Biofilm formation in Enterococci and Streptococci

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Table of contents

List of figures

List of tables

List of Abbreviations

ATP	Adenosine triphosphate
Acm	Adhesion of collagen from *E. faecium*
AS/Agg	Aggregation substance
N-terminal	Amino terminal
Amp	Ampicillin
AT	Autotransport
Bap	Biofilm associated protein
BSA	Bovine Serum Albumin
BHI	Brain-Heart Infusion broth
CFU	Colony Forming Unites
CRC	Colorectal Cancer
CLSM	Confocal Laser Scanning Microscope
CV	Crystal Violet
c-di-GMP	Cyclic diguanylate monophosphate
Cyl	Cytolysin
D-Ala-D-Ala	D-alanyl-D-Alanine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
D-Lac	D-Lactate
D-ser	D-serine
ER	Endoplasmic Reticulum
Esp	Enterococcal surface protein
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
E. coli	*Escherichia coli*

ECM	Extra Cellular Matrix
eDNA	Extracellular DNA
EPS	Extracellular Polymeric Substances
GI	Gastrointestinal Tract
GelE	Gelatinase
GBAP	Gelatinase Biosynthesis-Activating Pheromone
GSP	General Secretion Pathway
GBS	Group B Streptococci
His	Histidine
Caco-2-cells	Human colorectal adenocarcinoma cells
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LPS	lipopolysaccharide
LB	Luria-Bertani
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecules
MIC	Minimum Inhibitory Concentration
NGM	Nematode Growth Medium
OD600	Optical density at 600 nm
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
P	Prokaryotic
Lsp	prolipoprotein Signal peptidase
PMF	Proton Motive Force
R	Resistant
RNA	Ribonucleic acid

Sec	Secretory-pathway
S	Sensitive
SprE	Serine protease
SPase	Signal peptidase
SP	Signal peptide
NaCl	Sodium Chloride
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
S. aureus	*Staphylococcus aureus*
THB	Todd-Hewit broth
TSB	Trytpone Soya broth
TSB-G	TSB containing 0.25% Glucose
Tat	Twin-arginine translocation
TPS	Two-Partner protein Secretion
UV	Ultraviolet Light
UTI	Urinary Tract Infection

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Declaration

The work presented in this thesis is original work conducted by myself under the supervision of Dr. Albert Bolhuis. All resources of information has been acknowledged by means of references. None of this work has been used in any previous application for a degree at this or any other university or institute of learning.

Abstract

Enterococci are intestinal facultative anaerobic strains which recognized as opportunistic pathogens. The ability to form biofilms is an important virulence trait that has been reported for Enterococci. Biofilm formation showed differences between *E. faecalis* strains. However, several factors were involved in this process e.g. the presence of virulence factors, hydrophobicity and heterogeneity. Interestingly, we demonstrated for the first time a biochemical test for a cell surface protein in biofilm formation: addition of the purified N domain of Esp (EspN) to *E. faecium* E1162Δesp resulted in the restoration of biofilm formation.

Streptococcus bovis also, is an intestinal facultative anaerobic bacterium. This organism also has been reported as an opportunistic pathogen causing multiple diseases such as septicemia and endocarditis associated with colorectal cancer (CRC). Although the association of *S. gallolyticus* infection with CRC is a major issue, the mechanisms behind this link are still unclear. This link between CRC and the virulence of *S. bovis* strains was studied in more detail in a collaborative project with Dr Harold Tjalsma. The Tjalsma group mainly focussed on host-pathogen interactions, whereas we analysed biofilm formation of *S. bovis* strains as well as their pathogenicity using the *in vivo C. elegans* infection model.

Our biofilm showed that *S. bovis* strains form biofilms particularly well on collagen-rich surfaces at least indicate why there is this association. *C. elegans* experiments also showed that pathogenicity of *S. bovis* strains is more similar to *E. faecalis* than to *E. faecium* in which both *S. bovis* and *E. faecalis* have a slow mode of killing that is absent in *E. faecium*. Full genome sequences of S. gallolyticus UCN34 strain have revealed the presence of a number of potential collagen-binding proteins (e.g., *gallo_2179*) that are related to the MSCRAMMs family. However, we successfully cloned the *gallo_2179* gene in an enterococcal expression vector, and demonstrated transcribed in *E. faecalis*. Unfortunately, this strain did not form better biofilms on a collagen surface, suggesting either that not sufficient amount of the protein was made, or that the protein is not functional in *E. faecalis*. In addition, a bioinformatics analysis was

performed to identify putatively secreted proteins in *S. gallolyticus.* Proteins that were expected to be found include for instance three collagen-binding proteins, amylase, tannase and beta lactamase.

Chapter 1: Introduction

1.1. General

Enterococci are facultative anaerobic Gram positive bacteria which usually occur in pairs or short chains; they are belong to the group of lactic acid bacteria, many of which produce bacteriocins (Fisher and Phillips, 2009). Enterococci were initially classified in the genus *Streptococcus*. When in 1930 the Lancefield serological typing system was introduced, they were put into group D streptococci. However, it was also realized that these organisms were somewhat different from other streptococci. For instance, they are tolerant to a wider range of adverse conditions and could grow at temperatures between 10 to 45˚C, but also survive at a relatively high temperature up to 60˚C for 30 minutes, tolerate to grow in 6.5% Sodium salt (NaCl), in pH 9.6 and in 40% bile salt. Genomic analysis in the 1980s made it clear that these organisms were distinct from streptococci and were therefore given the genus name *Enterococcus* (Fisher and Phillips, 2009; Cetinkaya et al., 2000).

Enterococci are natural inhabitants of the human and animal's gastrointestinal (GI) tract and oral cavity. They also can be found in water, soil and plants. In addition, they have been used in food fermentation and human probiotics (Franz et al., 1999; Gaspar et al., 2009). However, recently Enterococci recognized as an opportunistic pathogens which cause disease such as bacteremia, wound infection, endocarditis and pelvic and urinary tract infection (Gaspar et al., 2009).

The most commonly encountered enterococcal species are *Enterococcus faecalis* and *Enterococcus faecium*, which are responsible for 80-90% and 10-20%, respectively of human enterococcal infection (Mohamed and

Huang, 2007). Importantly, enterococcal infections are particularly problematic because of their multiple antibiotic resistance (Cox et al., 2005).

1.2. Biofilm formation

A biofilm is a population of cells that adhere irreversibly on a range of biotic and abiotic surfaces and which is encased in a matrix of exopolymeric substances (EPS; Mohamed and Huang, 2007). In nature, bacterial cells exist in biofilms much more frequently than as planktonic (free floating) cells. Also, biofilms can comprise one or multiple species of microorganisms (O'Toole et al., 2000, Prakash et al., 2003). Biofilm may form on variety of surfaces including living or dead tissue, indwelling medical devices, water pipes, natural aquatic systems and contact lenses (Prakash et al., 2003)

Biofilm-associated cells are physiologically different from planktonic cells. This is governed by several features, such as the very slow rate of growth of cells within biofilms, a fast genetic exchange, and cell-cell communication through quorum sensing. This leads to, for instance, an up to 1000-fold increase in antibiotic resistance (Raffa et al., 2005, Donlan, 2001, Donlan, 2002). In addition, biofilms provides protection for cells from environment stress including UV light, antibiotic, shear forces and host immune defense (Prakash et al., 2003).

Resistance against antimicrobial occurs to several reasons. Firstly, EPS act as a barrier which prevents the penetration of some of antibiotics. Also, the

negative charge of EPS matrix contributes in antibiotic resistance through binding directly to these compounds (Mah and O'Toole, 2001, Donlan, 2002, Prakash et al., 2003). Secondly, cells within the biofilm are characterized by slow growth and therefore have a slow metabolic rate which explains their resistance to antibiotics that inhibit processes in actively dividing cells, such as cell wall synthesis, or transcription/translation. Also, the close proximity of cells within the EPS matrix enables conjugation of plasmids, some of which encode genes for (multiple) antibiotic resistance. Thirdly, some antibiotics are inactivated in the EPS by secreted enzymes such as β-Lactamase (Soto, 2013, Mah and O'Toole, 2001). In addition to antibiotic resistance, cells in biofilm are also toleratant to host immune defense systems such as phagocytosis, as for instance neutrophils are unable to make proper contact and phagocytose cells that are embedded in EPS (Prakash et al., 2003).

Note that biofilm formations also have many beneficial functions in the environment. For example, biofilms formed on the Rhizosphere plant roots provide water stability for the plant. Also, biofilms are being utilized in ground water treatment with, for instance, contamination from petroleum compounds (Davey and O'Toole G, 2000).

The biological cycle of biofilm development takes place in a series of stages starting with initial attachment, followed by microcolony formation, biofilm maturation and dissolution (Fig 1.1; O'Toole et al., 2000).

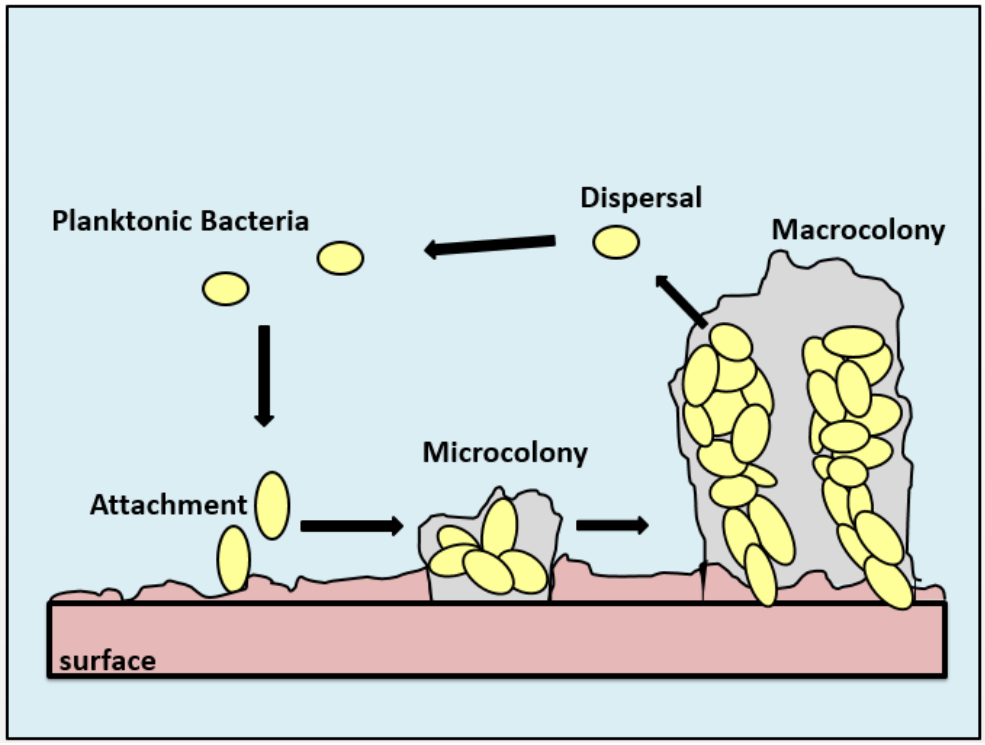

Figure 1.1 simplified model for biofilm formation stages. See text for more details.

1.2.1. Initial attachment

Initial attachment of planktonic bacteria to a surface depends on several factors including cell surface properties, substratum and environment conditions (O'Toole et al., 2000, van Merode et al., 2006b). Bacterial adhesion is mediated by electrostatic, Lifshitz- van der Waals, Lewis acid-base interaction and hydrophobic forces. These interactions along with cell surface proteins react to overcome the repulsion of the net negative charge surface (van Merode et al., 2006b).

Several cell surface components have been noted to promote the initial adhesion for example, flagella, lipoproteins, polysaccharides and fimbriae in Gram negative bacteria whereas autolysin , biofilm associated proteins

(BaP) and adhesin in Gram positive bacteria (Hall-Stoodley and Stoodley, 2002, Lejeune, 2003, Lasa and Penades, 2006).

Interestingly, several reports have shown that genes encoding some cells components such as flagella in *E. coli* are repressed after attachment as these genes are only required in the early stages but are not required in development of biofilm, indicating genetic changes during biofilm formation (Hall-Stoodley and Stoodley, 2002, Monds and O'Toole, 2009, Beloin and Ghigo, 2005).

Furthermore, the characteristics of the substratum and the medium also have an effect on bacterial attachment. It has been shown that bacterial attachment is enhanced with increased roughness and hydrophobicity of the surfaces (O'Toole et al., 2000). Other environmental factors that can affect initial bacterial colonization include flow velocity, pH, nutrient levels, cation concentration and temperature (O'Toole et al., 2000, Beloin and Ghigo, 2005). In addition, the presence of a conditioning film especially with liquid-solid surfaces may alter the surface properties. This film, which may contain both organic and inorganic material depends strongly on the existing environment (Pringle and Fletcher, 1983).

1.2.2. Microcolony formation and maturation of biofilm

Once bacterial cells attach to the surface irreversibly, more bacteria will adhere to the monolayer. Furthermore, as cells on the surface will divide to form microcolonies which may contain around 100 cells in a cluster (Monds and O'Toole, 2009).

At this stage the bacteria start to produce Extracellular polymeric matrix (EPS) which facilitates cells adhesion and provides protection against antibiotics and host immune defense. EPS is a very strongly hydrated matrix which contains water channels that allow inflow of oxygen and nutrients, and outflow of byproducts within the biofilm (Donlan, 2002, O'Toole et al., 2000, Molobela et al., 2010). In addition, it provides structure to the biofilm. EPS primarily consist of polysaccharides, lipids, proteins and extracellular DNA (eDNA; Aguilera et al., 2008, Flemming et al., 2007, O'Toole et al., 2000). However, variation in these components is governed by several factors including nutrient availability, shear forces, temperature and the organism within the biofilm (O'Toole et al., 2000, Sutherland, 2001, Donlan, 2002).

As growth population increased within biofilm the cells density will increase allowing cell-cell communication through quorum sensing mechanisms. As a result of high cell density, cells release chemical signalling molecules, called autoinducers. Once these reach a critical concentration, the signals then activate quorum sensing mechanisms to regulate genes expression (Raffa et al., 2005, Miller and Bassler, 2001). These expression genes have an important role in many processes, including further development of biofilms, increased virulence, sporulation, protective bioluminescence and competence for the uptake of DNA (Raffa et al., 2005, Hancock and Perego, 2004, Dunn and Handelsman, 2002).

Continuous growth of the cells within the biofilm leads to development of a 3-dimensional complex biofilm containing macrocolonies that are separated by water channels (Hall-Stoodley et al., 2004, Kaplan, 2010). Also, the biofilm structure may interact with nonmicrobial particles depending on the

environment surrounding the biofilm. For example, biofilms developing on heart valves may consist of bacterial cells, EPS, fibrin and erythrocytes, which the latter may protect the biofilm from host defense such as leukocytes. If such colonization is indeed not cleared it results in infective endocarditis, a potentially dangerous disease (Donlan, 2002, Durack, 1975).

1.2.3. Biofilm detachment

Microbial cells in biofilm may disperse either in individual colonies or as a small portion of the biofilm (shearing dispersal), a mechanism which could be either passive or active. Active biofilm dispersal is initiated by the microbial cells themselves, by for instance release of molecules such as D-amino acids or polyamines (Kolodkin-Gal et al., 2012). Passive dispersal is mediated by signals in the biofilm, environmental changes or physical forces (Kaplan, 2010, Donlan, 2002). Factors induce detachment of biofilm include changing in nutrient and oxygen level, pH, temperature, quorum sensing, EPS degrading enzymes and various signalling molecules such as nitric oxide (Kaplan, 2010, Karatan and Watnick, 2009).

Physical dispersal has been classified into three processes: shearing, abrasion and sloughing dispersal. Shearing dispersal is removing of small biofilm portion due to the stress of the fluid flow on the surface. Abrasion removal when particles of biofilm colloid in the fluid bulk causing detach of the biofilm. The third type of biofilm dispersal called sloughing happens when there is nutrient or oxygen depletion, leading to detachment of larger chunks of the biofilm that can colonize other surfaces and start another

biofilm cycle, or develop as planktonic type bacteria (Choi and Morgenroth, 2003, Stoodley et al., 2001, Donlan, 2002).

1.2. Antibiotic Resistance in Enterococci

The mechanisms by which enterococci can resist multiple antibiotics have been categorized into two mechanisms: (i) intrinsic resistance to vary of antibiotics (ii) acquired resistance including the ability to transfer this resistance to the other species (Mundy et al., 2000).

Intrinsic resistance depends on the innate ability of bacteria to tolerate antibiotics and can depend on e.g. poor penetration of antibiotics into the cell or lack of a target for an antibiotic (Jankoska et al., 2008). In the case of enterococci, cells contain penicillin binding proteins (PBPs) which have a low affinity to several β-lactams antibiotics such as penicillin and cephalosporins. This resistance is in particular due to production the low-affinity of PBP5 (Cetinkaya et al., 2000). Enterococcal resistance to β-lactams is varies, but the lowest resistance is for in particular ampicillin (Cetinkaya et al., 2000).

Enterococci also have resistance to aminoglycosides either moderate resistance due to poor permeability or high resistance due to production of inactivating enzymes (Cetinkaya et al., 2000). Enterococci resistance to aminoglycosides depends on the production of the inactivating enzyme. For example, strains that produce a bifunctional enzyme such as 2”-phosphotransferase-6’-acetyltransferase mediates high level resistance to gentamicin, kanamycin and amikacin.

Strains producinge 3'-phosphotransferase-III are also resistant to kanamycin, but more susceptible to gentamycin. Also, some strains are resistant to streptomycin through production of streptomycin adenyltransferase (Herman and Gerding, 1991), but these are still susceptible to gentamycin (Herman and Gerding, 1991, Cetinkaya et al., 2000). Therapeutic combination of aminoglycosides and β-lactams or glycopeptides (cell wall synthesis inhibitor) may overcome the tolerance against aminoglycosides (Cetinkaya et al., 2000).

Enterococci can acquire antibiotic resistance through either exchange of resistance genes carried on a plasmid or transposon, or via mutation (Cetinkaya et al., 2000). Innate low ampicillin resistance through production of low affinity PBP5s has previously been mentioned. High ampicillin resistance usually refers to acquired resistance obtained through one of two mechanisms. Firstly, in most cases in *E. faecalis* there is production of β-lactamases, which were originally acquired from the *S. aureus* β-lactamase operon (Rice, 2001, Rice and Marshall, 1992). Secondly, mutations or alterations in the amount of production of PBP5s can also lead to high level resistance. An increase in the expression genes encoding PBP5s is most commonly found in *E. faecium* (Cetinkaya et al., 2000, Grayson et al., 1991).

1.3. Vancomycin resistance Enterococci (VRE)

Vancomycin resistance enterococci (VRE) were first reported in Europe during mid-1980s. VRE initially disseminated in Europe when an analogue drug for vancomycin (Avoparcin) was widely used in farm animals, which

attributed in community-acquired vancomycin resistance. In contrast, in the US no glycopeptides have been used in farms. However, the great use of vancomycin in hospitals leads to the emergence of VRE even though they do not have reservoir of VRE among the community (Tacconelli and Cataldo, 2008, Stobberingh et al., 1999, Mascini and Bonten, 2005). Nowadays, VRE is causing clinical problems and a major concern in medical practice due to the ability to transfer the vancomycin resistance across other bacteria such as methicillin resistance *Staphylococcus aureus* (MRSA; Zirakzadeh and Patel, 2006), for which vancomycin is one of the few "last-resort" treatment options.

Vancomycin act as a cell wall synthesis inhibitor through it is binding to the D-alanyl-D-alanine (D-ala-D-ala) the terminus of peptidoglycan precursor, which inhibits attachment of the transglycosylase enzyme to the peptidoglycan precursor (Arthur and Courvalin, 1993). There have been described six phenotypes of vancomycin resistance; VanA, VanB, VanC, VanD, VanE and Van G (Reynolds and Courvalin, 2005).

The resistance mechanisms occurs when the terminal residue of the peptidoglycan precursor D-alanine is replaced either by D-lactate(D-lac) which present in VanA, VanB and VanD, or by D-serine (D-ser) which present in VanC, VanE and VanG (Reynolds and Courvalin, 2005). Another resistance mechanism is elimination of the target residue with two enzymes D,D-carboxypeptidase or/and D,D-dipeptidase leading to remove C-terminal D-Ala (Reynolds et al., 1994).

Among several types of Vancomycin resistance in enterococci, VanA and VanB are the most common cause of clinical problems due to their ability to transfer to other bacteria (Cetinkaya et al., 2000, Sood et al., 2008) .

VanA induces high resistance against vancomycin and teicoplanin, whereas VanB is resistant to vancomycin only (Sood et al., 2008). However, both phenotypes are acquired and present in *E. faecalis* and *E. faecium* (See table 1.1). The other phenotypes are also all acquired except VanC. The latter is also found in other enterococci such as *E. gallinarum, E. casseliflavus* and *E. flavescons,* whereas VanD is found in *E.* faecium, and VanE and VanG in *E. faecalis* (Sood et al., 2008, Zirakzadeh and Patel, 2006, Cetinkaya et al., 2000).

Table 1.1 characteristics of VRE phenotypes.

	Phenotypes					
Characteristic	VanA	VanB	VanC	VanD	VanE	VanG
Vancomycin	R	R	R	R	R	R
Teicoplanin	R	S	S	S	S	S
Genetic - determination	Acquired	Acquired.	Intrinsic	Acquired.	Acquired.	Acquired
Transferable	Yes	Yes	No	No	No	No
Enterococci existence	*E. faecalis, E. faecium*	*E. faecalis, E. faecium*	*E. gallinarum, E. casseliflavus and E. flavescons*	*E. faecim*	*E. faecalis*	*E. faecalis*

R=resistant, S=sensitive.

1.4. Pathogenicity and Virulence of Enterococci

As mentioned previously, enterococci are found in the gastrointestinal (GI) tract as a part of the gut flora and they are normally harmless. However, during the last few decades enterococci have become a major cause for a variety of human infections (Jett et al., 1994).

Virulence of enterococci species is mediated by many factors, including their ability to colonize the GI tract and adherence to the extracellular matrix proteins (such as collagen) and epithelial cells (Fisher and Phillips, 2009). In addition, using broad spectrum antibiotics contribute intestinal overgrowth of enterococci, which then leads to their colonizing and translocation through the epithelial cells to the liver and spleen. Dissemination of enterococci may cause several serious infection such as bacteremia and endocarditis (Jett et al., 1994).

Several enterococcal virulence factors have been reported to have important roles in biofilm development and pathogenesis of *E. faecalis*, including cell wall adhesion proteins and secreted proteins (Pires-Boucas et al., 2010). These include for instance, aggregation substance AS, Enterococcal surface protein Esp, *E. faecalis* endocarditis associated antigen A (EfaA), adhesion of collagen of *E. faecalis* Ace, gelatinase GelE and the toxin Cytolysin (see table 1.2).

Adhesion to host extracellular matrix components (ECM) is the first step for pathogens to mediate infection. This is important in promoting enterococci to colonize host vascular tissues through interactions between enterococcal surface proteins and host proteins such as collagen and laminin. In fact, many studies have reported surface proteins containing an

immunoglobulin-like fold which are named MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules; (Nallapareddy and Murray, 2006). Indeed, many well-characterized surface proteins in Gram-positive bacteria (such as streptococci, staphylococci and enterococci) are MSCRAMMS (Walsh et al., 2008, Sillanpää et al., 2009). These proteins share several characteristics including an N-terminal signal peptide sequence followed by an A-domain which consist of one or multiple subdomains, each of which adopt a IgG-like fold (immunoglobulin G-like). Following the A-domain is a series of repeated sequences that is referred to as the B-domain. The C-terminal has a so-called LPXTG motif which is required for cell wall anchoring that is accomplished by a specific enzyme called a sortase (Hendrickx et al., 2009, Liu et al., 2007).

E. faecalis Ace shares sequence similarity with *S. aureus* MSCRAMM Cna. Characteristics of these proteins including an N-terminal signal peptide followed by A-domain then B-domain and C-terminal. Based on the studies on *S. aureus* Cna, it was shown that the collagen-binding activity is located in the A-domain (Hendrickx et al., 2009, Liu et al., 2007). Ace has been reported to contribute in pathogenicity in a rat endocarditis model and it has been demonstrated that during endocarditis there is a higher level of *ace* expression than in laboratory conditions (Singh et al., 2010). Also, the same study confirmed inhibition of *E. faecalis* collagen adherence by using active and passive immunization based on the collagen binding domain of Ace. Thus, this study did not only indicate the importance role of this protein in endocarditis, but also showed promising therapeutic strategies against *E. faecalis* endocarditis.

AS is an *E. faecalis* surface-anchored protein which is encoded by pheromone-responsive plasmids (Dunny, 1990). It facilitates transfer of plasmids between cells by allowing the adherence of the donor bacterium cells to recipient cells (Dunny, 1990). In addition, aggregate substances mediate adherence into renal and intestinal epithelial cells and cardiac vegetation and also, enhance biofilm formation by cell aggregation (Kreft et al., 1992, Chow et al., 1993).

Enterococcal surface protein Esp is a cell wall protein. Studies observed an association of Esp in the initial attachment and biofilm formation of *E. faecalis* on abiotic surfaces (Toledo-Arana et al., 2001). Furthermore, it has been shown that Esp promotes the colonization and persistence of *E. faecalis* in urinary tract infection in animal model (Shankar et al., 2001).

E. faecalis endocarditis antigen A (EfaA) amino acid sequence analysis showed 55% to 60% similarity to a group of streptococcal proteins (FimA from *Streptococcus parasanguis*, SsaB from *Streptococcus sanguis*, ScaA from *Streptococcus gordonii*, and *PsaA from Streptococcus pneumonia*), which have been shown to be involved in adhesion in endocarditis (Lowe et al., 1995). A study using a peritonitis mouse model, mice injected with an *E. faecalis* mutant lacking *efaA* showed prolonged survival compared to mice injected with wild type *E. faecalis*, suggesting an important role of EfaA in disease (Singh et al., 1998a).

GelE is a secreted zinc-metalloprotease (gelatinase) that shares similarity with *P. aeruginosa* Elastase and *S. aureus* Aurolysin (Potempa and Pike, 2009). Its gene, g*elE* is an operon with the serine protease *sprE* with the latter located immediately downstream from *gelE* (Gaspar et al., 2009).

GelE and SprE expression is controlled by the *fsr* (faecal streptococci regulator) locus, which contains four genes: *fsrA*, *fsrB*, *fsrC* and *fsrD*. These genes are part of a quorum sensing two-component system (Sava et al., 2010, Fisher and Phillips, 2009). As mentioned previously, activation of this system will lead to up- and down-regulation of the expression of other genes, and this happens when the concentration of the autoinducer peptide outside the cell reaches a minimum threshold (quorum) level that is usually only reached at high cell densities.

Gelatinase biosynthesis-activating pheromone is an autoinducer peptide encoded by *fsrD*. FsrB is responsible of exporting and cyclization of this FsrD peptide. Accumulation of the peptide in the extracellular space can be sensed by histidine kinase sensor FsrC leading to activation of the response regulator FsrA (Sava et al., 2010, Thomas et al., 2008). This process then results in the expression of GelE and SprE, which are located downstream from *fsr* locus.

Gelatinase is able to degrade several substrates such as casein, gelatin, fibrin and other immune peptides (Thurlow et al., 2010). Also, GelE has been shown to have a role in development of biofilms of *E. faecalis* (Hancock and Perego, 2004). The mechanism by which GelE contributes in biofilm formation is unknown. However, there are a number of theories of how GelE promotes biofilm formation, one being that GelE increases cell surface hydrophobicity by cleaving cell surface proteins at hydrophobic residues and therefore, enhanced cell attachment to the surface (Carniol and Gilmore, 2004). Several studies have reported a reduction in biofilm formation of *E. faecalis* mutant lacking *gelE* (Thomas et al., 2008, Mohamed et al., 2004). Also, GelE has been shown to contribute to

virulence in mouse models in peritonitis, endocarditis and endophthalmitis (Singh et al., 2005, Singh et al., 1998b, Engelbert et al., 2004).

Cytolysin or hemolysin are other secreted proteins which are related to *S. pyogens* streptolysin (Cox et al., 2005). Production of these lytic enzymes involves several genes that are encoded either on a plasmid or on the chromosome (Haas et al., 2002). Cytolysin is expressed as two peptide subunits, CylLL (the long subunit) and CylLS (the small subunit). Production and activation of cytolysin involves several stages. The two subunits LL and LS are synthesized ribosomally and then post-translationally modified by the protein CylM. Next, the modified peptides are proteolytically cleaved and secreted from the cell by CylB (an ABC transporter). The secreted peptides are then activated by further cleaved by CylA (a serine protease). Cells producing cytolysin could be protecting themselves by CylI which is located in the cell wall. Production of cytolysin is regulated by CylLs, which can can interact with the regulatory protein CylR1 on the cell membrane (Haas et al., 2002, Jett et al., 1992).

