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Genetic and phenotypic characterization of the RyhB regulon in *Salmonella* Typhimurium

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ABSTRACT

Salmonella encodes two homologs of RyhB, a small RNA (sRNA) involved in iron homeostasis. In *Salmonella* Typhimurium, the expression of both RyhB-1 and RyhB-2 is negatively regulated by the Fur repressor, while stationary phase is the primary signal inducing RyhB-2 expression. To identify the target mRNAs of RyhB-1 and RyhB-2, 9 predicted target genes were analyzed by quantitative RT-PCR to monitor differential transcript levels between wild type and each of three mutants (Δ*ryhB-1*, Δ*ryhB-2* and Δ*ryhB-1*Δ*ryhB-2*) under conditions that maximize the expression of both sRNAs. Our results, along with bioinformatic predictions, suggest that the genes *acnA*, *sodB*, *ftn*, *STM1273.1n*, and *acnB* are the primary targets of at least one of these sRNAs. To understand the biological roles of the RyhB regulon, the aforementioned deletions were created in either wild type or Δ*fur* backgrounds and were subjected to various phenotypic assays. The results showed that these sRNAs are singularly or additively involved in the expression of multiple phenotypes, including acid resistance, resistance to hydrogen peroxide, and sensitivity to bactericidal antibiotics. The results support a model whereby RyhB-1 and RyhB-2 have a global regulatory effect on diverse cellular pathways in response to multiple environmental cues via post-transcriptional regulation of distinct sets of overlapping targets.

1. Introduction

Small noncoding RNAs (sRNAs) are widespread in bacteria and function as regulators of diverse cellular pathways. Most sRNAs interact with specific target mRNA transcripts through imperfect base-pairing. The transcription of sRNAs is often regulated by environmental signals and the produced sRNAs act on target mRNAs to regulate either transcript stability or protein synthesis at a post-transcriptional level (Waters and Storz 2009). Although protein-based regulation appears to be the primary regulatory mechanism in bacteria, the prevalence of sRNA-mediated regulation implies a unique and distinctive role for sRNAs in the complex global regulatory system of bacteria that differs from the regulation by protein regulatory factors. Indeed, both bioinformatic and experimental analyses show several aspects of sRNA-mediated regulation that are distinct from those of protein-based regulation (Levine et al. 2007; Mitarai et al. 2007, 2009; Shimoni et al. 2007). However, the details of sRNA-mediated regulation continue to be explored for its unique contribution to regulatory networks in bacteria.

In the human bacterial pathogen *Salmonella* Typhimurium, at least 70 sRNAs have been predicted and validated to date (Sittka et al. 2008; Vogel 2009). For the majority of these sRNAs, the biological function and mRNA targets remain unknown. In *S.* Typhimurium, several sRNAs, including CyaR, GcvB, InvR, IsrJ, RybB, MicA, RyhB, CsrB, and CsrC, have been characterized for their biological implications beyond confirmation of transcript messages (Vogel 2009). RyhB is of particular interest in many aspects: RyhB was first discovered in *Escherichia coli* as an abundant sRNA species (Wassarman et al. 2001). RyhB is a member of the Fur (Ferric Uptake Regulator) regulon and therefore is repressed by the iron-activated Fur repressor in *E. coli*. Additionally, RyhB was found to be the missing link that mediates the positive regulation of certain genes in *E. coli* by the Fur repressor (Massé and Gottesman 2002). In the presence of high iron concentrations, the activated Fur represses transcription of genes whose promoter contains the consensus FUR box sequence. Since most mRNAs targeted by RyhB are repressed at the translation level, these target genes are positively regulated by the activated Fur repressor through RyhB. Sequence homologs of *E. coli* RyhB have been found in many closely related bacteria, including *Salmonella*, *Klebsiella*, *Yersinia pestis* (Massé and

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Table 1
Bacterial strains and plasmids used in this study.

Strain and plasmid[a]	Genotype or relevant characteristics	Source or reference
***Salmonella* Typhimurium**		
14028	*Salmonella* Typhimurium	ATCC[b]
JS566	14028 Δ*fur-42*::*tetAR*	Ellermeier and Slauch (2008)
JS569	14028 Δ*rfrB-2*::Cm (=Δ*ryhB-2*::Cm)	Ellermeier and Slauch (2008)
ST193	14028 pKD46	This work
ST237	14028 Δ*ryhB-1*::Km	This work
ST279	14028 Δ*fur-42*::*tetAR*, Δ*ryhB-1*::Km	This work
ST280	14028 Δ*fur-42*::*tetAR*, Δ*ryhB-2*::Cm	This work
ST282	14028 Δ*ryhB-1*::Km Δ*ryhB-2*::Cm	This work
ST285	14028 Δ*fur-42*::*tetAR*, Δ*ryhB-1*::Km, Δ*ryhB-2*::Cm	This work
ST289	14028 Δ*fur*::Km	This work
ST290	14028 Δ*ryhB-2*::Km	This work
ST372	14028 Δ*fur-42*::*tetAR*, Δ*ryhB-1*::Km, pJXK11	This work
ST374	14028 Δ*fur-42*::*tetAR*, Δ*ryhB-2*::Cm, pJXK12	This work
Plasmid		
pKD4	*bla* FRT *ahp* FRT PS1 PS2 oriR6K	Datsenko and Wanner (2000)
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS	Datsenko and Wanner (2000)
pJXK11	*ryhB-1* complementation plasmid	This work
pJXK12	*ryhB-2* complementation plasmid	This work

[a] All strains are isogenic derivatives of ATCC 14028 (see Section 2).
[b] ATCC, American Type Culture Collection.

