Shuttle expression plasmids for genetic studies in *Streptococcus mutans*

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A set of shuttle plasmids containing four different constitutive promoters was generated to facilitate overexpression of foreign and native genes in streptococci, such as *Streptococcus mutans*. The four promoters that were chosen were: P_{amin} , P_{spac} , P_{23} and P_{veg} . These promoters are active in many Gram-positive bacteria, and allow various levels of gene expression depending on the host bacterium. Shuttle plasmids were constructed based on two types of broad-host-range replication origins: a rolling-circle replicon (pSH71) and a theta replicon (pAM β 1). Shuttle plasmids derived from the pAM β 1 replicon were generated to avoid the structural and segregational stability problems associated with rolling-circle replication, since these problems may be encountered during large gene cloning. In a complementation assay, we used one such plasmid to express a gene *in trans* to show the utility of these plasmids. In addition, a series of plasmids was generated for the expression of recombinant proteins with an N-terminal 6 $\times$ His tag or a C-terminal Strep-tag fusion, and, using a gene derived from *S. mutans*, we showed a high level of recombinant protein expression in *S. mutans* and *Streptococcus pyogenes*. Since these plasmids contain broad-host-range replication origins, and because the selected promoters are functional in many bacteria, they can be used for gene expression studies, such as complementation and recombinant protein expression.

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INTRODUCTION

The genus *Streptococcus* constitutes a wide variety of bacteria ranging from beneficial organisms, such as *Streptococcus thermophilus*, to pathogenic organisms such as *Streptococcus mutans*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* (Facklam, 2002). Like many other bacteria, streptococci have developed complex regulatory networks of gene expression that allow them to survive under diverse environments. To understand how the regulatory networks function, and to study interconnection among the various networks, one needs to express the regulatory genes under heterologous promoters that are separated from the regulatory controls of the genes. For expression of genes at different levels, foreign promoters with varying strength are normally employed. While the availability of foreign promoters for gene expression is not an issue for Gram-negative bacteria, such as *Escherichia coli*, or for the well-studied Gram-positive bacterium *Bacillus subtilis*, for many other Gram-positive bacteria, including streptococci, the availability of suitable promoters for gene expression remains a challenge.

In many instances, promoters isolated from one Gram-positive organism have been shown to work efficiently in

other Gram-positive organisms. For example, the *veg* promoter (P_{veg}) of *B. subtilis* is also functional in *S. pyogenes* (Opdyke *et al.*, 2003). Similarly, lactococcal promoters P_{23} and P_{59} are functional in many Gram-positive bacteria, including *Staphylococcus aureus* (Que *et al.*, 2000). In addition, there are a few inducible heterologous promoters, such as those derived from nisin or tetracycline genes (Apfel *et al.*, 2001; Eichenbaum *et al.*, 1998), that have been shown to work in *S. pneumoniae* or *S. pyogenes*; however, these promoters have high basal levels of expression, and a very low titrable range, and this makes them incompatible for regulated gene expression studies.

In the case of *S. mutans*, which is the principal aetiological agent of human dental caries (Loesche, 1986), gene expression studies remain very difficult because of the limited availability of heterologous promoters. Moreover, there has not been any comparative study regarding gene expression under different promoters in this pathogen. Here, we constructed a series of shuttle plasmids that contain four different promoters derived from *B. subtilis* (P_{veg} and P_{spac}), *Lactococcus lactis* (P_{23}) and *S. pneumoniae* (P_{ami}). Using a *gusA* reporter gene, we showed that each of the four promoters is active in *S. mutans*, as well as in *S. pyogenes*. Furthermore, using these promoters, we also constructed vectors that allow the expression of a gene of interest as N- or C-terminal-tagged fusion proteins.

Abbreviations: CSP, competence-stimulating peptide; GUS, β -glucuronidase; MCS, multiple-cloning site; MU, Miller units; RCR, rolling-circle replicon.

