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A *rns*-Like Regulatory Gene for Colonization Factor Antigen I (CFA/I) That Controls Expression of CFA/I Pilin

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Colonization factor antigens (CFA) are needed for adherence of human enterotoxigenic *Escherichia coli* (ETEC) strains to their hosts. The CFA/II antigens, CS1 and CS2, which are found in some ETEC strains, require the plasmid-encoded gene *rns* for expression (J. Caron, L. M. Coffield, and J. R. Scott, Proc. Natl. Acad. Sci. USA 86:963–967, 1989). Other ETEC strains express CFA/I, whose synthesis and assembly require genes on two unlinked regions (regions 1 and 2) of a plasmid (G. A. Willshaw, H. R. Smith, and B. Rowe, FEMS Microbiol. Lett. 16:101–106, 1983). We report that CFA/I region 2 DNA can substitute for *rns* to cause expression of CS1 and CS2. The *cfaR* gene in region 2 is defined by a mutation abolishing both expression of CFA/I and complementation of a *rns* mutant for expression of CS1 or CS2. In a strain containing only region 1, complementation for expression of CFA/I by a plasmid containing *rns*$^+$ is inefficient but is adequate to cause hemagglutination by the CFA/I adhesin.

Enteric bacteria, such as *Escherichia coli*, may cause diarrheal disease in humans and animals when they are able to colonize the intestine and produce enterotoxins (2, 14, 22). Such enterotoxigenic *E. coli* (ETEC) strains carry colonization factor antigens (CFA) associated with different serologically typable pili (16), whose adhesins provide attachment specificity. The adhesins of CFA/I and CFA/II pili cause hemagglutination of human and bovine erythrocytes, respectively, and the adhesins of both pili recognize and bind to human enterocytes.

The CFA originally designated CFA/II (6) was found to be composed of three morphologically distinct entities: CS1, CS2, and CS3 (23). Antiserum directed against CS1 pili detects a predominant 16-kilodalton (kDa) antigen, and antiserum directed against CS2 detects a major pilin antigen of 15 kDa, whereas that directed against CS3 recognizes a 14-kDa major antigen (23). CFA/I pili contain a major pilin antigen of 15 kDa (8).

The regulatory and structural genes for the synthesis and assembly of pili from animal and human *E. coli* are usually found on contiguous regions of either chromosomal or plasmid DNA (1, 13, 17, 18). However, the genetic organization of the loci required for the expression of both CFA/I and CFA/II pili differs. For CFA/I, two widely separated DNA regions, designated regions 1 and 2, are needed (21, 25). Although region 1, which contains the major pilin structural gene, and region 2 are quite distant from each other, they are on the same plasmid (25).

For the CFA/II pili, expression of CS1 and CS2 requires DNA regions which are genetically unlinked. The presence of either CS1 or CS2, which seem to be mutually exclusive, is correlated with the serotype and biotype of ETEC strains. The expression of these pilins requires the plasmid-encoded *rns* gene, but neither the CS1 nor CS2 pilin structural genes are found on the same plasmid as this gene (3).

The Rns protein, which is a member of the AraC family of positive regulators, activates CS1 at the transcriptional level (J. Caron, J. S. Swartley, J. Perez-Casal, and J. R. Scott, manuscript in preparation). In ETEC strains isolated from humans, *rns* is apparently the first such regulator for a pilin gene to have been identified (3). To determine whether pili of other human ETEC strains have *rns* homologs, we initiated a hybridization study (J. Caron, D. R. Maneval, J. B. Kaper, and J. R. Scott, submitted for publication). In the present communication we define a gene, *cfaR*, that is homologous to *rns* and that is required for production of CFA/I pilin.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strains 58R80 (21), JM83 (15), and CH734 (4) were used as hosts for molecular cloning and expression. LMC2 and LMC10 are restriction-and-modification-negative, plasmid-cured, wild-type *E. coli* strains (3) which express CS2 and CS1 pili, respectively, when *rns* is supplied in *trans*. The plasmids pEU2030 and pEU2130 contain the *rns* gene (Table 1), whereas pEU2034 contains a *rns-1* frameshift mutation shown to prevent the expression of CS1 and CS2 (3). The plasmids pNTP513 and pNTP503 (25), obtained from H. R. Smith, contain, respectively, CFA/I region 1 and CFA/I region 2 DNA cloned on compatible vectors (25). Assuming sequence similarity with *rns*, we constructed a mutation in pNTP503 by digestion with *Bgl*II, filling in of the 3′ recessed ends, and religation, thereby generating a 4-base-pair insertion mutation contained in pNTP503B. The sequence of this gene, recently determined by Savelkoul et al. (P. H. M. Savelkoul, G. A. Willshaw, M. M. McConnell, H. R. Smith, A. M. Hamers, B. A. M. van der Zeijst, and W. Gaastra, Microb. Pathog., in press), indicates that this insertion causes a frameshift.

