Coordinate Regulation and Sensory Transduction in the Control of Bacterial Virulence

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Genes and operons that encode bacterial virulence factors are often subject to coordinate regulation. These regulatory systems are capable of responding to various environmental signals that may be encountered during the infectious cycle. For some pathogens, proteins that mediate sensory transduction and virulence control are similar to components of other bacterial information processing systems. Understanding the molecular mechanisms governing global regulation of pathogenicity is essential for understanding bacterial infectious diseases.

BACTERIAL PATHOGENS ARE HIGHLY ADAPTED MICROORGANISMS with a survival strategy that requires multiplication on or within another living organism. It is the particular strategy used for this purpose that distinguishes pathogens from innocuous commensals or bacteria that cause disease primarily in compromised hosts. Mechanisms for establishment within a suitable niche, nutrient acquisition, and avoidance of immune clearance may go unnoticed during asymptomatic infection or cause overt harm during disease. Disease may therefore develop as a consequence of the specific microbial design used to outwit the host and multiply.

The host-microbe interaction that occurs during pathogenesis is a dynamic process in which the infecting organism encounters diverse environmental conditions. Competitive growth and survival in different locations within the host, as well as the transition to and from an external reservoir, require adaptive responses on the part of the bacterium. These and other selective pressures appear to have directed the evolution of specialized regulatory systems controlling the expression of virulence factors.

Microbial pathogenicity is a complex phenotype that involves the products of many genes. The multifactorial nature of pathogenicity is well illustrated by *Bordetella pertussis*, the causative agent of human whooping cough (*1*). Adherence to ciliated epithelial cells in the upper respiratory tract, evasion of host defenses, and production of local damage and systemic disease requires several factors with a variety of biological activities (*2*). Attachment to cilia is probably mediated by filamentous hemagglutinin and pili. Pertussis toxin, extracellular adenylate cyclase toxin, dermonecrotic toxin, and tracheal cytotoxin are involved in subsequent stages of infection and disease. Most of these virulence factors are members of a global regulatory network, and their expression is determined by a common regulator in response to environmental conditions.

In this review, the control of bacterial virulence is examined from the viewpoint of a stimulus-response pathway (*3*). There is evidence that bacterial pathogenesis is a process in which the infectious agent is constantly sensing its surroundings and responding in an appropriate manner. The response often involves coordinate alterations in the expression of sets of genes and operons encoding virulence factors. From a biological standpoint, this is not surprising. It does, however, have important application to our understanding of bacterial pathogens and the pathogenesis of infectious diseases.

Although our focus is primarily on human infectious agents, work over the last several years with a phytopathogen has been informative. The analysis of crown gall tumor formation by members of the genus *Agrobacterium* supports a model for prokaryotic information transfer (*4, 5*). We therefore begin with a consideration of tumorigenesis by these organisms.

Agrobacterium and a Model for Sensory Transduction

Agrobacterium tumefaciens is a soil bacterium that genetically transforms susceptible plant cells to induce tumors (*6*). This process involves the transfer of a piece of DNA, the T-DNA, from a large virulence plasmid to the nuclear genome of the infected cell. In plant cells, the T-DNA encodes and expresses enzymes used in the production of opines and the biosynthesis of growth hormones (*6*). Opines serve as efficient carbon, nitrogen, and energy sources for the infecting organisms. Induction of auxin and cytokinin synthesis results in neoplastic growth and an expansion of the population of plant cells producing nutrients for the infecting bacteria.

Ti (tumor-inducing) plasmids also carry a *vir* region, consisting of a cluster of operons encoding products required for T-DNA transfer. The *vir* region is organized into six complementation groups representing separate transcriptional units that span approximately 35 kilobases (*7*). Mutations in the *virA*, *B*, *D*, and *G* loci result in avirulence, whereas mutations in *virC* and *virE* cause attenuated virulence (*8*). Transcription of all of the *vir* loci is induced by signal molecules (phenolic compounds) present in exudates of wounded plants (*9–11*).

The *vir* operons of Ti plasmids constitute a single regulatory network, a regulon (*3*), subject to positive transcriptional control by the products of *virA* and *virG*. These genes are positively autoregu-

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lated and encode proteins that respond to a sensory stimulus provided by wounded host cells (*5, 9, 11*). The nucleotide sequences of *virA* and *virG* have been determined, and the deduced amino acid sequences are similar to sequences of various two-component prokaryotic regulatory systems that also respond to environmental stimuli (*5, 12, 13*). The current model for the action of these proteins is in fact derived largely from observations of amino acid sequence homology (*4, 5, 14*) (Fig. 1).

The predicted product of *virG* is a 30-kilodalton protein homologous over its entire length to OmpR, SfrA, and PhoB, and sharing amino-terminal homology with CheB, CheY, NtrC, SpoOA, and SpoOF (*5, 13*) (Table 1). All of these are regulatory proteins found in a variety of bacterial species, and many of them appear to carry out their role in conjunction with a sensory component (*4, 5, 14*). For example, OmpR controls transcription of porin genes (*ompC* and *ompF*) in response to EnvZ-detected changes in osmolarity (*15*).