METHODS

Bacterial strains, growth conditions and genetic transformation. *E. coli* strains DH5 α and NEB5 α were grown in Luria–Bertani medium supplemented, when necessary, with ampicillin (100 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹), erythromycin (300 μ g ml⁻¹) or chloramphenicol (20 μ g ml⁻¹). *S. mutans* UA159 and *S. pyogenes* JRS4 were routinely grown in Todd Hewitt medium (BBL; Becton Dickinson) supplemented with 0.2% yeast extract (THY). When necessary, kanamycin (300 μ g ml⁻¹), chloramphenicol (20 μ g ml⁻¹) or erythromycin (5–10 μ g ml⁻¹) was included in the growth medium. *S. mutans* was transformed with the addition of competence-stimulating peptide (CSP), as described previously (Biswas *et al.*, 2007b). *S. pyogenes* cultures for transformation were grown overnight in THY broth in static conditions at 37 °C. The overnight cultures were then diluted 100-fold, and grown at 37 °C until the OD₅₉₅ reached approximately 0.6. Cultures were then harvested, washed three times with ice-cold glycerol (10%, w/v), and resuspended in 10% glycerol to 1/100th of the original culture volume. A 100 μ l aliquot was electroporated with approximately 5 μ g plasmid DNA, using an Eppendorf electroporator at the 1.75 kV setting.

Construction of promoter plasmids for chromosomal integration. Plasmid pIB107 was selected to construct plasmids for chromosomal integration with a *gusA* reporter fusion (Biswas & Biswas, 2006). DNA fragments containing the promoter of interest were amplified from the appropriate plasmids containing the desired promoters, digested with *Bam*HI and *Xho*I, and cloned into *Bam*HI/*Xho*I-digested pIB107. For cloning of the *P_{ami}* promoter into pIB107, the primer pair Pami-Bam-Apa-F and Pami-Xho-R2 (for all the primers, see Table 1) was used to amplify a 55 bp fragment containing the promoter from the pAL2 plasmid (Beard *et al.*, 2002). To clone the *P_{spac}* promoter into pIB107, plasmid pEU308 (Eichenbaum *et al.*, 1998) was used as a template to amplify a 202 bp fragment with Pspac-Bam-Apa-F and Pspac-Xho-R2 primers. A 180 bp fragment containing the *P₂₃* promoter was amplified from the

pOri23 plasmid (Que *et al.*, 2000) using P23-Bam-Apa-F and P23-Xho-R2 primers, for cloning into pIB107. Finally, the *P_{veg}* promoter region (180 bp) was amplified from pJRS1315 plasmid (Opdyke *et al.*, 2003) using primers Pveg-F1 and Xho-Pveg-R2, and cloned into pIB107. The resultant plasmids were linearized with *Bgl*I, and transferred to UA159 by transformation, as described previously (Biswas & Biswas, 2006). The presence of the promoters in the chromosome of UA159 was verified by PCR amplification and DNA sequencing.

Construction of shuttle plasmids with heterologous promoters.

To construct shuttle plasmids containing the various promoters under study, plasmid pJRS1315 was chosen. This plasmid is a derivative of pLZ12 (Perez-Casal *et al.*, 1991), and it replicates via rolling-circle mechanism using the pSH71 replicon, which is active in both Gram-negative and Gram-positive bacteria (de Vos, 1987). pJRS1315 also contains a chloramphenicol-resistance gene, which is active in both Gram-positive and Gram-negative bacteria, and the *P_{veg}* promoter from *B. subtilis* (Moran *et al.*, 1982). To facilitate promoter cloning, an intermediate plasmid, pIB144, was generated by cloning a *Bam*HI/*Hind*III-digested fragment amplified from pIB81, which is a derivative of pASK43 (IBA) carrying the *S. mutans covR* gene, into *Bam*HI/*Hind*III-digested pJRS1315 (Biswas *et al.*, 2007a). Thus, plasmid pIB144 contains the *P_{veg}* promoter with extra restriction sites. Different promoter fragments, along with two small fragments carrying multiple-cloning sites (MCS), were cloned into pIB144 in two steps. First, the promoters of interest were amplified using the following primer pairs: *P_{ami}*, Pami-Bam-Apa-F and Pami-Bam-R1; *P_{spac}*, Pspac-Bam-Apa-F and Pspac-Bam-R1; and *P₂₃*, P23-Bam-Apa-F and P23-Bam-R1. Amplified PCR fragments were digested with *Apa*I and *Bam*HI, and cloned into *Apa*I/*Bam*HI-digested pIB144, so that the resident *P_{veg}* promoter was replaced with the respective promoter of interest. For the *P_{veg}* promoter, pIB144 was used directly for the second step, which involved cloning of DNA fragments containing two types of MCS into the intermediate plasmids. An MCS

Table 1. Oligonucleotides and templates for PCR amplification of promoter regions

