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Virulence gene regulation inside and outside

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Much knowledge about microbial gene regulation and virulence is derived from genetic and biochemical studies done outside of hosts. The aim of this review is to correlate observations made *in vitro* and *in vivo* with two different bacterial pathogens in which the nature of regulated gene expression leading to virulence is quite different. The first is *Vibrio cholerae*, in which the concerted action of a complicated regulatory cascade involving several transcription activators leads ultimately to expression of cholera toxin and the toxin-coregulated pilus. The regulatory cascade is active *in vivo* and is also required for maintenance of *V. cholerae* in the intestinal tract during experimental infection. Nevertheless, specific signals predicted to be generated *in vivo*, such as bile and a temperature of 37 °C, have a severe down-modulating effect on activation of toxin and pilus expression. Another unusual aspect of gene regulation in this system is the role played by inner membrane proteins that activate transcription. Although the topology of these proteins suggests an appealing model for signal transduction leading to virulence gene expression, experimental evidence suggests that such a model may be simplistic. In *Streptococcus pyogenes*, capsule production is critical for virulence in an animal model of necrotizing skin infection. Yet capsule is apparently produced to high levels only from mutation in a two-component regulatory system, CsrR and CsrS. Thus it seems that in *V. cholerae* a complex regulatory pathway has evolved to control virulence by induction of gene expression *in vivo*, whereas in *S. pyogenes* at least one mode of pathogenicity is potentiated by the absence of regulation.

Keywords: *Vibrio cholerae*; ToxR; *Streptococcus pyogenes*; virulence; regulation

1. INTRODUCTION

Studies on how virulence traits are regulated in bacterial pathogens have generally been guided by either of two intuitions. The first is that genes encoding virulence factors are in fact regulated and that, knowing the virulence gene products, it should be possible to work backwards to the regulatory factors and thereby to the *in vivo* regulatory parameters. The second guiding intuition has been that correct assumptions may be made about the *in vivo* environment and these will aid in identifying both virulence factors and their regulatory parameters. An example of the first class of intuition was the identification of the regulator for the cholera toxin genes of *Vibrio cholerae*, after it had been demonstrated that high-level expression of cholera toxin is not constitutive (Pearson & Mekalanos 1982). Thus Miller & Mekalanos (1984) cloned the relatively weak promoter for the cholera toxin genes (*ctxAB*) into *Escherichia coli* as an operon fusion to *lacZ*, and subsequently identified a regulatory gene, *toxR*, whose expression in this *E. coli* background resulted in elevated *ctx-lacZ* expression. From this simple experiment sprouted a vast field of observations concerning the regulation of cholera toxin, as well as of several other factors important in the pathogenesis of cholera (Skorupski & Taylor 1997). This system will be explored in more detail in § 3(a).

An example of the second intuition underlying regulatory studies in pathogenesis led to classic work on *Shigella* spp., including the observation that cell invasion by these organisms is temperature regulated. Genes encoding temperature-regulated phenotypes were therefore targeted by using a promoterless *lacZ* gene engineered into a transposable element. Screening was for fusions with high activity at 37 °C and low activity at 25 or 30 °C (Maurelli *et al.* 1984; Maurelli & Curtiss 1984; Maurelli & Sansonetti 1988). This approach led to the identification of several virulence genes encoded on a virulence-associated plasmid, which have been well characterized in the ensuing time-period, and also, eventually, to regulatory elements that control expression of these genes including VirF, VirB and H-NS (Dorman & Porter 1998).

The difference between these examples, of course, is in the relative extent of knowledge available prior to when studies of gene regulation were imposed on each system. Cholera toxin, the signal virulence determinant in *V. cholerae*, had been studied for a long time prior to cloning the genes and subsequent analysis of their regulation. Although the fact of its environmental regulation was well established by the work of Richardson and his colleagues (Evans & Richardson 1968; Callahan & Richardson 1973; Richardson 1969), the study of gene

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regulation proceeded once ToxR was identified as being required for cholera toxin expression. After that came the assumption that environmental regulation by parameters such as pH, temperature and osmolarity probably proceeds in some fashion through the action of ToxR (Miller *et al.* 1987; Miller & Mekalanos 1988).

Shigella, on the other hand, was less well understood in terms of the factors that contribute to its pathogenicity when the development of gene fusion technology allowed for isolation of temperature-regulated genes. But by focusing on the likely signals that regulate virulence based on the observation that growth temperature influences cell invasiveness, the same outcome as the cholera story was achieved; regulatory elements were identified and the conditions under which they operate to control gene expression were assumed.

Notwithstanding the level of knowledge already in hand upon initiation of regulatory studies, each of the two approaches outlined above is based on a 'virulence-factor' model: that known virulence factors may lead the investigator to the regulatory system controlling them.

What is the value of analysing the animating assumptions that have led to the understanding we currently have of gene regulation in pathogenesis? Because this analysis helps put into perspective the fact that in the post-genomic era the assumptions that underlie regulatory studies in pathogenesis will probably be entirely different ones. To be sure, future work using genomic approaches will take advantage of the large body of work that has been done in gene regulation not only in pathogenic organisms, but also in non-pathogenic organisms. Thus, it is now possible to search complete genomes for homologies to known regulatory elements and knockout each one that is discovered in order to assess its role in virulence. This method, a 'virulence regulator' approach, requires that there be some way for the investigator to analyse the mutant phenotypes, and probably the best way is to use a reliable animal model to ascertain the virulence phenotype of the knockout strains. If introducing a mutation into a homologue of a regulator gene results in altered virulence, then it may be concluded that the gene in question probably regulates another gene that is required for virulence. Once that knowledge is obtained, further molecular or genetic approaches are then necessary to identify those genes and their products.