Table 1.2 Summary for *E. faecalis* virulence factors.

Virulence factor	**Role in virulence**
Surface associated proteins	
AS	• Biofilm formation • Adhesion and invasion of endothelium cells • Attachment to ECM
Esp	• Biofilm formation • Endocarditis and Urinary tract infection
EfaA	• Experimental peritonitis
Ace (MSCRAMMs)	• Attachment to ECM • Endocarditis
Secreted proteins	
GelE and SprE	• Biofilm formation • Experimental endocarditis, peritonitis and endophthalamitis • Virulence in *C. elegans*
Autolysin	• Biofilm formation
Cytolysin	• Tissue damage • Virulence in *C. elegance* • Experimental endocarditis

1.5. *Streptococcus bovis*

Streptococcus bovis is a facultative anaerobic, spherical and Gram positive lactic acid bacterium (Herrera et al., 2009). *S. bovis* belongs to the group D streptococci based on its cell wall polysaccharide antigen (Lancefield, 1933). These bacteria are considered as normal flora in both animal and human GI tract (Herrera et al., 2009). However, *S. bovis* has been implicated in several human diseases such as bacteremia, meningitis and endocarditis (Songy et al., 2002, Gavin et al., 2003). In addition, *S. bovis* can cause disease to animals including septicemia in pigeons, bovine mastitis and acute acidosis, bloat and liver abscesses in ruminants (Rusniok et al., 2010, Herrera et al., 2009).

Multiple studies showed the association between *S. bovis* and colon cancer in humans, with the first study already being published in 1951 (McCoy and Mason, 1951). However, the mechanisms behind this link still unclear (Rusniok et al., 2010). The association may simply be that alteration of the environment in the colon due to a tumour provides a niche more suitable to colonization, which then leads to translocation of the pathogens into blood stream. This, in turn, can then result in other diseases such as endocarditis. Indeed, there is a very high risk of patients with *S. bovis* endocarditis also having colon cancer (Boleij et al., 2011b).

Another option on the link between colon cancer and *S. bovis* may be that the organism exacerbates tumour development. Indeed, it has been suggested that it contributes in carcinoma by stimulation the COX2 cyclooxygenase 2 pathway resulting in cell proliferation (zur Hausen, 2006, Tjalsma et al., 2006). The final option is that *S. bovis* causes of colon

cancer. Bacteria causing cancer is not unprecedented as, for instance, *Helicobacter pylori* appears to be the cause of gastric adenocarcinoma and was declared a human carcinogen in 1994 (McColl, 2010). However, there is at present no clear evidence that *S. bovis* is the cause of colon cancer.

Recently, *S. bovis* has been divided into four biotypes(see chapter 6 for details; (Schlegel et al., 2004). One of the species is *S. gallolyticus* which belongs to *S. bovis* biotype I (mannitol fermentation positive). The name *gallolyticus* refer to its tannase activity and therefore, decarboxylate gallate which is a derived organic acid from tannin degradation (Rusniok et al., 2010). Also, *S. gallolyticus* be able to express bile salt hydrolase and amylase (Chamkha et al., 2002). The aforementioned association between *S. bovis* infections and colon cancer appears to be clearly linked to this biotype I. It was shown that association between *S. bovis* type I and bacteremia and endocarditis is 94%, and that this is 71% with bacteremia and colon cancer. In contrast, the association of *S. bovis* biotype II is only 18% with bacteremia and endocarditis, and 17% with bacteremia and colon cancer (Ruoff et al., 1989).

Studies on *S. gallolyticus* virulence factors are still largely unknown. However, a study on *S. gallolyticus* strain isolated from pigeon have described five serotypes and shown that these species all produced a polysaccharide capsule (De Herdt et al., 1992). Also, electro-microscopically studies on *S. gallolyticus* isolated from pigeons showed the presence of capsule and pili structure which been hypothesized to be involved in virulence (Vanrobaeys et al., 1999). Further evidence on factors playing a role in pathogenicity is presented in chapter 6, whereas some potential extracellular virulence factors are identified in chapter 3.

Similar to other Gram positive bacteria including staphylococci, streptococci and enterococci, *S. bovis* can mediate endocarditis by its ability to adhere to ECM proteins (Sillanpaa et al., 2008). A study on *S. gallolyticus* isolated from human with endocarditis identified indeed the presence of collagen binding protein Acb (adhesion to collagen of S. bovis) and other ECM binding proteins (Sillanpää et al., 2009). Furthermore, a full genome analysis of *S. gallolyticus* UCN34 strain isolated from human with endocarditis associated with colon cancer revealed the existence of capsular polysaccharides, pili and ECM binding proteins (Rusniok et al., 2010). The presence of capsules may provide protection for the pathogen from immune host defense and cell surfaces proteins contribute in bacterial adhesion to host tissues (Rusniok et al., 2010).

1.6. The aims of this thesis

Enterococci have became a major clinical problem because of their antibiotic resistance and ability to transfer resistance genes among other bacteria. The first part of this thesis is to study and identify virulence factors and properties in particularly *E. faecalis* strains. Our aim was to characterise in particular three clinical isolates from biliary stents that differed in the presence of a few critical virulence factors that have been mentioned above (Esp, GelE and AS). The genomes of these strains have not been sequenced, and these studies aimed in testing a number of factors, including testing the presence or absence of further virulence factors, pathogenicity and biofilm formation. In addition, we analysed the role of

Esp in biofilm formation, mainly using an *E. faecium* strain for which an *esp* mutant is available.

The second part was to examine the ability of several *S. bovis* isolates to form biofilms and factors that play a role in that. We were in particular interested in the role of the presence of collagen in this process, as some of the isolates are associated with diseases such as endocarditis or colon cancer. Collagen is a major constituent of heart valves, whereas it may also be exposed on tumours in the colon. Furthermore, using bioinformatics we analysed the (putative) secretome of *S. bovis*; as some of the proteins in this secretome may indeed represent (novel) virulence factors. Finally, pathogenicity of *S. bovis* strains was tested using the *C. elegans* nematode model.

Chapter 2: Materials and Methods

2.1. Chemicals

All chemicals and bacteriological media used in this study were purchased from Sigma-Aldrich, Oxoid or Fisher Scientific, unless noted otherwise. Custom oligonucleotides were synthesized by Sigma-Aldrich.

2.2. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in table 2.1 and table 2.2. *Enterococcus* and *Streptococcus* strains were cultured in Brain Heart Infusion (BHI) or Tryptone-soya-broth (TSB). For biofilm assays, TSB supplemented with 0.25% glucose (TSB-G) was used. For pathogenicity testing, strains were cultured in BHI agar supplemented with the appropriate antibiotics. *E. coli* strains (OP50, HB101 and BL21 (DE3)) were maintained on Luria-Bertani (LB) medium. For assays involving nematodes (section 2.7), nematode growth medium (NGM) agar plates were used for growth of *E. coli (Brenner, 1974).E. coli* BL21 (DE3) was grown in Luria-Bertani Medium (LB) supplemented with Kanamycin (50 µg/ml). Spectinomycin (60 µg/ml) and Chloramphenicol (5 µg/ml), were added to LB medium to prevent the loss of plasmids derived from pAT79.

Table 2.1 a list of bacterial strains used during this project.

Strains	Important characteristics	Reference
***E. faecalis*:** BS12297	Isolate from clogged biliary stents. Esp+, GelE-	(van Merode et al., 2006b)
BS11297	Isolate form clogged biliary stents. Esp+, GelE+	(van Merode et al., 2006b)
BS385	Isolate form clogged biliary stents. Esp-, GelE-	(van Merode et al., 2006b)
E. faecalis ATCC 19433		ATCC (American type culture collection)
E. faecium: E1162	Clinical Blood isolate (CC17). AmpR, Esp+	(Thomas et al., 2008)
E1162Δ	E1162 strain with esp gene deleted	(Thomas et al., 2008)
TX1130	Healthy volunteer faecal isolate. AmpS, Esp-	(Lasa and Penades, 2006)
***S. bovis*:** SB1293	Biotype I (*gallolyticus* subsp. *gallolyticus*)	H. Tjalsma (Nijmegen)
SB1294	Biotype II/2 (*S. gallolyticus* subsp. *pasteurianus*)	H. Tjalsma (Nijmegen)
NTCT8133	Biotype II/1 (*S. infantarius*)	H. Tjalsma (Nijmegen)
S. macedonicus		H. Tjalsma (Nijmegen)
S. gallolyticus NTB1	Biotype I (*S. gallolyticus* subsp. *gallolyticus*)	H. Tjalsma (Nijmegen)
S. gallolyticus UCN34	Biotype 1(*S. gallolyticus* subsp. *gallolyticus*)	H. Tjalsma (Nijmegen)

Lactobacillus plantarum	WCFS1	(Kleerebezem et al., 2003)
Salmonella typhimurium NTB6	Clinical isolate, Radboud collection	H. Tjalsma (Nijmegen
C. elegans	AU37 (*glp-4, sek-1*)	CGC(CaenorhabditisGeneticsCenter)
Escherichia coli *E. coli* OP50	Standard strain to maintain *C. elegans*	CGC (Caenorhabditis Genetics Center)
Eschericia coli NTB5	Clinical isolate, Radboud collection	H. Tjalsma (Nijmegen
E. coli BL21 (DE3)	F^- *ompT gal dcm lon* $hsdS_B(r_B^- m_B^-)$ λ(DE3[*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*])	(Studier and Moffatt, 1986)
E. coli HB101	F^- mcrB mrr hsdS20($r_B^- m_B^-$) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm^R) glnV44 λ^-	Smith et al ,1989
NovaBlue Giga singles competent cells	For routine cloning	Novagen

- Abbreviation: gelatinase E (*gelE*), enterococal surface protein (*esp*), AmpR (ampicillin resistance), *glp-4, sek-1* (MAPK kinase deficiency and temperature-sensitive sterile), Spc^r, cat^r specinomysin and chloramphenicol resistance, respectively.

Table 2.2 a list of plasmids used during this project.

Plasmids	Details	Reference
pAT79	*E. coli* shuttle vector containing *Ori*R from pAMβ1, *Spc*^{r}, *lacZ*,P2 and *cat*^{r}	(Depardieu et al., 2003)
pAT-gallo2179	pAT79 containing collagen-binding protein gallo2179 from *S. gallolyticus* UCN34	This study
pET28a	Km, oriR pBR322, origin f1, promoter T7, coding sequence His-Tag, terminator T7, lacZa	Novagen,R&D Systems
pET-Esp-n	N-terminal domain Esp in pET28a	This study

2.3. Biofilm formation assay

2.3.1. Crystal violet biofilm assay

Biofilm formation assays were essentially performed as described before with some minor modifications (Heikens et al., 2007). Strains were grown overnight in TSB-G broth at 37^{o}C. Next day the cultures were diluted in TSB-G to 10^7 cfu/ml and dispensed in 96-well microtiter plate (Costar). Next, the microtiter plates were incubated at 37^{o}C for 24 hours on 3D plate rotator (Grant-Bio; 30prm). After that the cell suspension was removed and the plates were washed twice with 0.9% NaCl and inverted to dry at room temperature for 1 hour. Following this 150 µl of crystal violet solution (CV; Prolab Diagnostics)

was added to the wells and was allowed to stain for 15 min. After staining, CV was removed and the wells were washed 3 times with 0.9% NaCl. The bound CV was then solubilized by adding 200 µl of ethanol-acetone (80:20 v/v). The absorbance of CV was read at 595 nm on the plate reader (Versa max Tunable microplate reader).

To test effect of various compounds on biofilm formation, 100 µl of cells were mixed with 100 µl of the relevant compound diluted in TSB-G to the relevant concentration.

For biofilm formation on collagen-coated plates, the assays were performed similarly to the crystal violet assay except that the assay was either performed in pre-coated 96-well microtiter plates (coated with collagen I or collagen IV; Becton Dickinson, Bio-Coat), or by using "home-made" coated plates. In case of the latter, 64 µl of rat tail collagen or 100 µl, 100 µg/ml, of collagen I (Sigma) was added in the wells of a microtitre plate, and the plate was left overnight at 25^{o}C in a laminar flow hood. Next day, the plates were rinsed with Phosphate buffered saline (PBS; 8g/l NaCl, 0.2g/l KCl, 1.44g/l Na_2HPO_4, 0.24g/l KH_2PO_4 pH7.4) and allowed to dry before starting the assay.

2.3.2. Confocal laser scanning microscopy (CLSM)

Bacteria were grown in TSB containing 0.25% glucose overnight at 37ºC. Next day the cultures were diluted in TSB-G to 10^7cfu/ml. Sterile coverslips (polyvinyl; Fisher Scientific) were placed in each well of a 6-well plate and then 2 ml of diluted culture plus 2 ml TSB-G medium was added. Biofilms were then grown on the coverslips for 24 hours at 37ºC on a 3D rotator (30 rpm). Next, coverslips were removed and washed twice with 0.9% NaCl. Finally, the coverslips were transferred to a 55 mm petri dish. 800 µl of Syto9 stain (Sigma) was added to each coverslip and stained in the dark for 10 minutes before being washed as described above.

To visualise biofilm formation on a collagen-coated surface, coverslips were covered with collagen in 0.1 M acetic acid, similar as described in section (2.3.1), and left overnight at room temperature in a laminar flow hood. Next day the coverslips were washed three times with PBS to remove the excess acetic acid. Biofilms were then grown on the coverslips as described above. Images were collected using LSM510META Zeiss confocal laser scanning microscope, laser including the argon laser and the helium laser with wave length of 488nm and 543nm, respectively.

2.4. Protease assay

2.4.1. Azocasein assay

This assay was done as described by (Denkin and Nelson, 1999) with some modifications. Bacteria were cultured at 37^{o}C overnight in BHI, The following day the culture suspensions were centrifuged for 10 minutes at 2,500 g and the cell pellet was discarded. 150 µl of the supernatant was added to 250 µl 2% azocasein in PBS and incubated for 2 hours at 37^{o}C . Next, the assay was terminated by adding 1.2 ml 10% trichloroacetic acid (TCA). An enzyme blank was prepared by mixing buffer, enzyme, TCA and the substrate in the same order. Next, the samples were centrifuged for 10 minutes at 5000g , and 1.2 ml from the supernatant was added to 1.4 ml 1M NaOH, followed by measuring the absorbance at 440 nm.

2.4.2. Milk – TSB agar method

This has been done by spot around 5 µl of an overnight bacterial culture on a TSB-agar plate supplemented with 1%-1.5% of skim milk(Thomas et al., 2008), followed by incubation at 37^{o}C for overnight.

2.5. Minimum inhibitory concentration (MIC) tests

The MIC test was done as described by (Andrews, 2001). In a 96-well microtiter plate 100 µl of the compound to be tested (0.5-512 µg/ml) was mixed with 100 µl of medium containing 10^{5} cells/ml and then the plate was

incubated for 18 hours at 37^{o}C. The MIC is defined as the lowest concentration without visible growth.

2.6. Cell surface hydrophobicity determination

This test was adapted from (Tendolkar et al., 2004). Bacterial strains were grown overnight in TSB-G at 37^{o}C containing the compound. The next day the culture was diluted 1:50 in 5 ml fresh media and the culture then incubated for 4 hours at 37ºC. Next, 1 ml of the culture was centrifuged to harvest the bacteria. The bacterial pellet was washed twice with 1.2 ml PUM buffer (0.15 M potassium phosphate, 0.3 M urea, 6.7 mM $MgSO_4$, pH 7.1) and then resuspended in 1.2 ml PUM buffer. The optical density was adjusted to 1 OD at 400 nm. 200 µl of n-hexadecane was added and the suspension was mixed and incubated for 10 minutes. The absorbance of the aqueous layer was measured at 400 nm. The percentage hydrophobicity was calculated by using the following formula: [1-(final OD400/ initialOD400)] *100

2.7. Nematode killing assay

C. elegans strain AU37 was maintained and propagated on *E. coli* OP50 as previous described by(Brenner, 1974) with some modifications. For infection with enterococci or streptococci, antibiotics were added to the BHI medium to prevent growth of *E. coli*. Kanamycin (30 µg/ml) was used with all *E. faecalis* strains and *S. bovis* strains.

Erythromycin (4 µg/ml) was used for *E. coli* NTB5, and streptomycin was used with *E. coli* HB101 and *Salmonella typhimurium* NTB6. For age synchronisation, eggs were collected by treatment of gravid adults with bleach solution (1 ml bleach - 5% hydrochlorite - plus 0.5 ml 5M NaOH). Eggs were washed with M9 buffer and incubated in tube on rotary mixer for overnight at room temperature. Next day, the L1 larvae were deposited on NGM plates with *E. coli* HB101 and grown for 48-52 hours. Between 20 and 30 *C. elegans* L4 or young adult hermaphrodites were transferred from a lawn of *E. coli* HB101 to a lawn of the bacterium to be tested and incubated between 8-12 hours at 25°C and for anaerobic killing assays bacteria were grown on plates in anaerobic GasPak (Becton Dickinson) before starting the experiment. Infected worms were then washed and transferred into a well of a 12-well plates, with each well containing 1:10 BHI (diluted in M9 buffer) with an appropriate antibiotic. Animals were examined for 7 days with a dissecting microscope for viability. Worms were considered dead when they did not respond to touch with a platinum wire pick. Each experimental condition was tested in triplicate(Jansen et al., 2002).

2.8. DNA techniques

2.8.1. Plasmid purification

Plasmids were isolated using the Nucleospin Plasmid Kit (Macherey-Nagel) by following manufacturer's instructions.

2.8.2. Chromosomal DNA extraction

DNA of Gram-positive bacteria was extracted by phenol method as described before by(Shankar et al., 1999) with some modifications. Bacterial strains were grown overnight in BHI supplemented with 2.5% glycin at 37°C, and the next day cell pellets obtained by centrifugation were suspended in 1 ml TES (50 mM NaCl, 100 mM Tris, 70 Mm EDTA) containing 25% sucrose plus 100 µl lysozyme (10 mg/ml) and 50 U mutanulysin. This was incubated for 2 hours at 37°C. Next, 20 µl of proteinase K (20 mg/ml) and 50 µl of 10% SDS were added, and the samples were incubated for another hour at 55°C. After the cells lysed, DNA was purified by adding one volume of phenol-chloroform–isoamylalcohol (25:24:1), followed my mixing and centrifugation for 5 min at 10,000g. The top layer was removed and extracted with phenol/chloroform/isoamylalcohol twice more. The DNA was precipitated by adding 0.1 volumes of 3M Na acetate and 0.6 volume of isopropanol centrifugation at 12,000g 1minute. Finally, the DNA pellets were washed with 80% ethanol, dried at room temperature and resuspended in 150 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA pH 8).

2.8.3. Polymerase chain reaction (PCR)

PCR was performed with an Eppendorf Mastercycler gradient machine (Eppendorf). PCR reactions were set-up using either Taq One polymerase (New England Biolabs) or KAPA2G robust (KAPA biosystems) by following manufacturer's instructions. The primers used for PCR are listed in table 2.3.

Table 2.3 Primers[a] used in this study.

PCR	Forward primer (5' to 3')	Reverse primer (5' to 3')
GelE	GGATCGATCC**TCTAGA**GGAA AAGAAATAAAAGGAACTGG	CAGACTTTGC**AAGCTT**CATAAGA TTATGCCACTCCTTATCC
Gallo_2179	ATAT**GAGCTC**GGTTGCTAAT TGTGTGTGCTAATG	ATAT**TCTAGA**GATTGGTTTTACT TTAGATAAAATC
Gallo_0577	ATA**GAGCTC**GCTTGGCGAGC AATTTCTGACATG	ATAT**TCTAGA**CTAACATACCACT AGCAATTCTAC
Gallo_1570	ATAT**GAGCTC**CAATTGTTGT TGAATTCGTTGTCG	ATAT**TCTAGA**GTTAATTGTCAAC GTATAAGTTGA
Gallo_2032	ATAT**GAGCTC**GACAAGCCGT TCAAACAGAGATTG	ATAT**TCTAGA**CCAACAACATTGT GTCGAGTGACG
Ace	GGCGACTCAACGTTTGAC	GTAGGCGATTTGGCTGGA
Efa	TGGGACAGACCCTCACGAAT A	GGCTTGAACTTAGAAACAGGCG
CylB	GAGGTGTAATTATGAAAAGA TTGAAG	CAATTATACGGACTTTATCAATA TTTG
CylM	CTGATGGAAAGAAGATAGTA T	AAATGTAATCAGACCAACTCA
Agg	AAGAAAAAGAAGTAGACCAA C	TATTTACTTGTCTTGCCGTTT
EspN	ATATCCATGGGCGAACTAGT TAAAGCACAAGATG	ATATGTCGACAATATCTTTACTT ACAGTTACTGC
Gallo_2179	AAAA**GGATCC**GTGATAAAGT GGTTGCTAATTGTG	AAAA**GTCGAC**CTACTAGAGATTG GTTTTACTTTAG

[a]The restriction sites used for cloning are indicated in bold and underlined (*XbaI/HindIII* for GelE and *XbaI/SacI* and BamHI/SalI for *Streptococcus bovis* collagen binding proteins. gelatinase E (*gelE*), enterococal surface protein (*esp*), N-terminal of esp (espN) endocarditis specific protein (*efa*), collagen binding protein (*ace*), cytolysin transport protein (*cylB*), cytolysin immunity protein (*cylM*), aggregation substances (*agg*) and collagen binding proteins in *S. gallolyticus* UCN34 (*gallo*).

2.8.4. Restriction enzyme digestion

Restriction enzymes were purchased from New England BioLabs, and performed as recommended by the manufacturer. Enzymes used were: *Sac*I, *Xba*I, *Hind*III and *Bam*HI. DNA and plasmid were digested overnight and cleaned up using the Nucleospin Extract II Kit (Macherey-Nagel).

2.8.5. Ligation

DNA was ligated in to a digested vector using T4 ligase enzyme (New England Biolabs). Ligation reactions were set up following manufactures instructions and the reaction were incubated overnight at room temperature.

2.8.6. Agarose gel electrophoresis and Gel purification

Agarose gel electrophoresis was performed as described by (Sambrook and Russell, 2001).The 0.8% agarose gel stained with Ethidium bromide was run at 100V and DNA was photographed under UV light. DNA gel purification was carried out by using the Nucleospin Extract II Kit (Macherey-Nagel) following manufacturer's instructions.

2.8.7. Transformation of bacteria

2.8.7.1. Transformation of *E. coli*

NovaBlue GigaSingles compentent cells (Novagen) were transformed with plasmids or ligation mixtures according to the manufacturer's instructions.

2.8.7.2. Enterococci and Streptococci Transformation

Preparation of electrocompetent cells from enterococci and streptococci was performed as described by (Dunny et al., 1991), using a Bio-Rad electroporator. 5 µl of DNA was mixed with 100 µl of thawed cells. Cells were left on ice for 5 minutes, transferred into a 1 mm electroporation cuvette, and then pulsed at 1.25 kV. Cells were immediately diluted with Todd Hewitt Broth medium (THB) containing 0.5 M sucrose and then incubated at 37°C between 1.5-2 hours. Finally, 100 µl and 50 µl of these transformed cells were plated on THB agar with 20% sucrose containing the appropriate antibiotics and incubated for overnight at 37ºC.

2.8.8. Automated DNA sequencing

Eurofins MWG Operon, UK performed Samples sequencing. For sample preparation, 150 ng/µl of plasmid DNA and 15 pmols of primer were mixed in a total volume of 15 µl.

2.9. Protein expression and purification

2.9.1. Induced expression of *esp-n* in *E. coli* cells

E. coli BL21 (DE3) cells containing plasmid pET-Esp-n were grown in LB medium supplied with 50 µl kanamycin until an OD600 of 0.4. 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) was added and cells were grown for a further two hours at 37ºC. Cells were harvested by centrifugation and resuspended in 50 mM Na-phosphate pH 6.8 buffer containing 0.1 mg/ml lysozyme and protease inhibitor cocktail (Complete, EDTA-FREE, Roche Molecular Biochemicals). Cells were incubated on ice for 10 min and then were lysed by sonication for 5 minutes using a Branson Sonifier 250 set to a duty cycle of 30% and an output of 3. To remove cell debris, the lysate was centrifuged for 15 minutes at 5000 g and then the supernatant mixed with 1 volume 50% Ammonium Sulfate and left at room temperature for one hour. Proteins precipitating at 25% $(NH_4)_2SO_4$ were removed by centrifugation for 20 minutes at 6000 g.

2.9.2. Purification on phenyl sepharose column

Phenyl-sepharose column were obtained from GE health care. The protein sample (in 25% $(NH_4)_2SO_4$) was loaded on the column and the flow through were collected. Protein elution was performed by decreasing salt concentration using 25, 20, 15, 10, 5 and 0% of ammonium sulfate. Samples were verified on SDS-PAGE.

2.9.3. Protein dialysis

Dialysis of samples from the protein purification was performed using Snakeskin Pleated Dialysis tubing (10 kDa MWCO, Pierce), against buffer containing 50 mM Na-phosphate and 100 mM NaCl for overnight at 4 ºC.

2.9.4. **Protein concentration**

Protein concentration was determined using the BCA protein assay reagents from Pierce following manufacturer's instructions. Bovine serum albumin (BSA) was used to prepare standards of known protein concentration.

2.10. Protein gel techniques

2.10.1. SDS-polyacrylamide gel electrophoresis (PAGE)

Protein samples were mixed with loading buffer and boiled for 3 minutes prior to resolving the samples on SDS-PAGE as described by (Laemmli, 1970). For Esp, 10% acrylamide gels were used. A pre-stained protein marker (EZrun) was used from Fisher Scientific. After separation of protein samples on SDS-PAGE, the gel was fixed with 40% methanol and 10% acetic acid for 30 minutes. Then it was transferred to Coomassie stain (10% acetic acid, 0.025% Coomassie G-250) for one hour. Finally, the gel was transferred to destain solution (20% methanol, 7% acetic acid) for overnight.

2.11. RNA techniques

2.11.1. RNA extraction from Enterococci and Streptococci

Strains were grown to an OD600 of 0.5 in BHI medium. RNA were purified using RNA protect bacteria reagent and RNeasy protect bacteria Kits (Qiagen) following manufacturer's instructions. Then RNA were treated with 1 unit of DNase I (RQ1 RNase-Free DNase, Promega) per 1 µg of the sample and incubated for 30 minutes at 37ºC. Finally, the samples were incubated at 65ºC for 10 minutes to inactivate the DNase.

2.11.2. RT-PCR (Reverse Transcriptase PCR)

SuperScript III One step RT-PCR System (Invitrogen) was used to produce cDNA and amplify the resulting product in one step by following the manufacturer's instructions. The results were verified by agarose gel electrophoresis as described in section 2.9. Primers used for the one step RT-PCR are listed in table 2.4.

Table 2.4 List of primers used in RT-PCR only.

RT-PCR	Forward primer (5’ to 3’)	Reverse primer (5’ to 3’)
Gallo_2179	TCCCACAACAACATCCTCTGA	CGACTACCATCTACACCACCA
Gallo_2179	GTTTTGTTGGTGAGAGTGCCT	TCCCACAACAACATCCTCTGA

2.12. Statistical analysis

Differences between conditions were analysed using Student T-test. Significance difference was defined as a P-value <0.05, evaluation were performed using Excel 2007.

Chapter 3: Bioinformatics analysis of the extracellular proteome of *Streptococcus gallolyticus*

3.1. Introduction

Transport of bacterial polypeptides across the cytoplasmic membrane is mediated by a number of distinct processes. Two of these are general secretion pathways (GSP) which translocate several proteins. These are the Twin-arginine Translocation (Tat) pathway, which is able to transport fully folded proteins, and the Sec-dependent pathway, which transports unfolded proteins across the membrane (Bolhuis, 2002). There are also other systems such as the Autotransport (AT) and Two-Partner protein Secretion (TPS), which are involved in translocation of specific proteins (Hodak and Jacob-Dubuisson, 2007).

Many virulence factors, some of which may be involved in biofilm formation, are translocated proteins, and it is therefore of interest to analyse the proteome of pathogens for putative secretory proteins. An analysis of enterococcal extracellular proteome has already been performed (Meredith, 2013). The goal here was therefore to perform a similar analysis on *S. gallolyticus*. This chapter deals with the Sec-dependent pathway only, as streptococci, similar to enterococci, do not contain a Tat pathway.

A simple model for protein secretion through the Sec dependent pathway is shown in Figure 3.1. Secretory proteins are synthesized by ribosomes as pre-proteins with an N terminal signal peptide. This signal peptide is recognized by chaperones and the translocase machinery to be translocated to the *trans* side of the membrane. Then at the *trans* side of the membrane the signal peptide is cleaved off by signal peptidases (SPases) and the protein will fold to its active

form. This process utilises two energy sources: ATP hydrolysis and the proton motive force (PMF; (Schiebel et al., 1991). Translocase components include the core units SecY, SecG and SecE, and the ATP-driven motor of the Sec pathway, SecA. Most bacteria also contain accessory factors such as SecD and SecF, but the latter two appear to be absent in organisms such as enterococci, streptococci and lactococci (Nouaille et al., 2006).

