Development of genetic tools for *in vivo* virulence analysis of *Streptococcus sanguinis*

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Completion of the genome sequence of *Streptococcus sanguinis* SK36 necessitates tools for further characterization of this species. It is often desirable to insert antibiotic resistance markers and other exogenous genes into the chromosome; therefore, we sought to identify a chromosomal site for ectopic expression of foreign genes, and to verify that insertion into this site did not affect important cellular phenotypes. We designed three plasmid constructs for insertion of *erm*, *aad9* or *tetM* resistance determinants into a genomic region encoding only a small (65 aa) hypothetical protein. To determine whether this insertion affected important cellular properties, SK36 and its erythromycin-resistant derivative, JFP36, were compared for: (i) growth *in vitro*, (ii) genetic competence, (iii) biofilm formation and (iv) virulence for endocarditis in the rabbit model of infective endocarditis (IE). The spectinomycin-resistant strain, JFP56, and tetracycline-resistant strain, JFP76, were also tested for virulence *in vivo*. Insertion of *erm* did not affect growth, competence or biofilm development of JFP36. Recovery of bacteria from heart valves of co-inoculated rabbits was similar to wild-type for JFP36, JFP56 and JFP76, indicating that IE virulence was not significantly affected. The capacity for mutant complementation *in vivo* was explored in an avirulent *ssaB* mutant background. Expression of *ssaB* from its predicted promoter in the target region restored IE virulence. Thus, the chromosomal site utilized is a good candidate for further manipulations of *S. sanguinis*. In addition, the resistant strains developed may be further applied as controls to facilitate screening for virulence factors *in vivo*.

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INTRODUCTION

Streptococcus sanguinis is a member of the viridans group of streptococci, an important component of the normal human oral flora beginning with the emergence of teeth (Aas *et al.*, 2005; Caufield *et al.*, 2000), and an indicator of good oral health (Aas *et al.*, 2005; Marsh, 2003). *S. sanguinis* colonizes the tooth surface and serves as a scaffold in the development of dental plaque. Viridans streptococci can cause extra-oral pathologies including bacteraemia in neutropenic, onco-

haematological patients (Bochud *et al.*, 1994), and infective endocarditis (IE) in patients with predisposing cardiac damage (Moreillon & Que, 2004). A recent meta-analysis of population-based IE studies implicated viridans streptococci as the predominant cause of bacterial IE (Tleyjeh *et al.*, 2007). *S. sanguinis* is the viridans species most commonly associated with IE (Douglas *et al.*, 1993; Dyson *et al.*, 1999; Roberts *et al.*, 1979; Vlessis *et al.*, 1996). Despite advances in diagnostic methods, antimicrobial and surgical therapies, and complication management, the mortality and morbidity associated with IE have remained unchanged in the last two decades, thus prompting the need for identification of prophylactic and therapeutic targets (Moreillon & Que, 2004; Wilson *et al.*, 2008).

The rabbit model of IE, first introduced by Durack and Beeson, produces 'vegetations' composed of bacteria,

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Abbreviations: CI, competitive index; Cm, chloramphenicol; CV, crystal violet; Em, erythromycin; IE, infective endocarditis; Sc, spectinomycin; Tet, tetracycline; TH-HS, Todd–Hewitt horse serum; TSA, Tryptic Soy agar.

immune cells, and fibrin–platelet clot components that closely resemble those recovered from human patients; therefore, this model has been adopted for evaluation of virulence factors for bacterial IE in many studies (Durack *et al.*, 1973; Durack, 1975). Our group previously used a random signature-tagged mutagenesis approach to screen for *S. sanguinis* virulence determinants in the rabbit model of IE. The five attenuated mutants identified had transposon insertions in genes encoding housekeeping functions such as cell-wall synthesis, protein or nucleic acid synthesis, and survival in anaerobiosis (Paik *et al.*, 2005). The lack of identification of classical virulence factors, e.g. those facilitating adhesion in colonization, was surprising given the abundance of putative virulence factors predicted from the complete SK36 genome sequence (Xu *et al.*, 2007). This study presented the need for advancement of genetic tools to facilitate further screening of streptococcal factors required for development of disease. Of particular interest is the validation of virulence factors via complementation of inactivated genes, methods for exclusive strain selection from a multiple-strain pool, and an approach for introduction of a non-adjacent selective marker in an in-frame deletion mutant.

Limitations exist for strain selection by plasmid-borne markers during *in vivo* analyses, including loss of plasmids due to lack of selective pressure, plasmid transfer among genetically competent strains, and undue effects of plasmid copy number on gene expression and cell cycling. To circumvent these issues we sought to develop a system for single-copy gene expression in *S. sanguinis*. This strategy required identification of a suitable chromosomal site for ectopic expression of foreign genes.

