Adaptive gene expression of endocarditis-causing viridans group streptococci and *Staphylococcus aureus*

Aldwin J.M. Vriesema

P108

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Success is the ability
to move from failure to failure
without losing your enthousiasm.

(Winston Churchill)

Aan Martine

CONTENTS

CHAPTER 1

General Introduction

Infective endocarditis (IE) has long been recognized as an important human endocardial disease (1). In the preantibiotic era this disease always had a fatal outcome. After the discovery of penicillin in the late 1920s the mortality rates have dropped drastically. However, despite the availability of a broad range of different antibiotics and of novel surgical techniques nowadays, morbidity and mortality remain high.

The classification of IE as acute, subacute, or chronic, indicating the interval between the start of the clinical manifestations and death, dates back to the preantibiotic era. Acute endocarditis was defined as a rapid fatal disease with death occurring within several weeks, and was mostly caused by virulent microorganisms, including *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. Subacute and chronic endocarditis, mostly caused by viridans group streptococci, had a more indolent disease progression. However, objections to this classification, including the largely arbitrary division based on the duration of the illness, have resulted in new classification schemes. At present, IE is classified as native valve endocarditis (NVE), prosthetic valve endocarditis (PVE), or endocarditis in intravenous drug abusers (IVDU), and the microorganism involved defines the type of IE. Disease definition is mainly based on clinical and pathological findings, which are gathered into identification schemes like the Von Reyn or the more recent Duke criteria (2,3).

The demographic characteristics of IE have changed over the past decades, with different factors contributing. The age of the patients contracting disease has increased, with a growing number of patients over 60 years. Furthermore, the predominant type of underlying heart disease, as predisposing factor for IE, has changed. Rheumatic heart disease has decreased dramatically as underlying cause, compared to the incidence in the beginning of this century. Congenital heart disease is still a major clinical finding, mainly in pediatric patients with IE. In elderly patients the predisposing endocardial damage is mostly the result of mitral valve prolapse or degenerative heart disease. In addition, the implant of prosthetic heart valves, a surgical intervention applied worldwide nowadays, has been associated with the initiation of IE (PVE). In an increasing number of cases, especially in intravenous drug users, no underlying cardiac disease is present (4). The etiological agents involved in IE have also changed. Although the viridans group streptococci are still the predominant cause of NVE, the frequency of NVE cases caused by *Staphylococcus aureus* is increased over the last decade. This might complicate treatment of IE, as antibiotic resistance is a widespread phenomenon within this latter bacterial species. PVE is predominantly caused by coagulase-negative staphylococci in the first year after surgery.

Over the past century the pathogenesis of IE due to viridans group streptococci has been studied extensively. This has resulted in a detailed insight into the initiation and progression of IE (Fig.1). Initial damage to the endocardial lining or the heart valves leads to accretion of blood components, mainly blood platelets and fibrin, onto the lesion. The resulting thrombus (or vegetation) becomes colonized after adherence of bacteria which are transiently present in the bloodstream. Bacterial growth within such a vegetation induces ongoing local

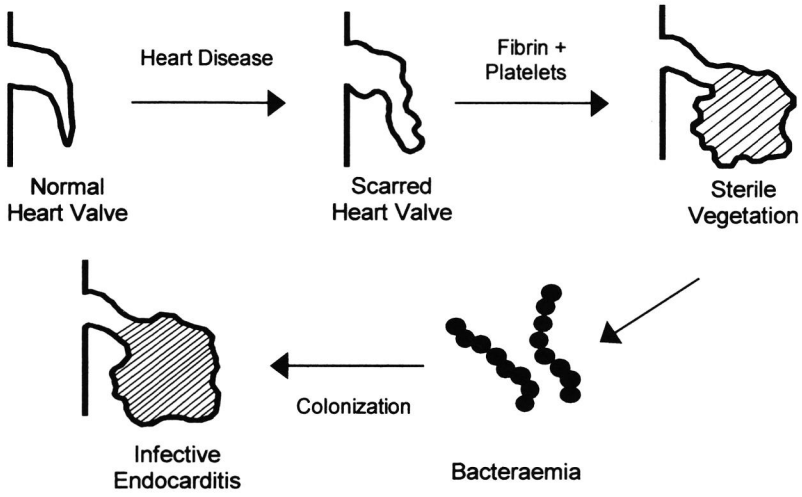

Figure 1.

Schematical depiction of the progressive steps in the pathogenesis of infective endocarditis. Modified from Durack and Beeson (5).

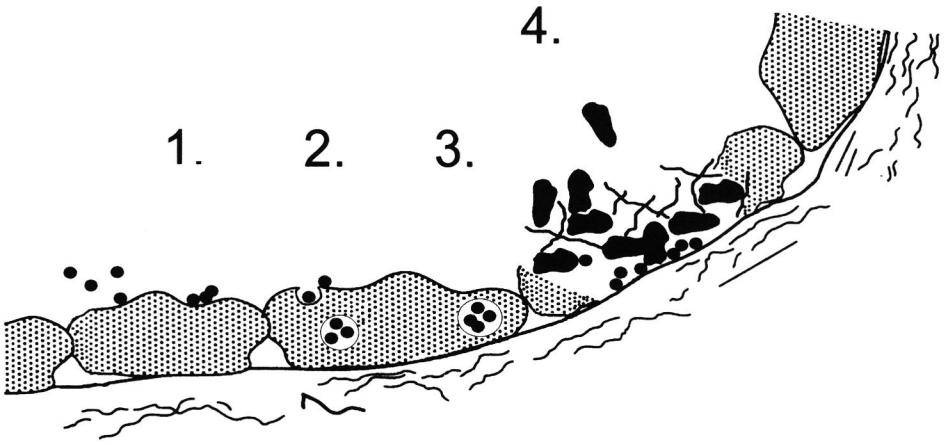

Figure 2.

The interaction of *Staphylococcus aureus* with human vascular endothelium. The successive events of (1.) bacterial binding, (2.) internalization, (3.) persistence, and (4.) cellular damage followed by platelet and fibrin deposition, result in the onset of infective endocarditis.

coagulation, leading to an increase in the size of the vegetation. Embolic events, major heart failure, and bacterial sepsis are the most often observed complications, causing severe morbidity and even death.

Underlying heart disease appears no strict prerequisite in the onset of IE. Strikingly, *S. aureus* is the predominant pathogen isolated from cases without known prior heart disease or heart abnormality, which is possibly due to the tropism of this microorganism for endovascular tissue (6). Binding of *S. aureus* to vascular endothelial cells is followed by uptake of the bacteria by these cells. *S. aureus* are able to persist in this cellular environment for prolonged periods of time, and eventually their presence leads to cellular damage. The exposure of the subcellular matrix triggers deposition of blood components, which then can lead to IE (Fig.2).

Most IE research over the past decade has either focussed on extracellular bacterial structures that are possibly involved in disease development, or on cellular and immunological host responses (7-11). The adaptive processes of the bacterium necessary for survival in hostile niches in the host, and presumably required for its virulence, remained largely unknown upto now. Recently two novel molecular genetic techniques, IVET (*In Vivo* Expression Technology) and STM (Signature Tagged Mutagenesis), were implemented in IE research (12-16). Both these techniques contemplate the detection of genes that are of importance for bacterial virulence or survival. Using IVET, promoter regions of genes can be identified that are activated under specific physiological conditions, through differential expression of two promoterless selection markers. STM involves the identification of genes that are important for bacterial survival, by detection of tagged transposon mutants which have reduced survival capacities under certain (*in vivo*) conditions.

Scope and outline of this thesis

Knowledge on the regulation of bacterial gene expression in specific niches in the human host can result in novel understandings regarding the development of IE. The scope of this thesis is, therefore, to obtain insight in the adaptive response of both viridans group streptococci and *S. aureus* to their environment. Assessment of the adaptive bacterial gene expression involved was carried out by both IVET and transposon-based techniques.

In Chapter 2 of this thesis an overview of the current knowledge on NVE, the virulence characteristics of the most often encountered pathogens, and a description of new research methods in this field are presented. The development of various molecular genetic tools required for the research on viridans group streptococci is described in Chapters 3 and 4. These tools were applied for the identification of genes from *Streptococcus gordonii* CH1 which are activated by specific environmental stimuli encountered in the host during the pathogenesis of IE, including neutral pH and iron limitation (Chapters 4 to 6). In addition, the growth characteristics of different viridans group streptococcal strains within platelet-fibrin thombi, which might represent major virulence determinants in IE, were studied in Chapter 7.

The interaction of *S. aureus* with human vascular endothelium, and the bacterial response to this niche, are addressed in Chapter 8. Finally, in Chapter 9 the significance of the obtained results in the light of infective endocarditis is discussed.

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CHAPTER 2

Native valve infective endocarditis: streptococcal and staphylococcal characteristics and the search for novel bacterial virulence traits

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1. Introduction

Since William Osler reported in his Gulstonian lectures in 1885 on what is nowadays known as infective endocarditis (IE) (138), many researchers have studied the pathogenesis of this disease. Although the incidence of IE has dropped in the post-antibiotic era, it still has high morbidity and mortality rates (62, 79, 83, 152, 160). Additionally, the rising frequency of antibiotic-resistance in microorganisms causing IE urges the need for new insights in the onset and development of this disease, by which new approaches for prevention and treatment of IE can be provided. Infective endocarditis can be categorized into native valve endocarditis (NVE), early and late prosthetic valve endocarditis (PVE), and endocarditis in intravenous drug users (IVDU). Although an increase is observed in both PVE and IVDU cases, NVE still remains the major clinical entity of IE (157) and will therefore be the focal point of this review. The review focusses on the pathogenesis of native valve infective endocarditis (NVE), the bacterial characteristics involved, and the interaction of those bacteria being a common cause of NVE with the various host factors encountered. Additionally, novel research methods and initial results obtained are discussed that can provide new insights into disease initiation and progression.

Epidemiology

The epidemiology of NVE has evolved over the past decades. Mitral valve relapse and degenerative valvular disease have replaced rheumatic heart disease and congenital heart defects as the most common predisposing conditions (125). Additionally, infection of the undamaged endocardial lining has been recognized as an important cause of NVE (125, 159). The incidence of IE in general varies between 0.7 and 6.8 per 100,000 population per year (41, 79, 189). Nowadays, the overall mortality ranging from 13% (79) to as high as 56% for patients with nosocomial endocarditis (54), is mainly the result of congestive heart failure (CHF), sepsis, and embolic events (152). Advanced age is considered an important risk factor for IE (41, 166, 194). Especially due to the growing proportion of elderly people in the population, IE is of increasing medical importance in most developed countries.

Etiology

In NVE, a subacute and an acute disease progression can be discriminated, determined in particular by the etiological agent (71). The most common bacteria causing NVE are viridans group streptococci (VS) and *Staphylococcus aureus* (79, 152). The high frequency of IE caused by these microorganisms may be due to the fact that Gram-positive bacteria are more capable of adhering to damaged endocardial surfaces than Gram-negative bacteria (61, 67, 114). Viridans group streptococci (VS), forming a significant part of the normal flora of the human oral cavity and upper pharyngeal tract (60), account for 30-60% of all NVE cases (45, 79, 152, 165). Disease progression of NVE caused by this group of bacteria is usually

subacute. The second most prevalent bacterial species in NVE is *S. aureus*. This species colonizes the nasopharynx and the upper pharyngeal tract in 30-50% of healthy adults (113). *S. aureus* is of increasing importance as etiologic agent in NVE, nowadays accounting for 25-35% of cases (9, 45, 79, 152, 198). Progression of NVE caused by this bacterium is mostly acute. In about 30% of the *S. aureus* NVE cases, no prior heart abnormalities are recognized (85, 125, 159). In these cases IE is suggested to be caused by the ability of *S. aureus* to directly interact with the undamaged endothelial lining of the heart (137).

Treatment and prevention of NVE; antibiotics and beyond

In the pre-antibiotic era the mortality rate for NVE was 100%. Mortality dramatically decreased with the introduction of antibiotics, and surgical intervention has offered additional chances for cure and long-term survival (53). Although guidelines for the antibiotic treatment of NVE are regularly revised (32, 161), antibiotic treatment is not always successful and sometimes even has adverse effects (149). This is in part caused by the development of tolerance and resistance of the causative microorganisms to the antibiotics (48, 59, 98, 121, 123). Additionally, the site of infection maybe incomplete sterilized due to restricted diffusion of the antibiotics used (11, 29, 31). Therefore, prolonged treatment for many weeks is required. A complication observed with prolonged use of antibiotics in IE patients, however, is the development of neutropenia, necessitating discontinuation of the therapy (15).

New therapeutic strategies have been developed in recent years to overcome some of these problems. The use of granulocyte colony stimulating factor (G-CSF), a hematopoietic glycoprotein hormone that promotes proliferation, differentiation, survival, and function of neutrophils, was demonstrated to accelerate disease clearance in neutropenic patients (15). Also the use of antiplatelet agents (aspirin, ticlopidine) and of tissue plasminogen activator (tPA) have been demonstrated in both animal models and in humans to have a positive effect on the treatment of NVE (55, 133, 134, 177). Recently, lysostaphin, a peptidase which specifically cleaves the interpeptide bridges of the *S. aureus* cell wall peptidoglycan, was found to be one of the most effective agents in sterilizing vegetations infected by methicillin resistant *S. aureus* in a rabbit model of IE (28). This observation underscores the potential use of such agents for therapeutical purposes in NVE.

Other new strategies for the prevention and treatment of IE include the development of specific vaccines. In animal models, vaccination with whole bacteria was found to reduce the development of IE caused by *Streptococcus sanguis* (44, 154), *Streptococcus mutans* (44), *Streptococcus pneumoniae* (1), and *Streptococcus defectivus* (162, 188). More recently, immunization with recombinant FimA, a surface-associated adhesin from *Streptococcus parasanguis* involved in the initial colonization of damaged heart tissue (17), was demonstrated to be very successful in reducing disease development in rats (196). Active immunization with the recombinant fibronectin binding protein (FnBP) from *S. aureus*, or passive immunization with IgG from rabbits immunized with a *S. aureus* type 5 capsular

polysaccharide conjugate showed similar results (105, 156). The RNAIII activating protein (RAP), involved in expression of RNAIII, has recently been demonstrated to be a good vaccine candidate against *S. aureus* infections (7).

Nevertheless, as for other prevention and treatment strategies, prevention by the vaccine approach is not always obtained. For example, rats actively immunized against type 1 or type 5 capsular polysaccharides from *S. aureus* were not protected against IE caused by the homologous strain (132). Although in most vaccination studies antibody responses are obtained, the antibodies are not always protective against infection (104), and the prevention by vaccination against IE is never 100%. Additionally, the efficacy of such vaccines in humans, and the effects on the normal human bacterial flora are largely unknown. These findings therefore stress the need for new insights into the pathogenesis of NVE, in order to come to novel treatment strategies.

2. Pathogenesis of NVE: bacterial virulence determinants and the bacterial interaction with the host

The initial event in the pathogenesis of NVE is the formation of a sterile vegetation (VG), a thrombus mainly consisting of platelets and fibrin (2, 42), a socalled nonbacterial thrombotic endocarditis (NBTE). VGs develop after injury of the heart valves or the endocardium. Such damage is most often caused by hemodynamic abnormalities, like regurgitant blood flows, or the presence of high-pressure gradients, due to narrow orifices associated with ventricular septum defects or valvular deformities (118). In patients with rheumatic heart disease, immune complex deposition can lead to the initial endocardial or valvular damage. Also many forms of exogenous stress, such as infection, hypersensitivity states, high cardiac output states, or hormonal manipulations, may lead to the production of NBTE. Damage of the endocardial lining exposes the subendothelial matrix (collagen, tissue factor) to the bloodstream, triggering the initiation of blood coagulation. This is accompanied by the deposition of fibrin and platelets from the bloodstream onto the endocardial lesion, through local activation of thrombin and by release of soluble factors such as ADP and thromboxane A_2 from activated platelets (16). The resulting VGs can become colonized by bloodborne bacteria during bacteremia, most often transient. The bacteria adhere directly to fibrin or activated platelets present in VGs, or indirectly via bridging molecules like fibrinogen, fibronectin, collagen, or laminin (discussed later). Rapid multiplication of the bacteria inside the thrombus, as demonstrated in the rabbit model of IE (36, 43), results in the accretion of more platelets and fibrin at the site of infection. Bacterial reseeding of the VG and dissemination to other organs occurs by transient release of bacteria from the VG. The growing VG can ultimately cause embolization or major heart failure.

Although NBTE in most cases is the first step towards NVE, not all NVE patients are known to have predisposing abnormalities prior to disease (125, 159). In these patients NVE is thought to result from direct interaction of the bacteria, particularly *S. aureus*, with the endocardial cells. Internalization of such bacteria by these endothelial cells eventually results in cellular damage due to bacterial toxin production (191) or through apoptosis of the host cells, induced by the presence of the bacteria (127, 201).

Many bacterial determinants of both VS and *S. aureus* have been characterized over the past years, that are known or suggested to be involved in the initiation or progression of NVE. In addition, the interaction of these bacterial (virulence) factors with cellular components of the host is of major importance in disease development, and has been extensively studied.

2.1 Viridans group streptococci in the pathogenesis of NVE

VS are part of the the normal oral microflora of humans, and are in general not regarded important pathogens. However, these bacteria are capable of inducing opportunistic infections, and are identified as the most prevelant etiologic agent in NVE. Various properties of VS play a role in the onset of NVE.

Exopolysaccharides

One of the first recognized virulence factors of VS involved in NVE is the ability to produce exopolysaccharides (EPS). These comprise capsular polysaccharides, which constitute the capsule or glycocalyx, and dextrans like glucans and fructans. The production of capsular polysaccharides by VS is found to correlate with signicantly larger VGs, and with a reduced ability to clear these VGs with antibiotics (33, 34, 129, 146). Glucans and fructans can only be formed from sucrose by bacterial glucosyl- and fructosyltranferases, and are therefore different from the capsular polysaccharides (13, 96). As sucrose is not present in the bloodstream, glucans and fructans presumably can not be produced by VS during growth inside VGs. The major contribution of these polysaccharides to virulence is considered to be their involvement in the initial binding of VS to the VG after they have entered the bloodstream (148), a crucial step in the initiation of NVE (2, 155, 155). An isogenic mutant of *S. mutans* V403, defective in three glucosyltransferases and one fructosyltranferase, showed a 46% reduction in IE development (131). In contrast, no difference in IE frequencies due to *S. gordonii* CH1 and its isogenic mutant, defective in its single glucosyltransferase, was observed (200). Therefore, the involvement of glucans and fructans in IE induction might not be the same for the various VS strains or species (168), which is illustrated by the fact that only 53% of VS isolates from IE cases were capable of producing EPS (139). Additionally, the *in vitro* capability to produce dextrans not necessarily correlates with the ability to cause IE (96).

Adhesins

Both VS and *S. aureus* are able to bind to many different host components, including matrix molecules like fibronectin, fibrinogen, laminin, collagen, vitronectin, elastin, and thrombospondin, and cellular components like platelets (reviewed in (4, 85, 96, 168)). The family of bacterial surface proteins that enable binding to extracellular matrix components is referred to as MSCRAMM (microbial surface components recognizing adhesive matrix molecules) (141, 142). Several VS adhesins for host cellular matrix molecules are implied to be involved in the pathogenesis of NVE (Table 1). The FimA adhesin of *S. parasanguis* can, apart from binding to saliva-coated hydroxyapatite, mediate binding to fibrin substrates (17). FimA was demonstrated to be a major virulence determinant in experimental IE, as both deletion of the corresponding gene and immunization of animals with the protein resulted in a dramatic decrease in the infective propensity by this strain (17, 196). FimA belongs to a group of related oral adhesins, which include the SsaB of *S. sanguis* (63) and the ScaA of *S. gordonii* (97), and FimA-like proteins are identified in many different VS species (196). Like FimA, these related proteins might be involved in onset of NVE, although this remains to be determined. In contrast, the antigen I/II or P1 protein of *S. mutans*, another bacterial adhesin important for oral colonization, was found not to play a role in IE (150).

VS are also able to bind laminin, a constituent of the basement membrane underlying the endothelium (176, 193). This binding may be of importance in disease initiation, as VS isolates from IE cases in general bind better to this matrix molecule compared to oral isolates (176). *S. mitis* strains express a limited number of laminin receptors, that bind laminin in a high-affinity interaction (176). The 145-kDa laminin binding protein of *S. gordonii*, which is also able to bind type I collagen, is recognized as a major antigen from the sera of patients with NVE (163). These results suggest a dinstinct function for the bacterial laminin binding proteins in NVE.

Another important matrix molecule is fibronectin. VS can bind to this component (112, 193, 202), which is an important constituent of endocardial VGs (69). In an early study, Scheld and coworkers reported a correlation between the fibronectin binding capacity of certain VS and endocarditis production in rabbits (153). Additionally, *S. sanguis* mutants reduced in their capacity to bind fibronectin, were also reduced in the ability to produce experimental IE (111).

In most VS teichoic acid and lipoteichoic acid (LTA) molecules extend from the cell membrane to the bacterial outer surface (175). LTA has been implied to be a virulence factor in IE (114), possibly by acting as an adhesin (82). LTAs are polymers of ribitol phosphate and glycerol phosphate, linked to glycolipids or glycophospholipids in the bacterial cell membrane (175), and being both polyanionic and hydrophobic in nature. Inhibition of LTA production by subinhibitory concentrations of penicillin resulted in a decreased binding of *S. sanguis* to platelet-fibrin surfaces *in vitro*, as well as in a decreased incidence of experimental IE in a rabbit model (114). More recently it was found that dextran-producing *S. sanguis* and

Table 1. Host components and VS adhesins or binding structures suggested to be involved in IE.

Host structure/matrix molecule	*Bacterial species*	Bacterial structure	IE "model"	Involvement in IE	References
vegetation	*S. mutans*	glucan/fructan	rat	yes	131
	S. gordonii	glucan	rat	no	200
platelet-fibrin surface	*S. sanguis*	LTA	rabbit	yes	114
salivary pellicle / fibrin	*S. parasanguis*	FimA	rat	yes	17, 196
salivary pellicle	*S. mutans*	P1 protein	rat	no	150
laminin	*S. mitis*	unknown protein	human	possible	176
	S. gordonii	145-kDa surface protein	NT	unknown	163
collagen type I	*S. gordonii*	145-kDa surface protein	human	possible	163
fibronectin	*S. sanguis*	unknown	rabbit	yes	153
	S. sanguis	unknown	rat	yes	111
vascular endothelial cells	*S. gordonii*	153-kDa glucosyltransferase	NT	unknown	185
	S. mutans	protein I/IIf and RGPs	NT	unknown	195
platelets	*S. gordonii*	PAAP	rabbit	yes	76

NT, not tested

S. mutans accumulate significantly more LTA at their surface, and that the amount of this cell surface LTA is reduced by dextranase treatment (81). Thus, the differences in IE induction due to variation in EPS production, as discussed before, possibly reflect differences in the amounts of LTA present at the bacterial surface.

Apart from binding to the VG, VS are also able to directly interact with vascular endothelial cells. Surface located glucosyltransferase of *S. gordonii* was found to mediate binding to human umbilical vein endothelial cells (HUVEC) (185). Additionally, protein I/II and rhamnose-glucose polymers (RGPs) of VS are involved in this process, resulting in the induction of cytokine production (IL-6 and IL-8) by the endothelial cells. Protein I/II binds through lectin interactions, and probably recognizes N-acetyl neuramic acid (NANA) and fucose as the cellular surface receptor (195). The role of this interaction in the initiation of IE remains to be established.

Bacterial toxins and exoenzymes

VS excrete an α-hemolysin, which *in vitro* degrades hemoglobin from red blood cells. The α-hemolysin of *S. gordonii* CH1 was demonstrated to be hydrogen peroxide (8). Although this toxin is a potential virulence factor, it is unknown whether this α-hemolysin is involved in the onset of NVE. Additionally, putative VS toxins have been identified that may cause fever and other septic signs in endocarditis patients (122, 178). LTA of VS might also exert such action (47).

VS produce several proteases and other enzymes that are of particular interest in IE (65, 89, 124, 167). *S. sanguis* has activities related to the formation (thrombin, activated protein X, and Hageman factor) and dissolution (kallikrein, plasmin, and activated protein C) of blood clots (124). Nevertheless, it remains to be determined if *in vivo* these activities enhance the progression of NVE.

Bacteria-platelet interactions

Platelets are important components in the development of IE. After the initial damage to the endocardial lining, platelets are bound to collagen in the subendothelial layer. Local thrombin activity induces aggregation of platelets, and initiates fibrin deposition onto the lesion. In addition, thrombin-activated platelets bind very efficiently to endocardial cells (87). The importance of normal platelet function in experimental IE was pointed out by the finding that pigs with a severe form of Von Willebrand disease failed to develop IE (86, 173).

NVE-causing VS strains can bind and activate platelets in the presence of plasma proteins (172, 174). This interaction *in vitro* leads to platelet degranulation and aggregation, resulting in the formation of microthrombi (75). The importance of bacteria-platelet interactions in IE in humans was demonstrated in a study of Erickson and Herzberg (76), who provided evidence for a relationship between the platelet interactive phenotype of *S. sanguis* strains and the clinical course of the disease. Although platelet aggregation by VS is not an absolute

prerequisite for IE development (120), strains capable of aggregating platelets caused significantly larger VGs with higher numbers of bacteria, more severe clinical symptoms including pulmonary hypertension and ischemia, and higher mortality (68, 76, 128). Additionally, IE patients suffering severe disease are more often infected with platelet-aggregating VS than patients with mild symptoms (40). Thus, the ability of VS to interact with platelets does seem to increase the bacterial virulence.

Erickson and Herzberg identified a platelet-interacting structure on the surface of *S. sanguis* (49, 50). This adhesin, platelet aggregation-associated protein (PAAP), is synthesized as a 150 kDa glycoprotein containing 40% carbohydrate, and contains a domain [PGE(P/Q)GPK] conform to the consensus motif of the platelet-interactive domain of collagens (51). *S. sanguis* carrying PAAP on its surface induced significantly larger VGs, a more severe clinical disease course, more gross lesions in different organs and greater mortality, as compared to a PAAP deficient strain or to the PAAP expressing strain pretreated with PAAP-specific antibodies (76). Expression of PAAP was upregulated upon growth in the presence of certain collagen types (52). In addition, its putative platelet receptor counterpart was identified using anti-idiotypical monoclonal antibodies (66).

Certain *S. sanguis* strains express ecto-ATPases, that can hydrolize ATP released from the platelets to ADP (116). As ADP is a platelet agonist, it can amplify the platelet aggregation reaction. EPS of VS reduced the bacterial binding to platelets, but different strains were used in this study (169). Therefore, the influence of strain-dependent factors may have influenced the findings. The bacteria-platelet interaction is multimodal, and involves, apart form bacterial components and cellular adhesins, specific plasma components such as fibrinogen, IgG, and complement (57, 58, 171, 174).

Platelets also have an important role in the host defense against invading microorganisms, as was recently reviewed (205). Already in the early seventies proteins were isolated from platelets, exerting an antimicrobial effect (39, 179, 199). Such proteins have been refered to as β-lysin (39), platelet bactericidal protein (199), platelet microbicidal proteins (207) or thrombocidins (36, 99, 100, 211). More of these proteins were recently purified from rabbit platelets after acid extraction or thrombin stimulation (210). The proteins are small in size (6 - 40 kDa) and cationic in nature, and are stored in the α-granules of platelets (35). In experimental IE studies it was found that these proteins influenced the onset of IE (35, 36). Indirect evidence for a role of these proteins in the initiation of IE development was found from studies on the effect of thrombocytopenia on streptococcal IE in rabbits (170). Thrombocytopenic rabbits were found to have higher bacterial densities of *S. sanguis* within VGs and higher numbers of bacteria per valve. Antimicrobial proteins from thrombin-stimulated platelets have been implied to be involved in the clearance of adhering VS from VGs in experimental IE (36). Thrombocidin resistant strains adhered less to VGs than thrombocidin sensitive strains (36). The susceptible strains, however, were cleared much more readily from VGs than the resistant strains. It can thus be concluded that there is a dual role

for blood platelets in the pathogenesis of IE, in that platelets can both stimulate and limit disease development.

2.2 *S. aureus* in the pathogenesis of NVE

In contrast to the rather avirulent VS, *S. aureus* is a pathogen involved in many different pathologies. Different *S. aureus* components were found to play a role in disease development in general (113), and several of these factors also appear to be involved in NVE.

Capsule

Most *S. aureus* strains produce a polysaccharide capsule, and 11 serotypes are known to date (104, 164). Type 5 and type 8 are the two major serotypes recovered from human disease, accounting for about 75-85% of all *S. aureus* isolates (26, 164). Circulating antibodies to these serotypes are used for the differential diagnosis of IE in patients with *S. aureus* septicemia (26). The role of *S. aureus* capsular polysaccharides in development of NVE appears to be opposed to that of EPS of VS, as capsule expression was found to attenuate virulence of *S. aureus* in the experimental rat model of IE (5). Isogenic mutants of serotype 5 and serotype 8 strains, deficient or negative in capsule production, had significantly lower ID_{50} numbers than capsule producing parent strains. Therefore, capsular polysaccharides of *S. aureus* may not directly be involved in NVE, but may influence disease indirectly through for example their antiphagocytic action (90, 180).

Cell wall

The cell wall of *S. aureus*, as of other Gram-positive bacteria, is predominantly composed of peptidoglycan, a layer consisting of polysaccharide backbones of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues that are linked by peptide bridges. The introduction of new moietes into this peptidoglycan layer during bacterial growth is in part regulated by the action of autolysins, via controlled digestion of the peptidoglycan layer. One study has indicated a possible role for autolysins in NVE (119). An autolysin-defective *S. aureus* mutant showed attenuated virulence in a rat model of IE, although the mechanism of this virulence attenuation is not known. Additionally, the combination of peptidoglycan and LTA can induce the production of different cytokines by monocytes (14). LTA and a specific part of peptidoglycan from *S. aureus* were recently found to synergize the initiation of shock and organ failure (91). These two components possibly also influence NVE development.

Adhesins

S. aureus has several adhesins for host cellular matrix molecules that are implied to be involved in the pathogenesis of NVE (Table 2). One of the matrix molecules to which *S. aureus* can bind is fibronectin (193). *S. aureus* mutants, reduced in their binding capacity to fibronectin, were also reduced in the ability to produce IE (102). Immunization with

staphylococcal fibronectin-binding protein (FnBP) protected against experimental IE, as bacterial densities in the VGs of immunized rats were over 100-fold lower than in non-immunized rats (156). However, although antibodies to the FnBP are present in patients with staphylococcal infections, these antibodies do not inhibit fibronectin binding *in vitro*. This is possibly due to conformational change of the ligand binding site of FnBP (19), which consists of several repeats. A protective role of these antibodies in IE in humans remains undetermined.

