An amino acid change near the carboxyl terminus of the *Streptococcus gordonii* regulatory protein Rgg affects its abilities to bind DNA and influence expression of the glucosyltransferase gene gtfG

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The *Streptococcus gordonii* glucosyltransferase structural gene, gtfG, is located immediately downstream from its positive transcriptional regulatory determinant, rgg. Recent genetic studies have indicated that the 3' end of rgg is involved either directly as a binding site or indirectly, e.g. by playing a role in secondary structure, in the interaction of Rgg with the gtfG promoter. A previously identified spontaneous mutant with a point mutation near the 3' end of rgg had only ~25% of the parental level of glucosyltransferase activity. To determine if this decreased activity was due to a change in the DNA binding site of trans-acting Rgg, or due to a change in the Rgg protein itself, complementation analyses and DNA-binding studies were performed. In Rgg-deficient strains, the chromosomal rgg point mutation did not influence the ability of plasmid-borne rgg to increase glucosyltransferase expression. However, plasmids carrying parental rgg were able to increase glucosyltransferase activity and expression of a gtfG promoter fusion to a greater extent than plasmids carrying the mutant allele, indicating that the mutant Rgg protein had decreased activity. The ability of NH2-terminal (hexahistidine) tagged proteins to bind to a 107 bp dsDNA fragment corresponding to the region immediately upstream of gtfG was demonstrated by surface plasmon resonance. Despite their differences in activity, both mutant and parental recombinant Rgg proteins bound to this dsDNA, albeit with different strengths. These studies provide insights into functional domains of *S. gordonii* Rgg which influence glucosyltransferase expression, and may have implications for Rgg-like regulatory proteins in related bacteria.

**INTRODUCTION**

Expression of the *Streptococcus gordonii* glucosyltransferase (GTF) structural gene, gtfG, is controlled by the upstream positive regulatory determinant, rgg, which encodes a predicted approximately 34 kDa cytoplasmic protein (Sulavik et al., 1992). An intergenic region of dyad symmetry separates the gtfG putative promoter and ribosome-binding site. Northern blot analyses have identified rgg- and gtfG-specific, as well as polycistronic rgg/gtfG, mRNA transcripts (Sulavik & Clewell, 1996). Recent genetic studies support the nucleotide-sequence-based prediction (Sulavik et al., 1992) that expression of rgg has cis effects on GTF activity. Distancing rgg from gtfG by insertion of an integrated vector results in decreased GTF activity, suggesting that translation of rgg and gtfG may be coupled (Vickerman & Minick, 2002). In addition, complementation studies indicate that the product of rgg acts in trans to increase gtfG transcription (Sulavik et al., 1992). The presence of rgg in the streptococcal plasmid pAMS57 causes both the parental strain Challis CH1 and strain DS512, which has a premature translational stop in rgg, to express GTF at levels significantly above wild-type (Sulavik et al., 1992). Recent studies of *S. gordonii* strains with nested chromosomal deletions have suggested that some of the nucleotides within the 3' end of rgg are necessary for trans-acting rgg to increase GTF activity (Vickerman & Minick, 2002). These studies support the model that Rgg binds to the DNA region surrounding the 3' end of rgg and the 5' end of gtfG to increase gtfG transcription. In addition, structural prediction algorithms (Bateman et al., 2000) indicate that Rgg has a helix–turn–helix motif near the amino terminus, suggestive of a DNA-binding function (Wintgens & Rooman, 1996).

