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Virulence regulons of enterotoxigenic *Escherichia coli*

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Abstract Enterotoxigenic *Escherichia coli* is frequently associated with travelers' diarrhea and is a leading cause of infant and childhood mortality in developing countries. Disease is dependent upon the orchestrated expression of enterotoxins, flexible adhesive pili, and other virulence factors. Both the heat-labile (LT) and heat-stable (ST-H) enterotoxins are regulated at the level of transcription by cAMP-receptor protein which represses the expression of LT while activating expression of ST-H. The expression of many different serotypes of adhesive pili is regulated by Rns, a member of the AraC family that represents a subgroup of conserved virulence regulators from several enteric pathogens. These Rns-like regulators recognize similar DNA binding sites, and a compiled sequence logo suggests they may bind DNA through both major and minor groove interactions. These regulators are also tempting targets for novel therapeutics because they play pivotal roles during infection. To that end, high-throughput screens have begun to identify compounds that inhibit the activity of these regulators, predominately by interfering with DNA binding.

Keywords ETEC · Enterotoxin · AraC · CRP · cAMP

Worldwide impact of diarrheal disease

Approximately 1.7 million people, primarily infants and children, perish from diarrheal diseases every year [1]. For citizens of low-income countries, diarrheal diseases are projected to remain among the ten leading causes of death through 2030 [1]. Although many viral and bacterial pathogens cause diarrhea, enterotoxigenic *Escherichia coli* (ETEC) is one of the most frequently encountered bacterial pathogens in low-income nations where it is estimated to kill between 300,000 and 700,000 children and infants every year [2–4]. An additional 280 million people will be sickened by ETEC annually including travelers to certain high risk areas of the world where infection rates may reach 50 % [5, 6]. Thus, it is not surprising that ETEC is practically synonymous with travelers' diarrhea. Although poor sanitation in low-income nations is a major contributing factor to ETEC infections, industrialized nations, including the United States, are not exempt from sporadic outbreaks [7, 8].

Despite considerable research and development, to date there are no commercially available ETEC-specific vaccines [9, 10]. Many vaccine formulations under development include pilins, protein subunits that polymerize to form pili, because they are surface exposed and antigenic. However, ETEC strain heterogeneity is a significant obstacle to the development of pilus-based vaccines because more than 22 different types of pili have been characterized [4, 11]. Many more have yet to be identified because ~50 % of ETEC isolates are nontypeable with regard to pili [12]. In addition, ETEC vaccines may need to be region-specific because ETEC strains are geographically diverse [13]. Discouragingly, an alternative and novel vaccine strategy that delivered toxin antigens via a dermal patch met with failure in Phase III clinical trials despite encouraging initial studies [14, 15]. Thus, the future prospects for ETEC vaccines remain uncertain.

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Enterotoxins

ETEC is noninvasive enteric pathogen that secretes heat-stable (ST) and/or heat-labile (LT) enterotoxins. Since either toxin is sufficient to cause diarrheal disease and ETEC strains are heterogenous, it is not surprising that ST^+ LT^-, ST^- LT^+, and ST^+ LT^+ have been isolated [16]. The ST's are expressed as ca. 72-amino-acid pre–pro toxins that are processed to peptides of 18 or 19 amino acids containing three intramolecular disulfide bonds which are essential for their activity [17]. Two ST types, ST-H and ST-P, have been isolated in clinical isolates from humans including the prototypical strain H10407 which carries both ST-H and ST-P [18]. Both types have the same biological activity. Upon maturation, ST's are secreted and bind to the extracellular receptor domain of the transmembrane protein guanylate cyclase C. This triggers the overproduction of cGMP within the cytosol [19, 20].