In a study of Moreillon and coworkers (130), staphylococcal clumping factor was found to play a specific role in the pathogenesis of IE. Clumping factor (ClfA) binds fibrinogen (72), and a different but closely related molecule (ClfB), which also binds fibrinogen, has recently been identified (46). ClfA defective mutants bound fibrinogen 100-fold less compared to the parent strain, and infectivity was approximately 50% reduced in the rat IE model. Complementation of the mutation restored both fibrinogen binding and infectivity (130).

S. aureus is able to bind collagen, and the *in vitro* binding capacity of *S. aureus* strains to this matrix molecule is strongly correlated with the presence of the *cna* gene encoding collagen adhesin (143, 151). Collagen binding can also be mediated by *S. aureus* protein A (193). The role of collagen binding in the pathogenesis of IE remains, however, unclear. In single as well as in mixed infections, a clinical *S. aureus* isolate outnumbered an isogenic mutant strain defective in collagen binding in VGs, 24 h after infection. However, one hour after challenge the numbers of both strains were the same. It was concluded that collagen binding enhanced bacterial propagation or survival, although it plays a limited role during the initial attachment (78). Recent reports indicate that collagen binding is equally prevalent among NVE strains as among strains isolated from patients with skin infections (80), and appears to be no major virulence factor specific for IE (151, 181).

In addition to binding to a VG present on an endocardial lesion, *S. aureus* is also able to directly interact with undamaged vascular endothelial cells (70, 115, 137). Four bacterial components (183) and two cellular proteins (84, 184) have been identified in this interaction, although the nature of these structures remains largely unknown. Plasma components, like fibronectin (70, 190) and fibrinogen (23), and the inflammatory mediator TNF-α (22) substantially promote the adherence of *S. aureus* to EC. Internalization of *S. aureus* by endothelial cells was recently found to induce cellular apoptosis (127), and thus could induce the initiation of IE *in vivo*.

Expression of the staphylococcal adhesins is predominantly regulated by the *sar* (staphylococcal accessory regulator) locus, and to a lesser extent by the *agr* (accessory gene regulator) locus. Mutants defective in the *sar* locus or *sar/agr* double mutants had lower infectivity rates than their parent strains (21, 25). Using transcriptional fusions of the three promoter regions of the *sar* gene with the green fluorescent protein (20), it was recently demonstrated that the expression of one of the three different *sar* transcripts is highly induced

Table 2. Host components and *S. aureus* adhesins or binding structures suggested to be involved in IE.

Host structure/matrix molecule	Bacterial structure	IE "model"	IE involvement	References
fibronectin	FnBP	rat	yes	102, 156
fibrinogen	unknown	rabbit	yes	153
	ClfA	rat	yes	130
	ClfB	NT	unknown	46
collagen	CnBP	rat	yes	78
	CnBP	human	no	80, 151, 181
	Protein A	NT	unknown	193
vascular endothelial cells	4 unknown proteins	NT	unknown	183
platelets	unknown	rat	yes	101, 134, 135, 177, 209

NT, not tested

in the VG, in particular at the vegetational surface (24). This might indicate a possible role for *S. aureus* adhesin expression at the site of infection, and thus in disease development.

From all studies on bacterial adhesive components it appears that binding to the VG and damaged tissue occurs through interactions of multiple adhesins with different matrix molecules. This sometimes makes it difficult to define the role of one specific adhesin in disease (56, 130, 202), and can also obscure the correlation between the presence of certain adhesins and their involvement in IE (80, 136, 151).

S. aureus toxins and exoenzymes

S. aureus is able to produce many different toxins. These include enterotoxins A-E, exotoxins α-δ, toxic shock syndrome toxin-1 (TSST-1), leukocidin, and epidermolytic toxins (104, 113). Some of these toxins (enterotoxins, TSST-1, exfoliative toxins) can act as superantigens by direct binding to MHC class II molecules, which results in extensive and uncontrolled T-cell activation (90). Two recent studies, however, showed that patients with subacute NVE are more often infected by *S. aureus* strains that do not produce any enterotoxin or α-toxin (80, 136). Hyperproduction of the α-toxin has even been found to reduce the potential to induce IE (12). Although staphylcococcal toxins are suggested to be virulence factors in other diseases, they thus seem of little importance in NVE, and may even be a disadvantage for *S. aureus*.

S. aureus produces several exoenzymes, including lipase, protease, and hyaluronidase, that are capable of destroying host tissues (104, 113). Although their role in NVE has not been studied, these enzymes can possibly influence IE development. Staphylococcal coagulase, an activator of prothrombin (113), has long been regarded as a major virulence factor in different disease settings. It is, however, not of major importance in the disease process of IE, because a coagulase-deficient isogenenic *S. aureus* mutant behaved similarly as the wild type strain in the experimental rat model of IE, as defined by infectivity, weights of infected VGs, bacterial concentrations within the VGs, and early mortality rates (6, 130).

Bacteria-platelet interactions

The ability of *S. aureus* to interact with platelets (27, 209) clearly plays a role in the pathogenesis of IE. Blocking platelet aggregation with inhibitors of the cyclooxygenase pathway, like for example aspirin or ticlopidine, was found to prevent growth of the VG and strongly reduced both the *S. aureus* numbers in the VG and the incidence of cerebral emboli (26, 101, 134, 135, 177). As is found for VS, the interaction between *S. aureus* and platelets also involves specific bacterial and cellular surface ligands as well as the platelet Fc receptor (27, 209).

The importance of resistance of *S. aureus* to the antimicrobial proteins from platelets in IE has been extensively studied. *S. aureus* isolates from IE cases appear to be less susceptible to the microbicidal action of these proteins than other isolates (10, 204, 206). The resistant

phenotype of *S. aureus* strains is, however, not correlated with the ability to bind and aggregate platelets *in vitro* (206). Relevance of *S. aureus* resistance to the antimicrobial proteins from platelets in IE development was provided in experiments using an *S. aureus* transposon mutant that showed a strongly reduced susceptibility to the antimicrobial proteins compared to its parental strain (38). Although IE induction rates of both strains were equivalent, the resistant mutant reached much higher densities in the VG than the parent strain (37, 38). Resistance to the platelet derived antimicrobial proteins is therefore suggested to be of major importance in the pathogenic process following adherence, like bacterial proliferation within the VG and hematogenous dissemination (37, 38). Apart from the antimicrobial action of these proteins, they also seem to be involved in reducing the capacity of bacteria to adhere to platelets, and block subsequent aggregation *in vitro* (208). Whether this is a mechanism that occurs *in vivo*, remains to be clarified. It is, however, clear that antimicrobial proteins from blood platelets are involved at one or more stages in the development of IE (35).

3. In search of new virulence factors

As discussed in the previous section, both VS and *S. aureus* express many different factors that have been experimentally confirmed or are suggested to be involved in the pathogenesis of IE. However, most of these characteristics are limited to the initial steps in the initiation and progression of IE. More refined mechanisms must exist that allow the bacteria to adapt to and to grow within the specific niche of a VG.

Novel techniques are therefore of increasing importance in studying bacterial pathogenicity. The focus of the last years has mainly been the identification of genes that are expressed in host cells and tissues or in animal models, and which specifically contribute to bacterial survival and virulence in the host (147, 182). These new insights must lead to an expanded knowledge of the regulation of bacterial virulence, and to the identification of targets for new antimicrobial therapies.

3.1 Novel approaches to study bacterial gene expression related to infectious diseases

In vivo complementation

In vivo complementation has been one of the early approaches in the identification of bacterial genes involved in pathogenic processes, and is especially useful when systems for allelic exchange are absent. This technique has successfully been applied in for example the identification of virulence genes of *Mycobacterium tuberculosis* (140). The requirement of

two genetically similar strains, one of which is virulent and the other is not, limits general applicability of the system.

Differential display

Differential display (DD) was developed to identify differences in eukaryotic gene expression, by separating and cloning mRNAs from differentially expressed genes by means of PCR (106). This system has now in addition been applied to identify cDNAs that are specific for bacteria grown in a complex (*in vivo*) environment, and which represent genes that are necessary for bacterial survival or growth under those conditions (103, 144).

In vivo expression technology (IVET)

In order to identify genes of which the expression is induced in the undefined *in vivo* environment (*ivi* genes), Mahan and coworkers developed IVET (*In Vivo* Expression Technology) (117). This is a promoter trap system that makes use of differential expression of two promoterless genes after genomic integration of a non-replicative vector. One gene is used to select for active promoters in the complex (*in vivo*) environment, whereas the other gene discriminates between induced and constitutive promoter activity under regular *in vitro* culture conditions. Extensive analyses have resulted in the identification of over 100 *ivi* genes from *S. typhimurium* that are induced during infection of BALB/c mice or after phagocytosis by cultured macrophages (73). The IVET system has broadly been adopted for research on Gram-negative (18, 92, 108, 197) and Gram-positive bacteria (110) in different disease settings.

Signature tagged mutagenesis (STM)

Signature tagged mutagenesis (STM) has originally been developed for the identification of genes from *S. typhimurium* that are strictly necessary for the *in vivo* survival in a murine model of typhoid fever (74). In contrast to most other selection systems, STM is based on a negative selection scheme. From a pool of tagged insertion mutants, clones are identified that show reduced survival rates in the infection model due to mutation of specific genes. This system has demonstrated its value for studying genes required for survival of Gram-positive bacteria like *S. aureus* (30, 126, 158) and *Streptococcus pneumoniae* (145) in different disease settings.

Differential fluorescence induction (DFI)

A relatively new technique is differential fluorescence induction (DFI) (186). This technique combines the green fluorescence protein (*gfp*) gene from *Aequorea victoria* (20) with FACS-scan analysis, to identify genes that are preferentially expressed when a bacterium associates with host cells. DFI has successfully been applied to assess induced gene expression in *S. typhimurium* upon macrophage infection, identifying several genes involved in intracellular

survival and virulence (186, 187). The use of FACS-scan analysis is a limitation of the system, as it can not be used to study bacterial responses in more complex environments.

Total genomic expession monitoring

Two methods have recently been described, that allow detection of the whole repertoire of gene expression as well as the levels of expression of those genes. SAGE (serial analysis of gene expression) was first used to characterize gene expression in human pancreas (192). The system is based on the detection of short, unique nucleotide sequence tags to identify transcripts, and concatenation of these tags followed by sequencing, to allow an efficient analysis of the total mRNA transcripts. This technique largely depends on effective mRNA isolation. As bacterial mRNAs do not carry a polyadenylation signal, which is exploited to isolate mRNA from eukaryots, application of this method to bacteria still requires extensive method development. The second approach is the micro-array technology (107). This technique involves hybridization of total mRNA populations to small, high-density arrays that contain thousands of synthetic oligonucleotides. Differences in gene expression patterns and levels of expressed genes of the budding yeast *Sacharomyces cerevisiae* grown in rich and in minimal medium could be quantitatively assessed (203). A prerequisite for both techniques is the availability of the complete nucleotide sequence of the organism studied. As an increasing number of bacterial genome sequences are becoming available (3), application of these techniques to study bacterial pathogenicity will only be a matter of time.

The identification of genes with no homology to known sequences or with homology to genes encoding proteins with unknown function, remains a major problem in any of the above approaches for pathogenicity research. In addition, homology of genes from different organisms does not necessarily imply the same function of the encoded proteins. Combination of these novel techniques with other molecular genetic and biochemical methods is pivotal for determination of the precise role and function of identified genes in bacterial pathogenicity.

3.2 Use of novel techniques in IE research

Two of the above mentioned novel techniques have been applied in the research on IE. IVET has been used in the study of two different IE pathogens. Induced gene expression of *Enterococcus faecalis* in a rat model of IE was determined (64) through a genetic recombination approach (18). This system uses a promoterless *tnp*R gene which encodes resolvase, a site-specific recombinase, and a kanamycin resistance marker cloned between two *res* sites on which the resolvase enzyme acts. Activation of the resolvase by an inducible promoter in front of the promoterless *tnp*R gene causes excision of the resistance marker, resulting in a kanamycin sensitive phenotype as an indication for prior gene expression. (18, 109). This approach has identified several induced *E. faecalis* genes after five days of infection in a rat model of IE, although the precise function of the encoding proteins in IE remains to be determined (109). The second species studied more extensively by IVET is the

VS strain *S. gordonii* V288 (77, 93-95). The system used in these studies comprises an integration vector with a dual (*amy-cat*) reporter gene for detection of inducible promoters. In a rabbit model of IE, 13 different inducible genes could be identified 3 days after infection. The inferred functions of these genes can be divided into six categories, being sugar metabolism, resistance to host defense, bacterial growth, transport, regulation, and adhesion (94). It is, however, unknown at what point during the infective process the identified genes are induced, and what the activating signal is.

More recently, STM has been implemented in IE investigations. The high-affinity proline permease (PutP) of *S. aureus* was identified to play a major role in a rabbit model of IE as well as in several other infection models (158). This showed the importance of proline scavenging by these bacteria in *in vivo* growth and proliferation. Analogs of this amino acid can possibly serve as new antistaphylococcal agents (158). The identification of PutP is a prime example of research using the new techniques that offers prospects to the development of novel antimicrobial approaches.

Further STM studies by Coulter and coworkers have identified many different *S. aureus* genes required for bacterial survival during IE and possibly involved in the pathogenesis of this disease (30). The genes identified to be important in a rabbit endocarditis model encoded three different transporter proteins, a lantibiotic homolog, a putative dihydroliposamide succinyltransferase, and a gene with no homology to known entries in the sequence databases. The transporters show homology to similar genes in *E. coli* and *B. subtilis*, and are involved in the peptide transportation. The gene homologous to the dihydroliposamide succinyltransferase of *B. subtilis* is implicated to be involved in staphylococcal capsule production. The lantibiotic homolog showed homology to the *Staphylococcus epidermidis* gene encoding epidermin. In addition, five *S. aureus* mutants impaired in anaerobic growth were found attenuated in the endocarditis model, implicating a low oxygen concentration in the VG (30). Despite the identification of these *S. aureus* genes, much additional research needs to be carried out to assess the actual role of these putative virulence genes.

4. Conclusions

Native valve infective endocarditis remains a major health problem because of high morbidity and mortality rates, especially in developed countries, due to an increase in the population at risk, e.g. the elderly. VS remain the most important etiological agents, and are found in cases with subacute progression of disease. The number of IE cases caused by *S. aureus* is, however, increasing steadily. *S. aureus* is often isolated from patients not known to have prior heart defects. *S. aureus* also causes a more rapid and destructive form of the disease. New diagnostic tools and therapies have facilitated disease detection and treament, but have not been able to eliminate IE.

The bacterial interaction with the host in IE is very complex and multimodal, involving many different bacterial and cellular components. Bacterial exopolysaccharides, cell wall components, adhesins, toxins, and exoenzymes are indicated in evasion of host defense, initial binding to the VG, and bacterial survival and proliferation. Especially the interaction with platelets, which have a function in both progression and limitation of disease, is of major importance in IE.

Novel techniques have been developed in recent years, that have rapidly extended our knowledge on bacterial adaptation and propagation in different disease settings. Two of these methods, IVET and STM, have also been implemented in pathogenicity research in IE, with promising results. With the complete bacterial genome sequences of several endocarditis pathogens becoming available, research on gene expression will be speeded up by the introduction of the micro-array technology. However, additional in-depth biological and biochemical studies on the function of identified bacterial genes and their products are required to determine their precise function in IE disease development, as homology comparisons alone comprise the danger of identifying only virtual virulence factors.

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CHAPTER 3

A simple procedure for the isolation of cloning vectors and endogenous plasmids from viridans group streptococci and *Staphylococcus aureus*.

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ABSTRACT

Isolation of plasmid DNA from viridans group streptococci is difficult, and plasmid preparations are often heavily contaminated with chromosomal DNA. We developed a simple protocol to isolate pure plasmid DNA for use in different molecular techniques, including automated sequencing. The protocol is also applicable for plasmid isolation from *Staphylococcus aureus*. In addition, the protocol allows isolation of pure endogenous plasmids from streptococci and *S. aureus*.

INTRODUCTION

Isolation of plasmid DNA from viridans group streptococci and various other gram-positive bacteria is hampered by the rigidity of the bacterial cell wall. Isolation protocols developed for viridans group streptococci and lactococci (1, 4-6) often yield plasmid DNA contaminated with large amounts of chromosomal DNA. To obtain pure plasmid DNA, such preparations need to be purified by CsCl-ethidium bromide buoyant density gradient centrifugation, which is a time-consuming and laborious step. The availability of a rapid plasmid isolation protocol yielding pure plasmids will facilitate selection and recovery of specific genes using plasmid-based cloning systems, abolishing the need for chromosomal integration of selected DNA fragments.

For our studies on infective endocarditis due to viridans group streptococci, we have developed a plasmid isolation procedure which allows isolation of pure plasmid DNA from these bacteria within several hours. The plasmid DNA is sufficiently pure for restriction analysis, subcloning and electrotransformation, and can be sequenced effectively. In addition, the method is also suitable for the isolation of pure plasmid DNA from *Staphylococcus aureus*.

RESULTS AND DISCUSSION

The protocol was developed using the endocarditis-causing *Streptococcus sanguis* strain U108 (3), carrying *Escherichia coli*-lactococcal shuttle vector pGKV210 (9). The final plasmid isolation procedure is presented in Table 1. Bacteria were grown without shaking in 15 ml of Todd-Hewitt broth (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 5 μg of erythromycin per ml and 0.5% glycine in 15-ml Falcon Bluemax polypropylene conical tubes (Becton Dickinson Labware, Franklin Lakes, N.J.) at 37°C overnight. The presence of glycine as a destabilizer of newly synthesized cell walls was crucial, since bacteria grown in broth not supplemented with glycine lysed poorly or not at all. Protoplast formation was accomplished by digesting the bacterial cell walls with mutanolysin (Sigma Chemical Co., St. Louis, Mo.) and an excess lysozyme (Sigma) in 100 μl of 50 mM Tris/HCl - 10 mM EDTA (pH 8.0) at 37°C for 2 h. Variations to this protocol such as shorter incubation time, excess of mutanolysin combined with a 10-fold reduced amount of lysozyme, or additional treatment of the cells with proteinase K resulted in less-pure plasmid preparations. Protoplasts were lysed by alkaline lysis, as described for *E. coli* (2), to obtain a cleared lysate. After centrifugation, two methods to purify the plasmid DNA from the cleared lysate were compared: (i) three phenol-chloroform extractions, followed by RNase A treatment for 1 h at 37°C and a fourth phenol- chloroform extraction to remove the RNase A;

Table 1. Protocol for isolation of plasmid DNA from viridans group streptococci and *S. aureus*.

Step	Volume
Protoplasting	
Centrifuge bacterial culture for 10 min at 4,000 x *g* in a Minifuge RF (Heraeus)	15 ml
Wash pelletted cells in H_2O and transfer suspension to a 1.5 ml microtube	1 ml
Centrifuge for 1 min at 14,000 rpm in a microcentrifuge (Eppendorf)	
Resuspend pelletted cells in 50 mM Tris/HCl-10 mM EDTA (pH 8.0)	100 µl
Add lytic enzyme mixture (lysozyme [50 mg/ml], mutanolysin [200 U/ml], lysostaphin [0.3 mg/ml])[a]	50 µl
Shake for 2 h at 37°C	
Add 50 mM Tris/HCl-10 mM EDTA (pH 8.0)	200 µl
Lysis	
Add 0.2 N NaOH-1 % sodium dodecyl sulphate	300 µl
Mix immediately by inversion	
Incubate for 10 min at room temperature	
Add 3 M K acetate	300 µl
Mix immediately by inversion	
Incubate for 15 min on ice	
Centrifuge for 15 min at 14,000 rpm	
Purification	
Transfer supernatant to a new microtube	
Extract once with phenol-chloroform (1:1, vol/vol) and once with chloroform	650 µl
Purify plasmid DNA from upper phase with a QIAGEN-tip 20 mini column	
Add isopropanol and mix by inversion	650 µl
Centrifuge for 15 min at 14,000 rpm	
Wash pellet with 70% ethanol	500 µl
Air dry pellet and dissolve in H_2O	30 µl

[a] Lysostaphin is not essential for the isolation of plasmid DNA from viridans group streptococci.

and (ii) one phenol-chloroform extraction and subsequent application of the lysate to a QIAGEN-tip 20 mini column, as described in the instructions of the manufacturer (QIAGEN GmbH, Hilden, Germany). The purities of the plasmid DNA obtained by both methods were similar, but the second method resulted in a two- to threefold-higher yield and was less time-consuming than the first method. Therefore, we used the second method as the standard purification step in our protocol (Table 1). Phenol-chloroform extraction of the cleared lysate prior to application onto the QIAGEN column is crucial, because if this step was omitted, plasmid isolates were always heavily contaminated (Fig. 1; lanes 3 and 4). Protocols supplied by QIAGEN GmbH for the isolation of plasmid DNA from either staphylococci or *Bacillus cereus* using a QIAGEN-tip 20 mini column were inappropriate to obtain purified plasmid DNA from *Streptococcus sanguis* U108. These protocols essentially differ from our protocol

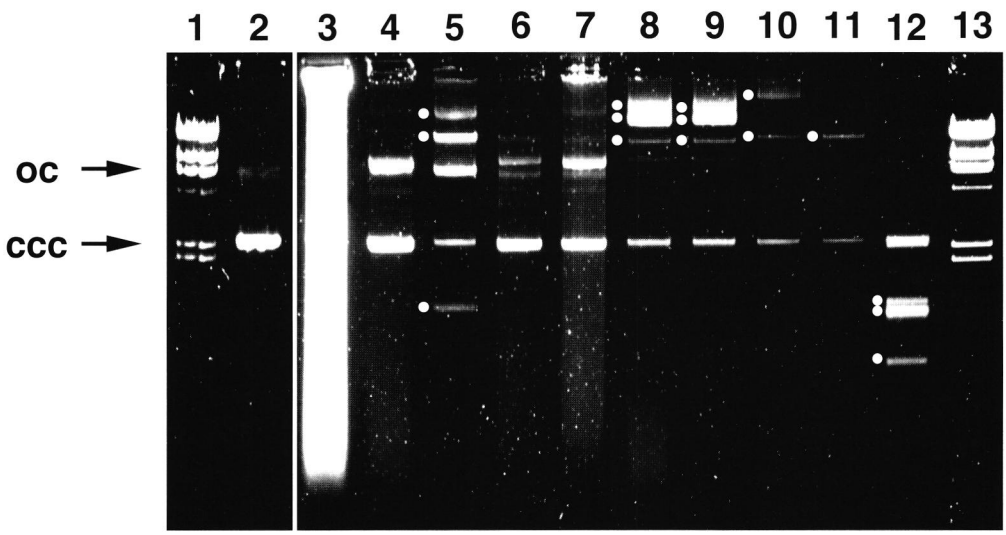

Figure 1.

Plasmid DNA isolated from three strains of viridans group streptococci and three *Staphylococcus aureus* strains. All strains harbored plasmid pGKV210; arrows indicate different conformations of this plasmid (oc, open-circle conformation; ccc, closed-circle conformation). Several strains carry one or more endogenous plasmids (lanes 5 to 12; white dots), which were coisolated by this isolation method. Lanes 1 and 13, *Hind* III-digested phage λ DNA; 2, pGKV210 isolated from *E. coli* (2) and purified by CsCl-ethidium bromide buoyant density centrifugation; 3 to 6, plasmid yields from the lytic enzyme mix without lysostaphin (pGKV210 purified from the cleared lysate of *Streptococcus sanguis* U108, either by direct application onto the QIAGEN-tip 20 column [lane 3] or after one phenol-chloroform extraction and application onto a QIAGEN-tip 20 column [lane 4]; pGKV210 isolated from *Streptococcus sanguis* J30 [lane 5] and from *Streptococcus mitis* S417 (lane 6]); 7 to 12, plasmid yields from the lytic enzyme mix with lysostaphin included (pGKV210 isolated from *Streptococcus mitis* S417 [lane 7], from *Staphylococcus aureus* A108218 [lanes 8 and 9], from *Staphylococcus aureus* ATCC 29213 [lanes 10 and 11] and from *Staphylococcus aureus* 42D [lane 12]; protoplasting periods were 2 h [lanes 8, 10, and 12] and 30 min [lanes 9 and 11].

in the following: (i) growth of the bacteria in broth not supplemented with glycine, (ii) a much shorter treatment time, (iii) treatment with much lower amounts of lysozyme, and (iv) no phenol-chloroform extraction prior to application of the lysate onto the QIAGEN column. Other published protocols (1, 4-8, 10) also failed to produce pure plasmid DNA from our streptococcal strains. Viridans group streptococci appear to be very resistant to lysis, and enzymatic treatment releases large amounts of degradation products, which strongly interfere with the purification procedure. Only by the unique combination of all steps described was it possible to overcome these problems and to obtain pure plasmid DNA from these gram-positive bacteria.

By use of the plasmid DNA isolation protocol (Table 1), 1 to 1.5 μg of the low-copy-number plasmid pGKV210 was obtained from an overnight 15-ml streptococcal culture. Plasmid DNA was also isolated from culture volumes as small as 5 ml, by using the volumes of reagents given in Table 1. For large-scale isolation of plasmids, volumes of reagents (Table 1) had to be increased proportionally with the increase in culture volume.

The isolated plasmid DNA was introduced into viridans group streptococcal strains by electro-transformation. Transformation-frequencies were similar to those obtained with CsCl-purified plasmid pGKV210 (unpublished data).

Plasmid pGKV210 isolated by our method was sequenced with the Taq Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, Calif.). The sequences from the plasmids purified either by CsCl or by our new method were easily readable, and the qualities of both sequences were similar (data not shown).

The plasmid isolation procedure (Table 1) was applied to two other viridans group streptococcal strains (Fig. 1, lanes 5 and 6) and three *Staphylococcus aureus* strains (Fig. 1, lanes 8 to 12) which had been transformed with pGKV210. Plasmids of high purity were obtained from all strains. The presence of lysostaphin in the lytic enzyme mixture was essential for isolation of pGKV210 from *Staphylococcus aureus* and did not influence isolation of plasmids from viridans group streptococci (Fig. 1, lanes 6 and 7). The use of lysostaphin as the single lytic enzyme in the protoplasting step of the *Staphylococcus aureus* strains (10) was insufficient for the isolation of pure plasmid pGKV210. In addition, a protoplasting period of 10 min (10) was too short for our *Staphylococcus aureus* strains. A protoplasting period of 30 min showed variable results for the strains (Fig. 1, lanes 9 and 11), whereas a period of 2 h always yielded well-purified plasmid DNA (Fig. 1, lanes 8, 10, and 12).

In addition to the cloning vector pGKV210, endogenous plasmids of different sizes present in several of the viridans group streptococcal and *Staphylococcus aureus* strains were isolated by our procedure (Fig. 1, lanes 5 to 12). Since isolation of endogenous plasmids is often difficult, this protocol can be of great value in the studies of such important topics as plasmid-borne virulence factors, antibiotic resistance, and spreading of such traits in the bacterial population.

In conclusion, our plasmid isolation protocol provides a simple method for the isolation of plasmid DNA from viridans group streptococci and *Staphylcoccus aureus* strains, yielding plasmid DNA of high purity which can readily be used for purposes like restriction analysis, subcloning, electrotransformation, and automated sequencing. This plasmid isolation procedure simplifies the selection and recovery of cloned genes using plasmid-based cloning systems in viridans group streptococci and other gram-positive bacteria and facilitates studies of endogenous plasmids which can also be isolated with this protocol.

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CHAPTER 4

Wide-host-range shuttle vectors for the screening of regulated promoter activity in viridans group streptococci; isolation of a pH regulated promoter

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ABSTRACT

Viridans group streptococci are major constituents of the normal human oral flora, and are also identified as the predominant pathogenic bacteria in native valve infective endocarditis. Little information is available regarding the regulation of gene expression in viridans streptococci, either in response to changes in the oral environment or during development of endocarditis. We therefore constructed a set of broad host range vectors for the isolation of promoters from viridans group streptococci, that are activated by specific environmental stimuli *in vitro* or *in vivo*. A genomic library of *Streptococcus gordonii* strain CH1 was constructed in one of the new vectors, and this library was introduced into the homologous bacterium using an optimized electroporation protocol for viridans group streptococci. As viridans streptococci entering the bloodstream from the oral cavity encounter an increase in pH, we selected promoters upregulated by this specific stimulus. One of the selected promoters was analysed in detail. This promoter showed homology to the promoter region of the *hyd*A gene from *Clostridium acetobutylicum*, of which the expression is known to be regulated by the environmental pH. The isolation of this pH-regulated promoter shows that *S. gordonii* can sense an increase in the environmental pH, which serves as a signal for bacterial gene activation. Furthermore, this demonstrates the usefulness of these new selection vectors in the research on adaptive gene expression of viridans streptococci, and possibly also of other Gram-positive bacteria.

INTRODUCTION

Viridans group streptococci (VS) are major constituents of the human commensal oral flora (15). They constantly have to adapt to the rapid changes in their natural habitat (4). Adaptation is characterized firstly by sensing of environmental changes, followed by signal transduction, that can result in the expression of genes of which the products are involved in the adaptive process (17, 31, 45). One of the environmental changes VS encounter is variation in the extracellular pH, which drops rapidly after carbohydrate consumption by the host. *Streptococcus mutans* responds to such a decrease in pH by rapid upregulation of the expression of several regulatory genes and of genes involved in stress responses, including *hcr*A, *grp*E, and *dna*K (23). However, knowledge on gene expression in other VS induced by pH or other environmental stimuli is scarce.

VS, like *Streptococcus sanguis*, *Streptococcus oralis*, and *Streptococcus gordonii*, are the most frequently encountered bacterial causes in native valve infective endocarditis (NVE) (10, 42). This disease is caused by the rapid growth and persistence of bacteria embedded in a platelet-fibrin thrombus (a vegetation) present on damaged endocardium or heart valves (12). Studies on virulence factors of VS in the pathogenesis of IE have mainly focussed on components involved in bacterial adherence to the vegetation. These include exopolysaccharides (7, 32) and adhesins for connective tissue proteins, for adhesive macromolecules present in plasma, and for blood platelets (3, 19, 25, 50). Little information is available on the regulation of these and possible other virulence factors of VS in the host.

Therefore, we have developed a plasmid-based selection system for the isolation of inducible VS promoters. The system is designed partly in analogy to the *In Vivo* Expression Technology (IVET) system (18, 29), since IVET has been shown to be a promising tool in the study of adaptive gene expression of VS in an experimental rabbit model of IE (20, 26-28). As VS experience an increase in the environmental pH from slightly acidic to neutral levels when entering the blood from the oral plaque (38), we used this selection system to identify genes of which the expression was influenced by this specific stimulus. A pH-regulated promoter of *Streptococcus gordonii* strain CH1 was isolated and characterized, showing that VS indeed recognize an increase in pH as a signal for adaptive gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

Bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. *Escherichia coli* strains BHB2600 and DH5α were cultured in Luria Bertani (LB) broth and on LB agar at 37°C. For

Table 1. Bacterial strains used in this study.

