR products from a mastermix control (H_2O), a negative control (a staphylococci, in house number 6-01) and a positive control (*E. faecium* DO, 32-56).
5) 0,5X TBE buffer was used as electrophoresis buffer. The running parameters were 120V for 45 min.
6) After the electrophoresis the gel was exposed to UV light to detect the DNA. The bands were compared to the 1kb+ molecular weight standard to decide the size of the products, and to see if the products might be the DNA of interest.

DNA sequencing

The sequencing was done using the procedure provided by the sequencing lab at the University of Tromsø.

Before the sequencing of PCR products primers and the remains of dNTPs should be removed to get rid of some potential contaminants and yield the best sequence possible. EXO/SAP is a common way to purify the PCR products before sequencing, the mixture is made out of equal amounts of exonuclease I (EXO) that remove the primers and shrimp alkaline phosphatase (SAP) that dephosphorylase the dNTPs to dNDPs and/or dNMPs.

The sequencing method used in this project was thermal cycle sequencing which has two main advantages over the Chain termination method; double stranded DNA can be used as a template (1) and only a little amount of DNA is needed for sequencing and therefore no cloning is needed to succeed (2). In each reaction only one strand is copied because of the use of just one primer. In other ways this method is built on the same principals as the

chain termination method: a small amount of ddNTPs (dedeoxynucleotides) is added to the reaction, when ddNTP is incorporated into the growing strand of DNA the elongation process stops due to the lack of the 3'-hydroxyl-group needed to form the connection with the next nucleotide [Brown 2006].

Procedure

1) Make the EXO/SAP mixture; calculate 1µl mixture pr sample (0,5µl EXO and 0,5µl SAP for each sample) and 2 – 4µl excess mixture. This was done on ice.
2) Add 1µl EXO/SAP to each sample while working on ice.
3) Run the samples at the following program to remove primers and dephosphorylase dNTPs (a PCR machine was used for this): 37˚C for 1minute → 37˚C for 1-hour → 85˚C for 15 minutes.
4) Set up the sequencing reaction as in table 13, work on ice.

Table 13- The sequencing reaction.

Reagent	Quantity
BigDye 3.1	1µl
Template (30-1200ng)	3-6µl
BigDye sequencing buffer (5X)	2,5µl
Primer (2 pmol/µl)	1,6µl
MQH_2O	up to 20µl
Total volume	20µl

5) Prewarm the PCR machine to 95˚C and insert the samples, run a PCR using the following program:
   96˚C for 1 minute
   25 cycles:
   96˚C for 10 seconds
   50˚C for 5 seconds
   60˚C for 4 minutes
   Cool the samples at 4˚C until the samples are removed from the machine.

6) When the PCR were finished the markings on the samples were checked and refreshed if needed. Then the samples were delivered to the Sequencing Lab Unit where the samples would be precipitated for the terminator reaction.

Analyzes of DNA sequences

The DNA sequences were analyzed by using Chromas Lite and BioEdit.

Procedure

1) Every sequence was searched for N's, which could be any of the four nucleotides. Each N was tried replaced with the correct nucleotide by checking which top beneath the N was the highest.
2) When finished in Chromas every sequence was saved as a fas file so the edited sequences could be opened in BioEdit.
3) In BioEdit each sequence from a reverse primer was reversed and complemented using the "the reverse complement" from the "Sequence" menu.
4) The sequences belonging to the same primer pair was aligned using a local alignment allowing terminal gaps.
5) The aligned sequences were then aligned with the appropriate given operons checking for mutations and orientation of the insert.
6) The sequences were also checked for the CACC overhang and ATG start codon.
7) When all the ends were checked the middle part of the pili genes were sequenced, checked and edited for N's in Chromas and the reverse primer sequences were reversed and complemented in BioEdit.
8) The sequences for each pili gene were put together aligning the partial sequences and making a consensus between each aligning. See figure 12, next page.

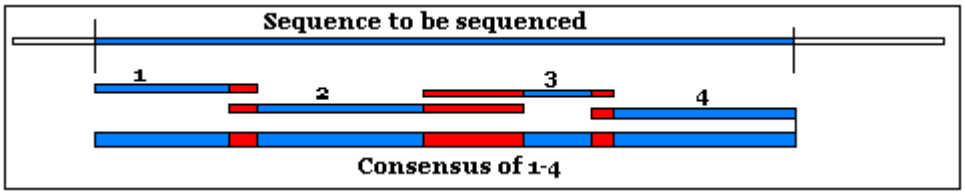

Figure 12- Putting together partial sequences to make a consensus sequence. The red areas are the overlapping sequences between the partial sequences.

9) The approximate size of the gene product was calculated using the option "amino acid composition" under the sequence menu.

Cloning with Gateway® Technology

This was done by Invitrogens pENTR Directional TOPO® cloning Kit and *E. coli* Expression System with Gateway® Technology.

The Gateway® technology cloning method contains three main steps; (1) TOPO® cloning of blunt ended PCR products into an pENTR TOPO vector to form an entry clone, (2) performing an LR recombination reaction between entry clone and a gateway ® destination vector to form an expression construct, and finally (3) introducing the expression construct into an appropriate host for expression and express the protein. [Snyder & Champness 2007,Invitrogens pENTR Directional TOPO® cloning Kit, *E. coli* Expression System with Gateway® Technology]. Figure 13 shows the principle behind the gateway technology.

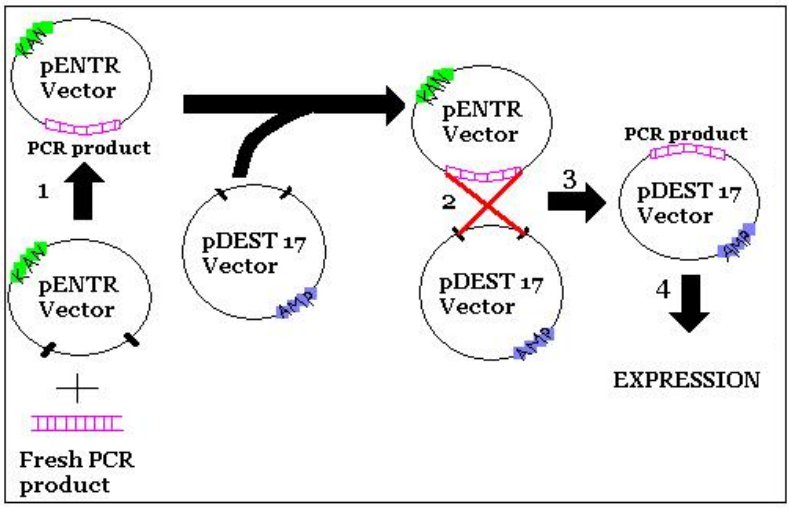

Figure 13 - An overview of the Gateway technology. 1) Topo cloning of blunt ended PCR products into pENTER, an entry vector. 2) LR recombination of the PCR product into a destination vector, pDEST17. 3) Transformation of the destination vector into an appropriate expression host. 4) Expression of the gene of interest.

Topo cloning relays on topoisomerase I from vaccinia viruses which has a strong sequence specificity and always makes a break next to the 3'T in the following 5bp sequence: 5'-(C/T)CCTTNNN-3'. Normally type I topoisomerase makes a single stranded break in one DNA strand, and while topoisomerase I stays attached to the 3'T, the other strand passes through the break to form or remove super coiling in the DNA molecule. But if the break is made within 10bp from the end the last part of the DNA falls apart leaving a 5'-overhang that can pair with a complementary 5'-overhang and topoisomerase will ligate the two molecules together [Snyder & Champness 2007].

The LR recombination reaction between the *att*L flanked sequence in the entry clone and the *att*R destination vector creates an *att*B-containing expression clone. With the help from an enzyme mix containing the bacteriophage λ integrase and excisionase proteins mediates the reaction, and the *E. coli* integration host factor protein; this enzyme mix is called LR Clonase™ II Enzyme Mix [Invitrogen LR Clonase manual].

In the pDEST17 vector the expression of the gene of interest is controlled by a strong bacteriophage T7 promoter that is recognized by the T7 RNA polymerase.

In BL21-AI™ *E. coli* strain the T7 RNA polymerase is inserted into the *ara*B locus of the *ara*BAD operon making it possible to induce expression of the T7 RNA polymerase by the sugar L-arabinose, and repress the expression by glucose. By transforming the pDEST17 with the gene of interest into BL21-AI competent cells that supplies T7 RNA polymerase it is possible to express the protein encoded by the inserted gene of interest by adding L-arabinose to the culture, or repress the expression of the gene by adding glucose [Invitrogens *E. coli* expression system with Gateway technology manual].

Topo® Cloning to Entry Vector and Transforming into One Shot® Competent *E. coli*

This was done by Invitrogens pENTR Directional TOPO® cloning Kit.

Procedure

1) The cloning reaction was mixed as in table 14. The molar ratio between the PCR product and vector should be 0,5:1 to 2:1 for the most effective cloning. The amount of PCR product was estimated by visualization in an agarose gel.

Table 14 Cloning reaction mix

Reagents	Volume
Fresh PCR product	0,5 – 4µl
Salt solution	1µl
Sterile water	Add to final volume of 5µl
TOPO® vector	1µl
Final volume	6µl

2) The reaction was gently mixed and incubated for 5 minutes in room temperature. After the incubation the cloning reaction was placed on ice.
3) 2µl of the TOPO® cloning reaction was added into a vial of One Shot® chemically competent *E. coli* cells, and mixed gently (not by pipetting).

4) The competent cells with the cloning reaction were incubated on ice for 5 – 30 minutes.
5) The cells were heat-shocked for 30 seconds at 42˚C without shaking. The cells were immediately transferred to ice after the heat-shock.
6) 250µl of room temperature S.O.C. medium was added to the cells. The tubes were capped tightly and shaken horizontally at 200 rpm in an incubation shaker for 1 hour at 37˚C.
7) 50µl were spread on one LB plate containing 50µg/ml kanamycin, and 150 µl were plated on another plate. The plates were incubated over night at 37˚C.
8) Single colonies were picked for plasmid isolation. The cells were incubated over night at 37˚C in 10-15 ml LB medium containing 50µg/ml kanamycin, the tubes were shaking at 220rpm in an incubation shaker.

LR- Recombination and Transforming Library Efficiency® DH5α™ Cells

This was done with *E. coli* Expression System with Gateway® Technology from Invitrogen. Transforming into DH5α-cells was done for progation and maintaining the plasmid.

Procedure

1) The contents in table 15 were mixed in 1,8ml eppendorf tubes in room temperature

Table 15 - shows the concentrations of entry clone and destination vector in the LR-recombination reaction.

Component	Sample	Positive Control
Entry clone (50-150 ng/ reaction)	1-7 µl	-
Destination vector (150 ng/µl if supplied by the kit)[1)]	1µl	1µl
pENTR™-gus (50ng/µl, positive control)	-	2µl
TE buffer, pH8,0	To 8µl	5µl (or to 8µl)

In this project the destination vector had to be isolated from its host and the amount of destination vector is approximately 150ng/ reaction.

2) The LR Clonase™ Enzyme Mix (supplied by the kit) was removed from the -20˚C freezer and thawed on ice, this took approximately 2 minutes.
3) The LR Clonase™ Enzyme Mix was vortexed briefly twice, approximately 2 seconds each time.
4) 2µl Clonase™ Enzyme Mix was added to each sample, the sample and clonase was mixed by pipetting up and down. The Clonase™ Enzyme Mix was immediately returned to the -20˚C freezer after use.
5) The reaction was incubated for 1 hour at 25˚C (room temperature).
6) 1µl Proteinase K solution (supplied by the kit) was added to each reaction, and the reaction was incubated for 10 minutes at 37˚C. (Can be stored up to 1 week at -20˚C before transformation.)
7) For each sample one tube of 100µl chemical competent DH5α *E.coli* cells were thawed on ice.
8) To each tube of chemical competent DH5α *E.coli* cells, 2-5µl LR recombination reaction was added; this was gently mixed (not by pipetting up and down!).
9) The cells were incubated on ice for 30 minutes on ice.
10) The cells were heat-shocked at 42˚C for 30 seconds without shaking, after the heat-shock the cells were immediately put on ice.
11) 450µl S.O.C. medium (supplied by the kit) with room temperature was added to the cells.
12) The tubes were tightly caped and shaken at 200rpm in an incubation shaker for 1 hour at 37˚C.
13) For each reaction 20µl and 100µl were spread out on selective LB plates containing 100 µg/ml ampicillin and incubated over night at 37˚C.
14) Cells were harvested for overnight cultures, plasmid purification and analyzes of the transformants. Analyzes were done by PCR with primers located within the insert.

Transforming BL21-AI™ One Shot® Cells

This was done by Invitrogens *E. coli* Expression System with Gateway Technology kit.