Promoter	Template DNA	Primer	Sequence (5'→3')*
<i>P₂₃</i>	pOri23	P23-Bam-Apa-F P23-Bam-R1 P23-Xho-R2	ACACAGGATCCGGGCCCGAAAAGCCCTGACAACC ACACAGGATCCAACATCATTGTGCATTCAT ACACACTCGAGAACATCATTGTGCATTCAT
<i>P_{ami}</i>	pAL2	Pami-Bam-Apa-F Pami-Bam-R1 Pami-Xho-R2	ACACAGGATCCGGGCCCGAAAATTTGTTTGCTTTT ACACAGGATCCTAGATTGACAACCATTATA ACACACTCGAGTAGATTGACAACCATTATA
<i>P_{spac}</i>	pEU308	Pspac-Bam-Apa-F Pspac-Bam-R1 Pspac-Xho-R2	ACACAGGATCCTGGGCCCTGCAGATGAATTAATT ACACAGGATCCAATTGTTATCCGCTCACAAAT ACACACTCGAGAATTGTTATCCGCTCACAAAT
<i>P_{veg}</i>	pJRS1315	Pveg-F1 Xho-Pveg-R2	ACACAGGATCCGGGCCCTGCAGAGGAGAGCTATGCGGG ACACACTCGAGATTTATTGTACAACACGAGCCCAT
MCS-A	–	MCS-F MCS-R	CGATCCCCGCGGTACCCGGGAATTCTAGAGCTCGAGATCTATCGATA AGCTTATCGATAGATCTCGAGCTCTAGAATTCGCCGGTACCCGGGG
MCS-B	pASK-IBA-43 +	Bgl-pASK43-F2 pASK-IBA-R	ACACAAGATCTCTTTAAGAAGGAGGATATACAAATGGCTAGCAGAGGATCGC CGCAGTAGCGGTAAACG
Others†	pIB175-pIB178 UA159	pJRS-F pJRS-R Eco-Smu487-F1 Bam-Smu487-R2	TAAGGCTATTGGTGTATTATGGC GTACAATTGTCATCACCATTCTT GCGGAATTCATGCTGATGAGTAAAAACAAAGTTATACTGG CGCGGATCCAACCTCTGTTGGTTTTGAAACAACAGC

*Bold letters indicate incorporated restriction sites.

†Primers used in the construction of various plasmids.

linker fragment was generated by annealing two primers, MCS-F and MCS-R, followed by cloning of the linker into *Bam*HI/*Hind*III-digested intermediate plasmids (or pIB144) to create pIB164 (*P_{ami}*), pIB166 (*P₂₃*), pIB168 (*P_{spac}*) and pIB170 (*P_{veg}*), all of which contain the MCS to facilitate cloning of foreign genes for expression under the promoters of choice. Another MCS was also amplified from pASK43, this time using *Bgl*II-pASK43-F2 and pASK-IBA-R primers and digestion with *Bgl*II and *Hind*III. It was then cloned into *Bam*HI/*Hind*III-restricted intermediate plasmids (or pIB144) to generate plasmids pIB163 (*P_{ami}*), pIB167 (*P_{spac}*), pIB165 (*P₂₃*) and pIB169 (*P_{veg}*). This MCS allows cloning of genes for expression under different promoters as His-tagged proteins.

Various promoter-containing shuttle plasmids were also generated based on a theta-replicating plasmid pOri23 (Que *et al.*, 2000). Plasmid pOri23 replicates in *E. coli* and Gram-positive bacteria, and contains an *ermAM* gene that confers erythromycin resistance in both *E. coli* and Gram-positive bacteria (see Fig. 2a). To clone heterologous promoters, primers pJRS-F and pJRS-R were used to amplify the *P₂₃* promoter using pIB166 as a template, or to amplify the *P_{veg}* promoter using pIB170 as a template. PCR fragments were then cloned into *Eco*RI/*Sal*I-digested and T4-blunted pOri23 to construct plasmids pIB184 (*P₂₃*) and pIB185 (*P_{veg}*). The correct orientation of the promoter fragments was verified by PCR analyses, followed by DNA sequencing. A fragment containing the ORF of the *covR* gene, which encodes a response regulator (Ajdic *et al.*, 2002; Biswas & Biswas, 2006), was amplified from UA159, digested with *Bam*HI and *Eco*RI, and cloned into *Eco*RI- and *Bam*HI-digested pIB184 to create pIB609 (I. Biswas, unpublished), which was used for the complementation study.