Strain	Reference
Escherichia coli DH5α	Gibco-BRL (Breda, The Netherlands)
Escherichia coli BHB2600	21
Streptococcus gordonii CH1	56
Streptococcus sanguis U108	8
Streptococcus sanguis S221	Culture collection
Streptococcus sanguis S263	Culture collection
Streptococcus bovis S225	Culture collection
Streptococcus bovis S228	Culture collection
Streptococcus mitior dx+ S195	Culture collection
Streptococcus mutans S39	Culture collection
Streptococcus mutans V403	32
Streptococcus oralis J30	8
Streptococcus oralis S223	Culture collection
Streptococcus oralis S235	Culture collection
Streptococcus oralis S245	Culture collection
Streptococcus salivarius S304	Culture collection
Streptococcus salivarius S310	Culture collection

Table 2. Plasmids used in this study.

Plasmid	Relevant features	Reference
pEC2A	*E. coli* plasmid, containing *trp'-'lacZ* fusion gene; 7.5 kb; Em^R	5
pIC20R	*E. coli* plasmid; 2.7 kb; Ap^R	30
pLITMUS28	*E. coli* plasmid; 2.8 kb; Ap^R	14
pMG36	lactococcal expression vector; 3.7 kb; Km^R	52
pMG36e	lactococcal expression vector; 3.6 kb; Em^R	52
pGKV210	lactococcal shuttle vector; 4.4 kb; Em^R	53
pORI19S	lactococcal shuttle vector; 3.9 kb; Sp^R	47
pSU31	pUC19 containing transcriptional terminator from *S. equisimilis* H46A; 6.2 kb; Ap^R	48
pMM201	pMG36e containing *Eco*RI-*Bam*HI part of pIC20R MCS and *trp'-'lacZ* gene from pEC2A; 6.6 kb; Em^R	This study
pMM206	derivative of pGKV210; deletion of *Eco*RI-*Bam*HI fragment; 4.4 kb; Em^R	This study
pMM210	pLITMUS28 containing 3.4 kb *Eco*RI-*Kpn*I fragment of pMM201; 6.2 kb; Ap^R	This study
pMM211	pMM210 with selfannealed primer AV7 cloned into *Kpn*I site; 6.2 kb; Ap^R	This study
pMM214	pMM211 with promoterless *aad*(9) (Sp) gene of pORI20 cloned into *Bam*HI site; 6.9kb; Ap^R	This study
pMM218	pMM214 with *Spe*I-*Eco*RI transcription terminator fragment from pSU31; 7.0 kb; Ap^R	This study
pMM219	pMM218 with promoterless *aph*III (Km) of pMG36 replacing the promoterless *aad*(9); 7.1 kb; Ap^R	This study
pMM223	pMM206 with 4.3 kb *Spe*I fragment from pMM218 in *Xba*I site; 8.1 kb; Em^R	This study
pMM225	pMM206 with 4.4 kb *Spe*I fragment from pMM219 in *Xba*I site; 8.2 kb; Em^R	This study
pMM239	pMM223 with streptococcal *rgg-gtf*G promoter fragment from *S. gordonii* CH1 in *Eco*RI-*Sal*I site; 9.2 kb; Em^R	This study
pMM240	pMM223 with *Sau*3A fragment containing constitutive promoter from *S. gordonii* CH1 in *Bgl*II site; 9.0 kb; Em^R	This study
pMM243	pMM223 with *Sau*3A fragment containing neutral pH inducible promoter from *S. gordonii* CH1 in *Bgl*II site; 8.6 kb; Em^R	This study

maintenance of plasmids ampicillin (50 μg/ml) or erythromycin, 150 μg/ml for strain BHB2600 and 300 μg/ml for strain DH5α, were added to the culture medium. Viridans group streptococcal strains (VS) were cultured in Todd Hewitt (TH)-broth and on TH-agar (Difco Laboratories, Detroit, MI) at 37°C in a 5% CO_2 atmosphere or anaerobically. When required, TH was supplemented with erythromycin (5 μg/ml).

MIC/MBC determination.

The MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) values of VS for spectinomycin were determined by microbroth dilution assays (33). Dilution series of spectinomycin or kanamycin, ranging from 25 - 1000 μg/ml, in TH broth supplemented with 5% horse blood were prepared in microtiter plates. To each well 100 μl of TH containing 10^6 bacteria was added, and the plates were incubated overnight at 37°C in a 5% CO_2 atmosphere. The MIC was defined as the lowest antibiotic concentration at which no growth was visible. To determine the MBC, 1 μl from each well without visible bacterial growth was cultured on blood agar plates. The MBC was defined as the lowest antibiotic concentration that reduced the bacterial inoculum at least 1,000-fold.

DNA isolation.

Plasmid DNA was isolated from *E.coli* using QIAGEN plasmid DNA isolation kits (QIAGEN GmbH, Hilden, Germany; Westburg, Leusden, The Netherlands), and from VS as described previously (55). Chromosomal DNA from VS was isolated using the Puregene Chromosomal DNA isolation kit for Gram-positive bacteria and yeast (Gentra Systems Inc., Minneapolis, MN).

Molecular cloning and DNA sequence determination.

DNA manipulations were done according to standard techniques (41). Plasmid DNA was introduced into *E.coli* by electroporation using the method of Dower et al. (11). DNA sequencing was performed by PCR-mediated *Taq* Dye Deoxy Terminator Cycle sequencing (Perkin Elmer, Foster City, CA) using an Applied Biosystems model 373 DNA sequencer.

Construction of selection vectors pMM223 and pMM225.

Plasmids used for the construction of the selection vectors pMM223 and pMM225 are listed in Table 2. The 3.3 kb *Bam*HI-*Hin*dIII fragment of pEC2A (5) containing the promoterless *trp'-'lacZ* fusion gene (9), was ligated to the *Eco*RI-*Bam*HI part of the pIC20R multiple cloning site (MCS) (30), and the product was introduced into the *Eco*RI-*Hin*dIII digested shuttle vector pMG36e (52) resulting in pMM201 (data not shown). To achieve higher cloning efficiencies, the MCS-*trp'-'lacZ* fragment was excised from pMM201 as an *Eco*RI-*Kpn*I fragment, and ligated to *Eco*RI/*Kpn*I-digested *E. coli* plasmid pLITMUS28 (14) creating pMM210 (Fig.1). Self-annealed primer AV7 (Table 3) was cloned into the *Kpn*I site of pMM210, replacing this site by an *Spe*I site (pMM211). The gene *aad*(9), originating from *Enterococcus faecalis* and conferring resistance to spectinomycin (28), was amplified as a promoterless gene from plasmid pORI19S (47) using primers AV3 and AV4 (Table 3). The 795 bp PCR fragment was digested with *Bam*HI, and cloned into the *Bam*HI site of pMM211, resulting in pMM214. A bidirectional transcriptional terminator of *Streptococcus equisimilis* H46A present on a 922 bp *Pst*I-*Hin*dIII fragment of plasmid pSU31 (48) was amplified using primers AV8 and AV9 (Table 2). The terminator fragment was digested with *Spe*I and *Eco*RI, and ligated in the reversed orientation in front of the selection cassette of pMM214 to generate plasmid pMM218 (Fig.1). For the construction of the second selection cassette, the *aph*III gene originating from the *Enterococcus faecalis* plasmid pJH1 and conferring resistance to kanamycin (51) was selected. It was amplified from plasmid pMG36 (52) as a promoterless gene using primers AV1 and AV2 (Table 3). The 855 bp product was digested with *Bam*HI and used to replace the *Bam*HI fragment of pMM218 comprising the promoterless *aad*(9) gene. The resulting plasmid was designated pMM219. The selection cassettes were excised as *Spe*I fragments from

pMM218 and pMM219 and ligated to *Xba*I-digested pMM206, a derivative of the lactococcal shuttle vector pGKV210 (53), to yield the vectors pMM223 (*aad*(9), Sp) and pMM225 (*aph*III, Km), respectively (Fig.1).

Table 3. Synthetic oligonucleotides used in this study[a]

Primer	Sequence (5'-3')	Restriction site
AV1	AACAGGATCCGGGGTATCTTTAAATACTGTAG	*Bam*HI
AV2	AACAGGATCC**CGGG**CTAGGTACTAAAACAATTCATCC	*Bam*HI/*Sma*I
AV3	AATTGGATCCCTAATCAAAATAGTGAGGAGG	*Bam*HI
AV4	AATTGGATCC**CGGG**TTTTTTTATAATTTTTTTAATCTG	*Bam*HI/*Sma*I
AV7	ATCATACTAGTATGATGTAC	*Spe*I
AV8	AACAGAATTCACGGTCTTCTAAAACGATG	*Eco*RI
AV9	ATGTCACTAGTCTCTACAAC	*Spe*I
AV13	GATAAGATCTTGACGGAGATTAGCAAAAAG	*Bgl*II
AV16	GATACTGCAGCCTTCTGAAAATAGTATAAAG	*Pst*I
AV19	CCTCCTCACTATTTTGATTAG	

[a] Oligonucleotides were obtained from Perkin-Elmer Nederland B.V. (Nieuwerkerk aan de IJssel, The Netherlands). Primers AV1/AV2, AV3/AV4, and AV8/AV9 were used to amplify the promoterless *aad*(9) gene, the promoterless *aph*III gene, and the streptococcal transcriptional terminator, respectively. Oligo AV7 was self-annealed and used in the construction of the cassettes (Fig. 1). Underlined and bold sequences are recognition sites for the indicated endonucleases. AV9 and AV19 were used for sequencing of the cloned chromosomal fragments.

Electrotransformation of viridans streptococci.

Electrocompetent VS cells were prepared according to Smith et al. (46), with minor modifications. Briefly, bacteria harvested from mid-log cultures in TH broth were washed 3 times in icecold, sterile distilled water, and 3 times in icecold 0.3 M sucrose/10% glycerol. Bacteria were resuspended in 1/500 volume of the latter solution, and 50 µl of this suspension was used for electroporation with a Bio Rad Gene-Pulser and Pulse Controller (Bio Rad Laboratories B.V., Veenendaal, The Netherlands). Immediately after electroporation, 0.95 ml of TH supplemented with 0.3 M sucrose was added, and the bacteria were incubated for 2 h at 37°C and subsequently plated on the appropriate selective agar medium. Colonies of VS transformants were visible after 24-48 h of incubation at 37°C in a 5% CO_2 atmosphere. To obtain maximal transformation frequencies, cuvettes with different electrode gap sizes (0.1 or 0.2 cm) were tested, and resistance setting (100Ω or 200 Ω), field strength (10 - 25 kV/cm), and type of electrical pulse (decayed pulse or squared pulse) were varied. In addition, the influence of procedures affecting VS cell wall integrity on electroporation efficiencies was tested. DL-threonine (40 mM), 1% (v/v) glycine, or sub-MIC penicillin G was added to the growth medium used for the preparation of competent VS cells, or competent VS were enzymatically treated with lysozyme (0.5 mg/ml; Sigma Chemical Co., St. Louis, MO) and mutanolysin (2.5 Units/ml; Sigma) for 1 h.

Natural transformation of *S. gordonii* CH1.

S. gordonii CH1 cells were made competent for natural transformation according to Jenkinson (24). Briefly, an overnight culture in BHY (Brain Heart Yeast; Brain Heart Infusion medium (Difco Laboratories, Detroit, MI) with 5 g/l of Yeast extract (Difco)) was diluted 100-fold in fresh BHY supplemented with 5% horse serum and 1% glucose, and incubated for 3 h at 37°C. This culture was diluted 100-fold in fresh medium and incubated for 1 h at 37°C. From this culture 1 ml aliquots were taken, 1.5 µg of plasmid DNA was added, and incubation was continued for another 3 h at 37°C. Aliquots of 10 µl were plated onto selective TH-agar, and incubated for 24-48 h at 37°C in a 5% CO_2 atmosphere to select for plasmid-containing transformants.

β-galactosidase activity assay.

β-galactosidase activity of the VS clones was determined using the fluorescent substrate fluorescein di-β-galactopyranoside (FDG; Molecular Probes Europe BV, Leiden, The Netherlands). Bacteria cultured overnight in TH containing erythromycin (5 μg/ml) were harvested by centrifugation, washed, and resuspended in STES buffer (10 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 20% sucrose) to an A_{620} of 1.0. Of this suspension 1.5 ml was centrifuged, the pellet was resuspended in 1 ml STES buffer supplemented with 50 mg/ml lysozyme and 200 U/ml mutanolysin, and this suspension was incubated at 37°C. After 2 h, 50 μl of 1% SDS and 50 μl of chloroform were added, samples were mixed for 10 s, and were left for 15 min at room temperature. Six replicate samples of 50 μl of this suspension were transferred to wells of microtiter plates, and 150 μl of Z buffer (40 mM NaH_2PO_4, 60 mM Na_2HPO_4, 10mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.0) containing 33 μM FDG were added to each replicate. At different time intervals, the emission was measured at 530 nm (bandpass = 30 nm) after excitation at 485 nm (bandpass = 20 nm) using a Cytofluor II fluorescence multi-well plate reader (PerSeptive Biosystems, Inc., Farmingham, MA). The β-galactosidase activity was plotted as arbitrary fluorescence units over time, and results are the average of six reactions.

Construction of *S. gordonii* CH1 genomic library.

A genomic library of *S. gordonii* CH1 was constructed using the selection vector pMM223. Vector DNA was digested with *Bgl*II and dephosphorylated using Calf Intestine Alkaline Phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany). Genomic DNA isolated from *S. gordonii* CH1 was digested to completion with *Sau*3A. Vector and chromosomal fragments were ligated, and the ligation mixture was introduced into *E. coli* BHB2600 by electroporation. Plasmid DNA was isolated from erythromycin resistant transformants constituting the genomic library, and introduced into the homologous streptococcal strain CH1 by electroporation.

Selection of pH-regulated promoters.

The streptococcal genomic bank was plated onto TH-agar of pH 7.3 supplemented with erythromycin (5 μg/ml, Em) for plasmid maintenance and spectinomycin (500 μg/ml, Sp) for selection of active streptococcal promoters. After anaerobic incubation at 37°C for 36 hours, colonies resistant to Em as well as Sp were replated onto TH-agar of pH 6.2, again supplemented with Em and Sp. As a control for the viability of the isolated *S. gordonii* clones, these were also restreaked onto TH-agar plates of pH 6.2 supplemented with Em only, and onto plates of pH 7.3 with Em and Sp. Plasmids were isolated from clones that failed to grow on the pH 6.2 agar but did grow on the agar of pH 7.3 in the presence of Sp. The fragments cloned in these plasmids were amplified by PCR using primers AV4 and AV9 (Table 3), and the PCR products were sequenced using primer AV19 (Table 3). The obtained sequences were analysed using the BLAST program (1).

Measurement of *in vitro* growth rate.

To determine the relative activity of promoters at different pH values, the growth rate of selected clones was determined in TH broth of pH 6.2 and pH 7.3, in the presence and absence of Sp. A single colony of each clone was cultured at 37°C in TH supplemented with Em for plasmid maintenance. Overnight cultures were diluted 100-fold in fresh TH containing both Em (5 μg/ml) and Sp (500 μg/ml), or Em alone. Growth was monitored by measuring the optical density at 620 nm (A_{620}) over time, and the mid-log phase doubling time ($t_{½}$) was determined. Relative promoter activity at pH 6.2 and 7.3 was expressed as the ratio of growth in the presence and absence of spectinomycin at each pH ($t_{½}$(+spec) / $t_{½}$(-spec)).

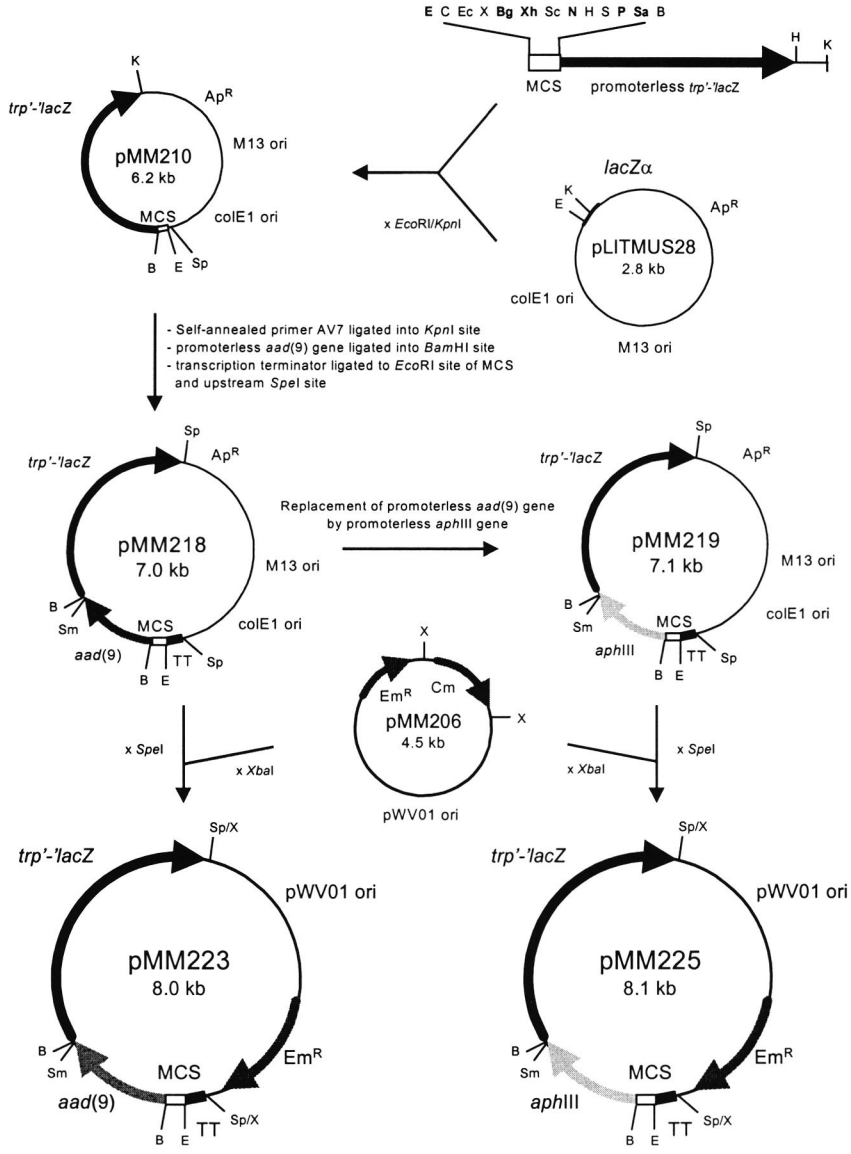

Figure 1.

Schematic representation of the construction of selection vectors pMM223 and pMM225. Details of the construction are described in the text. *aad*(9), promoterless spectinomycin resistance gene; *aph*III, promoterless kanamycin resistance gene; *trp'-'lacZ*, promoterless β-galactosidase fusion gene; MCS, multiple cloning site; TT, streptococcal transcription terminator; ori, origin of replication; Ap^R, ampicillin resistance gene; Em^R, erythromycin resistance gene; Cm, promoterless chloramphenicol acetyltransferase gene. B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; Ec, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; N, *Nru*I; P, *Pst*I; Sc, *Sac*I; Sa, *Sal*I; Sm, *Sma*I; S, *Sph*I; Sp, *Spe*I; X, *Xba*I; Xh, *Xho*I. Endonucleases printed in bold represent unique sites in the MCS.

RESULTS

New broad host range promoter-selection vectors pMM223 and pMM225.

We constructed a set of self-replicating broad host range promoter-selection vectors for Gram-positive bacteria (Fig. 1). Firstly, selection cassettes were constructed in *E. coli* plasmid pLITMUS28 (14). Each selection cassette contains two promoterless genes. The first gene is either a promoterless *aph*III gene (51), conferring resistance to kanamycin, or *aad*(9) (28) gene, conferring resistance to spectinomycin, for the selection of active promoters. The second gene is the *trp'-'lacZ* fusion gene encoding ß-galactosidase (9), to be used for discrimination between constitutive and induced promoter activity. In front of the two promoterless genes, the multiple cloning site (*MCS*) from pIC20R (30) was introduced for insertion of DNA fragments with possible promoter activity. A bidirectional transcriptional terminator (*T*) from *Streptococcus equisimilis* strain H46A (48) was cloned in front of the *MCS* to prevent possible readthrough into the promoterless cassette. The terminator was inserted in its reversed orientation, which has the highest termination activity in *E.coli* TG1 (48). The resulting selection cassettes have a total size of approximately 4.3 kb, and all components of the cassettes can be replaced or removed separately or in combination, using common restriction endonucleases (Fig. 1).

The cassettes were cloned as *Spe*I fragments into *Xba*I-digested plasmid pMM206, a derivative of the lactococcal shuttle vector pGKV210 (53), replacing the promoterless chloramphenicol acetyltransferase gene of pMM206. Plasmid pMM206 had been constructed by digestion of pGKV210 with *Eco*RI and *Bam*HI, filling in the ends with Klenow large fragment DNA polymerase I, and ligation of the blunt ends. This vector contains an erythromycin resistance gene for plasmid maintenance, and the broad host range origin of replication (*ori*) of plasmid pWV01 (27). The sequences of the new vectors were assembled from published sequences of the different fragments used, and sequences obtained after sequence determination of the borders of these fragments after ligation. These sequences of the vectors pMM223 and pMM225 have been assigned Genbank accession numbers AF076212 and AF076213, respectively.

Transformation of viridans streptococci.

We optimized the electroporation procedure using one of our standard *Streptococcus sanguis* test strains, strain U108, and shuttle vector pGKV210. Maximal efficiencies were obtained using cuvettes with a 0.1 cm electrode gap at a resistance setting of 100 Ω, a capacitance of 25 μF, and a field strength of 25 kV/cm (Fig. 2). At these settings time constants ranged from 2.0 - 2.5 ms. The number of transformants increased linearly with the plasmid DNA concentration over a range from 5 ng to 500 ng (data not shown). Despite the high field strength, survival rates of *S. sanguis* U108 generally were 70% or higher. Colonies of the U108 transformants were often variable in size, but the introduced plasmid pGKV210 could be isolated from the transformants in all cases.

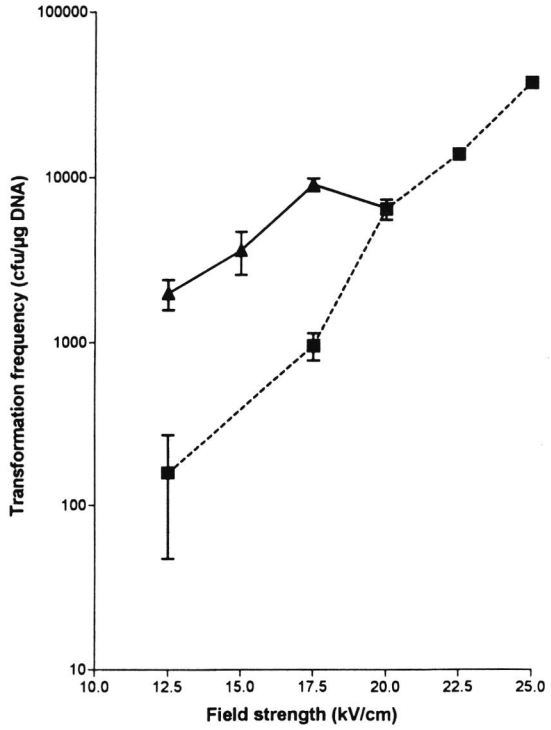

Figure 2.

Electroporation frequency of *S. sanguis* strain U108 as function of the field strength. The lactococcal shuttle vector pGKV210 was used as the donor DNA. Electroporation cuvettes with an electrode gap of 0.1 cm were used, at a resistance setting of 100 Ω (squares) or 200 Ω (triangles) and a capacitance of 25 µF.

Culturing of *S. sanguis* U108 in the presence of 1% (v/v) glycine (22) or 40 mM DL-threonine (6), or enzymatic treatment of the bacteria with lysozyme (37, 44) and mutanolysin reduced transformation frequencies at least 5-fold. Addition of sub-MIC penicillin G to the medium used to prepare electrocompetent cells (36) or the use of a squared instead of a decayed pulse (35), did not improve electroporation in *S. sanguis* U108.

To determine the transformability of other VS strains, several laboratory strains and clinical endocarditis isolates were electrotransformed with pGKV210 (Table 4). In these experiments, the field strength was reduced to 20 kV/cm, since at 25 kV/cm arcing regularly occurred. *S. gordonii* CH1 and *S. sanguis* U108 had the highest transformation frequencies. When tested at

25 kV/cm, transformation of *Streptococcus gordonii* CH1 was over 100-fold more efficient than of *S. sanguis* U108 (Table 4).

Subsequently, electroporation efficiencies of pGKV210, pMM223, and pMM225 were determined for both *S. sanguis* U108 and *S. gordonii* CH1. The highest frequencies were obtained with CH1 for all vectors tested (Table 5). As strain CH1 is known to be naturally transformable, efficiencies of the optimized electroporation procedure and of natural transformation were compared. The optimized electroporation procedure proved to be superior, especially for the newly constructed vectors (Table 5). Plasmid pMM223 and *S. gordonii* CH1 were chosen to assess the functionality of the selection system.

Table 4. Transformation of different viridans streptococcal strains with plasmid pGKV210, at a field strength of 20 kV/cm or at the maximal field strength of 25 kV/cm.

Strain	Frequency (cfu/μg)[a]	
	20 kV/cm	25 kV/cm
S. bovis S225	0	
S. bovis S228	20	
S. gordonii CH1	2.0×10^4	5.1×10^6
S. mitior dx+ S195	0	
S. mutans S39	1.4×10^3	
S. mutans V403	40	
S. oralis J30	4.5×10^3	
S. oralis S223	0	
S. oralis S235	40	
S. oralis S245	0	
S. salivarius S304	0	
S. salivarius S310	40	
S. sanguis U108	6.6×10^3	4.0×10^4
S. sanguis S221	80	
S. sanguis S263	4.2×10^2	

[a] Cuvettes with a 0.1 cm electrode gap were used.

Table 5. Efficiencies of transformation of pGKV210 and derived vectors into *S. sanguis* U108 and *S. gordonii* CH1.

Vector	***S. sanguis* U108**	***S. gordonii* CH1**	
	Electroporation	Electroporation	Natural transformation
pGKV210	4×10^4	5×10^6	2×10^5
pMM223	$< 10^2$	7×10^3	3×10^2
pMM225	$< 10^2$	5×10^3	$< 10^2$

Testing of expression vectors.

To determine the spectinomycin level required for the selection of active promoters, plasmid pMM239 was constructed. A 1.1 kb *rgg-gtfG* promoter fragment (49) amplified from the genomic DNA of *S. gordonii* CH1 using primers AV13 and AV16, was digested with *Bgl*II and

*Pst*I, and cloned into the multiple cloning site of pMM223. The MIC and MBC levels of *S. gordonii* CH1 harbouring either plasmid pMM223 or pMM239 were assessed. The presence of the active *gtf*G promoter in pMM239 was well detectable, since *S. gordonii* CH1 without plasmid or with pMM223 were susceptible for spectinomycin concentrations <25 μg/ml, whereas *S. gordonii* CH1 harbouring pMM239 was resistant to concentrations >500 μg/ml. Thus, the system allowed selection of active promoters.

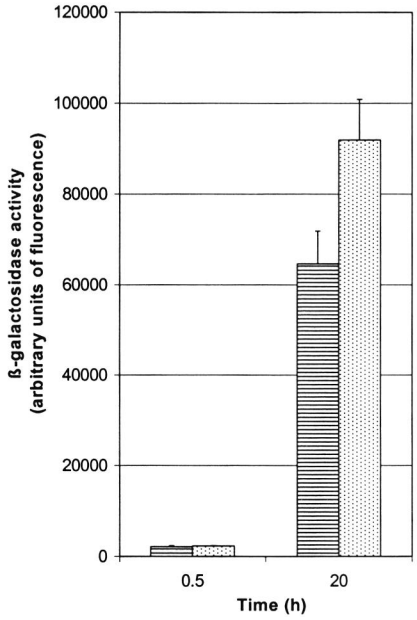

Figure 3.

Assessment of β-galactosidase activity in *S. gordonii* CH1 harbouring either plasmid pMM223 (no promoter; hatched bars) or pMM239 (active *rgg-gtf*G promoter; dotted bars) using the fluorescent substrate FDG.

Colonies of *S. gordonii* CH1 carrying either pMM223 or pMM239 plated onto agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 500 μg/ml), stained blue only very faintly after several days of incubation at 37°C. Therefore, bacteria were plated in 10 ml TH agar containing 500 μg/ml X-gal as an overlay on plates containing 10 ml TH agar, as described for *S. pneumoniae* (1). Both strains produced blue colonies after 2-3 days of incubation. As it was difficult to discriminate between presence and absence of an active promoter using this method, β-galactosidase activity was determined using the fluorescent substrate FDG. Despite the endogenous β-galactosidase activity of *S. gordonii* CH1, the presence of the *rgg-gtf*G promoter on plasmid pMM239 could be discriminated after 20 hours of incubation (Fig. 3). Although addition of 2% glucose to the growth medium did lower the

Stellingen behorende bij het proefschrift:

Adaptive gene expression of endocarditis-causing viridans group streptococci and Staphylococcus aureus

1. De rol van het methionine sulfoxide reductase (MsrA) van *Streptococcus gordonii* in de pathogenese van endocarditis, is niet alleen de veronderstelde bescherming tegen oxidatieve stress (*Kiliç et al., General Meeting of the ASM, 1997*) maar ook de betrokkenheid bij bacteriële vermeerdering.

   (*dit proefschrift*)

2. De hypothese dat bacteriële proteases van invloed zijn op de afbraak van vegetaties voor het vrijmaken van voedingscomponenten voor de bacteriën (*Goldstein et al, General Meeting of the ASM, 1999; Juarez and Stinson, Infect. Immun 67:271, 1999*), wordt alleen bevestigd door middel van wetenschappelijke onderbouwingen.

   (*dit proefschrift*)

3. Omdat het optimaliseren van sommige genetische tools vaak een tijdrovende bezigheid is, wordt de keuze van het te bestuderen micro-organisme grotendeels bepaald door de genetische toegankelijkheid ervan.

   (*dit proefschrift*)

4. Visualisatie is voor wetenschappers de belangrijkste graadmeter.

5. "Een oorvijg mag best, als de jeugd loopt te klieren" is mogelijk de enige manier om het wegglijdende normbesef in onze maatschappij tegen te gaan.

   (*korpschef P. van Zunderd, Volkskrant, 1999*)

6. Als werken een hobby wordt, is vrije tijd ver te zoeken.

7. De significante en positieve correlatie tussen de omtrek van de taille van een vrouw en het aantal zoons dat zij heeft (*Manning et al, Nature 399:214, 1999*), kan voor sommige vrouwen een reden zijn vermageringskuren te mijden.

8. Het dominant willen overkomen door het aannemen van een aggressieve houding is zelfs tijdens wetenschappelijke discussies niet ongebruikelijk.