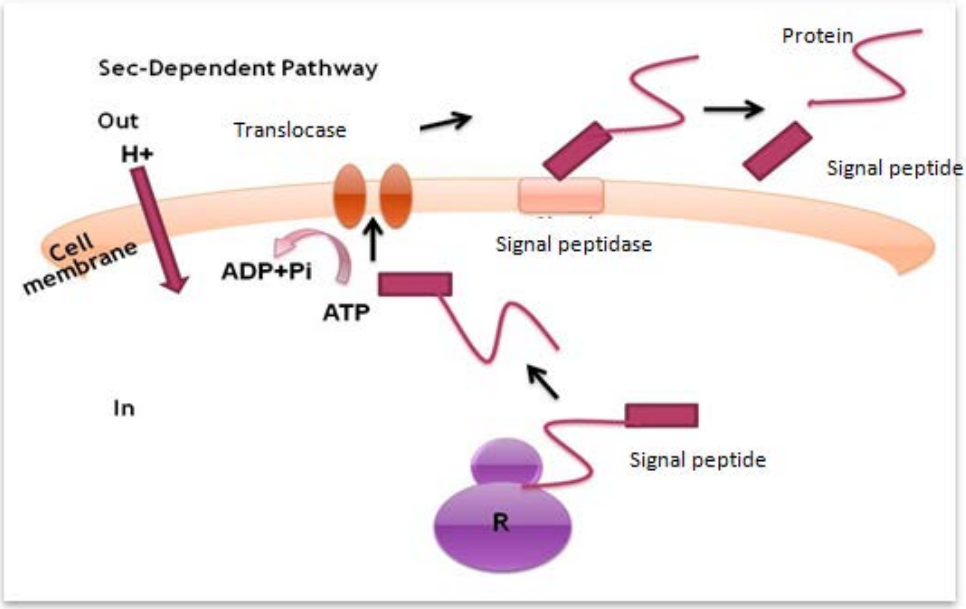

Figure 3.1. Protein translocation by the Sec-dependent pathway. See text for details.

Structural features of signal peptides play an important role in directing proteins to different pathways to cross the membrane. Therefore, there are four types of signal peptides (SP; Figure 3.2): archetypal (Bacterial signal peptide), lipoprotein, Tat and prepillin signal peptides (Paetzel et al., 2002); here we will mainly focus only on the archetypal signal peptides. The structure consists of three regions: N-, H- and C-regions. The N-terminal region consists of positive charged residues which promotes directing the signal peptide into the membrane. This is followed by a hydrophobic H-region (7-15 residues) which contributes to the formation of an α-helix that spans the membrane (Heijne, 1990, Paetzel et al., 2002). This is then followed by a more polar C-region, which contains the signal peptidase recognition site sequence, Ala-X-Ala, at position -1 and -3 relative to the cleavage site in the pre-protein (Paetzel et al., 2000). Lipoprotein signal peptides have a similar structure, but their signal peptidase recognition site has the lipobox residues Leu-(Ala, Ser)-(Gly, Ala)-Cys at -3 to +1 positions, with the Cys residue being modified with a lipo-moiety that anchors the protein to the membrane (Hayashi and Wu, 1990).

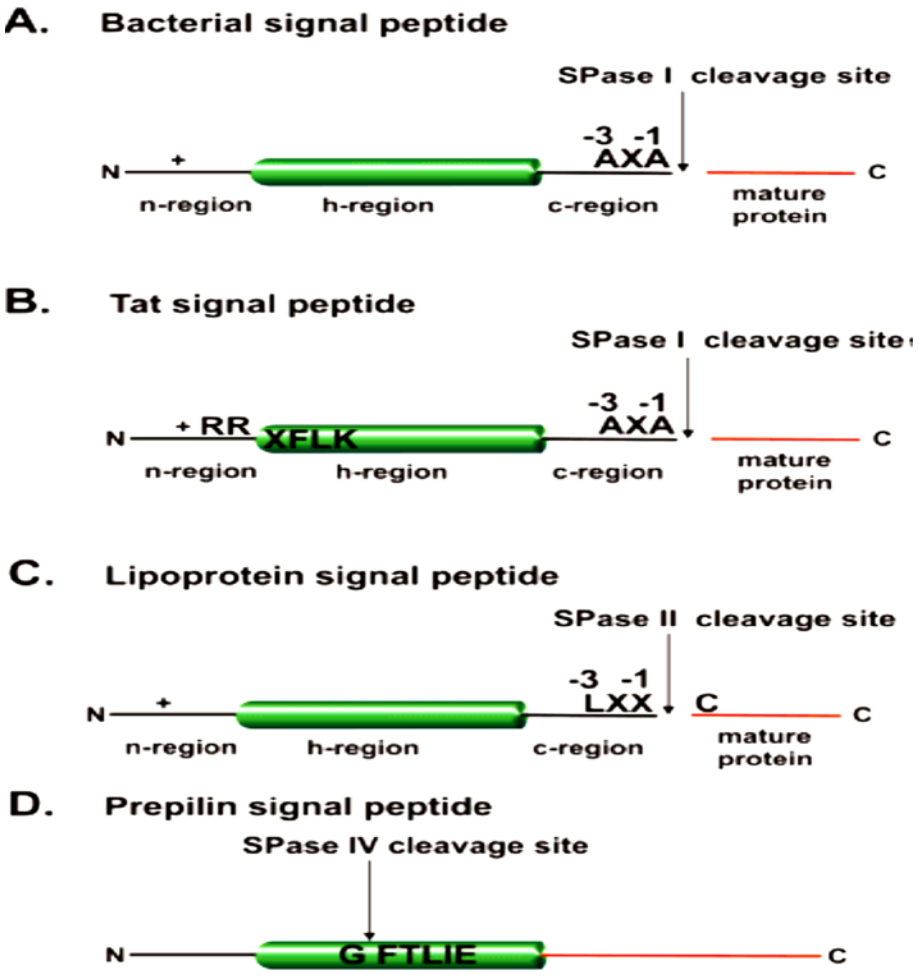

Figure 3.2 Types of bacterial signal peptides. A. Bacterial signal peptide (archetypal). B. Tat signal peptide. C. lipoprotein signal peptide. D. prepillin signal peptide. Bold letter (except X) represent the conserved amino acid residue and X represent non-conserved amino acid residue. Black arrows indicate cleavage sites. Taken from Paetzel et al., (2002), see text for details.

Signal peptides are removed during or shortly after translocation by SPases, of which there are two different classes: type I and type II signal peptidase. Both of their active sites are at the *trans* side of the membrane (Paetzel et al., 2002). SPase type I are essential for bacterial cells. They belong to the serine protease class of proteases and are highly efficient in target recognition. SPase type II, or prolipoprotein SPase (Lsp), remove the glycoside-modified prolipoprotein signal peptides that contain the lipobox with a Cys residue at position +1 (Tjalsma et al., 1999, Pragai et al., 1997).

Many bacteria contain only type I SPase, referred to as the prokaryotic (P) type. However, some bacteria have a second type, which is the Endoplasmic-Reticulum (ER)-type SPases (Tjalsma et al., 1998). P-type SPases are found in bacteria, mitochondria and chloroplast, whereas ER-Type are conserved in all three life domains (van Roosmalen et al., 2004). The best studied Gram-positive organism, *Bacillus subtilis,* has been shown to contain seven type I SPase genes. Five of these genes are chromosome encoded (*sipS, sipT, sipU, sipV and sipW*) and two (*sipP*) are located on plasmids (Tjalsma et al., 1997; Tjalsma et al., 1998). Four of the chromosomal genes are related to P-type SPase, whereas the SipW is related to ER-type SPase. However, it is not clear why such bacteria may have more than one type SPase and it has been suggested that presence of multiple types of SPase may be related to substrate specificity or that it simply increases the production capacity of pre-protein translocation and also may allow cells to adapt to the changing in environmental conditions (van Roosmalen et al., 2004). Interestingly, enterococci also have multiple SPases, with e.g. both *E. faecalis* and *E.*

faecium containing three, with one of these being of the ER-type and the two others of the P type (Meredith, 2013). Again, the reason why enterococci contain multiple type I SPases is unknown. *S. bovis* appears to contain only one P type SPase I, whereas it lacks an ER type SPase (data not shown). This is rather surprising as *S. bovis* is closely related *to E. faecalis* and *E. faecium*.

As mentioned previously into the introduction of this thesis, *S. bovis* (and in particular *S. gallolyticus*) has been associated with colon cancer, and there are several factors involved in the process of adhesion, invasion and colonising of host tissues. Therefore, it was interesting to use bioinformatics to analyse the secretion proteins in *S. gallolyticus.* We chose *S. gallolyticus* ATCC BAA-2069 for analysis; unfortunately, at the time of analysis, the proteome of *S. gallolyticus* UCN34 (one of the strains used in chapter 6) was not accessible, but these two strains are very similar as they are of the same biotype.

3.2. Identification of secretory proteins in *S. gallolyticus* via Sec-pathway

As mentioned before, Sec-dependent secretory proteins contain the signal peptide for their translocation. Firstly, we identified secreted proteins with a signal peptide using SignalP (V.4.0) software (Petersen et al., 2011). Membrane spanning domains of membrane proteins are very similar in signal peptides. The N-terminus of such proteins typically contain a positively charged N domain followed by a hydrophobic domain. The main difference is that they are not cleaved by SPases. However, that is not always clear, and for

that reason all SignalP–positive proteins were scanned for multiple membrane spanning domains with the server TMHMM (v 2.0; Krogh et al., 2001); all proteins with three multiple membrane spanning domains or more were removed. The final result is shown in Table 3.1, which represents the putative secretory proteins of *S. gallolyticus*.

The total number of proteins encoded by *S. gallolyticus* genome is 2271, and 90 of those (3.9%) are predicted to be secreted. 4 of those were hypothetical proteins, and the remainder have a predicted or known function. As expected, a number of transport proteins were observed. These included several ABC (ATP-binding cassette) transporters. The ABC transporters utilize ATP to translocate various substances across the bacterial membrane. They are of particular importance in the transport of various nutrients such as essential amino acids, as well as virulence factors (Moussatova et al., 2008). Note that there are a number of proteins that, either because of a very short signal peptide or based on their function are unlikely to represent genuine secretory proteins. These include for instance a DNA topoisomerase (presumably cytoplasmic), and YidC, which is a membrane protein involved in the insertion of other membrane proteins (Samuelson et al., 2000). All of these have been indicated in table 3.1 in italics. Proteins that were expected to be found included for instance three collagen-binding proteins (see chapter 6 for more details). Other proteins that one would expect to be translocated are degradative enzymes such as amylase, pullulanase, autolysin and beta-lactamase, the latter of which degrades beta-lactam compounds thus providing resistance to penicillin and related antibiotics. Also of interest is to note a

tannase. This is an enzyme involved in degradation of ester linkages in hydrolysable tannins, resulting in the production of gallic acid that distinguishes *S. gallolyticus* from other *S. bovis* strains. The name *gallolyticus* refers to tannase activity and therefore, decarboxylate gallate which is a derived organic acid from tannin degradation, was expected (Rusniok et al., 2010).

As mentioned, one of the secreted proteins included was autolysin, an enzyme involved in degradation of the cell wall. Apart from autolysin a number of other proteins associated with the bacterial cell wall were also seen. Examples are penicillin-binding protein (PBP), a peptidoglycan hydrolase and lysozyme. These proteins are very important in cell wall turnover, cell division and cell wall stress response mechanisms (Popham and Young, 2003, Smith et al., 2000). Proteins such as lysozyme may also aid in the bacterium's defense mechanisms, whereas PBPs are the target of β-lactam antibiotics; low affinity for β –lactams, or mutations that result in low affinity, in PBPs leads to resistance to beta-lactam antibiotics such as penicillin.

Amongst other secreted proteins, a number of competence-associated membrane nucleases were seen. These proteins have hydrolase activity and contain both metal ion binding and nucleic acid binding domains. Other proteins of note include several substrate binding proteins, which have also been found in *E. faecium* (Meredith, 2013), and proteins containing the aforementioned LPxTG motif (see chapter 4). These proteins are anchored to the cell wall by a specific enzyme, denoted as sortase, which is an extracellular protein also listed in Table 3.1.

The list also contains a number of lipoproteins. It is possible that the genome encodes more lipoproteins, but not all are picked up by the SignalP server as the H domain of several lipoproteins is rather short and sometimes more difficult to spot.

Several of the proteins in Table 3.1 may represent virulence factors, some of which are likely to be involved in biofilm formation. This includes for instance the collagen-binding proteins. Several of the hypothetical proteins may also be required for virulence, and it would require a systematic analysis by creating knock-outs combined with *in vivo* studies to test whether that is indeed the case. That would be a considerable effort, but initial *in vivo* studies could be performed with simple invertebrate infection models such as the *C. elegans* model as utilised in chapters 4 and 6.

Finally, one protein in the list, lactocepin, may even have prospects as a therapeutic agent. Lactocepin is a secreted protease that is also produced by a number of probiotic strains such as lactobacilli. In these organisms is has been shown that lactocepin specifically cleaves IP-10, a lymphocyte-recruiting chemokine, thereby reducing inflammation in patients with inflammatory bowel disease (von Schillde et al., 2012). However, whether this protein from *S. gallolyticius* has similar benefit as its counterpart from *Lactobacillus* requires of course further analysis.

3.3. Discussion

Translocation processes are essential for all organisms as they facilitate the transport of various proteins across the membrane. In opportunistic pathogens several of these proteins are virulence factors such as enzymes, exotoxins and biofilm-associated proteins. A detailed analysis of the secreted proteome of bacteria can help to understand the possible role and biochemical processes of that organism in a particular environment, and in the case of pathogens this would include the host. In this chapter a bioinformatics approach was taken to conduct an analysis of the extracellular proteome of *S. gallolyticus*. Various secretion mechanisms such as general secretion pathways of the Tat and Sec-dependent pathways were briefly discussed. Interestingly, *S. gallolyticus* does not possess a Tat pathway. Organisms that do contain this pathway are often capable of anaerobic respiration, a process that requires extracellular proteins which contain complex cofactors. These cofactors need to be incorporated into proteins in the cytoplasm; cofactor-binding requires partial or full folding of a protein, which thus necessitates a transport system that can handle folded proteins. The Sec system is only capable of transporting unfolded proteins, hence the need for the Tat system which is capable of translocation fully folded proteins (Robinson and Bolhuis, 2004). In contrast, Gram-positive bacteria such as lactococci, streptococci and enterococci lack electron transfer complexes, but instead generate energy via fermentative pathways, and it has been suggested that their simpler fermentative lifestyle is the reason that these organisms do not require a Tat pathway (Yen et al., 2002). For those reasons, this chapter focussed mainly on the Sec-dependent pathway, and in this context

a bioinformatics study was provided regarding proteins containing Sec-type signal peptides.

The secreted proteome was analysed using SignalP software to identify secreted proteins. These are distinguished by their tripartite nature with a positively charged N domain, a hydrophobic H domain, and a polar C domain where the signal peptide is processed resulting in release of the mature protein. In order to exclude genuine membrane proteins from those that were identified by SignalP, another software application, TMHMM, was used to scan for multiple membrane passing domains.

It was observed that 90 out of the 2271 proteins produced by *S. gallolyticus* were predicted to be secreted. The variety of proteins secreted enabled to obtain a paint general picture about the biochemical and pathogenic activities of *S. gallolyticus*. It was observed that it was putatively resistant to beta-lactam antibiotics due to the presence of the enzyme beta-lactamase. This particular strain was not investigated for antibiotic resistance, but of the strains of streptococci described in chapter 6, one (*S. bovis* NCTC 8133) was found to be ampicillin resistant (data not shown). The resistance of that strain may thus be the results of the production of a beta-lactamase homologous to the one identified here. The secreted proteins were also found to include various virulence associated proteins such as a number of potential biofilm associated proteins, mainly in the form of collagen adhesins. These play an important role in biofilm formation, which is a bacterial “lifestyle” that is very important from clinical perspective. It has even been suggested that biofilms are involved in the majority of human infections (Mohamed et al., 2004). This is very relevant

clinically, as biofilms have increased tolerance to antibacterial substances, and also horizontal transfer of genetic information such as antibiotic resistance frequently occurs in a biofilm environment (Mundy et al., 2000).

Not surprisingly, a number of proteins involved in the transport of various substances across the membrane were observed. Among these, there were a number of ABC transporters responsible for amino acid transport. A periplasmic component of a Proline/glycine betaine ABC transport system was observed. This transporter system helps to protect the bacterial cells from osmotic shock (Graham and Wilkinson, 1992). Furthermore, translocated proteins with an LPxTG motif were observed, and these thus become anchored to the cell wall via a sortase. An efflux protein was also observed; such a protein is often associated with the removal of unwanted substances and may play an important major role in antibiotic resistance (Morita et al., 2006).

Bacteria acquire their nutrients from the environment. Often, they are faced with the challenge of hydrolysing a complex substrate such as starch before it can uptake the released sugars and utilise these for its energy requirements. For such purposes, a number of extracellular enzymes are secreted from of the cell. These and other enzymes also marks the potential of *S. gallolyticus* as a bacterium of industrial importance, as it produces for instance amylase, pullulanase and tannase, all of which are widely used in e.g. the food industry (Hii et al., 2012). In addition, also lactocepin was identified, which is believed to possess potential therapeutic importance (von Schillde et al., 2012).

Unexpectedly, some proteins were identified that were not expected. For instance, a LytR family transcriptional regulator was observed. Transcriptional

regulators are mostly DNA binding proteins and are thus commonly found in the cytoplasm. LytR is a regulator that is part of the LytS/R two component system and is believed to play a role in biofilm formation in *Staphylococcus aureus* (Sharma-Kuinkel et al., 2009). The presence of this protein amongst the secreted proteins is thus surprising, and indicates that false-positive hits are conceivable. However, without experimental evidence we cannot exclude that LytR is a genuine secretory protein.

Overall, the results of the bioinformatics analysis of the secreted proteins of *S. gallolyticus* reveal some interesting information about the virulence and biochemical nature of the bacterium. The analysis enables to study the whole spectrum of proteins secreted by the bacterium and thus aids in identification of novel proteins, which might be of industrial or clinical importance. However, the results obtained needs to be validated by wet lab experiments to verify that these proteins are indeed produced and secreted to corroborate the bioinformatics data. In this respect it should also be noted that expression of genes encoding secretory proteins is likely to be regulated, and some genes may indeed only be expressed under specific conditions such as nutrient stress. For instance, it is conceivable that collagen-binding adhesins are only produced during early stages of biofilm formation and/or during an infection when collagen is present (see also chapter 6), thus helping the establishment of a biofilm. Novel bioinformatics tools have enabled analysis of a vast range of cellular products and components across all domains of living organism, and the techniques used in this study are important tools in the characterization of novel organisms.

Table 3.1 predicted secreted proteins in *S. gallolyticus.**

NCBI reference sequence	Predicted function	Secreted proteins ↓
F0VXQ7	*DNA topoisomerase*	*MATTTTT**K**APTAVKKSSK/KTT*
F0VYE1	Hypothetical secreted protein	M**KRK**AVFSLMLLACLVTGILGMNQIKNKDVVA/DDV
F0VTZ7	Membrane protein insertase YidC	M**KRKIK**LLGLSSLTLILLAACGRSEVTA/SST
F0VWB8	*Penicillin-binding protein 2B*	*MSF**KK**RLSKL/KFA*
F0VTT3	Putative glucan-binding protein D	MM**RK**VLQSILVTFLGLGLLLSAQKVEA/VDA
F0VX83	Tannase	MP**RKK**WFFTSSAVLLCSAMLLTACSSSSNSSTSSS/SSQ
F0VVG2	Alpha/beta hydrolase	M**RK**I**R**I**RKRR**VLLGIIALLFVVSVGA/SFY
F0VT98	Alpha-amylase	MVF**R**N**K**E**K**M**KKK**L**K**LGLGSALIFTILGTGTFVQVSVVNA/DTE
F0VS30	Amino acid ABC transporter, amino acid-binding protein	M**KI**K**K**IFLGVLALISVLTLAACGSSS/NEN
F0VXF0	Amino acid ABC transporter, extracellular amino acid-binding protein	M**K**F**KK**VLVGALALVSTLTLAACSSLSSKKA/TST
F0VY90	Amino acid ABC transporter, permease protein	MS**KKK**WIVAGGVVVALVAATVIGRQLTGKTTASA/QSS
F0VY86	Amino acid ABC transporter, substrate binding protein	M**KKK**V**K**WIISIIVVALVGLLIFDKVTKNTSEA/KSD
F0VY50	Amino acid ABC transporter, substrate binding protein	M**K**IV**KR**FLAIVSLLVVVLLVGCSTSKS/SSS
F0VRX4	Autolysin	M**H**L**KK**NVL**R**SCLLSPVIIGAFLSSSLVLA/DEN
F0VU99	Backbone pilus subunit (T6-antigen-like)	M**KK**L**K**LIFATFFAALFTFTGGKALA/YDI
F0VXU9	Basic membrane protein A	MN**KK**IVGLGLAAVATLALGACSRSNSSSSSSDSSVKA/AIV
F0VUN7	Beta-lactamase	M**KK**LFAVMLIPFFLTSLSVVSTEKTIALT/NEE
F0VYF9	Capsule biosynthesis protein CapA	MFD**R**IIY**KK**TTLACLSFLVISLLGSGVYSLA/FEK
F0VV45	cellobiose-specific IIC component	MS**K**ALIICVAGMSSSLMAQKTTDFF/KNQ
F0VW36	Collagen adhesin	MA**K**L**RK**ILIALLLLCSSIFSARVAFA/DTV
F0VSL2	Collagen adhesin	MM**RK**FI**R**IFLVLLTTLLTLTGISAKA/EED
F0VTR5	Collagen adhesin	M**KK**LFTILFVLLSVLCFLGGEKGVLA/ESN

NCBI reference sequence	Predicted function	Secreted proteins ↓
F0VWQ2	Competence associated membrane nuclease	M**K**I**K**TLSAISLTVIPSLILANFCHPFPSSQ/TNS
F0VWU5	Competence associated membrane nuclease	MA**KK**S**K**LS**K**QT**K**SLLSLVILLVGIGTGWVTISDS/NDP
F0VW92	Conserved hypothetical lipoprotein	M**KK**SC**K**ITVILFCSVLLLGACS/KKK
F0VUI4	Conserved hypothetical secreted protein	M**KK**FATSIIILISLVLVGCTHS/TDS
F0VWQ5	Cyclomaltodextrin glucanotransferase	M**KKR**IL**K**FCLGAATLTALMTVPFSQNIVSA/ESY
F0VTT6	D-alanine extramembranal transfer protein	ML**KR**LWQILGPVVCAVLIVLLVFICVPTANQ/KHD
F0VVI6	D-alanyl-D-alanine carboxypeptidase	M**KK**IFAFFAIFLTFLAVGSKVSA/DDD
F0VXL5	Dextransucrase	M**K**LLGGIME**KKI**H**Y**K**L**H**K**V**KK**QWVTIAVTTLALVVGLGAATSTQVVTA/DET
F0VYE6	Efflux transporter	MP**RR**S**K**A**K**MS**KK**T**K**GIIGAAAACFVVAGA/ALL
F0VX28	Ferrichrome ABC transporter, substrate-binding protein	M**KKK**LTLLLTAMMVLVMAVFLGACSSSSSSNSTSQSSNA/SEV
F0VSL1	Fimbrial subunit type 2	M**KK**L**K**LILATLLSILFAFTGVKAFA/DES
F0VV03	Fructan beta-fructosidase	M**KK**EQE**KK**CVNWFM**HKR**G**K**QWIYGCGVLVCGIVLGTVATPVMA/DEA
F0VXA9	fructose-specific IIB component	M**K**IVGITSCPAGLAHTPMAAKA/LEK
F0VXL8	Glucan-binding protein C	MF**KK**S**K**ETFYI**RK**LTIGVVSVAVAGLLAINNAQVNA/DET
F0VXL7	Glucosyltransferase-I	ME**KK**V**H**Y**K**L**HK**V**KK**QWVAIAVTSLALVGLGVSVPTQSVSA/DTT
F0VVJ3	Glycoside hydrolase family 5	MY**K**VT**K**NL**KK**ITTALILALVALFAIDSNTEVAYA/ATT
F0VWL3	Inner spore coat protein H	M**K**NNKSFLLVILLLVVTAGLVLIVGLFTNNDDD/DSS
F0VTG9	Iron (Fe+3) ABC superfamily ATP binding cassette transporter, binding protein	M**KK**FFAVLTTFLATFLLVACHNTSSTS/DDT
F0VWP6	Lactocepin	MD**KK**E**R**FSF**RK**Y**K**VGLVSVLVGAVFLAAGAGRVSA/DEL
F0VTS2	LPXTG-motif cell wall anchor domain protein	M**KK**FF**RR**EYLAQF**K**A**K**Y**KK**VIGAIAVIVVFVTTYALILPALTLDSNA/ANQ
F0VUD6	LPXTG-motif protein cell wall anchor domain protein	M**KK**QQTLAMVAVTTAVLAGAGTTTFA/DEV
F0VUL4	LysM and putative peptidoglycan-binding domain-containing protein 3	MQ**K**NTL**K**S**K**S**K**TI**K**LGVASAAFAAALIAPAVANA/DSY
F0VSF8	Lysozyme	M**RRR**I**K**PIVVVVFFALCGLLLVIGKAHS/DSL
F0VVM1	*LytR family transcriptional regulator*	*MKLGKKILLMI/AAI*

NCBI reference sequence	Predicted function	Secreted proteins ↓
F0VS37	Maltose/maltodextrin transport system substrate-binding protein	M**KK**NTW**KK**MVLAGAGLTLAGSVLVACSNSSSNSSS/SSS
F0VV55	Mannan endo-1,4-beta-mannosidase	M**KK**ALVGSLATLTVVAGLASAQGVKA/DEI
F0VTL0	*Membrane protein insertase YidC*	*M**KKK**LN**R**VLFSGLSLSLLFLLTGCVSR/DSS*
F0VV56	Multiple sugar-binding protein	M**KK**GLLTIGMTALAAVTLVGCSSG/SSD
F0VW93	N-acetylmuramidase/lysin, putative	M**KK**SMFG**R**EEQ**R**FGI**RK**YSVGVASVLIASVLFMGGQTVA/ADD
F0VSM2	Pectate lyase L	M**K**TT**K**QVLLVLFSAILMVGMTLAGMSSLKVDA/STT
F0VVU0	*Penicillin-binding protein 1A*	*MITIKKKITR**KRK**/KTN*
F0VS51	Peptide/nickel transport system substrate-binding protein	MEF**K**STW**KR**VGLGVVSLASAALLAACGNSSS/SSS
F0VS50	Peptide/nickel transport system substrate-binding protein	MEI**K**NW**KR**VGLGAVTLLSAAVLAACGNSSS/SSS
F0VYC2	Peptidoglycan hydrolase	MA**K**T**RKRK**V**RKK**A**RS**HRR**Q**KK**VP**K**WALYGMSLLAVLACVALVIFTYQAQDA/DST
F0VSN1	Peptidoglycan linked protein	M**KK**VIIS**H**YGLPIVKLMVILVVGILVLGISHTVQA/LTR
F0VTB3	Peptidyl-prolyl cis-trans isomerase A (Cyclophilin A	M**KK**FWSFGLMVLCLASLSGCESITRA/IRG
F0VVI7	Periplasmic oligopeptide-binding protein	MLL**KS**K**T**W**KR**IGLGAVTLVSAAVLAACGGSSS/SSS
F0VT16	Phosphate-binding protein pstS	M**KK**W**K**MLMLLAFVGVGVVLTACSK**S**NS/SES
F0VXW2	Phosphate-binding protein pstS	M**KK**M**KK**LSFLLLITSVSIILSGCASW/IDK
F0VVT2	Polar amino acid ABC uptake transporter substrate binding protein	M**KKRR**LLSFGFLFLLTLVLAACSNQSQSSG/KTV
F0VS32	Polar amino acid transport system substrate-binding protein	M**KK**FF**R**GFILLIIVGSLSACSSSTGVSQS/SIQ
F0VSQ7	Polar amino acid transport system substrate-binding protein	M**KK**VLLGLAVLLSASLSVHVEA/ADK
F0VTP3	Polar amino acid transport system substrate-binding protein	MTLM**KK**ILGVTGVALA/STT
F0VY99	Proline/glycine betaine ABC transport system,periplasmic component	M**K**N**KK**VISGALLVVILVAIVGGIWA/WRN
F0VSA1	Pullulanase PulA and related glycosidases	M**KK**VS**R**LSFLE**KR**QFFGI**RK**L**K**VGVASVAIATALFWSASLANSVSA/DQI
F0VUF5	Putative agglutinin receptor	M**K**NNNVVG**R**GYF**RK**S**K**AYGLVCGIALATAFLGTHVSA/DEV
F0VSX2	Putative aliphatic sulfonates-binding protein	M**K**N**KK**IA**RK**SFIFALLVCWIGVAFYGWKQTQA/DDS
F0VS33	Putative amino acid transporter, amino acid-binding protein	MIM**K**VS**K**LFGGLAVAAASLFLLSACGSSS/SED

NCBI reference sequence	Predicted function	Secreted proteins ↓
F0VXT1	Putative exopolysaccharide biosynthesis protein	M**R**FL**KKH**AYTLLFSTALLSANVYVLLKTFVIPSAVTKVAA/ETS
F0VTV6	Putative glucan-binding protein C	M**K**S**KH**YQALGIGLFSSLVLLTPQVMA/DET
F0VUC6	Putative glucan-binding protein D	M**KK**SIS**K**GIVLSAVSFLGVFAGSQVVSA/DTD
F0VSJ8	Putative glutamine transport system substrate-binding protein	M**KKK**LGLAILASLSLILLTLFAGKTTFA/DSV
F0VTQ2	Putative immunity/modification protein	M**KK**NVL**K**SIILLSATVLSMATVSVFA/DDE
F0VTS1	Putative major pilus subunit	MNNL**KK**ILTPFLTVLALVFVCGAVSA/QTV
F0VTS9	Putative manganese ABC transporter,substrate-binding liprotein and adhesin	M**KK**ITSLICLLLIICILGACA/TTR
F0VSM1	Putative pectate lyase related protein	M**KK**T**KR**VLSLMFSILLLVAMILTGVSLLKA/DTN
F0VT44	Putative secreted protein	MFMN**RK**TQLALATATVSGALLFSQVNADA/DTY
F0VWP9	Putative secreted protein	M**KK**GFYILTIAALSFTLTACS/QNS
F0VSD5	Putative thioesterase	M**KK**I**KK**FTLSFLLIIATLIAISGVVLHQKTYQA/SSE
F0VUD8	*Sortase A*	*MKKKIIMLLMV/MIG*
F0VV08	*Sugar-binding periplasmic protein*	*MTKLTKIKLW/LSV*
F0VU13	UTP--glucose-1-phosphate uridylyltransferase	M**RK**V**RK**AVIPAAGLGTRFLPATKALA/KEM
F0VV82	YvfO protein	M**K**IL**KK**IFLTTVALIGLAALGTTVTKA/DEF
F0VWM4	Zinc-binding protein adcA	M**KKK**FLLLINLVALLFAWQISHIKQVSA/DDK
F0VTP2	Lipoprotein	M**K**L**KK**LFGLASVAFASTVLLAACGSSS/SSS
F0VU52	Lipoprotein	M**KKK**VLSLIVTGFVATILTGCGASQV/ATS
F0VUG8	Putative lipoprotein	M**KKK**LLATLLAVMSVFLLVGCSSK/DDL
F0VWH8	Putative serine rich lipoprotein	M**KK**TVTYLALAATSVLFLTACSNNN/QES

*Signal peptide positive charged residues are indicated in bold letter, the H-domain is indicated in grey shading, the residues -1 to -3 positions relatively to cleavage site are underlined and the black arrow indicated SPase cleavage site. Letter in italics represent membrane proteins, See text for details.