For promoter expression studies, a *gusA* reporter gene was isolated from pJRS462 by *Sma*I and *Sac*I digestion, and it was cloned into *Sma*I/*Sac*I-digested pIB164, pIB166, pIB168 and pIB170 to generate pIB175 (*P_{ami}-gusA*), pIB176 (*P₂₃-gusA*), pIB177 (*P_{spac}-gusA*) and pIB178 (*P_{veg}-gusA*), respectively.

To generate promoter fusions in the theta replicon, plasmid pIB185 was used. Promoter sequences fused to the *gusA* gene were amplified directly using pJRS-F and pJRS-R primers from the plasmids pIB175, pIB176, pIB177 and pIB178, and they were cloned into *Apa*I/*Bgl*II-digested pIB185 to generate pIB188 (*P_{ami}-gusA*), pIB187 (*P₂₃-gusA*), pIB189 (*P_{spac}-gusA*) and pIB186 (*P_{veg}-gusA*), respectively.

A fragment containing the *smu487* gene, which encodes a response regulator (Ajdic *et al.*, 2002; Biswas *et al.*, 2008), was amplified from UA159 using primers *Eco*-*Smu487*-F1 and *Bam*-*Smu487*-R2. This fragment was restricted with *Eco*RI and *Hind*III, and cloned into *Eco*RI/*Hind*III-digested pIB169 to generate pIB181.

Assay for β -glucuronidase. The β -glucuronidase (Gus) assay was modified from a published method (Cutting & Vander-Horn, 1990). Bacterial cultures were grown in THY overnight at 37 °C with the appropriate antibiotics. Cultures were diluted 1 in 20, and grown at 37 °C until the OD₅₉₅ reached approximately 0.6. A 1 ml volume of culture was harvested, washed in saline, and stored at -20 °C until all samples were ready to be processed. The pellet was then thawed, resuspended in Z-buffer (Cutting & Vander-Horn, 1990) containing lysozyme (0.5 mg ml⁻¹ final concn), and incubated at 37 °C for 10 min. Triton X-100 was then added to a final concentration of 0.01 %, and the suspension was incubated on ice for 5 min. The enzymic reaction was initiated at 30 °C by addition of *p*-nitrophenyl- β -D-glucoside (Sigma) to a final concentration of 0.8 mg ml⁻¹; as soon as a yellow colour developed in the reaction mixture, the reaction was stopped by the addition of Na₂CO₃ to a final concentration of 0.25 M. The absorbance at 420 nm, and the time of Na₂CO₃ addition, were noted. Gus activity was expressed as [1000 $\times$ A₄₂₀]/[time (min) $\times$ cell OD₅₉₅] in Miller units (MU).

RESULTS

Measuring the relative strength of the heterologous promoters

We selected four different promoters, *P_{ami}*, *P_{spac}*, *P_{veg}* and *P₂₃*, for constructing shuttle plasmids suitable for gene expression. These promoters were chosen because they are highly expressed, and functional in various heterologous hosts (Barnett *et al.*, 2007; Eichenbaum *et al.*, 1998; Halfmann *et al.*, 2007; Opdyke *et al.*, 2003; Que *et al.*, 2000; Takahashi *et al.*, 2002). To evaluate the relative strength of these promoters, we made use of a previously described transcriptional reporter plasmid, pIB107, that allows for the fusion of promoters of interest to a promoterless *gusA* reporter gene (Biswas & Biswas, 2006). This plasmid also allows for insertion of the promoter-*gusA* reporter construct into the chromosome of *S. mutans* at the *smu1405* locus for single-copy expression. Expression of *gusA* was quantified from these promoters by measuring Gus activity. For comparison purposes, we also included two well-studied native promoters from *S. mutans*: *P_{gbpC}* and *P_{gtfB}* (Biswas *et al.*, 2007a; Biswas & Biswas, 2006). These promoter-*gusA* fusions were generated in the same way as the heterologous promoter fusions. As shown in Fig. 1, we found that each of the four promoters was functional in *S. mutans*, and expressed the *gusA* gene with a wide range of activity. The lowest activity, which was observed with the *P_{spac}* promoter (20 MU ml⁻¹), was comparable with the strength of the native promoter isolated from *gbpC* (Fig. 1). Since the expression of *gbpC* is moderately high, this suggests that the expression from the *P_{spac}* promoter is adequate for gene expression studies. On the other hand, the highest activity was observed with the *P_{veg}* promoter (104 MU ml⁻¹), and this was comparable with the expression from the *P_{gtfB}* promoter, which is highly expressed in *S. mutans* (Biswas & Biswas, 2006; Goodman & Gao, 2000; Li & Burne, 2001) (Fig. 1). Thus, our results showed that all four heterologous promoters were functional and highly active, making them suitable for gene-expression studies in *S. mutans*.