9. Verbeeldingskracht is belangrijker dan kennis.

(*Albert Einstein*)

10. Succes is de bekwaamheid om ondanks mislukking op mislukking je enthousiasme niet te verliezen.

(*Winston Churchill*)

11. Als Bill Gates van Joodse origine zou zijn, had Microsoft het millenium-probleem misschien nu niet gekend.

12. Elementen die bijdragen aan het ontstaan van infectieziekten (sociale factoren, demografische factoren, milieu factoren, en microbiële evolutie) zullen aanhouden of zelfs intensiveren in de 21ste eeuw.

(*Binder et al., Science 284:1311, 1999*)

Aldwin J.M. Vriesema

Amsterdam, 21 oktober 1999

endogenous β-galactosidase levels of *S. gordonii* CH1, it did not further improve the efficiency to detect an active promoter (data not shown).

Selection of pH-regulated promoters of *S. gordonii* CH1.

A genomic library of *S. gordonii* CH1 was constructed using *Escherichia coli* as an intermediate host, since direct transformation of ligation mixtures to *S. gordonii* CH1 resulted in low numbers of recombinant clones. The final streptococcal genomic library contained approximately 10^5 independent clones, with an average insert size of approximately 500 bp. Statistically, this library represents the entire genome of *S. gordonii* CH1 (41).

VS causing IE translocate from the oral plaque to the blood, and the bacteria will be exposed to a pH shift from pH 6.0 - 6.5 (38) to pH 7.3 - 7.4. Therefore, we selected promoters which were upregulated at pH 7.3. Of 143 individual clones that grew on TH agar of pH 7.3 supplemented with 500 μg/ml Sp, 5 clones were identified which hardly grew on TH agar of pH 6.2 when spectinomycin was present. The pH of the medium influenced the activity of ß-galactosidase (data not shown), hampering assessment of relative promoter activity. Therefore, the promoter activity at pH 6.2 and 7.3 of one of these clones was further tested by determination of the *in vitro* growth rate in the presence or absence of Sp. The growth rate was increased at pH 7.3, demonstrating the upregulation of the promoter at this pH (Table 6).

Table 6. Activity at pH 6.2 and 7.3 of a constitutive and of a pH-regulated promoter fragment isolated from *S. gordonii* CH1, recorded as the ratio of growth in medium with and without Sp[a].

pH	CH1 pMM240 (constitutive)	CH1 pMM243 (neutral pH-inducible)
6.2	0.95 ± 0.01	0.69 ± 0.04
7.3	0.98 ± 0.01	0.94 ± 0.01

[a] Growth was monitored by measuring the optical density at 620 nm (A_{620}) over time, and the mid-log phase doubling time ($t_{½}$) was determined. Relative promoter activity at the different pH values was expressed as the ratio of growth in the presence and absence of spectinomycin at each pH tested ($t_{½}$(+spec) / $t_{½}$(-spec)).

Sequencing of the pH-regulated promoter fragment and comparison to Genbank sequences revealed homology to the promoter region of the *hydA* gene of *Clostridium acetobutylicum* (Fig. 4). The -35 and -10 regions were identified at almost identical positions in both sequences. The extended -10 sequence present in the clostridial *hyd*A promoter region (5'-AatATga-3') (57), was found at the same position upstream of the -10 box of the streptococcal promoter. The inverted repeat sequences present upstream and downstream of the *Clostridium* promoter stretch, involved in catabolite repression (13, 16) and repression of the transcription of *hyd*A (16, 54) respectively, were however not identified in the streptococcal sequence. A possible Shine-Dalgarno sequence and translation start were found (Fig.4). The cloned fragment only

contained the sequence encoding the first 24 amino acids of the putative coding region following this translation start. This sequence did not show homology to any entry in the Genbank database, nor to the partly sequenced genomes of *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*.

```
pMM243 GATCAAAGTAACCATCAAATATGAATTTTGTCATTCCGTTGGTATTTTTCCAAATATATA  60
pMM243 GCGGACAATAACTGTTAAATAATTGGCCTTCAGATTTTTCAGAGATTAAATAAACTTTGA 120
pMM243 AAAACAAATCGTCGAATCCATCCATTAAATGACCTGTATTTCGGACACGTTGTCTAATGA 180
pMM243 TATCCATATCATAATCTGATGGTAATTTTATAGTATATTGCATTGCTTGCATAGCAGTAC 240

            **********                    -35
  hydA      GATTTGTGCTTCTTTTAACAAAATAAATTATTTAAAACATTTTAGACTTTATTTAA
              | |       || || |||  |||||| ||||| | ||  ||  |   || ||||
pMM243 CCCTTTTCTAATGAACTATTGACAGTATAAATGATTTATATCAAGTTGAATAATACTTAA 300

             -10                 ****************************
  hydA ATATGATATAATTATAAAATGTACGTAATATTTACGTTGATTAAACGTTAATTTTTTAAC
       | |||||||||||||| | |       |     |   || | | ||  |||||  | | |
pMM243 AAATGATATAATTATCATAAAAAGTGATAGGGGTGTTTTAAATGAATTTTAATGATATGAG 360
                                  SD          M  N  F  N  D  M  S

  hydA GAAGTTTATTAATATATTTTA
         | |||  | |  || | | |
pMM243 CATTTTTGTGACAATCTATGAGACAAGGTCAATAAACAAAAGTTCAAAGATC 412
        I  F  V  T  I  Y  E  T  R  S  I  N  K  S  S  K  I
```

Figure 4.

Nucleotide sequence of the isolated pH-regulated promoter fragment from *Streptococcus gordonii* CH1 pMM243, and homology to the promoter region of *hyd*A of *Clostridium acetobutylicum* ATCC 824. The sequence has been assigned Genbank accession number AF127175. The -35 and -10 boxes are underlined, and the extended -10 region, as described for *C. acetobutylicum* (57) is printed in italics. The inverted repeat sequences in the *C.acetobutylicum* sequence upstream and downstream of the promoter region are indicated by asterisks. A putative Shine-Dalgarno (SD) sequence and translational start site in the streptococcal sequence are printed in bold. The translated amino acid sequence of the putative open reading frame is shown.

DISCUSSION

Apart from being important colonizers of the oral cavity and upper pharyngeal tract in humans, viridans group streptococci are recognized as the most common bacterial agents causing native valve infective endocarditis (NVE) (10, 42). Little information is available regarding the adaptive potential in this group of microorganisms, either in their natural habitat or during the pathogenesis of IE. Of techniques to study adaptive gene expression (40), the IVET-system is most suited for selection of promoters of genes induced under specific *in vitro* conditions or in the complex *in vivo* environment (29). In its original design, the IVET-system used integration of a non-replicative vector into genomic DNA via a single cross-over. This approach requires

high frequencies of transformation, which are difficult to achieve in many Gram-positive bacteria. In addition, although the IVET-system was originally developed not to disrupt any bacterial genes (29), integration of a non-replicative vector containing a fragment without a promoter may still cause mutations. We therefore developed an IVET-based selection-reporter system using self-replicating plasmids to study inducible genes in viridans group streptococci. Plasmids pMM223 and pMM225 carry the replication origin (*ori*) of the lactococcal plasmid pWV01 (3-5 copies per cell in lactococci, streptococci, *B. subtilis*) (27). Therefore, these selection vectors are expected not to interfere significantly with regulation of gene expression in these Gram-positive bacteria. Promoterless genes conferring resistance to kanamycin (*aph*III) and spectinomycin (*aad*(9)) were used for selection of active promoters, as these antibiotics are bactericidal for our viridans streptococci (data not shown). In contrast, the use of bacteriostatic antibiotics and associated resistance genes (20, 26), like CAT86 in pGKV210, may result in erroneous selection of surviving bacteria that do not have a cloned active promoter.

Viridans group streptococci generally are refractory to electrotransformation. Weakening of cell walls by various methods, which increased transformation frequencies for several Gram-positive bacteria (6, 22, 36, 37, 44), did not improve transformation frequencies of our VS strains. With the optimized electroporation protocol transformation frequencies appeared to increase almost linearly with increasing field strength, and were maximal at 25 kV/cm (Fig. 2). At this maximum attainable field strength, only approximately 30% of the competent VS were killed. For *E. coli* maximal transformation frequencies are obtained when 50 to 75% of the cells are killed due to the electrical discharge (11). Thus, even higher transformation frequencies might be possible with equipment capable of generating higher field strengths. Transformation frequencies for *S. gordonii* CH1 and *S. sanguis* U108 were the highest of the 15 strains tested. Plasmid size and possibly the plasmid-encoded genes appeared to influence transformation efficiencies, as pMM223 and pMM225 had lower frequencies than pGKV210 in both *S. sanguis* U108 and *S. gordonii* CH1. Electroporation was superior to natural transformation of *S. gordonii* CH1 (Table 5), and frequencies were sufficiently high to construct a respresentative genomic bank.

Active promoters of *S. gordonii* CH1 could be detected using the promoterless spectinomycin resistance gene and promoterless *trp'-'lacZ* gene of pMM223. Detection of the induced expression of the *trp'-'lacZ* in this strain proved to be more difficult, although this gene has succesfully been used in detection of β-galactosidase expression of the closely related *S. pneumoniae* (5). *S. gordonii* CH1 harbouring pMM239, which carries the active *rgg-gtf*G promoter fragment (49), could not be discriminated from *S. gordonii* CH1 carrying pMM223 without promoter, either on X-gal containing plates or in agar overlays (1). Using the fluorescent substrate FDG, elevated β-galactosidase levels in lysates of bacterial clones harbouring pMM239 could be recorded after 20 hours of incubation (Fig. 3). Although the β-galactosidase expression of *S. gordonii* CH1 was subject to catabolite repression, as demonstrated by reduction of expression in the presence of 2% glucose, glucose did not

improve discrimination between presence and absence of an active promoter. The use of a streptococcal mutant with lower endogenous β-galactosidase activity, as described for *S. pneumoniae* (1), could be a suitable alternative. Secondly, the *E. coli lacZ* gene might be replaced by the recently described β-galactosidase gene of *Bacillus stearothermophilus* (38).

From a *S. gordonii* CH1 genomic library constructed in vector pMM223 a promoter was isolated of which the activity was upregulated by a shift from oral plaque pH (6.2) to blood pH (pH 7.3). This promoter has homology to the promoter region of the *hyd*A gene of *Clostridium acetobutylicum* ATCC824 (16), which encodes a putative hydrogenase with strong homology to the [Fe] hydrogenases from *Desulfovibrio* and other *Clostridium* species. The expression of *hyd*A is downregulated at the transcriptional level by a decrease in environmental pH (16). The repeat sequences located upstream and downstream of the clostridial *hyd*A promoter, suggested to be involved in catabolite repression (13, 16) and repression of the transcription of *hyd*A (16, 54), respectively, could not be identified in the streptococcal sequence.

The identity of the streptococcal gene under control of the *hyd*A promoter homolog remains to be determined. A possible ribosome binding site and start codon were identified (Fig. 5), but the downstream sequence did not show homology to a known nucleotide or protein sequence. This is presumably due to its small size. The clostridial *hyd*A coding region is located 98 nucleotides downstream of its -10 region, and the putative streptococcal *hyd*A homolog might also be located more distally from the identified promoter. However, the activity of this promoter was clearly regulated by the environmental pH, showing that our selection method allows identification of genes of which the expression is upregulated by changes in this environmental parameter.

In their study using an IVET chromosomal integration system, Kiliç and coworkers selected thirteen inducible promoters from *S. gordonii* V288 in a rabbit model of IE. The genes controlled by the isolated promoters encoded proteins involved in different cellular functions, including rapid bacterial growth and resistance to host defense (20, 26). A *hyd*A promoter homolog as identified in our study was not among these selected promoters. The environmental stimuli responsible for induction of those identified inducible genes are still unknown (20, 26).

To gain a more complete understanding of the regulation of expression of *in vivo* induced genes, and of other virulence genes, it is crucial to identify the stimuli responsible for their induction. To our knowledge this report is the first one on the isolation of a viridans streptococcal promoter of which the activity is directly influenced by a shift from oral to blood pH. Such a stimulus might also be an important signal for gene induction in other bacterial species. We are presently studying whether the expression of more VS genes is influenced in response to this pH change, and whether this response involves a common regulatory mechanism. These insights possibly also lead to a better understanding of the pathogenesis of IE.

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CHAPTER 5

A shift from oral to blood pH is a stimulus for gene expression of endocarditis-causing *Streptococcus gordonii*, and induces protection against oxidative stress by activation of *msrA*

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ABSTRACT

Viridans group streptococci (VS) from the oral cavity entering the bloodstream may initiate infective endocarditis (IE) if a damaged endocardium is present. In response to the new environment VS are expected to express specific genes, of which the products will enable the bacteria to survive and proliferate. We aimed to identify genes that are expressed in response to a defined environmental signal, and that might be involved in the pathogenesis of IE. Using a recently developed promoter-screening vector, we isolated five promoter fragments from the genomic DNA of *S. gordonii* CH1 of which the activity was upregulated when the pH increased from slightly acidic (pH 6.2) to neutral (pH 7.3) levels as encountered when bacteria enter the bloodstream from the oral cavity. No common regulatory sequences were identified in these promoter fragments that could account for the coordinate expression of the corresponding genes. One of the isolated fragments contained the promoter region and 5'-end of a gene that was highly homologous to the methionine sulfoxide reductase (*msrA*) of various bacterial and eukaryotic species. As expression of the *msrA* gene of *S. gordonii* strain V288 was, in addition, recently indicated to be induced in a rabbit model of IE, we isolated and characterized this gene. To define the function of the encoded protein, a chromosomal insertion mutant was constructed. This mutant was more sensitive to hydrogen peroxide, suggesting a role for the streptococcal MsrA in protecting against oxidative stress. Moreover, MsrA appeared to be important for growth of *S. gordonii* CH1 under aerobic and anaerobic conditions. Both these properties of MsrA may contribute to the virulence of *S. gordonii* in the pathogenesis of IE.

INTRODUCTION

Viridans group streptococci (VS), colonizing the teeth and oral mucosal surface of humans, are the most prevelant bacteria causing native valve infective endocarditis (IE), and are isolated from 40-60% of such patients (34). One of the early steps in the development of IE is that VS from the oral cavity gain access to the bloodstream, causing a transient bacteraemia. Subsequently, VS may adhere to a preformed cardiac vegetation, a meshwork of platelets and fibrin present on endocardial lesions (9). Several surface components of VS are thought to be involved in their adherence to the vegetations, like FimA of *Streptococcus parasanguis* (3, 35) and extracellular polysaccharides of various VS species (4, 30, 32). The adherent bacteria are able to multiply rapidly within the vegetation (5, 9).

After entering the bloodstream, the adaptation of VS to this new environment presumably involves the expression of genes, induced upon sensing of signals from the changed environment. One of these signals may be a change in the pH. Many bacteria are known to respond to pH changes. Most investigations have focussed on adaptive responses to a decrease of the pH. Acidification induces expression of specific genes in several bacterial pathogens, like *Salmonella typhimurium* (22) and *Vibrio cholerae* (6), and upregulates the expression of the major stress protein *dna*K in *Streptococcus mutans*, a member of the VS group (16). However, in case of VS entering the bloodstream, the bacteria experience an increase of the pH from slightly acidic (6.0-6.5) (27) in the dental plaque to near neutral in blood (7.3). As this stimulus is possibly involved in the induction of VS genes that might play a role in the colonization of the vegetation by VS, and therefore are involved in the pathogenesis of IE, we isolated promoters of which the activity was upregulated by this pH increase. One of the isolated fragments contained part of an *msrA* homolog, a gene of which the expression was recently found to be induced in *S. gordonii* V288 in the experimental rabbit model of IE (17). We therefore cloned and further characterized this putative *S. gordonii* virulence gene.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *Streptococcus gordonii* strain CH1 was cultured in Todd Hewitt (TH) broth (Oxoid, Basingstoke, Hampshire, England) or on TH-agar at 37°C in a 5% CO_2 atmosphere. TH broth and TH agar plates were supplemented with 5 μg of erythromycin (Em) or chloramphenicol (Cm) per ml, or 500 μg of spectinomycin (Sp) per ml when required. *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium or on LB-agar. When required, 100 μg/ml Em or ampicillin (Amp), and 10 μg/ml Cm were added.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant Characteristics	Reference or source
Strains		
Escherichia coli DH5α		Gibco-BRL, Breda, The Netherlands
Escherichia coli Top10F'		Invitrogen, Groningen, The Netherlands
Escherichia coli BHB2600		14
Streptococcus gordonii CH1		39
Streptococcus gordonii MM1	*S. gordonii* CH1 *msrA*::Em^R	This study
Plasmids		
pCR2.1	*E. coli* PCR cloning vector; 3.9 kb; Ap^R, Km^R	Invitrogen
pUC19	*E. coli* cloning vector; 2.8 kb; Ap^R	41
pMG36e	lactococcal shuttle vector; 3.6 kb; Em^R	12
pNZ124	lactococcal shuttle vector; 2.8 kb; Cm^R	29
pMM223	broad host range promoter trap vector; 8.0 kb; Em^R	36
pMM240	pMM223 with constitutive promoter fragment of *S. gordonii* CH1 in *Bgl*II site	36
pMM1221	pMM223 with neutral pH inducible promoter fragment SG_{P1221} in *Bgl*II site	This study
pMM1222	pMM223 with neutral pH inducible promoter fragment SG_{P1222} in *Bgl*II site	This study
pMM1223	pMM223 with neutral pH inducible promoter fragment SG_{P1223} in *Bgl*II site	This study
pMM1224	pMM223 with neutral pH inducible promoter fragment SG_{P1224} in *Bgl*II site	This study
pMM1225	pMM223 with neutral pH inducible promoter fragment SG_{P1225} in *Bgl*II site	This study
pMM1226	pUC19 with 2.0 kb *Eco*RI fragment containing 3'-end of *msrA* gene	This study
pMM1227	pCR2.1 with *msrA* homolog of *S. gordonii* CH1	This study
pMM1228	pMM1227 with Em^R gene of pMG36e cloned in unique *Hin*dII site of *msrA* homolog	This study
pMM1229	pNZ124 with *msrA* homolog of *S. gordonii* CH1 cloned in *Xba*I and *Hin*dIII sites	This study

DNA isolation, DNA manipulations, and bacterial transformation

Plasmid DNA was isolated from *E. coli* using the Wizard Plus SV miniprep DNA Purification System (Promega Corporation, Madison, WI), and from *S. gordonii* CH1 as described previously (38). Streptococcal chromosomal DNA was isolated using the Puregene Chromosomal DNA isolation kit for Gram-positive bacteria and yeast (Gentra Systems Inc., Minneapolis, MN), with some minor modifications. Lysozyme and mutanolysin were added to the lysis mixture of the DNA isolation kit at a final concentration of 5 mg/ml and 20 U/ml, respectively, and the period of incubation to obtain protoplasts was extended to 2 hours at 37°C. Routine DNA manipulations were performed as described by Sambrook et al. (31), and enzymes were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Transformation of *E. coli* was done by standard electroporation (7). *S. gordonii* CH1 was transformed using an optimized electroporation protocol for VS (36).

Genomic DNA library and selection of neutral pH inducible promoters

The construction of the novel broad host range selection vector pMM223 (Genbank Acc. No. AF076212), and of a genomic expression library of *S. gordonii* CH1 in this vector has been described elsewhere (36; Chapter 4). Briefly, genomic DNA of strain CH1 was digested with *Sau*3A, and fragments were ligated into the *Bgl*II site of pMM223. Recombinant plasmids, containing chromosomal fragments of 100-1.000 bps in size were introduced into the homologous host by electroporation. This *S. gordonii* library contained approximately 10^5 independent clones, statistically representing the entire genome (31).

To isolate neutral pH inducible promoter fragments from this library, 25 µl containing 2.5 x 10^5 clones was plated onto TH-agar of pH 7.3 supplemented with 5 µg of Em per ml for plasmid maintenance, and 500 µg of Sp per ml for selection of active streptococcal promoters. After incubation at 37°C for 36 h, colonies resistant to Em as well as to Sp were plated onto TH-agar of pH 6.2, again supplemented with Em and Sp to identify colonies susceptible to Sp at this lower pH. As a control for the viability of the isolated *S. gordonii* clones, these were also restreaked onto TH-agar plates of pH 6.2 supplemented with Em only, and onto plates of pH 7.3 with Em and Sp. From clones that failed to grow on the pH 6.2 agar, but which did grow on the agar of pH 7.3 in the presence of Sp, the cloned chromosomal fragments were amplified.

PCR amplification and DNA sequence analysis

Cloned chromosomal DNA fragments from selected *S. gordonii* CH1 strains were amplified from crude bacterial lysates by PCR (15), using primers AV9 (5'-ATGTCACTAGTCTCTACAAC-3') and AV4 (5'-AATTGGATCCCGGGTTTTTTTATAATTTTTT TAATCTG-3') and Taq DNA polymerase (Promega Corporation, Madison, WI). Amplicons were purified using the High Pure PCR product purification kit (Boehringer Mannheim), and sequenced by PCR-mediated Taq Dye Deoxy Terminator Cycle sequencing (Perkin Elmer, Foster City, Ca) on an Applied Biosystems (San Jose, CA) model 373 DNA sequencer. Primer AV9 or primer AV19 (5'- CTCCTCACTATTTTGATTAG-3'), annealing upstream and downstream of the unique *Bgl*II site of pMM223, respectively, were used to sequence the cloned fragments. Obtained sequences were analysed using the BLAST program (1). For identification of possible common sequence features, the CLUSTAL program was used (13).

Measurement of in vitro growth rate

To determine the relative activity of isolated neutral pH inducible promoter fragments, the growth rate in the presence and absence of Sp of the clones carrying these fragments was determined at both pH 6.2 and pH 7.3 (36). In short, a single colony of each clone was grown at 37°C in TH supplemented with Em for plasmid maintenance. After overnight incubation, cultures were diluted 100-fold in fresh medium containing Em and Sp, or Em alone. Growth was monitored by measuring the optical density at 620 nm over time, and the mid-log phase doubling time ($t_{½}$) was determined. Relative promoter activity at each pH was expressed as the ratio of growth in the presence and absence of Sp ($t_{½}$(+spec) / $t_{½}$(-spec).

Isolation and characterization of the streptococcal *msrA* gene

Chromosomal DNA of *S. gordonii* CH1 was digested to completion with *Hin*dIII, and the resulting fragments were self-ligated. Using primers AV40 (5'-CAAGCCCCAGAAACACCCGC-3') and AV41 (5'-CAGTGGGATACGCCAATGGAC-3'), corresponding to the complement of nucleotide 23-42 and to nucleotide 83-103 of the identified streptococcal *msrA* homolog, respectively (see Results), a fragment of approximately 3.5 kb was amplified. The purified amplicon was ligated into the PCR cloning vector pCR2.1, and introduced into *E. coli* TOP10F' cells. Part of this fragment, containing the 3'-end of the *msrA* gene, was subcloned as a 2.0 kb *Eco*RI fragment into pUC19 generating pMM1226. After digestion with *Bam*HI and *Sph*I, subclones with fragments of decreasing sizes were created by exonuclease III (Boehringer Mannheim) digestion according to standard procedures (31). Individual fragments were sequenced using the universal M13(-21) and M13(Reverse) primers, and the sequence of the streptococcal *msrA* homolog was compilated.

Primer extension assay

Total RNA was extracted from *S. gordonii* CH1 using the RNAeasy Mini Kit (Qiagen GmbH, Hilden, Germany). 10 µg of total cell RNA was used in the primer extension reactions. RNA was incubated for 5 min at 65°C with 0.2 pmol of primer AV40 in hybridization buffer (70 mM Tris-HCl [pH 8.3], 14 mM $MgCl_2$, 14 mM dithiothreitol) in a final volume of 14 µl. The mixture was gradually cooled to room temperature, and the volume was adjusted to 20 µl by the addition of dATP, dGTP, and dCTP to a final concentration of 100 µM, and dCTP to a final concentration of 10 µM. To this mixture 15 µCi of [α-^{32}P]dCTP with a specific activity of 3,000 Ci/mmol was added. cDNA was synthesized by the addition of 12.5 Units of avian myoblastosis virus reverse transcriptase (Boehringer Mannheim), and incubation at 42°C for 30 min. The reaction was terminated by the addition of 5 µl sequencing loading buffer. In addition, a sequence reaction was performed with the same primer, using the T7 Sequenase version 2.0 DNA sequencing kit (Amersham Life Science, Inc., Cleveland, OH) and [α-^{35}S]dATP. The primer extension reaction was electrophoresed on a 6% polyacrylamide - 7 M urea gel, parallel to the sequence reaction which served as marker for determination of the size of the synthesized cDNA.

Construction of an *S. gordonii* CH1 *msrA* deletion mutant

The complete *msrA* gene including its putative promoter sequence was amplified from the *S. gordonii* CH1 chromosomal DNA using the Expand long template PCR kit (Boehringer Mannheim), and primers AV45 (5'-AATTACTAGTGAAATGAAGAATATGGCTGGGTTGAGAAG-3') and AV46 (5'-ATATACTAGTGCCAACGCTCAGCAAAAAAGGCCTG-3'). The obtained amplicon of approximately 1.1 kb was cloned into pCR2.1, creating vector pMM1227. The erythromycin resistance gene of the broad host range vector pMG36e (12) was isolated as a 1.0 kb *Eco*RI – *Nsi*I fragment, and the sticky ends were filled in using Klenow fragment enzyme polymerase (Boehringer Mannheim). This fragment was ligated into the unique *Hin*dII site of the *msrA* gene in pMM1227, and the resulting vector pMM1228 was linearized with *Bgl*II. This linear plasmid DNA was introduced into *S. gordonii* CH1 by electroporation, and erythromycin resistant clones were selected on agar plates. Chromosomal integration of the *msrA* copy carrying the inserted erythromycin resistance gene was confirmed by Southern blotting. To complement the mutation, the *msrA* gene was obtained as an *Eco*RI fragment from pMM1227, and ligated into the unique *Eco*RI site of the broad host range vector pNZ124, resulting in plasmid pMM1229. After this construct was introduced into the insertion mutant and into the wild type, colonies resistant to Em and Cm were selected on TH agar plates.

Southern blotting

Southern blots were prepared according to standard procedures (31) using Zeta-probe membranes (Bio-Rad, Hercules, CA). DNA probes were random primed labeled with Digoxigenin-11-dUTP using the DIG system for filter hybridization (Boehringer Mannheim). Hybridization was done in DIG Easy Hyb hybridisation solution

(Boehringer Mannheim) at 60°C, and DIG-labeled nucleic acids were visualized with anti-DIG-HRP and CSPD as described by the manufacturer (Boehringer Mannheim).

Hydrogen peroxide inhibition assay

To test the susceptibility of bacteria to H_2O_2, a disk inhibition assay was performed, essentially as described by Moskovitz et al. (25). Bacteria were grown to stationary phase in TH broth. One ml of the bacterial suspension was added to 5 ml of liquid TH agar of 42°C, and poured onto TH agar plates. A 1,3-cm diameter filter disk (Whatman Scientific Ltd., Maidstone, UK) was placed on the plate and impregnated with either 20 µl of H_2O or 20 µl of a 30% H_2O_2 solution. The plates were incubated overnight at 37°C.

RESULTS

Isolation of pH-regulated promoters from *S. gordonii*

A genomic library of *S. gordonii* CH1 was used for the selection of neutral pH inducible promoters. A total of 146 Sp resistant colonies apparently carrying an active promoter fragment grew on TH-agar plates of pH 7.3 supplemented with Em (5 µg/ml) and Sp (500 µg/ml). The relatively limited number of Sp resistant clones was presumably due to the high antibiotic concentration used for selection. Two of the Sp resistant clones showed no growth on Sp containing TH plates of pH 6.2 (CH1 pMM1223, and CH1 pMM1224), and growth of three other clones was strongly reduced on these plates (CH1 pMM1201, CH1 pMM1202, and CH1 pMM1205). All five clones grew well on the two control plates. Growth of the other 141 Sp resistant clones on either of the three plates did not show any difference. This indicated that the five selected *S. gordonii* CH1 clones had a lower promoter activity at pH 6.2 than at pH 7.3.

Identification and characterization of the pH-regulated promoters

To identify the promoters of the five selected strains, the cloned genomic fragments were amplified by PCR and sequenced. Four of five promoter fragments showed sequence homology to known entries in the EMBL/Genbank/DDBJ databases (Table 2).

Table 2. Identified sequence homologies for the isolated neutral pH inducible promoter fragments from *S. gordonii* CH1.

Strain	Promoter	Database match[a]
CH1 pMM1221	SG_{P1221}	*cysK* of *Bacillus subtilis* (P37887)
CH1 pMM1222	SG_{P1222}[b]	*msrA* of *Streptococcus pneumoniae* (U41735)
CH1 pMM1223	SG_{P1223}	*hydA* of *Clostridium acetobutilicum* (U15277)
CH1 pMM1224	SG_{P1224}	no homology

[a] Accession number to EMBL/Genbank/DDBJ databases in parenthesis.
[b] This promoter fragment is identical to SG_{P1225}.

SG_{P1221} showed homology to the 5'-end of *cysK* from *Bacillus subtilis*, as well as to *cysK* homologs in several other bacterial species (*Mycobacterium*, *Escherichia coli*, *Salmonella typhimurium*). SG_{P1223} showed limited similarity to the promoter region of the *hyd*A gene of *Clostridium acetobutylicum* ATCC 824. We already isolated these promoter fragments in previous studies, using other experimental settings (36,37). The sequence within SG_{P1224} (Fig. 1) presumable responsible for the expression of the promoterless Sp gene of pMM223 did not have similarity to known sequences. Upstream and in the inverse orientation an open reading frame was located, of which the translated amino acid sequence was homologous to the N-terminal region of the 6-phosphate-beta-glucosidase of several bacterial species, including *B. subtilis* and *E. coli*. Several regions were identified in this fragment that could act as promoters driving either the expression of the promoterless Sp gene of pMM223 or of the oppositely oriented phospho-beta-glucosidase (*pbg*)-like gene (Fig. 1).

SG_{P1222} and SG_{P1225} appeared to be identical genomic DNA fragments. The sequence was highly homologous to the 5'-end of the methione sulfoxide reductase (*msrA*) gene from different bacterial and eukaryotic organisms. The translated sequence of this fragment showed strong identity to the N-terminus of the MsrA of *Streptococcus pneumoniae* (Swissprot database Acc. No. P35593). The upstream sequence was a possible open reading frame with over 85% identity at the protein level to the dihydroorotate dehydrogenase (PyrD) of *Streptococcus thermophilus* ST11 (EMBL database Acc. No. Y12213), an enzyme involved in the de novo biosynthesis of pyrimidine.

