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Published Ahead of Print 10 November 2006.

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The Rgg family of transcription regulators is widely distributed among gram-positive bacteria, yet how these proteins control transcription is poorly understood. Using Streptococcus pyogenes RopB as a model, we demonstrated that residues invariant among Rgg-like regulators are critical for function and obtained evidence for a mechanism involving protein complex formation.

RopB is necessary but not sufficient for activation of transcription (21) and may influence gene expression via its ability to influence the expression of other regulators (5). The integration of Rgg pathways with other regulatory pathways could also be established through protein-protein interactions. For example, RopB has been shown to associate with a negative regulator, LacD,1, which may be a mechanism for maintaining temporally controlled expression programs (16a).

Although the members of the Rgg family have been adapted to individual regulatory programs, it is likely that these proteins have a common structure and mechanism of action. Thus, an understanding of how any individual member functions may provide crucial insight into the general way that members of this family function. In S. pyogenes, RopB (also referred to as Rgg) influences the production of proteins during the transition to the stationary phase (3, 6), including the production of the secreted cysteine protease, SpeB (4, 18). Protease expression is dependent on RopB transcriptional activation (4, 18), and its activity is readily detectable, providing a convenient assay for modeling Rgg family function. The present genetic and biochemical studies were undertaken to determine if amino acids that are universally conserved throughout the Rgg family contribute to the ability of RopB to regulate SpeB expression in S. pyogenes. The results confirmed the importance of these invariant residues and provided insight for understanding the functional domains of RopB and related transcription factors.

**Conserved Rgg residues are essential for RopB function.** A recent influx of genomic information has revealed that the Rgg-like proteins constitute a large conserved family of regulators unique to gram-positive bacteria. To obtain insight into the common structure and mechanism of action that have been adopted by this group of proteins, the primary amino acid sequences of all predicted Rgg-like proteins for which protein sequence data were available were aligned by the Clustal W method (32). Proteins were considered candidates for this analysis if they were significantly homologous (E-value, <0.001) to the prototype protein from S. gordonii (accession number AAA26968) as determined by a BLAST analysis (8) of the GenBank database of microbial genomes (blastp; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). If sequence data were available for multiple isolates of a species, only one isolate was selected for analysis in order to avoid redundancy.
This search returned 57 open reading frames, and the alignment revealed numerous highly conserved and very similar amino acids, including three amino acids that were invariable (Fig. 1A; see the supplemental material).

We reasoned that because these residues were the most conserved, they might make an important contribution to the structure and/or function of Rgg-like regulators. To test this hypothesis, each of the invariant residues in RopB (G4, R11, and W142) (Fig. 2) was targeted for site-directed mutagenesis, and the ability of the mutant protein to function as an activator of speB transcription was assessed using protease indicator plates and a quantitative assay for activity in culture supernatants, as described previously (16, 18, 21). An expression vector encoding wild-type RopB with a C-terminal hemagglutinin (HA) epitope tag was used as the template for inverse PCR with the primers described in Table 1 to construct mutant derivatives with alanine substitutions (RopBG4A, RopB R11A, and Rop W142A) or a more conservative mutation at W142 (RopBW142F). The resulting plasmids were used to transform wild-type S. pyogenes strain HSC5 (14) or a RopB H11002 mutant derivative (MNN100 [21]) to generate the strains described in Table 2.

As noted above, RopB is required for speB expression (4, 18); therefore, a strain with a deletion in ropB produced only background levels of proteolytic activity compared to the wild-type strain (Fig. 1, compare the RopB H11002 strain with no plasmid to the wild-type strain with no plasmid). When wild-type ropB was introduced into the RopB H11002 strain on a plasmid, the protease activity was restored to wild-type levels (Fig. 1B and C, compare the RopB H11002 strain with the wild-type allele to the wild-type strain with no plasmid). The behavior of a RopB H11002 strain expressing RopBG4A was similar to that of the wild-type complemented strain (Fig. 1B, compare the RopB H11002 strain with the wild-type allele to the RopB H11002 strain with the G4A allele).
and was indistinguishable from that of the wild-type strain (Fig. 1C, compare the RopB/H11002 strain with the G4A allele to the wild-type strain with no plasmid). In contrast, strains expressing RopB alleles modified at the arginine (R11A) and tryptophan (W142A or W142F) residues were completely nonfunctional when they were examined on protease indicator plates (Fig. 1B, compare the RopB/H11002 strain with the R11A, W142A, or W142F allele to the RopB/H11002 strain with the wild-type allele) and quantitatively (Fig. 1C) (P < 0.001).

