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 CS1 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 CS1 pilin promoter, *P_{coo}*, and the *rns* promoter, *P_{rns}*, and at both promoters the upstream binding sites are required for full Rns-dependent transcription in vivo (15, 16). Downstream of *P_{rns}* 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sites, one of which is also required for positive autoregulation (16; G. P. Munson, L. G. Holcomb, and J. R. Scott, unpublished data).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, CsrR, and FapR from ETEC (3, 4, 10), AggR from enteroaggregative *E. coli* (17), PerA (BfpT) from enteropathogenic *E. coli* (EPEC) (7, 29), and VirF from *Shigella flexneri* (22). Like Rns, regulators CfaR, FapR, PerA, 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 affinity column was constructed by binding 5.4 nmol of purified MBP-Rns (15) via the MBP domain of the fusion protein to an amylose resin (New England Biolabs) in a 1.5-ml chromatography column equilibrated with binding buffer (10 mM Tris-Cl [pH 7.4], 50 mM KCl, 2 mM β -mercaptoethanol). Genomic DNA was isolated from ETEC

strain C921b-1 (O6:K15:H16) (27), which carries *rns* and expresses CS1 pili. The genomic DNA was digested to completion with *Sau3*AI, precipitated, and then suspended in binding buffer to a final concentration of 420 μ g ml⁻¹. A total of 2.7 mg of the digested DNA was passed through the MBP-Rns affinity column at a flow rate of 250 μ l min⁻¹ at 4°C. The column was then washed with 20 ml of binding buffer to remove unbound DNA. Bound DNA was eluted from the column by increasing the concentration of KCl in the binding buffer to 150 mM to disrupt Rns-DNA interactions (15). Eluant fractions that contained DNA, as determined by absorbance at 254 nm, were pooled and concentrated 10-fold by recovery on QIAquick PCR purification columns (Qiagen). The bound fragments were then ligated into the *Bam*HI site of cloning vector pUC19 (32) or pNEB193 (New England Biolabs) and transformed into *E. coli* strain DH5 α (23).

To determine the effectiveness of the MBP-Rns affinity column after just one round of selection, the cloned DNA fragments were recovered from 65 transformants and used in gel mobility assays with MBP-Rns as previously described (15). Some of the DNA fragments that were recovered from the MBP-Rns affinity column were not bound by MBP-Rns in this assay, and others, such as those carried by pEU2305, pEU2308, and pEU2312, required significantly more MBP-Rns to alter the DNA mobility than did *P_{rns}* DNA (Fig. 1). However some plasmids, such as pEU2304, were found to contain DNA fragments that were completely shifted by as little as 25 nM MBP-Rns (Fig. 1). The absence of the CS1 pilin promoter and *P_{rns}* (which have Rns binding sites near them) from this sample of 65 transformants suggests the need to screen more transformants to identify all of the genes of the Rns regulon and/or to perform multiple rounds of enrichment on the MBP-Rns affinity column.

Of the 65 plasmids that were screened by gel mobility assays, the apparent binding affinity of MBP-Rns was highest for the DNA fragment cloned into pEU2304 (Fig. 1 and data not shown); therefore it was characterized further. DNA sequence analysis revealed that pEU2304 carries three *Sau3*AI fragments from different regions of the *E. coli* chromosome. There are two potential Rns binding sites in one of these DNA fragments, which is 227 bp and which is from an intergenic region upstream of genes *yiiS* and *yiiT* (Fig. 2A). Additional gel mobility assays indicated that MBP-Rns binds to the region upstream of *yiiS*. Two *Sau3*AI fragments from the *yiiST* region (Fig. 2A) were amplified from genomic DNA of ETEC strain

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FIG. 1. Gel mobility assay of MBP-Rns binding to DNA fragments recovered from an MBP-Rns affinity column. The listed plasmids were digested with restriction enzymes to release their cloned inserts, which were then labeled with ³²P. The vector-derived 182-bp fragments were used as internal negative controls because they do not carry Rns binding sites. The 886-bp fragment released from pEU2087 was used as a positive control and carries the *ms* promoter and three Rns binding sites. Lanes 1, 2, 3, and 4 contain 0, 25, 50, and 100 nM MBP-Rns, respectively.

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

FIG. 2. Binding of MBP-Rns to the region upstream of *yiiS*. (A) The *yiiRST* region of *E. coli* K-12. Large arrows, open reading frames; small arrows, positions of predicted Rns binding sites (the direction indicates whether the consensus-like sequence (14) occurs on the coding or noncoding strand. *Sau*, *Sau*3AI. (B) Gel mobility assays to determine the relative affinities of MBP-Rns for the 227- and 292-bp *Sau*3AI fragments from the *yiiRST* region. Arrows, MBP-Rns complexes with DNA.

region upstream of *yjiS*, nucleotides -307 to -81 (relative to the beginning of the *yjiS* open reading frame). Moreover, the observation of two complexes between MBP-Rns and the 227-bp *yjiS* fragment suggests that this fragment carries two Rns binding sites.