Procedure

1) For each transformation one vial of BL21-AI™ One Shot® Cells was thawed on ice.
2) 5-10ng DNA in a volume of 1-5µl was added to each vial of BL21-AI™ One Shot® Cells, and mixed by gentle tapping.
3) The transformation mixture was incubated on ice for 30 minutes
4) The cells were heat shocked at 42ºC for 30 seconds, and the vials were immediately transferred to ice.
5) 250µl room temperate S.O.C. medium were added to the cells.
6) The tubes were tightly capped and incubated in 37ºC for 30 minutes, shaking at 200 rpm in an incubation shaker.
7) 20µl and 100µl were spread out on two different pre-warmed selective plates, containing 100 µg/ml ampicillin, and incubated over night at 37ºC.

Protein Isolation and Detection

The gene of interest is inserted in the T7 RNA polymerase, which is induced by L-arabinose, and repressed by glucose that acts as a repressor. The expression can therefore simply be regulated by the addition of L-arabinose to activate transcription, or the addition of glucose to repress the transcription [*E. coli* expression System with Gateway Technology].

Western blotting is useful for the identification and quantification of specific proteins, it relays on the transfer of electrophoretically separated proteins from the gel to a solid support, e.g. a membrane. To detect the proteins the membrane is probed with antibodies that react with antigenic epitopes displayed by the target protein [Sambrook & Russel 2001].

Antibodies are immunoglobulin's, and can be made to detect specific proteins. The antibody recognizes and binds to the antigens epitope that is a 5 to 8 amino acid long domain, normally located on the protein surface. There are two different types of epitopes; continuous, which mean that they are on the same peptide, or discontinuous, which mean that the amino acids that make up the epitope is brought together by the three dimensional structure. Antibodies might bind to fragments, denatured fragments or the native protein, depending on the antibody it self and the type of epitope it recognizes [Novus Biological antibody guide].

There are 5 classes of antibodies; IgM, IgD, IgA, IgG and IgE, all of these classes share the same basic structure: Two identical heavy polypeptide chains (440 amino acids) and two identical light polypeptide chains (220 amino acids) that are held together by both covalent and non-covalent disulfide bonds that form a symmetrical Y structure with identical halves see figure 14, the number and type of disulfide bonds varies between the classes. The antigen binding sites are located on the top ends of the Y structure, see figure 14 [Novus Biological antibody guide].

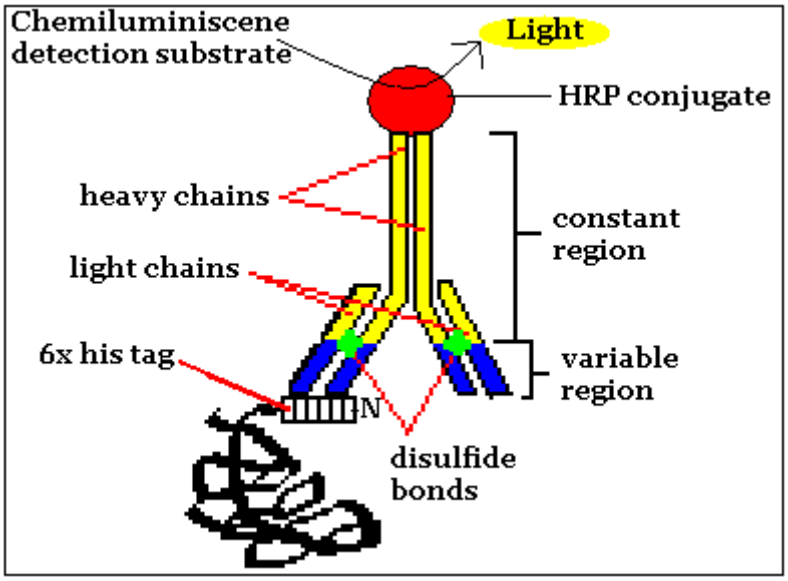

Figure 14- Blotting and detection with antibodies

His-tags are normally composed of six histidine residues and are the simples of all tags used for protein purification. The his-tag is most often located on the N or C terminus of the protein where it is less chance that it will influence the protein function. If a part of the protein is known to be non-essential for the function of the protein, the his-tag can be located in this part [Reece 2004].

His-tagged proteins can be easily purified in a one step purification. The imidazole rings in the histidines binds strongly metal ions like Ni^{2+}, this binding is non-covalent. By adding increasing concentrations of imidazole to the column will elute the his-tagged proteins, due to the competition for the Ni^{2+}, and the imidazoles stronger affinity [Reece 2004].

Pilot expression

This was done by Invitrogens *E. coli* Expression System with Gateway Technology kit.

Procedure

1) 4 transform ants were selected from the "BL21-AI™ One Shot® Transformation" procedure previously described.
2) The transformants were cultured in LB medium containing 100µg/ml ampicillin at 37˚C with shaking until the cultures reached an OD_{600} between 0,6 and 1,0. These cultures were used to inoculate fresh LB medium containing 100µg/ml ampicillin to an OD_{600} between 0,05 and 0,1 (this was approximately a 1:20 dilution of the initial culture). The volume of fresh culture were big enough to take samples for measurement of OD_{600} until it reached approximately 0,4, split the cultures in two, and take up to 5 time point samples of 500µl each.
3) The cultures were grown until mid-log phase, OD_{600} ~0,4, and split into two cultures.
4) To induce the culture L-arabinose was added to one of the cultures to a final concentration of 0,2%. The other culture was left uninduced.
5) 500µl aliquot was removed from each culture.

6) The aliquot was centrifuged for 30 seconds at maximum speed to pellet the cells, the supernatant was discarded and the pellets were frozen at -20˚C. These pellets were the time zero samples.
7) The rest of the cultures were incubated at 37˚C with shaking.
8) Time points were taken every hour for 4 hours, 500µl aliquot each time and treated as in step 6.

Preparing Samples for Water Soluble/ Insoluble Protein

This was done by Invitrogens pENTR Directional TOPO® cloning Kit.

Procedure

1) Lysis buffer were made.
2) Each pellet from the "Pilot expression" procedure were thawed and resuspended in 500µl of lysisbuffer.
3) The samples were frozen in dry ice; this was done by setting the samples in the ice and pouring ethanol over. This was repeated 3 times.
4) The samples were centrifuged at maximum speed for 1 minute at 4˚C to pellet the water insoluble proteins, and the supernatant were transferred to a new eppendorf tube and stored on ice.
5) 5 µl 2X SDS-PAGE sample buffer, 7 µl water and 8 µl supernatant were mixed and boiled for 5 minutes.
6) The pellets from step 4) were resuspended in 500µl 1X SDS-PAGE sample buffer and 20µl were taken out and boiled for 5 minutes.
7) 10µl of the supernatant sample and 5µl of the pellet sample were loaded onto an SDS-PAGE gel and run an electrophoresis.

SDS-PAGE electrophoresis

Procedure

1) A NuPAGE 4% -12% Bis-Tris gel was taken out of the bag, the gel cassette was washed with deionised water and the tape at the bottom was removed.
2) The comb was carefully removed from the cassette and wells were washed with 1X NuPAGE running buffer. The cassette was hold up-side down to remove excess buffer. This was repeated twice.
3) The outer chamber was filled with NuPAGE Mes running buffer 1X.
4) The inner chamber was filled with NuPAGE Mes running buffer 1X and 500µl antioxidant.
5) The gels were placed in the minicell, the wells facing the inner chamber. The dummy gel was only used when one gel was used.
6) 10µl of each pellet sample, 20µl of each supernatant sample, and 3µl MagicMarker Molecular weight standard were loaded on the gel. The molecular weight standard was loaded first, followed by uninduced samples and induced samples at the end.
7) The gel was run at 200V for 35 minutes.

InVision staining

Procedure

1) After SDS-Page electrophoresis (described above) the gel was fixed for 1 hour in fixing solution for NuPAGE® Novex gels.
2) The fixing solution was decanted and the gel was washed 2x 10 minutes in 100ml deionised water, the water was disposed of between the to washings.
3) The gel was covered in ready to use solution and InVision™ His-tag In-gel Stain and incubated for 1 hour.
4) The stain was disposed and the gel was washed in 20mM phosphate buffer, pH7,8
5) Picture of the gel was taken using…

Western blot

Procedure

1) SDS –Page electrophoresis.
2) 1X blotting buffer and 1X TBST was made during the gel run.
3) 5 blotting pads soaked in blotting buffer in a container. They had to be completely wet and free of air.
4) Two pieces of membrane were cut to the approximately same size as the gel, the right corner was cut off and the side that was going to lie against the gel was marked with a soft pencil. The membranes were put in separate holders containing blotting buffer while the SDS-page electrophoresis ran.
5) Four pieces of filter paper was cut to the same size as the blotting pads.
6) When the gel was finished the plates were carefully separated, the plate on top was removed, and the gel was on the bottom plate.
7) The wells and the gel foot were removed with a gel knife, and a filter paper moisturised in blotting buffer was placed on top of the gel. Air bubbles were removed by rolling a glass rod over.
8) The plate with the gel and filter paper was laid up side down in the hand, and the gel knife was used to carefully remove the last plate.
9) The wet membrane was put on the gel with the marked side down. And a filter paper moisturised in blotting buffer was placed on top of the membrane.
10) Two blotting pads was laid in the cathode core of the blotting chamber, the sandwich containing the gel and the membrane was put on top of the blotting pads, membrane side up. One blotting pad was put on top of the sandwich.
11) Steps 6-10 were repeated for the second gel.
12) The second gel sandwich was put on top of the blotting pad, membrane side up, and the last two blotting pads was put on top and the anode core was placed on top to close the blotting chamber. See figure 15 (next page) for the build up.
13) The blotting chamber was placed in the blotting module, while pressing it together.
14) The blotting chamber was filled with 1X blotting buffer, and the blotting module/outer chamber was filled with deionised water.

15) Blotting was run on 30V for 1 hour.

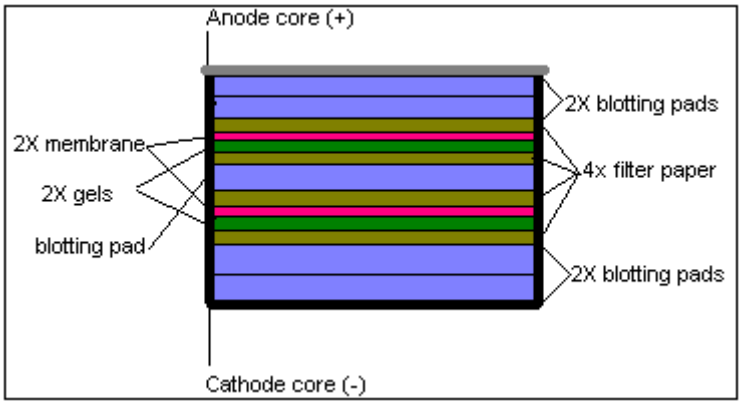

Figure 15 - The set up for western blotting with two gels

Immunodetection with anti-his antibodies

This was done using QIAexpress Detection and Assay handbook 10/2002 protocol 7, Immunodetection with Anti-his Antibodies or Anti-His HRP conjugate (Chemiluminicent method).

Procedure

The membranes were treated in different containers

1) The membranes were washed for 10 minutes in TBS buffer at room temperature.
2) The membranes were incubated in blocking buffer over night at 4ºC.
3) The membranes were washed twice for 10 minutes in TBS-Tween/Triton buffer at room temperature.
4) The membranes were washed for 10 minutes in TBS buffer at room temperature.
5) The membranes were incubated in Anti-His HRP Conjugate solution for 1 hour at room temperature.
6) The membranes were washed twice for 10 minutes in TBS-Tween/Triton buffer at room temperature.
7) Each membrane was incubated for 1 minute in 3,5ml of each bottle Super signal West Pico Chemiluminicent Substrate. And air-dried.

8) The dry membranes were taped in a cassette and a transparent was taped over the membranes.
9) In the dark (only red light on): Two layers of Chemiluminicent film were taped over the membranes. The first film was developed after 5 minutes, the next after 1 hour, if no signals new film were taped on, the third was developed after two hours and the last was developed the next morning.

Screening for Pili 1:44 and Pili 1:48

93 isolates were screened for pili 1:44 and 1:48, the PCR programs and primers used were those in table 12 for pili 1:44 and pili 1:48 products. The positive control was *E. faecium* DO, the negative control was *S. epidermis* and the water was used as mastermix control. E-Gel® *96* Gels was used.