Construction of rolling-circle-replicon-based shuttle plasmids containing different promoters

Some rolling-circle replicon (RCR) plasmids have a wide host range, and are known to replicate in both Gram-negative and Gram-positive bacteria (Gruss & Ehrlich, 1989). These are generally high-copy-number plasmids that are small in size, making them suitable for cloning purposes. To construct the shuttle plasmids, we chose an RCR-based vector, pJRS1315, which contains the pSH71 replicon, and which replicates well in *E. coli* and many streptococci (de Vos, 1987; Opdyke *et al.*, 2003). In addition, this plasmid encodes a chloramphenicol-resistance gene that is functional in both *E. coli* and Gram-positive bacteria (Fig. 2a). We cloned the promoters of interest in pJRS1315 by replacing the resident promoter

Fig. 1. Activity of various promoters in *S. mutans*. Promoter sequences were fused to a promoterless *gusA* reporter gene, and inserted into the *S. mutans* UA159 chromosome. Expression driven from the promoters was evaluated by measuring Gus activity at mid-exponential growth phase. The relevant regions of the promoters under investigation are shown. The -35 and -10 motifs, as well as the start site of transcription, are in bold. Underlined TG sequences indicate extended -10 regions. The Gus values are means ($\pm$ sd) for experiments repeated at least twice. Background values for the control strain containing the *gusA* reporter gene without a promoter were less than 0.5 MU ml⁻¹.

Promoter	-35	-10	Activity [Gus values (MU ml ⁻¹)]
P _{ami}	TTGATT ttttaatggataatgT <u>Ga</u> TATAAT ggttg G		72.5 $\pm$ 0.1
P _{spac}	TTGACT ttatctacaagggtgT <u>Gg</u> CATAAT gtgtgg A		20.5 $\pm$ 0.1
P ₂₃	ATGACA aaaagagaaaatttT <u>Ga</u> TAAAA tagtctt A		53.7 $\pm$ 1.7
P _{veg}	TTGACA aaaatgggctcgtgttg TACAAT aaatgt A		104.8 $\pm$ 0.9
P _{gfpC}	TTTGAA aaaatgtcttttttatT <u>Gt</u> TATAAT taaaatt G		23.1 $\pm$ 1.5
P _{gltB}	TTGTTT tattattagaagg TGTTACA att A		108.7 $\pm$ 6.1

and an unrelated gene (*sigX*) to generate intermediate plasmids. To facilitate the cloning of target genes in these intermediate plasmids, we introduced DNA fragments containing two distinct MCS. The first MCS fragment (MCS-A) allows for cloning using 11 unique restriction sites (Fig. 2c), while the other MCS fragment (MCS-B) is derived from an *E. coli* expression plasmid pASK43+ that allows for the flexible cloning of fragments of interest, and also allows the expression of genes as fusion proteins (Fig. 2c).

Plasmids containing MCS-A, along with their respective promoters, are shown in Fig. 2(b). To evaluate the efficacy of these plasmids, we expressed the *gusA* reporter gene (~2 kb) under the control of the promoters. Resultant plasmids containing the respective promoter fused to *gusA* were introduced into *S. mutans* and *S. pyogenes*, followed by measurement of Gus activity. As shown in Table 2, with the exception of P₂₃ promoter, the levels of Gus activities obtained from these promoters in *S. mutans* were lower compared with the activities obtained with single-copy expression from the integrated chromosomal location. The Gus activities were also lower in *S. pyogenes*; however, they were higher than the respective activities measured in *S. mutans*. In both *S. mutans* and *S. pyogenes*, the level of *gusA* activity from P_{spac} was lower (but above the background level) compared with the levels from the other promoters. Taken together, our results indicate that these shuttle plasmids can be used for the expression of genes in both *S. mutans* and *S. pyogenes*. However, our results also suggest that cloning of *gusA* may have caused some plasmid instability, since the overall *gusA* activity was lower than expected when expressed from the plasmid, as opposed to chromosomal single-copy expression.

