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Contribution of Invariant Residues to the Function of Rgg Family Transcription Regulators[∇][‡]

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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.

The Rgg-like regulators constitute a conserved family of proteins that modulate transcription in gram-positive bacteria. This group is widely distributed, and the members occur in both pathogenic and commensal species and include Rgg of *Streptococcus gordonii*, which is required for extracellular glucosyltransferase expression (29, 30); GadR of *Lactococcus lactis*, which is required for glutamate-dependent acid tolerance (27); MutR, which is required for expression of the mutacin lantibiotic, MutA, of *Streptococcus mutans* (23); and the plasmid-encoded LasX protein of *Lactobacillus sakei*, which regulates the synthesis of and immunity to the lantibiotic lactocin S (25, 28). Additional uncharacterized Rgg-like proteins are encoded by the genomes of *Streptococcus pneumoniae* (31), *Streptococcus agalactiae* (13), *Streptococcus oralis* (10), *Streptococcus sanguis* (34), *Streptococcus equi* (http://www.sanger.ac.uk), and *Listeria monocytogenes* (12), and some genomes, like those of *S. pyogenes* (9), *S. gordonii* (15, 33), *S. pneumoniae* (31), and *S. mutans* (1), contain multiple *rgg*-like genes.

How the members of this extensive family function to regulate gene expression is not well understood. Rgg-like proteins have a helix-turn-helix motif in the amino terminus of the polypeptide, which is a conserved DNA-binding domain found in other families of transcription regulators (17). Only recently has it been established that any Rgg-like proteins bind specifically to DNA to regulate transcription. For example, association with nucleic acid has been demonstrated for Rgg of *S. gordonii* (35), RopB (21), and LasX (24), but there is only a weak consensus binding site (24). The absence of a conserved regulatory motif in the promoter regions of genes regulated by Rgg-like proteins and the functional diversity of the regulated gene products suggest that Rgg-like proteins interact with additional regulatory networks to alter gene expression. Experimental data supporting this hypothesis were obtained in an analysis of the *speB* regulatory program in *S. pyogenes*, where

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.
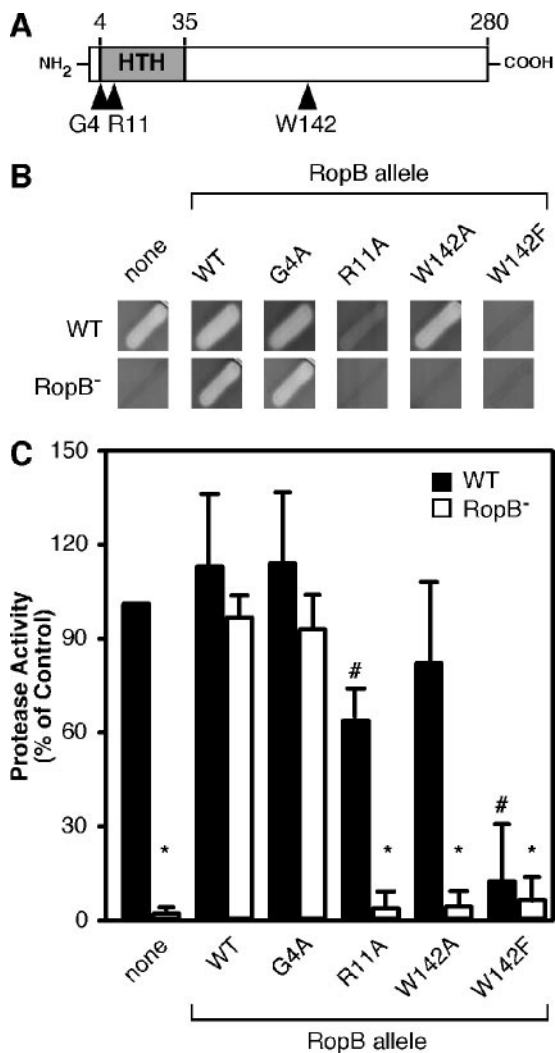
FIG. 1. Mutation of invariant residues results in functional deficiency and a dominant-negative phenotype. (A) Schematic diagram of RopB. The putative N-terminal helix-turn-helix (HTH) motif is shaded, and the invariant residues are indicated by arrows; the numbering is based on the RopB sequence (accession number NP_269988). (B) Proteolytic activities of various strains. The strains compared were the wild-type and *ropB* deletion (RopB⁻) strains (as indicated on the left) with no plasmid (none) or expressing a RopB allele (as indicated at the top). The results for a montage of colonies patched onto protease indicator plates are shown, and protease activity is indicated by a clear zone surrounding bacterial growth. (C) Quantitative protease activity with a fluorescein isothiocyanate-casein substrate in 8-h culture supernatant fluids. The activity is expressed as a percentage of the activity obtained for the wild-type strain with no plasmid, and the data are the means and standard deviations of at least three independent experiments with samples analyzed in triplicate. Statistically significant differences from the wild-type allele for the RopB⁻ and wild-type strains were calculated by using the unpaired Student *t* test and are indicated by asterisks ($P < 0.001$) and number signs ($P < 0.02$), respectively. WT, wild type.