Here, we describe the manipulation of a previously uncharacterized locus of *S. sanguinis* SK36 for introduction of antibiotic resistance determinants expressed from synthetic promoter elements optimized for *Streptococcus pneumoniae* or derived from *Enterococcus faecalis* (Claverys *et al.*, 1995; Dintilhac *et al.*, 1997; Hancock & Perego, 2004). A series of plasmid constructs was used for insertion of cloned antibiotic resistance determinants into this locus, and the resulting strains were tested for maintenance of relevant *in vitro* and *in vivo* properties. It was determined that insertional mutagenesis of the target site did not affect growth, biofilm formation or genetic competence. Importantly, virulence for IE was also maintained. The site was also used for ectopic expression of a mutated virulence gene, allowing for complementation of virulence. The applications of the target site explored here provide evidence that this locus may be exploited to further our understanding of *S. sanguinis* IE.

METHODS

Bacterial strains, growth conditions and chemicals. The bacterial strains and plasmids used in this study are listed in Table 1. *S. sanguinis* SK36, a human oral isolate, was obtained from Mogens Kilian (Kilian *et al.*, 1989). *S. sanguinis* strains were routinely cultured

in jars under reduced oxygen (~7.2% H₂, ~7.2% CO₂, ~79.7% N₂, ~6% O₂; referred to in the text as 6% O₂) or anaerobiosis (~10% H₂, ~10% CO₂, ~80% N₂, 0% O₂, maintained with a palladium catalyst) generated using an Anoxomat system (Mart). All culture media used were from Difco. *S. sanguinis* strains were grown in Brain Heart Infusion (BHI) medium for liquid culture, Tryptic Soy Broth with 1.5% Bacto Agar (TSA) for surface plating, or Tryptic Soy Broth with 1% low-melting-point agarose (National Diagnostics) for layer plating (Paik *et al.*, 2005). For transformation, *S. sanguinis* strains were cultured in Todd–Hewitt broth containing 2.5% (v/v) heat-inactivated horse serum (Invitrogen) (TH-HS) (Paik *et al.*, 2005). When required for selective growth of *S. sanguinis*, erythromycin (Em), spectinomycin (Sc) and tetracycline (Tet) were used at 10, 200 and 5 µg ml⁻¹, respectively. Electromax *Escherichia coli* DH10B (Invitrogen) was used as a bacterial host for plasmid construction. All *E. coli* strains were cultured in Luria–Bertani (LB) medium, or on LB containing 1.5% Bacto Agar. For selective culture of *E. coli*, Em, Sc, Tet, chloramphenicol (Cm) and kanamycin were used at 300, 100, 2.5, 5 and 50 µg ml⁻¹, respectively.

DNA isolation and manipulation. Chromosomal DNA was isolated from *S. sanguinis* SK36 according to the method described by Kitten *et al.* (2000). DNA isolation from agarose was routinely performed using Bio-Rad Quantum Prep Freeze 'N Squeeze columns. PCR products were purified by the MinElute PCR Purification kit from Qiagen. Quantitative reverse transcriptase PCR was performed with a 7500 Fast Real-time PCR System (Applied Biosystems). *S. sanguinis* strains were transformed as previously described (Paik *et al.*, 2005). Briefly, *S. sanguinis* was cultured in TH-HS to an OD₆₆₀ of ~0.07. Transforming DNA, 330 µl cells and 70 ng synthetic *S. sanguinis* competence-stimulating peptide (Gaustad & Havarstein, 1997) were transferred to 0.7 ml microcentrifuge tubes and incubated at 37 °C for 1 h. Cells were plated with appropriate selective antibiotics and grown at 37 °C for 48 h. The frequency of transformation was defined as the ratio of transformants to total c.f.u. The efficiency of DNA uptake was defined as transformants per µg of transforming DNA.

Plasmid and strain construction. Oligonucleotides used in this study are listed in Table 1. A schematic depicting antibiotic resistance plasmid construction is shown in Fig. 1. Primers 0169-Sall-up/0169-Sall-dn were used to PCR amplify the 0169 locus from SK36 chromosomal DNA. The PCR fragment was inserted into the Sall restriction site of pVA2606 – a non-replicating plasmid in *S. sanguinis* SK36 – to yield pJFP34. The Em resistance cassette *erm* (Claverys *et al.*, 1995) (kindly provided by D. Morrison, University of Illinois at Chicago) was amplified with primers Erm-NcoI-up/Erm-NcoI-dn, digested with NcoI, and inserted into a unique NcoI site within the 0169 insert of pJFP34 to yield pJFP36. Transformation of SK36 with pJFP36 produced strain JFP36, with the *erm* cassette integrated via double crossover at the 0169 locus.

To expand the applicability of the 0169 cloning site, a linker with *AscI* and *NotI* restriction sites was integrated into the NcoI site of pJFP34, to yield pJFP46. Complementary oligonucleotides 0169RE-up and 0169RE-dn (Table 1) were 5'-phosphorylated, combined, heat denatured and annealed, resulting in a 5' NcoI sticky-end overhang permitting ligation into the NcoI site of pJFP34. A base pair change at one end of the oligonucleotide 0169RE-dn abolished the second NcoI site, creating a series of unique cloning sites (*AscI*, *NotI* and NcoI).

