Adaptive gene expression of endocarditis-causing viridans group streptococci and Staphilococcus aureus
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CHAPTER 6

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

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ABSTRACT

We aimed to identify transcription signal sequences from *Streptococcus gordonii* strain CH1 by random chromosomal cloning. Five genomic fragments from a Sau3A digest, that constitutively activated transcription of a promoterless spectinomycin resistance gene in this strain, were isolated and characterized. Additionally, one promoter fragment was isolated which was specifically activated under iron-limiting conditions. A sequence motif with similarity to the consensus for Fur-binding regulatory DNA sequences (Fur box) in *Escherichia coli* was detected within the putative promoter region. The open reading frame downstream of this region possibly encodes a transmembrane protein involved in iron uptake.
INTRODUCTION

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

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

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

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions
S. gordonii CH1 (26) was cultured in Todd Hewitt (TH) broth and on TH-agar (Difco Laboratories, Detroit, MI) at 37°C in a 5% CO₂ atmosphere. When required, 5 µg ml⁻¹ erythromycin (Em) was added for plasmid maintenance, and 0.1, 0.5, or 2.0 mg ml⁻¹ spectinomycin (Sp) for selection of streptococcal promoters.

Molecular biology procedures
DNA manipulations were done according to standard techniques (23). Restriction enzymes, T4 DNA ligase, and Calf Intestine Alkaline Phosphatase (CIAP) were purchased from Boehringer (Boehringer Mannheim GmbH, Mannheim, Germany). For PCR, Taq DNA polymerase and dNTPs were obtained from Promega (Promega Corporation, Madison, WI). A genomic expression library of S. gordonii CH1 was constructed in the broad host range selection vector pMM223 (Genbank Acc. No. AF076212). This vector is derived from the lactococcal...
shuttle vector pGKV210 (25), and contains the promoterless \textit{aad}(9) gene of \textit{Enterococcus faecalis}, conferring resistance to spectinomycin, for selection of promoter activity (16). The construction of this vector has been described in detail elsewhere (Chapter 4). Fragments from a total \textit{Sau3A} genomic digest were ligated into the unique \textit{BglII} site of the multiple cloning site of pMM223, preceding the promoterless \textit{aad}(9) gene. Recombinant plasmids, containing chromosomal fragments in the range of 100-1000 bps, were then introduced into the homologous host by electroporation. The streptococcal library contained approximately $10^5$ independent clones, and statistically represents the entire \textit{S. gordonii} CH1 genome (23).

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

**DNA sequence analysis**

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

**RESULTS AND DISCUSSION**

**Isolation and characterization of \textit{S. gordonii} promoter sequences**

For the isolation of promoter sequences from \textit{S. gordonii} CH1, 25 µl of the genomic streptococcal library, containing approximately 2.5x$10^5$ clones, was plated onto erythromycin (Em)-containing TH agar. To select for promoters with different levels of activity, spectinomycin (Sp) was added to the agar at different concentrations. In duplicate experiments 350, 170, and 85 colonies were visible on plates with 0.1, 0.5, and 2 mg ml$^{-1}$ Sp, respectively, after 24 h. The reduction in the number of spectinomycin-resistant transformants at increasing Sp concentrations clearly demonstrated selection for promoters with increasing levels of activity. Colonies were randomly picked from agar plates containing the three different Sp concentrations, and cultured in TH broth containing 5 µg ml$^{-1}$ erythromycin. To analyse the strength of the isolated promoters, each colony was also plated onto TH agar containing Sp concentrations higher than that in the original selection plate, except when colonies had been grown on plates containing 2.0 mg ml$^{-1}$ Sp. Promoters of different strength were identified by this procedure (Table 1). To characterize the transcription signals, the cloned chromosomal inserts were amplified from bacterial lysates by PCR. Amplicons were analysed on agarose gels, and five fragments of different sizes were purified and sequenced.
Table 1. Characteristics of the isolated promoter sequences from *S. gordonii* strain CH1.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Size</th>
<th>Access. No.</th>
<th>Activity</th>
<th>Database match</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG&lt;sub&gt;p6&lt;/sub&gt;</td>
<td>0.55 kb</td>
<td>AJ243487</td>
<td>low</td>
<td>Transcription-repair coupling factor (Mfd; <em>B. subtilis</em>, P37474&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td>SG&lt;sub&gt;p2&lt;/sub&gt;</td>
<td>0.2 kb</td>
<td>AJ235898</td>
<td>intermediate</td>
<td>Response regulator (CiaR; <em>S. pneumoniae</em>, Q54954&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>SG&lt;sub&gt;p21&lt;/sub&gt;</td>
<td>0.75 kb</td>
<td>AJ236899</td>
<td>high</td>
<td>Topoisomerase IV subunit E and unknown protein (ParE and Orf2; <em>S. pneumoniae</em>, Z67739&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>SG&lt;sub&gt;p22&lt;/sub&gt;</td>
<td>0.3 kb</td>
<td>AJ236900</td>
<td>high</td>
<td>Cysteine synthase A (CysK; <em>B. subtilis</em>, P37887&lt;sup&gt;b&lt;/sup&gt;)</td>
</tr>
<tr>
<td>SG&lt;sub&gt;p32&lt;/sub&gt;</td>
<td>0.1 kb</td>
<td>AJ236901</td>
<td>high</td>
<td>No homology</td>
</tr>
</tbody>
</table>

<sup>a</sup> Accession numbers of the sequences of the different promoter fragments in the EMBL database

<sup>b</sup> The promoter activity is expressed as the highest spectinomycin concentration in the agar plate at which the recombinant bacterial clones were able to grow.