Procedure

1) Distilled water was added to the samples to a final volume of 22µl.
2) The Mother E-Base™ was plugged into an electrical outlet, and the pwr/prg (power/program) button was pressed and released to select program EG.
3) The gel was removed from the package and the comb was removed from the gel.
4) The gel was slided into the Mother E-Base™.
5) 20µl sample was loaded into each well. The loading had do be done within 30 minutes after the gel was removed from the package.
6) E-Gel® Low range quantitative DNA marker was loaded into the marker wells.
7) 20µl sample buffer was loaded into the empty wells.
8) The pwr/pgr button was pressed and released to start the run. After about 12 minutes the gel is finished, the pwr/pgr button was pressed and released again to stop the beeping.
9) Picture was taking using transilluminator (UV-light).

Estimating p-value by Using χ^2-test

The χ^2-test is used to check for correlation between two variables. In this sort of test the 0-hypothesis will always be "no correlation", and the hypothesis to be tested will be if there is a correlation, and the decision to be made is whether or not the 0-hypothesis is true. When the χ^2 is large, there is a correlation and the 0-hypothesis is rejected, the opposite is true when the χ^2 is small [Ringdal 2001].

There are two types of mistakes that can be made in a χ^2-test. One is when a true 0-hypothesis is rejected, it is called a type I fail, and the chance of doing this mistake is set by the α-value. Normally α=0,05, which mean that the researcher is prepared to accept a 5 % chance for the results to be wrong or random [Ringdal 2001].

The other mistake that can be made is a β-mistake, this occurs when a wrong 0-hypothesis are being kept. This mistake is strongly connected to the α-value, the lower this value is the bigger the chance of making the β-mistake is [Ringdal 2001].

The number of "test objects" also affects the outcome of a χ^2-test. If it is a large amount of samples tested, a small difference or a weak correlation can lead to a rejection of the 0-hypothesis. In a smaller collection of samples small differences or weak correlations will not have the same effect. This means that it is easier to reject the 0-hypothesis in a large collection, than in a smaller one, providing that all other factors are equal [Ringdal 2001].

The "degree of freedom" (df) were calculated by taking the number of columns minus one and multiplying it with the number of rows minus one, for this example: df = (2-1)x(2-1) = 1 [Griffiths et al. 2002].

The α value was set to α=0,05, which has become the standard α value [Ringdal 2001, Jekel et al. 2001].

E was calculated for all four possibilities using the formula (row total/study total)(column total/study total) x study total. Figure 16 shows an example of how a grid could look like, the estimated E is in parentheses.

	pili	non-pili	Totals
Human	33 (31)	44 (46)	77
Non Human	4 (6)	12 (10)	16
Totals	37	56	93

Figure16- Example of a grid with 4 variables used in the estimation of E. The numbers in the white cells is the observed number, and the numbers in parentheses are the estimated E.

The statically significance was estimated: $\chi^2 = \Sigma(O-E)-0,5)^2/E$, were O is the observed number in a class and E is the expected number of the same class. To correct for continuity in the χ^2 test, 0,5 was subtracted from the sum of (O-E), this will not make a big difference for large numbers [Jekel et al. 2001].

Results

For all PCR protocols: *E. faecium* DO was used as positive template, *Staphylococcus epidermis* or *E. faecium* BM 4105-RF were used as negative templates, and water was used as master mix control.

PCR Optimalization

The PCR protocols were optimized using *E. faecium* DO as positive template, *S. epidermis* as negative template, and H_2O as mastermix control for the primers marked in table 12. The Optimalization was done because of unspecific primer binding resulting in positive products in the negative control, and in some cases more than one DNA band in electrophoresis. Figure 17 shows one example of Optimalization results. The marked PCR programs in table 12 shows the annealing temperatures based on the Optimalization. The optimal annealing temperature was when the positive template was positive and the negative template was negative.

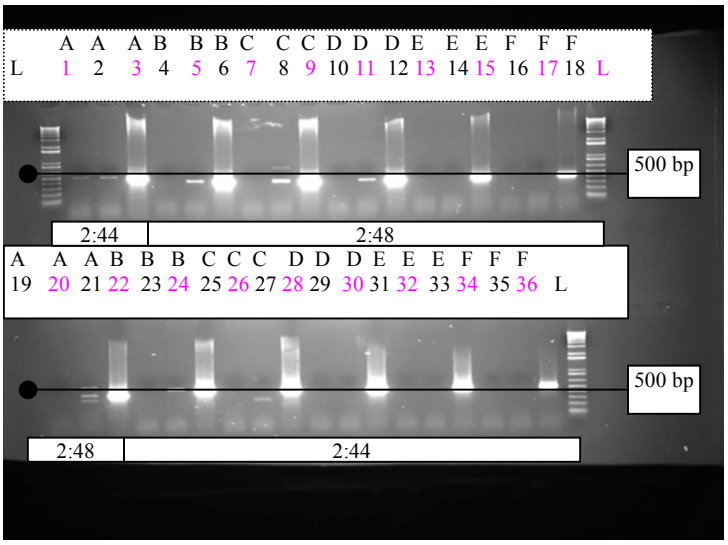

Figure 17- Optimalization results of the PCR programmes. Lane L is 1kb+ molecular weight standard, lanes: 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31 and 34 were mastermix controls (H_2O). Lanes: 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32 and 35 contains the negative template, and wells: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33 and 36 contains the positive template. Block A) 54ºC, B) 55,1ºC, C) 57,4ºC, D) 61,2ºC, E) 64,2ºC, F) 65,8ºC. 2:44 and 2:48 refers to the primer pairs listed in table 12.

The PCR results showed confirmed the *E. faecium* DO to be positive for the pili genes, after optimalization *S. epidermis* was negative for all pili genes.

E. faecium DO, *E. faecium* 64/3 and *E. faecium* BM 4105-RF were tested for both operon 44 and 48. In operon 44 *E. faecium* DO was positive for all genes, *E. faecium* 64/3 was positive for pili 1:44 and pili 3:44, and *E. faecium* BM 4105-RF was negative for all except pili 2:44. In operon 48 *E. faecium* DO was positive for all genes, *E. faecium* 64/3 was positive for pili 1:48, 2:48 and pili 3:48, and *E. faecium* BM 4105-RF was negative for all.

Cloning into pENTR

The PCR product of C3:44R1 and C3:48R1 were ligated into the pENTR vector, and transformed into OneShot competent cells. Confirmation of insert was done by PCR.

Both C3:48 and C3:44 were successfully cloned into the pENTR™ Topo vector by Topo® cloning and transformed into One Shot® Competent *E. coli* cells, C3:44 had 27 transformants, and C3:48 had 40 transformants.10 transformants from each transformation were checked by PCR with C3:44M and C3:48M1 primers (see table12 for primer pairs) to screen for the proper insert. Figure 18 shows the result from such a PCR. Clones in lanes 10-13 and 22-26 had the proper insert with expected size, and plasmids from clones 8-10 in lanes 11-13 and 18-20 in lanes 18-20 were purified for recombination with pDEST17 vector.

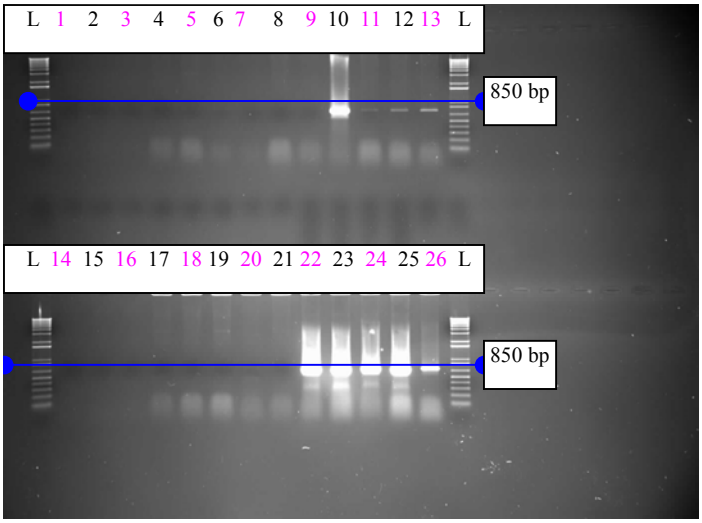

Figure 18- Amplification of inserts in the pENTR vector. Lane L is the 1 kb+ molecular weight standard. Lanes 1 and 14 are the mastermix control, 2 and 15 are the positive template (DO), 3 and 16 are the negative template. Pili 3:44M primers were used to check C3:44 transformants; lanes 4-13 is C3:44 transformants, lanes 10-13 is C3:44 transformants with insert. Pili C3:48M1 primers were used to check C3:48 transformants: lanes 14-26 is C3:48 transformants, lanes 16-20 is transformants with insert.

Plasmid Purification for Recombination into pDEST17

E.Z.N.A. Plasmid Miniprep protocol II was used for purification of for high copy number plasmids (pENTR vector), the NanoDrop was used to check the quality and amount of purified plasmid.

Table 15 (next page) shows the plasmid purification results for the clones used in later experiments. A 260/280 ratio greater than 1,8 indicates that the nucleic acid purity is greater than 90%. The concentration of plasmid pSI20C was not as high as expected, but it was high enough for later use. The concentration of plasmid A was as expected. 1,5 µl pSI20C was used for recombination with pDEST17, and 2 µl A was used for recombination with pDEST17, to fill the protocols demand of 50-150ng entry clone in 1-7µl sample.

Table 15- Quantity and purity of purified plasmids.

Plasmid	ng/µl	260/280[1]	H/L[2]	44/48[3]	Further use
pSI 20C	29,75	1,75	H	48	Transformation and LR recombination
A	49,18	1,99	H	44	Transformation and LR recombination

1 a 260/280 ratio greater than 1,8 indicates a nucleic acid purity greater than 90%
2 H = High copy number plasmids, L= Low copy number plasmids
3 44 = pili C3:44 clones, 48= pili C3:48 clones

Sequencing

PCR for sequencing was done by cycle sequencing programme where the template was PCR products or plasmid, and only one primer was used. The sequencing lab precipitated and used a terminator reaction for obtaining the sequence. Sequencing was done to confirm right orientation of the inserts, confirm that the length and sequence was the same as the operon 44 and 48. Sequencing was done from PCR products, and figure 19 shows the results from such a PCR. Products from *E. faecium* DO, wells 2, 6, 10, 14 and 18 were sequenced. The products were judged, based on figure 19, to have the expected size.

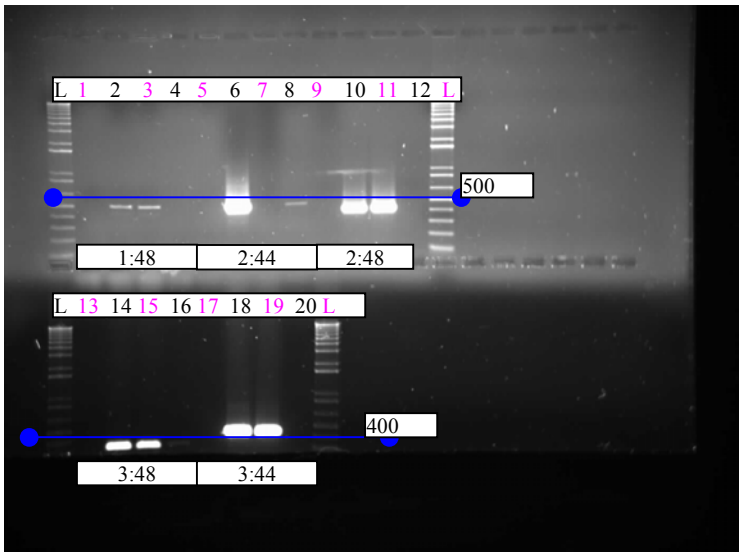

Figure 19- PCR products for sequencing. Lane L contain the 1 kb+ molecular weight standard. Lanes: 1, 5, 9, 13 and 17 contain the mastermix control. Lanes: 2, 6, 10, 14 and 18 contain the positive template. Lanes: 3, 7, 11, 15 and 19 contain the negative template (*E. faecium*64/3). Lanes: 4, 8, 12, 16 and 20 is the *E. faecium* BM 4105-RF. 1:48 and 2:48, 3:48, 2:44 and 3:44, refers to the primer pairs listed in table12.

All primers were used for sequencing and the size of the PCR products in table 16 are based on the sequences obtained. The PCR results showed that there were some differences between the expected size and the obtained size from sequencing, these differences was mainly in the ends, and some gaps were registered.

The difference between 10 PCR products and the expected size differed only by few nucleotides, less than 10. 5 PCR products differed with more than 10 nucleotides from the expected size, with pili C3:48R2 as the one with the biggest difference, this sequence had one strand successfully sequenced. One PCR product, pili 3:44 3', had the exact same size as expected.

Table 16- Size of PCR sequences.