In contrast to ST, LT is a large multimeric enterotoxin containing a pentamer of B subunits surrounding an enzymatic A subunit [21]. The holotoxin is assembled within the periplasm and transits the outer membrane through a type II secretion system [22]. Curiously, the majority of secreted LT remains associated with the bacterial cell because its B subunits bind LPS [23]. LPS bound toxin is only released from the cell as the pathogen sheds vesicles of outer membrane material. The exterior of these vesicles is coated with LT bound to LPS, while the interior contains LT and other periplasmic proteins captured during vesicle budding [24]. These vesicles deliver their cargo to host cells upon binding of GM1 gangliosides via a second binding pocket within the B subunits. Upon reaching the cytosol, the enzymatic A subunit catalyzes ADP-ribosylation of Gsα. This modification causes an overproduction of cAMP because Gsα regulates the activity of adenylate cyclase. Among other consequences, dysregulation of cGMP and/or cAMP synthesis disrupts the normal flow of chloride and other electrolytes across the cytoplasmic membrane ultimately leading to diarrhea.

We have shown that cAMP also plays a role in regulating the expression of both LT and ST-H. This is accomplished at the level of transcription by cAMP-receptor protein (CRP), a homodimeric bacterial transcription factor. Upon binding cAMP, CRP undergoes an allosteric conformational change that permits the transcription factor to bind DNA [25]. Depending on the location of its binding site(s) within a target promoter CRP may act as a repressor or activator [26]. For example, we have shown that CRP represses the expression of *eltAB*, the bicistronic message encoding the subunits of LT, by binding to an operator that is centered over the −35 hexamer of the *eltAB* promoter [27]. As expected from its location, occupancy of this operator excludes RNA polymerase from the promoter. In contrast, CRP activates the $estA_H$ (ST-H) promoter through occupancy of sites that facilitate productive interactions between the transcription factor and RNA polymerase (Bodero and Munson, unpublished). Thus, in the presence of cAMP the expression of ST-H is greater than that of LT because CRP represses *eltAB* and activates $estA_H$. In the absence of cAMP the opposite (LT > ST-H) is observed because CRP neither represses *eltAB* nor activates $estA_H$.

Either an endogenous or exogenous source may provide cAMP to CRP. Endogenous cAMP is synthesized in the bacterial cytosol by adenylate cyclase (CyaA). However, the activity of CyaA, and indirectly that of CRP, is inhibited by the phosphoenolpyruvate/sugar phosphotransferase system when glucose or other sugars are imported from the environment [28]. This inhibitory mechanism if referred to as catabolite repression and may influence the expression of ETEC virulence genes during infection because secreted enzymes degrade complex sugars and carbohydrates within the intestinal lumen to monosaccharides prior to absorption by host tissues [29]. Thus, ETEC may encounter a high-to-low gradient of glucose as it moves through the intestine. We and others have proposed that ETEC may use this gradient to temporally and spatially separate the expression of LT from ST-H [27, 30]. However, more recent studies suggest that the situation is likely to be more complex. For example, Kansal et al. have shown that ETEC undergo many transcriptional changes upon adherence to epithelial cells including depressed expression of *crp* relative to planktonic ETEC [31]. In addition, acidic pH has been shown to inhibit secretion of LT while alkaline pH promotes it [32]. Curiously, CRP also promotes secretion of LT even though it represses expression of LT at the transcriptional level [32]. Although the mechanism for this latter result is currently unknown, it provides a cautionary tale—enterotoxin expression does not necessarily correlate with enterotoxin secretion and delivery as the latter steps may be regulated independently of expression.

E. coli also have the capability to import cAMP synthesized by an exogenous source. The import of cAMP effectively overrides catabolite repression because the activity of CRP is solely regulated by the availability of cAMP. Theoretically it is possible that ETEC import cAMP from LT-intoxicated epithelial cells as they efflux cAMP in an attempt to maintain homeostatic levels [33, 34]. If true it would be a fascinating example of a host-pathogen feedback loop and suggest novel roles for LT and similar toxins. However, as noted above, it appears that the expression of enterotoxins and other virulence factors is modulated by several stimuli–few of which are clearly understood.