The VirG protein may also act in concert with a sensory component, which is most likely VirA. The deduced amino acid sequence of the *virA* gene product corresponds to a 92.4-kD protein with a predicted amino-terminal signal sequence. A hydrophobic stretch located about 270 residues from the amino terminus suggests a transmembrane domain, and VirA localizes to the inner membrane during cell fractionation (*12*). A region of about 250 amino acids near the VirA carboxyl terminus is homologous to carboxyl-terminal sequences of NtrB, CheA, EnvZ, CpxA, and PhoR. These are all sensor-transmitter proteins (Table 1), and the latter three may also span the membrane (*14*).

Functional analysis of individual systems as well as specific patterns of homology have led to the model for signal transduction summarized in Fig. 1. With EnvZ/OmpR, VirA/VirG, and homologous systems, the amino-terminal domain of the membrane protein is thought to be located in the periplasm and may have a specific sensory function. For example, phenolic compounds present in plant exudates could bind directly to this region of VirA. According to the model, a signal is then transmitted to the conserved carboxyl-terminal domain of the same protein. Transmission of this signal may occur through an allosteric alteration, although a change in monomer-oligomer equilibrium after stimulation also seems possible. The activated carboxyl-terminal portion of the sensor protein then transmits the signal to the amino-terminal domain of the regulator. Phosphorylation appears to be a common mechanism used for this purpose by two-component systems. NtrB and CheA are protein kinases that phosphorylate themselves as well as NtrC and CheB/CheY, respectively (*16, 17*). Furthermore, EnvZ has recently been shown to autophosphorylate in vitro (*18*).

The amino terminus of the regulator, once activated, could alter the function of its carboxyl-terminal domain to control DNA binding or some other function. It is interesting to speculate that sensory transduction by a two-component mechanism imparts a measure of flexibility to the system. Sensor/transmitter proteins may have the ability to interact with multiple regulatory components that share amino-terminal sequences. In addition, evidence for "cross talk"—that is, interactions between sensor and regulator proteins belonging to distinct two-component systems—has recently been obtained with purified chemotaxis and nitrogen regulatory proteins (*17*).

Sequence similarities found between sensor-regulator systems suggest that, during prokaryotic evolution, a single two-component motif has been continually adapted to link expression of sets of genes with specific environmental stimuli. Bacterial pathogens are no exception. Although sensory transduction by *Agrobacterium vir* products conforms well with the proposed model, functionally similar systems in *Vibrio cholerae* and *Bordetella pertussis* show some interesting differences.

Coordinate Regulation of Virulence by *Vibrio cholerae*

Cholera is a severe diarrheal disease caused by colonization of the human small bowel with enterotoxin-producing strains of *V. cholerae* (*19*). After contaminated food or water is ingested, the infecting organisms must survive the acidic *p*H of the stomach and reach the small intestine. The clearing action of peristalsis and mucus flow is countered by bacterial motility and chemotaxis. Bacterial protease, mucinase, and neuraminidase production may also aid in mucus penetration and nutrient acquisition. Eventually, the infecting cells reach the brush border of the intestinal epithelium, where they attach themselves by a mechanism that probably involves hemagglutinins and pili. Multiplication occurs on the mucosal surface and results in an adherent bacterial mass. During this process cholera toxin is produced, thereby further enhancing colonization as well as causing secretory diarrhea. It is evident that extremely diverse environmental conditions are encountered by organisms that utilize the fecal-oral route of transmission.

Cholera toxin is a major virulence factor of *V. cholerae* (*19*). The cholera toxin operon (*ctxAB*) is located on the chromosome within a genetic element that resembles a complex transposon and is subject to gene amplification during intestinal passage (*20*). Expression of the *ctx* operon in *V. cholerae* is dependent on a regulatory gene called *toxR* (*21*). Analysis of the *ctx* promoter region has shown that three to eight direct repetitions of the sequence TTTTGAT (the number of repetitions depends on the strain) appear to be required in *cis* for ToxR-mediated activation of *ctx* transcription (*22*). The *toxR* gene has been cloned in *Escherichia coli* by virtue of its ability to activate a transcriptional fusion between the *ctx* promoter and *lacZ* (*21*).

The transposon Tn*5* derivative Tn*phoA* (*23*), was used to identify the gene(*tcpA*) encoding the major subunit of a *V. cholerae* pilus (the

Table 1. Examples of two-component bacterial regulatory systems (*4, 5, 14*). All of the proteins listed contain the carboxyl-terminal sensor/amino-terminal regulator patterns of homology shown in Fig 1. Sensors directly perceive environmental stimuli and transduce the signal to a regulator. Alternatively, signal transduction by intracellular modulators such as NtrB and CheA require additional proteins that are responsible for the initial sensing event. These proteins are therefore more accurately described as "transmitter" proteins (*71*).