In either case, the virulence factor model or the virulence regulator model, the *in vivo* relevance of the regulatory system ultimately under consideration is not always straightforward, for even when a regulatory system is shown to be vital for *in vivo* effects leading to virulence, the precise mechanism for why this is so, or the signals that may impinge on the regulatory system, are not always obvious. This is particularly true for regulators of the so-called two-component family, in which a sensor kinase perceives a signal and transmits it through phosphorylation of a response regulator, which is typically a transcription factor. In this family of proteins, the sensor kinase is presumed to be the direct receiver of signals to which the system responds (Parkinson 1993; Parkinson & Kofoid 1992). In many cases of two-component regulators, however, the precise signals that initiate the cascade of events that leads to activation of the response regulator are very often not well understood.

A good example of this is in the well-studied Bvg system of *Bordetella* spp. The activity of the two-component system in these organisms, BvgS (sensor kinase) and BvgA (response regulator) is modulated by signals such as nicotinic acid, Mg^{2+} and temperature, although how these signals are actually identified by BvgS is less clear (Akerley & Miller 1996). Nevertheless, that signal transduction through this system is critical for establishing a normal host–pathogen interaction has been well demonstrated both by isolating mutants of BvgS that signal inappropriately, as if signals were present all the time or not at all (Miller *et al.* 1992), and by converting genes that are normally repressed by the Bvg system into ones that are activated by it. Such ectopic expression, as it has come to be called, profoundly disrupts normal host–microbe interaction (Akerley & Miller 1996; Akerley *et al.* 1995).

A more successful example in recent years of attempting to identify the signals that influence regulatory activity during infection has been the characterization of PhoP and PhoQ of *Salmonella* spp. This has been demonstrated to be a sensory (PhoQ)/response (PhoP) system that monitors the levels of available Mg^{2+} (and Ca^{2+}) and activates gene expression when those levels are low. This finding was triggered by the observation that among genes controlled by PhoPQ are a number of genes whose products are involved in magnesium transport (*mgt*). Subsequently it was demonstrated that magnesium induces a conformational change in PhoQ, the sensor kinase and that the PhoQ periplasmic domain binds magnesium in solution (Garcia *et al.* 1996; Vescovi *et al.* 1997). These observations indicate that magnesium is an important signal for this system and that at low levels, such as those postulated to occur intracellularly, PhoPQ activates virulence gene expression in *Salmonella*.

Two-component gene systems are appealing targets for study along the virulence regulator line because they are widely assumed to be sensory systems triggered by specific signals. Thus, if a two-component system regulates a particular virulence trait, the natural assumption is that the system is responding to some *in vivo* signal to do so. Added to this assumption in the overall appeal of studying two-component systems and their role in virulence is that both the sensor kinase and the response regulator are defined by specific motifs within the primary amino-acid sequence. These are related to domains of the proteins necessary for phosphorylation or for DNA binding and transcription control. It is therefore very straightforward to design primers for the polymerase chain reaction (PCR) that allow for amplification of all of the sensor kinases or response regulators of a given species, which has been done for a number of pathogenic bacteria (Wren *et al.* 1992). The homologies among two-component family members also make it easy to identify these proteins when analysing genome sequence data.

Recent work on a two-component system in *Streptococcus pyogenes*, CsrR and CsrS, suggests a different role for such proteins in governing virulence phenotypes of a pathogen. Rather than functioning as an activator of genes required for pathogenicity, CsrR represses transcription of the genes encoding a hyaluronic acid capsule (*hasAB*) that is strongly associated with abscess formation and subsequent disease in a mouse model of necrotizing

fasciitis (Bunce *et al.* 1992; Murley *et al.* 1999). This is an intriguing system as the pathogenesis of skin disease is dependent on acquiring inactivating mutations in *csrR* or the gene encoding its putative sensor kinase gene, *csrS*, thus raising the question of what signals the system may normally respond to and how this signalling may regulate important phenotypes in the interaction between host and pathogen.

After a period in which studies motivated by both virulence factor and virulence regulator models of gene discovery have identified a plethora of regulators required for virulence, demonstrated to be so by relevant animal, and sometimes human, models of infection, the question is less one of whether these are required *in vivo* as much as it is of how these proteins regulate gene expression *in vivo* and what signals may control their activity. Or, more properly, the question may be recast as the following: are signals that stimulate regulatory activity *in vitro* the same ones that stimulate such activity *in vivo*? It seems likely, from the *Salmonella* example, that the answer may ultimately be in the affirmative for all systems under investigation, but only after investigators figure out what the correct signals are. For the remainder of this paper, we will focus on attempts to address this question in two different systems of virulence regulation: the ToxR/ToxT system in *V. cholerae* and the CsrR system in *S. pyogenes*.