Plasmid pJFP56 was constructed by cloning of *aad9* – PCR amplified from pR412 (kindly provided by B. Martin and J-P. Claverys, Université Paul Sabatier, France) (Martin *et al.*, 2000) by primers Sc-NotI-up/Sc-NcoI-dn – into the pJFP46 *NotI* and NcoI restriction sites. Transformation of SK36 with pJFP56 produced the Sc-resistant strain JFP56.

Plasmid pJFP76 was constructed by amplifying the *tetM* cassette of pJM133 (kindly provided by M. Perego, Scripps Research Institute)

Table 1. Strains, plasmids and primers used in the present study

Strain, plasmid or primer	Description*	Reference
<i>S. sanguinis</i>		
SK36	Human plaque isolate	Kilian <i>et al.</i> (1989)
6-26	Cm ^r ; $\Delta nrdD::magellan2$, derived from SK36	Paik <i>et al.</i> (2005)
VT1614	Kn ^r ; $\Delta srpA::aphA-3$, derived from SK36	Plummer <i>et al.</i> (2005)
JFP36	Em ^r ; $\Delta 0169::pSerm$, derived from SK36	
JFP56	Sc ^r ; $\Delta 0169::aad9$, derived from SK36	
JFP76	Tet ^r ; $\Delta 0169::tetM$, derived from SK36	
<i>ssaB</i>	Cm ^r ; $\Delta ssaB::magellan2$, derived from SK36	Das <i>et al.</i> (2009)
JFP87	Sc ^r , Cm ^r ; $\Delta ssaB::magellan2$, $\Delta 0169::ssaB-aad9$, derived from the <i>ssaB</i> mutant	
JFP88	Sc ^r , Cm ^r ; $\Delta ssaB::magellan2$, $\Delta 0169::aad9$, derived from the <i>ssaB</i> mutant	
Plasmids		
pVA2606	Kn ^r ; pUC19-derived by replacement of <i>bla</i> with <i>aphA-3</i>	Paik <i>et al.</i> (2005)
pJFP34	Kn ^r ; derivative of pVA2606 with <i>0169</i>	
pJFP36	Kn ^r Em ^r ; derivative of pJFP34 with <i>erm</i>	
pJFP46	Kn ^r ; derivative of pJFP34 with <i>AscI</i> , <i>NotI</i> <i>0169</i> cloning site	
pJFP56	Kn ^r Sc ^r ; derivative of pJFP46 with <i>aad9</i>	
pJFP76	Kn ^r Tet ^r ; derivative of pJFP46 with <i>tetM</i>	
pJFP16	Kn ^r Cm ^r ; pVA2606 containing 3.3 kb <i>nrdD::magellan2</i>	
pJFP57	Kn ^r Sc ^r ; pJFP46 containing <i>ssaB-aad9</i>	Das <i>et al.</i> (2009)
pJFP87	Kn ^r Sc ^r ; pJFP57 with 149 bp upstream of <i>ssaACB</i>	
Primers†		
0169-SalI-up	CCGGTTCGACTGTCTATGGTGGACAGCGTCC	
0169-SalI-dn	CCGGTTCGACTCGTGACGGAAGCAGACTTGG	
pSerm-NcoI-up	CATGCCATGGCCGGGCCCCAAAATTTGTTTGAT	
pSerm-NcoI-dn	CATGCCATGGAGTCGGCAGCGACTCATAGAAT	
0169RE-up	CATGGGTGACGCGGCCGCTGCAGGCGCGCCTGCGAACA	
0169RE-dn	CATGTGTTTCGACGGCGCGCTGCAGCGGCCGCGTCACC	
Sc-NotI-up	CCGGCGGCCGCCGCTCTAGAACTAGTGGATCC	
Sc-NcoI-dn	TCTCGACCATGGCAATTTTTTATAATTTTTTAATCTG	
Tet-NotI-up	CAGTCGGCGGCCGCTAGGTTGATTTTCGTTT	
Tet-NcoI-dn	TCTCGACCATGGCTAGGTCACCTAAGTTATTTT	
AscI-ssaAP-up	CGCAGGCGCGCCCTTCTTCTCACTTTC	
ssaAP-dn	CCTAATTTTTTTCATCTCATACACCTCTA	
PssaB-up	GAGGTGTATGAGATGAAAAAATTAGGTTTTTTATCCCTGC	
StuI-ssaB-dn‡	CCTTAGAGAAGTACTTGAAGCAGCCTTC	
6-26-BamHI-up	AAGCGGATCCAGTACGCGCTGGGCGAAGACC	
6-26-BamHI-dn	TGGCGGATCCTTTCCACGCGCAGAGGGAC	
0169-F	ATGAAGCAAAAACCCGATGA	
0169-R	TTTTCTGGAAGCTGTTCTGGA	

*Cm, chloramphenicol; Kn, kanamycin; Em, erythromycin; Sc, spectinomycin; Tet, tetracycline.

