Molecular Microbiology (1992) 6(4), 451–458

MicroReview

Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*

Victor J. DiRita
Unit for Laboratory Animal Medicine and Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0614, USA.

Summary

Evolution of complex regulatory pathways that control virulence factor expression in pathogenic bacteria indicates the importance to these organisms of being able to distinguish time and place. In the human intestinal pathogen *Vibrio cholerae*, control over many virulence genes identified to date is the responsibility of the ToxR protein. ToxR, in conjunction with a second regulatory protein called ToxS, directly activates the genes encoding the cholera toxin; other ToxR regulated genes are not activated directly by ToxR. For some of these genes, ToxR manifests its control through another activator called ToxT. Expression of *toxT*, which encodes a member of the AraC family of bacterial transcriptional activators, is ToxR dependent and is modulated by *in vitro* growth conditions that modulate expression of the ToxR virulence regulon. Thus, as in other regulatory circuits, co-ordinate expression of several genes in *V. cholerae* results from the activity of a cascading system of regulatory factors.

Introduction

Virulence in *Vibrio cholerae*, an intestinal pathogen of humans, has been the subject of a great deal of study over the years. Much of this work has been directed towards understanding the biochemistry and regulation of the cholera toxin, a major virulence factor for this pathogen. Toxin production in *V. cholerae* is part of a regulated response in which several other virulence factors in addition to the cholera toxin are expressed. Production of all of these gene products is under the control of a central regulator, ToxR.

Received 25 September, 1991; revised and accepted 12 November, 1991. For correspondence Tel. (313) 936 3804; Fax (313) 936 3235. This article is dedicated to John Mekalanos on the occasion of his birthday, cheers John.

This type of co-ordinate control of virulence factor expression is a response conserved among many bacterial pathogens. My aim is to review what is understood about ToxR-controlled gene expression in *V. cholerae* and to put it into context with co-ordinate regulation in other well-studied pathogens. I hope that this will give the reader an appreciation of where the question of global gene regulation in bacterial pathogenesis stands at this point.

Cholera toxin and the *ctxAB* genes

Cholera toxin is an ADP-ribosylating enzyme composed, as are many such toxins, of A and B subunits; for a review on ADP-ribosylating toxins see Moss and Vaughan, (1983). The genes encoding the A and B subunits make up the *ctxAB* operon, which is part of a larger genetic element capable of *recA* dependent amplification in *V. cholerae* (Mekalanos, 1983; Goldberg and Mekalanos, 1985), and which has the features of a site specific transposable element (Pearson, 1989). The zonula occludens toxin (ZOT), which alters the tight junctions between intestinal epithelial cells, has recently been identified and is encoded by a gene adjacent to the *ctx* operon on the element (Fasano *et al.*, 1991). Amplification of the *ctx* element is selected for by growth in ligated rabbit ileal loops (Mekalanos, 1983), suggesting that, all other things being equal, elevated levels of cholera toxin and/or ZOT increase *in vivo* fitness of *V. cholerae*.

Pioneering work by S.H. Richardson and his colleagues showed that although strain differences sometimes make it difficult to generalize, *in vitro* synthesis of cholera toxin by cultures of classical *V. cholerae* strains is dependent on the growth medium and culture conditions (Evans and Richardson, 1968; Richardson, 1969). For example, there is a several-hundred-fold decrease in cholera toxin in cultures grown in L-broth with a starting pH of 8.5 as opposed to a starting pH of 6.5 (Miller and Mekalanos, 1988). Other environmental parameters that influence toxin production in the laboratory are temperature, osmolarity, and certain amino acids (asparagine, serine, glutamate, and arginine) present in glucose minimal medium (Taylor *et al.*, 1987; Miller *et al.*, 1987; Miller and Mekalanos, 1988, reviewed by DiRita *et al.*, 1990). As will be discussed in further detail below, the fact that

the growth environment modulates cholera toxin expression has led to the identification of other *V. cholerae* virulence factors based on their similar patterns of expression (Taylor *et al.*, 1987; Peterson and Mekalanos, 1988). Whether any of these signals operate *in vivo* is unknown, but the fact that *V. cholerae* cultures respond to them with expression of virulence genes demonstrates that the organism has a mechanism of sampling and responding to its environment. Further evidence that this ability is essential for *in vivo* survival comes from the observation that strains with lesions in the *toxR* gene, the product of which controls signal-dependent virulence gene expression (see below), are deficient in colonization of human volunteers (Herrington *et al.*, 1988).