2. MATERIAL AND METHODS

(a) *Construction of ToxR deletion derivatives*

PCR products with various amounts of *toxR* were generated using either Taq DNA polymerase (Gibco BRL, Grand Island, NY, USA) or the Expand^{TM} High Fidelity PCR System (Boehringer Mannheim, Indianapolis, IN, USA) using manufacturer's recommended protocols. PCR templates were pVJ21 (Miller *et al.* 1989) or chromsomal DNA from *V. cholerae* strain O395. Synthesized primers were engineered to have added recognition sequences for restriction endonucleases in order to facilitate directional cloning of the products. Products from the PCR were purified by gel electrophoresis followed by extraction with the QIAEX II system (Qiagen, Inc., Valencia, CA, USA). Cloning into expression plasmids was done using standard protocols (Sambrook *et al.* 1989)

(b) *Primer extension of mRNA*

RNA was isolated from bacteria using Trizol Reagent (Gibco BRL). Ten picomoles of primer were end-labelled using 50 µCi [γ-^{32}P]ATP using standard protocols (Sambrook *et al.* 1989). Approximately 2 pmol labelled primer were added to 30 µg RNA and diethylpyrocarbonate-treated water was added to a final volume of 20 µl. Primer extension was carried out using Superscript Reverse Transcriptase (Gibco BRL) as previously described (Higgins & DiRita 1994; Yu & DiRita 1999). Reaction mixtures were resolved in 8% denaturing polyacrylamide gels and visualized by autoradiography following standard protocols (Sambrook *et al.* 1989).

(c) *Mouse infections with group A streptococci*

A dermonecrotic mouse model, described by Barg and coworkers, was used to assess the level of pathogenicity of wild-type and *csrRS* mutants of MGAS166, an M1 SpeA2 group A streptococcus strain (Bunce *et al.* 1992; Heath *et al.* 1999). Unless noted, strains were harvested in mid-log growth and concentrated to produce inocula of specific numbers of microbes in 200 µl of suspension. This was injected into the right flank of four-week-old male crl:SKH1 (hairless; hrhr) BR mice (Charles River Laboratories, Wilmington, MA, USA). Mice were weighed before inoculation and every 24 h, and necrotic lesions were measured daily.

3. RESULTS AND DISCUSSION

(a) *Analysis of the ToxR regulon in* V. cholerae

Identification of ToxR as a major factor in virulence regulation in *V. cholerae* was a prime example of the virulence factor approach. The genes encoding cholera toxin, *ctxAB*, are not well expressed when cloned in the heterologous background of *E. coli*. This observation prompted a genetic screen for factors from *V. cholerae* which, when expressed in an *E. coli* strain having a *ctx-lacZ* operon fusion in it, would result in Lac$^+$ colonies due to activation of the fusion (Miller & Mekalanos 1984). From this screen arose clones that were shown to encode *toxR*, which was later demonstrated to be an unusual regulatory protein in that it resides in the inner membrane and has an amino-terminal, cytoplasmic domain that shares extensive and important homology with the DNA-binding–transcription-activation domains of several response regulator proteins in the two-component family. The carboxy-terminal domain of ToxR is in the periplasmic space and there it probably interacts with another protein called ToxS, also required for *ctxAB* expression (DiRita & Mekalanos 1991; Miller *et al.* 1989); the two proteins are encoded by an operon, *toxRS*. ToxS and the remainder of ToxR beyond the DNA-binding–activation domain do not share homology with other proteins that might provide intuition for how they function. Notably, they lack important conserved residues found in the phosphorylation-dependent two-component regulatory systems.

A search for gene fusions whose pattern of expression was similar to that of cholera toxin ultimately revealed several other genes that were also demonstrated to be regulated by ToxR. These include the toxin-coregulated pilus (TCP), the accessory colonization factor (ACF) and an outer membrane protein (OmpU) (Peterson & Mekalanos 1988; Miller & Mekalanos 1988; Skorupski & Taylor 1997). Another outer membrane protein, OmpT, is regulated oppositely to these other factors and we now know that its gene expression is repressed directly by ToxR (Li *et al.* 2000). Conditions for maximum expression of *ctxAB* in many strains of *V. cholerae* include a temperature of 25–30 °C and a relatively acidic pH (6.5 as opposed to 8.5). The temperature optimum in particular is counter-intuitive given that the organism expresses cholera toxin during infection of the small intestine at 37 °C. Also counter-intuitive is the fact that bile salts, which, *a priori*, might be considered as a potential signal for *in vivo* stimulation of virulence genes in *V. cholerae*, instead have a strongly repressive effect on expression of the ToxR regulon (Gupta & Chowdhury 1997; Schuhmacher & Klose 1999) (figure 1).

ToxR, although required for expression of cholera toxin, TCP, ACF and OmpU, actually controls expression of these genes indirectly through its ability to control expression of another activator, ToxT (DiRita *et al.* 1991).