When the relative transcript abundance of ropB and speB was determined by real-time reverse transcription-PCR performed as described elsewhere (2) using the primers listed in Table 1, we found that ropB transcription from a plasmid was increased relative to expression from the chromosome. However, the magnitudes of ropB overexpression were similar in all of the complemented RopB/H11002 strains (Fig. 2), and there was a correlation between protease activity and speB transcript abundance. In the strains that did not exhibit protease activity (e.g.,

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli strains</td>
<td></td>
<td></td>
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<tr>
<td>JL135</td>
<td>BL21(DE3)</td>
<td>pJL58</td>
<td>Expression of GST-RopB</td>
<td>This study</td>
</tr>
<tr>
<td>JL143</td>
<td>BL21(DE3)</td>
<td>PGEX</td>
<td>Expression of GST</td>
<td>Amersham 27-4587-01</td>
</tr>
</tbody>
</table>

S. pyogenes strains |
| HSC5 | Wild-type | None | Wild type | 14 |
| JL136 | Wild type | pLZ12 | HSC5 (vector) | This study |
| JL187 | Wild type | pL77 | HSC5(pRopB-HA) | This study |
| JL189 | Wild type | pL79 | HSC5(pRopB G4A-HA) | This study |
| JL191 | Wild type | pL92 | HSC5(pRopB R11A-HA) | This study |
| JL193 | Wild type | pL83 | HSC5(pRopB W142A-HA) | This study |
| JL216 | Wild type | pL105 | In-frame deletion of ropB | This study |
| MNN100 | HSC5 ropB<sub>54–274</sub> | none | MNN100 (vector) | 21; this study |
| JL410 | HSC5 ropB<sub>54–274</sub> | pLZ12 | MNN100(pRopB-HA; Km<sup>3</sup>) | This study |
| JL139 | HSC5 ropB<sub>54–274</sub> | pL60 | MNN100(pRopB-HA; Km<sup>3</sup>) | This study |
| JL207 | HSC5 ropB<sub>54–274</sub> | pL77 | MNN100(pRopB-HA) | This study |
| JL209 | HSC5 ropB<sub>54–274</sub> | pL79 | MNN100(pRopB G4A-HA) | This study |
| JL211 | HSC5 ropB<sub>54–274</sub> | pL92 | MNN100(pRopB R11A-HA) | This study |
| JL213 | HSC5 ropB<sub>54–274</sub> | pL83 | MNN100(pRopB W142A-HA) | This study |
| JL218 | HSC5 ropB<sub>54–274</sub> | pL105 | MNN100(pRopB W142F-HA) | This study |

 Gene designations are based on annotation of the SF370 genome of S. pyogenes.
the RopB\(^{-}\) strains with the R11A, W142A, and W142F alleles) the level of the speB transcript was reduced nearly 100,000-fold compared to the level in the wild type (Fig. 2). Thus, these data indicate that the mutations rendered RopB incompetent as a transcriptional activator. Although RopB\(_{G4A}\) was competent for promoting the production of protease activity, the allele was not as efficient at promoting speB transcription as the wild-type allele, as shown by a twofold decrease when the complemented RopB\(^{-}\) strain with the G4A allele was compared to the wild type (Fig. 2) \((P < 0.01)\) or the fivefold decrease observed when the RopB\(^{-}\) strain complemented with RopB\(_{G4A}\) was compared with the RopB\(^{-}\) strain complemented with RopB\(_{WT}\) (Fig. 2) \((P < 0.01)\).

The relative expression and stability of the wild-type and mutant epitope-tagged proteins in the RopB\(^{-}\) strain were assessed by Western blotting of whole-cell extracts (26) using polyclonal anti-HA antiserum (Sigma). This analysis revealed that RopB\(_{W142A}\) was probably unstable; however, substitution of another aromatic amino acid at position 142 and alanine substitutions at other positions were tolerated (Fig. 3A), indicating that the tryptophan contributes to both the stability of RopB and its ability to regulate gene expression. Introduction of alanine at position 142 did create a rare codon (7), raising the possibility that the reduced level of detectable RopB\(_{W142A}\) in streptococcal extracts was the result of translational inefficiency rather than protein instability. This interpretation is less likely, however, since expression of RopB\(_{R11A}\), which used the same rare codon, was efficient. Furthermore, a Western blot analysis of E. coli extracts was consistent with the S. pyogenes data (not shown), indicating that failure to detect RopB\(_{W142A}\) was due to a general problem rather than codon bias. Taken together, these data demonstrate that the conserved arginine (R11), tryptophan (W142), and, to a lesser extent, glycine (G4) are required for fully active RopB and are thus likely to be important for other Rgg-like regulators.

**Expression of nonfunctional RopB mutants inhibits native RopB function.** To obtain insight into the mechanism of RopB regulation, we examined whether the various mutants were dominant negative when they were expressed in trans. The RopB expression plasmids were used to transform the wild-type strain, and SpeB activity was measured on protease indicator plates (Fig. 1B) and quantitatively (Fig. 1C). As expected, overexpression of functional RopB had no significant effect on protease expression (Fig. 1B and C), compare the wild-type strain with no plasmid to the wild-type strain with the G4A and wild-type RopB alleles. In contrast, the presence of stable, nonfunctional RopB (R11A, W142F) was dominant negative, resulting in significantly reduced SpeB activity (Fig. 1C) \((P < 0.02)\). The stability of the dominant-negative proteins was confirmed by Western blot analysis (Fig. 3B). Taken together, these data suggest that RopB monomers may oligomerize or interact with another protein(s) in order to promote activation of speB transcription.