Expression of the *ctxAB* genes cloned in an *Escherichia coli* strain lacking any other *V. cholerae* genes is poor (Pearson and Mekalanos, 1982). By introducing a plasmid library of *V. cholerae* into an *Escherichia coli* strain harbouring a *ctx–lacZ* transcriptional fusion on its chromosome and scoring for transformants that expressed increased levels of β-galactosidase, Miller and Mekalanos (1984) identified a *trans* activator of the *ctx* promoter, encoded by the *toxR* gene. Sequences homologous to *toxR* are present in all *Vibrio cholerae* strains analysed to date (Miller and Mekalanos, 1985), including non-toxinogenic strains. Thus, while toxin genes may move from toxinogenic to non-toxinogenic strains by site-specific transposition (Pearson, 1989) their regulated expression by ToxR should be guaranteed. Regulatory parasitism of this nature, in which host mechanisms are used to control virulence genes present on a transmissible genetic element, has been observed with the Shiga-like toxin of *E. coli* and diphtheria toxin of *Corynebacterium diptheriae*. Both of these toxins are phage encoded and are controlled by host encoded *fur* and *dtxR* gene products, respectively (Calderwood and Mekalanos, 1987; Boyd *et al.*, 1990; Schmitt and Holmes 1991).

DNA binding by ToxR, a membrane spanning transcriptional activator

The *toxR* gene product is a transmembrane protein with a molecular size of approximately 32 kDa. DNA sequence analysis suggests that the first two-thirds of the protein are within the cytoplasm and are separated from a predicted periplasmic domain by a transmembrane stretch of hydrophobic, uncharged amino acids (Miller *et al.*, 1987). This structure was supported by analysing the properties of ToxR–PhoA fusion proteins (Miller *et al.*, 1987).

Miller *et al.* (1987) demonstrated that membrane fractions from *E. coli* cells expressing ToxR–PhoA fusion proteins specifically shift the gel migration of labelled DNA containing the *ctx* promoter. This intriguing result displayed several features of ToxR. The first is that ToxR activates *ctx* expression, most likely by binding directly to a specific element at the promoter. Support for this came from the observation that deletion of a tandemly repeated element (TTTTGAT) found upstream of *ctxAB* genes abolished gel retardation by ToxR–PhoA (Miller *et al.*, 1987).

Another feature of ToxR highlighted by the gel retardation experiment is that DNA binding is effected by the amino-terminal part of the protein. This is inferred because the experiment was performed with a ToxR–PhoA fusion protein in which the *C*-terminal periplasmic domain of ToxR had been substituted with alkaline phosphatase. This domain of ToxR shares similarity with the activator class of proteins that make up part of the two-component regulatory systems in prokaryotic signal transduction (J. F. Miller *et al.*, 1989a). Mutations at conserved amino acid residues within this region abolish both transcriptional activation and DNA binding (K. Ottemann and J. Mekalanos, personal communication).

That ToxR function is not greatly altered by replacing the *C*-terminal domain with alkaline phosphatase (Miller *et al.*, 1987; DiRita and Mekalanos, 1991) suggests either that the *C*-terminus of the protein is dispensable or that alkaline phosphatase adequately substitutes for the normal role of this domain. While the former possibility is unlikely (DiRita and Mekalanos, 1991), it has been suggested that the reason ToxR–PhoA fusion proteins retain ToxR function is that like many other DNA-binding regulatory proteins (Jones, 1990), active ToxR may be a dimer and alkaline phosphatase, which functions as a dimer (Schlesinger, 1967), acts as a dimerization domain for the ToxR moiety of the fusion protein. In this model, dimerization is a role played by the native *C*-terminus of ToxR.

The role of ToxS, an effector protein for ToxR function

Downstream of *toxR* is *toxS*, the second gene in an apparent operon (Miller *et al.*, 1989; DiRita and Mekalanos, 1991). *toxS* encodes a 19 kDa protein required by ToxR for activation of the *ctx* promoter (DiRita and Mekalanos, 1991). The properties of ToxS–PhoA fusion proteins suggest that ToxS is a membrane-associated protein that resides largely within the periplasm (DiRita and Mekalanos, 1991).

Since ToxS is in the periplasm, it seems likely that productive interaction between ToxR and ToxS takes place within this compartment. Consistent with this is that ToxR–PhoA fusions respond differently to ToxS, depending on the periplasmic location of the fusion junction (DiRita and Mekalanos, 1991). For instance, a fusion protein in which the entire ToxR *C*-terminus was replaced by

alkaline phosphatase exhibited wild-type levels of ToxR activity that were independent of ToxS (this sort of fusion protein was used by Miller *et al.* (1987) to characterize the DNA binding by ToxR). On the other hand, a fusion in which much of the *C*-terminus of ToxR is still present remained dependent on ToxS and also expressed decreased levels of alkaline phosphatase activity in the presence of ToxS (30 units versus 97 units in the absence of ToxS; DiRita and Mekalanos, 1991). This second type of fusion, termed a long fusion, was protected by ToxS from proteolysis in *E. coli* (DiRita and Mekalanos, 1991), indicating that direct interaction occurs between ToxR and ToxS. Given the structures of these two proteins, the likely place for such interaction is within the periplasm.