Bacterial strains were grown on Luria-Bertani (LB) (20), tryptone (20), or CFA (7) agar. Antibiotics were used for selection at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 40 μg/ml; and chloramphenicol, 30 μg/ml.

Recombinant DNA techniques. Transformations were done by using the rubidium chloride method (11). Other techniques used were as described by Maniatis et al. (12).

DNA colony blots. Colonies were grown overnight at 37°C on tryptone plates with appropriate antibiotics and blotted

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TABLE 1. Plasmids and relevant genotypes

Plasmid	Genotype	Vector	Drug resistance phenotype[a]	Reference
pEU2030	*rns*$^+$	pUC18	Ap^r	3
pEU2034	*rns-1*	pUC18	Ap^r	3
pEU2130	*rns*$^+$	pHSG298	Km^r	3
pNTP503	Region 2, *cfaR*$^+$	pBR325	Ap^r	25
pNTP503B	Region 2, *cfaR*	pBR325	Ap^r	This study
pNTP513	Region 1	pACYC184	Cm^r	25
pGCS755	*virF*	pBR322	Ap^r	5

[a] Ap^r, Ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant.

onto nitrocellulose filters. Filters were processed by lysing colonies in 0.5 N NaOH for 7 min and neutralizing the mixture in 1 M Tris, pH 7.5, for 2 min, followed by incubation in 1 M Tris with 1.5 M NaCl for 4 min. Filters were rinsed in 1 M Tris–1.5 M NaCl and then in chloroform prior to being baked in a vacuum oven for 2 h.

Filters were placed in polyethylene bags to which was added a prehybridization mixture containing 50% formamide, 0.75 M sodium chloride, 75 mM sodium citrate (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]), 5× Denhardt solution (12), 100 μg of denatured calf thymus DNA per ml, 200 μg of tRNA per ml, and 0.1% sodium dodecyl sulfate (SDS). The bags were sealed and incubated at 42°C for a minimum of 3 h. A 0.5- to 1-ng quantity of radiolabeled probe denatured at 100°C for 5 min was then added to the prehybridization mixture. The bags were resealed, and hybridization was allowed to proceed for 16 to 24 h at 42°C. The filters were washed (five times for 20 min each time) under stringent conditions in 0.1× SSC–0.1% SDS at 60°C and then exposed to Kodak XAR-5 film with a Lightning-Plus intensifying screen for 3 to 12 h at −80°C.

Probe. A 535-base-pair *Dra*I fragment was isolated from pEU2030 (Fig. 1) and radiolabeled in vitro by incorporation of [α-^{32}P]dATP to a specific activity of 10^8 cpm/μg by the random oligonucleotide priming method of Feinberg and Vogelstein (9).

Extracts for protein analysis. Heat extracts were prepared from overnight cultures grown on tryptone or CFA agar at 37°C by heating the cultures for 20 min at 60°C. Total cell extracts were prepared by resuspending cells from overnight cultures in 1 ml of LB broth and then lysing them by boiling them in a buffer containing 0.5 M Tris (pH 6.8), 10% SDS, 0.2 M EDTA, 30% glycerol, and 1% bromphenol blue dye. Cell concentrations were standardized by adjusting the optical density at 590 nm in LB broth. Proteins were separated on SDS–12.5% polyacrylamide gels, electroblotted to nitrocellulose, and reacted with polyclonal antiserum against CFA/I (a generous gift from James Kaper), CS1, or CS2 (3). Molecular size markers (Amersham) were used during electrophoresis to determine sizes of pilin antigens. Antigen-antibody complexes were allowed to react with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma) and visualized with BCI substrate (5-bromo-4-chloro-3-indolyl-phosphate-*p*-toluidine salt) (Bachem).