Chapter 4: Biofilm formation in Enterococci

4.1. Introduction

The ability to form biofilms is an important virulence trait that has been reported for enterococci. A biofilm is a sessile community of bacterial cells surrounded by extrapolymeric substance (EPS) which provides a strong framework and protection, and facilitates cell to cell communication. EPS is composed of different materials including proteins, nucleic acids and polysaccharides (Molobela et al., 2010) .

Enterococci are commonly found as intestinal microorganisms which also have been reported to cause infections such as endocarditis(Singh et al., 2010). Adhesion to host extracellular matrix components (ECM) is the first step for pathogens to mediate infection, and important in promoting enterococci to colonize host vascular tissues are interactions between enterococcal surface proteins and host proteins such as Collagen and laminin. In fact, many studies have reported surface proteins with characteristics similar to immunoglobulin-like fold which are named MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules; (Nallapareddy and Murray, 2006). Indeed, many well-characterized surface proteins in Gram-positive bacteria (such as streptococci, staphylococci and enterococci) are MSCRAMMS (Walsh et al., 2008, Sillanpää et al., 2009). These proteins share several characteristics including an N-terminal signal peptide sequence followed by an A-domain which consist of one or multiple subdomains, each of which adopt a IgG-like fold (immunoglobulin G-like). Following the A-domain is a series of repeated sequences that is referred to as the B-domain. The C-terminal has the

LPXTG like motif which required for cell wall anchoring (Hendrickx et al., 2009, Liu et al., 2007).

Genome sequencing of *E. faecalis* V583 and *E. faecalis* TX0016 revealed the presence of 17 and 15 MSCRAMMs, respectively. Three enterococcal MSCRAMMs have been studied in detail. Ace (adhesion of collagen from *E. faecalis*) was the first protein described among Enterococcal for interacting with collagen type I and IV, laminin and dentin. Acm (adhesion of collagen from *E. faecium*) interacts with collagen type I and lesser extent with type IV. Finally, Scm (second adhesion of collagen of *E. faecium*) binds to collagen type V and fibrinogen (Sava et al., 2010).

Furthermore, other virulence factors reported to have important roles in biofilm development and pathogenesis of *E. faecalis,* including cell wall adhesion proteins and secreted proteins (see section 4.2; (Pires-Boucas et al., 2010).

The aim of this chapter is to characterise three *E. faecalis* strains that were isolated from biliary stents. However, there are no genome sequences available for these strains. So, these are mainly preliminary studies aimed at testing a number of factors, including the presence of virulence factors, pathogenicity and biofilm formation.

4.2. Cell surface proteins and other virulence factors in *E. faecalis*

As mentioned before into the introduction of this thesis, that several virulence factors in Enterococci have been described, for instance, aggregation substance (AS), Enterococcal surface protein (Esp), *E. faecalis* endocarditis associated antigen A(EfaA), adhesion of collagen of *E. faecalis* (Ace), gelatinase (GelE) and the toxin Cytolysin (see chapter1 table 1.2).

AS is an *E. faecalis* surface-anchored protein which facilitates transfer of plasmids between cells by allowing the adherence of the donor bacterium cells to recipient cells (Dunny, 1990). In addition, aggregate substances mediate adherence into host tissues and, enhance biofilm formation by cell aggregation (Kreft et al., 1992, Chow et al., 1993).

Enterococcal surface protein (Esp) is a cell wall protein. Studies observed an association of Esp in the initial attachment and biofilm formation of *E. faecalis* on abiotic surfaces (Toledo-Arana et al., 2001).. More details on Esp are in chapter 5 of this thesis.

E. faecalis endocarditis antigen A (EfaA) amino acid sequence analysis showed 55% to 60% similarity to a group of streptococcal proteins, (FimA from *Streptococcus parasanguis*, SsaB from *Streptococcus sanguis*, ScaA from *Streptococcus gordonii*, and *PsaA from Streptococcus pneumonia*), which have been shown to be involved in adhesion in endocarditis (Lowe et al., 1995).

As mentioned early into the introduction of this chapter, that colonization of human tissue is occur via interaction between ECM protein ligands and the pathogen MSCRAMMs cell wall anchored proteins (Hendrickx et al., 2009).

In endocarditis, the disruption of the valvular endothelium leads to exposes of the underlying tissue which by deposition of the host proteins including ECM material such as, collagen and fibrin may become colonized by circulating bacteria (Singh et al., 2010). Previous studies have shown that Ace mediates *E. faecalis* attachment to immobilized collagen. The mechanism by which Ace could bind to collagen was named The Collagen Hug Mechanism (Hendrickx et al., 2009).

E. faecalis Ace shares sequence similarity with *S. aureus* MSCRAMM (Can). Characteristics of these proteins including an N-terminal signal peptide followed by A-domain then B-domain and C-terminal. Based on the studies on *S. aureus* Cna, it was shown that the collagen binding activity is located in the A-domain. However, A-domain consists of two subdomains, N1 and N2, which are predicted to adopt an open configuration to allow the collagen triple helix to "dock". As a result the MSCRAMM subsequently hugs around the collagen to "lock" between the two subdomains. Finally the structure stabilized by insertion of C-terminal "latch" extension of N2 in to the trench cleft of N1 subdomain (See figure 4.1; Hendrickx et al., 2009, Liu et al., 2007).

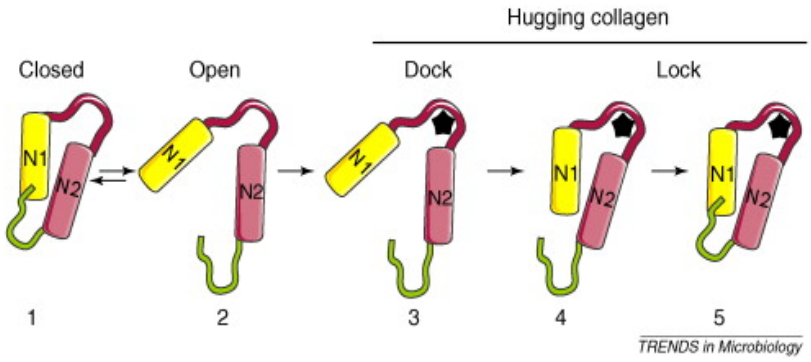

Figure 4.1 The collagen hug model, taken from Hendrickx et al., (2009).

E. faecalis Ace has reported to contribute in pathogenicity in a rat model endocarditis(Singh et al., 2010). Also, the same study confirmed inhibition of *E. faecalis* collagen adherence by using active and passive immunization based on the collagen binding domain of Ace. Thus, this study did not only indicate the importance role of this protein in endocarditis, but also showed promising therapeutic strategies against *E. faecalis* endocarditis.

Other virulence factors including secreted proteins been involved in pathogenicity of *E. faecalis* are gelatinase and serine protease which encoded by *gel*E and *spr*E, respectively, with both genes located on the same operon. Both secreted proteases are regulated by *fsr*, a regulatory two component system (see chapter 1; (Gaspar et al., 2009).

GelE is a secreted zinc-metalloprotease gelatinase. Its gene, g*elE* is an operon with the serine protease *sprE* with the latter located immediately downstream from *gelE* (Gaspar et al., 2009). Gelatinase is able to degrade several substrates such as casein, gelatin, fibrin and other immune peptides (Thurlow et al., 2010). Also, GelE has been shown to have a role in development of biofilms of *E. faecalis* (Hancock and Perego, 2004). However, there are a number of theories of how GelE promotes biofilm formation, one being that GelE increasing cell surface hydrophobicity by cleaving cell surface proteins at hydrophobic residues and therefore, enhanced cell attachment to the surface (Carniol and Gilmore, 2004). Another theory on the role of GelE was recently also proposed, (Thomas et al., 2008). In this, GelE either activates its own autolysin, or activates the autolysin localized on a sibling cell, resulted in

allolysis or fratricide “sibling-killing-sibling”. Both mechanisms result in cell lysis and subsequence release of extracellular DNA (eDNA) which is important in the development of biofilm formation.

Several studies have reported a reduction in biofilm formation of *E. faecalis* mutant lacking *gel*E (Thomas et al., 2008, Mohamed et al., 2004). Also, contribution of GelE in pathogenicity against *C. elegans* model has been demonstrated (Sifri et al., 2002).

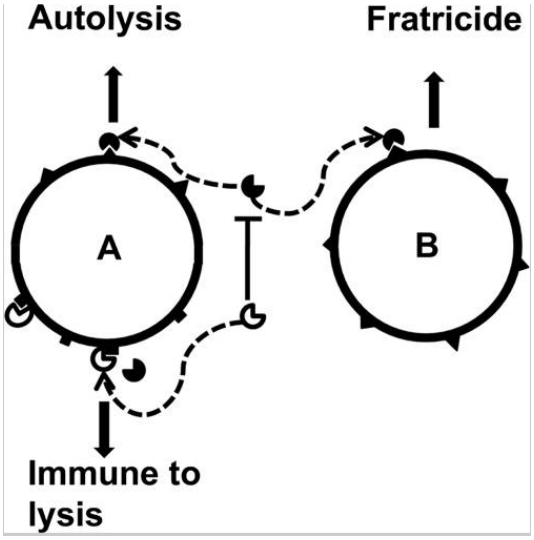

Figure 4.2 *E. faecalis* GelE activation autolysis model (Fratricide model). A, producer cell, B, sibling cell, GelE and SprE both illustrated by the black and the white spots, respectively. Active or inactive form of autolysin showed in ▲ and ■ respectively. Taken from Thomas et al., (2008).

Cytolysin or hemolysin are other secreted proteins which are related to *S. pyogens* streptolysin(Cox et al., 2005). Production of these proteins involves several genes that are encoded either on a plasmid or on the chromosome (Haas et al., 2002).

The Enterococcal hemolysin has been associated with lethality in endocarditis. A study done using a rabbit model with endocarditis was intravenously injected with an *E. faecalis* strain with/without defective in expression cytolysin. Vegetation on the heart valves associated with lethality were observed in 55% of animals injected with wild-type strains, whereas this was only 15% with strain defective in cytolysin expression (Cox et al., 2005).

Also, another study observed that *E. faecalis* cytolysin enhanced *C. elegans* nematode killing (Garsin et al., 2001). Beside that other studies showed the contribution of cytolysin in destroying and damaging of human erythrocytes and intestinal and retinal tissues (Jett et al., 1992, Haas et al., 2002).

4.3. Analysis of virulence factors in *E. faecalis* BS11297, BS12297, and BS385.

Three clinical isolates of *E. faecalis*, all obtained from biliary stents, were kindly provided by Dr Bastiaan Krom (University of Groningen). These strains have not been characterized extensively, and our first goal was to identify the presence or absence of a number of virulence factors that were described above. To determine the presence or absence of these virulence factors, PCR

reactions were performed, using *E. faecalis* V583 as a positive control. The primers used are listed in chapter 2 and the results are shown in Table 4.1.

Table 4.1 Detection of some virulence factors in *E. faecalis* strains by PCR.

Strain	*gelE*	*esp*	*efa*	*ace*	*cylB*
BS11297	+	+	+	-	+
BS12297	-	+	+	+	-
BS385	-	-	+	-	+

Absence (-) or presence (+) of genes encoding virulence factors as determined by PCR. gelatinase GelE, Enterococcal Surface Protein Esp, *E. faecalis* endocarditis Associated Antigen A EfaA, Adhesion of Collagen of *E. faecalis* Ace, and Cytolysin CylB.

Even though all three strains were isolated from biliary stents, they differed considerably in the virulence factors present. Efa was the only factor common to all three, but other factors were only found in two (Esp, CylB) or one (GelE, Ace) of the strains. The presence of GelE was also verified by growing the strains on TSB medium containing 0.5% milk powder. Only *E. faecalis* BS11297 formed a large clearing zone around the colony, indicating production of a secreted protease and confirming that only this isolate produces GelE protein (Figure 4.3).

BS11297 BS12297 BS385

Figure 4.3 *E. faecalis* BS11297, BS12297 and BS385 grown on 0.5% milk agar plates. The size of the clearing zone is proportional to the level of extracellular proteolytic activity.

4.4. Effects of sodium azide

4.4.1. Effects of sodium azide on enterococcal biofilm formation

Previous studies have shown that sodium azide acts as an inhibitor for Sec-dependent translocation by inhibition of the ATPase SecA(Miller et al., 2002). As most surface proteins in enterococci are predicted to be Sec dependent, it was anticipated that sodium azide could be used for preliminary studies into the importance of the Sec system in biofilm formation in *Enterococcus faecalis* strains by testing the effects of sodium azide (Pires-Boucas et al., 2010).

To get a first impression of biofilm formation of the *E. faecalis* strains, biofilms of the three clinical isolates were grown in 96-well plates. As shown in Figure 4.4 there is a great variation in the amount of biofilm formed between the strains, with *E. faecalis* BS12297 being able to form good biofilms, *E. faecalis* BS11297 forming very poor biofilms, and *E. faecalis* BS385 in between the two. This confirms earlier reports by Van Merode *et al,*. (2006) (van Merode et al., 2006b). To analyze the effects on perturbation of the Sec pathway, we first tested effects of different concentrations of sodium azide by analyzing its effects on the growth of one of the three strains. Enterococci are known to be very tolerant to sodium azide, and the minimal inhibitory concentration (MIC) for sodium azide was determined to be 12,800 µg/ml (for all three strains). This is very high compared to other bacteria; for instance, the MIC for *S. aureus* is 186 µg/ml and for *E. coli* is 34 µg/ml (Zhou et al., 2010). Growth curve of one of the strains is shown in Figure 4.4. Growth was slowed with increasing concentrations of azide and a concentration of 200 µg/ml of sodium azide was chosen for further analysis. Surprisingly, only in one of the strains (BS11297) was there a significant effect on biofilm formation (Figure 4.5). In the other two strains small differences were noted, but these were statistically not significant.

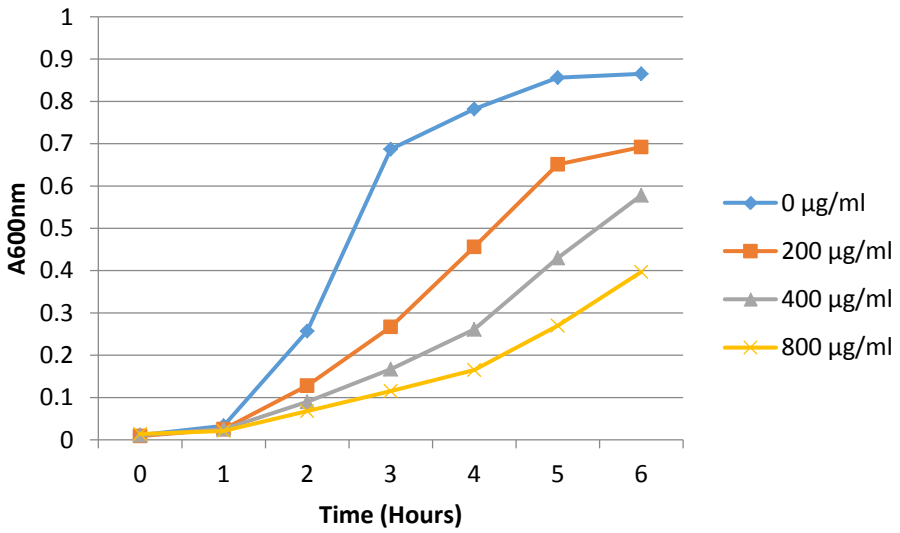

Figure 4.4 Effect of different concentration of azide on *E. faecalis* BS12297.

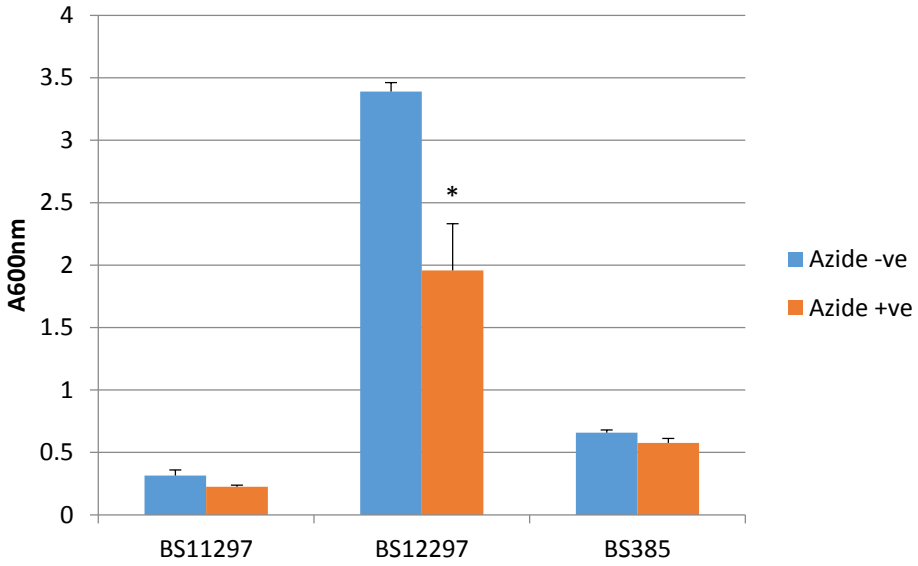

Figure 4.5 Biofilm formations in *E. faecalis* strains. BS12297 only showed a significant reduction with 200 µg/ml azide, the error bars represent the mean ± standard error. (n=4, *p=0.03).

4.4.2. Effects of sodium azide on hydrophobicity of Enterococci

Hydrophobicity of the cell surface is an important determinant of biofilm formation. This is easily measured by determining the distribution of cells between water and hexadecane layers. Figure 4.6 shows that hydrophobicity correlates with biofilm formation, with *E. faecalis* BS12297 being the most hydrophobic, BS11297 the least, and BS385 in between. In the case of BS12297 and BS385 hydrophobicity was affected, which could suggest that sodium azide affects the composition of the cell wall and thereby, at least in part explains the reduction in biofilm formation in these strains. In BS11297, which is a poor biofilm former, azide did not have an effect on hydrophobicity.

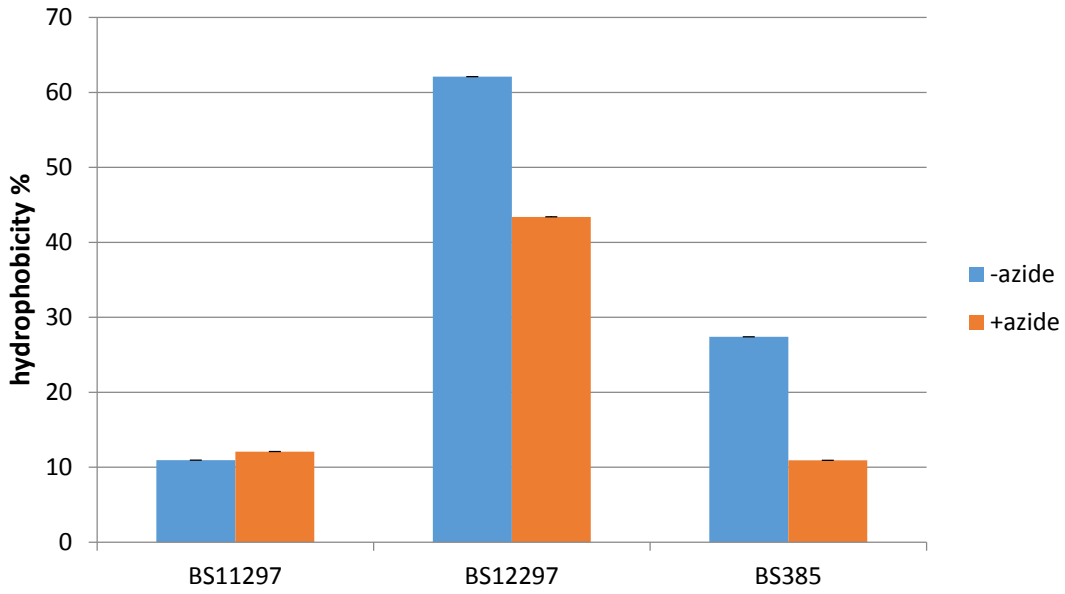

Figure 4.6 % of Cell surface hydrophobicity of *E. faecalis* (BS12297, BS385, and BS11297). Addition of 200 µg/ml sodium azide reduced the hydrophobicity in *E. faecalis* BS12297 and BS385 more than BS11297. The error bars represent the mean ± standard error. (n=6).

To test whether sodium azide indeed affects protein secretion in *E. faecalis*, the effects of this were investigated on the production of extracellular proteases in *E. faecalis* BS11297. Protease activity was determined with the substrate azocasein, in which the hydrolysis of casein leads to release of an azo dye that can be detected at 440 nm. Figure 4.6 shows that BS12297 protease production was not effected in the presence of sodium azide, corroborating the results found on hydrophobicity. BS11297 has significantly increased extracellular protease activity compared to BS12297 (figure 4.3 above and figure 4.7). This is very likely due to the presence of GelE in BS11297, whereas this protease is absent in BS12297 (van Merode et al., 2006b). Surprisingly, GelE was produced at a much higher level in BS11297 in the presence of azide. The reasons for this are unclear, but it could be speculated that azide causes a stress response that result in overexpression of proteases such as GelE. However, earlier findings showed that the presence of GelE stimulates biofilm formation(Thomas et al., 2008). This was not the case here as results showed that sodium azide increased GelE production in BS11297 (in which GelE is the major protease) while biofilm formation was somewhat reduced. The question is thus whether this is a direct effect of the amounts of GelE produced or whether there are other reasons for the effects of sodium azide on biofilm formation.

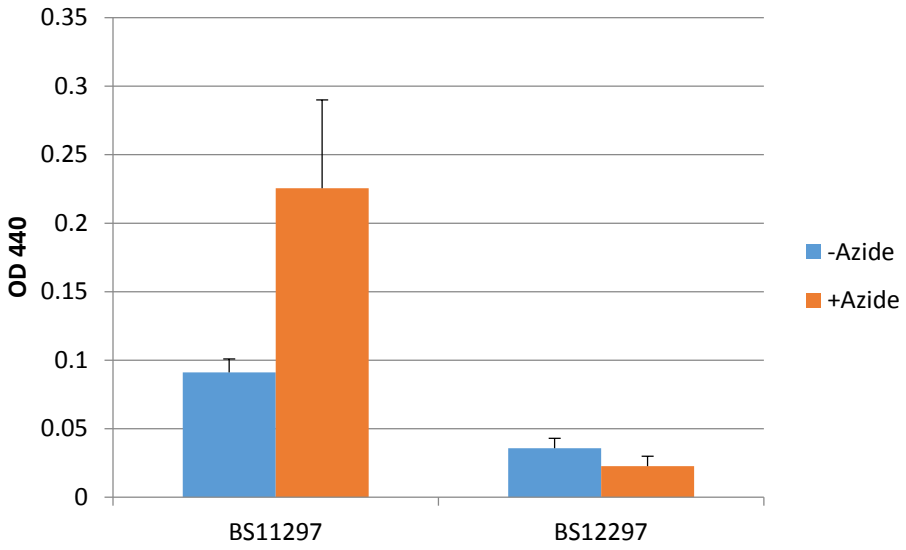

Figure 4.7 Protease activity produced by *E. faecalis* (BS11297 and BS12297). Presence of 200 µg/ml sodium azide decreased the protease activity in BS12297, whereas in BS11297 azide significantly ($p \leq 0.00075$) increased the protease activity. The error bars represent the mean ± standard error (n=6).

4.5. Presence of eDNA in the matrix of *E. faecalis* biofilms

Extracellular DNA may be one of the components of EPS, but its presence needs to be tested as it is not found in all strains. It was thus important to verify this by treating the biofilm of *E. faecalis* strains with DNase as reported previously (Tetz et al., 2009).

100 µl DNaseI was diluted in THB+0.25% glucose medium to a final concentration of 100KU, which was then added to 100 µl of an overnight culture and the Crystal violet assay were carried out as usual. As shown in figure 4.8, *E. faecalis* biofilms of all strains tested decreased when treated with DNaseI, although the effects in BS11297, BS385 and ATCC19433 were statistically not significant. However, in the strongest biofilm former, *E. faecalis* BS12297, the effect of DNase was significant (p=0.012) indicating that at least in this strain DNA forms a part of the EPS.

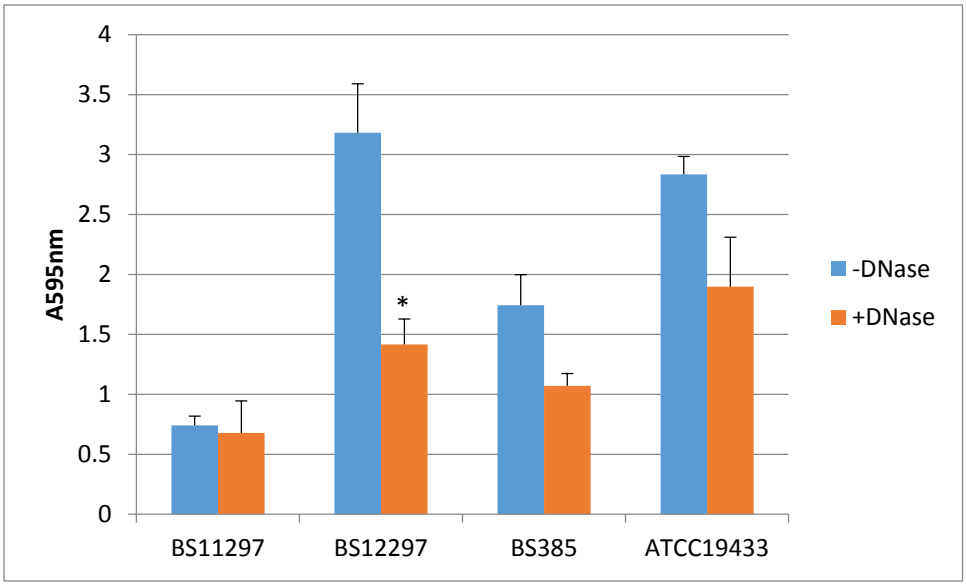

Figure 4.8 Biofilm formation in *E. faecalis* strains treated with DNaseI. BS12297 only showed a significant reduction with 100KU DNaseI, The error bars represent the mean ± standard error. (n=4, *p=0.012).