A long fusion protein was used to isolate *toxR* mutants that were 'blind' to the presence of ToxS; such mutant fusion proteins behaved in the presence of ToxS like the wild-type fusion protein did in the absence of ToxS (i.e., they exhibited elevated levels of alkaline phosphatase activity; DiRita and Mekalanos, 1991). The lesions leading to this phenotype were not in the periplasmic domain of ToxR, but within a cluster of 15 amino acid residues at one end of the region of similarity between the cytoplasmic domain of ToxR and other transcriptional activators in the two-component family (Miller *et al.*, 1987). Exactly why such mutants responded to this screen is not yet clear, but in keeping with the model that ToxR functions as a dimer, it was proposed that these lesions may define a region of ToxR critical to the ToxS-dependent dimerized form of the protein (DiRita and Mekalanos, 1991). The region identified by the ToxS blind mutants was defined as a 'linker' region (a term originally coined by Ames and Parkinson (1988) studying the transmembrane Tsr protein of *E. coli*; see below) connecting the DNA binding domain of ToxR and the transmembrane and periplasmic domains.

The cytoplasmic location of ToxS blind lesions in ToxR is reminiscent of mutations isolated in other prokaryotic signal-transduction systems. For instance, *mod* alleles of the *Bordetella pertussis bvg (vir)* locus, which lead to a signal insensitive phenotype (Knapp and Mekalanos, 1988), map in the cytoplasmic domain of BvgS, a transmembrane activator similar to the two-component regulatory proteins in other bacteria (J.F. Miller, personal communication). Likewise, in the Tsr protein of *E. coli*, which is a transmembrane protein that transmits signals relating to the chemotactic response, mutations with 'locked transducer' output have been isolated (Ames and Parkinson, 1988). Locked transducer mutants behave as if the chemotactic signal is present when it is not. Some of these mutants carry lesions in a linker region of the protein situated between the periplasmic receptor domain and the cytoplasmic signalling domain (Ames and Parkinson, 1988). ToxS blind mutants of ToxR, Mod mutants of BvgS, and linker region locked transducer mutants of Tsr may be forms of these transmembrane sensors that are impaired in recognition of periplasmic signals to which they normally respond. One difference between these mutant proteins is that ToxS-blind ToxR mutants lack ToxR function whereas the others (the Mod form of BvgS and the locked transducer form of Tsr) are constitutive for a function normally dependent on periplasmic sensing. Nevertheless, that the lesions in all of these are within their cytoplasmic domains may offer clues to the functional structure of these receptor proteins.

ToxR controls a virulence regulon in V. cholerae

Regulation of virulence in *V. cholerae* by ToxR extends to genes other than the *ctx* genes. In searching for Tn*phoA* fusions to genes that are regulated by the same *in vitro* conditions that regulate cholera toxin biosynthesis, Peterson and Mekalanos (1988) concluded that as many as 17 genes require ToxR in order to be expressed. Included in this regulon are genes encoding the TCP pilus colonization factor (Taylor *et al.*, 1987), an accessory colonization factor called ACF (Peterson and Mekalanos, 1988), and the outer membrane proteins OmpT and OmpU, which are subject to osmoregulation (Miller and Mekalanos, 1988). Serum resistance by *V. cholerae* is also a ToxR-regulated phenotype, as mutants of *V. cholerae* with lesions in *toxR* or in one of the *tcp* genes are approximately 10^5 times more sensitive to killing by anti-vibrio antibodies and complement (Parsot *et al.*, 1991). In addition, the *aldA* gene encoding the *V. cholerae* aldehyde dehydrogenase is controlled by ToxR, although *aldA* mutants were not defective in one animal model of *V. cholerae* colonization (Parsot and Mekalanos, 1991). (Whether the *zot* gene, encoding the recently described zonula occludens toxin, is part of the *toxR* regulon is unknown. However, Fasano *et al.* (1991) assayed *V. cholerae* supernatants for ZOT activity from cultures grown at 37°C, and at this temperature ToxR controlled gene expression is greatly reduced (see below). Also important to note is that the iron-regulated virulence gene, *irgA* (Goldberg *et al.*, 1990), is not a member of the ToxR regulon.) Thus, to a large extent, the virulence response in *V. cholerae* consists of regulated expression of a set of genes that are all under the general control of ToxR. The work to date has focused on membrane or exported gene products under ToxR control; the ToxR regulon might not yet be completely defined when one takes into account that cytoplasmic genes (other than *aldA*) may be members of the regulon.

This model of co-ordinate control of virulence gene expression by a 'master regulator' has been observed in many microorganisms, including *B. pertussis* (Weiss and Falkow, 1984), *Agrobacterium tumefaciens* (Stachel and

Zambryski, 1986), *Salmonella typhimurium* (S. I. Miller *et al.*, 1989), and *Staphylococcus aureus* (Recsei *et al.*, 1986).

Signal-dependent expression of ToxR regulon

Miller *et al.* (1987) showed that a *toxR* mutant of *V. cholerae* that produced no cholera toxin under any growth conditions could be complemented by *toxR* expressed from a plasmid, pVM7, on which *toxR* was apparently expressed from a plasmid promoter, and not the normal *toxR* promoter (Miller, 1985). In *V. cholerae toxR* mutants complemented with pVM7, regulation of cholera toxin synthesis in response to osmolarity and amino acid concentration mirrored that of the wild type (Miller *et al.*, 1987) indicating that, at least for these two parameters, environmental control of ToxR-regulated genes is not at the level of *toxR* expression.