†Underlined sequences correspond to restriction sites.

‡Primer 5'-phosphorylated.

(Hancock & Perego, 2004) with primers Tet-NotI-up/Tet-NcoI-dn, and ligating the digested fragment into the *NcoI* and *NotI* restriction sites of pJFP46. Transformation of SK36 with pJFP76 produced the Tet-resistant strain JFP76.

The Cm resistance determinant and approximately 1.4 kb of flanking DNA was PCR amplified from the chromosome of strain 6-26 (Paik *et al.*, 2005) by primers 6-26-BamHI-up/6-26-BamHI-dn. The PCR product was cloned into pVA2606 via *BamHI* restriction sites to create plasmid pJFP16.

An Sc-resistant derivative of pJFP46, pJFP57 (Das *et al.*, 2009), was used for cloning of *ssaB* with its predicted native promoter. Fig. 2 provides a schematic of the sequential cloning steps used. The

presumed promoter region of the *ssaACB* operon was amplified from SK36 chromosomal DNA by primers AscI-ssaAP-up and *ssaAP*-dn. The first 640 nucleotides of *ssaB*, from the start ATG to an intragenic *StuI* site, were amplified from SK36 chromosomal DNA by primers PssaB-up and StuI-ssaB-dn. Complementary overhangs between *ssaAP*-dn and PssaB-up fused the two amplicons in a third amplification reaction primed by AscI-ssaAP-up and StuI-ssaB-dn. Both the amplicon and the cloning vector, pJFP57, were digested with *AscI* and *StuI* for insertion of the presumed *ssaB* promoter adjacent to *ssaB*. An *ssaB* mutant (Das *et al.*, 2009) was transformed with the resulting plasmid, pJFP87, or the Sc resistance plasmid pJFP56, to yield JFP87 or JFP88, respectively. The nucleotide sequences of constructs described above were confirmed by DNA sequencing by

Fig. 1. Strategy for plasmid construction. Asterisks indicate restriction sites introduced by PCR. Open arrows indicate PCR amplifications; solid arrows indicate cloning steps. ORFs are shown in white; promoters driving expression of resistance determinants are represented by black circles; inverted repeat is abbreviated as IR. Unique restriction sites discussed in Methods are shown.

Fig. 2. Cloning of *ssaB* for complementation. Asterisks indicate restriction sites introduced by PCR. Open arrows indicate PCR amplifications; the ladder represents complementary base pairing of sequence overlaps introduced by PCR; solid arrows indicate cloning steps. ORFs are shown in white; the presumed promoter of the *ssaACB* operon is represented by a black circle; the *ssaACB* promoter fragment on pJFP57 is represented by a part-circle. Unique restriction sites discussed in Methods are shown.

the Nucleic Acids Research Facilities of Virginia Commonwealth University.

Overnight growth comparison in a microplate format. Late-exponential-phase *S. sanguinis* cultures were diluted 100-fold in BHI, and 200 μ l aliquots added to the wells of a 96-well microplate (Grenier). Growth at 37 °C under ambient atmosphere was monitored every 10 min over 20 h by measuring the OD₄₅₀ in a FLUOstar microplate reader (BMG Technologies). Cultures were mixed by orbital shaking (140 r.p.m.) with a width of 4 mm for 10 s prior to every recording.

RT-PCR. Trizol (Invitrogen) extraction was used for isolation of RNA from late-exponential-phase cells of SK36 cultured in BHI at 37 °C aerobically (225 r.p.m.), under 6% O₂, or anaerobically. RNA samples were purified and double-DNase I treated on RNeasy columns (Qiagen), and RNA quality assessed by the Experion RNA StdSens Chip (Bio-Rad). Reverse transcription was performed with pre-formulated beads from the Ready-To-Go You-Prime First-Strand reverse transcriptase kit (Amersham Biosciences), and primed by the random hexamer primer pd(N)₆. For each RNA sample a control reaction without reverse transcriptase was performed to monitor for DNA contamination. PCRs for quantitative analysis comprised 5 μ l RT² Real-Time SYBR Green/Rox PCR master mix (SuperArray), 10 pmol of the primer pair 0169-F/0169-R (Table 1) and 1 μ l cDNA template.

The cDNA template quantity was used for normalization of samples, with a previously described technique (Libus & Storchova, 2006). Briefly, an aliquot of each RNA sample was combined with RNA hydrolysis solution (1 mM EDTA, 100 mM NaOH) and heated for 20 min at 70 °C before and after reverse transcription. The samples were then neutralized by addition of 6 μ l 0.5 M Tris/HCl, pH 6.4, and then mixed with 200-fold diluted RiboGreen (Invitrogen) for fluorescence measurement at 485/520 nm in a FLUOstar microplate reader (BMG Technologies). The correction factor used for normalization was the calculated difference in sample fluorescence before and after reverse transcription. Following quantitative PCR, the transcript abundance value was divided by the correction factor of the corresponding sample. Transcript abundance was quantified based on a standard curve created by serially diluting a PCR amplification product of the gene and using it as template for the real-time reaction.

