In Vitro Identification of Rns-Regulated Genes

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To identify Rns-regulated genes, a maltose binding protein (MBP)-Rns fusion protein was used to isolate DNA fragments from enterotoxigenic Escherichia coli genomic DNA that carry Rns binding sites. In vivo transcription fusion analysis shows that Rns positively regulates the expression of the open reading frame of yiiS, which lies immediately downstream of one MBP-Rns-bound fragment.

In strains of enterotoxigenic Escherichia coli (ETEC) that produce CSI or CS2 pili, the expression of the pilin genes is positively regulated by Rns (2), a member of the AraC family of transcriptional regulators (6). In addition, Rns positively regulates its own expression (5). Rns binds upstream of the CSI pilin promoter, Peco, and the ms promoter, Pms, and at both promoters the upstream binding sites are required for full Rns-dependent transcription in vivo (15, 16). Downstream of Prns there are additional Rns binding sites, one of which is also required for positive autoregulation (16; G. P. Munson, L. G. Holcomb, and J. R. Scott, unpublished data).

Rns homologs have been identified in other strains of ETEC and in other bacterial enteric pathogens. These homologs include CfaR, CsVR, and FapR from ETEC (3, 4, 10). AggR from enteropathogenic E. coli (EPEC) (7, 29), and VirF from Shigella flexneri (22). Like Rns, regulators CfaR, CsVR, FapR, and AggR are required for the expression of pilin genes. However, Rns homologs regulate the expression of additional kinds of virulence genes as well. For example, in EPEC, PerA also regulates the expression of Ler, a second activator required for the expression of some virulence genes within the locus of enterocyte attachment and effacement (12, 24). In S. flexneri, VirF activates the expression of activator VirB (30) and icsA (virG), which is required for the polymerization of host cytosolic actin during cell-to-cell spread of the bacterium (11). Although ETEC strains are not thought to have homologs of Ler, VirB, or IcsA, these examples suggest that Rns may have additional regulatory targets that remain to be identified. Like pilin genes, other Rns-regulated genes may play important roles in ETEC pathogenesis. Therefore, the identification of these genes may provide a better understanding of ETEC virulence.

To isolate DNA fragments with Rns binding sites, a maltose binding protein (MBP)-Rns fusion protein was used to isolate DNA fragments from enterotoxigenic E. coli genomic DNA that carry Rns binding sites. In vivo transcription fusion analysis shows that Rns positively regulates the expression of the open reading frame of yiiS, which lies immediately downstream of one MBP-Rns-bound fragment.

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C921b-1 using primers designed from the published sequence of *E. coli* K-12. As little as 0.4 nM MBP-Rns was sufficient to produce complexes with the 227-bp DNA fragment (Fig. 2B). With increasing concentrations of MBP-Rns, the original shifted band was replaced by a second, lower-mobility complex (Fig 2B). A 292-bp fragment from the *yiiST* region has no predicted Rns binding sites and was used as a negative control. With this fragment, no complexes were seen until a very high MBP-Rns concentration was used. With both DNA fragments, high concentrations of MBP-Rns produced complexes that remained near the wells of the gel (Fig. 2B). These are most likely large aggregates formed by nonspecific protein-DNA interactions, which are expected at high protein concentrations. These results demonstrate that MBP-Rns binds to the...
region upstream of yiiS, nucleotides −307 to −81 (relative to the beginning of the yiiS open reading frame). Moreover, the observation of two complexes between MBP-Rns and the 227-bp yiiS fragment suggests that this fragment carries two Rns binding sites.

To determine if Rns affects the expression of yiiS in vivo, a yiiS-lacZ reporter prophage was constructed. Nucleotides −328 to +208, relative to the beginning of the yiiS gene, were amplified from chromosomal DNA of E. coli strain MC4100 (25) by PCR using primers yiiR-EcoRI (5′CGTGAATTCTG GTGATGATGCTTATCGATC) and yiiS-BamHI (5′TCCGG ATCCCTAATCTTATAGGCCACGCTTG). DNA sequence analysis revealed that there are no differences between the published sequences of E. coli K-12 and ETEC strain C921b-1 in this region. The resulting 540-bp product was digested with EcoRI and BamHI and ligated into the same sites of promoterless lacZ reporter vector pRS551 (26) to produce plasmid pEU2339. The yiiS-lacZ reporter fusion of pEU2339 was then crossed into χRS45 (26) by homologous recombination as previously described (15) to produce reporter phage χEU2339. PCR analysis indicated that the λ attP site was interrupted and thereby showed that only a single prophage had integrated at the chromosomal attB site.