Gottesman 2002), *Vibrio cholera*, *Photobacterium profundum* (Mey et al. 2005), *Vibrio vulnificus* (Alice et al. 2008), and *Erwinia chrysanthemi* (Boughammoura et al. 2008). All of the aforementioned RyhB homologs in these genera share the common feature of mediating positive regulation of their target genes by the Fur repressor and contributing to iron homeostasis as a member of the Fur regulon, except those in *Klebsiella*, *Yersinia*, and *Photobacterium*, which have yet to be studied.

Various phenotypes associated with the RyhB regulon have been reported in bacteria expressing RyhB homologs. In *E. coli*, growth defects of a *fur* deletion mutant solely relying on either pyruvate, succinate, or fumarate as the sole carbon source were restored when a second deletion in *ryhB* was introduced (Massé and Gottesman 2002). This observation suggests that the gene(s) required for growth on those carbon sources are the target(s) of RyhB-mediated repression in *E. coli*. Also, RyhB was shown to be essential for normal growth and survival during iron starvation in *E. coli* (Jacques et al. 2006). Bollinger and Kallio (2007) reported that acetate production is significantly decreased in the *E. coli ryhB* deletion mutant (Bollinger and Kallio 2007). A RyhB deletion of *V cholera* shows reduced chemotactic motility in low-iron medium and is unable to form a wild type biofilm (Mey et al. 2005). A RyhB homolog is essential to the virulence of *V. vulnificus* in an iron-loaded mouse infection model (Alice et al. 2008). In *Shigella flexneri*, the defect in acid resistance in Δ*fur* mutant was restored by the addition of a second deletion of *ryhB*, indicating gene(s) essential for acid resistance are translationally repressed by RyhB-1 (Oglesby et al. 2005).

Interestingly, functional homologs of RyhB with no sequence homology to *E. coli* RyhB have been identified in many other bacteria, including *Pseudomonas aeruginosa* (Wilderman et al. 2004), *Neiserria meningitis* (Mellin et al. 2007), *Bacillus subtilis* (Gaballa et al. 2008), and *Azotobacter vinelandii* (Jung and Kwon 2008). These functional homologs vary greatly in their DNA sequence, genomic location, mode of action, mRNA targets, and copy number. Nevertheless, they all share the common functional feature of mediating positive regulation of their target genes by the Fur repressor and contribute to iron homeostasis.

Although certain details of the RyhB regulon in *Salmonella* have been reported (Ellermeier and Slauch 2008; Padalon-Brauch et al. 2008), RyhB-mediated regulation has not been the subject of thorough investigation in *S.* Typhimurium. *S.* Typhimurium has two homologs of RyhB, termed RyhB-1 (96 nt) and RyhB-2 (98 nt), located separately on the genome. Previous study showed that RyhB-1 is strongly induced upon iron-depletion *in vivo* (Padalon-Brauch et al. 2008). The presence of the Fur box in the promoter region of *ryhB-1*, explains the Fur-mediated transcriptional regulation of RyhB-1. It was also shown that RyhB-1 was slightly induced by hydrogen peroxide (Padalon-Brauch et al. 2008). On the contrary, RyhB-2 expression is also slightly induced by activated Fur, but its primary inducing condition is the stationary growth phase. Interestingly, both RyhB-1 and RyhB-2 are strongly induced during macrophage infection (Padalon-Brauch et al. 2008), suggesting their roles during macrophage infection.

In this study, we attempted to characterize the regulatory mechanisms and biological roles of the RyhB regulon in *S.* Typhimurium in an effort to understand this complicated regulatory system mediated by two homologous sRNAs.

2. Materials and methods

2.1. Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are summarized in Table 1. *S.* Typhimurium ATCC 14028 was obtained from American Type Culture Collection (ATCC; Manassas, VA). The *fur* mutant (JS566) and *ryhB-2* mutant (JS569) were obtained from Dr. Slauch (University of Illinois, Urbana Champaign). For the generation of isogenic mutant strains, single deletion mutants (ST237, ST289 and ST290) were constructed using the λ Red recombination system (Datsenko and Wanner 2000) using olgonucleotides described in Table 2, followed by P22 transduction into a wild type background. All double (ST279, ST280 and ST282) or triple deletion mutants (ST285) were constructed by transferring the appropriate deletion(s) using P22 transduction. In order to construct the complementation plasmids, RyhB-1(121-bp long) and RyhB-2 (127-bp long) sequences were PCR-amplified using RyhB-1-*Eco*RI-FP & RyhB-1-*Xba*I-RP and RyhB-2-*Eco*RI-FP & RyhB-2-*Xba*I-RP primers, respectively. Following the gel-purification of the PCR products, the DNA fragments were digested with *Eco*RI and *Xba*I, and then cloned into plasmid pPROTet.E133 (Clontech) digested with the same restriction enzymes. The recombinant plasmids carrying either RyhB-1 or RyhB-2 genes were introduced into the respective single deletion (Δ*ryhB-1* or Δ*ryhB-2*) strains by electroporation.