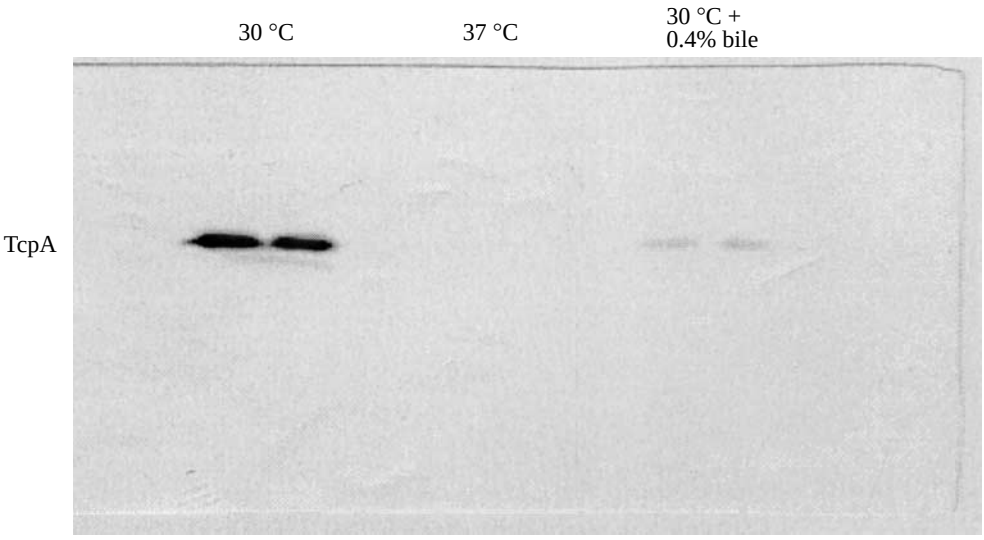

Figure 1. The effect of temperature and bile salts on expression of the ToxR-regulated TCP. Cultures were grown overnight in Luria–Bertani medium under the indicated conditions and lysed by boiling in sodium dodecyl sulphate polyacrylamide gel electrophoresis buffer. Equivalent amounts of protein were loaded on a 10% polyacrylamide gel and subjected to electrophoresis. The gel was blotted to nitrocellulose and probed with antibodies to the major TCP subunit, TcpA. Equivalent effects on cholera toxin were observed by enzyme-linked immunosorbent assay (not shown).

Regulation of *toxT* transcription is complex and requires another pair of membrane-localized regulatory proteins, TcpP and TcpH, which cooperate with ToxR and ToxS for activation of one of two *toxT* promoters. The other promoter controlling *toxT* expression is several kilobases upstream of the gene in front of the *tcp* operon, which contains the majority of genes for TCP synthesis. This promoter is controlled by ToxT itself. Thus *toxT* expression is controlled by a regulatory loop in which activation by ToxR and TcpP leads to expression from a proximal promoter and subsequent expression is controlled by ToxT from a more distal promoter (Brown & Taylor 1995; Yu & DiRita 1999; Medrano *et al.* 1999). Analysis of *toxT* expression from these different promoters *in vivo* is currently being investigated by Camilli and his co-workers using an *in vivo* reporter system (Merrell & Camilli, this issue).

Signals that control expression of the virulence factors may be overridden by constitutive expression of ToxT, implying that the signalling capacity for the system occurs prior to ToxT expression (DiRita *et al.* 1996). That this is probably so comes from the observation that expression of *tcpPH* is subject to conditional expression by two activators, AphA and AphB, leading to the hypothesis that regulated expression of *tcpPH* in turn controls regulated expression of *toxT* (Skorupski & Taylor 1999; Kovacikova & Skorupski 1999; Murley *et al.* 1999). This is not the case for bile salts, which repress virulence gene expression at the level of ToxT, as constitutive expression of ToxT does not overcome the effect of bile (Schuhmacher & Klose 1999). Regulatory signals controlling the ToxR regulon appear to be somewhat different between the two major epidemic biotypes of *V. cholerae*, classical and El Tor, with the latter biotype having more stringent growth requirements for activating the regulatory loop that leads to *toxT* expression (Medrano *et al.* 1999; Murley *et al.* 1999).

A paradox of this system is that although ToxR was originally identified for its ability to activate the cholera toxin operon in *E. coli* independently of other *V. cholerae* factors, mutants of *V. cholerae* lacking *toxT*, but expressing functional ToxR, do not express cholera toxin in the laboratory (Champion *et al.* 1997). Overexpression of ToxR in the *toxT* mutant background leads to a slight elevation of toxin production, but not to levels seen in wild-type cells. Experiments analysing expression of *ctx* using *in vivo* reporter systems will perhaps shed some light on this *in vitro* paradox.

The localization of several major regulatory factors, ToxR/ToxS and TcpP/TcpH, to the inner membrane leads naturally to the question of what role this placement may have in the regulation of gene expression. Some evidence suggests that the periplasmic domains of both ToxR and TcpP are important for signalling across the inner membrane, and this is indeed an appealing possibility given the receptor-like topology of these proteins. Replacement of the periplasmic domain of ToxR with the periplasmic protein alkaline phosphatase (PhoA) resulted in constitutive expression of cholera toxin, i.e. toxin expression occurred under normally non-permissive conditions (see below). This implies that the periplasmic domain of ToxR is a signal sensing domain that controls, for example, DNA binding or transcription activation by the cytoplasmic domain (Miller *et al.* 1987). Likewise, fusion of the periplasmic protein β-lactamase to the carboxy-terminal domain of TcpP led to constitutive expression of a *toxT–lacZ* gene fusion, again suggesting a role for the periplasmic domain, this time of TcpP, in signalling that leads to virulence gene expression (Häse & Mekalanos 1998).