Organism	Stimulus	Sensor/ transmitter	Regulator/ receiver	Response
A. tumefaciens	Plant exudate	VirA	VirG	Activation of virulence gene expression
E. coli	Changes in osmolarity	EnvZ	OmpR	Control of porin gene expression
	Dyes, toxic chemicals	CpxA	SfrA	Control of F factor *traJ* gene expression
	Phosphate	PhoR	PhoB	Control of phosphate-regulated genes
E. coli, B. parasponiae, and *K. pneumoniae*	Nitrogen	NtrB (NRII)	NtrC (NRI)	Control of nitrogen assimilation gene expression
E. coli and *S. typhimurium*	Repellents, attractants	CheA	CheB/Y	Chemotaxis: adaptation and motor control
B. subtilis	Nutrient deprivation	?	SpoOA/OF	Control of sporulation

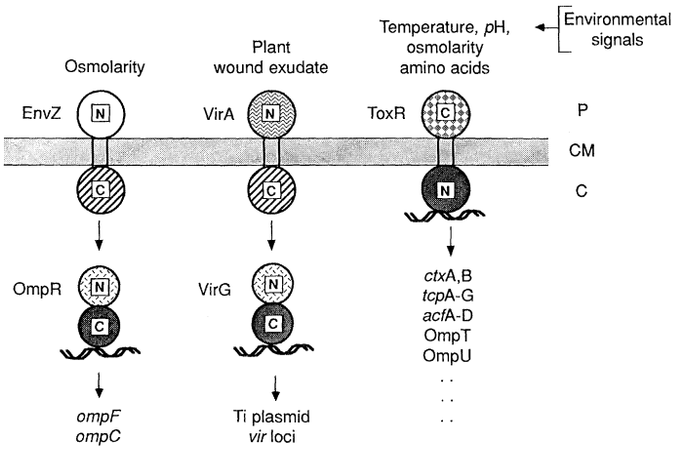

Fig. 1. Patterns of sequence homology observed between components of bacterial sensory systems. Details and functions of individual factors are presented in the text [see also (*4, 5, 14*) and Table 1]. In the two-component systems, EnvZ and VirA are sensor proteins that control the functions of the regulator proteins OmpR and VirG. In the case of ToxR, a single polypeptide is shown. Some environmental signals may act through the ToxR periplasmic domain while others may use membrane-associated or cytoplasmic regions. Identical patterns of shading indicate amino acid sequence homology. N and C refer to amino and carboxyl termini, respectively. The carboxyl termini of EnvZ and VirA contain homologous "transmitter" domains, and the amino termini of OmpR and VirG contain similar "receiver" domains (*71*). P, CM, and C designate periplasm, cytoplasmic membrane, and cytoplasm, respectively.

toxin coregulated pilus). Expression of *tcpA* is controlled by ToxR, and its product is essential for intestinal colonization (*24*). At least 14 genes, in addition to *ctxAB* and *tcpA*, are regulated by ToxR (*24, 25*). Seven of the genes are involved in assembly of pili (*tcpABCDEFG*) and a cluster of four genes (*acfABCD*) encode an accessory colonization factor (*25*). *Vibrio cholerae* strains carrying *toxR* null mutations are also deficient in expression of OmpU, a 38-kD outer membrane protein (*24, 26*). Conversely, production of OmpT (40 kD) appears to be negatively controlled by ToxR. These observations indicate that ToxR is a global regulatory protein. The recent demonstration that deletion mutations in *toxR* eliminate intestinal colonization in human volunteers provides further evidence for the importance of this regulatory system in cholera pathogenesis (*27*).

Coordinate regulation is also manifested at the physiological level, in that production of ToxR-regulated gene products is influenced by environmental signals. Indeed, most of the ToxR-regulated genes were first identified as Tn*phoA* fusions that responded to environmental signals known to affect the expression of cholera toxin. These include osmolarity, *p*H, temperature, and the addition of certain amino acids to cells grown in minimal media (*28*). With the possible exception of osmolarity, there is no clear evidence that the ToxR protein acts alone in sensing all of these environmental signals.

The nucleotide sequence of *toxR* has been determined, and the deduced amino acid sequence of 294 residues gives a protein with a predicted molecular mass of 32.5 kD (*22*). A stretch of 16 hydrophobic amino acids (position 183 to 198) forms a likely transmembrane domain. Fusion of the first 210 amino acids of ToxR with the carboxyl terminus of PhoA creates a hybrid protein with alkaline phosphatase activity, suggesting that at least a portion of the ToxR carboxyl terminus is located in the periplasm. Cell fractionation shows that the fusion protein is associated with the inner membrane (*22*). In addition, the ToxR-PhoA hybrid protein is still capable of activating *ctx* transcription in both *E. coli* and *V. cholerae* and binds to the *ctx* promoter region in vitro as demonstrated by gel retardation assays. *Vibrio cholerae* strains containing the *toxR-phoA* fusion on a plasmid and a *toxR* null mutation on the chromosome are no longer responsive to osmolarity, whereas the effects of *p*H and amino acids are retained. These observations suggest that *toxR* is a membrane protein with a cytoplasmic amino-terminal domain mediating both transcriptional control and DNA binding, and a periplasmic carboxyl-terminal domain involved in osmoregulation.