The efficacy of the plasmids containing MCS-B for the expression of genes as fusion-tagged proteins was evaluated. For this, we selected Smu487, which is a response regulator from *S. mutans* (Biswas *et al.*, 2008). A DNA

fragment containing the *smu487* ORF was cloned into the plasmid pIB169, which bears the P_{veg} promoter, to create plasmid pIB181, in which Smu487 is fused with the 6 $\times$ His tag. Plasmid pIB181 was introduced into *E. coli*, *S. mutans* and *S. pyogenes*, whole-cell lysates were prepared, and expression of His-Smu487 was monitored by Western blot analysis, using anti-6 $\times$ His antibody (Sigma). As shown in Fig. 2(d), His-Smu487 was expressed well in both *S. mutans* and *S. pyogenes*. The level of expression was about twofold higher in *S. pyogenes* compared with *S. mutans*. The expression of His-Smu487 was highest in *E. coli*, and this was about 5- to 10-fold higher than in the Gram-positive counterparts. Taken together, our results suggest that the plasmid sets generated here, with various promoters fused to MCS-B, can be used for expression of fusion proteins in streptococci, with high efficiency.

Construction of theta-replicon-based shuttle plasmids containing different promoters

Since we experienced some plasmid instability when we cloned the *gusA* gene into RCR-derived plasmids, we concluded that these plasmids may not be suitable for the cloning of certain genes, such as large genes, or genes with high G + C content (Gruss & Ehrlich, 1988). To circumvent this problem, we chose to use theta-replicating plasmids, which are structurally and segregationally more stable than RCR plasmids, and can accommodate large DNA inserts (Kiewiet *et al.*, 1993). To this end, we used plasmid pOri23, which is a derivative of pIL253, a relatively high-copy-number variant of pAM β 1 (Simon & Chopin, 1988). Plasmid pOri23 also contains the *colE1* replicon of pBluescript (Stratagene) for replication in *E. coli*, since pAM β 1 cannot replicate in *E. coli*, unlike pSH71. However, pAM β 1 can replicate in a wide range of Gram-positive bacteria, including streptococci (Luchansky *et al.*, 1988; Perez-Arellano *et al.*, 2001). Various pOri23 derivatives containing different promoters were generated, as described in Methods. These plasmids also contained

Fig. 2. RCR-based shuttle vectors suitable for gene expression in streptococci. (a) Generic physical maps of the RCR- and theta-based vectors illustrating the origins of replication and selectable markers for *E. coli* and Gram-positive bacteria. The locations of the promoters (P) upstream of MCS are shown. The RCR plasmids are based on the pSH71 replicon, and the theta plasmids are based on the pAM/β1 replicon. (b) RCR-based vectors, along with the corresponding promoters and MCS. B, *Bam*HI; H, *Hind*III; B*, hybrid *Bam*HI/*Bgl*II. (c) The DNA sequences encoded in the MCS regions. The sequence between *Bam*HI and *Hind*III of MCS-A, along with the unique restriction sites available for cloning, are shown in the upper panel. The sequence between hybrid *Bam*HI/*Bgl*II and *Hind*III of the MCS-B region, along with available restriction sites, are shown in the lower panel. The ribosome-binding site (RBS), 6xHis tag and Strep-tag sequence are underlined. The underlined GGATCT sequence indicates the *Bam*HI/*Bgl*II (B*) hybrid site. (d) Western blot detection of His-Smu487 expression. Whole-cell lysates were prepared from *S. pyogenes* (GAS), *S. mutans* (Smu) and *E. coli* containing plasmids with Smu487 (pIB181) and without Smu487 (pIB180) under the control of the *P_{veg}* promoter. Approximately 50 µg whole-cell lysate was separated by using 12% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was probed with anti-6xHis antibody.

MCS-A to aid in flexible gene cloning. To evaluate whether this series of plasmids can be efficiently used for gene expression in *S. mutans* and *S. pyogenes*, the *gusA* reporter gene was cloned into these plasmids, and introduced into these bacteria to quantify relative promoter strength. As shown in Table 2, high levels of *gusA* expression were observed in *S. mutans* from all of the promoters. The highest activity was obtained with *P₂₃* promoter (653 MU ml⁻¹), while the lowest activity was obtained with the *P_{spac}* promoter (41 MU ml⁻¹). Depending on the promoter used, the levels of Gus expression were approximately 2- to 10-fold higher compared with their chromosomally integrated counterparts (Fig. 1 and Table 2). Except for the *P_{spac}* promoter, which also showed lower

activity in RCR plasmids, higher Gus expression was also observed in *S. pyogenes* from the theta-replicating plasmids containing the promoters under study.