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  1 GATCAACCTGGCTTGGCTGGCAACTCCGCCTTTTGGAGTCACGTCTTGAACAGACAGGCC  60
    I  L  R  A  Q  S  A  V  G  G  K  P  T  V  D  Q  V  S  L  G
 61 TTTTCCCATTAGATTATAGGCACCCTCATATTGATTTGCCGCCGTTGCACCGCCCCAGAG 120
    K  G  M  L  N  Y  A  G  E  Y  Q  N  A  A  T  A  G  G  W  L
                          pbg
121 GAAATTTTTAGGAAATTTAGCCATTGCATTTCTCGCTTTCGTTTTTTCTTTGATTATATT 180
    F  N  K  P  F  K  A  M        SD     -35 P1        -10 Ppbg

181 ATAATCTTAGATGAAAATGTTTTCTTACACAATTCTCTTATATATTGATACTATTTTACT 240
    -10 P1          -35 P2                  -10 P2

241 ACAAGGAGCAGGGAATGCTAGATC 263
       SD         M  L  D
```

Figure 1.

Nucleotide sequence of the promoter fragment SG_{P1224}. Putative -35 and -10 promoter regions and Shine Dalgarno sequences (SD) are underlined. P_1 and P_2 are two possible promoter stretches driving expression of the promoterless Sp gene, and P_{pbg} is a putative promoter driving expression of the inversely oriented *pbg*-like gene. Translational start sites (ATG) are printed in bold, and partial open reading frames are shown. This sequence has been assigned Genbank accession number AF153503.

The inducibility of the selected clones was confirmed by determination of the ratio of the growth rates in the presence and in the absence of Sp. All clones showed a reduction in this growth rate ratio at pH 6.2 (Table 3). Although the activity of all promoters was upregulated by an increase in the pH, no general structure was identified in the sequences of the promoter fragments that might account for this regulation.

Table 3. Activity at pH 6.2 and 7.3 of a constitutive and of pH-regulated promoters isolated from *S. gordonii* CH1, recorded as the ratio of growth in medium with and without Sp[a].

Strain	pH 6.2	pH 7.3
CH1 pMM240	0.94 ± 0.01	0.98 ± 0.01
CH1 pMM1221	0.90 ± 0.03	0.99 ± 0.01
CH1 pMM1222	0.73 ± 0.09	0.93 ± 0.03
CH1 pMM1223	0.68 ± 0.05	0.86 ± 0.06
CH1 pMM1224	0.62 ± 0.05	0.82 ± 0.10

[a] Growth was monitored by measuring the optical density at 620 nm (A_{620}) over time, and the mid-log phase doubling time ($t_{½}$) was determined. Relative promoter activity at the different pH values is expressed as the ratio of growth in the presence and absence of spectinomycin at each pH tested ($t_{½}$(+spec) / $t_{½}$(-spec)). pMM240 contains a constitutive promoter of *S. gordonii* CH1.

Isolation and characterization of the *msrA* gene from *S. gordonii* CH1

As the activity of the *msr*A promoter homolog of *S. gordonii* V288 was recently found to be induced in the experimental rabbit model of IE (17), we further characterized the corresponding *S. gordonii* CH1 *msrA* homolog. The 3'-end of the CH1 *msrA* gene was amplified by inside-out PCR on a self-ligated *Hin*dIII digest of chromosomal DNA using primer pair AV40/AV41. After subcloning and exonuclease III treatment of the obtained 3.5 kb amplicon, a final fragment of approximately 1.2 kb was sequenced. This sequence contained the 3'-end of *msrA*, and overlapped the sequence of the SG_{P1222} promoter fragment, which allowed the assembly of a total sequence of 1782 nucleotides (Fig. 2).

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   pyrD
  1 GATCGGAAACGGCCTTTATATAGAAGATGAGTCAGTAGTCATTCGTCCGAAAAATGGCTT  60
      I  G  N  G  L  Y  I  E  D  E  S  V  V  U  R  P  K  N  G  F
 61 TGGAGGTATCGGTGGTCAGTACATCAAGCCGACCGCTCTGGCCAATGTCCATGCTTTTTA  120
      G  G  I  G  G  Q  Y  I  K  P  T  A  L  A  N  V  H  A  F  Y
121 CCAACGCCTGAAACCAGAAATCCAAATCATCGGAACTGGTGGTGTCTTGACAGGGCGGGA  180
      Q  R  L  K  P  E  I  Q  I  I  G  T  G  G  V  L  T  G  R  D
181 TGCTTTTGAGCATATCCTCTGTGGAGCCAGTATGGTGCAGGTCGGAACAACCCTTCATAA  240
      A  F  E  H  I  L  C  G  A  S  M  V  Q  V  G  T  T  L  H  K
241 AGAAGGAGTGGTAGCCTTCGAGCGCATCACCGCAAAACTCAAGACTATTATGGAAGAAAA  300
      E  G  V  V  A  F  E  R  I  T  A  K  L  K  T  I  M  E  E  K
301 AGGCTATGAAAGTCTGGAAGATTTCCGAGGAAAATTGAAATATATTGAGGAGTAATCTTC  360
      G  Y  E  S  L  E  D  F  R  G  K  L  K  Y  I  E  E  -
361 TATACAAGCGTTGTTGTGATTTGAAATGAAGAATATGGCTGGGTTGAGAAGTATTGAAAC  420
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                                                                *
421 CTAGCTTTTTCATATTATTTTGAAGAAAGTTCTAATCAAATTGCTATAATTTCGACTCCT  480
             -35                      -10
                                                  msrA
481 TTTATGAGAAAATAGACGCAAAGAAATGAGGTGTTTTTTATGGCTGAAATTTATCTAGCAG  540
                                SD          M  A  E  I  Y  L  A

541 GCGGGTGTTTCTGGGGCTTGGAAGAGTATTTCTCCCGCATTGAAGGTGTTAAGAAAACGA  600
    G  G  C  F  W  G  L  E  E  Y  F  S  R  I  E  G  V  K  K  T
601 CAGTGGGATACGCCAATGGACAGGTGGAATCAACCAACTACCAGCTGATTCATCAAACGG  660
    T  V  G  Y  A  N  G  Q  V  E  S  T  N  Y  Q  L  I  H  Q  T
661 ACCATGCAGAGACGGTTCATCTAATCTATGATGAAAAGCGGGTCAGCCTGCGGGAAATCC  720
    D  H  A  E  T  V  H  L  I  Y  D  E  K  R  V  S  L  R  E  I
721 TGCTTTATTATTTCCGAGTTATTGATCCTCTGTCGGTCAATAAGCAGGGAAATGATGTAG  780
    L  L  Y  Y  F  R  V  I  D  P  L  S  V  N  K  Q  G  N  D  V
781 GACGCCAGTATCGGACAGGTGTTTACTATACCAATCAAGCCGATAAGGCGGTCATTGAAC  840
    G  R  Q  Y  R  T  G  V  Y  Y  T  N  Q  A  D  K  A  V  I  E
841 AGGTCTTTGCTGAGCAGGAAAAGCAGTTGGGACAAAAGATTGCTGTTGAGTTGGAACCTT  900
    Q  V  F  A  E  Q  E  K  Q  L  G  Q  K  I  A  V  E  L  E  P
901 TGCGCCACTATGTCCTAGCCGAAGACTACCATCAGGATTATCTCAAGAAAAATCCCGGTG  960
    L  R  H  Y  V  L  A  E  D  Y  H  Q  D  Y  L  K  K  N  P  G
961 GCTACTGTCATATCAATGTCAATGACGCCTATCAGCCCTTGGTCGATCCTGGCCAATATG 1020
    G  Y  C  H  I  N  V  N  D  A  Y  Q  P  L  V  D  P  G  Q  Y
1021 AGAAACCTACAGATGCGGAACTGAAAGAACAGTTGACGCAAGAGCAATACCAGGTGACCC 1080
    E  K  P  T  D  A  E  L  K  E  Q  L  T  Q  E  Q  Y  Q  V  T
1081 AGCTCAGTGCGACAGAGCGTCCTTTCCACAATGCCTACAATGCCACTTTTGAAGAAGGAA 1140
    Q  L  S  A  T  E  R  P  F  H  N  A  Y  N  A  T  F  E  E  G
1141 TTTATGTTGATGTGACGACTGGGGAGCCTCTCTTTTTTGCCGGTGACAAGTTTGAGTCTG 1200
    I  Y  V  D  V  T  T  G  E  P  L  F  F  A  G  D  K  F  E  S
1201 GCTGCGGCTGGCCTAGCTTTAGTCGGCCTATTGCCAGAGAAGTTCTGAGATACTATGAAG 1260
    G  C  G  W  P  S  F  S  R  P  I  A  R  E  V  L  R  Y  Y  E
1261 ACAAGAGTCATGGCATGGAGCGGATTGAAGTGCGCAGCCGTTCTGGCAATGCCCATCTGG 1320
    D  K  S  H  G  M  E  R  I  E  V  R  S  R  S  G  N  A  H  L
1321 GCCATGTCTTTACTGACGGTCCAGAGTCAGCGGGCGGTCTTCGCTACTGCATCAACTCCG 1380
    G  H  V  F  T  D  G  P  E  S  A  G  G  L  R  Y  C  I  N  S
1381 CAGCTCTGCGATTTATTCCAAAAGAAAAAATGGAAGCAGAAGGTTATGCTTATCTACTTC 1440
    A  A  L  R  F  I  P  K  E  K  M  E  A  E  G  Y  A  Y  L  L

                 ------>          <------
1441 AACACATGAAATAAAAGGCCATCAGGCCTTTTTTGCTGAGCGTTGGCAAAAGTTTTTAGA 1500
    Q  H  M  K  -                                -35
                                                  orf
1501 TAAGGGATAATGATCATAATAAAAATGAGGAGTGAAAGATGAAATTAAAAAGATTTTCGA 1560
          -10                  SD          M  K  L  K  R  F  S

1561 CGATTTTACTTACTATTTTAACGATTATCACTCTAACTGCTTGTAAATCTAATGGTAAAG 1620
    T  I  L  L  T  I  L  T  I  I  T  L  T  A  C  K  S  N  G  K
1621 GGCAGGNTAATTCGAATAAACCAACTGAAGAAAGTTCAAGCTCAAAATCAGAAGAAAAGA 1680
    G  Q  X  N  S  N  K  P  T  E  E  S  S  S  S  K  S  E  E  K
1681 CTAAGTCAACTAGTAAAGAAGAGAAAACTAGTAAAGAGACCCAGTCGTCAAGTAAGGAAA 1740
    T  K  S  T  S  K  E  E  K  T  S  K  E  T  Q  S  S  S  K  E
1741 GTGGAGAGAAGGATAATTCTGGATTCTCAGAAAAAAGGGAAG 1782
    S  G  E  K  D  N  S  G  F  S  E  K  R  E
```

Figure 2.

Complete nucleotide sequence of the *msrA* gene from *S. gordonii* CH1, and partial sequences of a *pyrD* homolog and of a putative ORF, located upstream and downstream of the streptococcal *msrA*, respectively. Putative -35 and -10 promoter hexamers and Shine-Dalgarno sequences (SD) are underlined, and the transcriptional start of *msrA* is indicated with an asterisk. Inverted repeats, which might form a transcriptional termination stem-loop, are indicated with arrows. Translational start sites (ATG) are printed in bold, and the translated amino acid sequences of the *msrA* and of the partial open reading frames upstream and downstream of *msrA* are shown. Nucleotides 1 to 747 represent the sequence of the isolated promoter fragments SG_{P1222} and SG_{P1225}. This sequence has been assigned Genbank accession number AF128264.

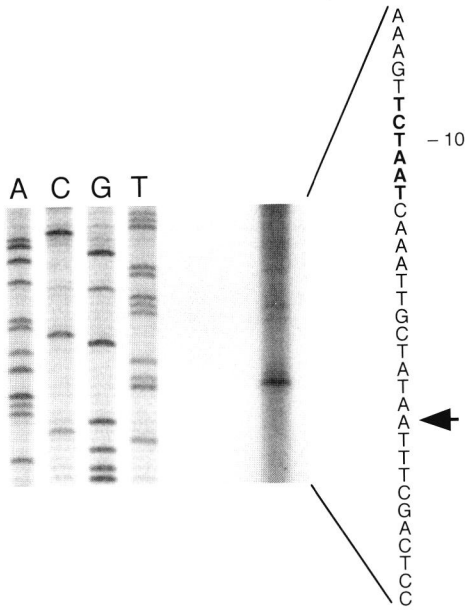

Figure 3.

Determination of the transcription start site of *S. gordonii* CH1 *msrA*. The transcription start site is indicated with an arrow, and the putative -10 region in the coding strand is presented in bold.

The *S. gordonii* CH1 *msrA* gene consisted of 933 nucleotides. A potential ribosome binding site was found 8 nucleotides upstream of the ATG translation start. Primer extension analysis revealed the transcription initiation site located 50 nucleotides upstream of the translation start of the gene (Fig. 3). Preceding this transcription start, putative -35 and -10 regions were identified. At the end of the gene inverted repeats, capable of forming a terminator stem-loop structure with a free energy of -11.4 kcal, were identified. The *S. gordonii* CH1 *msrA* encodes a putative protein of 311 amino acids with a predicted molecular mass of 35.7 kDa and pI of 5.35. Comparison of the translated amino acid sequence to entries in the databases revealed strong homology throughout the protein to other MsrA homologs (Fig. 4). There was 68% and 72.6% identity at the DNA and protein level, respectively, to the methionine sulfoxide reductase of *Streptococcus pneumoniae*. Upstream of the pneumococcal *msrA* sequence, so called Box elements are present that are possibly involved in regulation of gene expression (19). No such structures were detected upstream of the translational start site of the *msrA* gene of *S. gordonii* CH1.

Downstream of the *msrA* gene another possible open reading frame (ORF) was identified (Fig. 2). A putative ribosome binding site and -35 and –10 promoter regions were present in the intergenic region preceding this ORF. The open reading frame or its translated amino acid sequence did not have homology to any known sequences in the databases.

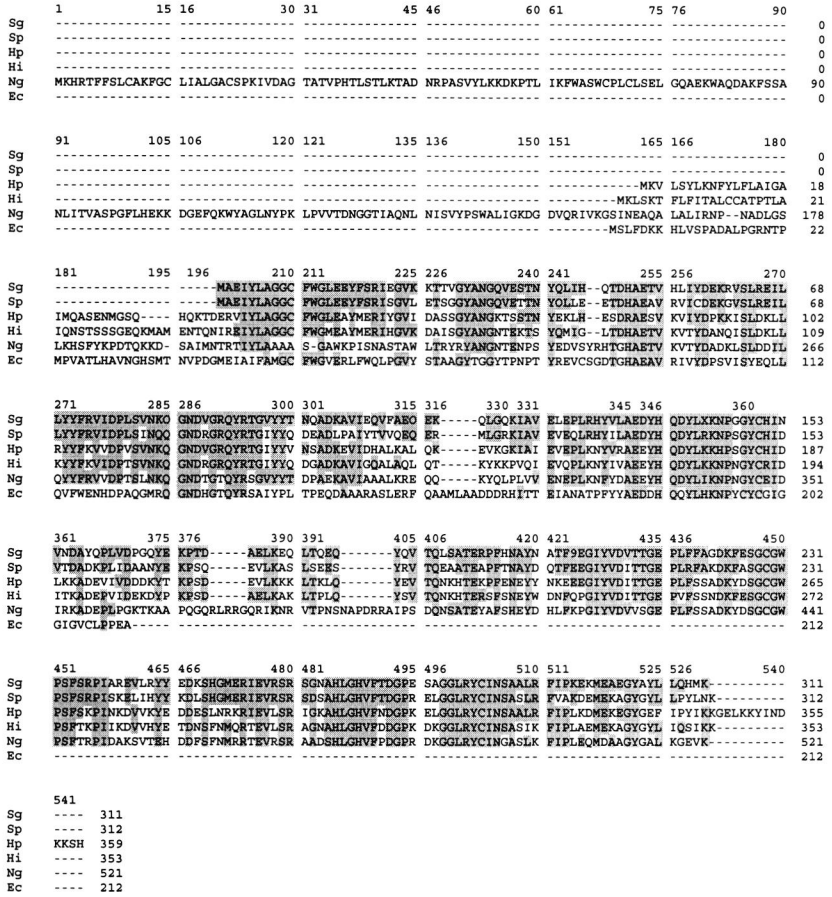

Figure 4.

Amino acid sequence alignment of MsrA of *S. gordonii* with MsrA proteins of *Streptococcus pneumoniae* (Sg), *Helicobacter pylori* (Hp), *Haemophilus influenzae* (Hi), and *Escherichia coli* (Ec), and with the homologous PilB of *Neisseria gonorrhoeae* (Ng). Amino acid sequence alignment was performed with the CLUSTAL program. Shaded boxes enclose residues of the MsrA from *S. gordonii* CH1 that are found at identical positions within one or more of the other MsrA sequences, or within Ng PilB.

Effect of *msrA* mutation on sensitivity to oxidative stress and on growth

An *msrA* mutant of *S. gordonii* CH1 was constructed by insertion of an Em resistance marker. Em resistant clones were tested for successful integration by Southern blotting. Strain MM1 was found to have the Em resistance gene inserted into the *msrA* gene, resulting in an increase in size by 1.0 kb of the chromosomal fragment hybridizing with the *msrA* probe (Fig. 5).

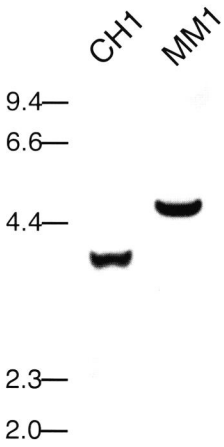

Figure 5.

Southern blot of *S. gordonii* strain CH1 and its *msrA* insertion mutant MM1. The hybridizing fragment in the wildtype strain is increased in size in the mutant strain by 1.0 kb, due to the inserted erythromycin resistance gene.

As MsrA is known to play a role in protection against oxidative damage in other bacterial and eukaryotic species (23-26, 40), sensitivity to oxidative stress of the *S. gordonii* CH1 *msrA* mutant was tested using an H_2O_2 disk inhibition assay (25). Growth of the mutant strain was more strongly reduced than observed for the parent strain when the disk was impregnated with 30% H_2O_2. Complementation of the mutation by introduction of an intact copy of the *msrA* gene on a low copy number plasmid into the mutant strain MM1 decreased the inhibition zone to that observed with the wild-type (Table 4). No growth inhibition was observed for either strain when the disk was impregnated with water. These data strongly indicate that absence of a functional *msr*A gene renders *S. gordonii* more susceptible to H_2O_2 stress.

Next, the growth rate in TH broth of the different strains was assessed, in order to define a possible influence of absence of a functional MsrA on bacterial multiplication. Growth of the mutant MM1 was strongly reduced compared to that of the wild-type strain CH1 when cultured at 37°C under either aerobic or anaerobic conditions. The growth rate of the *msrA* complemented mutant was almost identical to that of the wild-type strain CH1. These results imply a function of the streptococcal MsrA homolog in bacterial multiplication, in addition to its role in protection against oxidative stress.

Table 4. Effect of H_2O_2 treatment on the growth of *S. gordonii* CH1 and its *msrA* insertion mutant *S. gordonii* MM1.

Genotype		H_2O_2[a]	Area of growth inhibition(cm^2)[b]
msrA$^+$		-	0
msrA::Em^R		-	0
msrA$^+$		+	7.8
msrA::Em^R		+	11.2
msrA$^+$	(pMM1229, *msrA*$^+$)	+	7.8
msrA::Em^R	(pMM1229, *msrA*$^+$)	+	7.8

[a] In control experiments, the disks were impregnated with 20 µl of H_2O instead of H_2O_2.
[b] The amount of growth inhibition is expressed as the area of the clear zone minus the area of the disk, as no growth inhibition was observed under the control conditions.

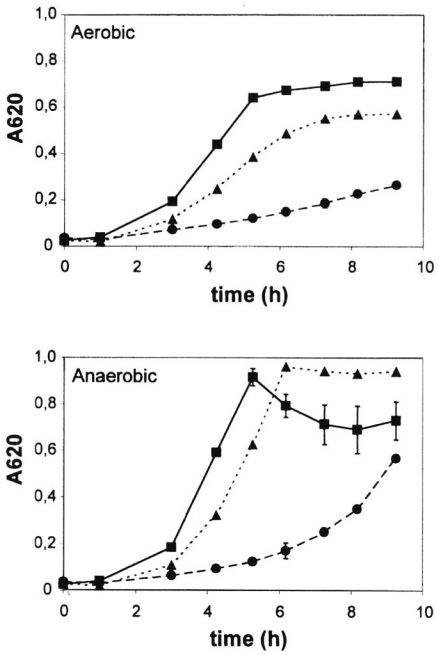

Figure 6.
Growth of *S. gordonii* CH1 (squares), its *msrA* mutant MM1 (circles), and the complemented mutant (MM1 pMM1229; triangles) under aerobic (upper) and anaerobic (lower) conditions in TH medium.

DISCUSSION

In this study we found that a slight increase in the environmental pH, as observed when VS from the oral cavity gain access to the bloodstream, induces or upregulates the expression of specific genes. Indeed, five clones were isolated from an *S. gordonii* CH1 expression library, containing a promoter of which the activity was upregulated when raising the pH from 6.2 to 7.3. No common regulatory sequences that might be involved in a coordinate pH regulated gene expression could be identified in the sequenced promoter regions. Another example of response to increase in pH by VS is intracellular thrombin-like activity of *Streptococcus sanguis*, which is reduced at acidic pH and is increased upon alkalification of the medium (20). Induction of gene expression upon increase of the environmental pH might, therefore, be a general response mechanism within the viridans group streptococci in order to survive when the bacteria translocate from the oral cavity to the blood.

One of the isolated pH-regulated promoter fragments, SG_{P1221}, showed homology at both the DNA and protein level to the cysteine synthase of *B. subtilis*. We have also identified this promoter fragment (EMBL database Acc. No. AJ236900) in recent screening experiments for constitutively active promoters from *S. gordonii* CH1 (37). In those experiments we used agar plates of pH 7.8, which explains the isolation of this promoter. In *B. subtilis*, CysK is expressed under normal laboratory conditions, but expression levels can be up- or downregulated by different environmental stimuli, e.g. cold shock, heat shock, and salt stress (11). In *S. gordonii* the level of expression of this gene is regulated by variation in the external pH, a stimulus which might also regulate expression of the *B. subtilis cysK*.

Fragment SG_{P1223} showed limited homology to the promoter region of the *hydA* gene from *Clostridium acetobutylicum* ATCC 824. Expression of this gene in *C. acetobutylicum* is known to be trancriptionally regulated by the environmental pH (10). SG_{P1223} was identical to a neutral pH inducible promoter fragment we have identified earlier (36), indicating reproducibility of the screening system.

One neutral pH inducible promoter fragment was isolated twice from the genomic DNA library (SG_{P1222} and SG_{P1225}). The fragment showed homology to the *msrA* gene found in many prokaryotic (*Escherichia coli*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*) (25, 40) and eukaryotic (*Saccharomyces cerevisiae*, rat, human) species (23, 24, 26). This gene encodes the methionine sulfoxide reductase, a protein involved in the reduction of oxidized proteins. Sulfur groups of methionine residues are highly sensitive to oxidation by oxygen radicals, and oxidized proteins are in general not functional. Reduction of oxidized methione residues by MsrA restores the protein function, thus decreasing the need for de novo protein synthesis (8). A second function recently suggested for MsrA is its involvement in the stabilization of adhesins. Mutation in *E. coli msrA* decreased fimbriae-mediated mannose-dependent agglutination of erythrocytes, and mutation of *S. pneumoniae msrA* caused

decreased binding to specific glycoconjugate-containing receptors on vascular endothelial and lung cells (40). Finally, the methionine sulfoxide reductase might also be involved in signal transduction, as it is highly homologous to PilB of *Neisseria gonorrhoeae* (40), the sensor component of the PilAB two-component regulator system (33). However, such a function could not be identified for the MsrA from *S. pneumoniae* (28).

The promoter of the *msrA* gene from *S. gordonii* V288 is activated *in vivo* in a rabbit model of endocarditis (17). In addition, methionine sulfoxide reductase has been demonstrated to be of importance for the survival of *S. aureus* in a murine bacteraemia model (21). Although an *S. aureus msrA* deletion mutant was not attenuated in its virulence in this model, in mixed infections the wild-type was almost solely reisolated (21). This indicates that the MsrA is beneficial for bacterial survival in this host.

We isolated and characterized the *msrA* gene of *S. gordonii* CH1. The transcriptional start site and putative -35 and -10 regions were identified. In *S. pneumoniae*, *msrA* is preceded by four conserved DNA sequences with strong identity to the consensus sequences of several pneumococcal Box elements (40). Box elements consist of various combinations of the three subunits boxA, boxB, and boxC, and are considered regulatory elements for coordinately controlled gene expression (19). Although boxA-like subsequences are widespread among bacteria (18), no homologs or other possible inverted repeats were identified upstream of *msrA* of *S. gordonii* CH1, indicating an alternative regulation of this gene.

MsrA of *S. gordonii* CH1 appeared to be involved in protection against oxidative stress, as growth of the *msrA* mutant strain MM1 on solid media in the presence of H_2O_2 was much more reduced than growth of wild-type CH1. This may well be of great importance for survival *in vivo*, as bloodborne bacteria are challenged by oxidative radicals produced by polymorphonuclear leucocytes and other cells of the host immune system (2).

In addition, MsrA was required for maximal growth, both under aerobic and anaerobic conditions. The observed growth reduction of the *S. gordonii* mutant under aerobic conditions was not caused by an increased sensitivity to oxidative damage, as a similar difference in growth rate between wild-type and mutant strain was observed when they were cultured under anaerobic conditions. Complementation of the mutation almost completely restored growth to wild-type levels. However, in *E. coli* mutation of *msrA* did not affect growth (25). It therefore seems that MsrA of *S. gordonii* CH1, in addition to having a function in protection against oxidative damage, plays an important role in bacterial growth. This phenomenon might also explain the above mentioned survival benefit of wild-type *S. aureus* in mixed infections with its *msrA* mutant in the murine bacteraemia model (21). In addition, MsrA will probably be of importance in IE, as rapid bacterial multiplication is a major characteristic of VS in the development of this disease (5, 9).

In conclusion, we have isolated several promoters from *S. gordonii* CH1 regulated by an increase in environmental pH, showing that such a stimulus is an extracellular signal for adaptive gene expression of VS. Mutation of the *S. gordonii* CH1 *msrA*, one of the genes

activated by blood pH, increased sensitivity to oxidative stress, and severly affected growth under both aerobic and anaerobic conditions. As these characteristics are important for bacterial survival *in vivo*, *msrA* presumably contributes significantly to the virulence of *S. gordonii* in the development of IE.

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CHAPTER 6

Isolation and characterization of promoter regions from *Streptococcus gordonii* CH1

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ABSTRACT

We aimed to identify transcription signal sequences from *Streptococcus gordonii* strain CH1 by random chromosomal cloning. Five genomic fragments from a *Sau*3A digest, that constitutively activated transcription of a promoterless spectinomycin resistance gene in this strain, were isolated and characterized. Additionally, one promoter fragment was isolated which was specifically activated under iron-limiting conditions. A sequence motif with similarity to the consensus for Fur-binding regulatory DNA sequences (Fur box) in *Escherichia coli* was detected within the putative promoter region. The open reading frame downstream of this region possibly encodes a transmembrane protein involved in iron uptake.

INTRODUCTION

Streptococcus gordonii belongs to the group of the viridans streptococci, commensals of the human oral cavity and oropharyngeal tract (7). In recent years, *S. gordonii* has been exploited in the field of oral vaccine development for use as a live, Gram-positive vector system. Expression of fusion genes of foreign antigens and streptococcal surface proteins resulted in surface exposure of these antigens (19, 22). Antigen delivery by these live *S. gordonii* vaccines induced not only excellent systemic but also efficient mucosal immune responses (18).

S. gordonii is also recognized as an opportunistic agent in infective endocarditis (IE), a relatively infrequent, but severe disease of the endocardial lining and heart valves (3). The disease is characterized by rapid growth of bacteria inside a thrombus predominantly composed of platelets and fibrin (vegetation) formed on an endocardial lesion (4). In order to survive and grow in this niche, the bacteria express specific genes (11, 14). Such genes will be activated in response to environmental signals, e.g. pH, low iron concentration, anaerobiosis, and temperature. These signals have been demonstrated to play a role in the expression of virulence factors of different bacterial species, including induction of M protein expression (by iron limitation) of the closely related *Streptococcus pyogenes* (17).

As more extensive knowledge about transcription signals from *S. gordonii* might be helpful for optimalization of heterologous protein expression in this bacterial species, we isolated and characterized promoter sequences with different activities from *S. gordonii* strain CH1, using a random chromosomal cloning approach. Additionally, since little information is available about the regulation of gene expression in *S. gordonii* by specific environmental signals, potentially involved in the pathogenesis of IE, we aimed to identify promoter sequences that were activated upon iron limitation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

S. gordonii CH1 (26) was cultured in Todd Hewitt (TH) broth and on TH-agar (Difco Laboratories, Detroit, MI) at 37°C in a 5% CO_2 atmosphere. When required, 5 μg ml^{-1} erythromycin (Em) was added for plasmid maintenance, and 0.1, 0.5, or 2.0 mg ml^{-1} spectinomycin (Sp) for selection of streptococcal promoters.

Molecular biology procedures

DNA manipulations were done according to standard techniques (23). Restriction enzymes, T4 DNA ligase, and Calf Intestine Alkaline Phosphatase (CIAP) were purchased from Boehringer (Boehringer Mannheim GmbH, Mannheim, Germany). For PCR, *Taq* DNA polymerase and dNTPs were obtained from Promega (Promega Corporation, Madison, WI). A genomic expression library of *S. gordonii* CH1 was constructed in the broad host range selection vector pMM223 (Genbank Acc. No. AF076212). This vector is derived from the lactococcal

shuttle vector pGKV210 (25), and contains the promoterless *aad*(9) gene of *Enterococcus faecalis*, confering resistance to spectinomycin, for selection of promoter activity (16). The construction of this vector has been described in detail elsewhere (Chapter 4). Fragments from a total *Sau*3A genomic digest were ligated into the unique *Bgl*II site of the multiple cloning site of pMM223, preceding the promoterless *aad*(9) gene. Recombinant plasmids, containing chromosomal fragments in the range of 100-1000 bps, were then introduced into the homologous host by electroporation. The streptococcal library contained approximately 10^5 independent clones, and statistically represents the entire *S. gordonii* CH1 genome (23).

After selection, inserts from plasmids were amplified from streptococcal lysates prepared as described (13). Primers used were AV9 (5'- ATGTCACTAGTCTCTACAAC-3'), annealing upstream of the multiple cloning site in which the chromosomal fragments were inserted, and AV19 (5'-CCTCCTCACTATTTTGATTAG-3') annealing at the 5'-end of the promoterless spectinomycin gene. Agarose gel electrophoresis was performed using the TAE buffer system (0.04 M Tris-acetate, 0.001 M EDTA, pH 8).