4.6. Biofilm formation of *E. faecalis* in the presence of collagen

As mentioned earlier in this chapter, many infections biofilms are formed on tissues in the body. A number of cell surface proteins are involved in this process, and one important group of such proteins are the aforementioned MSCRAMMs. Indeed, MSCRAMMs of streptococci and staphylococci have been identified to have a main role in adherence and colonization *in vivo* (Nallapareddy and Murray, 2006). We have shown that collagen-binding proteins are important in biofilm formation in a number of streptococcal strains (Sillanpää et al., 2009, Walsh et al., 2008; see chapter 6 in this thesis). Nothing is known about the capabilities of the *E. faecalis* strains described before with regards to adherence to collagen, and it was therefore of interest to test this.

In this experiment, biofilms were grown in 96-well plates pre-coated with collagen I, collagen IV, or in plates without coating. As shown in Figure 4.9 the presence of collagen did not significantly enhance biofilm in *E. faecalis* BS11297 and BS385. However, *E. faecalis* BS12297 formed more biofilm in the presence of collagen, particularly on collagen IV.

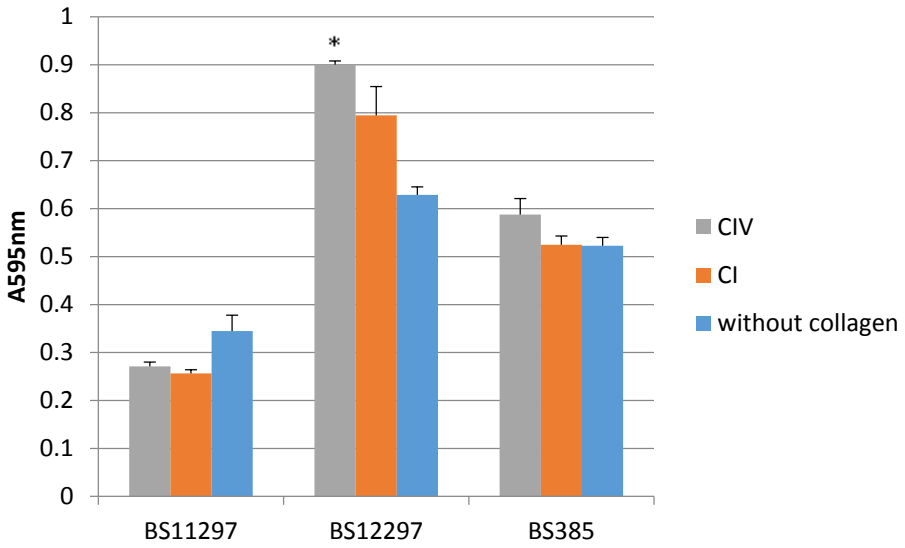

Figure 4.9 Biofilm formed by *E. faecalis* strains on collagen surface. Biofilm formation was tested in wells pre-coated with collagen I, IV and no collagen. BS12297 only showed a significant increase in the presence of collagen IV. CI and CIV= collagen type one and four, respectively. The error bars represent the mean ± standard error (n=12, *p= 0.008).

4.6. Cloning of *gelE*

To analyze the effect of the protease GelE on enterococcal biofilm formation and pathogenicity, *gelE* was cloned in an expression vector and transformed into *E. faecalis* strain lacking GelE. The purpose here was to (a) analyse the effects of overproduction of GelE on biofilm formation, and (b) test whether these effects could also be observed in strains normally not containing GelE. The latter could provide a means to test the need for other partners in the effects of GelE.

A 1573-bp long DNA fragment of *gelE* was amplified from genomic DNA of *E. faecalis* BS11297 using the KAPA2G robust PCR kit. The PCR product was digested with *Xba*I and *Sac*I and cloned in the shuttle vector pAT79, digested

with *Xba*I and *Sac*I, followed by electroporation in to *E. coli* competent cells with selection on LB medium with 60 µg/ml spectinomycin and 15 µg/ml chloramphenicol. Then plasmid DNA was purified using Nucleospin plasmid kit followed by electroporated in to *E. faecalis* BS385. Plasmids containing the *gelE* gene were obtained. The construct as intended is shown in Figure 4.10. However, upon sequencing it was noted that none of the constructs obtained contained a complete *gelE* gene and there were rearrangements within the plasmid, suggesting that cloning of a protease is deleterious to *E. coli* cells. Similar problems with cloning of foreign proteases has been observed before (Waschkowitz et al., 2009), and it was therefore decided not to persue this avenue further.

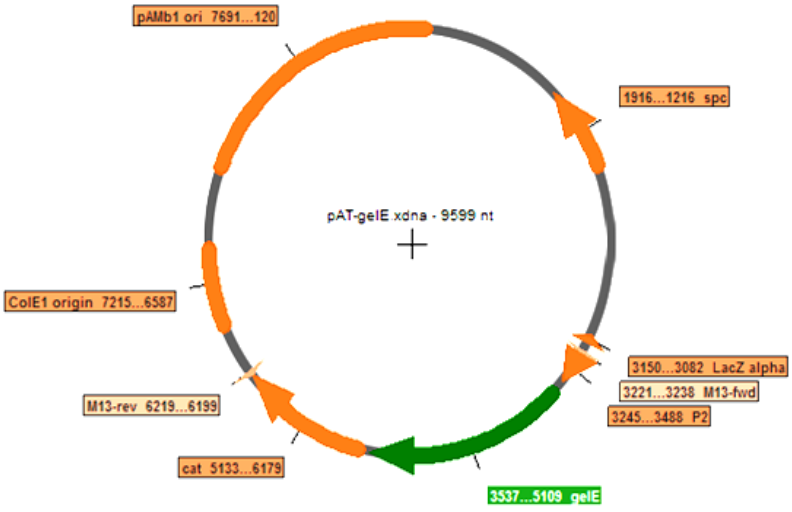

Figure 4.10 Vector pAT-gelE. *gelE* were ligated into the SacI, XbaI site of pAT79. The resulting construct formed pAT-gelE. *Spc* and *cat* encode spectinomycin and chloramphenicol resistance.

4.7. Pathogenicity of enterococci in a nematode infection model (*C. elegans*)

C. elegans is a soil nematode feed on *E. coli* and it has been fully genomic sequenced and studied. Moreover, for its small size, easy cultured and short life span, make *C. elegans* attractive resources for many researchers (Sifri et al., 2005, Mellies et al., 2006). Recent studies suggested that *C. elegans* can be used as *in vivo* infection model to detect pathogenicity for various microorganisms such as *Pseudomonas aeruginosa*, *E. coli* and *Staphylococcus aureus* (Lavigne et al., 2008). Bacterial pathogens kill nematodes either by "slow killing", by colonising and infecting, for instance, the gut of nematodes, or by "fast killing" through the production of toxins (Mellies et al., 2006).

The result in figure 4.11 shows the % of survival *C. elegans* after been infected with *E. faecalis* strains. *E. coli* HB101 used as non-pathogenic control strain. The lifespan of the nematodes was considerable shorter when infected with any of the *E. faecalis* strains, but in particular *E. faecalis* BS11297 and BS385 showed a high nematocidal activity.

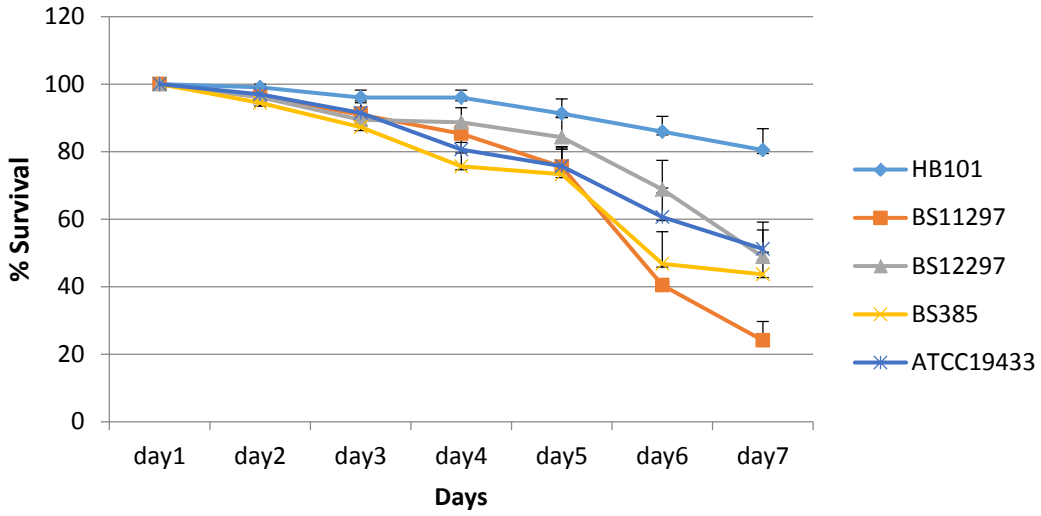

Figure 4.11 Survival of *C. elegans* after infected with *E. faecalis* isolates. *E. coli* HB101 is a non pathogenic control. The error bars represent the mean ± standard error. (n=9)

4.8. Discussion

Biofilm assays were performed on different *E. faecalis* strains, showing variation in biofilm formation between the strains. Several factors are involved in this process. *E. faecalis* BS11297 and BS12297 both contain the enterococcal surface protein Esp, but the latter formed significantly more biofilm in all the tests. A previous study on the cell culture surface charge heterogenicity reported that cultures of *E. faecalis* BS11297 are much more homogenous, i.e. showing little variation between cells, as compared to BS12297. It has been suggested that surface charge heterogeneity stimulates adhesion to the surfaces, explaining why the latter is better at forming biofilms (van Merode et al., 2006a). *E. faecalis* BS385 is also heterogeneous but lacks the surface protein Esp. The latter is important for biofilm formation,

explaining why BS385 was not able to form biofilms as good as BS12297 (van Merode et al., 2006b).

Furthermore, previous studies demonstrated the role of both extracellular proteases (GelE and SprE) in biofilm formation by providing eDNA resulted of activation of autolysin (Thomas et al., 2008). This was not observed here with isolate BS11297; this strain produces GelE, but it nevertheless formed very poor biofilms. Furthermore, DNaseI treatment of this strain showed only a marginal reduction in biofilm formation of *E. faecalis*, indicating that DNA is not a major component of the EPS in this isolate. This indicates that it is not easy to predict how well a strain can form biofilms by simply looking at the presence or absence of factors such as GelE or Esp, and that biofilm formation is a complex process depending on several factors. Thus, to study function of cell surface or extracellular proteins in biofilm formation it is clearly important to only compare strains with the same parental background. This explains other contradictory studies, with some showing that biofilm formation is independent from Esp and others showing that Esp is important (Toledo-Arana et al., 2001). One study showed that *E. faecalis* with mutations in the *fsr*-locus (which is involved in the regulation of e.g. *gelE*) or *gelE* resulted in poor biofilm formation. In the same study *fsr*-only mutants formed wild-type biofilm level by addition of purified GelE, suggesting that GelE alone could enhanced biofilm formation (Hancock and Perego, 2004). Effects of overproduction of GelE on biofilm formation have not been studied. An initial aim of our study was to investigate this, but we were unable to clone an intact *gelE* gene, possibly due to toxic effects of a protease in *E. coli*.

This part of the study was therefore not investigated further. What we did show here that hydrophobicity appears to be a better predictor of biofilm formation, which is important in bacterial adhesion on the substratum; the more hydrophobic the strain, the more biofilm it can form (Bruinsma et al., 2001).

Sodium azide (NaN_3) is a compound which used in laboratory and industrial applications for example it used as a preservative reagent (Marino et al., 2007). It is also a potent inhibitor of SecA, which is the central motor in Sec-dependent protein translocation. We have examined the effect of NaN_3 on *E. faecalis* strains in their biofilm formation, hydrophobicity and protease secretion. Our results suggested that the NaN_3 has reduced both biofilm formation and hydrophobicity in all strains. However, addition of NaN_3 did not have much effect on the protease activity in *E. faecalis* BS12297. In contrast, azide significantly increases protease (probably GelE) production in BS11297. The latter effect could be due to the stress of growing in the presence of azide. Indeed, a recent study reported that environmental stresses is often coupled to expression of virulence genes, which may be a survival strategy under adverse conditions (Lenz et al., 2010). Note however that it is not clear whether the stimulation of expression of *gelE* by azide is strain specific or more widely applicable, as other strains containing GelE have not been tested.

Enterococci have also been reported to have a putative collagen binding proteins, Ace and Acm for *E. faecalis* and *E. faecium*, respectively (Rich et al., 1999). Ace is a homologue of Cna from *S. aureus* (Rich et al., 1999, Garsin et al., 2001), which belongs to the family of MSCRAMMS. We have tested the biofilm formation of *E. faecalis* strains on collagen I and IV, and our results

showed that biofilm formation of neither BS11297 nor BS385 was influenced by the presence of collagen. Interestingly, BS12297 did form better biofilms on collagen (in particular collagen IV), and that was also the only strain, as confirmed by PCR, containing Ace.

Interestingly, our data confirmed *E. faecalis* virulence against *C. elegans* nematodes. The most pathogenic strain was BS11297, followed by BS385 and BS12297. This was in reverse order of the capability of biofilm formation of these strains, indicating that in the conditions tested biofilm formation *per se* is not a factor in the level of pathogencity. However, the three strains differed considerable in virulence factors present and, again, a straightforward comparison is therefore difficult to make. Nevertheless, the most pathogenic strain (BS11297) contained both cytolysin CylB and protease GelE, strain BS385 contained only CylB, and the least pathogenic strain (BS12297) lacked both of these proteins. Thus, it may be that in particular CylB and GelE play an important role in the pathogenicity in nematodes. This would need to be verified in future studies by mutating their genes

Chapter5: Role of Enterococcal Surface Protein Esp in biofilm formation

5.1. Introduction

As mentioned previously into the introduction of this thesis that sec-dependant pathway consist of many components react together to transport different proteins which contain a signal peptide across the cell membrane (Mori and Ito, 2001).

Many of virulence factors mentioned in this thesis are transported through sec machinery system for example, Esp, collagen binding proteins and AS. These proteins characterized by C-terminus LPxTG motif (x indicates for any amino acid) and therefore, attached to the cell wall by sortase (Hendrickx et al., 2009). This enzyme cleaves between the threonine and glysine residues in this motif to allow the proteins covalently immobilized the cell wall peptidoglycan (Hendrickx et al., 2009).

Enterococcal surface proteins (ESP) are large cell-wall protein with molecular mass approximately 202KDa, found both in *E. faecalis* and *E. faecium* . The proteins in both strains showed similar sequence identity of around 90% (Leavis et al., 2004).

The structure of Esp (figure 5.1) reveals some key features including a signal peptide, N-terminal domain, A,B and C repeats domains which contains cell wall anchoring motif and here with Enterococci the motif has[Y/F]PKTG sequence which the Leucine in position 1 has been replaced with either Tyrosine or Phenylalanine (Hendrickx et al., 2009). In spite the variation in the motif residue, Esp has been detected on the cell wall surface by experiments (Shankar et al., 1999, Heikens et al., 2007).

Figure 5.1 *E. faecium* E1162 Esp structure. The signal peptide motif YSIRK represent in purple, then N-terminal domain, A, B&C repeats represent in blue, red and green, respectively. FPxTG is cell wall anchoring motif.

It has been reported that N-terminal a lone is sufficient to mediate biofilm formation in *E. faecalis* (Tendolkar et al., 2005).

Esp shows similarity to other biofilm associated proteins in other organisms, which include BapA from *Salmonella enteritidis*, Lap from *Pseudomonas fluorescens* and *S. aureus* from Bap (Latasa et al., 2006, Lasa and Penades, 2006). For instance, the N-terminal domain of *E. faecalis* Esp has 33% identity with *S. aureus* Bap (Biofilm Associated Protein), and also the C-repeat region of these proteins showed similar levels of identity(Toledo-Arana et al., 2001). This C-repeats region has been shown similarity to repeats in the Rib and C alpha proteins in group B streptococcus (GBS) (Shankar et al., 1999). However, these proteins have different functions than the aforementioned proteins; both Rib and C alpha are surface expressed antigens, which have been shown to be involved in resistance against antibody-mediated immunity (Wästfelt et al., 1996, Madoff et al., 1996).

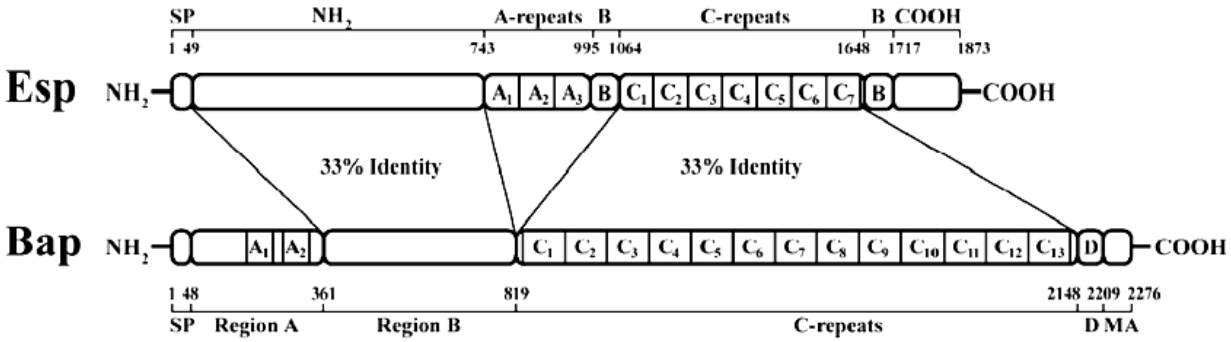

Figure 5.2 Structural similarities between Bap and Esp. Signal peptide (SP), membrane anchor (MA). Taken from Toledo-Arana et al., (2001).

Studies on Esp have shown variation in the number of A and C repeats between isolates as a result of homologous recombination. Strikingly, although (as mentioned above) the repeat domains are dispensable for biofilm formation, none of Esp positive isolates showed a complete A or/and C repeats loss, suggesting that they may have a role in maintaining Esp stability(Shankar et al., 1999). Also, variation in the repeats region showed no effect on isolates to form biofilm (Toledo-Arana et al., 2001). Furthermore, it has been observed in C alpha proteins that the shuffling of repeats is a technique for possible immune evasion (Madoff et al., 1996, Madoff et al., 1991).

Several studies have, as outlined above, shown the involvement of Esp in biofilm formation. However, all of these are based upon genetic studies, but the true biological function of Esp is not known. The main aim of the work in

this chapter was therefore to initiate biochemical studies on the Esp protein. As shown here we developed a biochemical assay to test the activity of the N-domain of Esp that may be used to further elucidate the function of Esp.

5.2. Esp expression analysis using SDS-PAGE

Firstly a fragment of the *esp* gene encoding the N-domain was cloned in the *E. coli* expression vector pET28a vector. The N-domain was expressed without its signal peptide (denoted EspN), but with a C-terminal 6His-tag to enable purification. The protein was expressed in *E. coli* BL21 (DE3) on incubation with 1 mM IPTG at 37˚C (see materials and methods chapter).

Initial trials to purify the protein on metal chelating columns were not successful as the protein failed to bind to columns loaded with either Ni^{2+} or Co^{2+}, indicating that either the His-tag is hidden within the protein, or perhaps cleaved off. Therefore an alternative purification strategy was developed (see materials and methods chapter). Firstly, ammonium sulphate was added to cell lysates to 25%, a concentration at which Esp does not precipitate. Then, lysate was loaded on a phenyl-sepharose column (bed volume ~ 5 mL), and proteins were eluted step-wise by decreasing ammonium sulphate concentration and the resulting fractions was resolved on SDS PAGE and visualized using coomassie stain. As can be observed in Figure 5.3, the Esp was not completely pure as some background bands were still visible. Also, it was decided to test the effect of limited protease Esp with trypsin 10 µg/ml for 1hour at 37˚C (Figure 5.4).

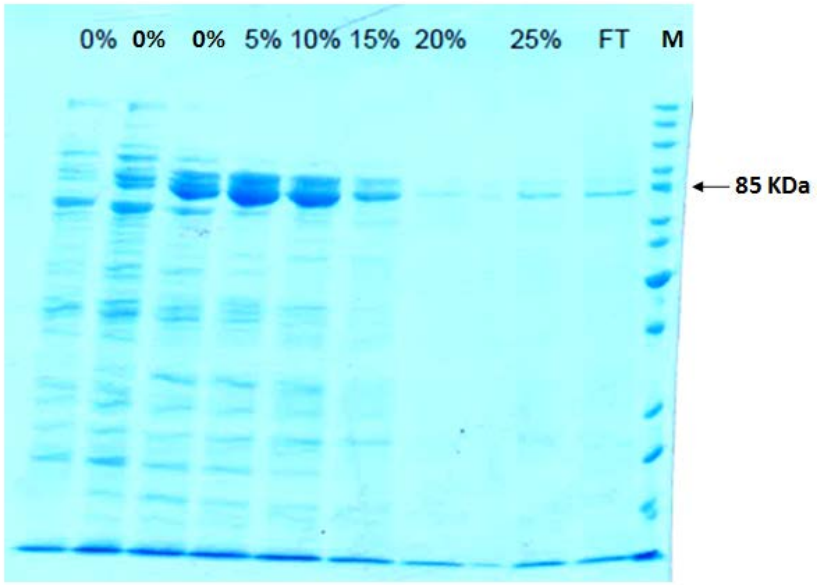

Figure 5.3 Purified fraction of Esp on SDS PAGE gel. .M, proteins marker, FT, flow through and the percentage represent the ammonium sulphate concentration.

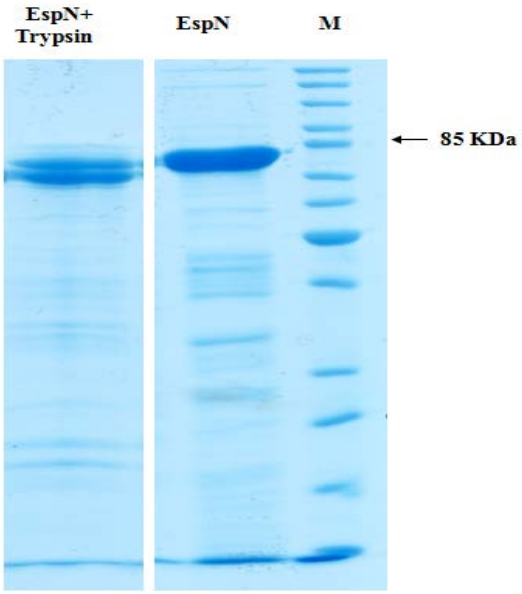

Figure 5.4 Purified EspN and EspN limited digested with trypsin. M, protein marker.

5.3. Effect of purified EspN on biofilm formation in *E. faecium*

To test the effect of addition of purified EspN on biofilm formation, it was decided to test that on *E. faecium* E1162, E1162Δ*esp* and *E. faecium* TX1330, the latter two of which lacking *esp*. Firstly, purified EspN was dialysed against buffer (containing 50 mM Na-phosphate and 100 mM NaCl) to remove ammonium sulphate. Then, a biofilm assay was carried out using the standard 96-well microtitre plate biofilm assay, but here 100 µl of a diluted overnight culture was added to 100 µl of purified EspN (200 µg/ml), after which cultures were incubated for 24 hours, followed by washing and crystal violet staining as usual. As a negative control, the Esp protein was treated with trypsin (10 µg/ml for 1hour at 37˚C), followed by inactivation of the trypsin with trypsin inhibitor.

Results in figure 5.5(a) shows that addition of EspN has significantly increased biofilm formation in both *E. faecium* E1162Δ*esp* and TX1330, both of which lack *esp*. Also, E1162 showed an increased in biofilm formation, but the difference observed was statistically not significant. In *E. faecium* E1162Δ*esp*, addition of trypsin-digested EspN did not alter biofilm formation significantly, indicating that the protein was inactivated by trypsin. In the wild-type E1162, the biofilm actually even reduced in the presence of trypsin-digested EspN, either suggesting that trypsin-treated EspN interfered with biofilm formation, or that the trypsin was not fully deactivated and that trypsin itself interfered with biofilm formation. Confusingly, in the case of TX1330 biofilm formation was still increased compared to the control (albeit not as high as with full

length Esp), indicating that the trypsin-treated EspN has in fact still some activity. Strikingly, when analyzing the trypsin-treated EspN by SDS-PAGE it was observed that about half of the amount of protein still appeared largely intact, while the other half was only of a slightly lower molecular weight (Figure 5.4). Also, it has been decided to look at the effect of pre-heated EspN on E1162Δ*esp* biofilm formation and results in figure 5.5(b) shows that pre-heated EspN for 10 minutes at 80˚C or 90˚C also were significantly reduced compared to untreated EspN, albeit that biofilm formation was slightly better than after trypsin treatment, indicating that heating does not denature all of the protein. The difference was however statistically not significant.

To analyse in more detail the nature of the trypsin-digested EspN, the samples were analysed by Fourier-Transform Icon Cyclotron Resonance (FTICR) spectrometry (the mass spec facility at the University of Edinburgh) to determine the exact size of EspN and trypsin-treated EspN. The size of EspN was determined to be 82510 Da. This was in fact larger than anticipated, and the actual size of EspN (including the 6His tag) is 80210 Da. The size determined was thus 2.3 KDa larger than expected. Unfortunately, the size of the trypsin-treated sample could not be determined. It should be noted that the size of the proteins is fairly large and it is on the border of what FTICR mass spec can determine accurately (Dr Logan Mackay, personal communication).

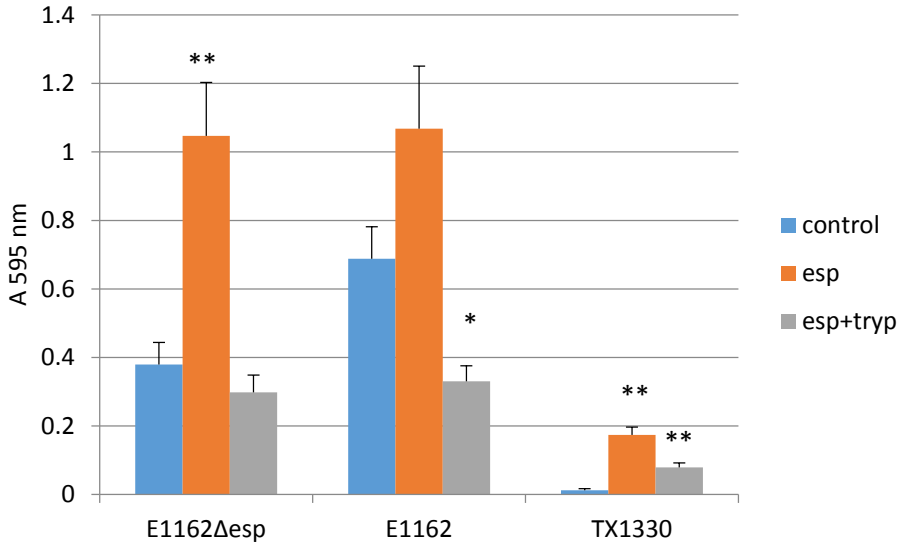

Figure 5.5a Effect of purified EspN and pre-digested EspN with trypsin on *E. faecium* strains. *E. faecium* strains, E1162 (+*esp*), E1162Δ*esp* and TX1330 both lacking *esp.* the error bars represent the mean ± standard error, (n=6, *p<0.05p<0.03).**

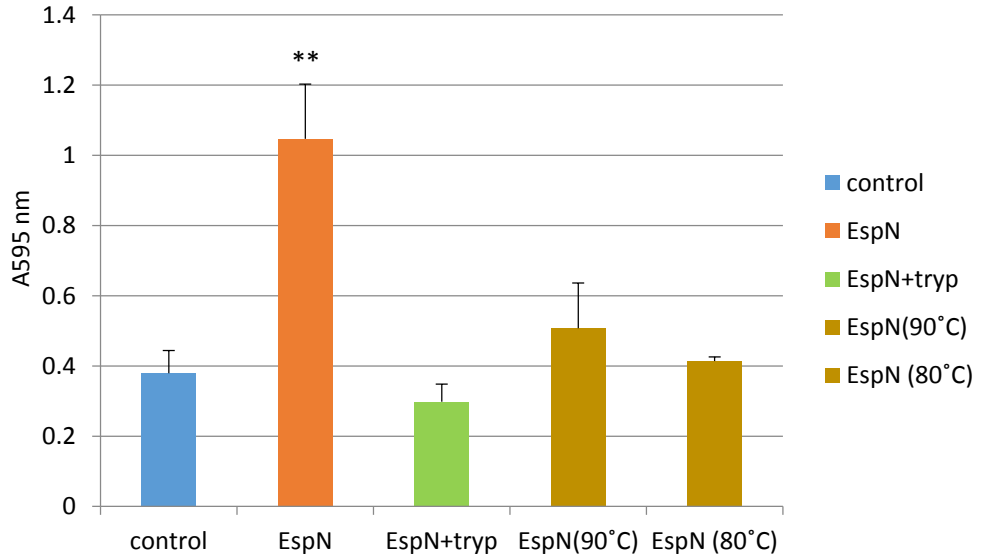

Figure 5.5b Effect of pre-heated purified EspN on *E. faecium* E1162Δ*esp.* the error bars represent the mean ± standard error, (n=6).