Expression of β-galactosidase was measured in E. coli K-12 strain MC4100(AEU2339 yiiS-lacZ) transformed with low-copy-number plasmid pEU2040, which carries rns expressed from its own promoter (20), or with vector pHSG576 (28) as a control. The resulting strains grew at similar rates (Fig. 3A), and expression of β-galactosidase increased as cells entered stationary phase (Fig. 3B). However, at all growth phases, strain MC4100(AEU2339 yiiS-lacZ)/pEU2040 expressed 1.6- to 2.3-fold more β-galactosidase than strain MC4100(AEU2339 yiiS-lacZ)/pHSG576. These results demonstrate that Rns positively regulates the expression of yiiS under these conditions although the increase in expression from the yiiS promoter in the presence of Rns is considerably less than that observed for other Rns-activated promoters (14–16). The increase in expression from the yiiS promoter is most likely the result of direct regulation by Rns because the MBP-Rns fusion protein bound to the region upstream of yiiS.

Both yiiS and yiiT, which begins 26 bp downstream of yiiS, have been characterized as part of an investigation of the stress-induced regulon of E. coli K-12 (N. Gustavsson, A. Dies, and T. Nyström, submitted for publication). YiiT has significant homology with UspA of E. coli (36% identity and 46% similarity over its entire length). UspA expression is highly induced in growth-arrested cells regardless of arresting conditions and enhances cellular survival under the stress conditions that were tested (18, 19). Expression of yiiT is also likely to be positively regulated by Rns because yiiT is transcribed on a polycistronic message from the promoter upstream of yiiS, as well as from its own promoter (Gustavsson et al., submitted). Whether the positive regulation of these genes by Rns contributes to the virulence of ETEC remains to be investigated.

The region upstream of yiiS that is bound by MBP-Rns in vitro is highly conserved (>97% identity) among ETEC strain C921b-1, E. coli K-12 (1), both of the sequenced EHEC O157:H7 strains (8, 21), and genomes of S. flexneri 2a, E. coli K1 meningitis strain RS218, and E. coli uropathogenic strain CFT7073, which are currently being sequenced by the University of Wisconsin-Madison E. coli Genome Project (http://www.genome.wisc.edu). Of these, only S. flexneri is known to carry a regulator, VirF (22), that is functionally interchangeable with Rns (14). It is possible that the region upstream of yiiS will also be conserved in other pathogenic strains of E. coli and S. flexneri that carry homologs of Rns such as CfaR and CsvR of ETEC and AggR of enterogroupaggregative E. coli. Since the predicted DNA binding domains of these regulators are nearly identical to that of Rns and since they have been shown to be functionally interchangeable with each other and/or Rns (3, 14, 17, 31), it seems likely that yiiS and yiiT will also be part of the regulons of these Rns homologs.

Among other DNA fragments captured by the MBP-Rns affinity column, we identified a fragment containing the promoter region of aslA that was captured twice and that carries a site to which MBP-Rns binds with high affinity (data not shown). This gene, which is conserved in different strains of E. coli, is homologous to that encoding AtsA, an arylsulfatase of Klebsiella pneumoniae (9). AslA has been shown to be important for E. coli K1 to invade human brain microvascular endothelial cells and to cause meningitis in a neonatal rat model (9). Thus, it is possible that aslA will be found to be a Rns-regulated virulence factor.

The excess mortality caused by enteric bacterial pathogens
and the problems attendant on the use of antibiotics make it important to try to identify new targets for therapeutic approaches and vaccine development. As demonstrated here, the MBP-Rns DNA capture method adds a valuable tool to aid in the identification of these targets. By passing genomic DNA from different bacterial strains through the MBP-Rns affinity column, it should be possible to identify strain-specific genes within the regulon of any regulator that is closely related to Rns (14). Additionally, as for yiiSt and potentially asfA, genes that are highly conserved among many bacteria may also be part of the Rns regulon. If these conserved genes are shown to be important for virulence of many different bacterial pathogens, their identification may allow the development of a single therapy capable of combating diverse bacterial diseases.

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