Parental levels of GTF allow *S. gordonii* cells growing in the presence of sucrose to synthesize 1,3-α- and 1,6-α-glucans that confer a sucrose-promoted phenotype (Spp+) to colonies on sucrose agar plates (Tardif et al., 1989). These glucans facilitate bacterial accumulation on surfaces and...
have been implicated as colonization determinants (Vickerman et al., 1991). Cells with decreased levels of GTF activity (Spp’−) are less able to grow in these bacterial biofilms in the presence of sucrose. The nucleotide sequence of one spontaneous Spp’− strain was examined and found to contain a point mutation near the 3’ end of rgg (Vickerman et al., 1997). This strain had only approximately 25% of the parental level of GTF activity (Tardif et al., 1989) and significantly decreased levels of gtfG transcript (Vickerman et al., 1995). To confirm that the rgg point mutation was responsible for the decreased GTF activity, strain CHC102 was constructed by allelic exchange (Vickerman et al., 1997). As expected, strain CHC102 had only approximately 25% of the parental level of GTF activity. The rgg allele of strain CHC102, designated rggC1, has a cytosine replacing the parental guanine at nucleotide number 811 in the 891 bp rgg ORF. This results in a codon change from a negatively charged aspartate to a positively charged histidine at amino acid number 271 of the encoded protein RggC1 (Vickerman et al., 1997). The computer-predicted pI of RggC1 is 6.12 compared with the parental Rgg pI of 5.78. The present genetic and biochemical studies were undertaken to determine if the decreased GTF activity in strain CHC102 was due to the change in the encoded RggC1 protein or due to the change in the chromosomal region near the 3’ end of rgg, which may function as a DNA-binding site for Rgg. The results provide insights into the binding interaction of Rgg with the chromosomal region upstream of gtfG, and have implications for understanding the functional domains and specificity of the Rgg protein.

**METHODS**

**Bacteria, medium and culture conditions.** Schematic diagrams of the *S. gordonii* strains used in these studies are shown in Fig. 1. All strains were stored at −70°C in 50% (v/v) glycerol. *S. gordonii* strains were grown in defined FMC (Terleckyj et al., 1975) or Todd–Hewitt (TH; Difco) medium and incubated at 36°C in an anaerobic chamber with a gas mixture of 85% N₂, 10% H₂ and 5% CO₂. Growth stage was determined by cell density readings at OD₅₂₀ or OD₆₀₀ (Pharmacia Ultraspec 2000 spectrophotometer) in FMC or TH medium, respectively. Sucrose-promoted colony phenotype [Spp’−, a hard, adherent colony, or Spp’+, a soft, moveable colony (Tardif et al., 1989)] was determined on 3% (w/v) sucrose TH agar plates incubated for 48 h at 36°C in 5% CO₂. *S. gordonii* strains carrying plasmids were selected and grown with 5 μg erythromycin ml⁻¹. RecA-deficient *S. gordonii* strains were selected and grown with 250 μg spectinomycin ml⁻¹. *Escherichia coli* strain DH5α (Invitrogen Life Technologies) used for plasmid construction, strain MC4100 derivatives used to measure lacZ activity (Sulavik & Clewell, 1996) and strain B834(DE3) (Novagen) for recombinant protein expression were grown in Luria–Bertani (LB) medium with appropriate antibiotics (100 μg ampicillin ml⁻¹, 20 μg chloramphenicol ml⁻¹ or 50 μg kanamycin ml⁻¹) and incubated aerobically at 37°C.

**DNA isolation and manipulations.** DNA preparation and cloning procedures were done according to standard molecular biology techniques (Ausubel et al., 1987). Modifications for *S. gordonii* included...
DNA binding and activity of S. gordonii Rgg

Plasmids for determining trans effects of Rgg. The rgg of strain CHC102, designated rggC1, was cloned into the streptococcal vector pVA749 (Macrina et al., 1981) to compare trans effects of rgg to those of rggC1 in S. gordonii. The oligonucleotide primers 5'-GGGATCCAGAGGATGAGTTTACCTGTAAC-3' and 5'-GGGATCCAGAGGATGAGTTTACCTGTAAC-3' were used in PCR with strain CHC102 chromosomal template to amplify rggC1 and its flanking region. The BamHI-flanked approximately 1-49 kb PCR fragment was cloned into a BamHI site which had been created in the HaeIII site (GGCC) of the streptococcal plasmid pVA749 by insertion of two nucleotides (AT). Plasmid DNA was sequenced to confirm PCR fidelity and orientation. The plasmids chosen for these studies, pMG1, which carried rggC1, and pAMS57 (Sulavik et al., 1992), which carries the parental rgg in the HaeIII site of pVA749, differed only by the expected point mutation in rgg and the two nucleotides (AT) at the engineered cloning site.