DNA sequence analysis

PCR products were purified using the High Pure PCR Product Purification kit (Boehringer). DNA sequencing of purified templates was performed by PCR-mediated *Taq* Dye Deoxy Terminator Cycle sequencing (Perkin Elmer, Foster City, CA, USA) on an Applied Biosystems 373 DNA sequencer, using primers AV9 and AV19. The obtained sequences were analyzed with the BLAST program (1).

RESULTS AND DISCUSSION

Isolation and characterization of *S. gordonii* promoter sequences

For the isolation of promoter sequences from *S. gordonii* CH1, 25 µl of the genomic streptococcal library, containing approximately $2.5x10^5$ clones, was plated onto erythromycin (Em)-containing TH agar. To select for promoters with different levels of activity, spectinomycin (Sp) was added to the agar at different concentrations. In duplicate experiments 350, 170, and 85 colonies were visible on plates with 0.1, 0.5, and 2 mg ml^{-1} Sp, respectively, after 24 h. The reduction in the number of spectinomycin-resistant transformants at increasing Sp concentrations clearly demonstrated selection for promoters with increasing levels of activity. Colonies were randomly picked from agar plates containing the three different Sp concentrations, and cultured in TH broth containing 5 µg ml^{-1} erythromycin. To analyse the strength of the isolated promoters, each colony was also plated onto TH agar containing Sp concentrations higher than that in the original selection plate, except when colonies had been grown on plates containing 2.0 mg ml^{-1} Sp. Promoters of different strength were identified by this procedure (Table 1). To characterize the transcription signals, the cloned chromosomal inserts were amplified from bacterial lysates by PCR. Amplicons were analysed on agarose gels, and five fragments of different sizes were purified and sequenced.

Table 1. Characteristics of the isolated promoter sequences from *S. gordonii* strain CH1.

Fragment	Size	Access. No.[a]	Activity[b]	Database match
SG_{P6}	0.55 kb	AJ243487	low	Transcription-repair coupling factor (Mfd; *B. subtilis*, P37474[c])
SG_{P2}	0.2 kb	AJ235898	intermediate	Response regulator (CiaR; *S. pneumoniae*, Q54954[c])
SG_{P21}	0.75 kb	AJ236899	high	Topoisomerase IV subunit E and unknown protein (ParE and Orf2; *S. pneumoniae*, Z67739[c])
SG_{P29}	0.3 kb	AJ236900	high	Cysteine synthase A (CysK; *B. subtilis*. P37887[c])
SG_{P32}	0.1 kb	AJ236901	high	No homology

[a] Accession numbers of the sequences of the different promoter fragments in the EMBL database

[b] The promoter activity is expressed as the highest spectinomycin concentration in the agar plate at which the recombinant bacterial clones were able to grow. Low, 0.1 mg/ml; intermediate, 0.5 mg/ml; high, 2.0 mg/ml

[c] Genbank accession number of matching sequence

SG_{P2}

```
  1 GATCCAAAAAATGATAAAAAAATTCTTTATTTTATAATAATCTTAGATGAAGCCAATTAG
                -35                    -10
                                ORF
 61 TATCTTATAGCAGGAGGAGAACAAGATGATTAAGATTTTACTAGTAGAAGACAACTTAGG
                  SD            M  I  K  I  L  L  V  E  D  N  L  G

121 GTTGTCAAACTCTGTATTTGATTTCTTAGATGACTTTGCGGATGTCATGCAGGTTTTTGA
181 TGGTGAAGAAGGTCTCTATGAAGCTGAAAGCGGTGTTTATGATC
```

SG_{P21}

```
  1 GATCCGATATACATACCTGGACGTTTACGTACCGCATCCAACCCTTCTAATACTTGAATG
                  PARE
 61 GCATCATCATTATAATTATTAATATTGATTTCCTTCTTTGCCACAAGGAACCTCCTGCAT
    A  D  D  N  Y  N  N  I  N  I  E  K  K  A  V          SD

121 TATTCATCCTTACTATCTTACAAGTTTTTATAAAATTTTGCAAAAGTTTTTGTCACAAAA
                                    -10                  -35
181 GCCTTGAAATAAACATTTTTATCGGATTAGACAAATAATTTACTAGATTGTTCAATACGA
                          -35                  -10
                                ORF2
241 AGCAAAATTCGTGATATAATAAAATTATGATAAATACAATTTTGGGCCTAATTTTGGCAT
                                  M  I  N  T  I  L  G  L  I  L  A
301 ATTTACTGGGTTCAATTCCAACTGGTTTATGGATTGGACAGATTTTCTTTAAAAAGAATT
361 TACGAGAATACGGTAGTGGAAATACGGGCACAACCAACACTTTCCGTATTTTAGGAAAGA
421 CAGCTGGAACTGATACTTTTGCAATCGACTTTCTAAAAGGAACTCTTGCAACCCTACTTC
481 CACTTTTTCTCCATATCAATGGTATATCACCCATGATTTTTGGTCTAATAGCTGTTCTTG
541 GCCACACCTTCCCAATTTTTGCTGAATTTAAGGGAGGCAAGGCCGTTGCTACAAGTGCAG
601 GTGTTGTTCNTGGATTTTCTCCCCTCTTCTTTTCCTATCTGATTATTATTTTTATTGTAA
661 CGCTTTACTTGGGAAGTATGATTTCTTTGGCTAGCATTGTTGTAGCTGGCTTTGCGATTA
721 TTTCCGTTCTTATATTTCCTTTATTAGGAATTATCCTTCCTAGTTACGATC
```

SG_{P29}

```
  1 GATCGAATTTTTCAATGGTAAAGTTCAGTTAAGCAACCAAGTAATTCGGCAAGTGGAAGT
                  ------------>  <------------
 61 TCCTATTATTTCTTGATATAAAAAAAAGCTATGATAGCCATAGCTTTTTTATATTTTACA
     P  I  I  S  -                                          -35
                                                        ORF
121 AATTCCAATTTTCATTTTATACTTTTCTTAATTCTATTTCATGAGGTGTAGTTATGTCTA
                        -10                    SD          M  S

181 AAATTTATAATAATATTACCGAACTAATCGGAAATACTCCCATTGTCAAACTAAATCATT
241 TGGTTCCTGATGGAGCAGCTGATGTATACGTGAAGTTAGAAGCCTTTAATCCTGGATC
```

SG_{P32}

```
  1 GATCTCTTTTTTGAACTCAGTTTTACCTATGAATAGTTTCTAATATATTGCTTTCATGGT
                                          -35
 61 ATACTATAGATGGATTAAGTCATTTGAAAAGCTTGAAGTATCTCTCTTTAGAAAGTGTGA
     -10

121 TC
```

Figure 1.

Nucleic acid sequences of isolated promoter fragments of *S. gordonii* CH1. Putative -10 and -35 regions and Shine-Dalgarno (SD) sequences are underlined, and inverted repeats are indicated by arrows. The amino acid sequences of the open reading frames are partly indicated, with the bold face printed nucleotides representing the start of the gene.

Four of the fragments showed high similarities to known sequences of different microorganisms (Table 1). Transcription signal sequences could be identified in most of the sequences (Fig. 1). SG_{P6}, a fragment with low promoter activity, showed strong similarity at the protein level to the transcription-repair coupling factor (Mfd) of *B. subtilis*, but no regular promoter sequence could be identified. As the homology was internal to the 3.5 kb *mfd* gene, with the obtained fragment being only 0.55 kb, it is likely that an unidentified promoter-like structure within this sequence was responsible for the observed low-level promoter activity.

The open reading frame (ORF) downstream of the intermediate strength promoter in SG_{P2} showed 97% identity over 46 amino acids (aa) to the CiaR response regulator of *Streptococcus pneumoniae*. This protein is part of the two-component signal-transduction system CiaR/CiaH, which is involved in competence and penicillin susceptibility (9). The *S. gordonii* homolog might have a similar role, as this species is also naturally competent (21) and is in general susceptible to penicillin (6).

The fragments SG_{P21}, SG_{P29}, and SG_{P32} had high promoter activity. The promoter fragment SG_{P21} contained two partial open reading frames, separated by an intergenic region. The two open reading frames showed homology to two different ORFs of *S. pneumoniae*, and the organization of the coding regions was similar in *S. gordonii* and *S. pneumoniae*. The ORF downstream of the *S. gordonii* promoter showed 71% identity over 168 aa to Orf2 of *S. pneumoniae*, a protein with unknown function (20). Upstream of the *S. gordonii* ORF and divergently oriented, a gene with homology to the 5'-end of the gene encoding the E subunit of topoisomerase IV gene (*par*E) of *S. pneumoniae* was identified on SG_{P21}, and the corresponding protein fragment was 100% homologous to the N-terminus of the *S. pneumoniae* ParE. Although an identical putative ribosome binding site (SD; Shine-Dalgarno

sequence) was identified preceding *par*E in both streptococcal species, no SD sequence was observed upstream of the translational start of Orf2 of either species.

The ORF downstream of the promoter region in SG_{P29} showed 65% identity to the cysteine synthase of *Bacillus subtilis*. Inverted repeats, capable of forming a stem-loop structure with a free energy of -14.4 kcal, were identified upstream of the *cys*K coding region. This stem-loop might function as a transcriptional terminator of the upstream located unknown open reading frame (Fig. 1). In *B. subtilis* CysK expression is altered by different environmental signals. Expression is increased after cold shock, reduced after heat shock, and completely absent after salt stress (8), but the underlying regulatory mechanism has not yet been determined. No common structures were identified in the sequence preceding the *cys*K genes of *B. subtilis* and *S. gordonii* that could be involved in the regulation of *cys*K expression under different environmental conditions.

Finally, a putative promoter sequence was identified on the 121 nucleotide SG_{P32} fragment, but the downstream sequence did not reveal any similarity to entries in the database.

In conclusion, we isolated presumably constitutive promoters from a genomic library of *S. gordonii* strain CH1 using the novel promoter screenings vector pMM223. Based on their capacity to induce a specific level of Sp-resistance, we could discriminate fragments with low, intermediate, and high promoter activity. As only a few *S. gordonii* promoter sequences have been reported up to now, it remains unclear which specific features within streptococcal promoters determine their strength. Nevertheless, the identified sequences might be of use in the field of oral vaccine development, for a more stable or for a higher level of expression of particular antigens. Furthermore, these promoters can possibly be applied in dairy and food industries, in which streptococci are used to express heterologous proteins.

Isolation of an iron limitation-regulated transcription signal

As iron limitation is a possible environmental signal for the expression of streptococcal virulence genes, we aimed to isolate promoter sequences from *S. gordonii* CH1 of which the activity was regulated by the external iron concentration. In an attempt to limit the amount of free iron, ethylenediamine di-o-hydroxyphenylacetic acid (EDDA; Sigma Chemical Co., St. Louis, MO, USA) was included in the TH agar. This iron chelator has been used to study hemin utilization of *S. pneumoniae* (24), a species closely related to *S. gordonii*. Although growth of *S. pneumoniae* was abolished at EDDA concentrations of 700 μM and higher (24), concentrations of up to 1,000 μM did not restrict the growth of *S.gordonii* CH1. Therefore, nitrilotriacetic acid (NTA), a chelator of divalent cations which has previously been used to study the iron acquisition and the iron starvation response of *Streptococcus pyogenes* (5), was added to the TH agar instead. At concentrations of 18 mM NTA or higher, growth of *S. gordonii* CH1 was completely abolished. Aliquots of 50 μl of the genomic library, containing approximately $5x10^5$ clones, were plated onto TH agar supplemented with 17 mM of NTA, 1 mM of $MgCl_2$, $ZnCl_2$, $CaCl_2$, and $MnCl_2$, and 0.5 mg ml^{-1} Sp for selection of iron limitation-

inducible promoters. To verify iron-limitation inducibility, colonies growing on these plates after 24 h were streaked on iron-limited TH agar plates, containing NTA (17 mM), cations (1 mM each), Sp (0.5 mg ml^{-1}) and Em (5 μg ml^{-1}), on TH agar with Sp and Em only, and on TH agar with Em as a control for growth. Clones that only grew on the Sp/Em containing agar plates in the presence of NTA were considered to carry an iron-limitation inducible promoter in front of the spectinomycin resistance gene.

```
   1 GATCTGAAAATCTCTAGAAGCGCTATTTTGGAGCTTGATAGGACAGATTTTTACGTGATT

                          -35                    -10
  61 TCATTACTTGTAGGTTTGTGTGTAATGGCTATAGAAAATGATAGTTTATAGGATAATAAG

 121 AAAAGTGGTTGTCATCAAATGATATGATTCTTAATGCTTGTGTCATCTGGCCTTGACAGA

 181 TTGTAACAATGAACATGCTATAATGTCTGCATGAAGACAGAAAATACAACTTTACATGCT
                                    M  K  T  E  N  T  T  L  H  A
 241 GTTAAGGCTCTAGCCTGTTTTAGTATCGTTTCTTTGCACTTTTTGTTACCAGGGGAATTT
      V  K  A  L  A  C  F  S  I  V  S  L  H  F  L  L  P  G  E  F
 301 GGTGTTTTTTATCAGATTGTGGCTCGTTTTGCAGTGCCCTTTTTCATGATGCTGTCGGGC
      G  V  F  Y  Q  I  V  A  R  F  A  V  P  F  F  M  M  L  S  G
 361 TATTATTCCTTTAATATTTCTAGGGGAAAAGTCAAGTATCGTCTCAAGCAAATGCTTCTT
      Y  Y  S  F  N  I  S  R  G  K  V  K  Y  R  L  K  Q  M  L  L
 421 TTGACAATCGCTAGTCTGATATTTTACTCGATTGTGCATTTTATTGACTTAGTACTGTCG
      L  T  I  A  S  L  I  F  Y  S  I  V  H  F  I  D  L  V  L  S
 481 GGAGAGCTGGCGGAAAAGATAGCAACTATAGACCTGTCTGATTTTGCCGATTTCTTCTTT
      G  E  L  A  E  K  I  A  T  I  D  L  S  D  F  A  D  F  F  F
 541 TTCAATAGTCCCAGAGATTTGATTGGTCCAGCTGCTACTCCGACCTGGTATTTGTTGGCT
      F  N  S  P  R  D  L  I  G  P  A  A  T  P  T  W  Y  L  L  A
 601 ATTTCCTATATTTACGGGCTTTATCTCCTTTTTTATAAGTATTTCCACCACCTGACCACC
      I  S  Y  I  Y  G  L  Y  L  L  F  Y  K  Y  F  H  H  L  T  T
 661 TTTGGTGTGTCTCTGATTCTGCTAGCTTTGGCTTTTTGTATCGAATTCAATACCAACAGT
      F  G  V  S  L  I  L  L  A  L  A  F  C  I  E  F  N  T  N  S
 721 ACCCTTTACTATCGAAATTTCCTGTTTATGGGTCTTCCCTTTTTCATTATGGGGATGCAG
      T  L  Y  Y  R  N  F  L  F  M  G  L  P  F  F  I  M  G  M  Q
 781 TTCGCCAAGTATCGGGAACGGATTTTAGCCTACGACTTATCATCTGCCAGGAAATGGGCT
      F  A  K  Y  R  E  R  I  L  A  Y  D  L  S  S  A  R  K  W  A
 841 ATTAGCTTGGGAATAATAGGTTTGATTCTGCTTGAATACTGTTTTATGGGAACAGAGCAC
      I  S  L  G  I  I  G  L  I  L  L  E  Y  C  F  M  G  T  E  H
 901 GACCTTTACCCCAGCACTCTTTTATCATCTAGTGCGATTTTCCTCTATGCGATC
      D  L  Y  P  S  T  L  L  S  S  S  A  I  F  L  Y  A  I
```

Figure 2.

Nucleotide sequence of the iron-limitation inducible promoter fragment SG_{P50}. Putative -10 and -35 regions are underlined. The amino acid sequence of the potential open reading frame is partly indicated, with the ATG transcription start printed in bold and the putative Fur box shaded. The sequence of SG_{P50} is deposited in the EMBL database under accession number AJ236902.

Of around 350 restreaked colonies, two clones were resistant to 0.5 mg ml^{-1} Sp only under the iron restricted conditions. The cloned fragments were amplified and sequenced, and found to be identical. The 954 bp fragment contained an open reading frame of 744 bp. No possible ribosome binding site preceding this open reading frame could be identified. The translated sequence of 248 amino acids (Fig. 2) had weak similarity to a transmembrane protein of a *S. pneumoniae* capsular type 33F strain, of which the corresponding gene is located within the capsular gene cluster (Llull, D., unpublished data; GenBank Acc. No. AJ006986). Iron-limitation inducibility of this gene has not been reported. Determination of possible transmembrane regions in the *S. gordonii* CH1 translated sequence using the TMpred program (12) indicated 8 putative transmembrane regions. Although cation-limitation inducible membrane transporter proteins of *S. gordonii* have been demonstrated to be involved in the uptake of metal ions (15), the precise nature and function of the protein encoded by the gene identified here remains elusive.

Putative –35 and –10 promoter regions and a translational start site could be identified in the sequence (Fig. 2). Within the promoter, a potential Fur binding regulatory sequence (Fur box) was present, with similarity to both the Fur box consensus of *Escherichia coli* (GATAATGATAATCATTATC) (2), and to the Fur box upstream of the superoxide dismutase (*sod*) gene of *Staphylococcus epidermidis* (GCTATATATAATAAT TACT) (10). This indicates that fur regulation might be operative in *S. gordonii*. Together with the identification of Fur-like proteins in *S. epidermidis* (10) and in *S. pyogenes* (Beall, B.W., unpublished data; GenBank Acc. No. U76538) this finding underlines the validity of the hypothesis that fur regulation may not be restricted to Gram-negative bacterial species (10).

The identified iron-limitation regulated promoter fragment of *S. gordonii* CH1 most likely is involved in gene expression in iron-restricted environments *in vivo*, like the bloodstream. The presence of a Fur box-like sequence in this promoter indicates that *S. gordonii* may have a Fur-like regulation system, and presumably additional iron limitation inducible genes. Such genes most likely will be activated during the pathogenesis of IE, and may play a role in bacterial virulence.

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CHAPTER 7

Rapid multiplication of endocarditis-causing viridans group streptococci in platelet-fibrin clots is dependent on plasma components and streptococcal protease activity

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ABSTRACT

Endocarditis-causing viridans group streptococci (VS) colonizing platelet-fibrin vegetations on heart valves are capable of rapid growth. In infected vegetations bacterial densities are extremely high. *In vitro*, 3 VS strains grew rapidly and to high densities in platelet rich plasma (PRP) clots, but also in platelet poor plasma (PPP) clots. To determine which host components are required for this rapid growth, *Streptococcus gordonii* CH1 was grown in platelet clots, fibrin clots, serum, and plasma. Since PRP, PPP, platelets, fibrin, or serum were not supportive for growth, the rapid multiplication required as yet unknown plasma components entrapped within the clot during coagulation. *S. gordonii* CH1 also grew well in fibrinolysed PPP clots. Therefore, growth promoting components apparently were not affected by proteases of the fibrinolytic cascade. Addition of protease inhibitors to fibrinolysed PPP clots largely blocked bacterial growth, indicating that bacterial protease activity is essential for growth of VS in vegetations.

INTRODUCTION

Infective endocarditis (IE) denotes infection of the endocardial lining and of the heart valves. Viridans group streptococci (VS) originating from the oral cavity and upper pharyngeal tract microflora are the most common bacteria causing native IE. During transient bacteremia due to traumatization or infection of the oral or pharyngeal mucosa, VS are able to bind to vegetations (VGs), platelet-fibrin thrombi present on previously damaged endocardial or valvular lesions. Subsequently, adherent VS multiply and colonize these VGs (1,2). Since clearance of bacteria within the VG by professional phagocytes is hampered (2,3), multiplication of VS continues until nutrient limitation occurs. Growth predominantly occurs just beneath the surface of the VG, while deep inside VGs clusters of bacteria, embedded in layers of fibrin, are metabolically inactive (2).

Several VS determinants are known to be involved in the initiation of IE. These include adhesins, providing VS the ability to adhere to different matrix molecules of the host (4,5), and resistance to antibacterial proteins from activated blood platelets, contributing to persistence of adherent VS (6,7,8). In contrast, characteristics of VS involved in the further progression of IE are scarcely studied. Such a characteristic may be the rapid multiplication of VS in the VG, as has been observed in experimental models of IE (1,6,7). It is conceivable that this rapid multiplication of VS, resulting in a high number of bacteria within 4 to 6 h after bacterial adherence to VGs (6), will contribute to their property to cause IE. By studying growth of VS in various human blood fractions in vitro, we aimed to identify factors either from the host or from VS that are supportive for the rapid bacterial growth.

MATERIALS AND METHODS

Bacteria and growth conditions

Streptococcus sanguis U108 (7), *Streptococcus oralis* J30 (formerly classified as *Streptococcus sanguis* II) (7), and *Streptococcus gordonii* CH1 (9) were cultured in Todd Hewitt (TH) broth and on TH-agar (Difco Laboratories, Detroit, MI) at 37°C. To prepare inocula for growth experiments, overnight bacterial cultures were centrifuged, the pellets were washed twice with phosphate-buffered saline (PBS, pH 7.4), and the bacteria were diluted in PBS to a final concentration of 2-4 x 10^6 cfu/ml. For each growth experiment, 25 µl of this suspension (0.5 - 1 x 10^5 cfu) was used as inoculum.

Reagents

Purified human fibrinogen was dissolved in 0.9% NaCl at a final concentration of 2 mg/ml. Human thrombin (CLB, Amsterdam, The Netherlands) was used at 10 U/ml, and tissue plasminogen activator (tPA; Boehringer Ingelheim B.V., Alkmaar, The Netherlands) at 0.1 mg/ml. One tablet containing a mixture of protease inhibitors (Complete™; Boehringer Mannheim GmbH, Mannheim, Germany) was dissolved in 5 ml of milliQ water, to obtain a 10x concentrated stock solution.

Preparation of human plasma, serum, and clots

After informed consent, blood from healthy volunteers was collected in polypropylene tubes containing a 3.2% buffered sodium citrate solution (blood-to-anticoagulant ratio, 9:1). Citrated blood was centrifuged for 10 min at 200xg, and three-fourth of the upper phase volume was collected to obtain platelet rich plasma (PRP). Platelet poor plasma (PPP) was collected after subsequent centrifugation of the remaining volume of blood for 15 min at 2,000xg. PRP and PPP clots were prepared by adding human thrombin and 1/10 volume of 0.5 M $CaCl_2$ to PRP and PPP, respectively. PRP and PPP serum were obtained after centrifugation of coagulated PRP and PPP for 10 min at 2,000xg. Fibrin clots were prepared by adding human thrombin and $CaCl_2$ to human fibrinogen suspensions. Fibrinolysis of PPP clots was initiated by addition of tPA. After incubation at 37°C for one hour the clots were completely lysed. The resulting suspension was designated as fibrinolate.

Bacterial growth experiments

Bacterial growth experiments were performed in 6 ml Falcon polypropylene tubes (Becton Dickinson Labware, Franklin Lakes, NJ) in duplicate. Twentyfive µl of bacterial inoculum was added to 250 µl of either of the different blood fractions. For reasons of comparison, identical VS inocula were added to 250 µl of TH broth. When required, 25 µl of the protease inhibitors stock solution was added. The final volume of all test samples, adjusted with sterile water if necessary, was 300 µl. After addition of the inoculum, coagulation of PRP, PPP, or fibrin to prepare clots was initiated, and continued for 90 min at room temperature. Then the clots were centrifuged briefly at 2,000xg and washed twice with PBS. PRP and PPP clots were subsequently submersed in 200 µl of fresh PPP, and fibrin clots in 200 µl of PBS. The test tubes were incubated at 37°C under continuous rotation (160 rpm). At various intervals, tubes were placed on ice, and ice-cold PBS was added to obtain a sample volume of 1.25 ml. The samples were ground using a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK). The resulting suspensions were sonicated for 30 s in a sonicator bath (47 kHz; Branson Europa B.V., Soest, The Netherlands), 10-fold serially diluted in PBS, and plated on blood agar. The grinding and sonication procedures did not influence survival of VS strains (6).

RESULTS

We first determined the growth of *S. sanguis* U108, *S. oralis* J30, and *S. gordonii* CH1 in PRP clots. The growth rates of U108 and J30 in PRP clots were similar to those in TH. *S. gordonii* CH1 grew even more rapidly in PRP clots than in TH broth (Fig. 1). After overnight incubation, the numbers of cfu of all three isolates in the PRP thrombi were at least 10-fold higher than those in TH-broth (Fig. 1).

To identify the components responsible for the rapid multiplication of VS, we studied the growth of *S. gordonii* CH1 in clots prepared from PRP, PPP, and fibrinogen, and in plasma, serum, and fibrinolate. *S. gordonii* CH1 was used in these experiments since this strain had an higher growth rate in the PRP thrombi than in TH-broth. Since logarithmic bacterial growth started approximately 0.5 h after incubation at 37°C (Fig. 1), the increase in bacterial numbers between 0.5 h and 5 h was used as a measure for the bacterial growth rate.

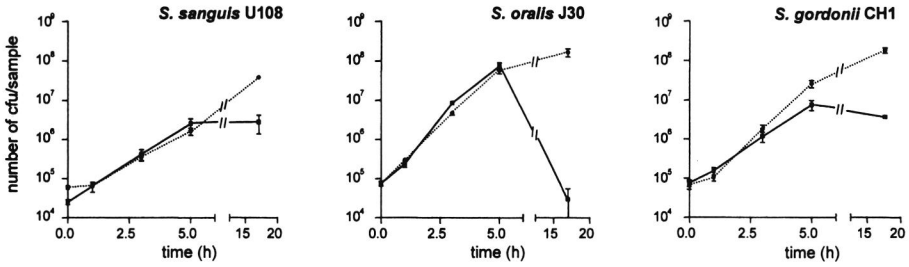

Figure 1.

Growth of viridans streptococcal strains *S. sanguis* U108, *S. oralis* J30, and *S. gordonii* CH1 in TH-broth (squares, solid line) and in PRP clots (circles, dashed line) over time.

Growth rates in PPP clots and PRP clots were essentially identical (Fig. 2A), and thus the number of platelets in the clots apparently had no influence on the bacterial growth. Soluble components released from thrombin activated platelets did not promote bacterial growth, since no increase in bacterial numbers was noted in PRP serum (Fig. 2A). Other soluble plasma or serum factors were not responsible for the growth-enhancing effect in clots either, as CH1 multiplied poorly in PPP (Fig. 2A), and the bacteria did not grow in PPP serum (data not shown) and in PRP serum. Furthermore, no growth was observed in clots prepared from human fibrinogen (Fig. 2A), implying that fibrin was not used as a nutrient source by *S. gordonii* CH1. Taken together, these data indicate that specifically PRP and PPP clots contain as yet unknown components, which are required for the rapid growth of VS. The availability of these clot components for the bacteria was only slightly affected by active fibrinolysis, as bacteria grew almost as well in a solution of degraded PPP clots (fibrinolate) as in intact PPP clots (Fig. 2A and 2B).

Finally, we determined whether the growth-enhancing components within the clots were directly available for bacterial growth or whether bacterial protease activity was required for further processing. To evaluate the role of bacterial proteases, the effect of host proteases was excluded by using PPP clot fibrinolates, which already contained all components resulting from the host fibrinolytic protease activity. Protease inhibitors were added to the fibrinolate before assessing growth of the bacteria. These protease inhibitors almost completely blocked the bacterial multiplication in PPP fibrinolate, whereas they only marginally influenced bacterial growth in TH-broth (Fig. 2B). Apparently, bacterial proteases are required for degradation of components present in the clot, that are not degraded by proteases from the host fibrinolytic pathway, providing nutrients required for the rapid bacterial growth within PRP or PPP clots.

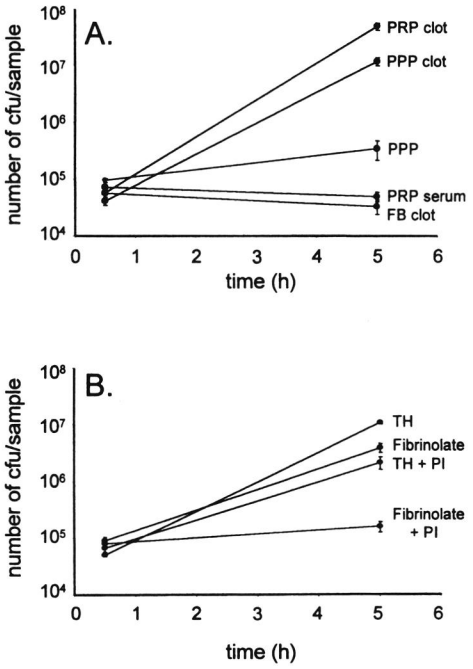

Figure 2.

Growth of *Streptococcus gordonii* CH1 in various clots and blood fractions, and in TH broth. PRP, platelet rich plasma; PPP, platelet poor plasma; FB, fibrin; TH, Todd Hewitt broth; PI, protease inhibitors.

DISCUSSION

Viridans group streptococci (VS) are commensals of the human oral cavity, and are in general not recognized as important pathogens. Nevertheless, VS are the most prevalent etiologic agents in native infective endocarditis (IE). Although several bacterial constituents are known to be involved in initiation of IE (4,5), little is known about the bacterial characteristics resulting in further progression of the disease. In the experimental rabbit model of IE, development of disease by VS is associated with rapid multiplication of the bacteria within the vegetation. Numbers of cfu of *S. oralis* J30 and *S. sanguis* U108 started to increase within VGs only 2 h after adherence (7). In the present study, rapid multiplication of these isolates was also observed in PRP plasma clots prepared in vitro, with similar growth rates as in the nutrient-rich TH broth. The more rapid growth of *S. oralis* J30 compared to *S. sanguis* U108, which was also observed in the experimental rabbit IE model (7), appeared to be a characteristic of this specific isolate. *S. gordonii* CH1, another VS strain often used in experimental IE studies (9), showed a similar rapid multiplication in PRP clots, and growth of

this strain in the clots was even faster than in TH broth. Additionally, bacterial densities in the platelet clots were substantially higher than in TH broth after overnight incubation. Growth of VS in the in vitro PRP and PPP clot model closely resembled the in vivo bacterial growth in experimental IE models (1,6,7).