A slightly different hypothesis is that localization to the membrane is important for the function of these activator proteins, and there is some evidence to support that. Several groups have produced different versions of ToxR that localize their DNA-binding–transcription-activation domain either to the cytoplasm or to the membrane, and these experiments have had different results (DiRita &

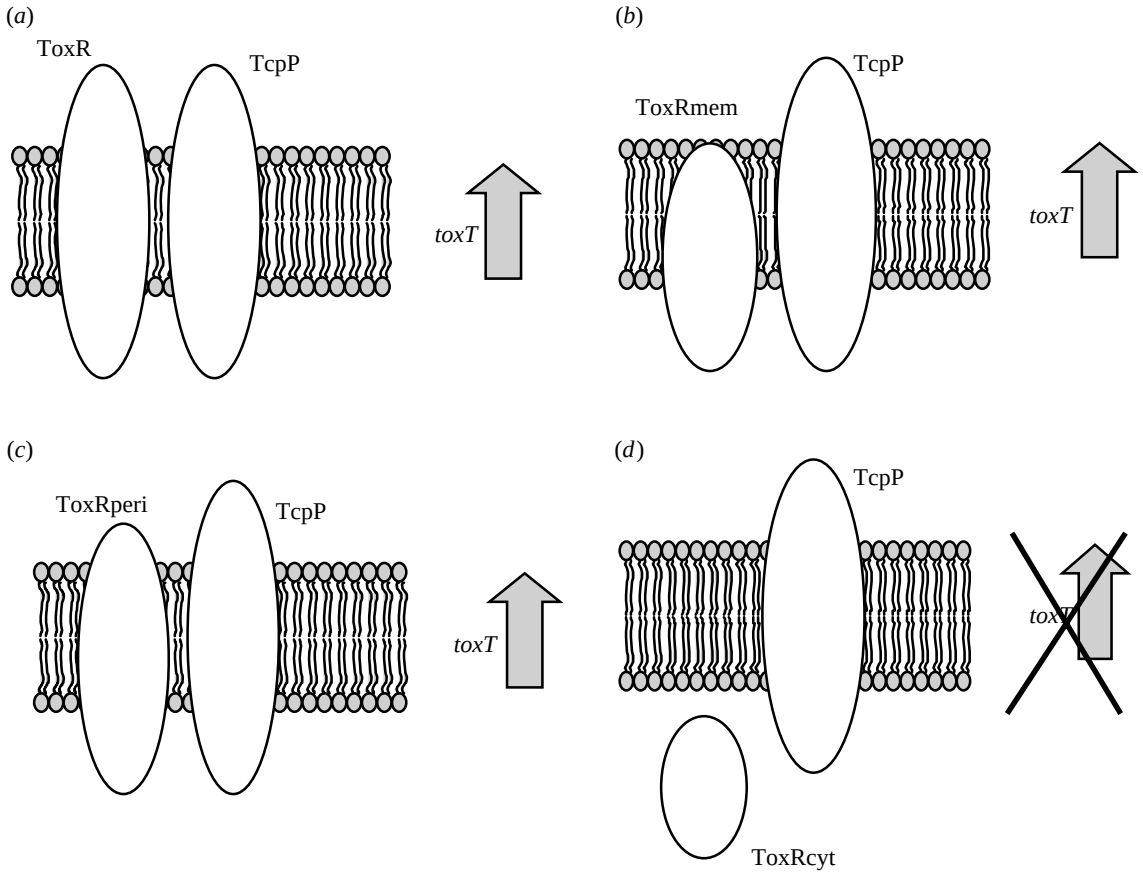

Figure 2. Membrane localization requirements. Summary of data from primer extension experiments monitoring transcription of *toxT* as described in the text. (*a–c*) Indicate the ability of membrane-localized forms of ToxR harbouring progressively less of the periplasmic domain to cooperate with membrane-localized TcpP for activation of *toxT* transcription. (*d*) Indicates that the cytoplasmic domain of ToxR (ToxRcyt), with only the DNA-binding–transcription motif, does not lead to TcpP-dependent activation. The cytoplasmic form does regulate the outer membrane proteins OmpU and OmpT like wild-type, as do all the other forms of ToxR that were tested.

Mekalanos 1991; Miller *et al.* 1987; Ottemann & Mekalanos 1995; Kolmar *et al.* 1995). These experiments have usually been predicated on the assumption that the multimeric structure of ToxR, which may be conferred by the periplasmic domain, is critical for ToxR function (DiRita & Mekalanos 1991; Miller *et al.* 1987; Ottemann & Mekalanos 1995; Kolmar *et al.* 1995). Therefore such experiments have often tested not solely the role of membrane localization, but also the multimerization status of the protein.

We approached the problem of membrane localization by constructing a series of ToxR derivatives with different degrees of the native protein beyond the DNA-binding–transcription-activation domain but otherwise having no fusion moiety. We specifically addressed the ability of these proteins to control TcpP-directed transcription activation of *toxT*. Based on its similarity to the OmpR response regulator of *E. coli*, this domain is predicted to have the structure of a winged helix, a motif in which an α-helix and two β-strands (which make up the wing) function in DNA recognition. When expressed in *V. cholerae* the winged helix of ToxR exhibited wild-type ToxR activity for regulation of OmpU and OmpT, but was unable to complement a *toxR* mutant for expression of cholera toxin or TcpA, the major subunit of the TCP (data not shown). Primer extension of *toxT* mRNA showed that activation of *toxT* transcription was severely diminished, which is probably the reason for diminished activation of toxin and TcpA. The results of these studies are summarized in figure 2, which indicates that membrane localization of the amino-terminal DNA-binding–transcription-activation domain of ToxR is required for activating *toxT* in a TcpP-dependent fashion.