5.4. Effect of purified EspN on hydrophobicity of *E. faecium*

As showed previously in chapter 4 with *E. faecalis* that hydrophobicity is an important factor biofilm formation, and strains lacking Esp are significantly less hydrophobic. It was therefore anticipated that addition of EspN would increase hydrophobicity of the strains, and this was measured. Surprisingly, as shown in Figure 5.6, addition of Esp did not alter hydrophobicity significantly and, if anything, appeared to slightly decrease hydrophobicity.

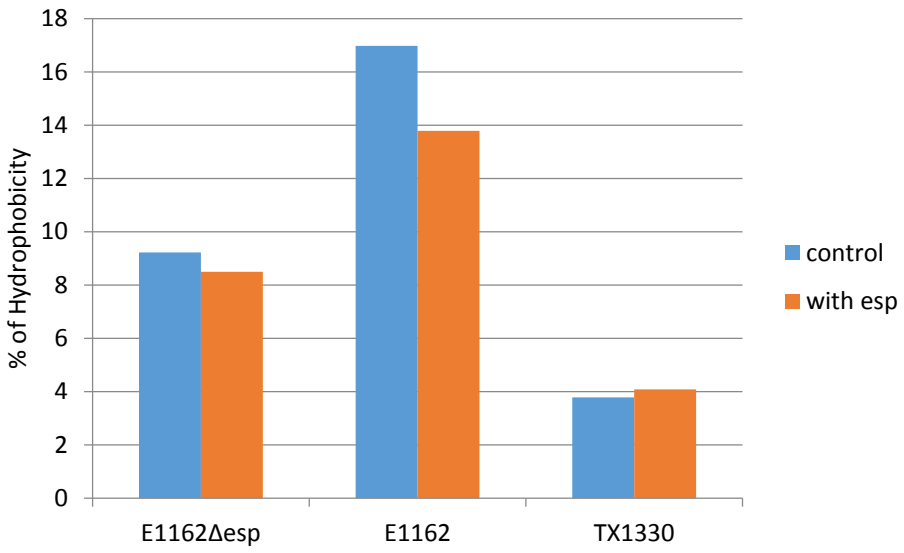

Figure 5.6 Effect of purified Esp on *E. faecium* strains Hydrophobicity.

5.5. Crystallisation of EspN

Initial attempts were made to crystallise the purified EspN protein. Four different screens (from Molecular Dimensions) were tested (at 50 nL scale) with the help of Dr Susan Crennell (Dept of Biology and Biochemistry, University of Bath). This involved 96 different buffers in each, with 3 different ratios of buffer:protein (1:2, 1:1 and 2:1), thus a total of 288 conditions per plate. Unfortunately, only in one condition (in the Morpheus screen), small crystals were obtained, and that appeared not to be reproducible when scaled up to a larger volume. Due to time constraints this was not pursued any further, but future studies on this should focus on, firstly, a higher level of purity of the protein and, secondly, a higher concentration of protein. In the condition tested the concentration of EspN was around 5 mg/mL, but a concentration of 10 mg/mL or higher might be more successful.

5.5. Discussion

Previous studies have shown biofilm formation for *E. faecalis* to occur both independently and dependent of the Esp presence, suggesting that there are other factors determined biofilm formation (Tendolkar et al., 2004, Toledo-Arana et al., 2001, Kristich et al., 2004). However, when cells have the same genetic background, removal of Esp clearly affect biofilm formation and the role of Esp in biofilm formation is therefore well-established. Esp also, showed an important role in biofilm formation in *E. faecium* (Heikens et al., 2007). Furthermore, it has been shown that Esp is important for the initial adherence,

colonization and persistence to gut cells (Heikens et al., 2007, Lund and Edlund, 2003).

All of the studies published so far were based on analyzing the phenotype of strains lacking Esp of parts therefore. The true function of Esp is not clear and a more biochemical approach is therefore important to study this protein in more detail. Here we showed for the first time that purified EspN is sufficient to stimulate biofilm formation in cells lacking Esp. Thus, even though Esp is unlikely to be an enzyme per se, we have now an assay to measure its activity. The purified protein used lacks the C-terminal repeats and cell-wall anchoring domain, further corroborating the genetic studies showing that the C-terminal repeat domains are not required for its function (Tendolkar et al., 2005). In the latter study the N domain of Esp still contained a membrane anchor.

The membrane anchor is lacking in purified EspN. However, the concentration used may be significantly higher than achievable *in vivo*, which could explain that we still observe stimulation of biofilm formation. It is also possible that EspN interacts with an as yet unknown partner in the cell wall to enable its function. That would confirm earlier suggestions that Esp indeed interacts with another component in enterococci, as Esp from *E. faecalis* is on its own is unable to stimulate biofilm formation in *Lactococcus lactis*, a bacterium that does not contain Esp-like proteins (Tendolkar et al., 2005).

We did not observe that adding EspN to *E. faecium* cells increased hydrophobicity. That was anticipated, as a strain lacking Esp is significantly less hydrophobic. However, the hydrophobicity measurements are performed in a buffer containing urea and it is conceivable that an interaction of EspN

with the components in the cell wall is not very strong and that this interaction is disrupted in the presence of urea. However, we have not yet tested other buffers or methods for measuring hydrophobicity.

In conclusion, this study demonstrated for the first time a biochemical test in which purified EspN was added to *E. faecium* strains lacking *esp* and resulting an increase in biofilm formation. This will enable future research to analyze the biological function of Esp in more detail.

Chapter 6: Biofilm formation in *Streptococcus bovis*

6.1. Introduction

Streptococci are a genus of Gram positive cocci that normally occur as natural flora on the skin, in the upper respiratory tract, or in the gastrointestinal tract of both humans and animals (Herrera et al., 2009). One of these is *Streptococcus bovis*, which_is an intestinal facultative anaerobic bacterium. This organism also has been reported as an opportunistic pathogen causing multiple diseases such as meningitis, septicemia and endocarditis (Poyart et al., 2002). Recently, *S. bovis* has been classified into three biotypes depending on their ability (biotype I) or inability (biotype II) to ferment mannitol. Also, *S.bovis* biotype II has been divided in to biotype II/1 (β-glucuronidase positive) and biotype II/2 (β-glucuronidase negative; Rusniok et al., 2010, Boleij et al., 2011a) as shown in table 6.1.

Several studies have shown that endocarditis cases are often linked with colorectal cancer (CRC) as first reported by (McCoy and Mason, 1951). Since then, several studies have shown full-bowel examination of colon cancer in 90% cases of patients with *S. bovis* infections (Vaska and Faoagali, 2009). As mentioned before in chapter1, that after Schlegel et al. suggested the new nomenclature of *S.bovis* strains (Schlegel et al., 2003) (see table 6.1), further studies showed that it is mainly *S.gallolyticus* subsp. *gallolyticus* being the major cause of endocarditis associated with CRC (Vaska and Faoagali, 2009, Corredoira et al., 2008).

Although the association of *S. gallolyticus* infection with CRC is a major issue, the mechanisms behind this link are still unclear (Rusniok et al., 2010). In a

healthy colonic environment the host has defence mechanisms against bacterial infection through secretion of mucus by Goblet cells to protect the epithelial cells and facilitate transit of bowel contents. Also, antimicrobial peptides, immunoglobulin A and cytokines secreted by enterocytes, act as protective agents against pathogens. However, CRC may lead to changes of physical barriers, including increased tight junction permeability and alteration in the mucus production and composition. This in turn could make the colon a more favourable environment for opportunistic pathogenic bacteria, leading to, for instance, increased translocation of the bacteria in to blood stream (Boleij et al., 2011a).

This link between CRC and the virulence of *S. bovis* strains was studied in more detail in a collaborative project (Boleij et al., 2011a) with Dr Harold Tjalsma (Radboud University Nijmegen Medical Centre, Nijmegen , The Netherlands). The Tjalsma group mainly focussed on host-pathogen interactions, whereas we analysed biofilm formation of *S. bovis* strains as well as their pathogenicity using the *in vivo C. elegans* infection model.

Table 6.1 Nomenclature of *Streptococcus bovis* Strains.

New Name	Old Name	Strains Used in Current Study
S. gallolyticus* subsp *gallolyticus	*S. bovis* biotype I	SB1293,SB 1294, NTB1, UCN34
S. infantarius* subsp *infantarius	*S. bovis* biotype II.1	NCTC8133
S. infantarius* subsp *coli	*S. bovis* biotype II.1	None
S. gallolyticus* subsp *pasteurianus	*S. bovis* biotype II.2	None
S. gallolyticus* subsp *macedonicus	*S. macedonicus*	CIP105685T (SM)

The Tjalsma group analysed several factors such as adhesion, invasion and translocation using differentiated caco2 cells (colorectal cancer cells). Furthermore, cellular immune responses to bacterial infection and bacterial surface structure images were analyzed. Strains used in this study were *S. bovis* strains (listed in chapter 2). Pathogenic control strains were *Salmonella typhimurium* (*ST*) and *E. faecalis* ATCC19433 (*EF*), whereas *Lactobacillus plantarum* (*LP*) and *E. coli* NTB5 (*EC*) were non pathogenic controls.

Figure 6.1 (Boleij et al., 2011) shows the adherence (panel A) and invasion (panel B) levels of *S. bovis* strains to caco2 cells after 2 hours of bacterial exposure. Adherence to the colonocyte cells experiment showed that *E. faecalis* has the highest adherence level about 80%. Non pathogenic strains *EC* and *LP* adhered moderately well (about 20-50%), whereas adhesion of *S. bovis* strains to the epithelial cells varied between strains. However, *S. gallolyticus* (*SG*) strains showed a low adhesion level compared to those of the other *S.*

bovis strains as well as the commensal *LP*. Notably, adherence of *SG* strains is similar to that of the pathogen *ST*, and it was suggested (Boleij et al, 2011) that this is a reflection of the inability of *SG* to efficiently colonise the human gut.

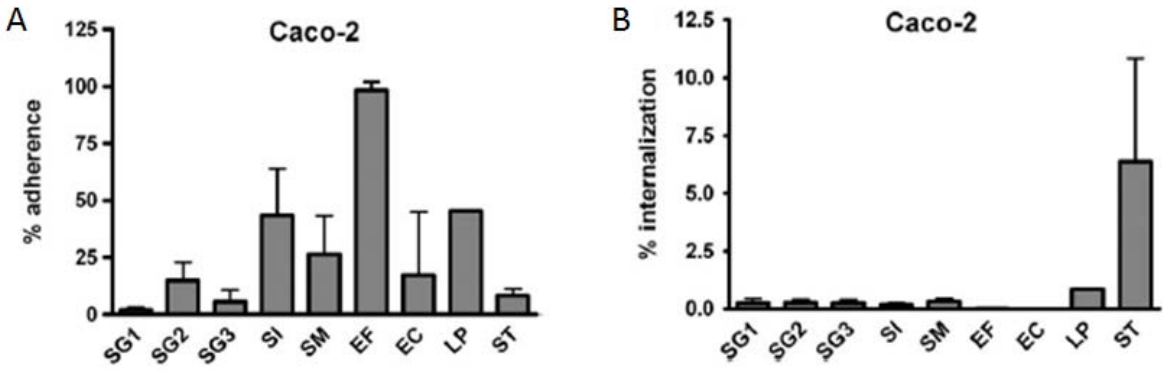

Figure 6.1 Bacterial adhesion (*A*) and invasion (*B*) of epithelial cells, see text for details, Taken from Boleij et al., (2011a). EC, *Escherichia coli*; EF, *Enterococcus faecalis*; LP, *Lactobacillus plantarum*; SG1, *Streptococcus gallolyticus* subsp *gallolyticus* UCN34; SG2, S. *gallolyticus* subsp *gallolyticus* 1293; SG3, S. *gallolyticus* subsp *gallolyticus* NTB1; SI, *Streptococcus infantarius* subsp *infantarius*; SM, *S. gallolyticus* subsp *macedonicus*; ST, *Salmonella typhimurium*.

After binding to colonic tissues, opportunistic pathogens use different mechanisms to cross the epithelial barrier and reach the blood stream. For example, *Salmonellae* can translocate through intestinal epithelial via a transcellular mechanism (Ibarra and Steele-Mortimer, 2009), whereas, group B streptococci use a paracellular translocation mechanism (Pezzicoli et al., 2008). A transcellular mechanism would involve invasion. As shown in Figure

6.1B, the *ST* strain was indeed capable of invasion, whereas none of the streptococcal strains or other controls were invasive. To analyze translocation capacity of *S. bovis* strains, the TJalsma group used Caco-2 trans-well cultures. Bacteria were added to the apical compartment of the cell culture and after incubation the numbers of viable bacteria in both the apical and basolateral compartments were counted by determining the colony forming units (CFU). It could thus be demonstrated that the *SG* strains (and in particular *SG*1), as well as the control EF strain were capable to translocation with up to around 19% of cells translocating (Figure 6.2). Notably, this was still considerably lower than for ST, of which 81% of cells translocated across a Caco-2 monolayer. Confocal microscopy experiments (Boleij et al, 2011; appendix A) further confirmed these results and showed that there was no passive leakage of cells across the monolayer, and it could thus be concluded that *S. gallolyticus* subsp *gallolyticus,* and to a lesser extend *S. infantarius* subsp *infantarius* utilize a paracellular mechanism to translocate across a polarized monolayer.

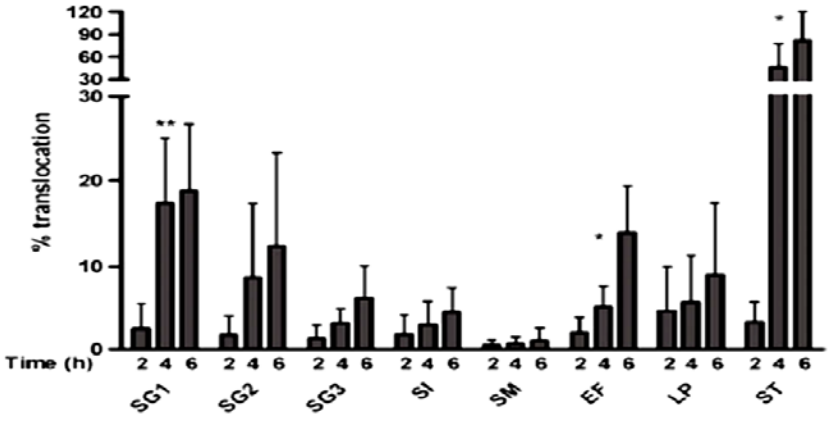

Figure 6.2 Translocation of indicated bacteria across epithelial monolayer was measured after 2, 4 and 6 hours.

Further experiments, figure 6.9, also showed that the *SG* strains were relatively invisible to the immune system as they did not elicit a significant epithelial innate immune response (measured through expression of IL-8 and IL-1b), and that many cells survived translocation (Boleij et al, 2011 and Appendix A). This thus enables the *SG* strains to enter the blood stream and cause, for instance, endocarditis in susceptible patients. In endocarditis, bacteria bind to extracellular matrix proteins and form a biofilm to enhance the surviving of vegetations on damaged valves (Vollmer et al., 2010).

Interestingly, the surface structure of *SM* and *SG* clearly differ as observed by electron microscopy (Boleij et al 2011 and Appendix A). This was further corroborated by the complete genome sequence of *S. gallolyticus* UCN34,*SG*3, which was recently been published (Rusniok et al., 2010); the sequence identified several genes in an *S. gallolyticus* operon that encodes proteins with a high similarity in sequence and organization with genes encoding *S. pneumonia* serotype 23F surface capsule. Moreover, the *S. gallolyticus* UCN34 genome also encodes other surface proteins that are homologous to staphylococcal collagen binding proteins. These four proteins are Gallo_0577, Gallo_1570, Gallo_2032 and Gallo_2179. However, only Gallo_2179 contains the collagen binding motif (Rusniok et al., 2010).

Our aim in this part of the project was firstly to test the capability of biofilm formation of the streptococcal strains, both on surfaces with and without collagen. Furthermore, the aim was to characterise the collagen-binding proteins and Gallo_2179 in particular. A final goal was to test the

pathogenicity of the streptococcal strains using a simple invertebrate model system that makes use of the nematode *Caenorhabditis elegans*.

6.2. Biofilm formation in *S.bovis*

6.2.1 Crystal violet and collagen binding assays

Some of the *S. bovis* strains can cause endocarditis, which is a biofilm-mediated infection of heart valves in which bacteria possibly bind to collagen. Therefore, biofilm formation assays were carried out in 96-well plates coated with collagen I or collagen IV, or without coating. As shown in Figure 6.3, initial experiments showed that these strains were not efficient in forming biofilms on uncoated plates when compared to an *E. faecalis* strain (see also chapter 4). Interestingly, several *S. bovis* isolates formed much better biofilms in the presence of collagen, and in most cases adherence to collagen I was better than to collagen IV. Exceptions were *SM*, which formed more biofilm on a collagen IV coated surface and *SI* which did not show any difference in biofilm formation on the different plates. The non-pathogenic control strain *LP*, a probiotic strain hardly formed any biofilm, irrespective of the presence or absence of collagen.

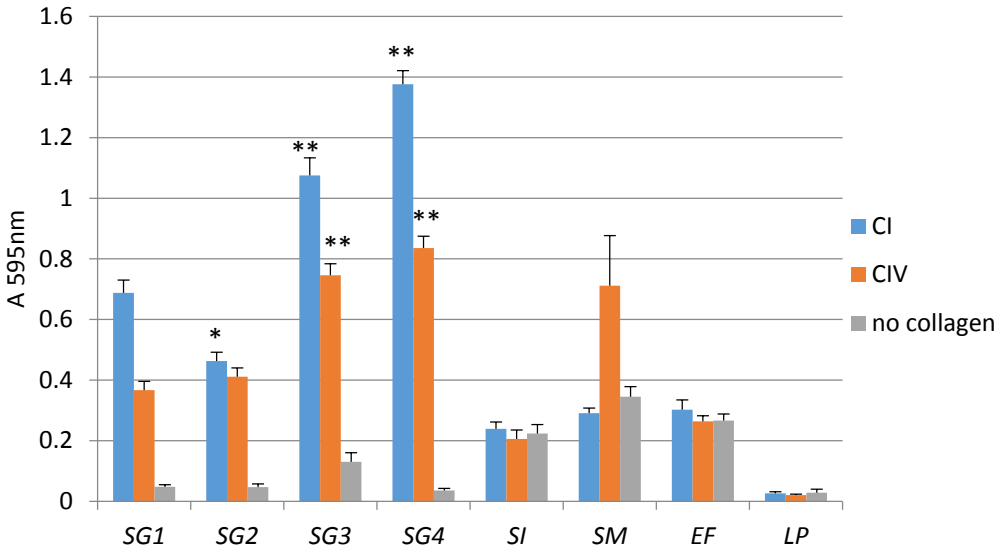

Figure 6.3 biofilms formed by *S. bovis* strains. SG1, SGUCN34; SG2, SB 1293; SG3 SG NTB1; SG4, SB 1294; SI, NTCT8133; SM, *S. macedonicus*; EF, *E. faecalis* ATCC 19433 and LP, *L. plantarum*. Biofilm formation was tested in wells coated with collagen I, IV and no collagen. The error bars represent the mean ± standard error. (n=12, *p≤0.05, **p≤0.01).

6.2.2 Effect of DNaseI on *S. bovis* biofilm formation

One component frequently found in biofilms is eDNA (see also chapter 4). To get an impression whether eDNA forms a part of the EPS in *S. bovis* biofilms, we analysed the effects of DNase on four of the *S. bovis* strains that form good biofilms on collagen I. 100 µl DNaseI was diluted in THB-G medium to a final concentration of 100 KU, which was then added to 100 µl of an overnight culture and the Crystal violet assay were carried out as usual. As shown in Figure 6.4, addition of DNaseI significantly decreased biofilm formation in all strains. In *SG*4 and *SG*2 biofilm formation were reduced by around 40%, while this was near 50% in the other strains tested.

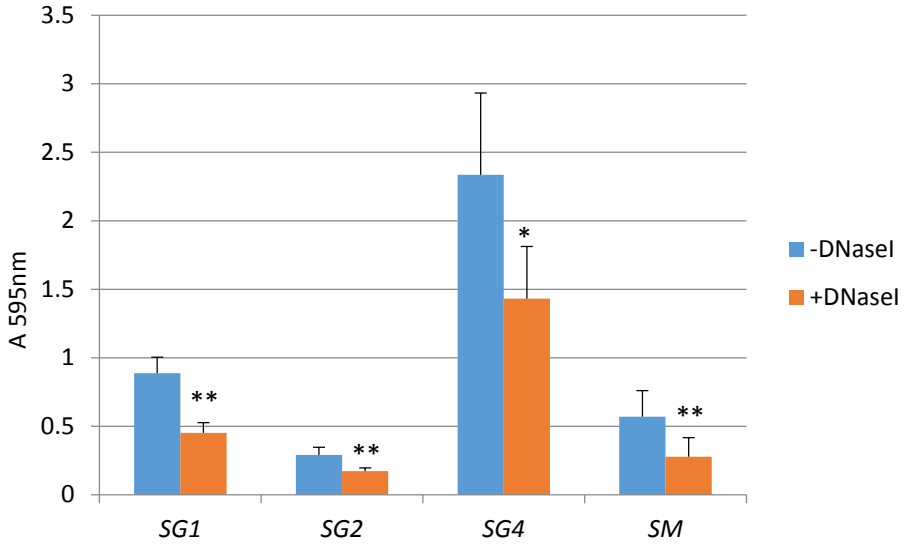

Figure 6.4 Biofilm formations in *S. bovis* strains. Strains were treated with 100 KU DNaseI. The error bars represent the mean ± standard error. (n=4, *p≤0.03, **p≤0.01).

6.3. Confocal Laser Scanning Microscopy (CLSM) images of *S. bovis* biofilms

To visualize *S. bovis* strains biofilm formed on collagen-coated surfaces, biofilms were grown on "home-made" collagen I pre-coated polyvinyl coverslips for 24-hours (see materials and methods chapter). Next, cells that adhered to the slides were stained with the dye Syto-9 and analysed with confocal laser scanning microscopy.

Here we decided to visualize only *SG*4, which formed the best biofilms on collagen I. As a control we also analysed *E. faecalis* BS385, which had shown no difference in biofilm forming in the presence or absence of collagen (see chapter 4).

As clearly shown in figure 6.7, in the presence of collagen I *SG*4 formed efficient biofilm, whereas without collagen only microcolonies were observed on the polyvinyl surface. In contrast, *E. faecalis* BS385 form biofilm in both cases.

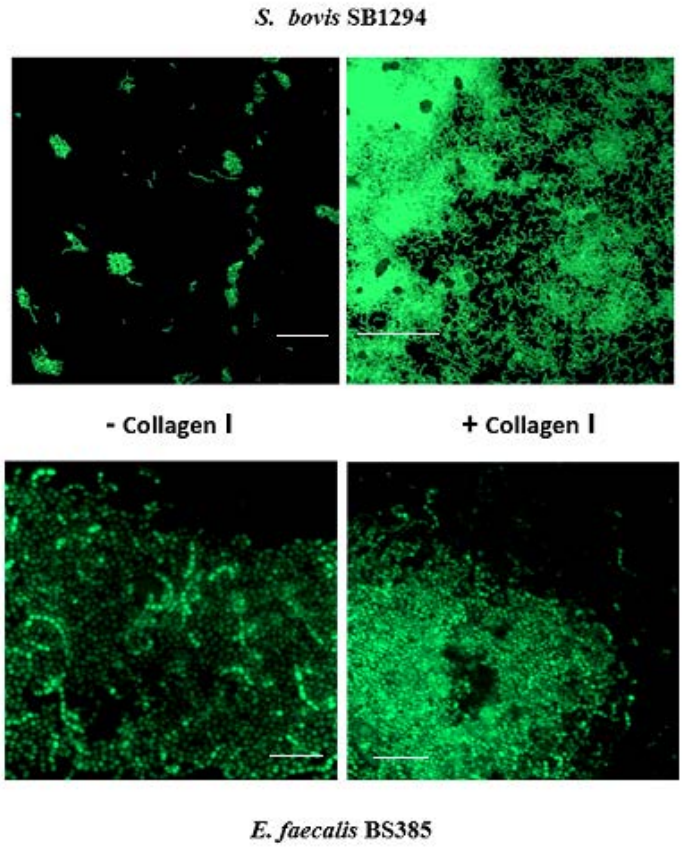

Figure 6.5 CLSM biofilm formation images for *S. bovis* SB1294, *SG4,* and *E. faecalis* BS385 in presence (+) or abcence (-) of collagen I.

6.4. Collagen binding protein *gallo_2179* cloning

As earlier shown in this chapter, *S. bovis* strains were observed to form good biofilms on collagen-coated surfaces. Recently, a study reported the complete genome sequence of *S. gallolyticus* UCN34, *SG*1, (Rusniok et al., 2010). The strain genome analysis revealed four genes encoding putative collagen binding proteins; however, only one of these genes encodes a protein with a collagen binding motif, *gallo*_2179. Our hypothesis was therefore that this protein was essential in biofilm formation on collagen-rich surfaces. As biofilm formation of *E. faecalis* ATCC19433 is not influenced by the presence or absence of collagen (see above), it was decided to clone the *gallo_2179* gene in this organism and test whether presence of this protein could stimulate biofilm formation on a collagen-coated surface.

A 1977-bp long DNA fragment of *gallo_2179* was amplified from genomic DNA of *S. gallolyticus* UCN34 using the TaqOne polymerase PCR kit (see chapter 2 for primers list). This amplified fragment was cloned into the enterococcal expression vector pAT79 using the *Bam*HI and *Sal*I restriction sites. The ligation product was used to transform *E. coli* cells which were grown on LB medium supplemented with 60 µg/ml spectinomycin and 5µg/ml chloramphenicol. Plasmid DNA was purified, and the correct product (denoted pATgallo_2179) was used to transform *E. faecalis* ATCC19433.

Biofilm assays using 96-well collagen-coated plate were performed using the standard crystal violet staining technique, comparing *E. faecalis* ATCC19433 and *E. faecalis* ATCC19433 (pAT*gallo*_2179). Unfortunately, no differences

were observed between the two strains (data not shown). This suggests that either *gallo_2179* gene is not transcribed in *E. faecalis*, that its mRNA is not translated, or that the gene product is not functional in *E. faecalis*. To test the first option, Reverse Transcriptase PCR (RT-PCR) was performed and the result, shown in figure 6.8, revealed that *gallo*_2179 mRNA is made in *E. faecalis* ATCC19444 (pAT*gallo*_2179). Thus, the remaining options are that the protein is either not made or not functional in *E. faecalis*, but due to time constraints this was not further tested. Note also that the RT-PCR technique is not quantitative, and it could also be that the quantities of mRNA produced are very low.

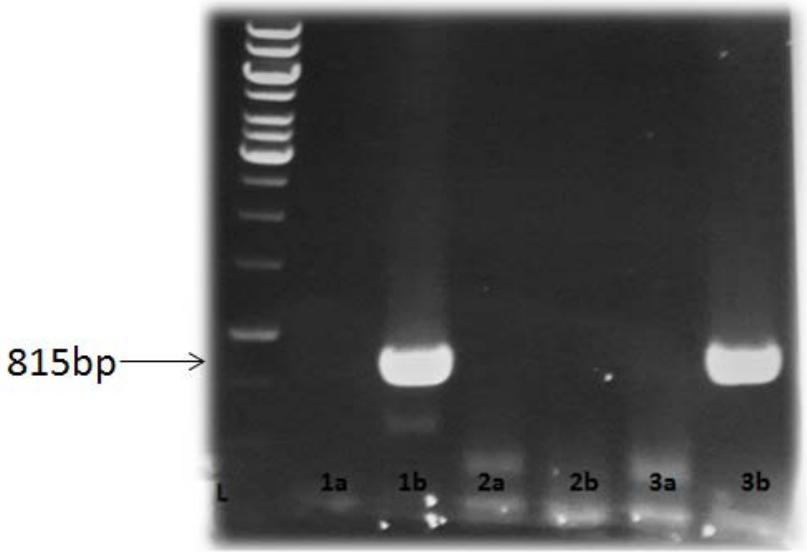

Figure 6.6 RT-PCR results for detect *gallo_2179* mRNA. L, DNA marker ladder; 1, *S. gallolyticus* UCN34 positive control; 2, *E. faecalis* ATCC19433; 3, *E. faecalis* ATCC19433 (pAT*gallo*_2179), see text for details. a, b related to the primers been used in this experiment.