Rns-dependent expression of adhesive pili

A critical event prior to or during an ETEC infection is the expression of pili which allow the pathogen to adhere to the host's intestinal mucosa and thus resist being flushed from the gastrointestinal tract. The archetypal ETEC pilus, CFA/I, is composed of the outer membrane usher CfaC, the major–in terms of abundance–pilin CfaB that polymerizes to form the shaft of the pilus, and the minor pilin CfaE that provides a ligand binding site at the pilus tip [35]. A periplasmic chaperone, CfaA, is required for pilus assembly but is not part of the final structure. Polymerization of the pilus into a helical filament occurs as the hydrophobic groove of one pilin subunit is filled by an amino-terminal strand of another in what is known as donor strand exchange [36, 37]. Remarkably, the pilus is not a static structure. Rather, it is capable of unwinding. This feat of flexibility likely dampens the effects of shear forces within the gut without breaking the linkage between pilin subunits nor pathogen attachment to host tissues [38].

The four genes encoding the CFA/I pilus are expressed as a polycistronic message from a single promoter. This promoter is activated by Rns, a 31-kDa protein that has alternatively been named CfaD or CfaR. In other ETEC strains, Rns activates the expression of CS1, CS2, CS3, CS4, CS14, CS17, CS19, and/or PCF071 pili [39–42] (Bodero and Munson, unpublished). Thus, a significant number of known pilus serotypes share a common regulator. As described below, this shared feature has implications for novel therapeutic strategies. We have shown by DNase I footprinting that all Rns-activated pilin promoters carry at least one Rns binding site that abuts the promoter's –35 hexamer [39, 43, 44]. One or more additional binding sites are typically located further upstream. However, not all promoters carry additional Rns binding sites.

The amino-terminal domain of Rns

Rns is a member of the AraC family of proteins [45]. Like most family members, Rns is composed of two domains joined by a linker that has been identified by insertional mutagenesis (Fig. 1) [46]. The amino-terminal domain of many AraC proteins contains a binding site for an effector molecule. For example, AraC represses the *araBAD* promoter in the absence of arabinose. Upon arabinose binding, AraC vacates the *araO2* operator to fully occupy the *araI* site. This reorganization results in activation of the *araBAD* promoter [47]. However, Rns is active when bacteria are cultured in either minimal or rich media. In addition, purified Rns does not appear to require a particular molecule to bind DNA in vitro as a variety of buffered salt solutions are sufficient. Thus, to date, there is no evidence that Rns requires an effector molecule for its activity. However, we cannot exclude the possibility that Rns binds an endogenous ligand, one that is synthesized by *E. coli* under a wide range of growth conditions and remains bound by Rns throughout protein purification. Alternatively, Rns may bind a host derived molecule that converts the protein from an active to an inactive state at some point during infection.

Given that some AraC family members lack an amino-terminal domain altogether, what is the function of the Rns amino-terminal domain if not ligand binding? We initially hypothesized that the domain was retained for dimerization as has been shown for other family members [45]. However, our genetic and biochemical studies found no evidence for Rns homodimers or other multimers [48]. That Rns may act as a monomer is consistent with the intrinsic asymmetry of its DNA binding sites (Fig. 2). Typically, the internal symmetry of a protein homodimer is reflected by internal symmetry within its DNA binding site. For example, CRP is a homodimer with twofold symmetry. This symmetry is mirrored within its binding sites which contain an inverted repeat, i.e., twofold symmetry [25]. Each subunit of the protein homodimer is responsible for contacting one half of the inverted repeat. In contrast, Rns binding sites are clearly asymmetric suggesting that Rns binds to DNA as a monomer. Other AraC family members are known to function as monomers. For example, the activators MarA, SoxS, and Rob lack the amino-terminal domain of most family members and are not surprisingly, monomeric [49–51]. Moreover, the DNA cocrystal of MarA reveals that a single monomer is responsible for all of the contacts within a given binding site [52]. Since MarA is exclusively monomeric, it is clear that multimerization is not a prerequisite for DNA binding and protein activity. Even when a family member contains an amino-terminal domain, it may not form dimers. For example, the virulence regulators PerA from enteropathogenic *E. coli* and InvF from *Salmonella typhimurium* are typical AraC family members in overall size and domain organization but function as monomers in vivo [53, 54].