FIG. 2. Mutant RopB proteins are defective for activation of *speB* transcription. The transcript abundance of *ropB* or *speB* in RopB⁻ bacteria expressing the RopB alleles indicated was determined by real-time reverse transcription-PCR performed as described previously (2). Bacteria were grown to the onset of the stationary phase in C medium for sample collection. The transcript abundance is expressed relative to the abundance in the wild-type strain with no plasmid, and the data are means and standard deviations of three independent experiments with samples analyzed in triplicate. An asterisk indicates a significant difference ($P < 0.01$) from the RopB⁻ strain expressing the wild-type allele; the arrows indicate a comparison of the *speB* transcript abundance in the RopB⁻ strain expressing the wild-type allele and the *speB* transcript abundance in the strain expressing the G4A allele ($P < 0.01$). WT, wild type.

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).

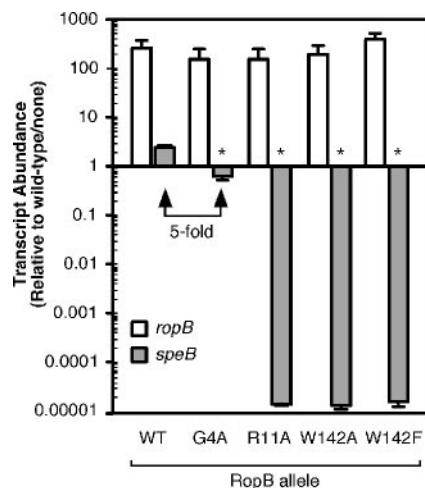We reasoned that because these residues were the most conserved, they might make an important contribution to the structure and/or function of Rgg-like regulators. To test this hypothesis, each of the invariant residues in RopB (G4, R11, and W142) (Fig. 2) was targeted for site-directed mutagenesis, 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 (RopB_G4A, RopB_R11A, and Rop_W142A) or a more conservative mutation at W142 (RopB_W142F). The resulting plasmids were used to transform wild-type *S. pyogenes* strain HSC5 (14) or a RopB⁻ 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⁻ strain with no plasmid to the wild-type strain with no plasmid). When wild-type *ropB* was introduced into the RopB⁻ strain on a plasmid, the protease activity was restored to wild-type levels (Fig. 1B and C, compare the RopB⁻ strain with the wild-type allele to the wild-type strain with no plasmid). The behavior of a RopB⁻ strain expressing RopB_G4A was similar to that of the wild-type complemented strain (Fig. 1B, compare the RopB⁻ strain with the wild-type allele to the RopB⁻ strain with the G4A allele)

TABLE 1. Primers used in this study

Function	Primer	Sequence[a]
RopB G4A mutagenesis	JLP47	ATGGA<u>AATTGC</u>TGAGACCGTTGAATTCATTAGGC
	JLP48	ATTCAAC<u>GGTCT</u>CAGCAATTTCCATATGTCAAG
RopB R11A mutagenesis	JLP49	TTCATT<u>GCGCA</u>TTCAAAAAACATTTCG
	JLP50	TTGAA<u>TGCGCA</u>ATGAATTCAACGG
RopB W142A mutagenesis	JLP51	CTTATT<u>AATATT</u>GAGACCGCGAGTCACTATGAGACTG
	JLP52	GACTCGC<u>GGTCT</u>CAATATTAATAAGATAGTTTG
RopB W142F mutagenesis	JLP53	TTATT<u>AATATT</u>GAGACCTTTAGTCACTATGAGACTG
	JLP54	CATAGTGACTAAA<u>GGTCT</u>CAATATTAATAAG
RopB-HA expression	JLP1	CATTCAACT<u>GGTCT</u>CGAATTAGGTACATGC
	JLP103	AAGCT<u>CTGCAG</u>TTA**AGCATAATCTGGAACATCATATGGATA**GGACAGTTTATGTTTAATG
Primers for pGEX-2TK/RopB	JLP99	GGCT<u>GGATCC</u>ATGGAAATTGGTGAAACC
	JLP100	TTAA<u>GGATCC</u>TCAGGACAGTTTATGTTTAATGGC
Real-time primers for *recA*	JLP32	AAGCCCTTGATGATGCTTTG
	JLP33	GGATAACCACCAGCTCCAAG
Real-time primers for *speB*	JLP17	GTCGGTAAAGTAGGCGGACA
	JLP18	GCCACCAGTACCAAGAGCTG
Real-time primers for *ropB*	JLP29	TGAACGGTGTTGTGTGTCTTT
	JLP30	TGGATCGTTTTGCAATTGAG