In contrast to the function of the ToxR winged helix domain alone, a construct in which the winged helix was expressed with the remaining cytoplasmic sequences as well as the transmembrane domain, but lacking any periplasmic domain, regulated *omp* gene expression like wild-type and also activated *toxT* transcription in a strictly TcpP-dependent manner. Analysis of other ToxR derivatives demonstrated that there are virtually no requirements for a periplasmic domain on ToxR, as constructs lacking this domain or having other sequences in place of the domain such as alkaline phosphatase, β-lactamase or the yeast leucine zipper domain GCN4 all activated *toxT* transcription and regulate *omp* gene expression like wild-type, similar to what has been observed by others, as described above. Each of these constructs demonstrated dependence on TcpP for their activity, suggesting that co-localization of the DNA-binding domain of ToxR and TcpP to the membrane is necessary and sufficient for activation of *toxT* and subsequent virulence gene regulation.

An appealing hypothesis for the function of this class of regulator is that the periplasmic domain is an important component of function, perhaps because it senses signals from the environment directly and transduces the signal by inducing conformational changes in the domain of the protein required for DNA binding and transcription activation. Evidence for this possibility comes from the observation that cholera toxin expression can be uncoupled from normal regulatory signals *in vitro* when its periplasmic domain was replaced by alkaline phosphatase. In this experiment, Miller *et al*. (1987) showed that toxin levels in cells expressing ToxR-PhoA were unaffected by growth at pH 8.5, a condition in which ToxR-regulated genes are not typically expressed. This result suggests that the periplasmic domain on ToxR may normally play a role in downregulating ToxR function.

Additional support for the periplasmic domain of membrane-localized activators acting as a sensor of environmental signals comes from work with two other such proteins, TcpP and the *E. coli* activator CadC. In the case of TcpP, Häse & Mekalanos (1998) mutagenized *V. cholerae* with the transposon Tn*bla*, which allows for identification of operon fusions to the periplasmic β-lactamase, and screened for isolates that expressed TCP constitutively, i.e. under conditions that typically lead to lack of TCP expression. Among this pool was a strain in which the periplasmic domain of TcpP had been fused to β-lactamase. The reason why this fusion results in an apparently constitutively active protein has yet to be determined, but among the possibilities are that the periplasmic domain regulates the ability of TcpP to activate *toxT* expression in coordination with ToxR under appropriate conditions, and that alteration of the periplasmic domain by fusion to β-lactamase may allow this signal detection process to be bypassed.

Evidence for a role in signal recognition by the periplasmic domain of a membrane-localized transcription activator is more strongly supported by studies with CadC, which regulates gene expression in *E. coli* at pH 5.8 in the presence of lysine. When these conditions are met, CadC activates expression of the *cadBA* operon, which expresses lysine decarboxylase, resulting in production of cadaverine from lysine. Mutant CadC proteins that activate *cadA* expression independently of pH or lysine have lesions in residues within the periplasmic domain of the protein, suggesting a possible direct role in signal recognition by this domain (Dell *et al*. 1994).

Another possibility for how signals may be recognized by this unusual class of proteins is that their membrane location *per se* is a component of their putative ability to sense signals. In this model, the membrane itself may be a co-factor in stimulating transcription activation, such that perturbations to the membrane structure, integrity or bioenergetics during *in vivo* growth, in the case of *V. cholerae*, may stimulate the activity of ToxR and/or TcpP. An intriguing result that supports this hypothesis is that, in their screen of Tn*bla* mutants constitutive for TCP production, Häse & Mekalanos (1999) identified insertions into the gene for a NADH:ubiquinone oxidoreductase (*nqr*). This gene product is probably responsible for the sodium motive force that powers the flagella in some *Vibrio* spp., including *V. cholerae* (Häse & Mekalanos 1999). Subsequent work by these investigators showed that flagellar motility is altered in a *V. cholerae nqr* mutant and that this correlated with upregulation of *toxT* transcription. In addition, upon chemical inhibition of the oxidoreductase, *toxT* transcription was also increased. These results extend previous studies showing that motility and expression of cholera toxin and TCP are oppositely regulated and suggest that TcpP-dependent activation of *toxT* expression is a crucial participant in this process (Häse & Mekalanos 1999).

(b) *Capsule production in group A streptococci as a result of regulatory mutations arising during* in vivo *growth*

To summarize work on the ToxR/TcpP system described above, it appears that membrane interactions between ToxR and TcpP, as well as membrane-dependent processes linked to flagellar motility are critical for expression of virulence genes in *V. cholerae*. We will turn our attention now to a very different regulatory system controlling invasive behaviour of a Gram-positive pathogen, *S. pyogenes*, or group A streptococci. This pathogen may cause a range of infections from relatively mild ones such as impetigo or pharyngitis to more severe and life-threatening ones such as toxic shock syndrome, necrotizing fasciitis and, through autoimmune sequelae, rheumatic fever. The focus in this review is its role in necrotizing fasciitis, an invasive and potentially rapidly progressing, grave disease that may be fatal if not aggressively treated.