This is not the case for all signals. At 37°C *in vitro*, expression of *ctxAB* and other ToxR-regulated genes is decreased a great deal relative to their levels of expression at 30°C (Richardson, 1969; J. Mekalanos, C. Gardel, R. Taylor, V. DiRita, K. Peterson, and C. Parsot, unpublished observations). This *in vitro* temperature effect may be due to the lack of other signals *in vitro* that are present at 37°C *in vivo*, but also is due at least partly to transcriptional control over the *toxR* promoter. Parsot and Mekalanos (1990) showed that directly upstream of and in the opposite orientation from the *toxR* gene is the *V. cholerae htpG* gene, which encodes a member of the Hsp90 family of heat-shock proteins. Growth at 37°C resulted in high-level expression of an *htpG–lacZ* gene fusion and decreased expression of a *toxR–lacZ* gene fusion, relative to fusion activity at 22°C (Parsot and Mekalanos, 1990). In an *E. coli* background, expression of the *toxR–lacZ* gene was decreased at both temperatures when the heat-shock sigma factor RpoH (σ-32) was supplied from an isopropyl-β-D-thiogalactoside (IPTG)-inducible promoter (Parsot and Mekalanos, 1990).

Based on these results, a model for heat-shock control over *toxR* expression was proposed by Parsot and Mekalanos (1990): elevated expression from the divergently expressed *htpG* gene occurs at 37°C at the expense of *toxR* expression owing to exclusion of RNA polymerase (σ-70), which likely recognizes the *toxR* promoter, by RNA polymerase (σ-32). According to this model, such regulation may have evolved to take advantage of an early stage of *V. cholerae* pathophysiology, in which stresses such as low pH, anaerobiosis, or starvation may induce the heat-shock response in an environment in which it would be inappropriate to express ToxR-activated genes. Passage through the stomach, which has a low pH due to gastric acid, may be the stage that induces such a response by infecting *V. cholerae* cells. If the organisms that survive this environment undergo a heat-shock response, concomitant down-regulation of the *toxR* gene may occur. This could be rapidly reversed after the shock simply by turnover of the heat-shock sigma factor, which occurs rapidly after heat shock (Grossman *et al.*, 1987; Strauss, *et al.*, 1987), without the need for dilution (by growth) of a putative repressor of *toxR* expression or induction of a new activator for *toxR*.

ToxR directs regulon gene expression indirectly through ToxT

While ToxR directly activates the *ctxAB* promoter, no other member of the regulon characterized in any detail to date is controlled by direct activation by ToxR. This led to the isolation of the *toxT* gene from *V. cholerae* strain 569B, which encodes a product that can activate ToxR-regulated genes in *E. coli* in the absence of other *V. cholerae* factors (DiRita *et al.*, 1991). An interesting aspect of this regulation is that both ToxR and ToxT activate *ctxAB* expression (DiRita *et al.*, 1991), suggesting that this promoter consists of regulatory sites for two different activators.

ToxR evidently directs its regulon by controlling expression of the *toxT* gene, the product of which then activates virulence genes. Northern blotting experiments showed that while wild-type *V. cholerae* strain O395 produced *toxT*-specific message under conditions in which the ToxR regulon is expressed, the *toxR* mutant strain JJM43 did not. In addition, a plasmid expressing *toxT* at high constitutive levels (see below) complemented expression of both cholera toxin and TcpA in the *toxR* mutant (DiRita *et al.*, 1991). These results indicate that while ToxR is normally required for activation of *toxT*, this requirement can be overridden if *toxT* is expressed from a constitutive promoter.

While temperature control over the ToxR regulon may be the result of antagonism from the heat-shock response (see above; Parsot and Mekalanos, 1990), signal transduction by ToxR in response to culture pH apparently involves its induction of *toxT* expression. As discussed earlier, ToxR-regulated genes are not expressed when cultures are grown with an initial pH of 8.5, compared with when they are grown with an intial pH of 6.5. This control over the ToxR regulon is evidently not at the level of *toxR* transcription, as Parsot and Mekalanos (1990) showed that β-galactosidase activity of a *toxR–lacZ* gene fusion was unaffected by the culture pH. Also, Miller *et al.* (1987) demonstrated that the *V. cholerae toxR* mutant O395-55 complemented with the plasmid pVM53-D (which expresses *toxR–phoA* from a plasmid promoter) retained the wild-type response to culture pH. Wild type *V. cholerae* O395 grown at pH 6.5 displayed *toxT* mRNA (by Northern blotting), but did not do so when grown at pH 8.5

(DiRita *et al.*, 1991). The *toxR* mutant JJM43 harbouring the constitutively expressing *toxT* plasmid synthesized both cholera toxin and *toxT* mRNA regardless of the culture pH. These observations indicate that ToxR (and ToxR–PhoA) function is modulated by culture pH, and that an important role for ToxR in directing co-ordinate gene expression is to activate *toxT* transcription under the proper conditions. Exactly how this is accomplished, either by ToxR directly or by ToxR acting through another regulator, is not yet clear.