Determination of GTF activity. Relative amounts of GTF activity for each strain were determined by measuring glucan synthesized in acrylamide gels, as described previously (Tardif et al., 1989). Briefly, strains were grown to the same mid-to-late exponential stage in FMC (OD520 value of ~1.6) and equal volumes of cell-free culture supernatants were run on SDS-8-75 % PAGE. After electrophoresis, gels were incubated overnight in 3 % sucrose, 0.5 % Triton X-100 in 10 mM sodium phosphate, pH 6.8, at 37 °C. Resulting glucan bands were stained with pararosaniline and densitometrically scanned to determine Intensity Units (Alpha Imager TM2200; Alpha Innotech). Band intensities reflected the relative levels of GTF activity. Results shown are representative of at least four independent experiments.

Determination of effects of plasmid-borne rgg on expression of gtfG promoter::lacZ fusions in E. coli. Derivatives of E. coli strain MC4100 with a promoterless lacZ (strain VA203) or gtfG promoter::lacZ fusion (strain VA204) in single copy in the att site were used to examine trans effects of rgg on gtfG promoter expression, as described previously (Sulavik & Clewell, 1996). To compare effects of rgg and rggC1, these genes were subcloned from pAMS57 and pMG1 into pBCKgG and pBCKgGc1, respectively. DNA ligation mixtures were transformed into DH5α and selected on chloramphenicol agar plates. After restriction digest mapping to confirm that rgg and rggC1 were in the same orientation, the plasmids were transformed into the kanamycin-resistant strains VA203 and VA204. After growth to mid-exponential phase in LB medium, β-galactosidase activity in chloroform-permeabilized cells was measured spectrophotometrically (Pharmacia Ultraspec 2000) using ONPG as the enzyme substrate (Miller, 1972). Total protein concentration was measured by the method of Markwell et al. (1978) and activity was expressed in Miller units (Miller, 1972). Comparisons between strains were done using a two-tailed Student’s t-test. Experiments were repeated a minimum of three times.

Expression and purification of recombinant Rgg proteins. Oligonucleotide primers 5'-AAGGATCCAGAGGATGAGTTTACCTGTAAC-3' and 5'-GGGATCCAGAGGATGAGTTTACCTGTAAC-3' (start and stop codons are italicized) were used in PCR with pAMS57 or pMG1 templates to amplify the rgg and rggC1 genes, respectively. The resulting PCR products were cloned into the in-frame, compatible BamHI and XhoI sites of pET28a (Novagen) to produce N- terminal hexahistidine-tagged recombinant proteins. E. coli B834 (DE3) cells (Novagen) transformed with pET28a carrying either rgg or rggC1 were grown at 37 °C in LB medium containing 50 μg kanamycin ml⁻¹ until an OD₆₀₀ value of approximately 0.6 was reached. Cultures were then induced with 1 mM IPTG for 18 h at 16 °C. Cells were harvested by centrifugation and resuspended in a lysis buffer [50 mM HEPES, 500 mM NaCl, 1 % (v/v) Triton X-100, 1 mM benzamide, 5 mM imidazole, pH 7.5] with 1 mM PMSF and 5 mM MgCl₂. After the addition of 2 mg RNase A ml⁻¹ and 500 U DNase, bacteria were lysed in a French pressure cell. The

Construction of S. gordonii strain CH51C1 by allelic exchange. To construct S. gordonii strain CH51C1, which had an engineered translational stop to encode a truncated Rgg, as in strain DS512, and also contained the point mutation at nucleotide number 811 in rgg, as in strain CHC102, PCR amplification was performed using strain CHC102 chromosomal DNA as template. The forward primer, 5'-TTGGATCCCTGCGAGGGTTTACCTGTAAC-3', had an engineered 5' XhoI site (underlined) compatible with SalI, an in-frame engineered translational stop (bold), and was designed to anneal immediately downstream of the SalI site of rgg (Fig. 1). The reverse primer, 5'-TTGGATCCCTGCGAGGGTTTACCTGTAAC-3', was designed to anneal to the SalI site at nucleotide number 750 in the gtfG ORF. The resulting approximately 1-1 kb PCR product was digested with XhoI and SalI and cloned into SalI–SstI-digested pMI2200 (Vickerman & Minick, 2002), a pGEM7 (Stratagene) derivative that carries an approximately 2-25 kb Apal–SstI fragment encoding the 3' end of the upstream determinant, htpX, rgg and the 5' end of gtfG of S. gordonii strain CH1. After confirmation of the correct nucleotide sequence, the resulting 2-25 kb fragment containing the inserted mutations was transformed into strain CH1 to allow homologous recombination between similar chromosomal and linear DNA on both sides of rgg. This resulted in integration of the desired sequence into the S. gordonii chromosome. Spp colonies, presumably arising from mutations in rgg, were selected on 3 % sucrose agar plates.