[a] Engineered restriction sites are underlined. The sequence for the influenza hemagglutinin epitope tag is indicated by boldface type.

and was indistinguishable from that of the wild-type strain (Fig. 1C, compare the RopB⁻ 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⁻ strain with the R11A, W142A, or W142F allele to the RopB⁻ 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⁻ 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 2. Strains used in this study[a]

Strain	Genotype	Plasmid	Description	Source or reference(s)
E. coli strains				
JL135	BL21(DE3)	pJL58	Expression of GST-RopB	This study
JL143	BL21(DE3)	PGEX	Expression of GST	Amersham 27-4587-01
S. pyogenes strains				
HSC5	Wild-type	None	Wild type	14
JL364	Wild type	pLZ12	HSC5 (vector)	This study
JL187	Wild type	pJL77	HSC5(pRopB-HA)	This study
JL189	Wild type	pJL79	HSC5(pRopB G4A-HA)	This study
JL191	Wild type	pJL92	HSC5(pRopB R11A-HA)	This study
JL193	Wild type	pJL83	HSC5(pRopB W142A-HA)	This study
JL216	Wild type	pJL105	HSC5(pRopB W142F-HA)	This study
MNN100	HSC5 *ropB*$_{\Delta6\text{-}274}$	none	In-frame deletion of *ropB*	21
JL410	HSC5 *ropB*$_{\Delta6\text{-}274}$	pLZ12	MNN100 (vector)	21; this study
JL139	HSC5 *ropB*$_{\Delta6\text{-}274}$	pJL60	MNN100(pRopB-HA; Km^r)	This study
JL207	HSC5 *ropB*$_{\Delta6\text{-}274}$	pJL77	MNN100(pRopB-HA)	This study
JL209	HSC5 *ropB*$_{\Delta6\text{-}274}$	pJL79	MNN100(pRopB G4A-HA)	This study
JL211	HSC5 *ropB*$_{\Delta6\text{-}274}$	pJL92	MNN100(pRopB R11A-HA)	This study
JL213	HSC5 *ropB*$_{\Delta6\text{-}274}$	pJL83	MNN100(pRopB W142A-HA)	This study
JL218	HSC5 *ropB*$_{\Delta6\text{-}274}$	pJL105	MNN100(pRopB W142F-HA)	This study

[a] Gene designations are based on annotation of the SF370 genome of *S. pyogenes*.
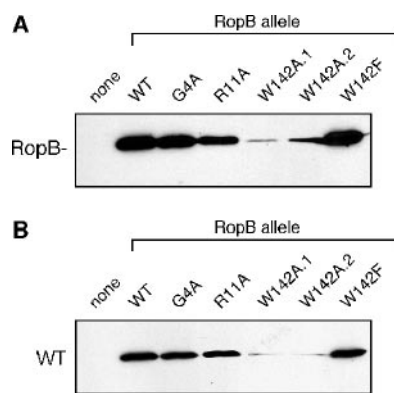
A

B

FIG. 3. Conserved tryptophan contributes to RopB protein stability and regulation: Western blot analyses of ectopically expressed RopB in *S. pyogenes* whole-cell lysates. The strains used were the strains described in the legend to Fig. 1; two independent isolates of the strains expressing RopB$_{W142A}$ were analyzed. Equivalent amounts of total protein were loaded in the lanes. WT, wild type.

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 antisera recognizing GST (1:10,000 dilution; Amersham) or HA (1:5,000 dilution; Sigma). In contrast to GST alone, the 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.
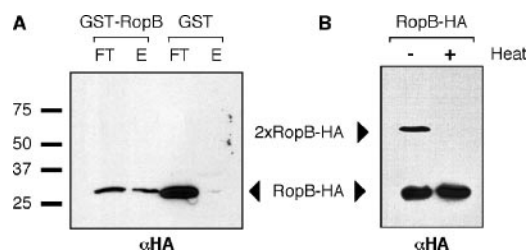
FIG. 4. Evidence of in vitro RopB dimerization. (A) RopB-HA from streptococcal whole-cell lysates was specifically bound by a GST fusion to RopB (GST-RopB). Extracts from *S. pyogenes* strain JL139 [RopB⁻(pRopB-HA)] were incubated with GST or GST-RopB, and specifically bound proteins were resolved by 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 (2×RopB-HA) 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 interface of a critical RopB-protein complex. Substitution of a nonhydrophobic residue can interfere with complex formation, resulting in an unstable protein. In this context, the decreased stability of RopB$_{W142A}$ is not surprising. Also consistent with this, we obtained evidence that RopB forms protein complexes in vivo through generation of dominant-negative mutant alleles and in vitro through pull-down assays with purified protein. These studies provide a foundation for investigation of the molecular mechanism of *speB* regulation and general insights into the specificity and functional domains of the family of Rgg-like proteins.

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