Nucleotide sequence analysis of the *toxT* clone isolated from *V. cholerae* 569B revealed an open reading frame representing a protein of 32 kDa with similarity to the AraC family of transcriptional regulatory proteins named for the regulator of the arabinose utilization operons in *E. coli* and *S. typhimurium* and consisting of members from different Gram-negative bacteria (Miyada *et al.*, 1980; Smith and Schleif, 1978; V. J. DiRita and J. Mekalanos, unpublished). Mutagenesis of this open reading frame by insertion of four base pairs abolished ToxT activity, and also resulted in loss of a polypeptide of approximately 35 kDa in an S30 transcription/translation extract (E. Nazareno and V. J. DiRita, unpublished). These results indicate that ToxT is a 32 kDa member of the AraC family of transcriptional control proteins.

Other virulence regulators in this family are the VirF protein (Cornelis *et al.*, 1989), which controls expression of thermoregulated virulence genes in *Yersinia enterocolitica* (reviewed by Cornelis *et al.*, 1987), the Rns(CfaD) protein responsible for production of colonization factor antigens in enterotoxinogenic *E. coli* (Caron *et al.*, 1989; Savelkoul *et al.*, 1990), and the ExsA protein that controls exoenzyme S synthesis in *Pseudomonas aeruginosa* (Frank and Iglewski, 1991). ToxT is therefore a member of a growing family of regulators that control gene expression, frequently under specific environmental conditions and in response to specific effectors.

Control over expression of these proteins by other transcriptional activators, as is the case with ToxR regulation of *toxT*, has not been reported before. The *ymoA* locus in *Yersinia* plays a role in controlling *virF* expression, perhaps by changing the relative DNA compaction in the cell at 25°C and 37°C (Cornelis *et al.*, 1991). Unlike wild-type cells, *ymoA* mutants expressed *virF* at 25°C; however, they induced further *virF* expression at 37°C (Cornelis *et al.*, 1991). Therefore, although an exhaustive search did not identify a positive thermoregulator of *virF* expression (Cornelis *et al.*, 1991), conditional regulation at the *virF* promoter other than that conferred by YmoA cannot be ruled out .

The unusual case of *V. cholerae* strain 569B

Transcriptional control of *toxT* may be a critical juncture in the overall regulation of virulence by ToxR, and is therefore of considerable interest at this time. It may be possible to test this notion by analysing a well-used laboratory strain of *V. cholerae*, strain 569B. This strain, from which *toxT* was originally isolated and characterized, is less subject to environmental modulation of virulence determinants than are other strains (Richardson, 1969). It carries a chromosomal deletion of the *toxS* gene (Miller and Mekalanos, 1985; Miller *et al.*, 1989) and at least some *toxR* mutants derived from it are unaffected with respect to cholera toxin and Tcp expression (DiRita *et al.*, 1991). These observations suggest that strain 569B has somehow become independent of ToxR and ToxS for expression of the ToxR regulon. The current strain 569B was isolated in the 1950s by passing an attenuated strain through rabbits in order to isolate a more virulent variant (Dutta and Habbu, 1955). Assuming that the pre-passaged strain was attenuated due to the *toxS* deletion (which is the case for *toxS* mutants derived from more typical strains; Pearson *et al.*, 1990), a potential site for a lesion that might overcome this attenuation is at the *toxT* promoter, resulting in constitutive expression. Such a mutation would be expected to lead to virulence gene expression independent of the presence of ToxR and ToxS. Pertinent to this possibility is the observation that recombination of the *toxS* gene onto the 569B chromosome in its normal location downstream of *toxR* did not restore the wild-type regulated phenotype (Pearson *et al.*, 1990; V. J. DiRita and J. Mekalanos, unpublished)

A great deal of data generated with the original *toxT* clone (pGJ40) from strain 569B are consistent with *toxT* expression in this plasmid originating from the *tet* promoter of the cloning vector (DiRita *et al.* 1991; see above). However, if strain 569B has acquired a *toxT* promoter up-mutation, it is possible that *toxT* expression from pGJ40 is due to a constitutive promoter inherent in strain 569B. However, preliminary results do not favour this possibility because some transposon insertions that abolish *toxT* activity from pGJ40 in *E. coli* map at the *tet* promoter (V. J. DiRita and J. Mekalanos, unpublished).