Biofilm formation assay. The method used for evaluation of *S. sanguinis* biofilm formation was modified from previously described protocols (Froeliger & Fives-Taylor, 2001; O'Toole & Kolter, 1998). Briefly, early-stationary-phase *S. sanguinis* strains were diluted 100-fold in chemically defined biofilm medium (BM) (Loo *et al.*, 2000) containing 1% (w/v) glucose or 1% (w/v) sucrose. Aliquots of 0.1 ml were transferred to the wells of a 96-well polystyrene microplate (Grenier) for incubation at 37 °C for 20 h under 6% O₂. Wells with media alone served as negative controls. For biofilm quantification, media and planktonic cells were removed from the wells by decanting and washing twice with deionized water (dH₂O). Wells were dried and stained with 1% (w/v) crystal violet (CV) for 15 min. Excess dye was removed with three dH₂O washes, and CV staining assessed via release of biofilm-associated dye into 30% (v/v) glacial acetic acid. The OD₆₀₀ of soluble CV was recorded. Statistical significance was determined by one-way ANOVA with a Tukey–Kramer multiple comparisons post-test, with significance defined as $P < 0.05$.

Competitive index studies in the rabbit model of IE. The rabbit model of IE was performed as previously described (Paik *et al.*, 2005). The protocol received Institutional Animal Care and Use Committee approval and complied with all applicable federal guidelines and institutional policies. A catheter was inserted through the internal

carotid artery of anaesthetized male, New Zealand White rabbits (weighing 3–3.5 kg) past the aortic valve to inflict minor valve damage. The catheter was sutured to remain in place for the duration of the experiment. The strains compared were co-inoculated (500 μ l of cells at an OD₆₀₀ of 0.8, corresponding to $\sim 1 \times 10^8$ c.f.u.) via an ear vein at 2 days post-catheterization. Bacterial amounts administered and recovered from rabbits were determined by c.f.u. enumeration. Ratios of JFP36, JFP56 or JFP76 to SK36 were determined following surface plating. Comparisons of JFP87 or JFP88 to JFP36 required layer plating (Paik *et al.*, 2005). Strain ratios in the inoculum were determined by selective plating in the presence or absence of selective antibiotics, as appropriate. Twenty hours post-inoculation, rabbits were sacrificed by injection of Euthasol (Virbac AH). The heart was removed and aortic valve vegetations excised and homogenized for selective plating with appropriate antibiotics. Plates containing the inoculum or vegetation homogenates were grown for 2 days at 37 °C under 6% O₂. Colonies were enumerated for determination of the competitive index (CI) value, which is defined as the mutant/wild-type ratio of the homogenate divided by the mutant/wild-type ratio in the inoculum. Since colony numbers are often highly skewed, log-transformed values were used to compute the CI value. Significance was determined by a paired *t*-test of log-transformed CI values, with $P < 0.05$ indicating significance.

RESULTS

Selection of an ectopic expression site for *S. sanguinis* SK36

We intended to develop a system for integration of recombinant genes into the chromosome of *S. sanguinis* SK36 without affecting phenotypes characteristic of this species. The SK36 genome is compact, with an average intergenic region of 115 bp, vs 130–177 bp for other streptococcal species with published genomes (Xu *et al.*, 2007). The proximity of ORFs meant that optimal candidate sites for intergenic insertion were fewer; however, we chose to target non-coding regions to minimize the effect of gene insertion on resident genes or operons. During initial annotation of the SK36 genome, no ORF was predicted for the 644 bp region between two hypothetical protein genes, SSA_0168 (0168) and SSA_0170 (0170). We therefore chose to utilize this region for integration of foreign genes. A 2.225 kb chromosomal fragment (Fig. 1) was cloned into the suicide vector pVA2606 to create pJFP34. A unique *Nco*I site within a predicted non-coding region of the insert was employed for cloning of *erm*, an Em resistance cassette expressed from a synthetic promoter. The promoter of *erm* is expressed in both streptococci and *E. coli*, and is a hybrid construct that combines the –35 region from the *S. pneumoniae* native *ami* operon promoter, pA, with the –10 region and 16 bp preceding *erm* on pAM β 1 (Claverys *et al.*, 1995). The resulting plasmid, pJFP36, was transformed into SK36 to generate JFP36. After creation of strain JFP36, a subsequent GLIMMER (Delcher *et al.*, 1999) analysis predicted the SSA_0169 (0169) ORF, bisected by the *Nco*I restriction site. This ORF encodes a 65 aa hypothetical protein that lacks homology to any proteins of known function and is 303 bp downstream from the 0168 gene. If 0168 and 0169 were

co-transcribed, then *0169* would be the last gene of the operon. *0169* and the gene downstream from it (*0170*) are separated by a predicted transcriptional terminator and are oppositely oriented. Therefore, it is unlikely that disruption of *0169* affects *0170* transcription.