Putative transformants were examined by Southern hybridization analysis (Ausubel et al., 1987) to confirm the presence of the upstream rgg mutation resulting from reigation of compatible SalI- and XhoI-digested DNA. SalI-digested chromosomal DNA was electrophoresed on 0-7 % agarose gels, transferred to Hybond-N membranes (Amerham) and probed with a double-stranded digoxigenin-dUTP-labelled DNA probe for rgg, using the Genius System (Roche Molecular Biochemicals), according to the manufacturer’s directions. Strains that showed loss of the rgg SalI site were chosen for nucleotide sequence analysis to confirm that in addition to the presence of the engineered translational stop codon, the strains also had the desired rgg point mutation.

Construction of RecA-deficient strains. To minimize the potential of recombination between plasmid-borne and chromosomal copies of rgg in complementation studies, RecA-deficient derivatives of strains CHC102 and CH51C1, designated CHC102R and CH51C1R, respectively, were constructed. The method used was that described previously to construct RecA-deficient derivatives of strains CH1 [parental RecA-deficient strain CH3 (Vickerman et al., 1993)], DS512 and CHAR1 [RecA-deficient strains DS512R and CHAR1R, respectively (Vickerman & Minick, 2002)]. Briefly, strains were transformed with pAM6200, an integrative plasmid carrying a spectinomycin-resistance determinant and an approximately 300 bp internal fragment of the S. gordonii recA gene. Expected integration of pAM6200 into the streptococcal chromosome, resulting in disruption of the recA gene, was confirmed by Southern hybridization analysis using the internal 300 bp recA fragment as a probe (Vickerman et al., 1993).

growing strains in 0-5 % (w/v) glycerine to weaken cell walls, incubating cells with mutanolysin and lysozyme to facilitate lysis, and making cells competent for transformation with horse serum, as described previously (Sulavik et al., 1992). E. coli transformations were performed using CaCl₂-competent cells. Plasmid DNA was prepared using purification columns (Qiagen) according to the manufacturer’s directions. PCR products were obtained using Elongase enzyme (Invitrogen Life Technologies) and oligonucleotide primers with 5' random nucleotides and engineered restriction sites to facilitate cloning. DNA fragments were eluted from gels with a Qiagen II kit (Qiagen).
lysate was centrifuged (15 000 g for 20 min at 4°C) and the supernatant was applied to an approximately 5–7 ml nickel-chelating Sepharose column (Amersham Pharmacia) pre-equilibrated with lysis buffer. The column was washed with 50 ml lysis buffer, then 100 ml of 500 mM NaCl/100 mM imidazole, pH 7-5. Histidine-tagged protein was eluted with a solution of 500 mM NaCl/500 mM imidazole, pH 7-5. Protein-containing fractions were pooled and dialysed against 500 mM NaCl/10 mM imidazole/5 mM EDTA/0-1 % (v/v) β-mercaptoethanol with a sample:dialysis buffer volume ratio of 1:100, with two changes at approximately 8 h intervals. Protein recovered in this manner could attain concentrations as high as 9 mg ml⁻¹, with a purity in excess of 99% as estimated by SDS-PAGE.

Surface plasmon resonance (SPR) studies. Binding interactions of Rgg and RggC1 recombinant proteins with DNA were monitored using the SPR method (Wang et al., 2000), which measured changes in refractive index that occurred when injected protein bound to DNA coupled to a biosensor surface. Double-stranded DNA fragments, representing the 107 bp region from the EcoRI site in rgg (Fig. 1) through the ATG start codon of gtfG, were made using a 5' biotinylated reading strand primer (Integrated DNA Technologies) hybridized to the complementary strand in a 1:1 ratio to ensure that all biotinylated oligonucleotides were in duplex form. The resulting dsDNA was purified by electrophoresis in a native 8% polyacrylamide gel (Ausubel et al., 1987).