Comparison with other bacterial polypeptides revealed significant sequence similarity between ToxR and *E. coli* OmpR, PhoM, PhoB, and SfrA proteins as well as the *Agrobacterium* VirG protein. This similarity lies within the amino-terminal 127 amino acids of ToxR and the carboxyl-terminal residues of the homologous polypeptides. Each of these proteins has been implicated in transcriptional control and, as discussed earlier, are members of two-component sensory systems. The conserved carboxyl-terminal domains of these proteins most likely mediate DNA binding and transcriptional regulation (*14*).

The pattern of homology displayed by ToxR is summarized in Fig. 1. Comparison of ToxR with VirA/VirG and EnvZ/OmpR reveals some interesting differences. First, the predicted orientation of ToxR is opposite that of VirA and EnvZ. Second, ToxR differs from the other systems in that the carboxyl-terminal domain of the sensory component and the amino-terminal domain of the regulatory component have been eliminated. Consequently, sensory stimuli perceived by ToxR may be directly transmitted to the DNA binding domain.

Although ToxR may be a one-component derivative of a two-component system, the possible involvement of additional factors should not be overlooked. For example, a second regulatory gene called *toxS* lies immediately downstream from *toxR* in the same transcriptional unit and under certain conditions is required concomitantly with *toxR* for activation of the *ctx* promoter in *E. coli* and *V. cholerae* (*29*). The deduced amino acid sequence of the *toxS* gene product and properties of ToxS-PhoA fusion proteins suggest that ToxS is also a membrane protein that may interact with the periplasmically located carboxyl terminus of ToxR (*29*).

A fundamental question that remains to be answered concerns the roles of temperature, osmolarity, *p*H, and amino acids as signals regulating the expression of virulence during the infectious cycle. It seems likely that signals other than temperature stimulate the activity of ToxR within the upper intestine since toxin production is reduced in laboratory media at 37°C. In contrast, the osmolarity of mucosal secretions and the likely presence of amino acids due to extracellular proteases correspond to conditions favoring toxin expression in pure culture. *Vibrio cholerae* survives well in estuarine and brackish water habitats, and the efficient transition from an environmental organism to a human pathogen probably requires sensory transduction by the ToxR regulatory system. It is difficult, however, to extrapolate observations obtained from laboratory analysis to events occurring in natural environments.

The *vir* Regulon of *Bordetella pertussis*

Bordetella pertussis is a highly adapted human pathogen. Whooping cough, the disease it causes, can be seen as occurring in two distinct phases. A local infection of the respiratory tract ultimately leads to systemic intoxication. Transmission is very efficient and takes place directly from one infected individual to another. Some of the virulence factors involved in this process were mentioned earlier and have been the subject of recent reviews (*1, 2*).

Two related forms of coordinate regulation control the expression of virulence in *B. pertussis*. In 1960, Lacey (*30*) reported that changes in growth environment caused reversible antigenic alterations that are correlated with virulence. In this and subsequent studies, $MgSO_4$, nicotinic acid, growth at 25°C (as opposed to 37°C), and

other environmental conditions decreased or eliminated expression of several virulence factors (*30, 31*). The second type of regulation, phase variation, is a reversible metastable transition from virulent (Vir^+) to avirulent (Vir^-) phases (*32*). Factors modulated by environmental conditions are also lost during this process. The proportion of Vir^- variants in a Vir^+ population is estimated at 10^{-3} to 10^{-6}, depending on the strain (*32*). Although a phenomenological description of phase variation and phenotypic modulation has been available for many years, the molecular details of these events are just beginning to be understood. Two classes of Tn*5* insertion mutations in the *B. pertussis* genome were shown to affect expression of virulence (*33*). One mutant class was deficient in one or two factors whereas a second class was missing all virulent phase markers. The second class identified a central regulatory locus, *vir*, that is required for virulence gene expression.

The *vir* region has been cloned and studied in *E. coli* (*34, 35*). The *vir* locus is closely linked to the *fhaB* locus, which encodes filamentous hemagglutinin (an adhesin), and cosmid clones containing both regions have been isolated (*34*). Expression of FhaB in *E. coli* was first detected antigenically and shown to require a 5-kb adjoining region corresponding to *vir*. Complementation analysis with *Bordetella* phase variants indicated that *vir* acts in trans and that an alteration in *vir* is responsible for phase variation.