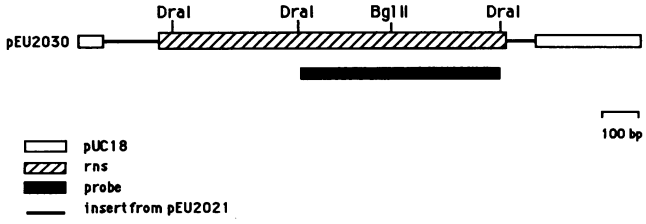

FIG. 1. Restriction map of a plasmid carrying the *rns* gene. Physical map of plasmid pEU2030 (3) containing a 1.2-kilobase DNA insert from pEU2021 which contains the *rns* gene (striped box). The line indicates flanking DNA sequences cloned from pEU2021 (3). The open box indicates the plasmid vector pUC18. The solid box indicates the 535-base-pair *Dra*I fragment used as the probe for *rns*.

Hemagglutination. In the presence of 0.1 M mannose, CFA/I strains were tested for hemagglutination by mixing equal volumes of a 3% suspension of washed human type A erythrocytes with pelleted bacteria that had been grown overnight at 37°C on CFA plates. Cell mixtures were rotated on a glass slide for 2 min at room temperature and observed for hemagglutination.

RESULTS

Homology with CFA/I. To determine whether the *rns* gene was unique to CS1 and CS2 strains, a probe which contains its C-terminal two-thirds (Fig. 1) was used for dot blot hybridization with DNA from various *E. coli* strains (J. Caron, D. R. Maneval, J. B. Kaper, and J. R. Scott, submitted for publication). As expected, DNA from strains that express CS1 and CS2 antigens hybridized with *rns*, while neither DNA from *E. coli* K-12 nor that from the vector showed homology. The *rns* probe also reacted with DNA from CFA/I strains. This homology was present both on the chimeric plasmid pNTP513, containing CFA/I region 1, and on pNTP503, containing CFA/I region 2.

Complementation of CS1 and CS2 expression by CFA/I region 2. To determine whether CFA/I region 1 and/or 2 would substitute for *rns* to allow expression of CS1 and CS2 antigens, complementation experiments were performed. Plasmids pNTP513 (region 1) and pNTP503 (region 2) were transformed in turn into both LMC10 (which has the CS1 structural genes) and LMC2 (carrying the CS2 genes). These strains were then assayed for CS1 and CS2 antigen expression by Western blot (immunoblot) analysis of surface proteins (heat extracts) and total cell extracts and by mannose-resistant hemagglutination of bovine erythrocytes.

In the absence of plasmid, neither CS1 nor CS2 antigens were found in LMC10 or LMC2 (data not shown), whereas pEU2030 (containing *rns*$^+$) caused expression of CS1 (data not shown) and CS2 (Fig. 2B, lanes 4 and 5). CFA/I region 1 did not substitute for *rns* to cause production of CS1 or CS2 (data not shown).

In contrast, CS1 (16 kDa) or CS2 (15 kDa) antigen was detected in the appropriate wild-type strain (LMC10 or LMC2) when plasmid pNTP503 (CFA/I region 2) was present instead of pEU2030 (Fig. 2A, lanes 1 and 2; Fig. 2B, lanes 2 and 3). The substitution of region 2 for *rns* is very effective, since the same amount of CS2 antigen was produced whether pNTP503 or pEU2030 was present in LMC2 (Fig. 2B; compare lanes 2 and 3 with lanes 4 and 5). The same is true for CS1 (data not shown). The distribution of pilin antigen between the cell surface (heat extracts) and the cytosol (total cell extracts) is about the same in cells containing either region 2 or *rns* (Fig. 2B; compare lanes 2 and 3 with lanes 4 and 5).