For preparation of biosensor surfaces with DNA, the biotinylated dsDNA was diluted to 1-5 nM in a buffer containing 10 mM HEPES, pH 7-4, 0-005 % polysorbate-20 and 1·0 M NaCl, and manually injected onto an immobilized Neutravidin surface of a BIACORE sensor chip B1 to ~450 Response Units using a BIACORE 3000 (Biacore). Neutravidin surfaces were prepared using an amine coupling reaction according to the manufacturer’s directions (BIAtechnology handbook). One flow cell was left undervisceratized to allow bulk refractive index change correction. Proteins were diluted in the running buffer containing 10 mM HEPES, pH 7-4, 150 mM NaCl and 0-005 % polysorbate-20. Following protein binding to DNA, regeneration was performed with a 30 s quick-injection of 0-25 % SDS in running buffer. For reproducibility, each experiment was repeated at least twice.

SPR analysis. Bia-evaluation software supplied by the vendor was used to analyse binding data using a simple Langmuir 1:1 model (Myszka, 2000). In the present experiments, the analyte is the recombinant protein, ligand is the DNA and Rₘₐₓ is the total surface-binding capacity. For the model [A] + [B] = [AB], where [A] = concentration of analyte, total ligand on the surface [B]₀ = Rₘₐₓ and initial response [AB]₀ = 0, local numerical integration for each curve was performed using the differential equation d[AB]/dt = k₄[A][B] - k₃[AB]. The fit of the data was reported as a mean sum of differences (Δ) between predicted and observed data and as standard errors (σ), values for the protein–DNA complexes were calculated as the k₄ (association rate constant) divided by the k₃ (association rate constant).

RESULTS

The chromosomal point mutation in rggC1 does not affect the ability of plasmid-borne rgg to increase GTF activity in Rgg-deficient strains

The point mutation at nucleotide number 811 in the rgg ORF of strain CHC102 has been previously demonstrated to result in an approximately 75% decrease in GTF activity compared with the parental strain CH1 (Vickerman et al., 1997). To determine if this chromosomal mutation affected the ability of plasmid-borne rgg to increase GTF activity, a RecA-deficient derivative was constructed for complementation studies. The resulting strain, CHC102R, was transformed with pAMS57 carrying the parental rgg. Although strain CHC102R(pAMS57) had a 5·36 ± 0·98-fold increase in GTF activity over the plasmid-free strain, it had only 62·7 ± 10·8 % of the activity of strains DS512R(pAMS57) and CHAR1R(pAMS57), which each had a chromosomally encoded truncated Rgg (Fig. 2a). It was possible that incomplete complementation of the chromosomal mutation was due to the chromosomally encoded RggC1 of strain CHC102R. RggC1 could potentially interfere with the plasmid-borne parental Rgg in strain CHC102R(pAMS57) in some way [e.g. by protein–protein interactions or by titration of DNA-binding site(s)]; apparently, the truncated Rgg encoded by strains DS512 and CHAR1 did not interfere with complementation. To control for this possibility, strain CH51C1, which contained the point mutation of rggC1 but did not encode the RggC1 protein, was constructed (Fig. 1). As expected, strain CH51C1, which encoded the same truncated Rgg as strains DS512 and CHAR1, had a similar low level of GTF activity (5·73 ± 3·75 % of the parental strain CH1). Furthermore, CH51C1R(pAMS57) had the same level (within 10%) of GTF activity as DS512R(pAMS57) and CHAR1R(pAMS57), indicating that the presence of a cytosine at nucleotide 811 in the rgg ORF did not affect the ability of plasmid-borne parental rgg to increase GTF activity (Fig. 2a). These results suggested that the basis of the decreased GTF activity in strain CHC102 is due to the change in the RggC1 protein, rather than a change in the chromosomal region upstream of gtfG that may serve as a binding site for trans-acting Rgg.