Product	Primers (Forward/Reverse)	Expected size (bp)	Size (bp) obtained from sequencing
Pili 1:44[1]	Pili 1:44F/ Pili 1:44R	370	372
Pili 2:44[1]	Pili 2:44F/ Pili 2:44R	497	500
Pili 3:44[1]	Pili 3:44F/ Pili 3:44R	420	421
Pili C3:44R1[1]	Pili C3:44F/ Pili C3:44R1	1682	1686
Pili C3:44R2[1]	Pili C3:44F/ Pili C3:44R2	1674	1610
Pili C3:44 5'	M13F/ Pili 3:44RL	720	739
Pili C3:44M	Pili 3:44FM/ Pili 3:44RM	756	755
Pili C3:44 3'	Pili 3:44 FR/ M13 R	699	699
Pili 1:48[1]	Pili 1:48F/ Pili 1:48R	523	522
Pili 2:48[1]	Pili 2:48F/ Pili 2:48R	418	409
Pili 3:48[1]	Pili 3:48F/ Pili 3:48R	323	322
Pili C3:48R1[1]	Pili C3:48F/ Pili C3:48R1	1857	1826
Pili C3:48R2[1]	Pili C3:48F/ Pili C3:48R2	1854	1222
Pili C3:48 5'	M13F/ Pili 3:48RL	650	497
Pili C3:48M1	Pili 3:48FM/ Pili 3:48RM	699	693
Pili C3:48M2	Pili 3:48FR/ Pili 3:48R	722	724
Pili C3:48 3'	Pili 3:48F/ M13R	746	735

1) Optimized PCR for these primers.

C3:44 3', C3:44 5', C3:48 3' and C3:48 5' were used to sequence the ends and confirm the orientation, the primers for these products are found in table 12. By sequencing C3:44M, C3:48M1 and C3:48M2 using the primers noted in table 12, the middle part of the sequences of the C3:44 and C3:48 genes. The full sequences were obtained by making a consensus of the sequences from the ends and the middle.

The sequencing showed that the pili C3:44 was almost identical to operon 44, that the right part of the genome had been amplified, and that the insert was inserted correct for further work. The estimated size of the protein encoded by the C3:44 sequence was approximately 136 kDa. The difference between operon 44 and the obtained sequence was the 5'C in the CACC in the beginning of the sequence.

The sequence for C3:48 is almost identical to operon 48, the difference was the ACC in the CACC. The sequence confirmed the orientation of the insert, and that the right part of the genome had been amplified. The estimated size of the protein encoded by the C3:48 sequence was approximately174 kDa.

The sequences obtained are found in appendix A, were C3:44 are aligned with operon 44, and C3:48 are aligned with operon 48. Primers used for cloning are written in blue, primers used for sequencing are written in green, and red markings indicate the differences between the operons and the obtained sequences.

Cloning into pDEST17

The cloning of the insert into pDEST17 vector was done by recombination between the pENTR and pDEST vector followed by transformation into DH5α chemical competent cells. Plasmids were purified from DH5α cells for transformation into BL21-AI cells for expression.

C3:44 were successfully recombined into the pDEST17 vector and transformed into DH5α competent *E. coli* cells for maintaining of the plasmid, the transformation resulted in 23 transformants. The presence of insert was confirmed in 2 out of 7 tested clones by internal primers, se figure 20. Lanes 10 and 11 in figure 20 shows C3:44 transformants with inserts, and plasmid was purified from these clones for transformation into BL21-AI cells for expression.

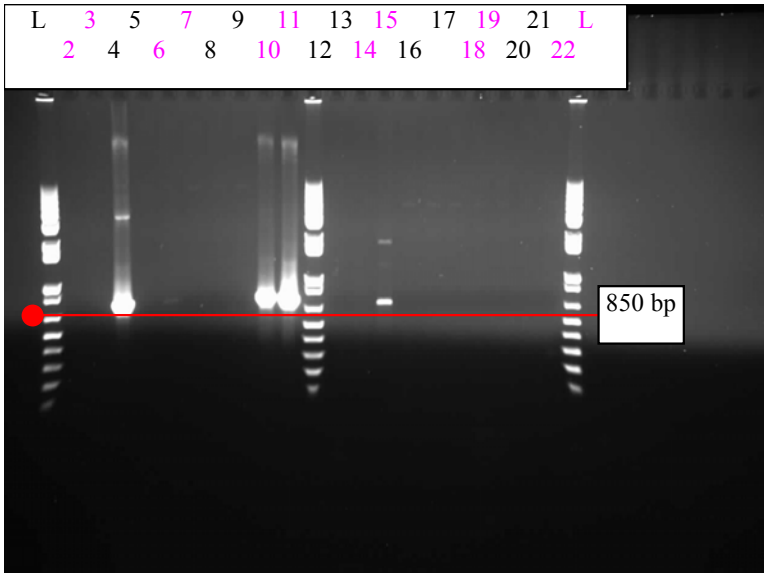

Figure 20- PCR amplification of inserts in the pDEST17 vector. Lanes L contains the 1kb+ molecular weight standard. Lanes 2 and 13 contain mastermix controls (H_2O), 3 and 14 contain negative controls, 4 and 15 contain positive controls. Lanes 5-11 contain C3:44 transformants, lanes 10 and 11 show transformants with insert, primers 3:44RM/FM were used. Lanes 16-22 contain C3:48 transformants, no clones with insert was confirmed, primers 3:48RM/FM were used, see table 12.

C3:48 clones gave colonies when transformed into DH5α competent *E. coli* cells, but PCR with internal primers did not confirm a successful recombination into pDEST17, no inserts were observed, se figure 20. After two attempts, the cloning of C3:48 into pDEST was terminated.

Purification of Plasmids for Protein Expression

Plasmid purification was done with the E.Z.N.A. Plasmid Miniprep protocol I for low copy number (pDEST vector). NanoDrop was used to check the quality and amount of

purified plasmid. The pDEST17 vector had to be purified; the same protocol was used for this purification.

Table 17 shows the plasmid purification results for the clones used in later experiments. A 260/280 ratio greater than 1,8 indicates that the nucleic acid purity is greater than 90%. A6 had to be diluted 1:1 with water, to obtain 5-10 ng DNA in 1-5 µl sample as required in the protocol, before transformation into BL21-AI cells and expressed.

Table 17- Quantity and purity of the purified plasmids

Plasmid	ng/µl	260/280[1]	H/L[2]	44/48[3]	Further use
pDEST 17	19,03	2,22	L	-	Vector
A6	13,47	1,94	L	44	Transformation and expression
20C1	10,7	1,94	L	48	Not used

1 a 260/280 ratio greater than 1,8 indicates a nucleic acid purity greater than 90%
2 H = High copy number plasmids, L= Low copy number plasmids
3 44 = pili C3:44 clones, 48= pili C3:48 clones

Expression of C3:44

The pDEST17 with the insert was transformed into BL21-AI™ competent cells for expression. The cells were lysed using a protocol to separate water soluble and insoluble proteins by centrifugation at 4˚C, and a western blot was performed to check if the protein was in the water soluble or insoluble fraction. Detection was done by chemiluminiscense. InVision staining was also performed to check the solubility of the protein, but this method failed.

Only pili C3:44 was cloned and expressed, the expression showed that the protein was water insoluble. Figure 21 shows an example of expression results. The protein seems to be approximately 100kDa, which is less than the estimated 135,5kDa. All three clones tested in this experiment expressed the pili protein in the pellet fraction, containing water insoluble proteins, from the induced cells.

The first western blot showed that more and more protein was produced for each time sample, just as one would expect, and the result showed that after two hours the induced cells had produced enough protein for detection and purification. After 3 hours a lot of incomplete proteins were observed as a short ladder (figure 21 is from the second western blot).

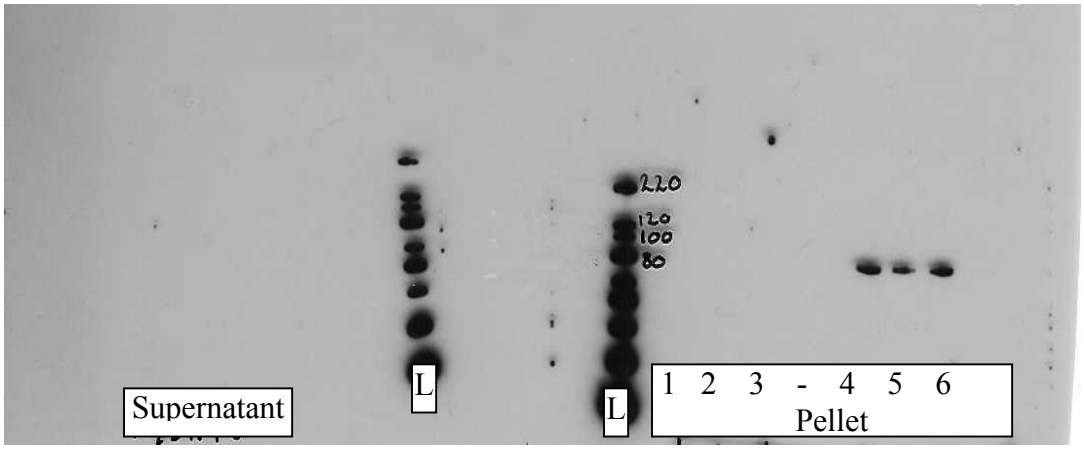

Figure 21- Western blot of a SDS-PAGE. The left side is the lysate/supernatant, the right side: Lane 1-3 contains the uninduced cells; lane 4-6 contains the induced cultures. The Magic Marker molecular weight standard is in the two lanes with several bands.

Prevalence of Pili Genes

The prevalence of pili loci were investigated by amplification of one selected region in each locus, 1:44 in operon 44 and 1:48 in operon 48. The collection of isolates was originally designed to check for VREfm [unpublished], and the same 96 well setup is used in this thesis.

The screening of 93 isolates was performed running two PCRs with pili 1:44 and pili 1:48 primers, the programs used are in table 12. After the PCR an E-gel was run to check if the isolates were positive or negative, no sizes were checked.

The df for the estimation of p-value was calculated to be 1, and $\alpha = 0,05$ for both screenings.

The results in table 18 shows that a total of 73 isolates were positive for pili, 67 (92%)of these were positive for 1:44, 37 (51%) were positive for 1:48, while 31 (42%) were positive for both. This gave a p-value of 0,05, and there is a correlation between the two genes.

Table 18- The overall result from both screenings.

Well	Reference number	Result 44[1)]	Result 48[1)]	Well	Reference number	Result 44[1)]	Result 48[1)]	Well	Reference number	Result 44[1)]	Result 48[1)]
1a	TUH 44-39	+	+	**5a**	TUH 32-79	-	-	**9a**	TUH N1-90	+	+
1b	TUH 44-29	+	+	**5b**	TUH 32-80	-	+	**9b**	TUH N2-1	+	-
1c	TUH 44-34	+	-	**5c**	TUH 340	+	+	**9c**	TUH N2-26	-	-
1d	TUH 7-57	+	+	**5d**	TUH 4-15	+	+	**9d**	TUH N2-78	-	-
1e	TUH 7-58	+	-	**5e**	TUH 4-21	+	-	**9e**	TUH N2-85	-	-
1f	TUH 41-64	-	+	**5f**	TUH 4-22	+	-	**9f**	TUH S1-102	-	-
1g	TUH 12-1	-	-	**5g**	TUH 4-41	-	-	**9g**	TUH S1-28	+	-
1h	TUH 12-16	-	+	**5h**	TUH 4-65	+	-	**9h**	TUH S1-7	+	+
2a	TUH 12-4	+	+	**6a**	TUH 44-23	+	-	**10a**	TUH S1-95	+	+
2b	TUH 2-18	+	+	**6b**	TUH 44-31	+	-	**10b**	K8-42	+	+
2c	TUH 2-19	+	-	**6c**	TUH 44-40	+	-	**10c**	K8-43	+	+
2d	TUH 2-21	+	+	**6d**	TUH 45-12	+	-	**10d**	K8-45	+	+
2e	TUH 2-8	+	+	**6e**	TUH 45-22	+	-	**10e**	K9-26	+	+
2f	TUH 32-56	+	-	**6f**	TUH 45-3	+	-	**10f**	K9-27	+	+
2g	TUH 32-61	-	-	**6g**	TUH 45-5	+	-	**10g**	K9-28	+	+
2h	TUH 32-62	-	+	**6h**	TUH 44-52	-	-	**10h**	K9-72	-	-
3a	TUH 32-63	-	-	**7a**	TUH 44-50	+	-	**11a**	K8-50	+	+
3b	TUH 32-64	+	-	**7b**	TUH 44-42	+	-	**11b**	TUH 2-40	+	+
3c	TUH 32-65	+	-	**7c**	TUH 44-47	+	-	**11c**	TUH 2-13	+	+
3d	TUH 32-66	+	-	**7d**	TUH 45-35	+	-	**11d**	TUH 7-56	+	+
3e	TUH 32-67	-	+	**7e**	TUH 45-25	-	-	**11e**	K8-61	-	-
3f	TUH 32-68	+	+	**7f**	TUH 45-30	-	-	**11f**	K8-62	+	+
3g	TUH 32-69	+	+	**7g**	TUH 7-15	+	-	**11g**	K8-64	+	-
3h	TUH 32-70	+	+	**7h**	TUH 7-55	-	-	**11h**	K8-74	-	-
4a	TUH 32-71	+	-	**8a**	TUH D2-34	+	-	**12a**	K8-69	-	+
4b	TUH 32-72	+	+	**8b**	TUH D2-6	+	-	**12b**	K8-68	-	-
4c	TUH 32-73	+	-	**8c**	TUH D2-69	+	-	**12c**	K9-10	-	-
4d	TUH 32-74	+	-	**8d**	TUH D2-91	+	-	**12d**	K18-79	-	+
4e	TUH 32-75	+	+	**8e**	TUH I1-82	+	-	**12e**	41-73	-	-
4f	TUH 32-76	+	-	**8f**	TUH N1-42	+	+	**12f**	Control	-	+
4g	TUH 32-77	+	+	**8g**	TUH N1-59	+	-	**12g**	Control	+	-
4h	TUH 32-78	-	-	**8h**	TUH N1-80	+	-	**12h**	Control	-	-

1) "-" is negative, "+" is positive

Table 22 and figure 19 shows the results for screening for pili 1:44. The 67 distinct bands show the location of the positive isolates.