However, the Smith laboratory has reported Rns homodimers and it is conceivable that a Rns homodimer spans two binding sites arranged as an inverted repeat [55]. If true the homodimer must be rather flexible because the distance between sites arranged as inverted repeats is variable. DNA looping could also accommodate the homodimer model at promoters with long spacers as the flexibility of DNA increases with length. However, we have also identified several promoters with an odd number of Rns binding sites [48] (Bodero and Munson, unpublished). In these situations, in vitro DNase I footprinting and gel mobility assays suggest that Rns can fill all sites simultaneously. In addition, promoters genetically engineered

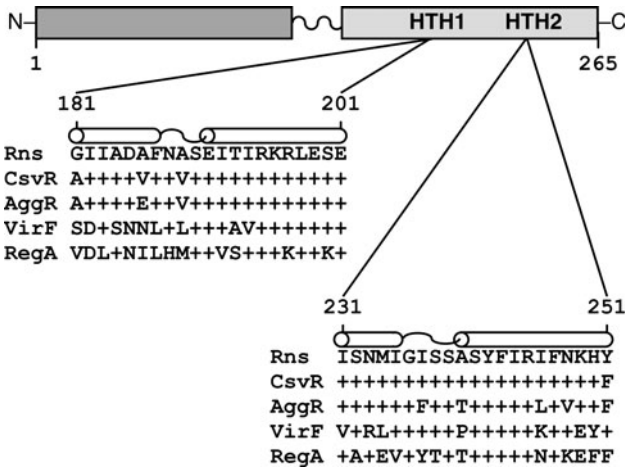

Fig. 1 Domain Organization of Rns. Rns is a 31-kDa protein consisting of two domains connected by a flexible linker region. The carboxy-terminal DNA binding domain contains two helix-turn-helix (HTH) motifs that are nearly identical to other virulence regulators within the AraC family including CsvR of ETEC, AggR of EAEC, VirF of *Shigella* sp., and RegA of *C. rodentium*. Numbering is relative to Rns (GenBank accession number AAA24419)

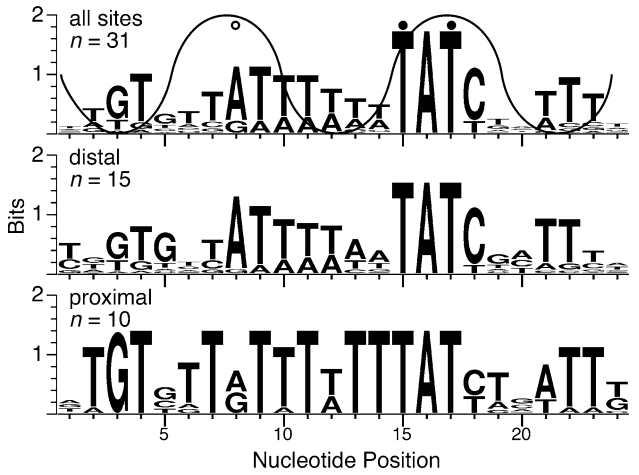

Fig. 2 Sequence logos of Rns binding sites. Thirty-one Rns binding sites have been identified by DNase I footprinting and site-specific mutagenesis. DNA helicity is represented by a sine wave with peaks and valleys corresponding to the major and minor groove, respectively. Positions of thymine C5-methyl groups that have been shown to interact with Rns by uracil interference are denoted by circles. *Filled circles* indicate positions on the forward strand, while an *open circle* indicates the reverse strand. As expected these positions coincide with sine wave peaks because thymine C5-methyl groups project into the major groove. The sequence logos also suggest Rns interactions with the minor groove at positions 2–4 and 21–23. Sequence conservation at these positions is unlikely to be the result of contamination by conserved promoter elements because the pattern is persistent when Rns binding sites are analyzed as subgroups containing promoter distal or promoter proximal sites. Six sites were excluded from the latter analyses because their associated promoters have not been mapped experimentally