6.5. Pathogenicity of *S. bovis* strains

6.5.1. *C. elegans* killing assay

In chapter 4, *E. faecalis* strains showed significant pathogenicity to *C. elegans*. To our knowledge *S. bovis* strains have never been analysed using this model system and it was therefore decided to test this. . Pathogenic controls were the enteric pathogens *Salmonella typhimurium* (ST) and *E. faecalis* ATCC 19433 (*EF*). The non-pathogenic strain used was *E. coli* HB101 (*EC*). The data in figure 6.5 demonstrate the survival of *C. elegans* after infection with the *S. bovis* isolates: *SG* strains, *SI* and *SM*.

Strains, *SG*1, *SG*2 and *SG*4 showing higher nematocidal activity against *C. elegans* with less than 15% survival at day 7. Other strains *SI* and *SG*3 also shows high level of virulence against *C. elegans,* with less than 30% *C. elegans* survival at day 7, while *SM* exhibited little degree of nematocidal activity.

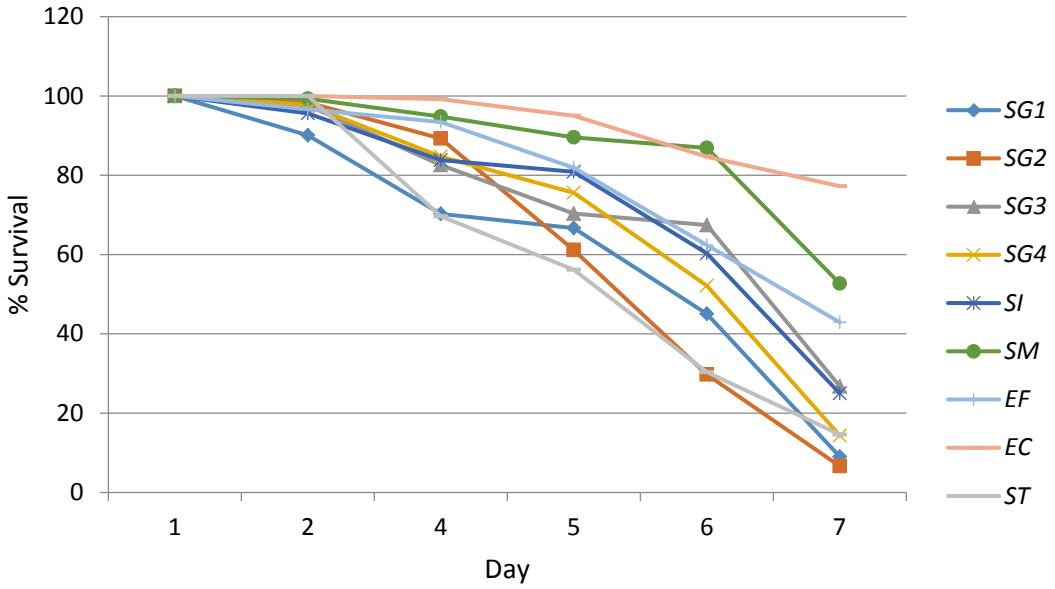

Figure 6.7 *C. elegans* killing assay for *S. bovis* strains. *EC, E. coli* HB101 is a non pathogenic control; *ST* and *EF* are pathogenic controls, (n=9).

6.5.2. *C. elegans* anaerobic killing assay

As mentioned previously in chapter 4, pathogens can kill nematodes in a slow mode (figure 6.5), or by a fast mode in which pathogens (e.g. *E. faecium*) can kill nematodes through the production of toxic compounds such as hydrogen peroxide (Jansen et al., 2002). To test whether *S. bovis* strains produce hydrogen peroxide or other toxic compounds in a similar manner as *E. faecium* E1162 (Meredith, 2013, Moy et al., 2004) bacteria were grown anaerobically on BHI plates to form a lawn. The next day *C. elegans* nematodes were deposited on the plates and their survival was monitored. This test was performed on selected *S. bovis* strains which form an efficient biofilm on collagen-rich surfaces (*SG*1, *SG*3, *SG*4 and *SM*).

Positive control was *E. faecium* E1162 and the negative control was *E. faecalis* BS385. As shown in Figure 6.6, , after a 2 hour incubation 100% of nematodes were still alive on the *S. bovis* lawns, while at that stage 60% of nematodes had died on the *E. faecium* lawn. Only after overnight incubation some death was observed (~35%), to a level similar to that of *E. faecalis*.

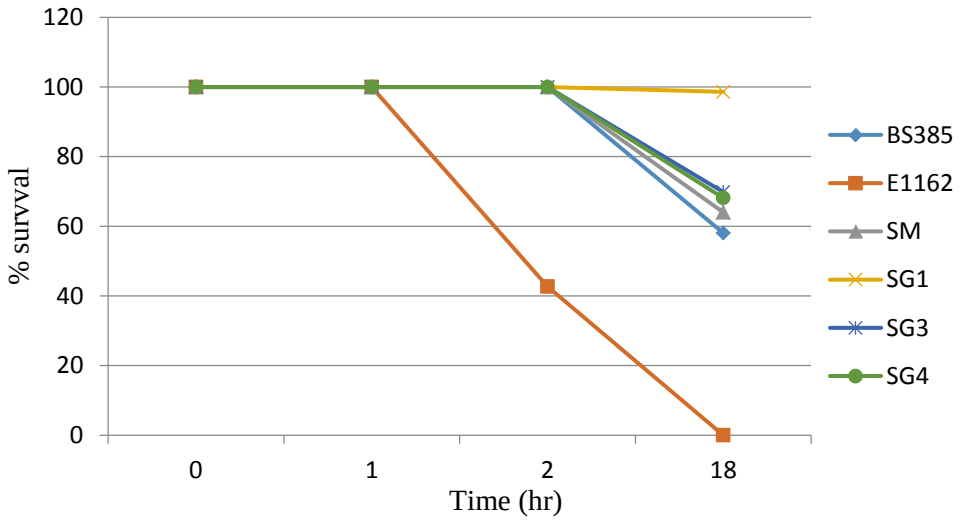

Figure 6.8 *C. elegans* anaerobic killing assay result for *S. bovis* strains. *E. faecalis* BS385 is a negative control and *E. faecium* E1162 is positive control.

6.6. Discussion

As mentioned in the introduction into this chapter, there is a clear association between CRC, endocarditis, and *S. bovis*. Virulence traits that are involved in this have been studied here and by the Tjalsma group. The conclusion of this study was that there are four essential steps in establishing endocarditis starting from intestinal tract (figure 6.9).

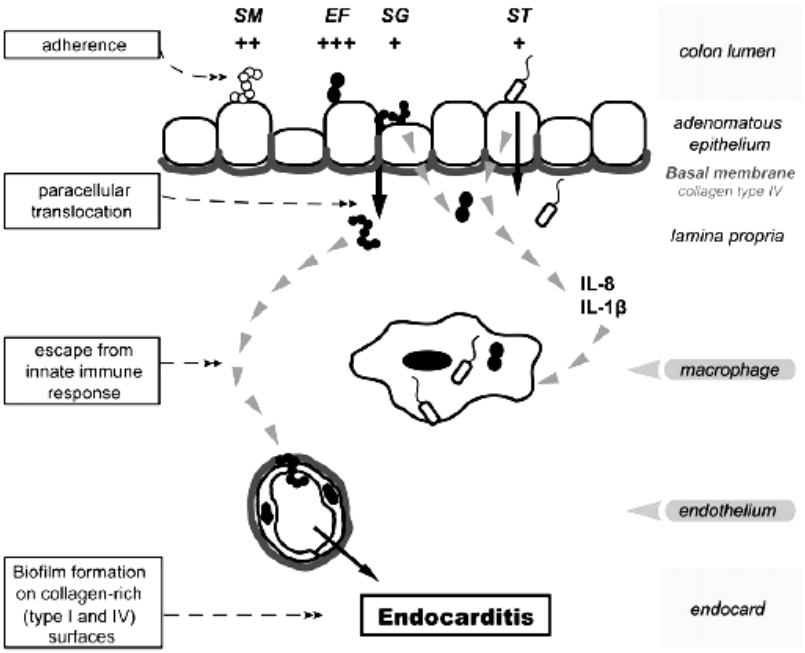

Figure 6.9 Model for the association of *SG* strains endocarditis and CRC. IL, interleukin, Taken from Boleij et al., (2011a).

Firstly, adherence and internalization of enterocytes or its extracellular matrix; secondly, paracellular translocation of the pathogenic bacteria through the epithelial barrier; thirdly, escaping the innate immune response; and fourthly, reaching blood stream and starting a secondary infection such as endocarditis.

Figure 6.9 shows a model for *S. bovis* strains endocarditis associated with colon lumen (Appendix 1). As shown previous, that *SG* strains displayed a low adherence to a healthy epithelial cell, however, in carcinoma epithelial cells exposed collagen IV which mediates the adherence of these bacteria and other pathogens, *ST* and *EF*, and translocation via paracellular mechanism except *SM* strain which able to only colonize epithelial cells. Upon infection, *SG* strain has the ability to escape from host immune defense, IL-8 and IL1β, to the blood stream. In contrast, pathogen strains such as *ST* and *EF* which induce immune response.

It is presently not clear whether *S. bovis* strains cause CRC, but our biofilm studies in which we showed that *S. bovis* strains form biofilms particularly well on collagen-rich surfaces at least indicate why there is this association. One possibility is that tumours in the gut merely provide an environment which is suitable to growth of *S. bovis*. Alternatively, it is possible that lesions in the gut provide a niche for *S. bovis* to adhere and form biofilms, and these might than exacerbate the damage leading to development of cancer. Indeed, a previous observation by Yantiss et al (2001) (Yantiss et al., 2001), showed that early colorectal tumor and polyps are characterized by continuously expression of collagen IV in the basement membrane in the mucosa. Thus, opportunistic pathogens such as *S. bovis* would find a good niche and environment to colonize and infect the colon, explaining why 10% of the normal population is colonised with *SG* strains, whereas these strains are found in 55% of CRC patients (Johansson et al., 2008).

It is at present unclear how much damage is created by colonisation of *S. bovis*, but our experiments with *C. elegans* show that there is sufficient damage in to cause death of the nematodes. These experiments also showed that pathogenicity of *S. bovis* strains is more similar to *E. faecalis* than to *E. faecium*. Firstly, both *S. bovis* and *E. faecalis* have a slow mode of killing that is absent in *E. faecium*. Secondly, of the three organisms only *E. faecium* has a fast mode of killing through the production of hydrogen peroxide (which accumulates during anaerobic growth; (Moy et al., 2004).

Upon adherence to tissues in the gut, *S. bovis* strains may reach the blood stream as outlined above. That enables the bacteria to reach other parts of the body and again, in particular, adhere to and form biofilms on surfaces that are rich in collagen. One such surface is the heart valve, which is rich in collagen I, thus explaining the association with CRC and encodarditis (Sillanpää et al., 2009)..

Interestingly, *SI* showed no differences in biofilm level with or without collagen, and this strain is not often found in endocarditis(Jean et al., 2004), corroborating our hypothesis on the link between collagen-binding and endocarditis. The Tjalsma group did observe translocation of *SI* to the blood stream, thus explaining the fact that *SI* is often found to cause to bacteremia in CRC patients(Jean et al., 2004).

As previously mentioned in this chapter, the full genome sequence UCN34 strain revealed the presence of a number of potential collagen-binding proteins that are related to the MSCRAMMs family (see chapter 4; Rusniok et al., 2010). Only one of these (Gallo_2179) contains a putative collagen binding

motif (Gallo_2179). We successfully cloned the *gallo_2179* gene in an enterococcal expression vector, and demonstrated that the gene was indeed transcribed in *E. faecalis*. However, this strain did not form better biofilms on a collagen-coated surface, suggesting either that not sufficient amount of the Gallo_2179 protein was made, or that the protein is not functional in *E. faecalis*. Insufficient amount of protein could either be due to low quantities of mRNA (our RT-PCR demonstrating presence of mRNA is not quantitative), inefficient translation of the mRNA, or instability and degradation of the protein. Lack of function of the protein could also have several reasons, including misfolding of the protein, improper localisation of the protein at the membrane, or the lack of a partner protein required for adherence to collagen. Further investigation of this would require a substantial amount of work including raising antibodies to determine e.g. the levels of protein produced and the localisation of the protein. Due to time constrains this was not pursued any further.

Chapter7: Final discussion

7.1. Final discussion

As mentioned in chapter 3, many virulence factors are secretory proteins that either function at the cell wall or in the extracellular milieu. For that reason a bioinformatics analysis was performed to identify putatively secreted proteins in *S. gallolyticus*; similar analysis have also been done for *E. faecium* (Meredith, 2013), whereas proteomic studies on extracellular proteins have been performed on *E. faecalis* (Shankar et al., 2012). In the same chapter, bioinformatics analysis of the *S. gallolyticus* genome revealed several secreted proteins. A number of those proteins are hypothetical proteins with unknown function and it would be interesting to discover more about these proteins. Such an approach was also used with *E. faecalis*, in which a hypothetical protein was shown to be a virulence factor involved in pili formation (which play an important role in adhesion and biofilm formation; Sillanpaa et al., 2004).

Biofilm assays were performed on different *E. faecalis* strains, showing variation in biofilm formation between the strains. Several factors were involved in this process for example the presence of virulence factors, hydrophobicity and heterogeneity.

One study showed that *E. faecalis* with mutations in the *fsr*-locus or *gelE* resulted in poor biofilm formation. In the same study *fsr*-only mutants formed wild-type biofilm level by addition of purified GelE, suggesting that GelE alone could enhanced biofilm formation (Hancock and Perego, 2004). In addition, studies on the role of Esp in biofilm formation resulted in

contradictory results with some showing that biofilm formation is independent from Esp and others showing that Esp is important (Toledo-Arana et al., 2001, Tendolkar et al., 2004, Kristich et al., 2004). However, as shown here (chapter 4), *E. faecalis* BS12297 is a much better biofilm former than BS11297, while both contain Esp, and BS11297 even contains GelE as well. It is thus clear that other factors play an important role as well and that capacity to form biofilms is not solely determined by the presence or absence of just a few factors. For instance, it has been shown that BS12297 shows more cell culture surface charge heterogeneity, which may stimulate adhesion to surfaces, whereas cultures of *E. faecalis* BS11297 are much more homogenous (van Merode et al., 2006b). In addition we have shown here also that hydrophobicity plays an important role, with the better biofilm formers being more hydrophobic. What exactly determines heterogeneity within cell cultures, or the hydrophobicity of cells is actually not well understood. For instance, strains lacking Esp are less hydrophobic (chapter 5), but Esp is not a hydrophobic protein and it is thus not clear why Esp makes cells more hydrophobic.

Other factors may also play an important role in biofilm formation in several bacteria the presence of extracellular DNA has been shown to be an important factor in biofilm formation. Interestingly, the effect of DNaseI treatment of this strain showed only a fairly mild reduction in biofilm formation of *E. faecalis*, indicating that DNA is not a major component of the EPS in *E. faecalis* isolate.

As several cell-surface proteins play an important role in biofilm formation, we analysed the effects of sodium azide; this inhibits ATPases but is particularly

active on the ATPase SecA, a central component in the Sec-dependent translocation pathway (Miller et al., 2002). As most surface proteins in enterococci are predicted to be Sec dependent, it was anticipated that sodium azide could be used for preliminary studies into the importance of the Sec system in biofilm formation in *Enterococcus faecalis* strains (Miller et al., 2002). Note of course that sodium azide is toxic to bacteria, but enterococci are somewhat of an exception as they are relatively tolerant to azide. Adding sub-lethal concentrations of sodium azide resulted in a significant decrease in biofilm formation of BS12297 (the strain which forms the best biofilms), whereas there was a small (but statistically not significant) decrease in the other isolates. Also, hydrophobicity was reduced in the presence of sodium azide in BS12297 and BS385. Thus indeed it seems that sodium azide influences the composition of the cell wall, resulting in a reduced biofilm formation. Confusingly, protease production was increased in BS11297 in the presence of sodium azide, possibly a stress-response effect, but this strain is a very poor biofilm former and azide did not have an effect on biofilm formation or hydrophobicity of this strain.

The clearest effect on the presence or absence of specific factors was observed in *E . faecium* E1162 and E1162Δesp; here we can compare these strains straightforward as both have the same parental background and thus differ only in the production of Esp. As demonstrated before, the strain lacking Esp shows a significantly reduced biofilm formation, and this strain is also significantly less hydrophobic. Here we also demonstrated for the first time a biochemical test for a cell surface protein in biofilm formation: addition of the purified N domain of Esp (EspN) to *E. faecium* E1162Δesp resulted in the restoration of

biofilm formation. This assay and the availability of purified protein will enable the further biochemical analysis on the role of this protein in biofilm formation in more detail.

In chapter 6, we demonstrated the ability of *S. bovis* strains to form biofilm on collagen coated surfaces. This provided an important clue towards the pathogenicity of these strains as, for instance, the heart valve is largely made of collagen thus explaining the link between endocarditis and *S. bovis* strains. Similarly, this could explain the link with colon cancer, as extracellular matrix proteins such as collagen may become exposed in tumours and/or lesions in the gut. Whether tumours provide a niche for *S. bovis* for colonization, or whether *S. bovis* infections of lesions could exacerbate development of such lesions into tumours remains to be investigated.

Recently, a full genome analysis has been identified for *S. gallolyticus* UCN34 (Rusniok et al., 2010), and one important protein identified in this was a gene encoding a putative collagen-binding protein denoted *gallo*_2197. Our aim was to clone the gene and then test its function in biofilm formation on collagen coated surfaces in *E. faecalis* ATCC19433, a strain in which biofilm formation is independent from the presence or absence of collagen. Unfortunately, no differences were observed between *E. faecalis* ATCC19433 and *E. faecalis* ATCC19433 (pAT*gallo*_2179). We did demonstrate (with RT-PCR) that the gene is expressed. From that we could conclude that either the quantity of mRNA produced is very low (the RT-PCR was not quantitative) or that gene is not functional in *E. faecalis*. The latter could be because of, for instance,

improper localisation of the protein, or lack of a protein partner that is only present in the parental *S. bovis* strain.

7.2. Future work

The mechanisms of factors involved in pathogenicity and biofilm formation remain unclear in both enterococci and streptococci. In future studies it will be interesting to discover more about their roles and whether they interact with other factors and for this genetic and biochemical analysis are required. A problem is, however, at present the lack of some genetic tools for enterococci and streptococci. Some tools are available but, for instance, there is a lack of efficient systems for inducible expression of genes. Several factors are of interest to study further. In particular the role of Esp is unclear; it is known that it is important in biofilm formation, but it is actually not known what it does. In this thesis the first tools and assays have been developed that will enable further biochemical studies. Understanding the process of biofilm formation at the molecular level will provide a platform for the development of new strategies for treatment of enterococcal infections, as it is clear that biofilms are an important component in the pathogenicity of enterococci.

For streptococci, the role of the collagen-binding protein is worthy of further investigation, as it may be an important virulence factor and thus also potential target for drug development. In the near future it should be investigated whether the collagen binding protein is indeed produced and translocated properly in *E. faecalis*; if so, we'd anticipate that it requires a binding partner.

Co-purification experiments or similar types of assays could then be used to identify and characterise that partner protein.

Appendix

MAJOR ARTICLE

Novel Clues on the Specific Association of *Streptococcus gallolyticus* subsp *gallolyticus* With Colorectal Cancer

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(See the editorial commentary by Hensler, on pages 1040–2.)

Background. The prevalence of *Streptococcus gallolyticus* subsp *gallolyticus* (*Streptococcus bovis* biotype I) endocarditis is in general low but very often linked to colorectal cancer. Therefore, this study aimed to reveal the virulence characteristics that distinguish this opportunistic pathogen from a panel of (closely related) intestinal bacteria.

Methods. The route of infection was reconstructed in vitro with adhesion, invasion, and translocation assays on differentiated Caco-2 cells. Furthermore, cellular immune responses upon infection and bacterial biofilm formation were analyzed in a comparative manner.

Results. *S. gallolyticus* subsp *gallolyticus* strains were demonstrated to have a relative low adhesiveness and could not internalize epithelial cells. However, these bacteria were uniquely able to paracellularly cross a differentiated epithelium without inducing epithelial interleukin 8 or 1β responses. Importantly, they had an outstanding ability to form biofilms on collagen-rich surfaces, which in vivo are found at damaged heart valves and (pre)cancerous sites with a displaced epithelium.

Conclusions. Together, these data show that *S. gallolyticus* subsp *gallolyticus* has a unique repertoire of virulence factors that facilitate infection through (pre)malignant colonic lesions and subsequently can provide this bacterium with a competitive advantage in (1) evading the innate immune system and (2) forming resistant vegetations at collagen-rich sites in susceptible patients with colorectal cancer.

The human intestinal tract is the habitat for several hundred different bacterial species with an increasing bacterial concentration and variability toward the distal colon. The commensal bacterial population aids human health by making dietary nutrients availableto the host, but it also prevents attachment and subsequent invasion of pathogenic bacteria [1]. Strikingly, however, the part of the intestine with the highest bacterial colonization, the colon, is also most affected by cancer, with 146,970 cases annually in the United States [2]. This, together with the fact that germ-free mice have lower rates of colon carcinogenesis [3], implies that intestinal bacteria play an important role in the development of colorectal cancer (CRC).

The gram-positive, opportunistic pathogen *Streptococcus bovis* is one of the few intestinal bacteria that have been consistently linked to CRC [4–6]. The first case report suggesting an association between *S. bovis* endocarditis and carcinoma of the sigmoid was already published in 1951 [7]. Since then, multiple studies have shown that a colon tumor or polyp was detected upon full-bowel examination in up to 90% [8] of patients with a *S. bovis* infection [5, 9]. Furthermore, fecal carriage of *S. bovis* in the healthy population is low but increases ~5-

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fold in patients with CRC [10]. After Schlegel et al introduced the new nomenclature of *S. bovis* strains [11] it became clear that *Streptococcus gallolyticus* subsp *gallolyticus* (*S. bovis* biotype I) (Table 1), a major cause of infective endocarditis, has the highest association with CRC [6, 8].

Although some studies have shown that *S. bovis* strains can directly promote carcinogenesis in a rat model for CRC [12, 13], an incidental relationship provides an alternative explanation for the association of *S. gallolyticus* subsp *gallolyticus* with CRC. In a normal healthy colonic environment the host has several defense mechanisms to shield itself from bacterial infection. Goblet cells within the polarized epithelium secrete a continuous layer of mucus that protects the epithelium and promotes transit of bowel contents [14], whereas enterocytes secrete antimicrobial peptides, cytokines and immunoglobulin A as preventive agents. However, CRC is characterized by several changes in this physical barrier including increased tight junction permeability [15] and altered mucus production and composition [16]. This distorted physical protection could make patients with CRC prone to rare opportunistic bacterial infections.

However, it is still unclear why *S. gallolyticus* subsp *gallolyticus* infections have such a high association with colon malignancies, whereas this is not the case for other (related) opportunistic pathogens that inhabit the human gastrointestinal tract. Therefore, the main aim of this study was to reveal the virulence characteristics that distinguish *S. gallolyticus* subsp *gallolyticus* from other bacteria to gain insight in how these features could specifically cause infections in patients with CRC. To this purpose, several host-pathogen interactions that are involved in this infective process were mimicked in vitro. These studies indicated that *S. gallolyticus* subsp *gallolyticus* avails of a unique repertoire of virulence characteristics that give it an advantage over related *S. bovis* strains and other intestinal bacterial species, to cross an epithelial layer, evade the immune system and form biofilms on collagen-rich surfaces.

MATERIALS AND METHODS

Cell Culture and Bacterial Strains

Colorectal adenocarcinoma cell lines HT-29 and Caco-2 (www.atcc.org) were cultured in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% fetal calf serum (FCS), 20 mmol/L HEPES, 2 mmol/L L-glutamine and 1× nonessential amino acids (Gibco) at 37°C/5% CO_2. The human monocytic cell line THP-1 was cultured in Roswell Park Memorial Institute 1640 medium (RPMI1640) supplemented with 10% FCS, 2 mmol/L L-glutamine, 1 μmol/L pyruvate, and 5 μg/mL gentamicin (Gibco). These media and culture conditions were used in experiments unless stated otherwise.

The following bacterial *S. bovis* strains were used, *S. gallolyticus* subsp *gallolyticus* UCN34 [17], *S. gallolyticus* subsp *gallolyticus* NTB1 (Radboud collection), *S. gallolyticus* subsp *gallolyticus* 1293 provided by Dr R. Zarrilli [18], *Streptococcus infantarius* subsp *infantarius* NCTC8133 [13] and *S. gallolyticus* subsp *macedonicus* CIP105685T (Pasteur collection). The new and old designations for *S. bovis* strains are depicted in Table 1.

Reference strains included *Enterococcus faecalis* 19433 (www.atcc.org), *Escherichia coli* NTB5, and *Salmonella typhimurium* NTB6 from the Radboud collection, *Lactobacillus plantarum* WCFS1 [19], and *Bacillus subtilis* 168 [20]. All strains were grown on Columbia blood agar or in brain-heart infusion broth (Difco) supplemented with 1% glucose at 37°C and 5% CO_2. *L. plantarum* was grown in de Man–Rogosa–Sharpe (MRS) broth at 37°C and 5% CO_2 and *E. coli* was grown at 200 rpm.

Adherence and Internalization Assay

Caco-2 and HT-29 cells were cultured in 24-well plates (Corning) to ~1 ×x 10^6 cells/well and infected with a multiplicity of infection (MOI) of 20. After 2 h of incubation, monolayers were washed 3 times with PBS to remove nonadherent bacteria and subsequently lysed in trypsin-PBS containing .025% triton-X100. Alternatively, extracellular adherent bacteria were killed with 200 μg/mL gentamicin and 50 μg/mL ampicillin for another hour to measure the amount of internalized bacteria. The amount of adherent or internalized bacteria was determined by counting colony-forming units. Adherence was expressed as a percentage of the inoculum, and internalization as the percentage of adherence.

Translocation Assay

Caco-2 cells were cultured on Transwell permeable supports with a polycarbonate membrane (3-μm pore size) (Corning).

Table 1. Nomenclature of *Streptococcus bovis* Strains

New Name	Old Name	Strains Used in Current Study
Streptococcus gallolyticus subsp *gallolyticus*	*S. bovis* biotype I	UCN34 (SG1), 1293 (SG2), NTB1 (SG3)
Streptococcus infantarius subsp *infantarius*	*S. bovis* biotype II.1	NCTC8133 (SI)
S. infantarius subsp *coli*	*S. bovis* biotype II.1	None
S. gallolyticus subsp *pasteurianus*	*S. bovis* biotype II.2	None
S. gallolyticus subsp *macedonicus*	*Streptococcus macedonicus*	CIP105865T (SM)

NOTE. Historically *S. bovis* strains were delineated into 2 biotypes according to their ability (biotype I) or inability (biotype II) to ferment mannitol [11]. The former *S. bovis* biotype I, *S. bovis* biotype II.2, and *S. macedonicus* are now designated in a single DNA cluster including 3 subspecies: *S. gallolyticus* subsp *gallolyticus*, *S. gallolyticus* subsp *pasteurianus*, and *S. gallolyticus* subsp *macedonicus*. *S. gallolyticus* subsp *gallolyticus* is most often linked with endocarditis-associated colonic cancer.

Transepithelial electrical resistance (TEER) measurements confirmed the formation of a polarized monolayer by a flattening of the TEER value (250–350 $\Omega^{*}cm^{2}$) at 21 days (Millipore ERS) [21]. Bacteria were added to the apical compartment (MOI, 50), and after incubation the numbers of viable bacteria in the apical and basolateral compartments were determined by counting colony-forming units. At every time point, medium in the lower compartment was replaced to prevent growth of translocated bacteria. Translocation was expressed as a percentage of the inoculum.