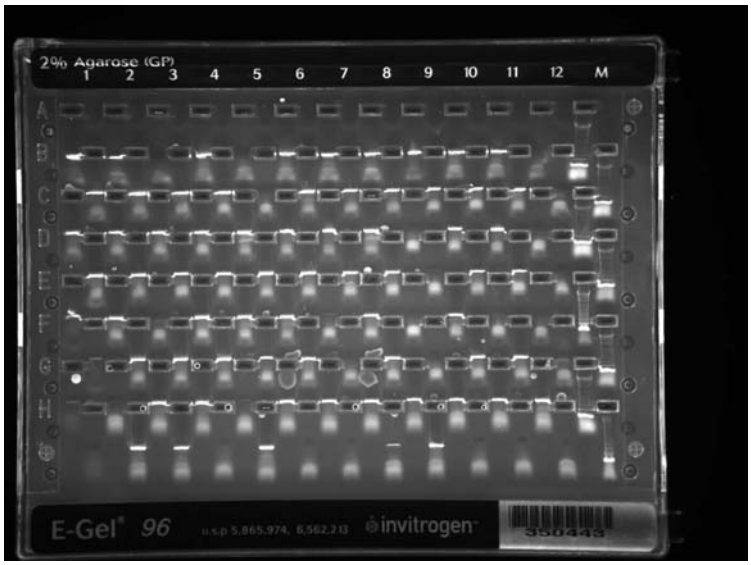

Figure 22 - 1:44 screening, distinct bands between wells indicate positive isolates. The M indicates the lane of wells with the molecular marker.

The p-values for pili 1:44 was above the α=0,05 for most of the χ^2- tests. There were a correlation between pili 1:44 and the hospital, for the hospital survey isolates with a p-value of 0,005, and a p-value of 0,05 for the hospital outbreaks isolates. There was also a correlation with isolates taken from other animal sources than faeces.

Table 19- The screening results from pili 1:44

Pili 1:44			Total	Positive	% of types	p-value
Total	Humans and animals	Isolates	93	67	72	-
Humans	**Sample source**	Human	77	55	71	0,1
	Origin	Community Survey	11	7	64	0,5
		Hospital Survey	7	1	14	**0,005**
		Outbreak	5	5	100	0,1
		Hospital outbreak	7	7	100	**0,05**
		Clinical	44	32	73	0,5
		Other	3	3	100	0,5
	Isolation site/sample type	Faeces	35	22	63	0,1
		Blood	17	14	82	0,1
		Urine	13	10	77	0,5
		Other	10	5	50	0,5
		From to sites	2	2	100	0,1
	Van Type	VanA and VanB	46	33	72	0,9
		VanA	37	26	70	0,9
		VanB	9	7	78	0,5
		None	31	18	58	-
	Geographical origin	USA	8	6	75	0,5
		Europe	68	48	71	0,5
		Scandinavia	48	36	75	0,1
		Norway	38	26	68	0,9
		Other	1	1	100	0,1
Animals	**Sample source**	Animals	14	11	79	0,5
	Epidemiology	Community survey	12	11	92	0,01
		Other	2	0	0	-
	Sample type	Faeces	12	9	75	0,1
		Other	2	2	100	**0,005**
	Van Type	VanA and B	9	7	78	0,5
		VanA	9	7	78	0,5
		VanB	0	0	0	-
		None	5	4	80	-
Other			2	2	100	-

The 37 distinct bands in figure 23 shows the location of the isolates positive for pili 1:48 in the screening.

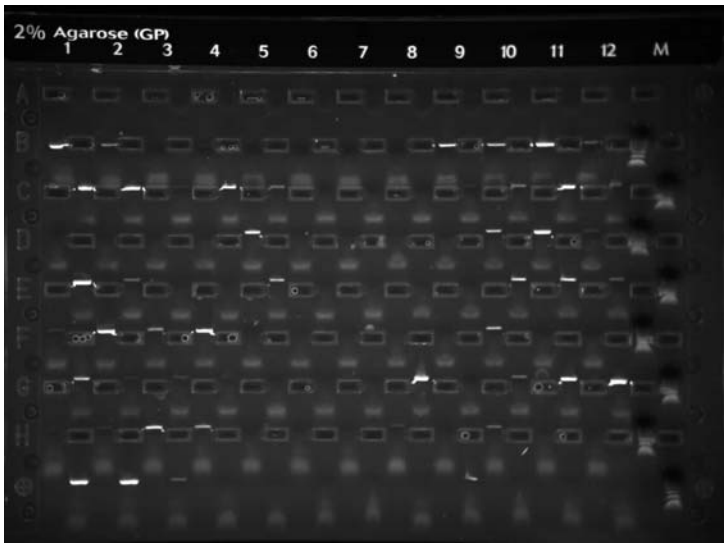

Figure 23- Screening for pili 1:48, a distinct band between wells indicates positive isolates. The M indicates the lane of wells with the molecular marker.

The results in table 20 shows that there are correlation between pili 1:48 and blood (p =0,025), vancomycin resistance (p=0,01), more concrete vanA (p=0,005), and animals (p=0,025), and for these tests the 0-hypothesis is rejected. For the other tests there were no correlation between pili 1:48 and origin and geographical origin, the p-values were all above 0,05.

Table 20- The screening results from pili 1:48

Pili 1:48			Total	Positive	% of types	p-value
Total	Humans and animals	Isolates	93	37	40	-
Humans	**Sample source**	Human	77	33	43	0,5
	Origin	Community Survey	11	4	36	0,5
		Hospital Survey	7	2	29	0,5
		Outbreak	5	3	60	0,5
		Hospital outbreak	7	3	43	0,5
		Clinical	44	20	45	0,5
		Other	3	1	33	0,5
	Isolation site/sample type	Faeces	35	18	51	0,1
		Blood	17	3	18	**0,025**
		Urine	13	5	38	0,5
		Other	10	5	50	0,5
		From to sites	2	2	100	0,5
	Van Type	**VanA and VanB**	46	25	54	**0,01**
		VanA	37	22	59	**0,005**
		VanB	9	3	33	0,5
		Non vanA and VanB	31		0	-
	Geographical origin	USA	8	3	38	0,5
		Europe	68	30	44	0,5
		Scandinavia	48	21	44	0,9
		Norway	38	18	47	0,5
		Other	1	1	100	0,1
Animals	**Sample source**	**Animals**	14	2	14	**0,025**
	Epidemiology	Community survey	12	2	17	0,1
		Other	2	0	0	0,1
	Sample type	Faeces	12	2	17	0,1
		Other	2	0	0	0,1
	Van Type	VanA and B	9	1	11	0,5
		VanA	9	1	11	0,5
		VanB	0	0	0	-
		Non VanA and VanB	5	1	20	-
Other			2	2	100	-

Discussion

The results showed that the PCR tools designed worked as planned for the primers. *E. faecium* DO was, as expected, positive for all pili genes. The results indicates that *E. faecium* BM 4105-RF might be negative for all loci tested for and could have been used as a negative control, while *E. faecium* 64/3 is positive for all, but pili 2:44.

PCR Optimalization

In *E. faecium* 64/3 strain every pili sequences except 2:44 were found, and in the *E. faecium* BM 4105-RF strain only 2:44 were found, this could indicate that the samples had been switched, but it was the same in every PCR run. Unspecific primer annealing is probably not the cause of these odd results, it was because of unspecific binding that the PCR programs had to be optimized, and the problem was solved after optimalization.

Even though *E. faecium* BM 4105-RF strain has tested positive for only 2:44, there is reason to believe that this strain really is negative for the pili genes, and could be used as a negative control.

Cloning into pENTR

Both sequences were confirmed in the pENTR vector by PCR, and pili C3:44 was confirmed in the pDEST17 vector with the same method. The number of transformants were low, this could be due to the DNA:vector ratio not being optimal, or because the treatment of the competent cells were too harsh.

Plasmid Purification for Recombination into pDEST17

The amount of purified plasmids was lower than the lowest amount expected for the high copy number plasmid pSI20C. This can be because of poor cell lysis due to bad

dispersion of the cells prior to the addition of Solution II or to short incubation in Solution II (E.Z.N.A plasmid Miniprep kit I & II manual). NanoDrop was used for quantification of the plasmid, this method is not an accurate method, it gives an idea of the concentration, and the concentration of the purified plasmids was probably higher than the estimated concentration. NanoDrop also register the amount of contamination by proteins, so the nucleic acid purity of the sample tested can be checked, this sort of contamination would affect the DNA concentration. The results from NanoDrop indicates that there is a low contamination of proteins.

The plasmid purity is not checked, the method does not differentiate between plasmid DNA and other DNA molecules, such as fragments. In some cases the NanoDrop gave a negative concentration of nucleic acid, and the blank control had to be measured to reset the control the samples were measured against. This should probably have been done between each sample, just to be sure.

Alternatively the DNA concentration could be measured by running an EtBr agarose gel electrophoresis, where one lane contains DNA with known concentration. Using this method expose the presence of other DNA molecules, but it would not say anything about protein contamination.

The concentration results from NanoDrop were used to estimate the volume of plasmid that had to be used to reach the recommended amount for transformation and recombination, and this could be a reason for transformation, and maybe recombination, failure. The amount and purity were important factors when the plasmids used for further use were selected.

Sequencing

Most sequences obtained had different length than the expected sequence length; this is due to the terminal N's that had too weak signals to be read and because of that was deleted to obtain a better alignment between the two strands. Most sequences had some

internal gaps, this was probably because of misreading of the N's in the sequence, or misreading of peaks that might have been background, or the nucleotide had been placed a bit too late or early compared to the peak.

Sequences used for sequencing C3:44 and C3:48 did not have the expected sequence length, but when the sequences were put together the lengths were as expected.

In addition to this not all sequences had a successful sequencing of the two strands, and therefore some lengths are based on only one strand. This was especially a problem for pili C3:48 R2, which had a long expected length and only one primer gave a sequence, when the sequences are long it is important to obtain sequences from both strands to get the best sequence.

When the gaps are spread the reading frame will be shifted after each gap, and the peptide sequence could be altered as a consequence of this. Three gaps in a row, or very near each other will not cause serious alteration in the peptide sequence.

The C3:44F/R1 primers yielded a longer sequence than expected, and C3:48F/R1 primers yielded a shorter sequence than expected, these differences might be due to weak signals in the sequence, and the length of the sequence itself. The amount of DNA in the sequencing reaction is crucial, too little and the signals get to weak for a reliable reading, too much and the signals gets to strong for a reliable reading, they disturb the sequence. This have probably been the reason for the bad sequences and the differences in expected size and obtained size. Most differences were due to terminal gaps, which could be in both ends of both strands, and therefore the sequence could be shortened in both directions and maybe in the middle if both sequences had bad overlapping ends. In some sequences there was not even possible to read the primer sequences, in some cases there was too much noise to see the highest top, in other cases there was just some lines on the bottom.