through deletion or oligonucleotide directed mutagenesis to carry a single functional Rns binding site retain Rns-dependent regulation [39, 56]. Thus, if Rns is a homodimer, it is not necessary for both subunits to contact DNA. Despite some ambiguity regarding the amino-terminal domain of Rns, it is nonetheless essential for its activity and we have shown that residues Ile14 and Asn16 are required for DNA binding [48]. Due to their location at the extreme amino-terminus of Rns, these residues are not thought to contact DNA directly. Rather, they may be required for inter-domain interactions as has been shown for residues within the flexible amino-terminal "arm" of AraC [57].

The DNA binding domain of Rns and its binding sites

More certain than the role of the Rns amino-terminal domain is its carboxy-terminal domain. Like most AraC family members, this domain contains two predicted helix-turn-helix (HTH) motifs. The MarA-DNA cocrystal reveals that MarA binds along one face of the DNA helix with each HTH placing a recognition helix within two adjacent major grooves [52]. Because the recognition helices are not identical, each interacts with a unique sequence of nucleotides. This observation also provides the structural basis for DNA binding site asymmetry. In the absence of Rns crystallography, we have used the uracil interference assay to probe the interactions of Rns with its DNA binding sites [43, 58]. Because uracil lacks the C5-methyl group of thymine, T-to-U substitutions that abolish DNA binding reveal the location(s) of essential C5-methyl groups. This assay is also specific for major groove interactions due to the orientation of the C5-methyl group within the DNA helix. Within each of four sites that were assayed, three C5-methyl groups were identified as being required for DNA binding by Rns. Two are contained within a TAT triplet that is invariant across all known Rns binding sites. The third is located on the opposite strand seven nucleotides upstream of the TAT triplet (Fig. 2). Although highly conserved, this third thymine is replaced with a cytosine in some Rns binding sites (Fig. 2). The spatial distribution of these C5-methyl groups places them in adjacent major grooves of the DNA helix separated by a minor groove. When coupled with a careful analysis of DNase I cleavage patterns of free and bound DNA, these results indicate that Rns, like MarA, binds along one face of the DNA helix with its recognition helices placed within two adjacent major grooves [43, 52, 58].

The HTH motifs of Rns have near identity with several other AraC family members including CsvR, AggR, RegA, and VirF (Fig. 1). CsvR may regulate the expression of CS5 pili in ETEC [59]. Likewise AggR is required for the

expression of AAF/I and AAF/II pili and other virulence factors in enteroaggregative *E. coli* [60, 61]. RegA of *Citrobacter rodentium*, a murine pathogen often used to model enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) pathogenesis because it carries many of the same virulence factors as the human pathogens, activates the expression of the *kfc* pilus operon and other known and putative virulence genes [62]. Unlike the other members of Rns-like regulators, VirF does not regulate the expression of pili. Rather, VirF is at the top of a regulatory cascade that is responsible for the expression of proteins required for the entry and spread of the *Shigella* sp. within epithelial cells [63]. Given the high degree of identify within the HTH motifs of these regulators, it is not surprising that we and others have shown that they recognize similar DNA binding sites [64–66].