Confocal Microscopy

Bacteria (1×10^{9}) were washed in PBS and labeled for 30 min at room temperature (RT) in PBS containing .5 mg/mL fluorescein isothiocyanate (FITC) (Sigma). Next, bacteria were extensively washed to remove nonbound FITC before infection (MOI, 50) of polarized Caco-2 monolayers on Transwell permeable supports. After 4 h of incubation in the dark monolayers were stained for confocal microscopy, as described in the Supplementary Information.

Phagocytosis Assay

Human monocytic THP-1 cells were seeded in 24-well plates at 50,000 cells/well in RPMI 1640 containing 1% FCS and were differentiated to macrophages by 50 ng/mL phorbol 12–myristate 13–acetate 24 h before phagocytosis assay. Next, bacteria were added (MOI, 50), spun at 400 *g* for 5 min, and incubated for 30 min to allow phagocytosis. Extracellular bacteria were killed with 200 μg/mL gentamicin and 50 μg/mL ampicillin, and after incubation viable intracellular bacteria were quantified by macrophage lysis with 1% saponin. Killing was expressed as the percentage of phagocytosed bacteria at $t = 0$.

Real-Time Polymerase Chain Reaction

Caco-2, HT-29, and THP-1 cells were washed and lysed in RLT lysis-buffer (RNeasy Mini Kit; Qiagen), and RNA extraction was performed according to Qiagen protocol. The RNA concentration and purity were evaluated with a NanoDrop Spectrophotometer (NanoDrop Technologies). Next, reverse-transcription polymerase chain reaction (PCR) (Iscript; Bio-Rad) was performed to synthesize 1 μg of complementary DNA under the following conditions: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Expression of interleukin (IL) 8 and *IL-1β* (gene expression assays Hs00174103_m1 and Hs00174097_m1; Applied Biosystems) was compared with expression of the gene for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; gene expression assay4310884E), using the following real-time PCR protocol: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C (7900 HT; Applied Biosystems). Data were analyzed via the ΔΔCt method using SDS software (version 2.2.1).

Biofilm Formation

Biofilm formation assays were essentially performed as described elsewhere, with some minor modifications [22]. Bacteria were cultured overnight in tryptone-soya broth containing .25% glucose, diluted to 10^{7} bacteria/mL and dispensed in polystyrene 96-well plates that were either coated with collagen type I or type IV or uncoated. Plates were incubated for 24 h at 37°C on a 3-dimensional plate rotator (30 rpm). The cell suspension was removed, and biofilms were washed 3 times with PBS. Then plates were dried for 1 h at room temperature, and biofilms were stained with crystal violet solution. After 15 min, excess crystal violet was removed, plates were washed 3 times with PBS, and crystal violet was dissolved in ethanol-acetone (80:20 vol/vol). The absorbance, which is representative of the amount of biofilm formed, was measured at 595 nm (A_{595}).

Electron Microscopy

Bacteria were grown in Todd-Hewitt medium and collected after overnight growth (stationary phase). Electron microscopic images were produced as described elsewhere [23]. For details, see Supplementary Information.

RESULTS

Adherence of *S. gallolyticus* Strains to Colon Epithelial Cells

The first important step to establish a gut-borne infection is adherence of bacterial cells to colonic tissue. Therefore, the binding capacity of *S. gallolyticus* subsp *gallolyticus* clinical isolates to colonocytes was compared with that of the pathogen *S. typhimurium*, the opportunistic pathogen *E. faecalis*, and nonpathogenic *E. coli* and *L. plantarum* strains (Figure 1A and B). These experiments showed that *E. faecalis* is by far the most efficient adhering bacterium to both HT-29 and Caco-2 cells, reaching adherence of about 80% and 98% of the inocula, respectively. The nonpathogenic strains *E. coli*, *S. gallolyticus* subsp *macedonicus*, and *L. plantarum* adhered moderately well (20%–50%) to the monolayers. Adherence of the *S. gallolyticus* subsp *gallolyticus* strains was similar to that of the pathogen *S. typhimurium*, all with adherence <15%. Thus, the adhesive properties of *S. gallolyticus* subsp *gallolyticus* strains resemble those of the pathogen *S. typhimurium* more than nonpathogenic bacteria. This may reflect one of the reasons that *S. gallolyticus* subsp *gallolyticus* cannot efficiently colonize a healthy human intestinal tract.

Paracellular Translocation of Colonic Differentiated Epithelial Cells by *S. gallolyticus* subsp *gallolyticus*

After adhesion to colonic tissue, several invading mechanisms can be used by (opportunistic) pathogens. For example, *Salmonellae* are efficient in transcellular crossing of intestinal epithelium [24], whereas paracellular crossing is described for group B streptococci [25]. To obtain insight into the translocation capacity of *S. bovis* strains, their internalization and translocation efficiencies were analyzed in Caco-2 Transwell cultures. As shown in Figure 2C, none of the *S. bovis* strains were

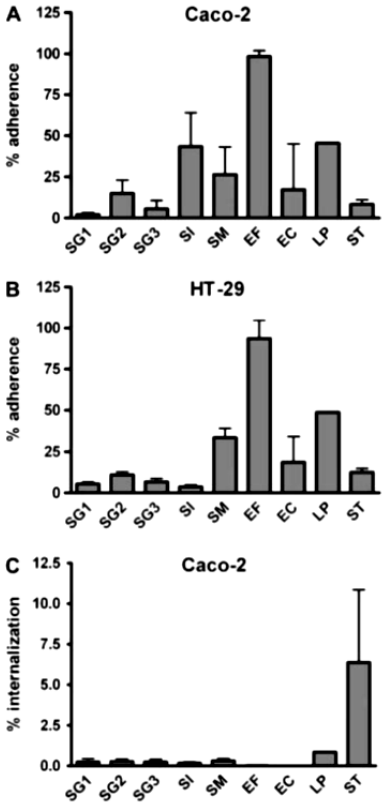

Figure 1. *A, B,* Bacterial adherence and invasion of epithelial cells. Adherence of indicated intestinal bacteria to Caco-2 (*A*) and HT-29 (*B*) colorectal cancer cells was analyzed after 2 h of bacterial exposure. *C,* Bacterial internalization after 2 h in Caco-2 cells. Adherence is presented as percentage of the bacterial inocula, and subsequent bacterial internalization as percentage of adherent bacteria. *EC, Escherichia coli; EF, Enterococcus faecalis; LP, Lactobacillus plantarum; SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius; SM, S. gallolyticus* subsp *macedonicus; ST, Salmonella typhimurium.*

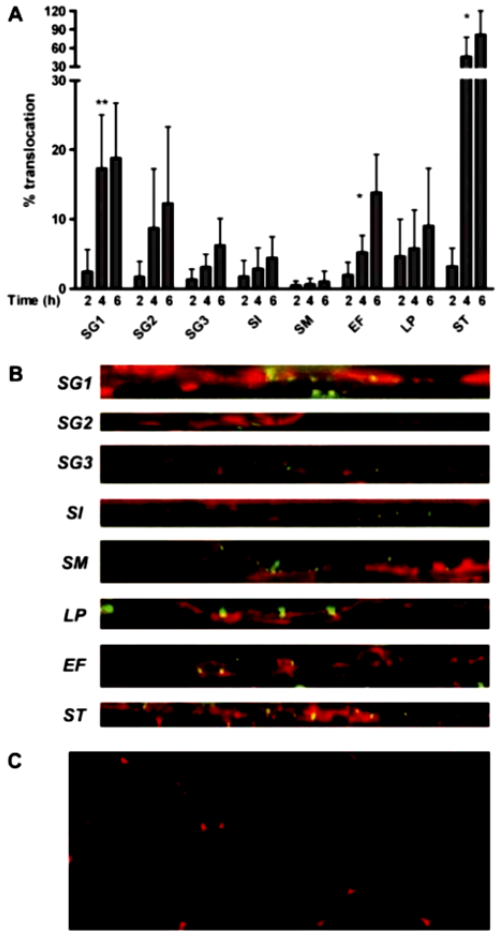

Figure 2. Bacterial translocation across an epithelial monolayer. *A,* Translocation of indicated bacteria across differentiated and polarized Caco-2 cells was measured after 2, 4, and 6 h. $*P < .05$; $**P < .01$ (significant increase in time; 1-way analysis of variance). *EF, Enterococcus faecalis; LP, Lactobacillus plantarum; SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius; SM, S. gallolyticus* subsp *macedonicus; ST, Salmonella typhimurium. B,* Confocal microscopy of fluorescein isothiocyanate–labeled bacteria (*green*) after translocation ($t = 6$ h). Cytoskeleton was stained with anti-actin antibodies (*red*). Top, lateral side; bottom, basolateral side of the epithelial monolayer. *C,* Zonula occludens 1 staining (*red*) of epithelial monolayer showing tight junction complexes in differentiated monolayer. Although *L. plantarum* displayed some discrepant results between translocation and confocal microscopy, it may be assumed that this bacterium cannot efficiently cross epithelial cells [26].

invasive (maximum, .2% of adherent bacteria), but 6%–19% of adhered *S. gallolyticus* subsp *gallolyticus* and *S. infantarius* subsp *infantarius* cells could translocate across the polarized and differentiated Caco-2 monolayer at efficiencies similar to those of the opportunistic pathogen *E. faecalis* (Figure 2A). In contrast, *S. gallolyticus* subsp *macedonicus* was unable to cross the differentiated monolayer (<2% of adhered bacteria), which clearly differentiates this strain from *S. gallolyticus* subsp *gallolyticus* and *S. infantarius* subsp *infantarius*. However, the data also showed that the only internalizing strain *S. typhimurium* displayed by far the highest translocation percentage (81%) of adhered bacteria.

Z-stack images made with confocal microscopy from the apical to the basolateral side confirmed that *S. gallolyticus* subsp *gallolyticus* (strains UCN34, 1293, and NTB1), *S. infantarius* subsp *infantarius*, *E. faecalis*, and *S. typhimurium* cells were indeed present at the basolateral side of the monolayer, whereas *S. gallolyticus* subsp *macedonicus* could be detected only at the apical side (Figure 2B). Zonula occludens 1 (ZO-1) visualization (Figure 2C) [26] and TEER measurements confirmed polarization and integrity of the monolayer during experiments, except for *S. typhimurium*, which induced a dramatic reduction in TEER after 6 h (data not shown) [27]. Therefore these data indicate that translocation of *S. gallolyticus* subsp *gallolyticus*, *S. infantarius* subsp *infantarius*, and *E. faecalis* cannot be attributed to passive leakage through a nonpolarized monolayer but instead constitutes an active process. Together, these data imply that *S. gallolyticus* subsp *gallolyticus* and *S. infantarius* subsp *infantarius*, but not *S. gallolyticus* subsp *macedonicus*, can translocate across a polarized epithelial monolayer via a paracellular mechanism.

Relative Invisibility of *S. gallolyticus* subsp *gallolyticus* to Epithelial Innate Immune System

When pathogens cross the intestinal barrier, the intestinal epithelium attracts macrophages by the production of alarm signals. To evaluate to which extent *S. bovis* strains induce an epithelial innate immune response, the expression of *IL-8* and *IL-1β* in Caco-2 cells was measured with real-time PCR on bacterial infection. As shown in Figure 3A and B, both interleukin *IL-8* and *IL-1β* were strongly increased 2 and 4 h after infection with *S. typhimurium* (maximum *IL-8*, 52-fold; *IL-1β*, 4-fold) and *E. coli* (maximum *IL-8*, 79-fold; *IL-1β*, 7-fold). The gram-positive strains *S. infantarius* subsp *infantarius*, *S. gallolyticus* subsp *macedonicus*, and *E. faecalis* also significantly increased *IL-8* messenger RNA levels after 4 h of infection but to a lesser extent than gram-negative strains (maximum induction, 6-fold). Surprisingly, however, all 3 *S. gallolyticus* subsp *gallolyticus* strains did not elicit a significant *IL-8* or *IL-1β* response (Figure 3A and B), similar to the probiotic bacterium *L. plantarum*.

To investigate to what extent *S. gallolyticus* subsp *gallolyticus* can withstand phagocytosis, *S. gallolyticus* subsp *gallolyticus* UCN34 cells were exposed to THP-1 derived macrophages. This experiment showed that ~14% of the *S. gallolyticus* subsp *gallolyticus* cells were still viable after 24 h (Figure 3C) in contrast to 0% of *L. plantarum* and *B. subtilis* cells, which were used as positive controls for bacterial killing [28, 29], whereas the pathogen *S. typhimurium* killed and escaped from macrophages within 5 h after infection (data not shown) [30]. However, no macrophage killing was observed by *S. gallolyticus* subsp *gallolyticus*, and bacterial cells remained confined within the macrophage. Accordingly, macrophages responded adequately to *S. gallolyticus* subsp *gallolyticus* infection by a 4-fold up-regulation of *IL-8* and a 3-fold up-regulation of *IL-1β* on the

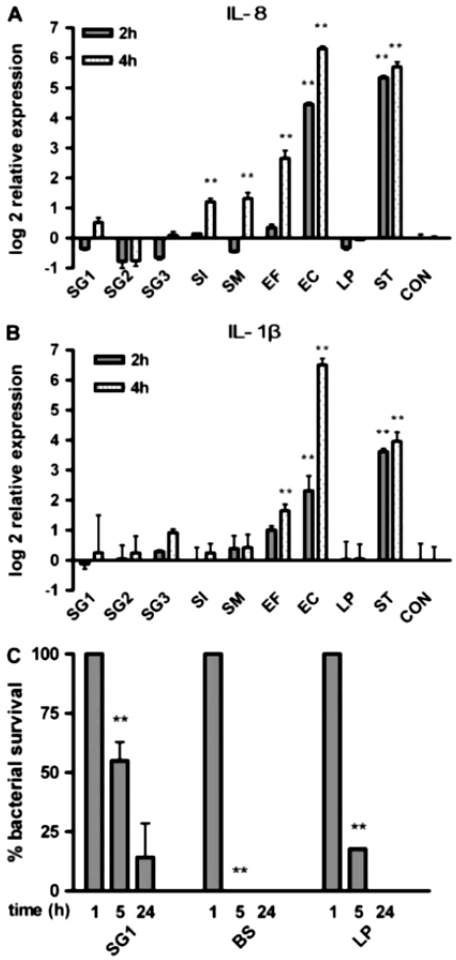

Figure 3. Bacterial interactions with innate immune system. *A, B,* Epithelial interleukin (IL) 8 (*A*) and *IL-1β* (*B*) immune response in Caco-2 cells elicited by indicated bacterial strains after 2 and 4 h of incubation. Data are presented as $\log_2$ values of fold changes. $^{**}P < .01$ (significant inductions; 2-way analysis of variance with Bonferroni posttests). CON, control; *EC, Escherichia coli; EF, Enterococcus faecalis; LP, Lactobacillus plantarum; SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius; SM, S. gallolyticus* subsp *macedonicus; ST, Salmonella typhimurium. C,* Bacterial killing by phorbol 12–myristate 13–acetate–stimulated THP-1 macrophages was analyzed after 5 and 24 h by counting colony-forming units of viable bacteria. $^{**}P < .01$ (significant reductions in time; 2-way analysis of variance with Bonferroni posttests). *BS, Bacillus subtilis.*

messenger RNA level after 4 h, similar to the response to *S. typhimurium*. Together, these findings indicate that *S. gallolyticus* subsp *gallolyticus* strains are relatively invisible to epithelial innate immunity upon infection, which could prolong their survival by the delayed recruitment of macrophages in the lamina propria.

Biofilm formation by *S. gallolyticus* subsp *gallolyticus* on Collagen-Coated Surfaces

After entry into the human body and escape from the immune system, *S. gallolyticus* subsp *gallolyticus* has the opportunity to establish endocarditis in susceptible patients. For endocarditis, it is known that bacterial binding to extracellular matrix proteins and biofilm formation are important characteristics to facilitate survival of bacterial vegetations on damaged or prosthetic heart valves [31, 32]. As shown in Figure 4, all *S. gallolyticus* subsp *gallolyticus* strains were indeed efficient in forming biofilms on surfaces coated with collagen I or IV (A_{595}, .4–1.4), while this was clearly not the case for uncoated polystyrene surfaces (A_{595}, <.15). In contrast, *S. gallolyticus* subsp *macedonicus*, *S. infantarius* subsp *infantarius*, and *E. faecalis* could form a biofilm on polystyrene surfaces (A_{595}, .2–.4) irrespective of the presence of collagens. In contrast, the probiotic bacterium *L. plantarum* did not form a biofilm under any of the tested conditions. These data demonstrate that *S. gallolyticus* subsp *gallolyticus* strains have exclusive features that enable them to form biofilms on collagen-rich surfaces.

DISCUSSION

In the present study, we reconstructed the route of gut-borne bacterial infections in patients with CRC. Basically there are 4 key events in establishing endocarditis from the intestinal tract: (1) fixing a dependable connection with the enterocyte or its extracellular matrix, (2) translocation of the epithelial barrier, (3) evasion of immune cells in the lamina propria, and (4) survival in the bloodstream and ability to establish a secondary infection. By comparative bacterial virulence analysis, we provided new clues on the underlying mechanism that specifically causes the increased incidence of clinical *S. gallolyticus* subsp *gallolyticus* infections in patients with CRC.

Focusing on the initial step of gut-borne infections, adhesion of *S. gallolyticus* subsp *gallolyticus* to epithelial cells can be categorized as low compared with related *S. bovis* strains and other intestinal bacteria. Genome exploration of *S. gallolyticus* subsp *gallolyticus* revealed that it contains a capsular operon that is highly similar in its organization to *S. pneumonia* serotype 23F [17], whereas *S. gallolyticus* subsp *macedonicus* contains a different capsule operon (P. Glaser, unpublished data). The diverse surface structures that are likely to determine the distinct adhesive properties of *S. gallolyticus* subsp *gallolyticus* and *S. gallolyticus* subsp *macedonicus* to epithelial cells are clearly visualized by electron microscopy (Figure 5). In general, capsular polysaccharides are known to negatively affect bacterial adhesion to host cells but may also shield the bacterial cell from the immune system and thereby be an important virulence factor [23, 33, 34]. In fact, encapsulation of *S. gallolyticus* subsp *gallolyticus* strains has already been shown to contribute to virulence in pigeons [23].

Our data clearly showed that *S. gallolyticus* subsp *gallolyticus* can translocate across an intestinal epithelial layer, whereas it was unable to invade epithelial cells as do pathogenic bacteria, such as *Salmonellae* [24]. Genome exploration of *S. gallolyticus* subsp *gallolyticus* revealed that this bacterium contains 3 pilus operons [17] with homology to the pilus backbone of group B streptococci that are known mediators of paracellular translocation [25, 35]. Strikingly, none of these operons are present in the genome of *S. gallolyticus* subsp *macedonicus* (P. Glaser,

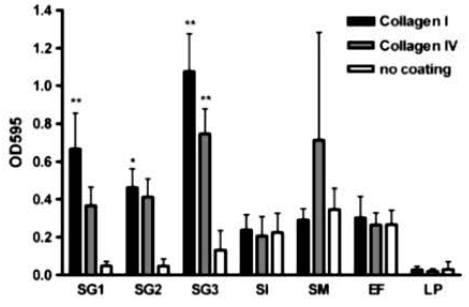

Figure 4. Bacterial biofilm formation. Biofilm formation of gram-positive bacteria on uncoated polystyrene and collagen type I– and type IV–coated surfaces. $^{*}P < .05$; $^{**}P < .01$ (2-way analysis of variance with Bonferroni posttests). *EF, Enterococcus faecalis; LP, Lactobacillus plantarum; SG1, Streptococcus gallolyticus* subsp *gallolyticus* UCN34; *SG2, S. gallolyticus* subsp *gallolyticus* 1293; *SG3, S. gallolyticus* subsp *gallolyticus* NTB1; *SI, Streptococcus infantarius* subsp *infantarius; SM, S. gallolyticus* subsp *macedonicus*.

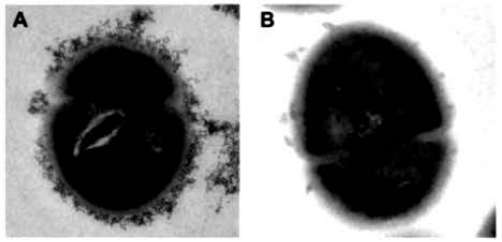

Figure 5. Distinct surface structure of *Streptococcus bovis* strains. Electron micrographic image of representative cells from the *Streptococcus gallolyticus* subsp *gallolyticus* UCN34 (*A*) and *S. gallolyticus* subsp *macedonicus* (*B*) strains, illustrating the different surface structures of these closely related strains.

unpublished data), for which no translocation was observed. These data suggest that pilluslike surface structures of *S. gallolyticus* subsp *gallolyticus* are important determinants for entry into the human body.

The third crucial step to establish an infection is the escape from the host immune system. On passage of the intestinal wall by a pathogen, immune cells in the lamina propria are normally alerted by the production of (for example) interleukin 8 and 1β originating from epithelial cells [36, 37]. In this study, we showed that epithelial cells were relatively unresponsive to *S. gallolyticus* subsp *gallolyticus* compared with other intestinal bacteria, which will delay *IL-8* and *IL-1β* gene expression on its infection. Notably, a functional Toll-like receptor 2 pathway is present in Caco-2 cells [38, 39], which is underscored by *IL-8* and *IL-1β* induction after exposure to *E. faecalis*. This implicates that the unresponsiveness to *S. gallolyticus* subsp *gallolyticus* is not due to lack of Toll-like receptor 2–mediated recognition of gram-positive bacteria. In contrast, macrophages infected by *S. gallolyticus* subsp *gallolyticus* yielded immune responses similar to other bacterial strains, and phagocytosed *S. gallolyticus* subsp *gallolyticus* were unable to escape from macrophages. Together, these findings suggests that the increased incidence of these infections in patients with CRC relates (in part) to a reduced epithelial immune response to these bacteria and subsequent delayed recruitment of tissue macrophages but not to resistance to macrophage-mediated killing itself, which increases the chance that *S. gallolyticus* subsp *gallolyticus* will reach the circulation after translocation of the bowel wall.

The final phase in the infective process toward bacterial endocarditis is survival in the bloodstream and infection of the heart endothelium. In general, gram-positive bacteria are relatively resistant to complement killing [40]. The fact that silent *S. gallolyticus* subsp *gallolyticus* infections can occur is nicely illustrated by the fact that this bacterium was found in the blood of a "healthy" blood donor who appeared to have a colon malignancy upon endoscopic examination [41]. Furthermore, we have shown elsewhere that patients with early-stage CRC can have increased antibody titers against *S. gallolyticus* subsp *gallolyticus* antigens without clinical signs of infection [42, 43]. Our observation that these bacteria have an advantage over other gram-positive intestinal bacteria in forming biofilms on collagen types I and IV may be crucial for explaining the pathology of *S. gallolyticus* subsp *gallolyticus* endocarditis. The aforementioned pilus structures may also, in addition to their role in paracellular transport, play an important role in the binding of these bacteria to extracellular matrix proteins [31, 44, 45], which is especially evident from the fact that one of the *S. gallolyticus* subsp *gallolyticus* pilus operons encodes a collagen-binding protein [17, 32]. Thus, a prerequisite for *S. gallolyticus* subsp *gallolyticus* to establish a clinical infection in patients with CRC seems to be the coincidental presence of collagens at the secondary infection site, including damaged heart valves, hepatic cirrhosis, and total knee replacements [46], which could explain the low co-occurrence of *S. gallolyticus* subsp *gallolyticus* infections in patients with CRC (estimated at <.1%). Although *S. infantarius* subsp *infantarius* has a similar translocation efficiency, it lacks the improved ability to form biofilms on collagen, in line with the fact that *S. infantarius* subsp *infantarius* is less often found in endocarditis but is more often the cause of bacteremia in patients with CRC [47].

Based on our current comparative virulence analysis, the most outstanding characteristic of *S. gallolyticus* subsp *gallolyticus* is its ability to form biofilms on collagen-rich surfaces. This finding inspired us to mine the scientific literature for additional links between *S. gallolyticus* subsp *gallolyticus*, collagens, and CRC. Intriguingly, this yielded previous histologic observations that polyps and early colorectal tumors are characterized by a continuous expression of collagen type IV in basement membranes that surround the crypts in the mucosa of hyperplastic polyps [48]. Accordingly, the collagen type IV containing basement membrane showed sawlike protrusions into the basal parts of the adenomatous epithelium [49]. In this situation, opportunistic pathogens such as *S. gallolyticus* subsp *gallolyticus*, which have low adhesion to epithelial cells, could gain a competitive advantage in colonizing these (pre)malignant sites. This may very well explain why these bacteria colonize only 10% of the normal population, compared with >55% of patients with CRC [14]. Furthermore, recent molecular analyses have indeed pointed toward increased colonization of CRC tissue by *S. gallolyticus* subsp *gallolyticus* strains [50] (unpublished observations).

Summarizing the above, we hypothesize that 2 surface features of *S. gallolyticus* subsp *gallolyticus* are the main determinants for its specific association with endocarditis and CRC. First, it contains a polysaccharide capsule that lowers its adhesive capabilities to epithelial cells, but this same capsule allows it to stay invisible for the host immune system for a prolonged period of time. Second, *S. gallolyticus* subsp *gallolyticus* contains pilus-like structures that facilitate colonization of polyps and adenomatous epithelium, paracellular translocation and the formation of resistant vegetations on collagen-rich sites. A model that summarizes these virulence features is depicted in Figure 6. It goes without saying that our experiments did not fully mimic the complex ecosystem of the gut or the human immune system and that future studies should concentrate on the actual role of the virulence factors highlighted here (ie, capsular polysaccharides and pili) in invasion, immune evasion, or biofilm formation. Preferably this would be done by evaluating mutant *S. gallolyticus* subsp *gallolyticus* strains in live infection models. Nevertheless, our in vitro approach allowed us to gain new insights into the infective mechanisms used by *S. gallolyticus* subsp *gallolyticus* in patients with CRC, which provides clear leads for these future explorations. Finally, our study underscores the importance of proper microbiologic

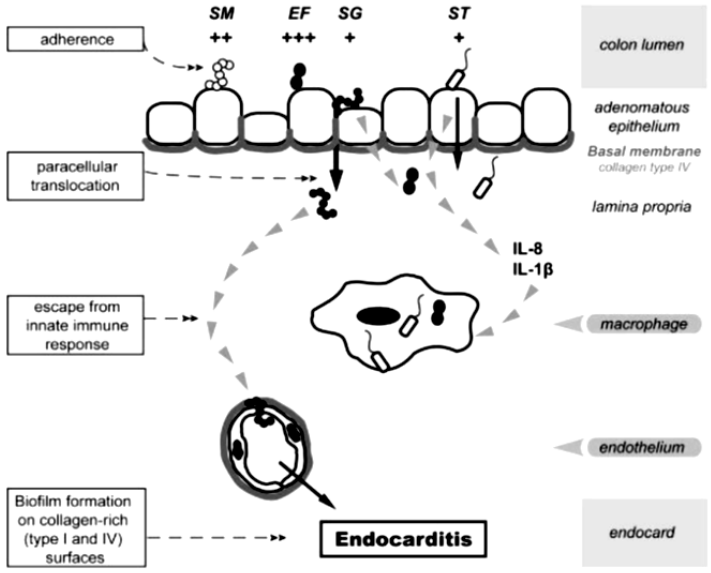

Figure 6. Model for specific association of *Streptococcus gallolyticus* subsp *gallolyticus* (*SG*) endocarditis with colorectal cancer (CRC). Based on our data and that of others, we postulate that *S. gallolyticus* subsp *gallolyticus* is an inefficient colonizer of a healthy intestinal tract and that it benefits from adenomatous epithelial tissue with displaced collagen type IV expression to translocate the epithelium via a paracellular mechanism. In comparison, the closely related strain *S. gallolyticus* subsp *macedonicus* (*SM*) is very effective in adhesion but unable to cross an epithelial layer. On infection, *S. gallolyticus* subsp *gallolyticus* is relatively invisible for the epithelial immune system causing a delayed recruitment of tissue macrophages, which increases its chances to reach the bloodstream. In contrast, other invading bacteria, such as *Salmonella typhimurium* (*ST*) and *Enterococcus faecalis* (*EF*), induce a (strong) epithelial immune response in which these infections are readily cleared by attracted macrophages. It is important to note that *S. gallolyticus* subsp *gallolyticus* infections remain subclinical in most individuals owing to low virulence in humans. However, in a very small fraction of patients with CRC and coincidental collagen depositions at, for example, damaged heart valves, these infections can become clinically manifest through the effective formation of resistant bacterial vegetations, which present as endocarditis. IL, interleukin.

classification of *S. bovis* subspecies. Because only *S. gallolyticus* subsp *gallolyticus* seems to have virulence characteristics that clearly associate endocarditis with underlying colon malignancies, the specific diagnosis of *S. gallolyticus* subsp *gallolyticus* infection might become a valuable tool for the early detection of CRC.

Supplementary Data

Supplementary data are available at http://www.oxfordjournals.org/our_journals/jid/online.

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