To demonstrate that these plasmids can be used for complementation studies, we complemented a *covR*-deficient strain of *S. mutans*. *CovR* is an important response regulator that regulates many virulence-associated genes in *S. mutans* (Biswas *et al.*, 2007a; Biswas & Biswas, 2006). The *covR*-deficient strain selected for our study was IBS132, and this also contains a *P_{gbpC}*-*gusA* reporter fusion (Biswas *et al.*, 2007a). The promoter *P_{gbpC}* is negatively regulated by *CovR*; therefore, *CovR* complementation can be studied by measuring transcription from *P_{gbpC}*. To

Table 2. Gus activity values obtained from various promoter-containing RCR-based and theta-based vectors

Mean values ($\pm$ SD) are based on measurements of at least three independent cultures at mid-exponential growth phase.

Promoter	Gus activity (MU ml ⁻¹)	
	<i>S. mutans</i>	<i>S. pyogenes</i>
RCR-based plasmid		
P _{ami}	29.9 $\pm$ 1.8	150.2 $\pm$ 1.6
P _{spac}	6.3 $\pm$ 0.3	9.9 $\pm$ 0.4
P ₂₃	191.0 $\pm$ 8.0	21.5 $\pm$ 1.4
P _{veg}	8.7 $\pm$ 0.7	42.1 $\pm$ 2.6
Theta-based plasmid		
P _{ami}	128.9 $\pm$ 2.5	125.9 $\pm$ 2.8
P _{spac}	41.1 $\pm$ 6.0	2.1 $\pm$ 0.3
P ₂₃	653.3 $\pm$ 0.3	517.9 $\pm$ 24.9
P _{veg}	179.5 $\pm$ 15.5	109.7 $\pm$ 7.7

achieve complementation, *covR* was expressed from plasmid pIB609, which contains *covR* under the control of the P₂₃ promoter in plasmid pIB184.

Transcription from P_{gbpC} was measured from the wild-type (IBS131), the *covR* mutant (IBS132), and the complemented IBS132/pIB609 strain. For comparison purposes, we also included an IBS132 strain containing plasmid pIB30, which expresses *covR* under its own promoter (Biswas *et al.*, 2007a). As expected, Gus activity was fourfold higher in IBS132 (mean $\pm$ SD, 109.4 $\pm$ 1.4 MU ml⁻¹), as compared with the wild-type IBS131 (27.1 $\pm$ 1.2 MU ml⁻¹). When *covR* was expressed *in trans* in IBS132 from plasmid pIB609, the Gus activity value decreased approximately threefold to 37.1 $\pm$ 0.7 MU ml⁻¹. This reduction was similar to the values that were obtained when *covR* was expressed from its own promoter in plasmid pIB30 (34.9 $\pm$ 0.1 MU ml⁻¹). However, the Gus activity in the IBS132 strain containing plasmid vector pIB184 without *covR* was 108.3 $\pm$ 1.9 MU ml⁻¹. Thus, our results indicate that expression of *CovR* from pIB609 is similar to the expression from pIB30. Therefore, the promoter-containing theta-replicating vectors described here can be used for genetic studies in streptococci.

DISCUSSION

We have explored the possibility of using four heterologous promoters for the expression of genes in streptococci. The promoters that were selected for this purpose originate from various Gram-positive bacteria, and are known to work in heterologous hosts (Barnett *et al.*, 2007; Eichenbaum *et al.*, 1998; Halfmann *et al.*, 2007; Opdyke *et al.*, 2003; Que *et al.*, 2000; Takahashi *et al.*, 2002). We found that each of the four promoters was functional in *S. mutans*, as well as in *S. pyogenes*, as expected. In *S. mutans*, the highest activity was obtained with the P_{veg}