Among a variety of virulence-associated traits that have been characterized in *S. pyogenes*, expression of a hyaluronic-acid capsule is tightly associated with the more severe pathogenicity attributed to the organism. A locus called *csrRS* controls expression of the gene for hyaluronic-acid synthesis, *hasA*. The *csrRS* operon encodes a typical two-component regulatory system including a putative sensor kinase, CsrS and a response regulator CsrR. As opposed to the positive form of regulation by ToxR/TcpP/ToxT in *V. cholerae*, CsrRS regulates capsule production in *S. pyogenes* through repression. Thus, strains carrying mutations in the regulatory locus express higher levels of capsule and are more virulent in a mouse skin abscess model of necrotizing infection (Heath *et al*. 1999). Along with capsule production, other virulence-associated traits are upregulated in *csrRS* mutants of *S. pyogenes*. These are a cysteine protease called pyrogenic exotoxin B, encoded by *speB*, and a gene associated with production of streptolysin S, an oxygen-stable haemolysin, encoded by *sagA*, a streptokinase and a mitogenic factor (Heath *et al*. 1999; Levin & Wessels 1998; Ashbaugh *et al*. 1998).

(i) In vitro *effects of* csrRS *mutation*

CsrRS controls these genes at the level of transcription so that in *csrRS* mutants elevated mRNA for each is detected. In addition, *csrRS* mutants express increased amounts of *csrRS* mRNA, suggesting autoregulation (figure 3). Purified CsrR binds to the promoters of *hasA*, *sagA*, and *speB*, but only upon phosphorylation, which may be effected presumably by CsrS *in vivo* but can be done using acyl phosphate *in vitro* (Bernish & Van de Rijn 1999; A. Miller, N. C. Engleberg and V. J. DiRita, unpublished data). Despite the autoregulation of *csrRS* observed by primer extension analysis of mRNA isolated from organisms grown *in vitro*, phosphorylated CsrR does not bind to

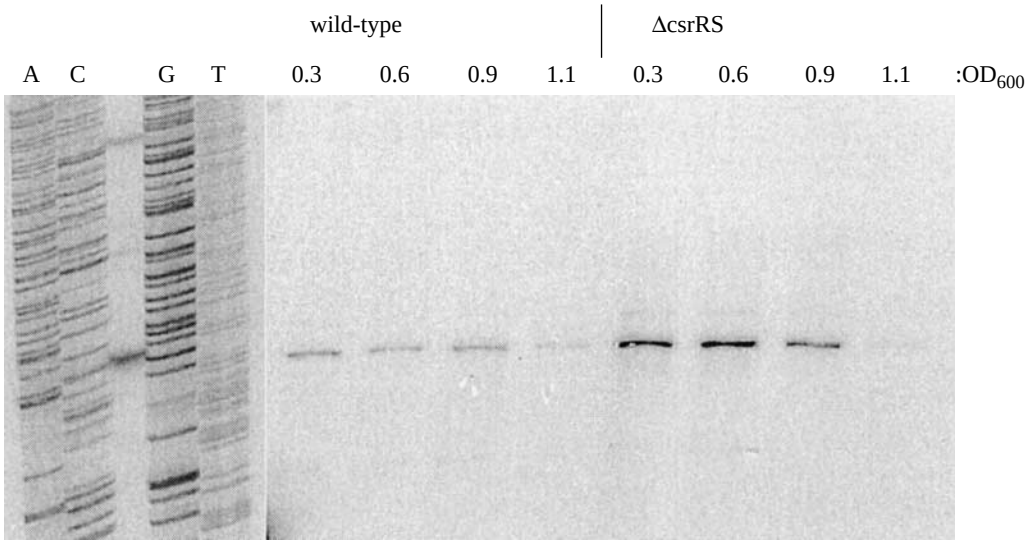

Figure 3. Primer extension of *csrR*. RNA was isolated from samples taken from cultures of wild-type and *csrRS* mutant *S. pyogenes*, collected at different points in the growth curve (indicated by the OD_{600} in the figure). The RNA was used in primer extension analysis using a primer that corresponds to a sequence within the *csrR* gene upstream of the deletion in the mutant strain.

the *csrRS* promoter indicating that regulation of its own operon may involve factors in addition to CsrRS.

Genes regulated by CsrRS are expressed in wild-type cells during late logarithmic growth, similar to regulation of virulence genes in the Gram-positive pathogen *Staphylococcus aureus*. In *csrRS* mutant cells, both the timing and the magnitude of expression is altered with genes being expressed to higher levels beginning at an earlier time in the growth cycle (Heath *et al.* 1999). We hypothesize that phosphorylation of CsrR by CsrS may be regulated during growth of the cells such that during early logarithmic growth a signal leading to CsrS kinase activity is present, thereby resulting in CsrR phosphorylation and repression of virulence genes. As cells progress through the growth curve this signal may be lost and CsrR may become unphosphorylated and derepression of capsule, SpeB and other genes occurs.