A gene in CFA/I region 2 needed for antigen expression. An insertion mutation constructed in region 2 (contained in pNTP503B; see Materials and Methods) reduced expression of CFA/I to background levels (see below). In addition,

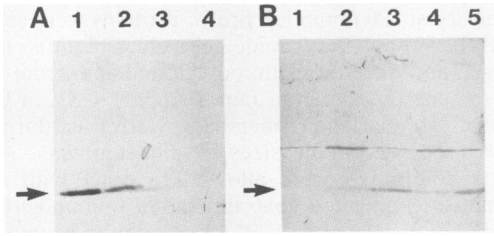

FIG. 2. CS1 and CS2 antigen production from strains carrying either pNTP503 (*cfaR*$^+$) or pNTP503B (*cfaR*). Pilin proteins from heat extracts of *E. coli* harboring various plasmids were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with CS1 (A) or CS2 (B) antiserum. Bound antibody was detected with goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase. Odd-numbered lanes contain 2 μl of heat extracts; even-numbered lanes contain 2 μl of total cell extracts. The following strains were used. (A) Lanes: 1 and 2, LMC10 containing pNTP503; 3 and 4, LMC10 with pNTP503B. (B) Lanes: 1, LMC2 with pNTP503B; 2 and 3, LMC2 with pNTP503; 4 and 5, LMC2 with pEU2030. The arrows indicate CS1 or CS2 pilin antigen.

pNTP503B did not cause production of CS1 (Fig. 2A, lane 3) or CS2 (Fig. 2B, lane 1) pilin antigens above the background levels found in the strain not carrying a plasmid (data not shown). We designate the gene defined by this mutation *cfaR*.

Complementation of CFA/I expression by *rns*. If *rns* were functionally equivalent to *cfaR*, then it should be able to substitute for region 2 DNA for production of CFA/I antigen. To test this, pEU2030 (*rns*$^+$) was introduced into strain 58R80 carrying pNTP513 (region 1). This strain expressed CFA/I pilin (Fig. 3, lanes 5 and 6), but at a level reduced by about 20- to 100-fold from that of the same strain carrying pNTP503 (region 2) in place of pEU2030 (Fig. 3, lanes 1 to 4).

It seemed possible that CFA/I region 2 genes other than *cfaR* might be needed for the higher level of antigen present in 58R80(pNTP513) containing pNTP503 than in 58R80 (pNTP513) containing pEU2030. To test this, strain 58R80 containing pNTP513 (region 1) and pEU2130 (*rns*$^+$) was transformed with pNTP503B (region 2, containing the *cfaR* mutation) and the amount of CFA/I pilin was determined by

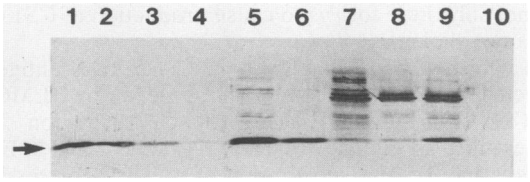

FIG. 3. CFA/I pilin production in *E. coli* K-12 strains harboring various plasmids. Proteins from heat extracts were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with CFA/I antiserum. Lanes 1 to 4 contain 1 μl each of heat extracts from 58R80(pNTP513)(pNTP503) at the following dilutions: lane 1, 1:20; lane 2, 1:100; lane 3, 1:400; lane 4, 1:800. Heat extracts of strain CH734 or strain 58R80 carrying other plasmids contained the following amounts of protein: lane 5, 5 μl of 58R80(pNTP513)(pEU2030); lane 6, 1 μl of 58R80(pNTP513)(pEU2030); lane 7, 5 μl of 58R80(pNTP513)(pNTP503B); lane 8, 5 μl of CH734(pNTP513)(pNTP503B)(pEU2130); lane 9, 5 μl of 58R80 (pNTP513); lane 10, 5 μl of 58R80(pNTP503). The arrow indicates the major CFA/I pilin antigen.