As *0169* was predicted to encode a small, non-conserved hypothetical protein, we assessed whether this site was transcribed by RT-PCR. We determined that transcription of *0169* occurs, and varies depending on the oxygen tension when *S. sanguinis* SK36 is grown aerobically, anaerobically or under 6% O₂. The least transcript was detected in cells cultured under reduced oxygen conditions (6% O₂ and anaerobiosis) that are standard in laboratory culture of this facultative anaerobe. Transcription of *0169* increased 80-fold in *S. sanguinis* SK36 cultured aerobically (data not shown). An *in vitro* growth comparison of *S. sanguinis* SK36 and JFP36 was performed to determine whether interruption of *0169* by *erm* affected aerobic growth. As shown in Fig. 3, JFP36 and SK36 exhibited identical growth curves, suggesting that mutation of *0169* did not influence growth of JFP36.

Characterization of JFP36 biofilm formation

S. sanguinis gene products contributing to biofilm formation have been identified by *in vitro* assays under both static and dynamic conditions (Black *et al.*, 2004; Ge *et al.*, 2008). The sessile mode of growth is particularly relevant to *S. sanguinis* in complex dental plaque; yet evidence suggests that biofilm-related characteristics may contribute to IE. Previous studies have demonstrated that exopolysaccharide production by *S. sanguinis* enhanced adherence to valve tissues, and that streptococci engulfed within the vegetation adopt biofilm-related properties of

Fig. 3. Growth comparison of *S. sanguinis* strains. Aerobic growth of SK36 (○), JFP36 (□), JFP56 (▼) and JFP76 (◆) at 37 °C was measured in a microplate growth assay in triplicate; the mean and standard deviation are shown at each time point. Growth every hour up to stationary phase is shown to emphasize similarities between strains during exponential growth.

increased antibiotic resistance (Cremieux *et al.*, 1989; Durack, 1975; Ramirez-Ronda, 1978). A previously described static biofilm assay was used to investigate the impact of *erm* insertion in JFP36 on biofilm development (Froeliger & Fives-Taylor, 2001). *S. sanguinis* strains SK36 and its isogenic *srpA* mutant, VT1614 (Plummer *et al.*, 2005), served as reference controls. The *Streptococcus parasanguinis* *SrpA* homologue Fap1 was previously demonstrated to be required for biofilm formation in the presence of glucose (Froeliger & Fives-Taylor, 2001). JFP36 biofilm formation was indistinguishable from that of SK36 (Fig. 4) in the presence of glucose and sucrose, and for both JFP36 and SK36, biofilm formation in 1% glucose was significantly greater than that of VT1614 ($P < 0.001$). Therefore, mutation of *0169* did not alter biofilm development under the conditions tested.

Genetic competence of JFP36

The capacity for genetic manipulation is invaluable for studying microbial pathogenesis. *S. sanguinis* is naturally competent and readily transformable when provided with extracellular DNA in the presence of competence-stimulating peptide and animal serum. A potential future application of JFP36 is for in-frame deletion mutagenesis of genes of interest. Transformation frequency and efficiency were calculated for JFP36 to determine whether this application is feasible. The plasmid used as transforming DNA, pJFP16, gives reproducibly high transformation frequencies and efficiencies, and integrates the *cat* gene into the *nrdD* chromosomal locus in single copy. As shown in Table 2, the transformation efficiency and frequency of JFP36 were comparable to that of SK36, thus confirming that genetic competence was retained.

Fig. 4. Biofilm formation is independent of *0169* expression. Stationary-phase *S. sanguinis* cells were subcultured in BM containing 1% glucose or 1% sucrose. Biofilm formation following overnight growth at 37 °C and 6% O₂ was measured by CV staining. Assays were performed in triplicate; mean values and standard deviations are shown. Asterisks indicate a significant difference ($P < 0.001$).

Table 2. Genetic competence is retained in JFP36

<i>S. sanguinis</i> strain	Transformation frequency (%) ^{*†}	Transformation efficiency [c.f.u. (μg DNA) ⁻¹] ^{*‡}
SK36	27.38	8.6×10^7
JFP36	27.13	8.25×10^7

^{*}Values computed from duplicate experiments.

[†]Ratio of transformants to total c.f.u.

[‡]Number of transformants per μg pJFP16.

Analysis of JFP36 competitiveness *in vivo*

S. sanguinis JFP36 was next tested for competitiveness in the rabbit model of IE by the CI assay. Early-stationary-phase SK36 and JFP36 were co-inoculated into catheterized rabbits and infection was monitored 20 h post-inoculation. The CI value was determined by dividing the ratio of JFP36 to SK36 recovered from the rabbit by the ratio of JFP36 to SK36 in the inoculum. *S. sanguinis* SK36 lacks a selective marker; therefore, plating in the presence and absence of Em was used to enumerate JFP36 c.f.u. and total c.f.u., respectively. SK36 c.f.u. were derived by determining the difference between total c.f.u. and JFP36 c.f.u. A mean CI value of 1.2 was obtained from four rabbits (Table 3). Relative to an equal competitiveness value of 1, this CI value confirms that JFP36 is equally competitive to SK36 ($P=0.52$). Therefore manipulation of the *0169* locus did not alter competitiveness.