Not all *toxR* mutant derivatives of 569B are unaffected with respect to cholera toxin production. One of the original low toxin mutants, M13, derived by Finkelstein *et al.* (1974) from strain 569B, is complemented for cholera toxin production by cloned *toxR* (Miller and Mekalanos, 1984), suggesting that in this mutant ToxR is not dispensable. However, M13 was isolated after heavy NTG mutagenesis and screening for decreased cholera toxin production (Finkelstein *et al.*, 1974), and it is therefore possible that a *toxR toxT* double mutation was actually isolated. Complementation to Ctx^+ with plasmid-encoded *toxR* should occur even in a *toxT* mutant, since both ToxR and ToxT activate the *ctx* promoter (DiRita *et al.*, 1991). We have recently cloned the *toxT* gene from strain O395

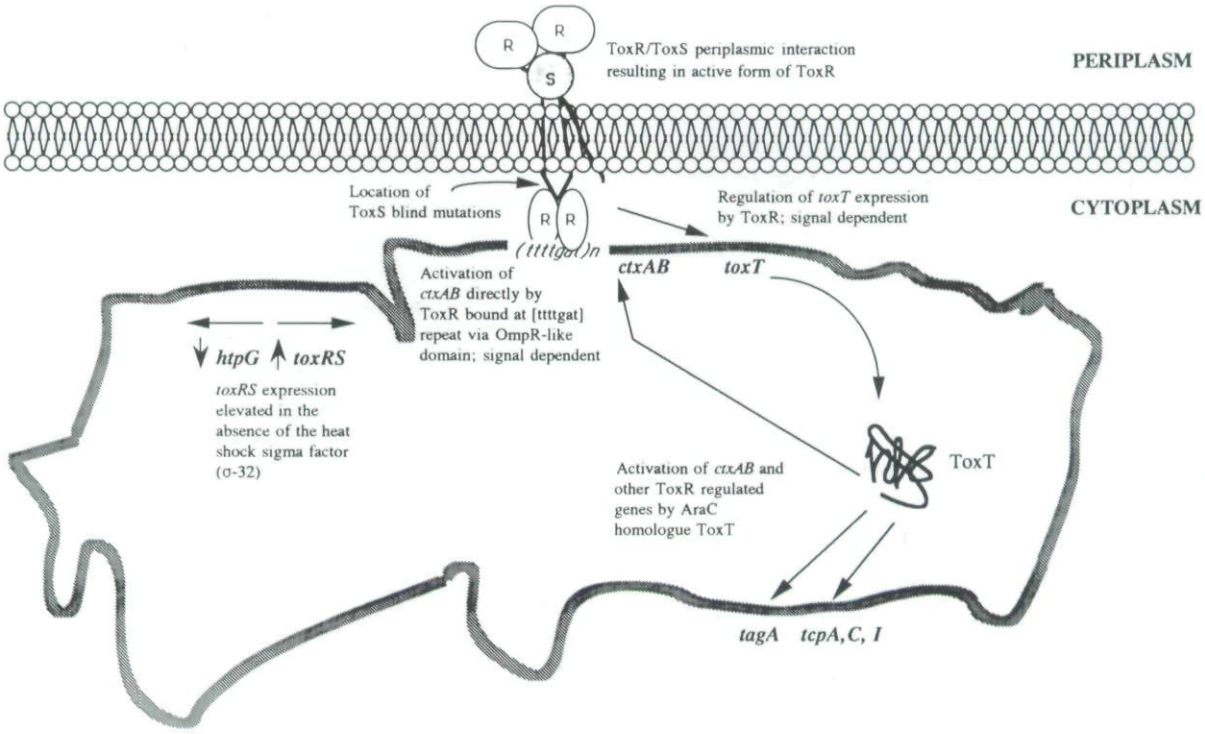

Fig. 1. Model for the ToxR regulatory system of *V. cholerae*. See text for details.

(D. Higgins and V.J. DiRita, unpublished), a classical *V. cholerae* strain that exhibits more typical regulation of ToxR-regulated virulence genes, and studies aimed at comparing *toxT* expression in O395 and 569B are currently underway. If the phenotype of 569B is due to a mutation leading to unregulated expression of *toxT*, this might be reflected in sequence differences in the *toxT* promoters from the two strains. Another test of this will be to determine whether *toxT* mRNA levels remain unaffected in *toxR* mutants of strain 569B.

Conclusions

A model for regulated expression of ToxR controlled genes is shown in Fig. 1. This model proposes that the system is essentially a cascade in which active ToxR activates expression of *toxT* and the *toxT* product then activates expression of other genes in the regulon. The cascade may be multilayered, given that the ToxT-activated *tcpI* gene is itself evidently a regulator of Tcp expression (Taylor, 1989). Gene dosage may also be a level of control over the cholera toxin genes that does not exist for other members of the regulon, since these are subject to amplification *in vivo* (Mekalanos, 1983).

The model suggests that conditions leading to active ToxR include the presence of ToxS in the right environment for ToxR function. As discussed above, many signals affect expression of the ToxR regulon, and, with the exception of the heat-shock response, these probably act at some level after *toxR* expression. This is particularly the case for elevated pH, at which *toxR* expression is unaffected but the regulon is off. That certain environmental signals affect the function of ToxR, and not its production, suggests that ToxR might have some other role, as yet unidentified, in the absence of such signals.