To determine if Rns affects the expression of *yjiS* in vivo, a *yjiS-lacZ* reporter prophage was constructed. Nucleotides -328 to $+208$, relative to the beginning of the *yjiS* gene, were amplified from chromosomal DNA of *E. coli* strain MC4100 (25) by PCR using primers *yjiR-EcoRI* (5'-CGTGAATTCTG GTGATGATGCTTATCGATC) and *yjiS-BamHI* (5'-TCCGG ATCCTTAATCTTATAGGCCACGCTGG). 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 *yjiS-lacZ* reporter fusion of pEU2339 was then crossed into λ RS45 (26) by homologous recombination as previously described (15) to produce reporter phage λ EU2339. PCR analysis indicated that the λ *attP* site was interrupted and therefore 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(λ EU2339 *yjiS-lacZ*) transformed with low-copy-number plasmid pEU2040, which carries *ms* 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(λ EU2339 *yjiS-lacZ*)/pEU2040 expressed 1.6- to 2.3-fold more β -galactosidase than strain MC4100(λ EU2339 *yjiS-lacZ*)/pHSG576. These results demonstrate that Rns positively regulates the expression of *yjiS* under these conditions although the increase in expression from the *yjiS* 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 *yjiS* promoter is most likely the result of direct regulation by Rns because the MBP-Rns fusion protein bound to the region upstream of *yjiS*.

Both *yjiS* and *yjiT*, which begins 26 bp downstream of *yjiS*, have been characterized as part of an investigation of the stress-induced regulon of *E. coli* K-12 (N. Gustavsson, A. Dies, and T. Nystrom, submitted for publication). *YjiT* 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 *yjiT* is also likely to be positively regulated by Rns because *yjiT* is transcribed on a polycistronic message from the promoter upstream of *yjiS*, 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 *yjiS* 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 Univer-

FIG. 3. Rns regulation of *yjiS* in vivo. (A) Growth curves for *E. coli* strains MC4100(λ EU2339 *yjiS-lacZ*)/pEU2040 (circles) and MC4100(λ EU2339 *yjiS-lacZ*)/pHSG576 (squares) grown aerobically at 37°C in Luria-Bertani medium with 40 μ g of chloramphenicol ml $^{-1}$. Optical density of each culture was measured in the spectral range of 640 to 700 nm with a Klett-Summerson photoelectric colorimeter. Plasmid pEU2040 carries *ms* expressed from its own promoter and is a derivative of low-copy-number vector pHSG576. (B) Aliquots of each culture were removed at the times indicated and assayed for β -galactosidase activity as previously described (13). For each time point the means and standard deviations of three enzymatic determinations are shown. Shaded bars, MC4100(λ EU2339 *yjiS-lacZ*)/pEU2040; unshaded bars, MC4100(λ EU2339 *yjiS-lacZ*)/pHSG576.

sity 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 *yjiS* will also be conserved in other pathogenic strains of *E. coli* and *S. flexneri* that carry homologs of Rns such as CfaR and CsrR of ETEC and AggR of enteroaggregative *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 *yjiS* and *yjiT* 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 *yjiST* and potentially *aslA*, 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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REFERENCES

- Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277**:1453–1474.
- Caron, J., L. M. Coffield, and J. R. Scott. 1989. A plasmid-encoded regulatory gene, *ms*, required for expression of the CS1 and CS2 adhesins of enterotoxigenic *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **86**:963–967.
- Caron, J., and J. R. Scott. 1990. A *ms*-like regulatory gene for colonization factor antigen I (CFA/I) that controls expression of CFA/I pilin. *Infect. Immun.* **58**:874–878.
- de Haan, L. A., G. A. Willshaw, B. A. van der Zeijst, and W. Gastra. 1991. The nucleotide sequence of a regulatory gene present on a plasmid in an enterotoxigenic *Escherichia coli* strain of serotype O167:H5. *FEMS Microbiol. Lett.* **67**:341–346.
- Froehlich, B., L. Husmann, J. Caron, and J. R. Scott. 1994. Regulation of *ms*, a positive regulatory factor for pili of enterotoxigenic *Escherichia coli*. *J. Bacteriol.* **176**:5385–5392.
- Gallegos, M. T., R. Schleif, A. Bairoch, K. Hofmann, and J. L. Ramos. 1997. *Arac/XylS* family of transcriptional regulators. *Microbiol. Mol. Biol. Rev.* **61**:393–410.
- Gomez-Duarte, O. G., and J. B. Kaper. 1995. A plasmid-encoded regulatory region activates chromosomal *eaeA* expression in enteropathogenic *Escherichia coli*. *Infect. Immun.* **63**:1767–1776.
- Hayashi, T., K. Makino, M. Ohnishi, K. Kurokawa, K. Ishii, K. Yokoyama, C. G. Han, E. Ohtsubo, K. Nakayama, T. Murata, M. Tanaka, T. Tobe, T. Iida, H. Takami, T. Honda, C. Sasakawa, N. Ogasawara, T. Yasunaga, S. Kuhara, T. Shiba, M. Hattori, and H. Shinagawa. 2001. Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12. *DNA Res.* **8**:11–22.
- Hoffman, J. A., J. L. Badger, Y. Zhang, S. H. Huang, and K. S. Kim. 2000. *Escherichia coli* K1 *aslA* contributes to invasion of brain microvascular endothelial cells in vitro and in vivo. *Infect. Immun.* **68**:5062–5067.
- Klaasen, P., and F. K. de Graaf. 1990. Characterization of FapR, a positive regulator of expression of the 987P operon in enterotoxigenic *Escherichia coli*. *Mol. Microbiol.* **4**:1779–1783.
- Lett, M. C., C. Sasakawa, N. Okada, T. Sakai, S. Makino, M. Yamada, K. Komatsu, and M. Yoshikawa. 1989. *virG*, a plasmid-coded virulence gene of *Shigella flexneri*: identification of the *virG* protein and determination of the complete coding sequence. *J. Bacteriol.* **171**:353–359.
- Mellies, J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper. 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Mol. Microbiol.* **33**:296–306.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Munson, G. P., L. G. Holcomb, and J. R. Scott. 2001. Novel group of virulence activators within the AraC family that are not restricted to upstream binding sites. *Infect. Immun.* **69**:186–193.
- Munson, G. P., and J. R. Scott. 1999. Binding site recognition by Rns, a virulence regulator in the AraC family. *J. Bacteriol.* **181**:2110–2117.
- Munson, G. P., and J. R. Scott. 2000. Rns, a virulence regulator within the AraC family, requires binding sites upstream and downstream of its own promoter to function as an activator. *Mol. Microbiol.* **36**:1391–1402.
- Nataro, J. P., D. Yikang, D. Yingkang, and K. Walker. 1994. AggR, a transcriptional activator of aggregative adherence fimbria I expression in enteroaggregative *Escherichia coli*. *J. Bacteriol.* **176**:4691–4699.
- Nystrom, T., and F. C. Neidhardt. 1992. Cloning, mapping and nucleotide sequencing of a gene encoding a universal stress protein in *Escherichia coli*. *Mol. Microbiol.* **6**:3187–3198.
- Nystrom, T., and F. C. Neidhardt. 1994. Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. *Mol. Microbiol.* **11**:537–544.
- Perez-Casal, J., J. S. Swartley, and J. R. Scott. 1990. Gene encoding the major subunit of CS1 pili of human enterotoxigenic *Escherichia coli*. *Infect. Immun.* **58**:3594–3600.
- Perna, N. T., G. Plunkett III, V. Burland, B. Mau, J. D. Glasner, D. J. Rose, G. F. Mayhew, P. S. Evans, J. Gregor, H. A. Kirkpatrick, G. Posfai, J. Hackett, S. Klink, A. Boutin, Y. Shao, L. Miller, E. J. Grothbeck, N. W. Davis, A. Lim, E. T. Dimalanta, K. D. Potamouis, J. Apodaca, T. S. Anantharaman, J. Lin, G. Yen, D. C. Schwartz, R. A. Welch, and F. R. Blattner. 2001. Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* **409**:529–533.
- Sakai, T., C. Sasakawa, S. Makino, and M. Yoshikawa. 1986. DNA sequence and product analysis of the *virF* locus responsible for Congo red binding and cell invasion in *Shigella flexneri* 2a. *Infect. Immun.* **54**:395–402.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Sanchez-SanMartin, C., V. H. Bustamante, E. Calva, and J. L. Puente. 2001. Transcriptional regulation of the *orf19* gene and the *tir-cesT-eae* operon of enteropathogenic *Escherichia coli*. *J. Bacteriol.* **183**:2823–2833.
- Silhavy, T. J., M. L. Berman, and L. W. Enquist. 1984. Experiments with genes fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Simons, R. W., F. Houtman, and N. Kleckner. 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**:85–96.
- Smyth, C. J. 1982. Two mannose-resistant haemagglutinins on enterotoxigenic *Escherichia coli* of serotype O6:K15:H16 or H-isolated from travellers' and infantile diarrhoea. *J. Gen. Microbiol.* **128**:2081–2096.
- Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh. 1987. High-copy-number and low-copy-number plasmid vectors for *lacZ* alpha-complementation and chloramphenicol- or kanamycin-resistance selection. *Gene* **61**:63–74.
- Tobe, T., G. K. Schoolnik, I. Sohel, V. H. Bustamante, and J. L. Puente. 1996. Cloning and characterization of *bfpTVW*, genes required for the transcriptional activation of *bfpA* in enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **21**:963–975.
- Tobe, T., M. Yoshikawa, T. Mizuno, and C. Sasakawa. 1993. Transcriptional control of the invasion regulatory gene *virB* of *Shigella flexneri*: activation by *virF* and repression by H-NS. *J. Bacteriol.* **175**:6142–6149.
- Willshaw, G. A., H. R. Smith, M. M. McConnell, and B. Rowe. 1991. Cloning of regulator genes controlling fimbrial production by enterotoxigenic *Escherichia coli*. *FEMS Microbiol. Lett.* **66**:125–129.
- Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–119.