Positive regulation of the pertussis toxin operon (*ptx*) by *vir* occurs at the transcriptional level (*36*). In *B. pertussis*, toxin gene fusions have in addition demonstrated that a region located 170 bp upstream from the mRNA start site is required for transactivation (*37*). The *ptx* operon is not transcribed in *E. coli* even in the presence of *vir* (*38*). In contrast, we constructed a *vir*-dependent regulatory system in *E. coli* by using single-copy transcriptional fusions between *fhaB* and *lacZ* (*35*). β-Galactosidase activity is increased several hundredfold by the presence of *vir* in trans on multicopy plasmids. Phenotypic modulation by $MgSO_4$, nicotinic acid, and temperature is observed in *E. coli*, both by measurement of transcriptional fusion activity and the production of FhaB antigen (*34, 35*). The observation that phenotypic modulation occurs in *E. coli* indicates either that all of the required functions are encoded by *vir*, or that the *vir* regulatory system is able to act in conjunction with *E. coli* sensory components. The apparent lack of activation of the *ptx* promoter by *vir* in *E. coli* also shows that *ptx* and *fhaB* differ in their requirements for transcription.

Tn*phoA* fusions have been used to identify genes regulated by modulation signals in *B. pertussis* (*39*). This analysis identified two groups of genes, called *vag* and *vrg*, which are activated or repressed by the *vir* locus, respectively. Several *vag* genes correspond to the structural genes for virulence factors; the function of the *vrg* genes is unknown. Mutations that cause constitutive expression of *vag* genes in the presence of one of several different modulators have been isolated and shown to map to the *vir* region (*39, 40*). These data provide additional evidence that modulation by signals like temperature, $MgSO_4$, and nicotinic acid require proteins encoded by *vir*.

The nucleotide sequence of the *vir* region has been determined, and several interesting observations have resulted (*41, 42*). Three open reading frames of 209, 275, and 936 amino acids are tandemly arranged and could encode polypeptides of 23, 30, and 102 kD, respectively. Sequence analysis of a series of spontaneous phase variants indicates that the third open reading frame (936 amino acids) is subject to frameshift mutations (*42*). Insertion of a guanine residue within a string of six G's accompanies the transition from vir^+ to vir^-, and removal of a G residue is associated with reversion to vir^+. The biological significance of this frameshift event, as well as phase variation, is under investigation.

Polypeptide sequences in the *vir* region show homology to members of the sensor/regulator paradigm (*41, 42*) (Fig. 1). The exact pattern of homology, however, is somewhat unexpected. In the largest open reading frame, amino acids 442 to 635 match most of the conserved sequences shared by the carboxyl termini of the sensor/transmitter class of proteins. This region follows a predicted transmembrane domain starting at position 240. The unique aspect of the pattern is the presence of an additional region bearing similarity to the amino-terminal domain of OmpR and related regulator proteins. This is located between the sensor homology and the carboxyl terminus. It therefore appears that *B. pertussis* has combined two domains normally found on different proteins into a single polypeptide chain. The proposed function of the carboxyl terminus of the sensor is to transmit a signal to the amino terminus of the regulator. The effect of combining these domains into a single protein remains to be determined.

The amino-terminal half of the first *vir* open reading frame is also homologous to the amino termini of regulator class proteins. When overproduced in *E. coli* through the use of exogenous promoters, the 23-kD *vir* polypeptide is capable of activating *fhaB* transcription in the absence of other *vir* gene products (*43*). The first open reading frame may therefore encode a regulatory protein subject to control by a sensor component.

Although neither the relevant signals nor the responses that occur in vivo are known, the ability of *B. pertussis* to sense its surroundings is likely to be of central importance during the course of infection. The dramatic serologic changes that result from cultivation under modulating conditions have been called "antigenic elimination" (*2*) and have been proposed as a mechanism for the establishment of a carrier state. It has also been suggested that environmental conditions arising during disease provide modulating signals and down-regulation of attachment factors. This may in turn promote transmission to a new host by allowing organisms to be expelled from an infected individual (*44*). In either case, evasion of the immune response would result. It is not unreasonable to propose that selective forces generated by the human immune system have played a role in the evolution of virulence regulation by *B. pertussis*. The function of *vrg* loci, which are expressed when genes for virulence factors are not expressed, may provide clues to the biological significance of the *vir* regulon and the modulation response.

Regulation by Iron

Normally, extracellular locations in the mammalian host are severely iron restricted (*45*). The iron-binding protein lactoferrin is found in milk as well as in respiratory, intestinal, seminal, and cervical secretions. Transferrin performs the same function in serum. High-affinity binding proteins therefore withhold the iron that is necessary for microbial multiplication.

A coordinated response of many bacteria to low iron includes production of iron-binding ligands (siderophores) and proteins involved in uptake of iron-siderophore complexes (*45*). These virulence-enhancing systems can be acquired by *E. coli* and other enteric organisms though the acquisition of plasmids and transposons. In *E. coli*, the expression of chromosomal and plasmid-encoded siderophore-based uptake systems is controlled by the *fur* gene product, a global regulator that represses transcription of several loci in the presence of iron (*46*).