Plasmid-borne rggC1 is unable to increase GTF activity to the same extent as plasmid-borne parental rgg

To determine the ability of RggC1, encoded by the mutant rggC1 allele, to influence GTF activity, pMIC1 was transformed into S. gordonii RecA-deficient strains CHR3 and DS512R. Although CHR3 encodes the parental Rgg and strain DS512 encodes a truncated Rgg, both strains have the same putative chromosomal DNA-binding sequence for trans-acting Rgg; the chromosomal sequences are identical, starting immediately downstream of the rgg SalI site through gtfG (Fig. 1). As noted above (Fig. 2a), pAMS57, which carries the parental rgg allele, was able to increase GTF activity in strain DS512R to the level seen in strain CHR3(pAMS57), consistent with previously reported data (Vickerman & Minick, 2002). However, pMIC1 was only able to increase activity 1·22 ± 0·14-fold from the level seen in plasmid-free strain CHR3, and 3·38 ± 0·93-fold from the level seen in plasmid-free strain DS512R (Fig. 2b). These differences were significantly less (P ≤ 0·0001 for both strains) than the 2·95 ± 0·32 increase in GTF activity due to the presence of pAMS57 seen in strain CHR3, and the 15·45 ± 0·88-fold increase due to the presence of pAMS57 in strain DS512R (Fig. 2). Thus, plasmid-borne rggC1 was unable to increase GTF activity to the same extent as plasmid-borne parental rgg.
Plasmid-borne *rggC1* is unable to increase transcription of *gtfG*::*lacZ* fusions to the same extent as parental *rgg*

Since expression of *rgg* has both *cis* and *trans* effects on *gtfG* expression in *S. gordonii* (Vickerman & Minick, 2002), independent measurements to clarify the ability of *trans*-acting *rgg* and *rggC1* gene products to increase activity of the *gtfG* promoter were performed in *E. coli*. Plasmids carrying these genes were transformed into strains VA203 and VA204. These strains had a promoterless *lacZ* or a *gtfG* promoter::*lacZ* fusion, respectively, chromosomally integrated in single copy into the *att* site. β-Galactosidase activities observed in strains VA203 and VA204 carrying pBCRgg were consistent with previously published results (Sulavik & Clewell, 1996). The presence of pBCRgg in the *gtfG* promoter fusion strain VA204 resulted in an approximately 6·38-fold increase in β-galactosidase activity compared with activity seen with the pBCSK vector alone (Table 1). Strain VA204(pBCRggC1) showed only an approximately 5·36-fold increase in levels of β-galactosidase activity compared to levels seen in VA204(pBCSK) (Table 1). These results suggested that although the product of *rggC1* increased the ability of the *gtfG* promoter to express *lacZ*, it was not able to achieve the same level as plasmid-borne parental *rgg*.

**SPR studies indicate that Rgg and RggC1 differ in their abilities to bind to the DNA region upstream of *gtfG***

To confirm results of the genetic studies that suggested that the *rgg* gene product interacts with the 107 bp chromosomal region upstream of *gtfG*, DNA-binding studies were performed using SPR. Results indicated that when biotinylated dsDNA representing this region was coupled to a biosensor surface, recombinant Rgg was able to recognize and bind to this DNA preferentially compared with the non-specific binding to a control polyA/polyT dsDNA fragment (Fig. 3a). Binding of Rgg to the 5' biotinylated 107-mer ssDNA was too weak to be reliably detected under conditions used for measuring binding of protein to dsDNA.

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**Fig. 2.** GTF activities gels. Bacteria were grown in FMC medium to the same mid-exponential stage and equal volumes of culture supernatants were run on SDS-8.75 % PAGE. Gels were incubated with sucrose and Triton X-100 detergent at 37°C. Synthesized glucan bands were treated with periodic acid and stained with pararosanineline. The position of the 174 kDa native protein band is indicated. Lower molecular mass active forms of GTF are thought to be due to the degradation of the native enzyme by endogenous proteases, as described previously (Grahame & Mayer, 1984). RecA-deficient strains were used for complementation analyses with the RecA-deficient parental strain CHR3 as the positive control. Gels shown are representative of a minimum of four independent experiments. (a) GTF activities of *S. gordonii* strains with chromosomal mutations in *rgg* complemented by plasmid-borne parental *rgg* in pAMS57. (b) Abilities of plasmid-borne *rgg* in pAMS57 and *rggC1* in pMIC1 to increase GTF activity in the parental derivative strain CHR3 and strain DS512R, which encodes a truncated chromosomal *rgg*.
Table 1. Regulation of gtfG::lacZ transcriptional fusion by plasmid-encoded Rgg and RggC1 in E. coli