TABLE 2. Complementation for CFA/I and CFA/II antigen expression by relevant plasmids

Additional plasmid(s)	Relevant genotype	CFA/I expression[a]		CFA/II antigen expression	
		MRHA[b]	Antigen[c]	CS1[d]	CS2[e]
pEU2030	*rns*$^+$	+	+[f]	+	+
pEU2034	*rns-1*	−	−	−	−
None	Region 1	−	+[g]	−	−
pNTP503	Region 2, *cfaR*$^+$	+	+	+	+
pNTP503B	Region 2, *cfaR*	−	+[h]	−	−
pNTP503B + pEU2130	Region 2, *cfaR* *rns*$^+$	ND[i]	+[h]	ND	ND
pGCS755	*virF*	−	ND	−	−

[a] Strain 58R80(pNTP513) with the additional indicated plasmid(s) was used to test CFA/I expression.
[b] MRHA, Mannose-resistant hemagglutination of human erythrocytes.
[c] CFA/I antigen detected by Western blots.
[d] Measured in strain LMC10 by Western blots.
[e] Measured in strain LMC2 by Western blots.
[f] At least 20-fold lower than in 58R80(pNTP513)(pNTP503); see Fig. 3.
[g] At least 100-fold lower than in 58R80(pNTP513)(pNTP503); see Fig. 3.
[h] 2,000-fold lower than in 58R80(pNTP513)(pNTP503); see Fig. 3.
[i] ND, Not determined.

quantitative comparison of Western blots. Not only did the presence of pNTP503B not increase the amount of CFA/I over that extracted from the strain not carrying this plasmid, but the amount of CFA/I actually appeared smaller (Fig. 3, lanes 5 and 8).

By increasing the amount of heat extract loaded in a well of a Western blot, we were able to detect CFA/I antigen from 58R80(pNTP513) in the absence of pNTP503 (Fig. 3, lane 9). Addition of pNTP503 increased the level of detectable antigen by at least 100- to 500-fold (Fig. 3, lanes 1 to 4). When the *rns* gene on plasmid pEU2030 was added to strain 58R80(pNTP513), there was an increase of about fivefold in the amount of CFA/I antigen (Fig. 3; compare lane 6 with lane 9). Similar results were obtained from total cell extracts (data not shown).

Complementation by *rns* for CFA/I hemagglutination. The ability of *rns* to complement region 1 for the adherence function of CFA/I was assayed by hemagglutination (Table 2). Strain 58R80(pNTP513) (region 1) did not hemagglutinate human erythrocytes. However, when pEU2030 (*rns*$^+$) was added to strain 58R80(pNTP513), hemagglutination was observed, indicating that the CFA/I adhesin was produced. The *rns* gene was responsible for this since no hemagglutination was seen when the plasmid pEU2034 with the *rns-1* mutation was used instead. These results suggest that *rns* is interchangeable with region 2 for production of the adhesin of CFA/I pili (Table 2), even though less major pilin antigen was produced when *rns* was present than was produced when region 2 was present.

Absence of complementation by *virF* for CS1 and CS2 expression. In *Yersinia enterocolitica*, another human pathogen that causes diarrhea, the outer membrane proteins (YOPs) appear to be virulence determinants. VirF, which like Rns is a member of the AraC family of positive regulators, activates YOP expression at the transcriptional level (5). *E. coli* and *Y. enterocolitica* are both adapted to grow in the human intestinal tract at 37°C, and at lower temperatures some of their virulence determinants are not expressed. These include the YOP class of surface proteins and many types of *E. coli* pili. Because of their similarities, we tested the ability of *virF* to substitute for *rns*. In a *rns* mutant strain

that carries the structural genes for CS1 or CS2, these pilin antigens are not expressed when *virF* is supplied in *trans* (Tables 1 and 2).

DISCUSSION

For the expression of CS1 and CS2, the unlinked, plasmid-located *rns* gene is needed in addition to the structural genes for the pili (3). CFA/I hemagglutination also requires two regions of unlinked DNA (25). Region 1 alone (pNTP513), which contains the structural gene for the major pilin subunit, leads to production of CFA/I antigen detectable in minicells (24) and in heat extracts containing the proteins released from the cell surface (Fig. 3, lane 9). However, in the absence of region 2, strains that contain region 1 do not show mannose-resistant hemagglutination (24) (Table 2). We have constructed an insertion mutation in region 2 that decreases the CFA/I antigen level in heat extracts 2,000-fold below the wild-type level. This mutation defines the gene *cfaR* required for hemagglutination and for maximal expression of CFA/I.