Development and characterization of JFP56 and JFP76

We chose to adopt the *0169* locus for expression of additional resistance markers, *aad9* and *tetM*, to increase the applicability of this tool. An oligonucleotide linker was inserted at the *NcoI* site of pJFP34 to create an abbreviated multiple cloning site including *AscI*, *NotI* and *NcoI* (Fig. 1). The *aad9* and *tetM* markers were integrated at the *NotI* and

NcoI restriction sites to create plasmids pJFP56 and pJFP76, respectively.

Expression of *aad9* is controlled by a synthetic promoter that includes the 5' region of the native *ami* operon pneumococcal promoter, pA, fused to the 3' region of the *aad9* promoter to permit optimal expression of Sc resistance in *S. pneumoniae* (Dintilhac *et al.*, 1997) (similar to the promoter of *erm*, described above). The pJFP56 insert maintained the synthetic *aad9* promoter adjacent to the *aad9* gene. SK36 was transformed with pJFP56 to create the Sc-resistant strain, JFP56.

The *tetM* gene from pJM133 was utilized for development of the Tet-resistant SK36 derivative (Hancock & Perego, 2004). *tetM* originates from the Tn916 composite conjugative transposon of *Ent. faecalis* (Senghas *et al.*, 1988). In pJM133, *tetM* transcription is controlled by a native *aad9* promoter of the *Ent. faecalis* plasmid pD255 (Podbielski *et al.*, 1996). The promoter controlling expression of *tetM* is constitutively expressed in both *E. coli* and streptococci and therefore ideal for our purposes. SK36 was transformed with the *tetM*-containing plasmid, pJFP76, for isolation of the Tet-resistant strain JFP76. DNA sequencing of *tetM* in the *0169* locus of JFP76 indicated that it shared greater identity with *tetM* of the *S. pneumoniae* Tn1545 determinant (GI:48189) than with the *tetM* gene of Tn916. The mosaic nature of *tetM* has been previously investigated. Allelic variation observed in nature was attributed to homologous recombination between two distinct *tetM* alleles, one chromosomally encoded by *Staphylococcus aureus*, and Tn1545 of *S. pneumoniae*. The evolution of mosaic *tetM* alleles would have been facilitated by the conveyance of these determinants on mobile conjugative transposon elements (Oggioni *et al.*, 1996).

An *in vitro* growth comparison of JFP56 and JFP76 via the microplate growth assay verified that insertion of the different resistance determinants at the *0169* locus did not affect the growth phenotypes of these strains, in that growth patterns observed were identical to SK36 (Fig. 3).

In vivo competitiveness of JFP56 and JFP76 in the rabbit model of IE was determined by CI comparison to the parental strain, SK36, as described above for JFP36. JFP56 was equally competitive to SK36, with a mean CI value of 1.1 ($P=0.6$) (Table 3). JFP76 appeared significantly more competitive than SK36, with a mean CI value of 2.1 ($P=0.003$) (Table 3). This was a surprising result, and we wondered whether it might be due to differences in efficiency of plating for JFP76 on TSA+Tet in the inoculum compared to the recovery pools. Colonies from the inoculum and recovery pools collected from TSA agar were patched onto TSA±Tet. The CI value for JFP76 calculated by this method was 0.91 (Table 3). This suggests that variations in plating efficiency for JFP76 were indeed responsible for the apparent increased CI value obtained for this strain. This result also suggests that JFP76 would not be an ideal competitor strain for measuring small

Table 3. Competitiveness of SK36 derivatives *in vivo*

Mutant	Competitor [*]	No. [†]	CI [‡]
JFP36	SK36	4	1.2 (0.42–1.7)
JFP56	SK36	3	1.1 (0.78–1.6)
JFP76	SK36	3	2.1 (1.9–2.2); 0.91§
JFP88	JFP36	4	5.7×10^{-5} (2.1×10^{-3} – 2.0×10^{-6})
JFP87	JFP36	3	0.49 (0.30–0.65)

^{*}Reference strain of wild-type competitiveness.

[†]Number of rabbits tested.

[‡]Mean CI values, with range in parentheses. Bold CI values are significantly different from 1 ($P<0.05$).