**Evidence for RopB dimerization in vitro.** The dominant-negative phenotype indicated that there was a potential for RopB-RopB interaction. This was not surprising, as it is typical for the functional unit of DNA-binding transcriptional regulators to be a dimer (17). It has been shown previously that RopB specifically interacts with DNA in the speB promoter that contains inverted repeats (21). In addition, analysis of conserved domains in the RopB primary amino acid sequence revealed a putative DNA-binding helix-turn-helix motif at the N terminus (20). Plasmid pJL58 encoding a glutathione S-transferase (GST) fusion to the N terminus of RopB, was constructed and expressed in E. coli using the pGEX-2TK expression vector (Amersham) and the primers described in Table 1. For expression and purification of the recombinant fusion protein and GST alone we followed the recommendations of the manufacturer (Amersham).

The fusion protein was used as bait for an interaction with proteins in a whole-cell extract (26) prepared from S. pyogenes expressing epitope-tagged RopB (RopB-HA). Glutathione-Sepharose beads were incubated with the GST-RopB fusion protein or GST and washed twice with phosphate-buffered saline prior to addition of streptococcal extracts. The extracts were incubated with the GST-fusion protein-coated beads and washed three times with phosphate-buffered saline, and bound proteins were eluted with reduced glutathione (10 mM) and subjected to immunoblotting with polyclonal anti-HA antiserum (Sigma). This analysis revealed that GST-RopB bound a protein which was the size of RopB-HA that reacted specifically with the anti-HA sera, indicating that there was a specific interaction between the two differentially tagged RopB derivatives (Fig. 4A). Western blot analysis showed that streptococcal extracts in the absence of RopB-HA did not react with the anti-HA antisera used in these studies (Fig. 3). Further characterization of purified GST-RopB and extracts containing RopB-HA demonstrated that a product with a molecular weight consistent with a dimer was detectable by Western blot analysis of unheated samples, and this product disappeared when samples were heated in sample buffer prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4B and data not shown). Taken together, these data provide evidence that RopB forms homodimers in vitro, consistent with data for other proteins that bind DNA via a helix-turn-helix motif. These data also support a model of RopB dimer-dimer interaction or another oligomeric complex that includes a RopB-RopB interaction.
FIG. 4. Evidence of in vitro RopB dimerization. (A) RopB-HA from streptococcal whole-cell lysates was specifically bound by a GST fusion to RopB (GST-RopB). Extracts from S. pyogenes strain JL139 [RopB (pRopB-HA)] were incubated with GST or GST-RopB, and specifically bound proteins were resolved by SDS-PAGE and immunoblotted with HA-specific antibody (αHA). The flowthrough fraction (FT) contained proteins that did not bind the immobilized GST bait, and the elution fraction (E) contained the GST bait and bound proteins. (B) A whole-cell lysate of S. pyogenes strain JL139 was prepared and heated for 10 min at 95°C or not treated prior to resolution by SDS-PAGE and immunoblotting with HA-specific antibody. The positions of molecular weight standards are indicated on the left in panel A, and the positions of bands corresponding to the expected sizes of a RopB-HA monomer and dimer (280 kDa) are indicated between the panels.

Implications for the Rgg protein family. In the present report, we show that common Rgg-like sequence motifs are essential for RopB function, which may indicate a common mechanism used by members of the Rgg-like family to process regulatory signals. The mechanism of Rgg family regulation has yet to be fully elucidated, but the data indicate that it likely involves interaction with both DNA and other proteins. For example, two of the invariant residues, G4 and R11, are located within the predicted DNA-binding domain. While the role of glycine has not been well studied, structural analysis of other XRE-type helix-turn-helix domains has revealed that arginine in this position contributes to the stability of the DNA-protein complex through formation of a fold-stabilizing salt bridge with a conserved glutamic acid residue (22) or side chain contacts with phosphate groups in target DNA (11, 36–38). Invariant tryptophan residues are often involved in protein-protein interactions (19), and W142 may localize to the DNA-protein complex through formation of a fold-stabilizing salt bridge with a conserved glutamic acid residue (22) or side chain contacts with phosphate groups in target DNA (11, 36–38). Invariant tryptophan residues are often involved in protein-protein interactions (19), and W142 may localize to the DNA-protein complex through formation of a fold-stabilizing salt bridge with a conserved glutamic acid residue (22) or side chain contacts with phosphate groups in target DNA (11, 36–38).

We thank M. Neely for providing strain MNN100 (21). We also thank Travis Jewett and L. David Sibley for providing reagents and technical advice. This work was supported by Public Health Service grant AI4643303 to Michael G. Caparon.

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