Neither platelets nor fibrin, the major constituents of VGs in vivo, were accountable for the observed enhanced growth of *S. gordonii* CH1. Although both plasma and serum are rich in soluble constituents, including glycoproteins that might function as nutrients for VS, rapid bacterial growth was not supported by these blood fractions either. Therefore, the rapid bacterial growth must depend on plasma components entrapped in the clots. The nature of these specific components remains to be determined. Since a VG is not only a site of local coagulation but also of fibrinolysis (10), we argued that fibrinolysis might influence growth of VS by altering the growth supportive clot components. This appeared not to be the case, as bacterial growth was almost as fast in total fibrinolates as in intact PPP clots. Alternatively, fibrinolysis might be required to release the growth-supportive components from the clot. Any of the growth-stimulating clot components present in the fibrinolate required further processing before they could be used by the bacteria, since rapid growth in fibrinolate was almost completely blocked by the addition of protease inhibitors. As the proteases of the fibrinolytic pathway had already actively degraded the clot to completion before addition of the protease inhibitors, the proteases involved in degradation of the clot components apparently were of bacterial origin. VS are known to produce many different protease and glycosidase acitivities (11,12,13). Proteases of VS have also been implicated in formation (thrombin-, Hageman factor-, activated factor X-like activities) and dissolution (plasmin-, kallikrein-, activated protein C-like activities) of clots (11). Straus identified four excreted proteases from an *S. sanguis* endocarditis isolate, of which two were able to degrade human serum albumin (14). Recently, an extracellular serine-type protease of *S. gordonii* CH1 was purified that hydrolyzes type IV collagen and collagen analogues (15). This protease is secreted under conditions considered to represent nutritional conditions at the endocardial thrombus. We now have shown the importance of VS protease activity for processing of clot components required for their growth, whereas such protease activity is not required for bacterial growth in TH broth. The involvement of specific bacterial proteases in intravegetational growth implies that these enzymes may indeed contribute to the virulence of VS in the pathogenesis of IE.

We conclude that specific components of plasma clots, other than platelets and fibrin, serve as nutrients for bacteria within a VG, and are required for rapid bacterial multiplication. VS proteases convert these components within the VG to nutrients supporting the rapid bacterial proliferation. These proteases might therefore serve as targets in the development of new strategies for the treatment of IE.

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CHAPTER 8

Altered gene expression of *Staphylococcus aureus* upon interaction with human endothelial cells

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ABSTRACT

Staphylococcus aureus is isolated from a large number of patients with infective endocarditis who are not known with predisposing heart abnormalities. It has been suggested that the infection is initiated by the direct binding of *S. aureus* to human vascular endothelium. To determine the mutual response of the endothelial cells and the bacteria, we studied the interaction between *S. aureus* and human vascular endothelium (HUVEC). Scanning electron microscopic analyses showed that binding of *S. aureus* to HUVEC mainly occurred via thread-like protrusions extending from the cell surface. Bound bacteria appeared to be internalized via retraction of the protrusions into newly formed invaginations of the endothelial cell surface. The growth phase of *S. aureus* had major impact on the interaction with HUVEC. Logarithmically growing bacteria showed increased binding to, and were more readily internalized by HUVEC compared to stationary phase bacteria. To assess the bacterial response towards the cellular environment, an expression library of *S. aureus* was used to identify genes of which the expression was induced after 4 hours of exposure to HUVEC. The identified genes could be divided into different categories based on the function of the encoded proteins (transport, catabolism, biosynthesis, and DNA repair). Further analyses of 5 of the *S. aureus* transposon clones showed that both HUVEC and human serum serve as stimuli for triggering gene expression in *S. aureus*.

INTRODUCTION

Infective endocarditis (IE) due to *S. aureus* is an acute infection of the heart. *S. aureus* IE frequently has a fulminant course and mortality is upto 40%. Approximately half of the patients have no known history of heart disease or heart damage (12, 21, 31, 33). It is assumed that these patients develop IE due to the ability of *S. aureus* to directly interact with the undamaged endocardial lining.

S. aureus has a tropism for endocardial tissue (20), and it adheres much more readily to vascular endothelial cells (EC) than other bacterial species (24). Following initial binding, adherent *S. aureus* are actively internalized by the EC. Intracellular *S. aureus* reside and persist in phagosome-like vacuoles (2, 10, 20, 24) (10, 20, 24). Although in EC the phagosomes were found to fuse with lysosomes, no bacterial degradation could be observed (20). The intracellular presence of *S. aureus* eventually leads to cell destruction (6, 20, 34), due to the direct action of bacterial toxins (35), or through induction of apoptosis of the EC (22). Damage of the vascular endothelial lining exposes the subendothelial matrix to the bloodstream, causing deposition of platelets and fibrin, and can then result in the onset of IE.

A recent study indicated that *S. aureus* responds to the complex *in vivo* environment by altering its gene expression (18). It remained, however, undetermined what the *in vivo* stimuli are to which *S. aureus* responds. Contact with eukaryotic host cells has recently been identified as a new signal for bacterial pathogens, resulting in the expression of genes that are specifically required for survival or virulence (7, 27, 38). As interaction of *S. aureus* with vascular endothelial cells might be a primary step in the pathogenesis of *S. aureus* IE, the present study focuses on the initial process of colonisation and invasion of the endothelial lining by *S. aureus*. In particular we aimed to identify *S. aureus* genes of which the expression is specifically induced in the presence of EC in order to understand the mechanism by which these pathogens enhance their pathogenicity. This was done by studying the response of *S. aureus* towards human umbilical vein endothelial cells (HUVEC), and analysing *S. aureus* genes of which the expression was induced upon exposure to HUVEC.

MATERIALS AND METHODS

Reagents and media

M199 medium was purchased from Life Technologies (Grand Island, NY). Human serum (HS) was prepared from blood collected from healthy donors, and heat-inactivated at 56°C for 30 min (HSi). Lysostaphin was from Sigma Chemical Co. (St. Louis, MO), gelatin from Difco Laboratories (Detroit, MI), L-glutamin from Flow Laboratories (Irvine, U.K.), penicillin G from Brocades Pharma B.V. (Leiderdorp, The Netherlands), streptomycin from Gist-Brocades N.V. (Delft, The Netherlands), and amphotericin B from Squibb B.V. (Rijswijk, The Netherlands). Endothelial cell (EC) growth factor was prepared from bovine hypothalamus as described previously (3).

Human umbilical vein endothelial cells (HUVEC)

HUVEC were isolated from the human umbilical cord vein by digestion with 0.1% collagenase as described previously (3). Cells were cultured to confluency in M199 cell culture medium, i.e. M199 medium supplemented with 10% HSi, 1 mM L-glutamine, 0.1 mg/ml streptomycin, 100 U/ml penicillin G, 100 U/ml amphotericin B, 0.1 mg/ml EC growth factor, and 5 U/ml heparin in plastic tissue culture dishes (Falcon no 3080, Becton Dickinson, Lincoln Park, NJ) in a 5% CO_2 atmosphere at 37°C.

***Staphylococcus aureus* inoculum preparation**

Staphylococcus aureus strain RN4220, a restriction negative mutant used for general cloning purposes (17), was used in these experiments. Inocula for infection of HUVEC were prepared from either overnight or early-exponential (3 h of growth) cultures grown by shaking at 37°C in Nutrient Broth no.2 (Oxoid Ltd, London, U.K.). The bacteria were harvested by centrifugation at 1,500 x *g* for 10 min, washed twice in 0.9% NaCl, and resuspended in M199 medium supplemented with 0.1% (w/v) gelatin (gelatin-M199). *S. aureus* were opsonized by incubation with 20% (v/v) fresh HS in gelatin-M199 at 37°C under rotation for 30 min. Bacteria were washed once with gelatin-M199, and suspended in M199 plus 10% HSi. Numbers of colony forming units (cfu) were determined by plating of serial dilutions in phosphate-buffered saline (PBS, pH 7.4).

Infection of HUVEC with *S. aureus*

HUVEC cultures grown to confluency on gelatin-coated glass coverslips in 24-well tissue culture plates were washed with M199 cell culture medium without antibiotics. When confluent, each well contained about 2 x 10^5 EC. Subsequently, 1 ml of opsonized *S. aureus* in M199 plus 10% HSi was added, and HUVEC cultures were incubated at 37°C in a 5% CO_2 atmosphere. Infection was allowed to proceed for one hour. Cell monolayers were then washed twice with prewarmed M199 of 37°C to remove extracellular bacteria, incubated with 2 U/ml of lysostaphin for 5 min at room temperature to lyse remaining cell-bound *S. aureus*, and then washed twice again with M199 cell culture medium of 37°C. Determination of the percentage of infected HUVEC and assessment of the number of intracellular bacteria was done by light microscopy and by plating of HUVEC lysed by the addition of 1 ml H_2O, respectively. For light microscopical counting, EC monolayers on 0.5% gelatin-coated glass coverslips were fixed by incubation in methanol for 15 min, and stained with Giemsa stain for 15 min (2). EC were scored positive for infection when intracellular bacteria could be distinguished, and results are the average of counting 100 microscopic fields of vision containing approximately 15 – 20 EC per field.

Scanning electron microscopy

HUVEC grown to confluency on gelatin-coated glass coverslips in culture dishes were infected for one hour with overnight grown *S. aureus*. After washing as described above, the EC were fixed overnight in a cacodylate-buffered (pH 7.4) gluteraldehyde/formaldehyde mixture (26). Subsequently, coverslips were thoroughly rinsed with cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol, and dried with hexamethyl disilasane. Samples were examined in a Philips XL20 scanning electron microscope (Philips, Eindhoven, The Netherlands).

Construction of a *Tn*917-*lacZ* transposon bank

A genomic expression library of *S. aureus* RN4220 was constructed using the transposon *Tn*917-*lacZ* containing vector pLTV1 (13) essentially as described by Camilli et al. (5). Vector pLTV1 was introduced into *S. aureus* RN4220 by electroporation (30). Final freezer stocks from this *S. aureus* bank were stored at -70°C. Over 99% of the bacteria from these stocks were tetracyclin sensitive and erythromycin resistant, indicating loss of pLTV1 and insertion of the transposon into the chromosome. The randomness of insertions was checked by Southern blotting (15).

Selection of HUVEC-inducible *S. aureus* genes

The approach for the selection of inducible *S. aureus* genes is schematically depicted in Figure 1. The staphylococcal expression library was plated onto LB agar plates containing 250 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) (Boeringer Mannheim). After overnight incubation at 37°C, white colonies were transferred to single wells of a 96-well microtiterplate containing 100 µl LB broth supplemented with erythromycin (Masterplate). Early log cultures, obtained by subculturing overnight cultures from the wells of the masterplates in LB with erythromycin for 3 h, were used to infect HUVEC cultured in the same format (10^7 cfu/well and 3 - 4 x 10^4 HUVEC/well, respectively). After 4 h of incubation, HUVEC were lysed by the addition of lysis reagent (Boehringer Mannheim). The remaining bacteria were washed once in 0.9% NaCl, resuspended in 100 µl of lysis reagent (Boehringer Mannheim), and lysed by addition of 2.5 U of lysostaphin and incubation at 37°C for 30 min. Bacterial β-galactosidase activity was determined using a chemiluminescent β-galactosidase reporter gene assay (Boehringer Mannheim). The assays were performed in opaque-coloured 96-well microtiterplates (Boehringer Mannheim) to reduce background, and chemiluminescent signals were detected in a Lumi-Imager (Boehringer Mannheim). To obtain higher signals, several of the selected *S. aureus* clones were retested by culturing HUVEC in 24-wells cell culture plates (2 x 10^5 HUVEC/well) and using higher start inocula (5 x 10^7 cfu/well). As controls, bacteria were cultured in M199 medium with or without HSi or in LB medium.

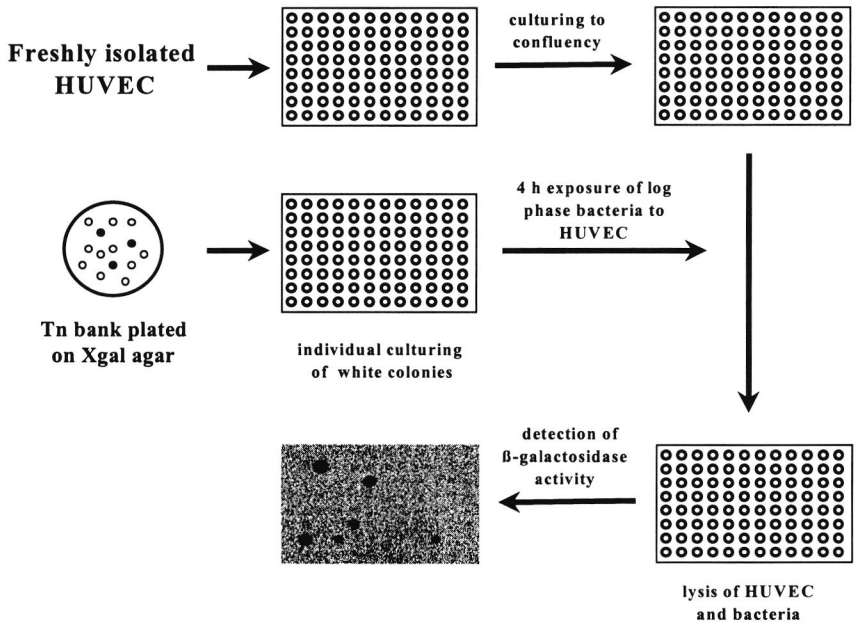

Figure 1.

Schematic representation of the selection strategy for the identification of *S. aureus* gene expression, as described in the Materials and Methods section. The black dots on the final photographic image represent those *S. aureus* clones positive for β-galactosidase activity after exposure to HUVEC, as detected using a chemiluminescent β-galactosidase gene assay and a Lumi-Imager.

DNA manipulations, transformation, and sequencing

DNA manipulations were done by standard techniques (29). Chromosomal DNA was isolated from *S. aureus* using the Puregene Chromosomal DNA isolation kit for Gram-positive bacteria and yeast (Gentra Systems Inc., Minneapolis, Mn) with lysostaphin at a final concentration of 5 U/ml. Chromosomal DNA fragments were self-ligated using T4 DNA ligase (Boehringer Mannheim). Plasmid DNA was introduced into *E.coli* BHB2600 (11) or DH5α (Gibco-BRL, Life Technologies, Breda, The Netherlands) by electroporation (9). Plasmid DNA was isolated from *E. coli* using the Wizard Plus minipreps kit from Promega Corporation (Madison, WI). DNA sequencing was performed using the PCR-mediated *Taq* Dye Deoxy Terminator Cycle sequencing kit (Perkin Elmer, Foster City, CA), and primer AV33 (5'- CAC AAT AGA GAG ATG TCA GCG -3'). Reactions were analysed on an Applied Biosystems (San Jose, CA) model 373 DNA sequencer. The obtained sequences were compared to entries in the Genbank database using the BLAST program (1).

RESULTS

Influence of bacterial growth phase on HUVEC infection

Expression of the various adhesive structures of *S. aureus*, such as protein A, clumping factor, and adhesins for fibronectin, fibrinogen, and collagen, is maximal in the early and mid logaritmic growth phase, and is down-regulated in the stationary phase (19). We therefore infected HUVEC with either early logaritmic phase or stationary phase *S. aureus* RN4220 using various inocula. The percentage of infected cells and the numbers of intracellular bacteria per cell increased with increasing inoculum size irrespective the bacterial growth phase. Logarithmic phase *S. aureus* were much more infective than stationary phase bacteria, since inocula of logarithmic phase bacteria required to obtain the same percentage of HUVEC infection were much smaller than those of stationary phase bacteria (Fig. 2). The number of bacteria per cell was also higher with the logarithmic phase bacteria than with the stationary phase bacteria. This was most pronounced at the higher inocula (Fig. 2).

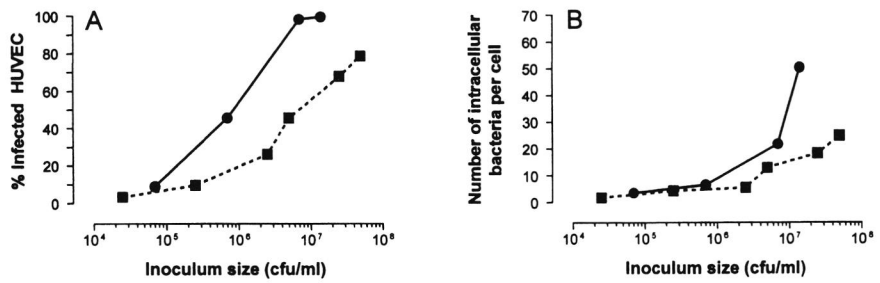

Figure 2.

Infection with different inocula of either logaritmic phase (circles) or stationary phase bacteria (squares) by (A) determination of the percentage infected cells and (B) the bacterial numbers per cell. Values represent the average of duplicate experiments.

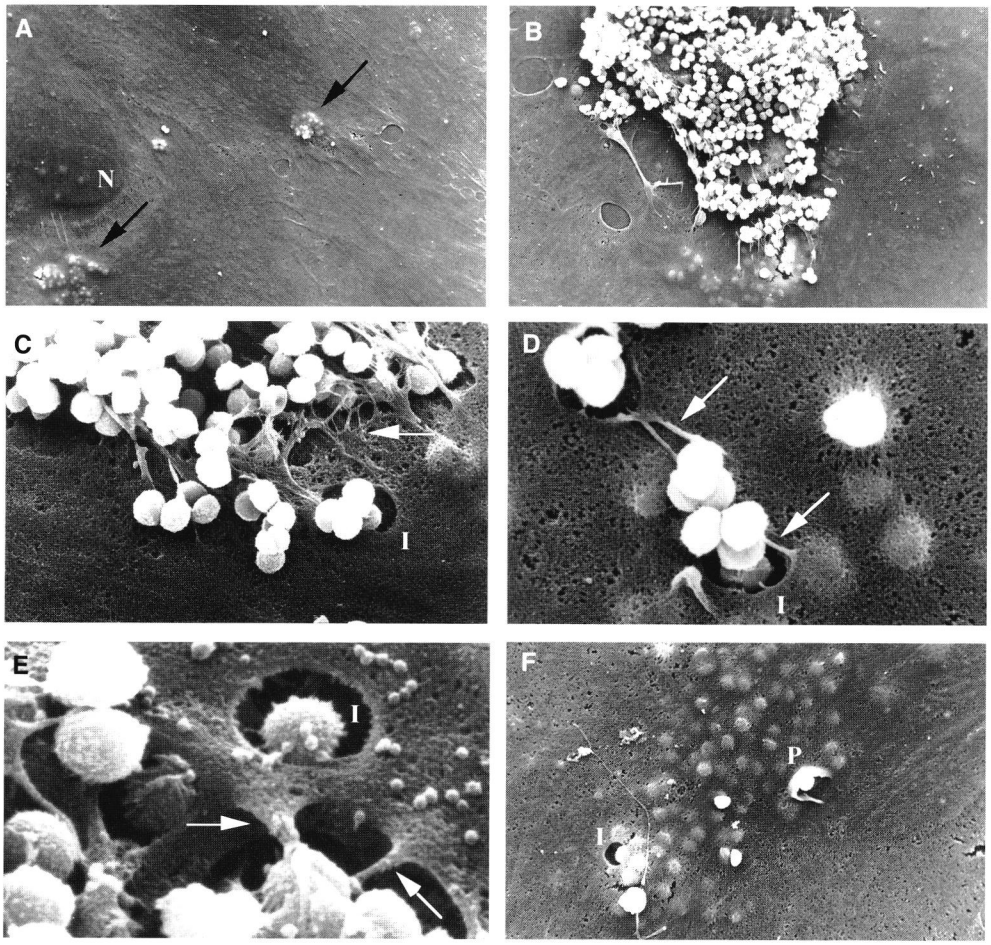

Figure 3.

SEM analysis of the interaction between HUVEC and *S. aureus* RN4220, one hour after infection. (A) Bacteria were internalized by HUVEC and could be observed within de EC, as indicated by the black arrows. (B) Most cell-surface bound *S. aureus* were present as clusters of various sizes. (C-E) *S. aureus* were mainly bound to thread-like protrusions (white arrows) extending from the cell surface, which appeared to be internalized or retracted into opened invaginations of the cellular surface. (F) Occasionally, pseudopod-like structures were observed during uptake of the bacteria by HUVEC. N, cell nucleus with several nuclei; I, invagination of the cellular surface; P, pseudopod-like membrane structure. Magnifications were (A) 1,500x, (B) 3,000x, (C) 10,000x, (D) 15,000x, (E) 30,000x, and (F) 5,000x.

SEM analysis of *S. aureus* – HUVEC interaction

The primary contact of *S. aureus* with HUVEC and the subsequent bacterial internalization was studied by scanning electron microscopy (SEM). After one hour of infection with 10^7 bacteria and removal of non-adherent *S. aureus*, internalized bacteria were observed within HUVEC (Fig. 3A). Most cell-bound *S. aureus* were present as clusters of various sizes (Fig. 3B), and bacteria were attached to thread-like protrusions extending from the endothelial cells (Fig. 3, C-E). Uninfected EC did not show such protrusions (not shown). Higher magnification revealed close contact between bacteria and cells via these protrusions (Fig. 3, D and E). The protrusions with adherent bacteria seemed to be internalized or possibly retracted into already opened invaginations of the endothelial cell surface (Fig. 3, C-E). Although occasionally bacteria were internalized by HUVEC through cup-shaped uptake processes with pseudopod-like structures (Fig. 3F), this was only in a minority of the phagocytotic processes (not shown).

Isolation of inducible *S. aureus* genes upon HUVEC exposure

As the bacterial response towards a new environment is, at least in part, accomplished by altering gene-expression profiles, we determined the induced gene expression of *S. aureus* RN4220 in the presence of HUVEC (see Materials and Methods). Since the number of intracellular *S. aureus* in this experimental setup was too low to assess bacterial β-galactosidase activity, the β-gal activity of the entire bacterial inoculum after 4 h of HUVEC exposure was determined. From a total of approximately 800 bacterial clones that were white on X-gal containing agar plates, 41 were identified that showed β-galactosidase activity when exposed to HUVEC. This indicated that transposon integration in the selected clones had occured in a gene of which the expression was induced under these conditions. To determine the site of transposon integration, genomic DNA was isolated from these clones, digested with *Eco*RI, and selfligated. Because of the presence of only one *Eco*RI site within the integrated vector pLTV1, selfligation results in plasmids that contain vector sequence necessary for plasmid replication, as well as chromosomal DNA of *S. aureus* upstream of the promoterless *lacZ* gene upto a chromosomally located *Eco*RI site. After transformation of *E. coli* BHB2600 with these ligation mixtures, erythromycin resistant colonies were selected on erythromycin containing LB-agar. Plasmid DNA was isolated from these colonies, digested with *Eco*RI to remove possible multiple chromosomal inserts, and the inserts upstream of the promoterless *lacZ* were sequenced.

A total of 33 different insertions were identified within the 41 isolated clones, and in 19 of these clones the identified *S. aureus* sequence showed homology at the protein level to entries in the database (Table 1). These sequences could be divided into four different categories according to their function. Four were sequences of transporter proteins, involved in amino acid (BrnQ), peptide (OppD, OppF) or sugar transport (FruA). Homology to two different peptidases was found, of which N-acyl-L-amino acid amidohydrolase was identified in six of the isolated

Table 1. Characterization of the isolated *S. aureus* sequences.

Classification	Strain	No.[a]	Blast description[b]
Transporters	4-C1/5-H9[c]	3	(U87144) branched-chain amino acid carrier protein; BrnQ (*S. aureus*)
	4-H11	1	(Z99111) PTS fructose-specific enzyme IIBC component; FruA (*B. subtilis*)
	5-C6	2	(X89237) oligopeptidepermease; OppF (*S. pyogenes*)
	8-C2	1	(AE001293) oligopeptide transporter ATPase; OppD (*C. trachomatis*)
Peptidases	5-G3/3-D11/9-D10[c,d]	6	(D90917) N-acyl-L-amino acid amidohydrolase; Ama (*C. synechocystis*))
	5-H3	1	(Z99110) similar to oligoendopeptidase; YjbG (*B. subtilis*)
Biosynthetic routes	8-C1	2	(P39593) hydroxyethylthiazole kinase (thiamin biosynthesis); ThiM (*B. subtilis*)
	9-B12	1	(B26532) prephenate dehydrogenase (tyrosine biosynthesis); TyrA (*B. subtilis*)
	5-B6	2	(P23630) diaminopimelate decarboxylase (DAP-lysine biosynthesis); LysA (*B. subtilis*)
	6-C6	1	(Q57865) dihydrodipicolinate reductase (DAP-lysine biosynthesis); DapB (*M. jannaschii*)
	8-H10[d]	1	(P08495) aspartokinase (DAP-lysine biosynthesis); Ask2 (*B. subtilis*)
	7-H6	1	(Q59291) aspartate-semialdehyde dehydrogenase (DAP-lysine biosynthesis); Asd (*C. jejuni*)
DNA repair	7-B11	1	(Z99115) UV-damage repair protein; UvrX (*B. subtilis*)
Unknown / hypothetical	5-H6	1	(Z99121) similar to hypothetical protein; YvgP (*B. subtilis*)
	6-G10	1	(Z99114) unknown; Yoj0 (*B.subtilis*)
	10-G2	2	(P37572) putative DNA repair protein; RadA (*B. subtilis*)
No database match[e]		14	

[a] Number of recovered strains with transposon integrated within the same gene.
[b] Determined by comparison of the sequences and coding regions with the EMBL Genbank DNA database, using the BLASTX and N determinations ($> e^{-10}$).
[c] Strains with transposon integration at different position within the same gene.
[d] Low homology scores (e^{-3} to e^{-9}).
[e] Homology scores with no significant database match ($< e^{-3}$).

sequences with three different transposon insertion positions within the gene. Homologs of 6 proteins which are involved in different biosynthetic routes, e.g. amino acid and nucleotide biosynthesis, were found. Strikingly, four of these were enzymes involved in the synthesis of lysine from aspartate (Fig. 4). One of the insertions was in a gene encoding a UV-damage repair protein. Finally, three of the identified sequences showed homology to unknown or hypothetical proteins from *Bacillus subtilis*, and fourteen did not give any significant database match.

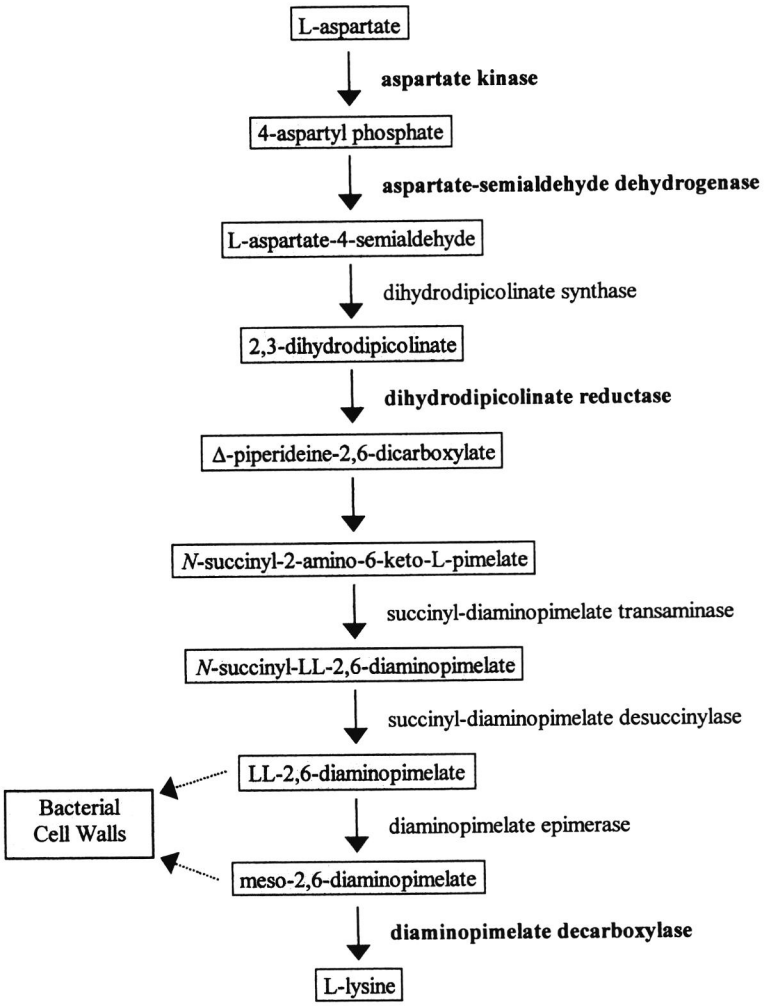

Figure 4.

Successive steps in the biosynthesic pathway of L-lysine from L-aspartate. The intermediates LL-2,6-diaminopimelate and meso-2,6-diaminopimelate are incorporated into bacterial cell walls. Expression of the genes encoding the bold face printed enzymes was found upregulated in *S. aureus* exposed to HUVEC.

Determination of inducing stimulus

The stimulus resulting in the induced bacterial gene expression was investigated in more detail. From each of the 4 categories (see above and Table 1) one clone was chosen randomly. These clones were 7-B11, 8-C2, 8-H10, and 9-D10. In addition, clone 4-G8 (no database match) was used. We first assessed the β-galactosidase activities in these clones cultured in M199 medium, or in M199 medium supplemented with 10% HSi, and compared these to the activity in LB. Clones 7-B11, 8-C2, 8-H10, and 9-D10 had no detectable β-galactosidase activity when grown in LB-broth, whereas clone 4-G8 had very limited activity (data not shown). All five strains showed strongly induced β-galactosidase expression when cultured in plain M199 medium. Although due to the detection limit of the assay the exact induction levels could not be determined, levels were in most cases more than 100-fold increased. In addition to the strong induction by the M199 medium, four of the tested clones showed even higher levels of β-galactosidase activity due to the presence of HSi in the M199 medium (Table 2). This indicates that components from serum can be stimuli for gene induction in *S. aureus*.

Table 2. Induction levels of gene expression in five of the isolated *S. aureus* RN4220 clones when cultured in the presence of heat inactivated human serum.

Clone	Homolog	Fold induction in M199 + Hsi[a]
8-C2	OppD	2.1
9-D10	Ama	1.0
8-H10	Ask2	1.3
7-B11	UvrX	3.0
4-G8	unknown	3.1

[a] Induction is expressed as fold increase in β-galactosidase activity of bacteria cultured for 4 h in M199 supplemented with 10% heat inactivated human serum compared to that of bacteria cultured for 4 h in M199.

Next, we determined if monolayers of HUVEC presented a signal to *S. aureus* resulting in induction of bacterial gene expression. The β-galactosidase activity of bacteria exposed to HUVEC in M199 cell culture medium supplemented with 10% HSi was determined, and compared to the β-galactosidase activity of *S. aureus* incubated in the same medium in the absence of HUVEC. Four clones showed increased β-galactosidase activities in the presence of HUVEC (1.4 to 4.0-fold), whereas 8-C2 showed no difference under these conditions (Table 3).

This indicates that *S. aureus* responds to signals from HUVEC, resulting in the expression of a specific subset of *S. aureus* genes.

Table 3. Induction levels of gene expression in five of the isolated *S. aureus* RN4220 clones when cultured in the presence of HUVEC.

Clone	Homolog	Fold induction in presence of HUVEC[a]
8-C2	OppD	1.0
9-D10	Ama	2.1
8-H10	Ask2	2.7
7-B11	UvrX	4.0
4-G8	unknown	1.4

[a] Induction is expressed as fold increase in β-galactosidase activity of bacteria cultured for 4 h in the presence of HUVEC compared to that of bacteria cultured for 4 h in the absence of HUVEC.