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chromosomal fusion genotype*</th>
<th>Plasmid</th>
<th>β-Galactosidase activity (Miller units)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA203</td>
<td>Tl–lacZ</td>
<td>pBCSK</td>
<td>4.97 ± 2.4</td>
</tr>
<tr>
<td>VA203</td>
<td>Tl–lacZ</td>
<td>pBCRgg</td>
<td>3.83 ± 1.8</td>
</tr>
<tr>
<td>VA203</td>
<td>Tl–lacZ</td>
<td>pBCRggC1</td>
<td>5.61 ± 2.6</td>
</tr>
<tr>
<td>VA204</td>
<td>Tl–gtfG::lacZ</td>
<td>pBCSK</td>
<td>58.9 ± 8.4</td>
</tr>
<tr>
<td>VA204</td>
<td>Tl–gtfG::lacZ</td>
<td>pBCRgg</td>
<td>376.2 ± 16.4</td>
</tr>
<tr>
<td>VA204</td>
<td>Tl–gtfG::lacZ</td>
<td>pBCRggC1</td>
<td>315.8 ± 21.6§</td>
</tr>
</tbody>
</table>

* Tl is four tandem copies of the E. coli rrnB terminator upstream of a promoterless or gtfG promoter::lacZ fusion in the att site of MC4100.
† Activity from mid-exponential cells expressed in Miller units (Miller, 1972).
‡ Diffs from results seen in strain VA204 carrying pBCSK vector control; P < 0.0001, n = 4.
§ Diffs from results seen in strain VA204 carrying pBCRgg; P ≤ 0.0038, n = 4.

**DISCUSSION**

Expression of the S. gordonii GTF structural gene is controlled by the upstream determinant rgg, the first identified (Sulavik et al., 1992) member of a family of streptococcal positive regulatory genes. Genome sequencing projects have identified a number of rgg-like genes in streptococci and related bacterial species, although characterization of the specific regulatory mechanisms of these genes has been limited. Those rgg-like genes that have been characterized appear to positively regulate transcription of adjacent genes encoding extracellular proteins (Sulavik et al., 1992; Lyon et al., 1998; Sanders et al., 1998; Qi et al., 1999). It appears that the regulated genes are preceded by DNA inverted repeats, suggestive of regulatory-factor-binding sites. However, at the present time, nucleotide similarities or shared patterns among these upstream regions of dyad symmetry or promoter sequences have not been recognized. Recent studies indicate that one of the rgg-like genes in Streptococcus pyogenes, designated ropB (Lyon et al., 1998) or rgg (Chaussee et al., 1999), is involved, not only in the regulation of the adjacent speB which encodes a major virulence determinant, but also appears to affect the expression of a number of extracellular proteins (Chaussee et al., 2001) and may be involved in global regulatory networks (Chaussee et al., 2002). The Rgg proteins from these different species share a number of conserved regions (Vickerman et al., 2001) including the helix–turn–helix motif near the amino terminus. Although the presence of a putative DNA-binding motif (Wintgens & Rooman, 1996) as well as genetic data from these different streptococcal species (Lyon et al., 1998; Sanders et al., 1998; Qi et al., 1999) suggested that rgg-like gene products interact with DNA near promoter regions to influence transcription, the present studies are the first demonstration of DNA binding by an Rgg-like protein.

Although previous studies in S. gordonii had demonstrated that deletion of a 264 bp DNA region flanking the rggC1 point...
mutation did not affect the ability of plasmid-borne rgg to increase GTF activity (Vickerman & Minick, 2002), the possibility existed that the deletion in strain CHAR1 allowed an upstream DNA region to act as an artificial binding site for Rgg. However, the present genetic studies suggest that parental rgg in pAMS57 can complement strain CH51C1, which had the chromosomal point mutation. This supports previous studies that had implicated only the involvement of the chromosomal region downstream of the rgg EcoRI site with Rgg (Vickerman & Minick, 2002). Accordingly, a 107 bp dsDNA fragment, representing the rgg EcoRI site through the start codon of gtfG, was used in Rgg–DNA-binding studies. Because the recombinant Rgg protein was relatively insoluble in the low salt conditions necessary for classical electrophoretic mobility shift assays (Ausubel et al., 1987), SPR was used to measure the protein–DNA-binding interaction. The very dilute solutions used in this method minimized protein solubility and aggregation problems (Green et al., 2000), so that in addition to monitoring binding in real time, kinetic studies were possible. The recombinant Rgg specifically bound to the 107 bp duplex at levels above the binding to the random poly dA/dT control dsDNA, supporting the Rgg–DNA-binding model for gtfG regulation.