(ii) In vivo *behaviour of* csrRS *mutants*

Capsule production by group A streptococci is an important determinant of pathogenicity and strains that express it exhibit the most severe level of virulence in animal models (Ashbaugh *et al.* 1998; Heath *et al.* 1999). Given that CsrRS is a repressor of capsule expression, how does capsule become expressed during *in vivo* growth? One hypothesis is that conditions *in vivo* are such that CsrR is non-phosphorylated and therefore unable to bind DNA and repress *hasA* transcription. That wild-type cultures of group A streptococci grown in static broth prior to inoculation of mice may cause formation of skin abscesses (but not typically more severe, invasive necrotizing disease) suggests that growth conditions may indeed affect the level of virulence and that this may be through control by CsrR, although there is no direct evidence for the latter hypothesis. Another possibility to account for activation of *hasA* and other genes normally repressed by CsrR–P is that mutations in the *csrRS* locus may arise during infection and that these strains lead to elevated levels of tissue invasiveness.

In support of this latter hypothesis is the fact that strains with lesions in *csrRS* are more virulent in a dermo-necrotic mouse model (Heath *et al.* 1999). Significantly more damage, assessed by three different parameters (24 h weight loss, presence of lesions and necrosis within lesions), is noted than when wild-type organisms are injected. Whereas mice injected with 4×10^6 wild-type organisms gained approximately 1 g in weight over 24 h after infection (similar to sham-inoculated controls), mice injected with *csrRS* mutants lost over 3 g of weight in the same period. Likewise, all animals infected with *csrRS* mutants developed skin lesions (compared with only one-third of those infected with wild-type); all of these lesions became necrotic by 72 h while none of the lesions caused by wild-type did (table 1) (Heath *et al.* 1999). These data suggest that derepression of the *csrRS* regulon is an important aspect for an infection to develop into a more life-threatening necrotizing disease. Of interest to us in helping to explain the pathogenesis of lesion formation was the fact that animals infected with double *csrRS*/*hasAB* mutants, which lack the genes for capsule production, nevertheless produced some lesion formation in infected animals, suggesting that factors other than capsule are involved in the dermo-necrotic phenotype.

The development of necrotizing skin infection by the *csrRS* mutant strains is associated with growth of the organisms within the lesion itself. To ask whether the environment within the lesion caused by the mutant was permissive for growth of wild-type organisms, we co-infected mice with both *csrR* mutant and wild-type organisms in numbers at which the wild-type is usually cleared from the animal. Mice developed lesions from this combination of organisms, and bacteria of both genotypes were isolated in numbers significantly higher than the input dose of each (Heath *et al.* 1999). From this experiment we conclude that the presence of the mutant strain influences the survival of the wild-type within the lesion, suggesting that a factor (or factors)

Table 1. *Patterns of mouse skin lesion formation by wild-type and* csrRS *mutants of* S. pyogenes

(Adapted from Heath *et al.* (1999).)

inoculum	genotype of strain	24 h weight gain	no. with lesions/no. inoculated	no. with necrosis/ no. with lesions
2×10^5	uninoculated	0.5 ± 0.6	0/8	0/0
	csrRS$^+$	0.9 ± 0.4	0/6	0/0
	ΔcsrRS	-2.1 ± 1.7	3/6	3/3
	ΔcsrRS, ΔhasAB	-0.4 ± 1.3	0/6	0/1
2×10^6	uninoculated	0.6 ± 0.07	0/6	0/0
	csrRS$^+$	-0.4 ± 1.2	0/6	0/0
	ΔcsrRS	-4.3 ± 0.6	6/6	6/6
	ΔcsrRS, ΔhasAB	-3.4 ± 0.8	6/6	5/6

other than capsule may contribute to survival within the lesion.

Given that encapsulated strains are hypervirulent and also grow very well within lesions, we asked whether spontaneous *csrRS* mutants could be isolated during infection in the hairless mouse skin model. To do this, wild-type organisms were mixed with Cytodex beads, which enables lesion formation by the wild-type upon subcutaneous inoculation of hairless Br mice (Heath *et al.* 1999). Organisms were isolated from the blood and spleen of these infected animals and screened on plates for capsule production, an easily detectable phenotype on agar media. Six organisms with elevated capsule production were isolated and the nucleotide sequence of the *csrRS* locus was determined in each. All of the isolates had mutations of various classes in either *csrR* or *csrS*. In addition, a hypermucoid isolate from a cutaneous abscess also carried a lesion in the locus as did a spontaneous hypermucoid strain isolated *in vitro*.

These findings suggest that the hypermucoid phenotype of *csrRS* mutants is strongly linked to invasive disease with group A streptococci. Thus, this system represents a rather unusual form of regulation of virulence in a bacterial pathogen, in that a regulator must be inactivated for maximum levels of virulence to occur. There are several questions remaining regarding the CsrRS system of virulence regulation. At present, for example, whether there are important determinants of pathogenicity regulated by CsrRS that we are not currently aware of is not known. Additionally, given that CsrRS is a two-component system we assume that regulatory signals exist and hypothesize that they influence the activity of CsrS for phosphorylation of CsrR. During *in vivo* growth of the wild-type strain, signals very likely keep capsule and other CsrR-regulated genes repressed. Another important question in this system is whether the wild-type organisms play any role in pathogenicity, given that it would appear that infections leading to necrotizing disease are mixed infections of both wild-type and mutant origin. This might be addressed, for example, by determining whether or not mixed infections progress to severe necrotizing disease faster than do clonal infections with only *csrRS* mutants.

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