DISCUSSION

In patients without predisposing underlying heart disease, development of *S. aureus* IE is believed to be due to a direct interaction of the bacteria with endothelial cells (EC) of the endocardium. EC actively internalize *S. aureus* (24), but the precise nature of this interaction is largely unknown.

S. aureus express several different adhesins which might play a role in the interaction with EC. Expression of these adhesins is maximal during logaritmic growth, and down-regulated at the late exponential and stationary phase (19). The growth phase of *S. aureus* influenced the interaction with HUVEC, with both the number of infected cells and the total numbers of intracellular bacteria per cell being higher with logarithmic phase *S. aureus* than with stationary phase bacteria. Expression of adhesins and toxins in *S. aureus* is largely regulated by the *agr* locus (14, 19), which is partly absent in restriction negative mutant *S. aureus* RN4220 (23). The observed differences in infectivity between early-log phase and stationairy phase *S. aureus* indicate that regulation of the expression of bacterial structures involved in EC binding may not depend on *agr* solely. In addition, RN4220 induced killing of most EC upon infection within 24 h, as was also found for the virulent peritonitis isolate *S. aureus* CAPD but not for the avirulent *S. aureus* strain 42D (32). It thus seems that, although *S. aureus* RN4220 misses part of one of

its regulatory loci, this strain still behaves much like a virulent *S. aureus* strain in HUVEC infections.

One hour after exposure of HUVEC to *S. aureus in vitro*, SEM showed both surface-bound and intracellular bacteria. Surface-bound *S. aureus* were mostly observed in clusters, resembling the patchy manner of binding of *S. aureus* to cultured human valvular endothelial cells, rabbit endovascular tissue, and human aortic tissue (20), and suggesting a nonuniform distribution of cellular receptors on the HUVEC surface. Thread-like protrusions extending from the surface of the HUVEC, to which most of the surface-bound bacteria were attached, appear to be specifically induced by the bacteria, since such structures were never observed on non-*S. aureus* exposed HUVEC. As the protrusions of the HUVEC show similarity to structures identified on the surface of endothelial cells from human aortic walls (20), formation of such structures might be a general response of vascular EC to the presence of *S. aureus*. The specific bacterial stimulus reponsible for the induction of the threat-like protrusions is unknown. Internalization of bound bacteria appeared mostly to occur via retraction of the thread-like protrusions into opened invaginations of the EC membrane. Cup-shaped internalization processes (10, 24) were observed, however very infrequently. Although it is known that internalization of *S. aureus* by HUVEC involves cytoskeletal rearrangements (2, 10, 20), the exact uptake mechanism including the formation of the observed protrusions remains to be elucidated.

Studies on the interaction of bacteria with vascular EC have mainly focussed on cellular reponses, including EC surface receptor and cytokine expression, and induction of apoptosis (2, 4, 36, 37), whereas little attention has been paid to the bacterial responses in the interaction with EC. This study is the first report describing the identification of *S. aureus* genes of which the expression was induced upon exposure of the bacteria to HUVEC, using a transposon-based expression library of *S. aureus* RN4220. There are some limitations of using a transposon-based system, including the possibility of gene disruption or bias for transposition. However, except for genes that are essential for bacterial maintenance, induction of disrupted genes will still result in detectable promoter activity in the EC interaction model. Additionally, as Southern blotting has indicated randomness of transposon integration (15), this approach is suitable for the identification of inducible *S. aureus* genes and can give new insights in the bacterial response towards its environment.

Most of the genes identified by our screening method are homologous to genes involved in amino acid and cell wall synthesis, transport of a number of (macro-) molecules, and DNA repair and recombination. It is striking that none of the sequences found in our study was identified in a recent study on induced gene expression of *S. aureus* in an experimental murine abscess model using IVET (18). This suggests a specific response of *S. aureus* to stimuli from the *in vitro* HUVEC model. The similarity between our study and the IVET study was the inability of detecting genes encoding classical virulence factors (e.g. adhesins or toxins). As expression of such factors is regulated (19), the corresponding genes must already be expressed when *S. aureus* are cultured on laboratoria media.

The induction of the identified bacterial genes was the result of the combined interaction of bacteria with HUVEC, e.g. extracellular, cell-associated, and internalized bacteria. HUVEC and human serum could act as stimuli for the upregulation of expression of several *S. aureus* genes. The presence of HUVEC increased expression of an *ask2* homolog, encoding an aspartate kinase involved in the conversion of L-aspartate to L-lysine. Interestingly, genes encoding three other enzymes of this biosynthesis route were also identified. Activation of this biosynthetic pathway can cause alterations in the cell wall, which may be beneficial, or even required, for bacterial survival in the presence of HUVEC, as found in *in vivo* infection models (9,24). Expression of a putative *ama* gene, encoding an N-acyl-L-amino acid amidohydrolase and catalyzing the hydrolysis of N alpha-acylated amino acids, was also increased due to the presence of HUVEC. The specific function of this protein in the response to HUVEC is unknown.

Expression of the *oppD* (clone 8-C2) and of the unknown sequence (clone 4-G8) was increased in the presence of human serum and not of HUVEC. *OppD* is part of an oligopeptide ABC transporter operon of *S. aureus*, and encodes one of the ATP binding proteins of the transporter complex. Mutations in this operon strongly decreased viability of *S. aureus* in different experimental infection models, including the rabbit endocarditis model (8), possibly due to defective import of peptides used by *S. aureus* for growth. The observed upregulation of the OppD expression could be the result of the presence of such peptides in human serum. Oligopeptide transporters are also involved in adherence to host cells, resistance to host defensins, and production of toxins (16, 25, 28). Further research is required to establish the possible role of OppD in the interaction of *S. aureus* with HUVEC.

Finally, expression of a gene encoding a putative DNA repair protein was increased in the presence of both serum and HUVEC, suggesting that in the bloodstream *S. aureus* are subject to increased environmental stress.

In conclusion, we have assessed the cross-talk between HUVEC and *S. aureus*. The EC react to the bacteria by extending large, thread-like protrusions from their surface. Furthermore, we have shown for the first time that *S. aureus* reacts to HUVEC and to human serum by activating genes potentially involved in survival and adaptation to the host. Further studies on the exact nature of the inducing signals and on the role of the induced genes will elucidate the mechanism of *S. aureus* survival and growth in interaction with EC. These insights may contribute to an understanding of the pathogenesis of *S. aureus* IE in patients without prior heart disease.

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CHAPTER 9

General Discussion and Summary

Although the pathogenesis of Infective Endocarditis (IE) has intrigued researchers over the past era, only few pieces of the complex puzzle on initiation and progression of this disease have been unraveled. It is now known that many different microorganisms, including bacteria, fungi, and protozoan, are able to cause IE. Nevertheless, Gram-positive cocci have always been the most prominent pathogens isolated from IE patients, indicating a specific role for these bacteria in IE.

Research on IE, and more specifically on native valve IE (NVE), has therefore mainly focussed on viridans group streptococci (VS) and *Staphylococcus aureus*, the most frequently identified pathogens in IE. Different adhesive structures have been identified on the surface of these groups of bacteria, suggested or proven to be involved in binding to the vegetation, one of the early events in the onset of NVE. These include adhesins for different matrix molecules like fibronectin, collagen, laminin, and for specific receptors on different types of host cells, like vascular endothelial cells and platelets (Chapter 2).

Numerous of these "classical" virulence determinants have been extensively studied. The adaptive responses of the bacteria to their new environment, required to survive and proliferate, have sofar received little attention. The work presented in this thesis, therefore, was initiated to obtain more insight in the bacterial adaptive processes, by studying the regulation of bacterial gene expression of VS and *S. aureus* in response to specific environmental stimuli. The possible role of the observed bacterial responses in the development of IE will be discussed.

Regulation of gene expression

Most bacteria are well equiped to adapt to changes in their environment, and adaptation is in part accompanied by altering gene expression patterns (7, 14). As VS are able to survive and multiply in different niches, including the hostile environment of the bloodstream and within the vegetation, we have studied changes in their gene expression due to specific stimuli from these milieu's. For this purpose, we first developed several new genetic tools (Chapters 3 and 4). An IVET approach was chosen to assess regulated gene expression. This system, that is based on the differential expression of two reporter genes by wich regulated promoter activity can be identified, has proven its value in research on different bacterial pathogens (8, 12, 17). One must, however, bear in mind that this approach also has its limitations. As difference in expression levels between standard *in vitro* culture conditions and the *in vivo* milieu are determined, specific virulence characteristics that are constitutively expressed, or of which expression is only fine-tuned under both conditions are missed.

IVET has also been used to study changes in gene expression of VS in the complex *in vivo* environment during the pathogenesis of IE (9, 11). This has resulted in the identification of several regulated VS genes, but it is unknown at what point during the infective process the identified genes are induced, and what the activating signal for gene induction is. We chose to identify genes of which the expression was specifically upregulated by defined signals from

the bacterial milieu *in vitro*. Both a slight increase in the pH (Chapters 4 and 5) and iron limitation (Chapter 6), stimuli encountered by VS entering the bloodstream, were found to cause upregulation of the expression of several genes from *Streptococcus gordonii* CH1. The nature of the identified genes indicates that changes in the metabolic pathways are very important features of bacterial adaptation, which is in accordance with the results of the *in vivo* studies (8, 9, 11, 12, 17).

Strikingly, in these latter studies and our own studies only very few genes that correspond to "classical" virulence genes have been identified. It might thus be necessary to expand the concept of virulence genes. In this regard, a clear example of a new virulence factor is the bacterial methionine sulfoxide reductase. By molecular approaches, this protein has been identified to play a role in the pathogenesis of IE caused by *S. gordonii* (9, 11), as well as in *S. aureus* and *S. pneumoniae* infections (13, 19). Characterization of the *msrA* of *S. gordonii* CH1, of which expression was found to be induced by a slight increase in the environmental pH (Chapter 5), showed the importance of this VS gene in protection against oxidative stress as well as for bacterial multiplication. The availability of a site-directed *msrA* mutant strain (Chapter 5) will allow a proper assessment of the contribution of MsrA to the pathogenesis of IE.

Bacterial growth

Colonization of the sterile vegetation (VG) is an important process in the initiation of IE. The initial binding of bacteria to the VG is followed by proliferation, resulting in rapid bacterial multiplication within such thrombus. The numbers of bacteria increase rapidly within several hours after colonization (2, 4). Especially within the group of VS, which are in general not regarded as major pathogens, this characteristic might be of crucial importance in the development of IE. The rapid bacterial growth is not prevented by the human immune system, as phagocytic cells appear to be obstructed in their capacity to enter the vegetation and to kill bacteria within a platelet-fibrin meshwork (3). As little attention has been paid in literature to bacterial growth within the VG, this feature was adressed in Chapter 7.

Although vegetations mainly consist of platelets and fibrin, these components did not support rapid bacterial growth. Other, unknown plasma components present within the VG served as growth-stimulating nutrients. Bacterial protease activity appeared necessary for the rapid multiplication. Many reports have indicated a possible function of streptococcal proteases in IE, but none of these studies have provided any proof for this assumption (5, 10, 16, 18). Therefore, our findings are the first to indisputably support the involvement of VS proteases in intravegetational growth. However, it remains to be established what the exact nature is of the growth-supportive vegetational components and of the bacterial proteases involved. In addition, the role of other bacterial features, like for the identified MsrA (Chapter 5), in rapid bacterial multiplication *in vivo* still must be assessed.

S. aureus and vascular endothelium

Although the presence of a vegetation on the endocardial lesion has long been thought an essential prerequisite for the initiation of IE, an increasing number of individuals who contract IE are not known with valvular or endocardial defects. It is suggested that this is attributable to the ability of the microorganisms involved to directly interact with endothelial cells of the endocard. Gram-positive cocci, including VS and *S. aureus*, adhere much more readily to heart valves *in vitro* than other bacterial species (6, 15). Especially in patients with IE due to *S. aureus* this seems an important interaction, as about 30% of these IE patients are not known to have predisposing endocardial damage. The interaction between *S. aureus* and vascular endothelial cells has therefore been studied extensively. Especially the response of the vascular cells during this interaction has been characterized, and involves binding and uptake of bacteria followed by induction of cellular receptors and cytokine expression (1). However, the response of the bacteria in their interaction with the endothelial cells, and the subsequent adaption to the intracellular environment are largely unknown.

By using a transposon-based reporter system, several genes were identified of which the expression was upregulated in the presence of vascular endothelial cells (Chapter 8). Strikingly, all identified genes encode functions for survival and adaptation (biosynthesis routes, transporters, peptidases, DNA repair) to the cellular environment, while no genes encoding known *S. aureus* virulence determinants were found. This implies that either these latter functions are not operative in infection of vascular endothelium, or that those characteristics are constitively expressed and thus not identified by this approach.

Retesting of five of the isolated clones has shown that both vascular endothelial cells and serum serve as signals for *S. aureus* gene expression. It remains to be established which cellular and serum components are the actual signals. This study, however, clearly shows the importance of the choice of the reference growth condition, that serves to determine the basal level for bacterial gene expression. Although gene expression in the retested clones was induced by both endovascular cells and serum, it appeared that the cell culture medium itself already induced a significant level of expression. This underscores that one must be very careful in extrapolating *in vitro* observations to possible *in vivo* functions.

Conclusions

The pathogenesis of NVE remains a very complex process, with multiple bacterial and cellular components involved. More advanced molecular biological techniques are nowadays extensively used to obtain a more refined knowledge on this interaction. Especially bacterial gene expression studies, as presented in this thesis, have been of major importance in recent IE research. Nevertheless, studying regulation of gene expression alone does not suffice. The identification of homology to genes of other organisms might not imply a conserved function for the encoding proteins. Also the identification of genes with homology to sequences encoding proteins with unknown function or with no homology to known sequences, hampers

research on NVE pathogenesis. Furthermore, gene expression itself does not necessarily result in protein expression. However, gene expression studies can lead to the discovery of novel characteristics that might have potential for the development of new therapeutic treatments for this disease. In order to further unravel the pathogenic processes involved in NVE, we see an important potential for these novel molecular techniques when combined with thorough biological and biochemical characterizations

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CHAPTER 10

Nederlandse Samenvatting
(voor niet-ingewijden)

Endocarditis is een serieuze infectie van de hartkleppen en van het endocard, de binnenbekleding van het hart. Vele wetenschappers hebben zich in de afgelopen eeuwen bezig gehouden met het bestuderen van deze ziekte. Ondanks dat dit tot diverse inzichten heeft geleid over het ontstaan en de progressie van de ziekte, zijn vele stukken van de puzzel nog steeds niet opgelost. Het doel van het in dit proefschrift beschreven onderzoek was met behulp van moderne genetische technieken inzicht te krijgen in hoe de belangrijkste bacteriële veroorzakers van endocarditis zich kunnen aanpassen aan de specifieke omstandigheden in het hart en vervolgens in staat zijn endocarditis te veroorzaken. Dit om enerzijds meer inzicht te krijgen in de ziekte zelf en anderzijds informatie te vergaren die kan leiden tot de ontwikkeling van nieuwe therapieën voor deze ziekte.

Het onstaan van endocarditis

Er worden verschillende vormen van endocarditis onderscheiden. Het meest voorkomend is natieve klep endocarditis (NVE). Deze vorm ontstaat bij mensen die een beschadiging hebben opgelopen aan de hartkleppen of aan het endocard. Deze beschadiging kan ontstaan door bepaalde ziektes (zoals rheumatische aandoeningen of infecties) of kan het gevolg zijn van een aangeboren hartdefect. Om deze beschadiging te laten helen zorgt het lichaam ervoor dat er een wondkorst wordt gevormd. Deze wondkorst wordt een vegetatie (VG) genoemd en bestaat voornamelijk uit twee verschillende bloedcomponenten: fibrine draden en bloedplaatjes. Bacteriën die zich tijdelijk in de bloedbaan bevinden (na bijvoorbeeld tandextractie of het poetsen van de tanden) kunnen in een dergelijke vegetatie worden ingevangen. Bacteriën afkomstig uit de mondholte (viridans groep streptococcen) en uit de neusholte (*Staphylococcus aureus*) zijn de belangrijkste veroorzakers van deze vorm van endocarditis, al zijn in principe alle micro-organismen (bacteriën, gisten, schimmels) in staat als ziekteverwekker op te treden. Eenmaal ingevangen in de vegetatie kunnen de bacteriën niet meer door het menselijk afweersysteem worden herkend en opgeruimd. Vanwege de overvloed aan voedingsstoffen zijn de bacteriën in staat zeer snel te groeien. Het gevolg van deze snelle groei is dat de afzetting van fibrine en bloedplaatjes op de vegetatie continueert, zodat deze toeneemt in grootte. Dit kan uiteindelijk leiden tot een embolie (het loslaten van een stuk van de vegetatie, wat elders kan leiden tot zuurstoftekort door afsluiten van een bloedvat) of zelfs tot hartfalen.

Endocarditis kan verder ontstaan als gevolg van de implantatie van een nieuwe hartklep en wordt dan prosthetische klep endocarditis (PVE) genoemd. De wondkorst die wordt gevormd op de plaats van implantatie is dan het begin van de ziekte. Indien dit binnen een jaar na de operatie gebeurt, spreekt men van vroege PVE. In dit geval zijn bacteriën afkomstig van de huid (*Staphylococcus epidermidis*) de belangrijkste veroorzakers. Als PVE meer dan één jaar na de operatie optreedt, spreekt men van late PVE. In dat geval zijn viridans groep streptococcen en *Staphylococcus aureus* de belangrijkste veroorzakers.

Een derde vorm van de ziekte wordt gevonden bij mensen die intraveneus drugs gebruiken (IVDU). Deze mensen hebben over het algemeen geen hartbeschadiging. Het wordt verondersteld dat in deze gevallen de bacteriën in staat zijn direct een interactie aan te gaan met de cellaag van het endocard grenzend aan het bloed (het endotheel). Met name *Staphylococcus aureus* is hiertoe in staat. De binding leidt tot opname van de bacteriën door de endotheelcellen, en het verblijf van de bacteriën in deze cellaag resulteert uiteindelijk in schade aan de cellaag. Deze celschade alsmede de aanwezigheid van afweercellen van het immuunsysteem zorgen uiteindelijk ook weer voor de vorming van een vegetatie, en daarmee het ontstaan van endocarditis.

Onderzoek aan de bacteriële veroorzakers van endocarditis

Zoals reeds aangegeven zijn bacteriën uit de mondholte (viridans groep streptococcen) en uit de neusholte (*Staphylococcus aureus*) de belangrijkste verwekkers van endocarditis. Beide groepen beschikken over een arsenaal aan eigenschappen waarmee zij in staat zijn het menselijk afweersysteem te omzeilen en vervolgens de ziekte te initiëren. Specifieke structuren op de buitenzijde van de bacteriën (adhesines) zijn betrokken bij de binding van de bacteriën aan de vegetatie of aan het endotheel, en een belangrijk deel van het endocarditis-onderzoek in de afgelopen decennia heeft zich hierop gericht (zie Hoofdstuk 2). Er is echter nog steeds zeer weinig bekend over het verdere verloop van deze ziekte. Met name de manier waarop de bacteriën in staat zijn zich aan te passen aan hun nieuwe omgeving en zich vervolgens kunnen vermeerderen was tot op heden een 'black box'.

Een mogelijkheid om het aanpassingsvermogen van de bacteriën te bestuderen is te kijken over welke eigenschappen de bacterie beschikt in die specifieke omgeving. De informatie voor deze eigenschappen is vastgelegd op zogenaamde genen (gedefinieerde stukken DNA op een chromosoom). Activatie van een gen (gen-expressie) vindt plaats vanaf een specifiek deel gelegen voor het gen; de promoter. Deze activatie kan uiteindelijk leiden tot de productie van een specifiek eiwit, dat verantwoordelijk is voor een bepaalde bacteriële eigenschap. Sommige van deze eiwitten zijn onder alle omstandigheden van belang voor het functioneren en overleven van de bacteriën, en de voor deze eiwitten coderende genen worden continue tot expressie gebracht (constitutieve genen). Er zijn echter ook eiwitten die alleen onder bepaalde omstandigheden nodig zijn. De bijbehorende genen worden dan ook alleen in die specifieke situaties tot expressie gebracht (reguleerbare genen). Door de patronen van gen-expressie onder verschillende omstandigheden te bestuderen kan men een indruk krijgen welke bacteriële eigenschappen van belang zijn onder welke omstandigheden.

Om de gen-expressie van viridans groep streptococcen en *Staphylococcus aureus* te kunnen bestuderen zijn eerst een aantal genetische werktuigen ontwikkeld (Hoofdstuk 3 en 4). Vervolgens is gekeken of hiermee genen geïdentificeerd konden worden van viridans groep streptococcen die tot expressie gebracht werden onder invloed van een specifieke stimulus. Een bacterie is in staat de omgeving te herkennen door het oppikken van signalen uit die

omgeving, zoals bijvoorbeeld de aanwezigheid van bepaalde voedingsstoffen, de zuurstofspanning, of de zuurgraad. Deze signalen zorgen ervoor dat in de bacterie bepaalde genen tot expressie worden gebracht. Veranderingen in deze signalen kunnen dus leiden tot verandering van het gen-expressie patroon. Als model signaal is eerst gekozen voor een verandering in de zuurgraad (ofwel 'pH'). De pH in de mondholte ligt normaal gesproken tussen de 6 en 6.5 (licht zuur), terwijl de pH van het bloed 7.3 - 7.4 (neutraal) is. Bacteriën die vanuit de mondholte in het bloed terecht komen, moeten zich dus aanpassen aan deze verandering. Inderdaad konden een aantal genen geïdentificeerd worden waarvan de expressie door dit specifieke signaal werd gereguleerd (Hoofdstuk 4 en 5). Een van deze genen bleek te coderen voor een eiwit (MsrA; methionine sulfoxide reductase) dat betrokken is bij de bescherming van de bacterie tegen zuurstofradicalen. Zuurstofradicalen vormen een van de afweermechanismen van het menselijk lichaam tegen binnendringende micro-organismen. Deze radicalen zorgen ervoor dat bacteriële eiwitten beschadigd raken, waardoor deze eiwitten niet meer functioneren en de bacterie dus dood gaat. Het MsrA is in staat de beschadiging aan deze eiwitten te herstellen. Het bleek echter dat dit eiwit nog een tweede funtie had, namelijk bij de vermeerdering van de bacteriën.

Het tweede omgevingssignaal dat bestudeerd is, is de beschikbaarheid van vrij ijzer (Hoofdstuk 6). IJzer is cruciaal voor bacteriën, omdat een groot aantal eiwitten dit element nodig heeft om correct te kunnen functioneren. Inderdaad kon in de viridans groep streptococ *Streptococcus gordonii* een gen gevonden worden dat tot expressie werd gebracht door de afwezigheid van vrij ijzer. In de promoter gelegen voor het gen werd een regio geïdentificeerd die sterk lijkt op regio's in genen van andere bacteriën, die specifiek gereguleerd worden door dit signaal. Het gen bleek mogelijk te coderen voor een eiwit dat aan de buitenzijde van de bacterie zit en betrokken is bij transport van ijzer de bacterie in. Deze hypothetische functie zal nog bevestigd moeten worden.

Bacteriële groei

Een belangrijke karakteristiek van viridans groep streptococcen en *Staphylococcus aureus* in het ontstaan van endocarditis is hun snelle groei na te zijn ingevangen in de vegetatie. Deze snelle groei kan niet worden tegengegaan door het menselijk afweersysteem, omdat de vegetatie de afweercellen tegenhoudt en daardoor het doden van de bacteriën voorkomt. Vooral voor de viridans groep streptococcen, die in het algemeen worden gezien als belangrijke ziekteverwekkers, is dit waarschijnlijk een cruciale eigenschap. Hoewel het gegeven van snelle groei reeds lang bekend is, heeft dit in de literatuur weinig aandacht gehad. Daarom is hier dieper op ingegaan in Hoofstuk 7.

Allereerst werd de groeisnelheid van een aantal verschillende viridans groep streptococcen in een, in een reageerbuis gemaakt, bloedstolsel bepaald. Het bleek dat de snelle groei die door andere onderzoekers geobserveerd werd in een vegetatie in het konijnenmodel van endocarditis, eveneens te zien was in dit "in vitro" (laboratorium) modelsysteem. Nader

onderzoek leerde dat de belangrijkste componenten van dit bloedstolsel (vegetatie), zijnde fibrine en bloedplaatjes, niet verantwoordelijk waren voor de snelle groei. Verder waren oplosbare componenten uit het bloed eveneens niet in staat de bacteriën aan te zetten tot de snelle groei. Andere, tot nu toe nog onbekende componenten die vanuit het bloed op het stolsel worden afgezet lijken verantwoordelijk voor dit fenomeen. Deze componenten bleken verder niet direct beschikbaar voor de bacteriën, maar dienden eerst nog omgezet te worden tot bruikbare voedings-componenten door middel van specifieke bacteriële eiwitten (proteases). Het blokkeren van de functie van deze proteases gaf een bijna volledige groei-stop te zien. Deze proteases dienen daarom mogelijk als nieuwe doelen in de ontwikkeling van strategieën voor de behandeling van endocarditis.

***Staphylococcus aureus* en het endotheel**

Ondanks dat de aanwezigheid van een vegetatie een eerste vereiste lijkt voor het ontstaan van endocarditis, blijkt bij een groeiend aantal mensen bij wie endocarditis wordt gediagnostiseerd (drugs gebruikers, maar ook gezonde personen) dergelijke schade voorafgaand aan de ziekte niet aanwezig te zijn. Zoals reeds aangegeven wordt in deze gevallen verondersteld dat endocarditis het resultaat is van een directe interactie van de bacteriën met de cellaag van het endocard grenzend aan het bloed (endotheel). Met name *Staphylococcus aureus* is zeer goed in staat om een interactie met deze cellaag aan te gaan. Er is reeds veel onderzoek gedaan naar de gevolgen van deze interactie vanuit de kant van de cellaag. Gebleken is dat de cellen de bacteriën actief opnemen en vervolgens als reactie op de aanwezigheid van de bacteriën zowel eiwitten op het celoppervlak laten verschijnen als verschillende eiwitten uitscheiden. Dit zijn specifieke signalen om het afweersysteem te informeren dat er een infectie gaande is. De aanwezigheid van deze bacteriën zorgt er uiteindelijk voor dat er een vegetatie gevormd wordt (ofwel door activatie van bepaalde witte bloedcellen, ofwel door het induceren van schade aan de endotheelcellen), wat uiteindelijk resulteert in endocarditis.

De reactie van *Staphylococcus aureus* op de interactie met de endotheelcellen was tot nu toe vrijwel geheel onbekend. Vandaar dat deze interactie nader is bestudeerd vanuit het oogpunt van deze bacterie (Hoofdstuk 8). Het bleek dat deze interactie in belangrijke mate bepaald werd door de groei-fase van de bacterie, aangezien groeiende (logaritmische fase) bacteriën veel beter bonden aan en veel beter werden opgenomen door de cellen dan uitgedeelde (stationaire fase) bacteriën. Fotografische opnamen gemaakt met behulp van een electronenmicroscoop lieten verder zien dat de bacteriën het endotheel aanzetten tot het maken van lange uitlopers, waarmee vervolgens de bacteriën werden opgenomen. Daarnaast is ook gekeken naar de genen die *Staphylococcus aureus* specifiek tot expressie bracht in de aanwezigheid van deze endotheelcellen. Een totaal van 33 verschillende genen werd gevonden, waarvan er 19 codeerden voor bekende eiwitten van *Staphylococcus aureus* of van andere bacteriën. Deze konden worden ingedeeld op basis van hun functie in 1.) transport eiwitten, 2.) peptidases, 3.) eiwitten betrokken bij verschillende biosynthese routes, en 4.)

eiwitten betrokken bij de reparatie van DNA. Met name de vorming van de celwand blijkt belangrijk in de interactie met endotheelcellen, aangezien vier verschillende genen uit de syntheseroute van lysine (een belangrijke component van bacteriële celwanden) teruggevonden werden.

Nadere bestudering van een aantal van de geïdentificeerde genen gaf aan dat niet alle genen specifiek door het endotheel werden geactiveerd. Ook serum (bloed zonder cellen en stollingsfactoren), dat nodig is voor het kweken van de endotheelcellen, bleek als een dergelijke stimulus te kunnen optreden. Echter, beide stimuli bleken in staat genen van *Staphylococcus aureus* te activeren die mogelijk betrokken zijn bij overleving van de bacteriën en bij aanpassing aan de nieuwe omgeving. Nadere studies naar de componenten van endotheelcellen en serum welke verantwoordelijk zijn voor deze activatie van gen-expressie zullen leiden tot een beter begrip van het ontstaan en verloop van endocarditis in deze groep van endocarditis-patiënten.

Concluderend kan gezegd worden dat het ontstaan en de progressie van natieve klep endocarditis een complex proces is. Met behulp van nieuwe, geavanceerde technieken, zoals beschreven in dit proefschrift, zijn we in staat een dieper inzicht te krijgen in het aanpassingsvermogen van de bacteriën die deze ziekte veroorzaken, alsmede met betrekking tot de interactie die ze aangaan met bepaalde componenten uit hun omgeving (bijvoorbeeld endotheel). Deze nieuwe inzichten kunnen leiden tot mogelijkheden voor het ontwikkelen van nieuwe therapieën voor de behandeling van endocarditis.

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Aldwin

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 26 september 1970 te Roosendaal en Nispen. Na het behalen van het VWO diploma aan de Katholieke Scholengemeenschap in Etten-Leur, begon hij in 1988 met de studie biologie aan de Landbouw Universiteit in Wageningen. Van 1991 tot 1994 werkte hij achtereenvolgens in de laboratoria van Prof. dr. W.M. de Vos (Afdeling Bacteriële Genetica), Dr. A.J.M. Debets (Afdeling Populatie Genetica), en Dr. D. Zuidema (Afdeling Baculovirologie). In deze periode was hij eveneens gedurende 7 maanden werkzaam in het laboratorium van Prof. dr. J.N. Reeve (Afdeling Microbiologie) aan de Ohio State University, Columbus, Ohio, USA. Het doctoraal examen werd afgelegd in juni 1994.

Van juli 1994 tot december 1998 was hij werkzaam als assistent in opleiding bij de afdeling Bacteriologie van de vakgroep Medische Microbiologie van het Academisch Medisch Centrum (AMC) in Amsterdam, onder begeleiding van Dr. S.A.J. Zaat en Prof. dr. J. Dankert, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd.

Vanaf april 1999 is hij aangesteld als post-doctoraal medewerker bij de afdeling Moleculaire Genetica & Gentechnologie van TNO Voeding te Zeist. Het huidige onderzoeksproject, dat valt binnen het onderzoeksprogramma van het Wageningen Center for Food Sciences (WCFS), beoogt het bestuderen van *Lactobacillus* soorten, bacteriën die gebruikt worden als pre- en probiotica in de voedingsindustrie.

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