At this point, we do not know whether ToxR regulation of *toxT* expression is direct or indirect. Sequence analysis of several hundred base pairs upstream of *toxT* from strain O395 (which regulates the ToxR regulon in a typical fashion) has not revealed the TTTTGAT repeated element required for ToxR activation of the *ctxAB* promoter, although elements bearing strong consensus homology to the standard prokaryotic –35 and –10 regulatory motifs are detected upstream of *toxT* (D. Higgins and V.J. DiRita, unpublished). This indicates that ToxR might control *toxT* expression through another protein; genetic experiments using *toxT–lacZ* gene fusions will answer this question.

Other pathogenic organisms use regulatory cascades to control virulence gene expression and these have been defined to greater or lesser degrees. In the well-studied pathogen *B. pertussis*, many genes are controlled by the *vir* locus (Weiss and Falkow, 1984), consisting of the *bvgS* and *bvgA* genes (Stibitz and Yang, 1991), which are

sensor and activator components, respectively, of the two-component system of bacterial activators (Arico *et al.*, 1989). A mutation at the *vir* locus abolishes expression of several genes, but only the gene encoding the filamentous haemagglutinin is activated directly by BvgA (Stibitz *et al.*, 1988, J. F. Miller *et al.*, 1989b).

In a very elegant series of experiments aimed at defining proteins that bind the promoter of the pertussis toxin gene, a 23 kDa protein apparently distinct from BvgA was identified in Vir^+ cells by Huh and Weiss (1991). This protein, termed Act, also binds the promoter for the gene encoding adenylate cyclase toxin, another member of the Vir regulon (Huh and Weiss, 1991). Analogous to the ToxR cascade, expression of Act (which is presumed to activate the promoters to which it binds) is predicted to be Vir regulated (Huh and Weiss, 1991).

In *Staphylococcus aureus*, the expression of several virulence genes is controlled by the *agrA* locus, made up of the *agrA, agrB,* and *agrC* genes (Peng et al., 1988; Janzon and Arvidson, 1990). AgrA, a member of the two component family, controls expression of δ-lysin, encoded by the *hld* gene (Janzon and Arvidson, 1990). Strong evidence suggests that the *hld* mRNA plays a regulatory role for expression of other genes in the AgrA regulon (Janzon and Arvidson, 1990; this reguatory mRNA is also referred to as RNAIII; Vandenesch *et al.*, 1991). In this novel system, control by the central regulator AgrA is somehow delegated through the mRNA of one of the genes in the regulon.

Exactly why microorganisms 'go virulent' when encountering a human host, given that this response is detrimental to the host, is often the subject of teleological and highly speculative debate. What is clear is that whether cascades of the type described above are involved or whether different, but equally complex, schemes are at work (Deretic *et al.*, 1991), tightly managed regulatory systems have evolved to allow pathogens to control precisely when virulence genes will be expressed. It is safe to say that since constitutive expression of virulence genes is rarely observed, it is probably almost never advantageous (Miller and Mekalanos, 1990). This suggests that virulence regulation has co-evolved with host environments and that a complete understanding of virulence regulation will involve a better understanding of these environments. Many investigators are now studying the interaction of microbes with host tissues and cell types, but specific *in vivo* signals that feed through known regulatory pathways have only rarely been determined. In fact, what might be the most well-understood use of authentic environmental cues (exclusive of iron) by a pathogen is the recognition by the plant pathogen *A. tumefaciens* of phenolic compounds at wound sites by the *virA/virG* two-component system (Stachel and Zambryski, 1986). However, as the regulatory machinery in other pathogens is dismantled and understood, and as investigators continue to be creative and bold in their approaches, we will no doubt soon have greater insight into the nature of specific *in vivo* signals and how they are processed.

Acknowledgements

I am grateful to John Mekalanos, Theresa Finn, Mike Koomey, Karen Ottemann, and Darren Higgins for critical comments on the manuscript. I also thank Jim Kaper, Jeff Miller, Claude Parsot, and Alison Weiss for communicating results prior to publication.