A number of toxins produced by pathogenic bacteria are also induced in environments containing low amounts of iron. It is interesting that transcription of bacteriophage-encoded Shiga-like toxin type I in *E. coli* is repressed by Fur in the presence of iron (*47*). In *E. coli*, the *fur* product can also function as an iron-dependent repressor of the diphtheria toxin promoter cloned from *Corynebacterium diphtheriae* corynephage β (*48*).

Expression of exotoxin A, elastase, and alkaline protease by *Pseudomonas aeruginosa* is also negatively controlled by iron (*49*). The product of the *toxR* gene [alternatively designated *regA* (*50*)], activates transcription of *toxA*, the locus that encodes exotoxin A (*51*). Transcription of both *regA* and *toxA* is reduced when cells are grown in medium containing high concentrations of iron (*50*).

Yersinia, Shigella, and *Salmonella*

Human infections caused by members of the genus *Yersinia* range from bubonic plague (*Y. pestis*) to acute gastroenteritis (*Y. enterocolitica*) and mesenteric adenitis (*Y. pseudotuberculosis*). All three species display an intracellular stage during the disease cycle, and harbor plasmids (60 to 76 kb) required for full virulence (*52*). The expression of several virulence-associated factors encoded by these plasmids responds to temperature and calcium as extracellular signals (*52*).

The presence of *Yersinia* virulence plasmids has been associated with several temperature-dependent properties that are expressed at 37°C but not at 25°C (*53*). These include a nutritional requirement for Ca^{2+}, synthesis of antigens V and W, serum resistance, hemagglutinin production, autoagglutination, and synthesis of several outer membrane proteins (YOPs) encoded by the plasmid. In addition, Ca^{2+} has a regulatory role in that maximal production of YOPs and V and W antigens occurs at 37°C in its relative absence (*54*). The virulence plasmid of *Y. pestis* carries several *lcr* genes that are responsible for regulating gene expression in response to temperature and Ca^{2+} (*54*). The products of these loci act in trans to control transcription of several unlinked genes and operons. The regulation of the plasmid-encoded virulence genes in *Yersinia* is complex, and the molecular mechanisms responsible for mediating the sensory response have not been fully described.

Yersinia pestis grows and multiplies within the phagolysosomes of macrophages (*55*). In order to probe this environment for its effect on expression of virulence loci, Pollack and co-workers (*56*) constructed a strain containing a *yopK-lacZ* transcriptional fusion. On a per cell basis, β-galactosidase levels increased nearly tenfold after internalization by macrophages as compared to growth at 37°C in medium containing 2.0 m*M* Ca^{2+}. This is a surprising result since phagolysosomes harboring *Y. pestis* had been assumed to contain millimolar levels of Ca^{2+} because of the presence of an adenosine triphosphate (ATP)–dependent Ca^{2+} pump. Thus, it is possible that *Yersinia* modifies its intracellular environment or that signals other than calcium are important within the phagolysosome.

Each of the *Yersinia* species discussed above alternates between growth at low temperature and high temperature during the infectious cycle (*52*). *Yersinia pestis* multiplies in a flea vector, whereas *Y. enterocolitica* and *Y. pseudotuberculosis* are found in contaminated food and water. Elevated temperature may therefore be a signal indicating presence within the mammalian host. It is tempting to speculate that the Ca^{2+} response allows differentiation between extracellular and intracellular environments. The *Y. pseudotuberculosis inv* gene, which encodes a protein that confers the ability to invade mammalian cells in tissue culture, is also temperature regulated, but in a manner that is opposite that of the virulence plasmid–encoded functions (*57*). Presumably, the regulatory profiles of *Yersinia* virulence factors relate to stage-specific requirements for pathogenesis.

Thermoregulation is a common form of virulence control and is also observed with members of the genus *Shigella*. The ability of these organisms to cause bacillary dysentery is associated with their capacity to enter and multiply within colonic epithelial cells (*58*). Although expression of genes located on the chromosome as well as on a large plasmid (180 to 215 kb) is required for full virulence, the plasmid itself is sufficient to promote invasion of HeLa cells by *E. coli* K12 (*59*).

When grown at 37°C, strains of *Shigella flexneri*, *S. sonnei*, and *S. dysenteriae* invade tissue culture cells and produce keratoconjunctivitis in guinea pigs. Growth at 30°C, however, results in avirulent organisms that produce negative results in both assays (*60*). Isolation of a *lacZ* transcriptional fusion to a *S. flexneri* plasmid *vir* locus led to the detection of temperature-regulated transcription (*61*). Transposon Tn*10* mutagenesis of a strain carrying a *vir-lacZ* operon fusion was then used to select regulatory mutants constitutive for the Lac^+ phenotype at the nonpermissive temperature of 30°C (*62*). This resulted in the identification of a chromosomal locus, *virR*, that is proposed to encode a trans-acting repressor that controls expression of at least one *vir* gene and several virulence plasmid–encoded polypeptides.