It was suggested by Willshaw et al. (25) that CFA/I region 2 was needed for transport and/or assembly of pili since it was required for hemagglutination but not for antigen production in minicells. We find that the distribution of CFA/I between the cytosol (total cell extracts) and the surface (heat extracts) of the cells is the same with and without region 2 (data not shown), indicating that there is no intracellular antigen accumulation. However, the 2,000-fold reduction in the level of the CFA/I antigen in the *cfaR* mutant would be consistent with the possibility that *cfaR*$^+$ is required for transport or stability of this antigen. Because of its homology to *rns*, however, we feel instead that it is more likely that *cfaR* is a positive regulator of the region 1 CFA/I gene(s). This is supported by the recent findings of Gaastra and co-workers (P. H. M. Savelkoul, G. A. Willshaw, M. M. McConnell, H. R. Smith, A. M. Hamers, B. A. M. van der Zeijst, and W. Gaastra, in press).

The homology that we observed between CFA/I region 2 and *rns* is probably due to the *cfaR* gene because *cfaR* is functionally similar to *rns*. The *cfaR*$^+$ gene, but not the *cfaR* mutant allele, can substitute for *rns* in stimulating production of CS1 and CS2 (Table 2). However, *rns* is not completely interchangeable with *cfaR*; when *rns* is used in place of region 2, at least 20-fold less CFA/I is produced. The addition of the other region 2 genes to a strain containing region 1 and *rns* does not increase CFA/I expression. The decrease in CFA/I pilin observed when pNTP503B (region 2 with the *cfaR* mutation) is added to a strain containing pEU2130 (*rns*$^+$) and pNTP513 (region 1) might be caused by reduced gene dosage since pEU2130 and pNTP503B are mutually incompatible. Alternatively, the decrease in CFA/I pilin could be caused by negative complementation; i.e., the mutant *cfaR* gene product could be blocking activation by Rns.

Although it causes production of at least 20-fold less CFA/I antigen than does a functional region 2, the *rns* gene effectively substitutes for *cfaR* to cause hemagglutination. There are two possible explanations for this phenomenon. Either the lower level of CFA/I antigen is sufficient to cause detectable hemagglutination or, in the absence of *cfaR*, some other gene product needed for hemagglutination is present in limiting quantity and the Rns protein increases the expression of this product adequately to allow CFA/I pilin function.

Region 1 and either region 2 or *rns* are required for expression of adherence by CFA/I pili. The specific CFA/I adhesin responsible for hemagglutination is not encoded on region 2 because the *E. coli* K-12 strain 58R80 carrying pNTP513 (region 1) and pEU2030 (*rns*$^+$) hemagglutinates human erythrocytes. This indicates that the CFA/I adhesin is encoded either on region 1 or on the *E. coli* K-12 chromosome.

The *rns* probe was also found to hybridize with CFA/I region 1 DNA, although *cfaR*, the CFA/I *rns* homolog, is present in region 2. However, unlike region 2, region 1 DNA is unable to complement a *rns* mutant strain for CS1 or CS2 expression. Nevertheless, a strain containing region 1 alone [58R80(pNTP513)] produces CFA/I pilin antigen, although at a level at least 100-fold lower than when region 2 is present. Thus, the *rns* homolog in region 1 may be expressed poorly or may differ in structure so that it is less efficient in activation of the structural gene for the CFA/I antigen.

Because of their surface location and immunogenicity, pilin antigens appear to be good candidates for vaccines against bacterial pathogens. However, the development of pilin vaccines is complicated by the diversity of their specific adhesins. Many of the pili that are needed by diarrheagenic bacterial pathogens to adhere to the human intestine require the presence of a positive regulator for their expression. The *cfaR* gene represents the fourth such case, the others being *rns* and the *virF* genes of *Yersinia* spp. (5) and of *Shigella flexneri* (19) and *S. sonnei* (10). An alternative approach to treatment or prevention of diarrheal diseases caused by these bacteria, therefore, might be based on an analog that acts as a competitive inhibitor for the positive regulator. All of these positive regulatory genes display homology at the protein level and are members of the AraC family. Although *cfaR* and *rns* are not functionally identical, their similarity suggests that development of a single agent that interferes with the action of both might be possible.

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