References

Ames, P., and Parkinson, J.S. (1988) *Cell* **55:** 817–826.
Arico, B. *et al.*, (1989) *Proc Natl Acad Sci USA* **86:** 6671–6675
Boyd, J. *et al.* (1990) *Proc Nat Acad Sci USA* **87:** 5968–5972.
Calderwood, S.B., and Mekalanos, J.J. (1987) *J Bacteriol* **169:** 4759–4764.
Caron, J. *et al.* (1989) *Proc Natl Acad Sci USA* **86:** 963–967.
Cornelis, G. *et al.* (1987) *Rev Infect Dis* **9:** 64–87.
Cornelis, G. *et al.* (1989) *J Bacteriol* **171:** 254–262.
Cornelis, G. *et al.* (1991) *Mol Microbiol* **5:** 1023–1028.
Deretic, V. *et al.* (1991) *Mol Microbiol* **5:** 1–7.
DiRita, V.J., and Mekalanos, J.J. (1991) *Cell* **64:** 29–37.
DiRita, V.J. *et al.* (1990) In *The Bacteria, vol. XI.* Iglewski, B.H., and Clark, V.L. (eds.) Orlando, Florida: Academic Press, pp. 355–376.
DiRita, V.J. *et al.* (1991) *Proc Natl Acad Sci USA* **88:** 5403–5407.
Dutta, N.K. and Habbu, M.K. (1955) *Brit J Pharmacol* **10:** 153–159.
Evans, D.J. and Richardson, S.H. (1968) *J Bacteriol* **96:** 126–130.
Fasano, A. *et al.*, (1991) *Proc Natl Acad Sci USA* **88:** 000–000.
Finkelstein, R. *et al.* (1974) *J Infect Dis* **129:** 117–123.
Frank, D.W. and Iglewski, B.H. (1991) *J Bacteriol* **173:** 6460–6468
Goldberg, I., and Mekalanos, J.J. (1985) *J Bacteriol* **165:** 723–731.
Goldberg, M.B. *et al.* (1990) *Infect Immun* **58:** 55–60
Grossman, A.D. *et al.* (1987) *Genes Dev* **1:** 179–184.
Herrington, D. *et al.*, (1988) *J Exp Med* **168:** 1487–1492.
Huh, Y.J. and Weiss, A.A. (1991) *Infect Immun* **59:** 2389–2395.
Janzon, L. and Arvidson, S. (1990) *EMBO J* **9:** 1391–1399.
Jones, N. (1990) *Cell* **61:** 9–11.
Knapp, S., and Mekalanos, J.J. (1988) *J Bacteriol* **170:** 5059–5066.
Mekalanos, J.J. (1983) *Cell* **35:** 253–263.
Miller, J.F. *et al.* (1989a) *Science* **243:** 916–922.
Miller, J.F. *et al.* (1989b) *J Bacteriol* **171:** 6345–6348.
Miller, S.I. and Mekalanos, J.J. (1990) *J Bacteriol* **172:** 2485–2490.
Miller, S.I. *et al.* (1989) *Proc Natl Acad Sci USA* **86:** 5054–5058.
Miller, V.L. (1985) Ph.D. thesis, Harvard University, USA.
Miller, V.L., and Mekalanos, J.J. (1984) *Proc Natl Acad Sci USA* **81:** 3471–3475.
Miller, V.L., and Mekalanos, J.J. (1985) *J Bacteriol* **163:** 580–585.

Miller, V.L., and Mekalanos, J.J. (1988) *J Bacteriol* **170:** 2575–2583.
Miller, V.L. *et al.* (1987) *Cell* **48:** 271–279.
Miller, V.L. *et al.* (1989) *J Bacteriol* **171:** 1288–1293.
Miyada, C.G. *et al.* (1980) *Nucl Acids Res* **8:** 5267–5274.
Moss, J., and Vaughan, M. (1983) In *Posttranslational Covalent Modifications of Proteins.* Johnson, B.C. (ed.) New York: Academic Press, pp. 329–342.
Parsot, C., and Mekalanos, J.J. (1990) *Proc Natl Acad Sci USA*
Parsot, C., and Mekalanos, J.J. (1991) *J Bacteriol* **173:** 2842–2851
Parsot, C. *et al.* (1991) *Proc Natl Acad Sci USA* **88:** 1641–1645.
Pearson, G.D.N. (1989) Ph.D. thesis, Harvard University, USA.
Pearson, G.D.N. *et al.* (1990) *Res Microbiol* **141:** 893–899.
Pearson, G.D.N., and Mekalanos, J.J. (1982) *Proc Natl Acad Sci USA* **79:** 2976–2980.
Peng *et al.* (1988) *J Bacteriol* **170:** 4365–4372.
Peterson, K.M., and Mekalanos, J.J. (1988) *Infect Immun* **56:** 2822–2829.
Recsei, ?? *et al.* (1986) *Mol Gen Genet* **202:** 58–61.
Richardson, S.H. (1969) *J Bacteriol* **100:** 27–34.
Savelkoul, P.H. *et al.* (1990) *Microb Path* **8:** 91–99.
Schlesinger, M.J. (1967) *J Biol Chem* **242:** 1604–1611.
Schmitt, M.P., and Holmes, R.K. (1991) *Infect Immun* **59:** 1899–1904.
Smith, B.R., and Schleif, R. (1978) *J Biol Chem* **253:** 6931–6933.
Stachel, S.E., and Zambryski, P. (1986) *Cell* **46:** 325–333.
Stibitz, S., and Yang, M.-S. (1991) *J Bacteriol* **173:** 4288–4296.
Stibitz, S. *et al.* (1988) *J Bacteriol* **170:** 2904–2913.
Straus, D.B. *et al.* (1987) *Nature* **329:** 348–351.
Taylor, R.K. *et al.* (1987) *Proc Natl Acad Sci USA* **84:** 2833–2837.
Taylor, R.K. (1989) In *Genetics of Bacterial Diversity.* Hopwood, D.A. and Chater, K.F. (eds.), London: Academic Press, pp. 309–329.
Vandenesch, F. *et al.* (1991) *J Bacteriol* **173:** 6313–6320.
Weiss, A.A., and Falkow, S. (1984) *Infect Immun* **43:** 263–269.