- Low, 0.1 mg/ml; intermediate, 0.5 mg/ml; high, 2.0 mg/ml

<sup>c</sup> Genbank accession number of matching sequence
Promoter sequences from Streptococcus gordonii

**Figure 1.**
Nucleic acid sequences of isolated promoter fragments of *S. gordonii* CH1. Putative -10 and -35 regions and Shine-Dalgarno (SD) sequences are underlined, and inverted repeats are indicated by arrows. The amino acid sequences of the open reading frames are partly indicated, with the bold face printed nucleotides representing the start of the gene.

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

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

The fragments SG$_{P21}$, SG$_{P29}$, and SG$_{P32}$ had high promoter activity. The promoter fragment SG$_{P21}$ contained two partial open reading frames, separated by an intergenic region. The two open reading frames showed homology to two different ORFs of *S. pneumoniae*, and the organization of the coding regions was similar in *S. gordonii* and *S. pneumoniae*. The ORF downstream of the *S. gordonii* promoter showed 71% identity over 168 aa to Orf2 of *S. pneumoniae*, a protein with unknown function (20). Upstream of the *S. gordonii* ORF and divergently oriented, a gene with homology to the 5'-end of the gene encoding the E subunit of topoisomerase IV gene (parE) of *S. pneumoniae* was identified on SG$_{P21}$, and the corresponding protein fragment was 100% homologous to the N-terminus of the *S. pneumoniae* ParE. Although an identical putative ribosome binding site (SD; Shine-Dalgarno...
sequence) was identified preceding parE in both streptococcal species, no SD sequence was observed upstream of the translational start of Orf2 of either species.

The ORF downstream of the promoter region in SGp2 showed 65% identity to the cysteine synthase of Bacillus subtilis. Inverted repeats, capable of forming a stem-loop structure with a free energy of -14.4 kcal, were identified upstream of the cysK coding region. This stem-loop might function as a transcriptional terminator of the upstream located unknown open reading frame (Fig. 1). In B. subtilis CysK expression is altered by different environmental signals. Expression is increased after cold shock, reduced after heat shock, and completely absent after salt stress (8), but the underlying regulatory mechanism has not yet been determined. No common structures were identified in the sequence preceding the cysK genes of B. subtilis and S. gordonii that could be involved in the regulation of cysK expression under different environmental conditions.

Finally, a putative promoter sequence was identified on the 121 nucleotide SGp32 fragment, but the downstream sequence did not reveal any similarity to entries in the database.

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

Isolation of an iron limitation-regulated transcription signal

As iron limitation is a possible environmental signal for the expression of streptococcal virulence genes, we aimed to isolate promoter sequences from S. gordonii CH1 of which the activity was regulated by the external iron concentration. In an attempt to limit the amount of free iron, ethylenediamine di-o-hydroxyphénylacetic acid (EDDA; Sigma Chemical Co., St. Louis, MO, USA) was included in the TH agar. This iron chelator has been used to study hemin utilization of S. pneumoniae (24), a species closely related to S. gordonii. Although growth of S. pneumoniae was abolished at EDDA concentrations of 700 μM and higher (24), concentrations of up to 1,000 μM did not restrict the growth of S. gordonii CH1. Therefore, nitrilotriacetic acid (NTA), a chelator of divalent cations which has previously been used to study the iron acquisition and the iron starvation response of Streptococcus pyogenes (5), was added to the TH agar instead. At concentrations of 18 mM NTA or higher, growth of S. gordonii CH1 was completely abolished. Aliquots of 50 μl of the genomic library, containing approximately 5x10⁵ clones, were plated onto TH agar supplemented with 17 mM of NTA, 1 mM of MgCl₂, ZnCl₂, CaCl₂, and MnCl₂, and 0.5 mg ml⁻¹ Sp for selection of iron limitation-
inducible promoters. To verify iron-limitation inducibility, colonies growing on these plates after 24 h were streaked on iron-limited TH agar plates, containing NTA (17 mM), cations (1 mM each), Sp (0.5 mg ml\(^{-1}\)) and Em (5 \(\mu\)g ml\(^{-1}\)), on TH agar with Sp and Em only, and on TH agar with Em as a control for growth. Clones that only grew on the Sp/Em containing agar plates in the presence of NTA were considered to carry an iron-limitation inducible promoter in front of the spectinomycin resistance gene.

Figure 2.

Nucleotide sequence of the iron-limitation inducible promoter fragment SG\(_{50}\). Putative -10 and -35 regions are underlined. The amino acid sequence of the potential open reading frame is partly indicated, with the ATG transcription start printed in bold and the putative Fur box shaded. The sequence of SG\(_{50}\) is deposited in the EMBL database under accession number AJ236902.
Of around 350 restreaked colonies, two clones were resistant to 0.5 mg ml\(^{-1}\) Sp only under the iron restricted conditions. The cloned fragments were amplified and sequenced, and found to be identical. The 954 bp fragment contained an open reading frame of 744 bp. No possible ribosome binding site preceding this open reading frame could be identified. The translated sequence of 248 amino acids (Fig. 2) had weak similarity to a transmembrane protein of a \(S.\) \textit{pneumoniae} capsular type 33F strain, of which the corresponding gene is located within the capsular gene cluster (Llull, D., unpublished data; GenBank Acc. No. AJ006986). Iron-limitation inducibility of this gene has not been reported. Determination of possible transmembrane regions in the \(S.\) \textit{gordonii} CH1 translated sequence using the TMpred program (12) indicated 8 putative transmembrane regions. Although cation-limitation inducible membrane transporter proteins of \(S.\) \textit{gordonii} have been demonstrated to be involved in the uptake of metal ions (15), the precise nature and function of the protein encoded by the gene identified here remains elusive.

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

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

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REFERENCES