To date, we have identified 31 Rns binding sites in ETEC through DNase I footprinting and site-specific mutagenesis. The Rns binding sites were aligned to each other and used to generate a sequence logo for Rns-like regulators (Fig. 2) [67]. As noted above, the TAT triplet at positions 15-17 are invariant and each position conveys approximately two bits of information, the maximum possible for DNA. Another maximum is observed at positions 8 and 9. Position 8 coincides with the location of the third thymine C5-methyl group identified by uracil interference as contributing to DNA binding. Because this third position is not invariant, it conveys less than 2 bits. The intervening minor groove coincides with decreased information conservation but is nonetheless nonrandom with a clear predominance of A and T nucleotides. Unexpectedly, we observed additional information conservation at positions 2–4 and 21–23. Although the two resulting logos are not identical, this pattern persisted when two subsets of binding sites, one containing only the most promoter proximal site of each promoter and another containing promoter distal sites, were used to generate logos (Fig. 2). Thus, it is unlikely that this conservation arises from sequences that are bound by RNA polymerase because it is observed with both the promoter proximal and promoter distal binding sites. This suggest that Rns may make minor groove contacts when these positions are projected along the DNA helix. This is also consistent with the $\sim$1 bit of information at these positions in the combined logo, the maximum amount of information that can be conferred within the minor groove of B-form DNA [68].

The information contained within the Rns binding logo can be applied to predict Rns-regulated genes in silico. For example, we have successfully used a smaller set of binding sites to identify the Rns-regulated genes *nlpA* and *cexE* [44, 56]. Unusually, Rns binds to a site overlapping the ATG start codon of *nlpA* where it occludes RNA polymerase from the *nlpA* promoter. To date, *nlpA* is the only gene known to be repressed by Rns. The binding site and gene are conserved in EAEC and as expected, expression of *nlpA* is also repressed by AggR [56]. NlpA is an inner membrane protein that has been implicated in the biogenesis of outer membrane vesicles raising the possibility that Rns and AggR may modulate the production of these pathologically important structures [69]. CexE encodes a periplasmic protein whose expression is dependent upon occupancy of a Rns binding site that abuts the promoter's −35 hexamer. The function of CexE is unclear; however, it has been shown to be packaged within OMVs [70]. Therefore, it is possible that CexE is delivered to the eukaryotic cytosol alongside LT. The extent of the Rns regulon is currently unknown; however, our bioinformatic analyses of ETEC genomes for Rns binding sites predict that many other genes, both characterized and uncharacterized, are regulated by Rns. The accuracy of these predictions is currently under investigation and will benefit from a better understanding of the importance of the residues conserved at positions 2–4 and 21–23. In theory, the inclusion of these positions should produce more accurate predictions of DNA binding sites. However, the decision to exclude or include these positions must await a biochemical and genetic analysis of their significance.

Chemical inhibition of virulence regulons

High-throughput screens of vast small molecule libraries have led to the identification of novel compounds that inhibit the activity of Rns-like regulators. One study identified 238 "hits" within a library of 42,000 compounds that appear to prevent the activation of the *virB* promoter by VirF [71]. Follow-up studies decreased the number of hits tenfold, leaving a promising set of lead compounds with the potential to be developed into novel therapeutics. Alternatively, a single molecule (SE-1) was screened for efficacy against VirF activity [72]. SE-1 was originally identified as an inhibitor of RhaS and RhaR, AraC regulators with little homology to VirF. Remarkably, SE-1 was found to inhibit VirF activity in vivo and block DNA binding in vitro. Thus, SE-1 may yet prove to be broadly effective against many AraC family members. Another compound, regacin, has been shown to block DNA binding of RegA [73]. This compound was developed and optimized after discovery of a lead compound within a library of $\sim$12,000. A subsequent screen of chemical analogs led to the identification of regacin, an inhibitor with greater efficacy than the original. Oral administration of regacin significantly attenuated the ability of *C. rodentium* to colonize mice. It appears likely that regacin will also block colonization by *rns* + ETEC because regacin has been shown to also inhibit Rns activity. Although considerable

work remains to be done before any of these lead compounds are developed into practical therapeutics, they are a clear illustration of basic research priming the translational pipeline.

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Conflict of interest None.

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