There might be other reasons for bad sequences. Contamination of the template with for example other DNA extracts, an extra primer and proteins will affect the out coming sequence. Unspecific primers can also cause problems because they bind to different places in the genome, and not necessarily within the region to be sequenced, and leads to a disturbing background that can be difficult to segregate from the sequence [The DNA Sequencing Core Facility]

Cloning into pDEST17

Pili C3:48 insert was not confirmed in the pDEST17 vector, but the transformation yielded some colonies on selective plates, so the transformation was successful, and the orientation of the insert was confirmed in pENTR, the orientation should not shift when the insert is recombined between the to vectors, but a new check of orientation should have been done just in case the insert orientation shifted during the recombination.

There might have been some problem with the LR-recombination reaction setup for pili C3:48, the donor:receptor vector ratio might have been wrong, but the amount recommended in the protocol was used, which indicate that it could be an idea to optimize the reaction, or that the NanoDrop results differed from the actual concentration.

Pili 1:44 was recombined, transformed and expressed using the same protocol used for pili C3:48, the LR-recombination and transformation of the two genes were done at the same time, so the same mistakes or changes from the original protocol were done for both clones.

Expression

The protein expression showed that pili 1:44 protein was expressed in amounts suitable for purification, it created distinctive bands in the chemiluminicent detection, it was a bit smaller than expected and it was water insoluble; this might be because of the hydrophobic domain and the following charged tail, which groups the pili proteins with

the CWAPs. The hydrophobic domain and the charged tail anchor the pili to the cell wall [Fischetti et al. 1990, Boekhorst 2005] and this anchoring to the wall can have been too strong and made it difficult not to pellet the pili protein with the water insoluble proteins. A relatively easy way to check this is to boil the cell wall fraction and run a SDS PAGE and look for a several distinctive bands were the difference in size is the same as the size of one subunit [Scott & Zähner 2006].

A way of getting a water soluble protein could be to reduce the coding region to be expressed, so that the LPXTG motif is excluded, in this way the hydrophobic domain and the charged tail would not be expressed. A reasonable place to end the region to be expressed would be after the T in the LPXTG motif, since this is were sortase works to remove the hydrophobic domain and charged tail, and link the subunits to each other.

To avoid cloning and expression, a defined region of the putative pili protein could be synthesized *in vitro*. Hendrickx et al [2008] used a 15 amino acid long peptide sequence to produce antibodies for electron microscopy, but they did not achieve successful results for C3:44 using this method.

Another way of making antibodies that will recognise the protein is to make a fusion protein that is soluble and purify it. A fusion protein is the product of two different genes that are ligated together [Nelson & Cox 2005].

The protein could be in the water insoluble fraction because of failed cell lysis. Using another lysis buffer, or using glass beads or sonication together with lysis buffer could confirm an eventual lack of lysis and lyse the cells.

The overall goal is to purify the protein and produce antibodies for immunogold labelling and electron microscopy, where C3:48, from PGC3, would appear as a flexible structure over 1μm at the poles of the cells, and uniformly distributed over the surface of single cocci [Hendrickx et al. 2008]. The C3:44 from PGC2 has not yet been identified in electron microscopy.

Further approaches

The main work for this thesis had to be terminated when the protein turned out to be in the water insoluble fraction after the pilot expression and cell lysis. To overcome this problem other lysis methods can be tried out, the LPXTG can be left out of the region to be expressed as discussed above, a fusion protein can be made or cloning of a specific peptide sequence can be tried. When the protein is water soluble it can be purified, there are many kits that purify proteins with different tags, and the most used is the small his-tag. The purified protein is used to make antibodies for screening for the protein in electron microscope.

When preparing the cells for transmission electron microscopy it is important to be aware of the possibilities for artefacts in the final result, every step in the preparation can cause an artefact in the final result [Bozzola and Russel 1999].

When immunolabeling is preformed for electron microscopy, tagged secondary antibodies are often used in addition to the primary antibodies. Reasons for this is that the use of primary antibodies can cause problems for the antibodies ability to bind to the antigen because of the tag that have to be used for detection. Another problem with using primary antibody is the difficulty of obtaining a conjugation between the tag and antibody, and it is often inefficient.

The secondary antibodies do not cause these problems, and they enhance the signal because more than one molecule can bind to the antigen (primary antibody), so more tags are connected to the antigens in the cells [Bozzola and Russel 1999]. These things have to be in mind when deciding if only primary antibody should be used, or if a secondary antibody should be used in addition to the primary. The concentration of antibodies has to be adjusted, this easiest done by checking a series of preparates with different antibody concentrations in the microscope.

When pili is confirmed there could be put some effort in looking for the other pili proteins coded for in the identified gene clusters, PGC1, PGC2, PGC 3 and PGC4, check for their localization in the cell, or on the cell, and screen for the presence of these genes. There could also be put some effort in detection of the sortase that has been identified.

Prevalence of Pili Genes

The overall results indicates that pili are a virulence factor, due to the correlation of pili 1:44 to the hospital, and the correlation of pili 1:48 to the isolates from blood, and the isolates with vancomycin resistance. Both structures showed correlation to factors outside the hospital, and humans; pili 1:44 had a correlation to samples from other sources than faeces, and pili 1:48 showed a correlation to animals, this indicates that pili is important also outside the hospital.

Pili 1:44 Prevalence

The prevalence of pili genes showed that pili 1:44 is correlated to the hospital environment, but not to clinical isolates. The correlation was in hospital survey isolates and in hospital outbreak isolates, suggesting that pili 1:44 is important for *E. faecium* survival in the hospital environment, and for causing infections when under the right circumstances.

The correlation of *E. faecium* to hospital outbreaks could also indicate that *E. faecium* is easily transferred between infected patients; this transformation can be indirect via medical devices, the clothes and hands of health care personnel or by surfaces that is often in contact with other persons, both health care personnel and other patients. The transfer between patients can also be more direct, like direct contact between the patients.

The correlation between 1:44 and the hospital survey supports the idea of *E. faecium* being able to survive in the hospital environment, and that the transfer via indirect routes is possible, and even probable. The expression of pili in the hospital environment can be

an advantage in maintaining the population, in the acquisition of new mechanisms that can make them even more potent to humans. Which support the idea of pili 1:44 being important for the survival in the hospital environment.

Pili 1:48 prevalence

The expression of pili 1:48 showed a correlation to blood, vancomycin resistance and animals. Pili 1:48 is thought to be a subunit of the pili C3:48 structure, which has been shown to be a long flexible structure [Hendrickx et al.. 2008].

The expression of pili in *E. faecium* isolates from blood indicates that this long flexible structure is an advantage in this environment. The pili can help *E. faecium* to attach to blood cells or to the blood vessel it-self, and pili could help the bacteria to translocate from the blood vessel into new sites in the body and cause infection there.

The p-values also indicate that there are correlations between pili 1:48 and vancomycin resistance, preferably vanA. This could be a problem if *E. faecium* with pili and vancomycin resistance is the cause of bacteriemia, or other blood borne diseases. The enterococcal infection would be harder to treat because of the vancomycin resistance, and the pili would make it easier for the enterococci to adhere to host cells, or translocate to other sites.

The correlation between pili and animals could mean that the structure is an advantage in other environments than the hospital. The isolates from animals were all, but two, from community survey, and there is no information about *E. faecium* in clinical isolates from animals, and therefore nothing can be said about animal invasive *E. faecium*, and no conclusion about the function of pili in animal pathogens. The material does not include the information about what sort of animal origin the isolates come from, so the correlation might be false.

Differences Between 1:44 and 1:48

The pili 1:44 is more spread than pili1:48, with 67 (92%) positives out of total 73 positives, with 36 (49%) isolates positive for only 1:44. The 1:48 has 37 (51%) positive isolates out of the 73 positive isolates, and only 6 (8%) isolates with only pili 1:48. These results indicate that pili 1:44 is more spread than pili 1:48; it might also indicate that pili 1:44 is more useful for the enterococci than pili 1:48, or that pili 1:44 is an older gene cluster that have had more time to spread in different environments and over geographical areas.

The environments the pili are expressed in have an influence of the rate the genes will spread, and how fast they will spread to other areas. Pili 1:44 is found in the hospital environment, this will make it easier to spread to other hospitals via persons travelling between hospitals, like for example patients that are transferred from one hospital to another.

Pili 1:48 is expressed in the blood, and are dependent on contact between the bacteria in some waste from the body, and something in the environment that will transfer it to other areas. This "something" could for example be clothing, floor, health-care personnel or visitors, but these things would somehow have to get contamination and not wash it of or getting rid of it some other way before the bacteria are out in the environment.

Hendrickx et al. 2008 showed that pili1:48 was more spread than pili 1:44, which is the opposite of the results in this thesis, this can reflect the isolates used for the screenings influenced the results. The isolates in this thesis was originally designed for another work [unpublished], and strains interesting for that work is included in this material. This material is unbalanced with regard to *E. faecium* isolates from animals.

There is a correlation between the two genes, pili 1:44 and 1:48. This indicates that both pili could be physically linked by being located near each other on the same genetic element, or that they are exposed to the same selective pressure. If they had been located on the same genetic element, all isolates would probably have tested the same for both

pili 1:44 and pili 1:48, this is not the case, so the genes must be exposed to the same selective pressure, even though they are expressed in complete different environments.

The reason for their correlation could be as simple as pili 1:44 provides an advantage between hosts to infect, and that pili 1:48 is not needed in this environment. When *E. faecium* infect the host another type of pili is needed, and then 1:48 is expressed and not 1:44. This would make it an advantage for the *E. faecium* to have both pili, and they are selected together by the ability to survive in both the hospital environment and in blood.

The difference in distribution indicates that the pili have different functions for the survival. Or it could indicate that they are expressed under different conditions. The hospital environment is probably a cold environment compared to the blood, which would indicate that pili 1:44 is expressed under colder conditions than 1:48. According to Hendrickx et al [2008] pili 1:48 will not be completely expressed at 21ºC (room temperature), but monomeric forms of C3:48 was observed. When the cells were grown at 37ºC the C3:48 was expressed as a fully polymerised pili structure. This support the results of this thesis, that indicate that the two pili genes are expressed at different temperatures, because the environments they are correlated to have different temperatures, and that pili 1:48 is dependent on higher temperatures than 1:44, all though Hendrickx et al [2008] do not say anything about pili 1:44.

Consequences of Pili

The consequences of having pili associated with enterococci that are specialists in surviving in the hospital environment, like CC17, could be severe. The CC17 are already known for their antibiotic resistance and putative pathogenicity island containing the esp, acm and probably hyl_{fm} genes [Hendrickx et al. 2007], which cause problems in treating infections by these bacteria.

Pili are thought to be an advantage for bacterial adherence to host tissue and some pili structures are involved in biofilm formation (ebp) [Singh et al 2006], this could make it

easier for the enterococci to infect a host, and withstand treatment, due to the biofilms protection against antibiotic effects [Merode et al. 2006].

Pili is also thought to be involved in cell-cell communication, and plasmids and other genetic elements can be transferred to other bacteria by conjugation, enterococci are known to transfer mobile genetic elements to other bacteria, this could be other enterococci species, Gram-positive bacteria or Gram-negative bacteria [Donelli et al. 2004]. This transference to other species can be fatal if the receiver of the genetic elements is a human pathogenic bacterial strain like for example MRSA.

Pili have been associated with biofilm production in *E. faecium* [Singh et al. 2006], and this in connection with vancomycin resistance could cause a problem in treating *E. faecium* infections, and the combination could be an extra advantage for survival, both in/on the host and in the surroundings. The results from this work do not indicate this to be a problem, yet. The gene correlated with vancomycin resistance was not correlated with the surrounding environment, and the gene that had a significant correlation to the hospital environment did not show any correlation to vancomycin resistance.

The antibiotic pressure in the hospital setting is high, and screenings for resistance to other antibiotics in the isolates could reveal more about their adaptation to this environment, and indicate if it is a hospital subpopulation in development or if it is an existing subpopulation, like CC17 isolates.

Concluding remarks

1. *To design PCR tools for amplification of putative pili coding sequences from E. faecium.*

   This was successfully done. The PCR programs obtained was used for sequencing and cloning. Not all primers were optimalized, only the primers used for cloning (C3:44R1 and C3:48R1), pili 3:48 F and pili 3:48R, these were optimalized because of problems with unspecific binding in the negative control, and to some extent in the positive control.

2. *To perform TOPO-cloning of amplicons into pENTR-vector by directional insertion, and to recombine the inserts into pDEST17 for expression.*

   Both genes to be cloned, C3:44 and C3:48, were successfully cloned into the pENTR vector and transformed into TOP10 chemically competent *E. coli* cells. Plasmids from clones with confirmed insert was purified from overnight cultures of these cells, the overnight cultures was also used to make a freeze culture for long time storage.