§CI value determined for JFP76 following patching of colonies from TSA plates onto TSA±Tet.

changes in virulence. It is worth noting, however, that this effect would likely be less if the strain to which it was being compared were resistant to another antibiotic. When comparing an antibiotic-resistant strain (such as JFP76) to SK36, the number of SK36 colonies on plates with no antibiotics must be calculated by subtracting the expected number of colonies of the competitor strain from the total number of colonies observed. Thus, an underestimate of the true number of competitor colonies also results in an overestimate of the number of SK36 colonies, magnifying the error in calculating their ratio. The error is similarly magnified if the number of competitor colonies is overestimated. This potential error can only be avoided by the cumbersome task of patching colonies recovered from antibiotic-free plates onto plates $\pm$ antibiotic. This highlights the utility of creating antibiotic-resistant virulent competitor strains such as JFP36 and JFP56 for virulence assays, since the amount of the competitor strain in a mixed-strain pool can be directly enumerated.

Adoption of 0169 for avirulent mutant complementation

To date, the most attenuated *in vivo* phenotype has been observed for an SK36 *ssaB* (*SSA_0260*) transposon-insertion mutant, which exhibited a $\sim 10\,000$ -fold reduction in endocarditis virulence when compared to JFP36 by the CI assay (Das *et al.*, 2009). To determine whether 0169 could be employed for ectopic expression of a complementing gene, the predicted promoter region of the *ssaACB* operon was fused to *ssaB*. The entire intergenic region separating *ssaA* and *pepO*, the upstream ORF in the opposite orientation, was joined with the first 640 bp of *ssaB* by gene splicing by overlap extension (Horton, 1995). Cloning of this amplicon replaced a partial *ssaACB* promoter fragment in a previously constructed plasmid, derived from pJFP46 (Das *et al.*, 2009). The *ssaB* mutant was transformed with the resulting construct, pJFP87, to create JFP87. A Sc-resistant, *ssaB*⁻ strain, JFP88, was created by transformation of the *ssaB* mutant with pJFP56.

Competitiveness of the two strains was then assessed relative to the Em-resistant control JFP36, by the CI assay. Since both mutant strains were Sc-resistant, the same antibiotics (Sc and Em) were used for strain selection in both experiments. The *ssaB* mutant JFP88 exhibited a mean CI value of 5.7×10^{-5} (Table 3). Ectopic expression of *ssaB* in the 0169 locus in JFP87 restored the CI value to 0.49. Although this value is less than 1, the difference was not significant ($P=0.099$) and represents an 8500-fold restoration of competitiveness compared to the *ssaB* mutant JFP88 (Table 3). As expected, the difference in competitiveness of JFP87 and JFP88 was significant ($P=0.0093$; unpaired *t*-test with Welch correction for unequal variances). Immunoblot analysis indicated that SsaB expression was not fully restored in JFP87 (data not shown), perhaps due to misidentification of the exact boundaries of the suspected promoter or ribosome-binding

site, or to other aspects of *ssaB* regulation that are not understood. It is also worth noting that overexpression of SsaB from a plasmid in the *ssaB* mutant background in another study resulted in SsaB expression equal to or greater than that of the wild-type strain, yet the CI value for the complemented mutant was also less than 1 (CI=0.29; Das *et al.*, 2009). Therefore, expression of *ssaB* from the ectopic chromosomal locus in JFP87 was as effective as, or more effective than a plasmid construct in restoring virulence, and was subject to none of the problems associated with plasmid use, including the need for maintaining antibiotic selection and the possibility for recombination between the complementing gene on the multi-copy plasmid and the mutant gene on the chromosome.

Concluding remarks

The publication of the *S. sanguinis* SK36 genome sequence in 2007 opened the possibility for greater characterization of this species, with this strain serving as the model. Of particular interest is identification of *S. sanguinis* virulence determinants that may serve as targets for prophylaxis of IE. In keeping with this goal we have identified a locus that may be used for genetic manipulation, including complementation of a mutation elsewhere on the chromosome, without directly affecting important cellular phenotypes including *in vitro* growth, genetic competence, biofilm formation, and competitiveness in the rabbit model of IE. While interruption of 0169 had no effect on any *S. sanguinis* phenotypes investigated here, it would be prudent to verify that any other phenotypes investigated with these strains are likewise unaffected. Preliminary PCR screening for the 0169 locus in other *S. sanguinis* strains revealed that the site is not conserved in ATCC 10556, yet is present in an endocarditis isolate (unpublished data). Incorporation of *erm* by transformation of the clinical isolate with pJFP36 was possible, suggesting this locus can be adopted for ectopic gene expression in *S. sanguinis* strains other than SK36. In *S. sanguinis* strains lacking the 0169 site, the resistance cassettes utilized here may be inserted at a different chromosomal site, as we have demonstrated that these markers are expressed in *S. sanguinis*, as in *S. pneumoniae* (Claverys *et al.*, 1995; Martin *et al.*, 2000) and *Ent. faecalis* (Hancock & Perego, 2004). The overall prevalence of the target site among *S. sanguinis* isolates remains to be determined; nevertheless, the genetic tools described here will facilitate phenotypic analysis of strain SK36 derivatives and possibly other *S. sanguinis* strains as well.

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