Although the *virR* locus is chromosomal, the regulated properties reported thus far are plasmid encoded. The size of the regulon, the locations of regulated genes, and the mechanisms of control remain to be established. The mutant isolation procedure used to identify *virR* would not have identified genes encoding positive regulatory elements. Transcriptional fusions provide a powerful method for the analysis of thermoregulated virulence in the *Shigellae*.

Invasion of intestinal epithelial cells is an early stage in the passage of pathogenic *Salmonella* species from the mucosal surface to the underlying tissues (*63*). Enteric fever, gastroenteritis, and bacteremia share a requirement for this process. As expected, the invasive phenotype of *Salmonella* is complex and multigenic. Using a tissue culture system, Finlay *et al.* (*64*) showed that the ability of *S. choleraesuis* and *S. typhimurium* to adhere to and enter epithelial cells is an inducible event. An interesting aspect of this observation is that induction requires cell-cell contact between the bacteria and structures on the epithelial cell that are sensitive to both trypsin and neuraminidase. Both induction and repression of the synthesis of several proteins was observed and does not require the presence of the large *S. typhimurium* virulence plasmid. Although the regulatory mechanisms involved in sensing the epithelial cell surface and regulating protein synthesis are currently under investigation, candidate regulatory loci have been identified by single transposon insertions that eliminate adherence as well as induction.

Survival of *Salmonellae* in the phagolysosomes of macrophages is also critical to the pathogenesis of enteric fevers. A regulatory locus (*phoP*) is necessary for full virulence of *S. typhimurium* in mice (*65, 66*). The *phoP* product regulates the expression of genes encoding factors that protect *Salmonellae* from the bactericidal activity of macrophage cationic proteins (*65*). Mutants have been isolated that carry Tn*phoA* and *lacZ* fusions to PhoP-regulated genes, and some of these mutants have virulence defects (*66*). The nucleotide sequence of the *phoP* gene has been determined and it encodes a polypeptide that shows striking similarity to OmpR and other regulator class proteins (*65, 66*). In addition, a second gene (*phoO*) lies immediately downstream from *phoP*. The *phoO* gene encodes a membrane protein with homology to the putative carboxyl-terminal kinase domains of EnvZ and related sensor proteins. This suggests that *phoP* and *phoO* define a two-component regulatory system that controls virulence in *Salmonella* (*66*).

Staphylococcus and *Streptococcus*

Although our discussion thus far has been limited to Gram-negative organisms, evidence for global regulation of virulence factors has also been obtained for at least one Gram-positive bacterial pathogen. *Staphylococcus aureus* synthesizes a number of extracellular proteins that are toxic to man and other animals and are

important during pathogenesis. A regulatory locus controlling production of at least 12 different exoproteins has been identified. This locus, alternatively called *agr* (*67*) and *exp* (*68*), acts at the transcriptional level in regulating alpha-toxin, toxic shock syndrome toxin, and protein A. For the group A streptococci, positive regulation of the M protein has been described (*69*). This surface protein is an important virulence factor and confers resistance to phagocytosis. A locus designated *mry* is required for transcription of the gene encoding M protein. Whether *mry* also controls expression of other streptococcal virulence determinants remains to be determined.

Conclusions and Perspectives

A consideration of the sensory signals affecting the regulation of bacterial pathogenicity reveals some general themes as well as unique features. Temperature must be considered as a major cue. The rationale for this is easy to surmise since the internal temperature of the mammalian host is frequently higher than that of the surrounding environment. The exceedingly low availability of free iron in host tissues appears to be another environmental signal that can trigger the coordinate expression of virulence determinants. The significance of other factors such as calcium (*Yersinia*), or $MgSO_4$ and nicotinic acid (*Bordetella*) requires careful characterization of the composition of the relevant host environment during disease.

Bacterial pathogenesis requires a large assembly of virulence factors that may not be simultaneously needed, or advantageous, during all stages of infection. We might therefore expect to observe coordinate regulation as well as control at a more individual level. As illustrated by catabolite repression in *E. coli*, membership in a regulon does not preclude specific forms of operon control (*70*).

From an evolutionary standpoint, the relative genetic locations of regulatory loci and the genes they control is informative. The frequent involvement of accessory genetic elements, such as plasmids, bacteriophages, and transposons, in coding for virulence factors is well known. The regulation of many of these factors by chromosomal loci is now becoming apparent. Examples cited herein include cholera toxin, which is encoded by a transposon-like structure and is regulated by the chromosomal *toxR* gene, plasmid virulence loci of *Shigella* that are negatively regulated by the chromosomal *virR* locus, and regulation of the bacteriophage-encoded Shiga-like toxin operon in *E. coli* by the *fur* gene product. In each case, genes on an accessory genetic element have become subject to a host-encoded sensory control system.