   Only C3:44 was successfully recombined into pDEST17, and transformed into DH5α chemically competent cells for maintaining the pDEST17 vector with the confirmed inserts. C3:48 formed colonies after the recombination and transformation, but no inserts were confirmed, and the experiments involving C3:48 stopped at this point.

3. *To purify the putative pili proteins by affinity chromatography and inject rabbits for production of polyclonal antibodies.*

   Before the expression, plasmids had to be isolated from the DH5α cells and transformed into BL21-AI cells containing the T7 promoter. The expression of C3:44 pili protein showed that the protein is water insoluble, and the work for detection of

pili in *E. faecium* was terminated. The reason for the protein being water insoluble are probably the hydrophobic domain, which LPXTG is a part of, and the charged tail that is used to keep the protein in the cell wall long enough for sortase to bind and link the protein to another pili subunit.

This problem can be avoided by cutting of the G in the LPXTG motif, or cutting the whole motif of. Other ways of avoiding this problem is to clone a part of the gene to get a peptide sequence, or fuse the protein to make it soluble. The protein has to be soluble for purification.

4. *To detect pili by electron microscopy with immunogold labeling*.

   Because of the protein being water insoluble it was not purified and it was not possible to produce antibodies, and the electron microscopy work for this thesis was not accomplished.

5. *To design PCR tools for screening of pili genes in a collection of E. faecium isolates*.

   PCR tools for screening of the pili genes were successfully done. Before screening the PCR programs was optimalized, due to problems with unspecific primer binding to the negative control, and to some extent in the isolated *E. faecalis* DNA.

   The results showed that pili 1:44 is correlated to isolates from hospital survey and hospital outbreak. 1:44 was also shown to correlate to isolates taken from animals, from sources other than faeces.

   Pili 1:48 was shown to correlate to isolates from blood and animal faeces, it was also shown to be a correlation between pili 1:48 and vancomycin resistance.

   Pili 1:44 and pili 1:48 are thought to be correlated to each other; this is based on the results in this thesis. The correlation is probably due to the same selective pressure.

The results also indicate that the two genes have different roles in *E. faecium*, due to the correlation to different environments. Pili 1:44 is thought to be expressed under room temperature, this is assumption is not tested, but based on the correlation to isolates from hospital survey. Pili 1:48 has been shown to down regulate the expression under room temperature, and be expressed at 37ºC [Hendrickx et al. 2008].

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Figures:

Figure 1 Population structure
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Figure 3 - 1kb+ molecular weight standard (downloaded 01.12.08).
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Figure 4 - MagicMarker molecular weight standard.
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Figure 5- the pENTR vector (downloaded 07.11.2008).

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Figure 6- the pENTR vector, sequence (downloaded 22.11.2008)
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Figure 7- the pDEST vector (downloaded 07.11.2008):
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Figure 8- The pDEST17 vector sequence (downloaded 22.11.2008)
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Figure B1- p-values (downloaded 14.11.2008)
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Appendix A: DNA sequences

Pili C3:44/ Contig 651 alignment with primers. Primers written in blue were used for cloning, while primers written in green were used for sequencing, the red markings indicates the differences between contig 651 and the obtained C3:48 sequence. The M13 primers are not included because they were located in the vector. For more information about the primers see tables 5 and 12.

```
                Pili C3:44F →
C3:44   1     CACCGGAATG AGTGTACTGT TACTCGTTAC TGCGGGAATA GGGATTTTCG GGAACGGAAA ACTAGTCAAA
651     1     CACGGGAATG AGTGTACTGT TACTCGTTAC TGCGGGAATA GGGATTTTCG GGAACGGAAA ACTAGTCAAA

C3:44   71    GCCGAAGAAG TCGCACAAAA ACCAAGTGAA GTGACGATTA CATTACACAA AAAAGGTTTC TCATCTGTTC
651     71    GCCGAAGAAG TCGCACAAAA ACCAAGTGAA GTGACGATTA CATTACACAA AAAAGGTTTC TCATCTGTTC

C3:44   141   CGGAAGAACG ACCGAATAGC GGATTGGTTT CTACGGATTT TGGTGAAGAA AATATTCCAG GAGTCGATTT
651     141   CGGAAGAACG ACCGAATAGC GGATTGGTTT CTACGGATTT TGGTGAAGAA AATATTCCAG GAGTCGATTT

C3:44   211   TGATTTGTTT GACGTAACGG AAGTATATTA CGATTTGATT CGAGATAATC CTTTAACACC GGAACGAGAA
651     211   TGATTTGTTT GACGTAACGG AAGTATATTA CGATTTGATT CGAGATAATC CTTTAACACC GGAACGAGAA

C3:44   281   GACGGTCTTA ATTCTGCCGA AGCGATTGAA TGGATTCAAA AACGGCACAC AGAAAGTTGG TTTTTAAAAT
651     281   GACGGTCTTA ATTCTGCCGA AGCGATTGAA TGGATTCAAA AACGGCACAC AGAAAGTTGG TTTTTAAAAT

C3:44   351   ATCGACTGAC AAGTATTGAC AAACAAACAA CGAATGAAGC TGGAGAAGCG GTATTCTCTA CCGTCCAAGT
651     351   ATCGACTGAC AAGTATTGAC AAACAAACAA CGAATGAAGC TGGAGAAGCG GTATTCTCTA CCGTCCAAGT

C3:44   421   GACGGAAGAA GCACCTTCTT CTAGAGATAA AGTCTATCTT TTCTTAGAAA CTTATTCGCC AGCTCATATT
651     421   GACGGAAGAA GCACCTTCTT CTAGAGATAA AGTCTATCTT TTCTTAGAAA CTTATTCGCC AGCTCATATT
                Pili 3:44FM →
C3:44   491   TCCAGAATCG CGTCTCCAAT GGTTGTTATG ATGCCAGTGA TGATGCCTGA TATGGTGGAT GGCGTGTGGG
651     491   TCCAGAATCG CGTCTCCAAT GGTTGTTATG ATGCCAGTGA TGATGCCTGA TATGGTGGAT GGCGTGTGGG
              ← Pili 3:44 RL
C3:44   561   ATGGTTCTAC ATGGAAAGAC ACATACAATA CAGATGTGCA TTTGTATCCG AAAAACGAAA TCAGAGAAGC
651     561   ATGGTTCTAC ATGGAAAGAC ACATACAATA CAGATGTGCA TTTGTATCCG AAAAACGAAA TCAGAGAAGC

C3:44   631   AGATAAACAG ATGAATGTAG AGGAAAGCGA CCTTAGACAA GTAACGATTA TTAATGAAGC TGGAGAACAA
651     631   AGATAAACAG ATGAATGTAG AGGAAAGCGA CCTTAGACAA GTAACGATTA TTAATGAAGC TGGAGAACAA

C3:44   701   GAAACGATTT CTTATATTGA TCTAGAAAGA GGAAAAACGG CTTCTTACAC GATCACAGCA CCTATTCCTT
651     701   GAAACGATTT CTTATATTGA TCTAGAAAGA GGAAAAACGG CTTCTTACAC GATCACAGCA CCTATTCCTT

C3:44   771   ATTTCATCGA TTCTGTCCTT GAAAACGGAA GCGCTGTTAT CAAAAATTAC AAAATCACAG ATACGCCTAC
651     771   ATTTCATCGA TTCTGTCCTT GAAAACGGAA GCGCTGTTAT CAAAAATTAC AAAATCACAG ATACGCCTAC

C3:44   841   AGTTGGTCTA ACTTATTATG ATCAGGAAAT AGAAGTGCGA GCCGGCGAGA CGATTTTAAC AAAAGGACAA
651     841   AGTTGGTCTA ACTTATTATG ATCAGGAAAT AGAAGTGCGA GCCGGCGAGA CGATTTTAAC AAAAGGACAA

C3:44   911   GATTATATAG TAGAAGTAGT AAGCAACGGG TTTGTCGTGA CGATTTTGAC AGAAGAAAAT GGAGTAGCCA
651     911   GATTATATAG TAGAAGTAGT AAGCAACGGG TTTGTCGTGA CGATTTTGAC AGAAGAAAAT GGAGTAGCCA

C3:44   981   AAGTAGATAC TTTAGGTAGA TTAGCAGATG CACGTGGAGG AGATTTAACG ATCACGTATA ATTTGAAGGT
651     981   AAGTAGATAC TTTAGGTAGA TTAGCAGATG CACGTGGAGG AGATTTAACG ATCACGTATA ATTTGAAGGT

C3:44   1051  TTCCACAGAA CTAGAAGCGG ATGATTTCCA TAACAACACA GCGGTTATTG AAATCGGACG AAATGATGAA
651     1051  TTCCACAGAA CTAGAAGCGG ATGATTTCCA TAACAACACA GCGGTTATTG AAATCGGACG AAATGATGAA
                                              Pili 3:44FR →
C3:44   1121  TTTGATTATG AAGAAGGAGT AGAGCCGCCA GAGAAAGTCA CAACAGGTGG AAGAAAGTTT GAAAAATATG
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651     1121 TTTGATTATG AAGAAGGAGT AGAGCCGCCA GAGAAAGTCA CAACAGGTGG AAGAAAGTTT GAAAAATATG

C3:44   1191 ATGCATCAAG CAGTGAATTG CTTAAAGATG CAAGATTCGA ATTGTGGAAT GAAGACCGTT CTGAATACGC
651     1191 ATGCATCAAG CAGTGAATTG CTTAAAGATG CAAGATTCGA ATTGTGGAAT GAAGACCGTT CTGAATACGC
                                        ← Pili 3:44FM
C3:44   1261 AATATTTTAT AAAGGGGAGT CGCCTTTAGC TGTTTATGAA TCAGGAGCAG ATAGAATTGA GTGGGCAACA
651     1261 AATATTTTAT AAAGGGGAGT CGCCTTTAGC TGTTTATGAA TCAGGAGCAG ATAGAATTGA GTGGGCAACA

C3:44   1331 AGCGGACAAG CAACAGAATT TGTCGCAGAT GGAAATGGAT ACTTTGAGGT TCAAGGGTTA GATTATGGAA
651     1331 AGCGGACAAG CAACAGAATT TGTCGCAGAT GGAAATGGAT ACTTTGAGGT TCAAGGGTTA GATTATGGAA

C3:44   1401 CCTATCAAAT GAAAGAGACC ATGGCGCCAG AAGGTTATGT ACTTCCAACA GGTGAGGCAG CCTTTACTGA
651     1401 CCTATCAAAT GAAAGAGACC ATGGCGCCAG AAGGTTATGT ACTTCCAACA GGTGAGGCAG CCTTTACTGA

C3:44   1471 ATTTATCATC TCTTATGGAA GCTATAATGA AGAAATTCAG ATCGTAGGAG TAGAAAATCC AGGGCCAGAA
651     1471 ATTTATCATC TCTTATGGAA GCTATAATGA AGAAATTCAG ATCGTAGGAG TAGAAAATCC AGGGCCAGAA

C3:44   1541 AGAGTACCGA ATATGAAACG CGGGTCACTT CCTGCTACTG GTGGGAACGG GCTCTTGGCA TTCTTACTAA
651     1541 AGAGTACCGA ATATGAAACG CGGGTCACTT CCTGCTACTG GTGGGAACGG GCTCTTGGCA TTCTTACTAA
                                                ← Pili C3:44 R1
C3:44   1611 TCGGAATAAG TTTGATGATT GGAGCGTACA GCTGGTACCG TAAATCTAAA ATGAAGTCTG AAGTATAA
651     1611 TCGGAATAAG TTTGATGATT GGAGCGTACA GCTGGTACCG TAAATCTAAA ATGAAGTCTG AAGTATAA
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This sequence will give a gene product with the approximate size of 135473,55 Da

Pili C3:48/ Contig 657 alignment with primers. Primers written in blue were used for cloning, while primers written in green were used for sequencing, the red markings indicates the differences between contig 657 and the obtained C3:48 sequence. The M13 primers are not included because they were located in the vector. For more information about the primers see tables 5 and 12.

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                Pili C3:48 F →
C3 48  1    CACCATGTTA GGAGTCCTTT TCCTTATTT T ACCATTACTC ACAAACAGCT TCGGCGCAAA AAAAGTGTTT
657    1    CGTTATGTTA GGAGTCCTTT TCCTTATTT T ACCATTACTC ACAAACAGCT TCGGCGCAAA AAAAGTGTTT

C3 48  71   GCAGAGGAGA CAGCAGCTCA AGTCATCCTT CATAAAAAGA AAATGACTGA TTTACCCGAT CCTTTAATCC
657    71   GCAGAGGAGA CAGCAGCTCA AGTCATCCTT CATAAAAAGA AAATGACTGA TTTACCCGAT CCTTTAATCC

C3 48  141  AAAACAGCGG GAAAGAAATG AGCGAATTCG ATCAATACCA AGGATTAGCC GATATTTCAT TTTCAGTTTA
657    141  AAAACAGCGG GAAAGAAATG AGCGAATTCG ATCAATACCA AGGATTAGCC GATATTTCAT TTTCAGTTTA

C3 48  211  TAACGTCACT CAAGAATTTT ATGCGCAACG AGATAAAGGA GCGTCCGTGG ATGCAGCAAA ACAAGCAGTC
657    211  TAACGTCACT CAAGAATTTT ATGCGCAACG AGATAAAGGA GCGTCCGTGG ATGCAGCAAA ACAAGCAGTC

C3 48  281  CAGTCTTTGA CTCCTGGTAC ACCAGTTGCT TCAGGAACGA CAGATGCTGA TGGAAATGTC ACTTTATCTT
657    281  CAGTCTTTGA CTCCTGGTAC ACCAGTTGCT TCAGGAACGA CAGATGCTGA TGGAAATGTC ACTTTATCTT
                                                          Pili 3:48FM →
C3 48  351  TACCTAAAAA ACAAAATGGG AAAGATGCAG TCTACACGAT CAAAGAAGAA CCAAAAGACG GAGTGTCAGC
657    351  TACCTAAAAA ACAAAATGGG AAAGATGCAG TCTACACGAT CAAAGAAGAA CCAAAAGACG GAGTGTCAGC

C3 48  421  TGCCGCAAAC ATGGTTTTAG CTTTCCCTGT ATATGAGATG ATCAAACAAG CAGATGGCTC TTATAAATAC
657    421  TGCCGCAAAC ATGGTTTTAG CTTTCCCTGT ATATGAGATG ATCAAACAAG CAGATGGCTC TTATAAATAC
          ← Pili 3:48RL
C3 48  491  GGGACAGAAG AACTAGATAC TATCCATCTC TACCCTAAAA ATACAGTCGG TAATGATGGA ACGTTGAAAG
657    491  GGGACAGAAG AACTAGATAC TATCCATCTC TACCCTAAAA ATACAGTCGG TAATGATGGA ACGTTGAAAG

C3 48  561  TTACAAAAAT CGGTACTGCC GAAAACGAAG CACTAAATGG AGCAGAATTT ATTATTTCTA AAGAAGAAGG
657    561  TTACAAAAAT CGGTACTGCC GAAAACGAAG CACTAAATGG AGCAGAATTT ATTATTTCTA AAGAAGAAGG

C3 48  631  AACACCAAGC GTCAAAAAAT ACATCCAAAG TGTCACAGAT GGATTGTACA CTTGGACAAC TGATCAAACC
657    631  AACACCAAGC GTCAAAAAAT ACATCCAAAG TGTCACAGAT GGATTGTACA CTTGGACAAC TGATCAAACC

C3 48  701  AAAGCCAAAC ATTTCATTAC TGGTCATTCT TATGACATCG GCAACAATGA CTTTGCCGAG GCATCTATTG
657    701  AAAGCCAAAC ATTTCATTAC TGGTCATTCT TATGACATCG GCAACAATGA CTTTGCCGAG GCATCTATTG

C3 48  771  AAAAAGGCCA GTTGATCGTT AATCATTTAG AAGTTGGAAA ATATAATTTA GAAGAAGTAA AAGCTCCTGA
657    771  AAAAAGGCCA GTTGATCGTT AATCATTTAG AAGTTGGAAA ATATAATTTA GAAGAAGTAA AAGCTCCTGA
                                                    Pili 3:48FR →
C3 48  841  TAATGCGGAA ATGATTGAAA AGCAAACAAT CACGCCTTTT GAGATCCTGG CAAATAGCCA AACACCAGTA
657    841  TAATGCGGAA ATGATTGAAA AGCAAACAAT CACGCCTTTT GAGATCCTGG CAAATAGCCA AACACCAGTA

C3 48  911  GAAAAGACCA TCAAAAATGA TACGTCTAAA GTTGATAAAA CAACACCTCA ATTGAATGGA AAAGATGTCG
657    911  GAAAAGACCA TCAAAAATGA TACGTCTAAA GTTGATAAAA CAACACCTCA ATTGAATGGA AAAGATGTCG

C3 48  981  CAATCGGTGA AAAAATTCAA TATGAGATTT CTGTCAATAT CCCATTAGGT ATCGCTGATA AAGAAGGAAC
657    981  CAATCGGTGA AAAAATTCAA TATGAGATTT CTGTCAATAT CCCATTAGGT ATCGCTGATA AAGAAGGAAC
                                          ← Pili 3:48RM
C3 48  1051 GCAAAACAAG TACACAACAT TCAAACTTA T CGATACTCAT GACGCTGCTT TAACATTTGA TAATGATTCT
657    1051 GCAAAACAAG TACACAACAT TCAAACTTA T CGATACTCAT GACGCTGCTT TAACATTTGA TAATGATTCT

C3 48  1121 TCAGGAACGT ATGCTTATGC CTTATATGAT GGAAATAAAG AAATCGACCC AGTAAATTAT TCTGTCACTG
657    1121 TCAGGAACGT ATGCTTATGC CTTATATGAT GGAAATAAAG AAATCGACCC AGTAAATTAT TCTGTCACTG

C3 48  1191 AGCAAACAGA CGGATTCACG GTTTCAGTTG ATCCGAATTA TATTCCTTCA TTAACTCCTG GCGGTACATT
657    1191 AGCAAACAGA CGGATTCACG GTTTCAGTTG ATCCGAATTA TATTCCTTCA TTAACTCCTG GCGGTACATT
                                    Pili 3:48F →
C3 48  1261 GAAATTCGTT TACTATATGC ATTTGAACGA AAAAGCAGAT CCAACCAAAG GATTTTCTAA CCAAGCAAAT
657    1261 GAAATTCGTT TACTATATGC ATTTGAACGA AAAAGCAGAT CCAACCAAAG GATTTTCTAA CCAAGCAAAT
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C3 48  1331 GTCGATAACG GGCATACAAA TGATCAAACA CCACCGTCAG TCGATGTCGT TACTGGGGGC AAACGATTTG
657    1331 GTCGATAACG GGCATACAAA TGATCAAACA CCACCGTCAG TCGATGTCGT TACTGGGGGC AAACGATTTG

C3 48  1401 TTAAAGTAGA TGGTGACGTT ACATCAGACC AAACACTTGC TGGAGCAGAA TTCGTCGTTC GTGATCAAGA
657    1401 TTAAAGTAGA TGGTGACGTT ACATCAGACC AAACACTTGC TGGAGCAGAA TTCGTCGTTC GTGATCAAGA

C3 48  1471 TAGTGACACA GCGAAATATT TATCGATCGA CCCATCCACA AAAGCCGTCA GCTGGGTATC GGCGAAAGAA
657    1471 TAGTGACACA GCGAAATATT TATCGATCGA CCCATCCACA AAAGCCGTCA GCTGGGTATC GGCGAAAGAA
                                                    ← Pili 3:48R
C3 48  1541 TCAGCAACGG TTTTTACAAC CACAAGTAAC GGTTTAATCG A TGTGACAGG TCTAAAATAT GGCACGTACT
657    1541 TCAGCAACGG TTTTTACAAC CACAAGTAAC GGTTTAATCG A TGTGACAGG TCTAAAATAT GGCACGTACT

C3 48  1611 ATCTGGAAGA AACGAAAGCG CCAGAAAAAT ATGTTCCATT AACAAACCGT GTAGCATTTA CTATCGATGA
657    1611 ATCTGGAAGA AACGAAAGCG CCAGAAAAAT ATGTTCCATT AACAAACCGT GTAGCATTTA CTATCGATGA

C3 48  1681 ACAATCTTAT GTAACAGCAG GACAGTTGAT TTCTCCTGAA AAAATACCAA ATAAACACAA AGGTACACTT
657    1681 ACAATCTTAT GTAACAGCAG GACAGTTGAT TTCTCCTGAA AAAATACCAA ATAAACACAA AGGTACACTT

C3 48  1751 CCTTCAACAG GCGGTAAGGG AATCTATGTG TATATCGGTG CAGGAGTAGT CCTTCTACTG ATTGCTGGAC
657    1751 CCTTCAACAG GCGGTAAGGG AATCTATGTG TATATCGGTG CAGGAGTAGT CCTTCTACTG ATTGCTGGAC
                          ← Pili C3:48R1
C3 48  1821 TGTACTTTGC TAGACGCAAG CACAGTCAGA TTTAG
657    1821 TGTACTTTGC TAGACGCAAG CACAGTCAGA TTTAG
```