promoter in the single-copy expression system. The level of expression of the P_{veg} promoter was about fivefold higher than expression from the P_{gbpC} promoter, which is a moderately expressed native promoter (Biswas *et al.*, 2007a; Li & Burne, 2001; Sato *et al.*, 2000; Yoshida & Kuramitsu, 2002). However, the expression level of the P_{veg} promoter was almost equivalent to that of the P_{gtfB} promoter, which is a highly expressed native promoter (Biswas *et al.*, 2007a; Biswas & Biswas, 2006; Li & Burne, 2001; Sato *et al.*, 2000; Yoshida & Kuramitsu, 2002). The P_{veg} promoter, which originated from *B. subtilis* (Moran *et al.*, 1982), contains a perfect -35 motif (TTGACA) and a near-perfect -10 motif (TACAAT) of the canonical σ A consensus. The other promoters were also highly active in the single-copy expression system, with highest expression observed from P_{ami} followed by P₂₃ and P_{spac}. Although the strength of the P_{spac} promoter was the lowest of the promoters tested, expression from P_{spac} was comparable with expression from the P_{gbpC} promoter. The P_{spac} promoter was originally constructed as an inducible promoter system for gene expression in *B. subtilis*, and it has been shown to work in *S. pyogenes* (Eichenbaum *et al.*, 1998); however, the level of induction is much lower in *S. pyogenes* than in *B. subtilis* (Eichenbaum *et al.*, 1998). We also found that, in *S. pyogenes*, P_{spac} promoter activity was very low compared with the other promoters.

We observed that when the promoters were present on RCR-based plasmids, with the exception of P₂₃, the activities of the promoters on the plasmids were much lower than when they were integrated in the *S. mutans* chromosome for single-copy expression. On the other hand, increased expression from the P₂₃ promoter was observed when P₂₃ was present on each of the RCR plasmids. RCR plasmids are known to exhibit structural or segregational instability after the cloning of relatively small DNA fragments (Gruss & Ehrlich, 1988). This is due to the nature of the replication mechanism involving ssDNA intermediates; this mechanism can lead to plasmid deletions (Michel & Ehrlich, 1986) or generation of high-molecular-mass plasmid multimers that are structurally unstable (Gruss & Ehrlich, 1988). Some DNA sequences as small as 7–10 bp in length can trigger high-molecular-mass plasmid multimer formation in bacteria (Biswas *et al.*, 1995; Dabert *et al.*, 1992). We speculate that the low level of promoter activity observed in the RCR plasmids is due to plasmid instability triggered by the cloning of DNA fragments containing the promoter sequences. Although the RCR plasmids suffer from this instability problem, in some instances, RCR plasmids are better suited for cloning and gene expression when compared with theta-replicating plasmids. For example, RCR plasmids are easier to introduce into various hosts compared with theta-replicating plasmids (Luchansky *et al.*, 1988; O'Sullivan & Fitzgerald, 1999; Turgeon *et al.*, 2006). Moreover, theta-replicating plasmids can lead to slower growth of the host, in addition to their limited host range, compared with RCR plasmids (O'Sullivan & Fitzgerald, 1999).

Therefore, the RCR-based shuttle plasmids constructed here are useful, despite potential risks associated with plasmid instability.

The theta-replicating plasmid that was used for construction of our shuttle plasmids is derived from pIL253, which contains a pAM β 1 replicon (Simon & Chopin, 1988). Plasmid pIL253, and its derivatives, are known to be present as a moderate-copy-number plasmid in Gram-positive bacteria (Simon & Chopin, 1988); however, the copy number of this plasmid can vary depending on the host (Perez-Arellano *et al.*, 2001). Since these plasmids can accommodate large DNA inserts, and are stable, they are the preferred choice for gene expression, specifically for those streptococci that are naturally transformable, such as *S. mutans*.

A series of shuttle plasmids was also designed so that proteins of interest could be expressed as tagged fusion proteins, as both N-terminal- and C-terminal-tagged proteins. The efficacy of the system was verified by the expression of Smu487 as a 6 $\times$ His-tagged protein in the three hosts *S. mutans*, *S. pyogenes* and *E. coli*. Smu487 was expressed well in each of the three hosts, and was easily detected, although the expression was highest in *E. coli*. When it comes to protein expression and purification in Gram-positive bacteria, the choice of available systems is very restricted (Brockmeier *et al.*, 2006; Fujimoto & Ike, 2001). Moreover, the available systems may not work in pathogenic streptococci, such as *S. mutans*. The gene-expression system developed here can be used for the successful expression and purification of proteins from Gram-positive bacteria, including pathogenic streptococci. This expression system should greatly facilitate gene regulation studies in this group of bacteria. Regulatory proteins may be associated with other indigenous accessory factors, and therefore purification from autologous hosts, rather than *E. coli*, offers immense advantages.

In conclusion, we report here a set of easy-to-clone vectors that permit high-level gene expression in *S. mutans*. Since these plasmids are based on constitutively expressed promoters that are carried by broad-host-range-based vectors, they can be used in many Gram-positive pathogens for gene-regulation studies. We are currently developing an inducible system that will allow us to further fine-tune the gene expression in streptococci.

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