The $K_D$ values for Rgg and RggC1 suggest that the protein–DNA complexes are less stable than those seen with some transcriptional activators or repressors, although dissociation rate constants are similar (Poon et al., 2001; Stockley et al., 1998). However, because of different functions of bacterial regulatory proteins, it is not necessarily valid to compare $K_D$ values among protein–DNA complexes. As in all in vitro systems, there are inherent limitations in interpretation of results. The $K_D$ ranges seen in the present SPR studies with purified recombinant Rgg may be influenced by several factors. In vivo, Rgg may form complexes with intracellular components such as sigma factors, RNA polymerase, or additional, as-yet-unidentified proteins. Indeed, previous genetic studies have indicated that distally located genes, which could potentially encode regulatory cofactors, influence rgg or gtfG expression (Vickerman et al., 1995, 1997). It is possible that such factors may influence the stability of the Rgg–DNA binding. In addition, it is probable that only a small number of nucleotides directly interact with the helix–turn–helix motif of Rgg (Wintgens & Rooman, 1996). In the semi-solid phase technology used in the SPR studies, the relatively large number of nucleotides in the 107 bp dsDNA fragment may sterically hinder the binding and stability of the protein–DNA complex. Additional studies to delineate the DNA region within the 107 bp sequence that interacts with Rgg are in progress. Identification of specific nucleotides involved in the Rgg–DNA interaction may demonstrate as-yet-unidentified general patterns in DNA regions regulated by Rgg-like proteins.

The basis for the decreased gtfG transcription and resulting decreased GTF activity (Vickerman et al., 1995) in strain CHC102 appears to be due to a change in the encoded Rgg protein. Comparison of Rgg-like sequences indicates that the amino acid change in RggC1 is located in a fairly conserved region near the carboxyl terminus (Vickerman et al., 2001); no function has yet been assigned to this domain. Although RggC1 has the parental sequence in the helix–turn–helix putative DNA–binding region (amino acids 11–64; Bateman et al., 2000), RggC1 was more efficient at binding DNA than the parental Rgg in the SPR studies. Although the molecular mechanism has not yet been determined, the increased DNA binding ability of RggC1 may be due to a conformational change in the protein that influences the DNA binding interaction at biochemical (e.g. due to local charge effects) or mechanical (e.g. torsional effects) levels (Travers & Muskeliashvili, 1998). Point mutations that result in bacterial proteins that are defective in transcriptional initiation or activation, yet do not show decreased DNA-binding abilities, have been previously described in other bacteria (Gerber & Hinton, 1996; Kelly & Hoover, 1999). Accordingly, despite its increased DNA binding ability in SPR studies, RggC1 appears to be defective in transcriptional activation. The plasmid pMIC1, carrying rggC1, was unable to increase GTF activity in S. gordonii strains to the same extent as pAMS57, carrying rgg. Furthermore, plasmids carrying these rgg alleles differed in their ability to increase expression of gtfG promoter fusions in E. coli. Although these latter studies must be interpreted with care due to differences in promoter recognition in E. coli and streptococcal species (Dillard & Yother, 1991), they nevertheless independently support the relative activities of the Rgg and RggC1 proteins for transcriptional activation of gtfG (Sulavik & Clewell, 1996). It is not clear if the point mutation in rggC1 causes a direct change in an RggC1 domain involved in transcriptional activation, or if the changes observed in DNA binding and transcriptional activation are due to indirect conformational effects on the RggC1 protein. Nevertheless, identification of an amino acid essential for maintaining transcriptional activating activity in S. gordonii Rgg not only provides insights into the control of gtfG expression, but also may provide general insights into the specificity and functional domains among the family of Rgg-like proteins.

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