This sequence will give a gene product with the approximate size 173998,33 Daltons

Appendix B: Estimation of P-Values

Table B1- Calculation of χ and reading of p-value for screening 1:44

Type	$\Sigma=((O-E)-0,5)^2/E$	p
Human	1,067	0,1
Community Survey	0,281	0,5
Hospital Survey	10,685	0,005
Outbreak	3,067	0,1
Hospital outbreak	3,984	0,05
Clinical	0,377	0,5
Other	0,368	0,5
Faeces	1,659	0,1
Blood	1,814	0,1
Urine	0,559	0,5
Other	0,534	0,5
From two sites	2,046	0,1
VanA and VanB	0,191	0,9
VanA	0,063	0,9
VanB	0,562	0,5
USA	0,350	0,5
Europa	0,562	0,5
Scandinavia	1,448	0,1
Norway	0,166	0,9
Other	2,207	0,1
Community survey	7,199	0,01
Other	-	-
Faeces	2,109	0,1
Other	7,604	0,005
VanA and B	0,493	0,5
VanA	0,493	0,5
VanB	-	-
Animal pili	0,571	0,5-

Table B2- Calculation of χ and reading of p-value for screening 1:48.

Type	$\Sigma=((O-E)-0,5)^2/E$	p
Human	1,942	0,5
Community Survey	0,361	0,5
Hospital Survey	0,877	0,5
Outbreak	0,767	0,5
Hospital outbreak	0,160	0,5
Clinical	0,342	0,5
Other	0,523	0,5
Faeces	1,970	0,1
Blood	5,844	0,025
Urine	0,238	0,5
Other	0,321	0,5
From to sites	2,937	0,1
VanA and VanB	6,248	0,01
VanA	8,062	0,005
VanB	0,554	0,5
USA	0,275	0,5
Europa	0,554	0,5
Scandinavia	0,101	0,9
Norway	0,676	0,5
Other	2,056	0,1
Animals	4,740	0,025
Community survey	2,274	0,1
Other	2,274	0,1
Faeces	2,274	0,1
Other	2,274	0,1
VanA and B	0,694	0,5
VanA	0,694	0,5
VanB	-	-

Figure B1- Table over critical values for χ^2 distribution.

Genetics

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CHI-SQUARE TO P-VALUE CONVERSION TABLE

Dr. Rick Hershberger - California University of Pennsylvania

	P-values												
	0.995	**0.990**	**0.975**	**0.950**	**0.900**	**0.750**	**0.500**	**0.250**	**0.100**	**0.050**	**0.025**	**0.010**	**0.005**
Degrees of Freedom	Chi-square values												
1	0.00004	0.00016	0.00098	0.00393	0.01579	0.10153	0.45494	1.32330	2.70554	3.84146	5.02389	6.63490	7.87944
2	0.01003	0.02010	0.05064	0.10259	0.21072	0.57536	1.38629	2.77259	4.60517	5.99146	7.37776	9.21034	10.59663
3	0.07172	0.11483	0.21580	0.35185	0.58437	1.21253	2.36597	4.10834	6.25139	7.81473	9.34840	11.34487	12.83816
4	0.20699	0.29711	0.48442	0.71072	1.06362	1.92256	3.35669	5.38527	7.77944	9.48773	11.14329	13.27670	14.86026
5	0.41174	0.55430	0.83121	1.14548	1.61031	2.67460	4.35146	6.62568	9.23636	11.07050	12.83250	15.08627	16.74960
6	0.67573	0.87209	1.23734	1.63538	2.20413	3.45460	5.34812	7.84080	10.64464	12.59159	14.44938	16.81189	18.54758
7	0.98926	1.23904	1.68987	2.16735	2.83311	4.25485	6.34581	9.03715	12.01704	14.06714	16.01276	18.47531	20.27774
8	1.34441	1.64650	2.17973	2.73264	3.48954	5.07064	7.34412	10.21885	13.36157	15.50731	17.53455	20.09024	21.95495
9	1.73493	2.08790	2.70039	3.32511	4.16816	5.89883	8.34283	11.38875	14.68366	16.91898	19.02277	21.66599	23.58935
10	2.15586	2.55821	3.24697	3.94030	4.86518	6.73720	9.34182	12.54886	15.98718	18.30704	20.48318	23.20925	25.18818