Although considerable progress is discernible, the ultimate goal of achieving a detailed understanding of bacterial gene regulation during the disease process remains somewhat illusory. The use of molecular techniques to identify virulence factors and regulatory mechanisms is powerful. These methods, however, are limited by the unnatural habitats and inappropriate signals frequently provided to bacteria during laboratory cultivation and analysis. Despite this, the potential benefits from our attempts are important. The identification of new genes involved in virulence by virtue solely of their coordinate regulatory properties is one such example. The analysis of gene regulation in pathogenic bacteria will hopefully lead to improved methods for vaccine production and treatment of bacterial infections. The possibility of targeting therapeutic approaches to the regulatory systems controlling bacterial virulence is becoming a new and exciting option. An understanding of coordinate regulation and sensory transduction is crucial for understanding the events that occur during the pathogenesis of bacterial infectious diseases.

Note added in proof: Cornelis and co-workers (*72*) have demonstrated that *virF*, a *Y. enterocolitica* virulence plasmid gene, is a transcriptional activator of the *yop* regulon. The *virF* gene product is homologous to AraC (*E. coli* and *S. typhimurium*), and its expression appears to be autoregulated. VirF is responsible for the effect of temperature on Yop production, although its role in the response to calcium is unclear.

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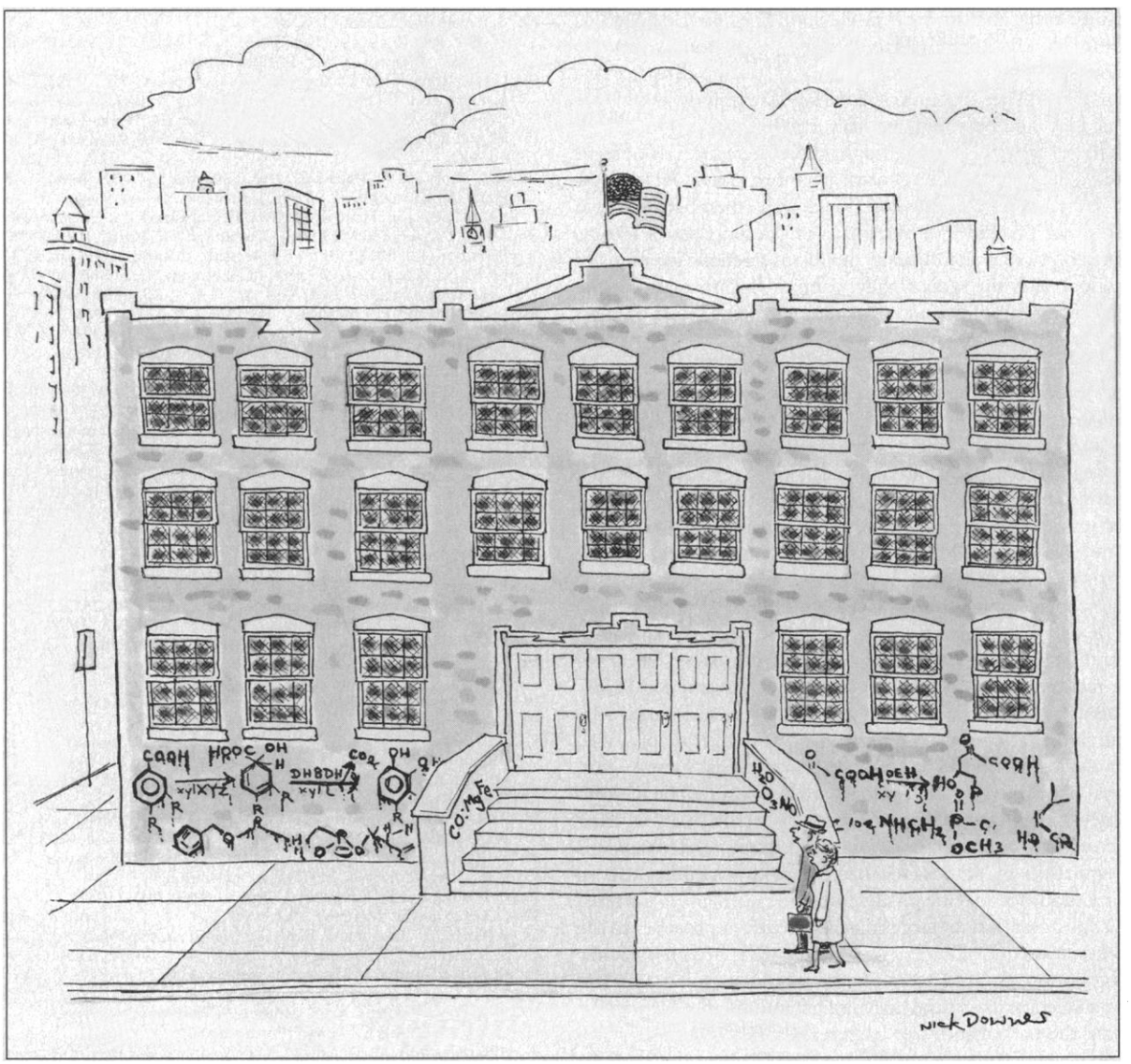

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