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MINIREVIEW

Environmental Signals Controlling Expression of Virulence Determinants in Bacteria

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INTRODUCTION

The study of microbial pathogenesis offers many unique research opportunities. The interaction between host and pathogen during disease is a dynamic battlefield where the microbe's clever strategies for survival and multiplication meet head on with the formidable defenses of the immune system. It is no wonder that the tactics employed by both participants in this struggle provide such interesting research topics for scientists of diverse disciplines. Major advances, fueled largely by the application of a genetic approach, have been made in the biochemistry, immunology, and cell biology of the host-parasite interaction. Among the many new insights is the recognition that bacterial pathogens have evolved highly sophisticated signal transduction systems controlling the coordinate expression of virulence determinants. In this minireview, I will discuss emerging themes that characterize these virulence regulatory systems and speculate on how these control strategies might contribute to the overall success of the microbe during pathogenesis. I refer the reader to several other full-length reviews and minireviews of areas related to microbial virulence and coordinate regulation (21, 24, 27, 28, 30, 52, 74). Because of minireview guidelines and space limitations, I have focused only on recent published observations and therefore apologize to my many colleagues who are not cited but who did contribute significantly to the topics discussed here.

As a prelude to a discussion of virulence regulation, it is helpful to consider briefly what defines a virulence factor (21, 27). Virulence as a concept is intrinsically coupled to disease and is therefore most easily measured in terms of morbidity and mortality. However, one must consider that the degree of host injury does not necessarily correlate with evolutionary success for a pathogenic microbe. Survival and multiplication are clearly the priorities for the microbe, while disease is simply a manifestation of the complex interactions required to accomplish these two goals within the milieu of host tissues.

I favor an expansive view of virulence determinants which includes all those factors contributing to infection as well as to disease, with the exception of "housekeeping" functions that are required for efficient multiplication on nonliving substrates. While some determinants fit comfortably into such a scheme (e.g., adherence to host tissues, production of host-specific toxins, invasion into host cells, and resistance to host defense mechanisms), others are in a gray area bordering on housekeeping functions. Thus, bacterial factors that facilitate the acquisition of iron in the host are arguably virulence factors given the prodigious effort the host makes to withhold this critical nutrient from invading microbes (17). However, conditions can be established in vitro under which the same virulence factors are required for growth on, for example, iron-limiting laboratory media. Likewise, the fact that mutations in genes encoding the enzymes of aromatic compound biosynthesis render many bacteria attenuated could qualify these enzymes as virulence factors. Such mutational studies are also of profound interest in understanding the metabolism of microbes during disease and as a practical approach to vaccine development. Interestingly, the coordinate regulation of a potential housekeeping function with a clear-cut virulence determinant can help support the function's role as virulence factor (e.g., regulation by iron of both siderophore biosynthesis and cytotoxin production in *Corynebacterium diphtheriae* or *Escherichia coli*). Thus, understanding the regulation of virulence properties can help us define what constitutes a potential virulence factor and indeed can facilitate the identification of new virulence factors on the basis of only their regulatory properties (45, 49, 53, 58, 62).

EVIDENCE FOR COORDINATE REGULATION

The multifaceted nature of the host-parasite interaction indicates that more than one virulence determinant is typically involved in pathogenesis. This prediction has been supported by numerous studies showing that specific virulence determinants (e.g., adhesins, invasins, toxins, capsules, etc.) contribute to unique steps in the pathobiology of microbes. These studies have further established that the expression of dissimilar virulence determinants is frequently coordinately controlled by a common regulatory system. Table 1 shows a partial list of the organisms that have virulence factors coordinately regulated by the same environmental signal(s). In virtually all of these examples, this coordinate regulation has as its basis a common regulatory system which controls the expression of genes encoding these virulence determinants. As discussed below, the molecular level of this control is usually transcriptional, but more than one DNA-binding regulatory protein can be involved.

REGULATION OF VIRULENCE BY SPECIFIC HOST PRODUCTS

Clearly, an effective mechanism for inducing expression of virulence genes could involve the sensing of "signature" molecules produced specifically by host tissues. As yet this pattern of regulation has not been recognized in any human pathogens, but it has been clearly demonstrated in two different phytopathogens. In the case of *Agrobacterium tumefaciens*, acetosyringone or related phenolic compounds together with a diverse group of monosaccharides have been shown to function as host-specific inducers of virulence

TABLE 1. Environmental signals controlling the expression of coordinately regulated virulence determinants in bacteria

Organism(s)	Environmental signal(s)	Reference(s)
Agrobacterium tumefaciens	Phenolic compounds, monosaccharides, pH, phosphate	3, 10, 14, 80
Bacillus anthracis	CO_2	5
Bordetella pertussis	Temperature, SO_4, nicotinic acid	42, 45, 51, 65
Corynebacterium diphtheriae	Iron	11, 59, 69
Escherichia coli	Iron, temperature, carbon source	4, 13, 36, 37, 77
Listeria monocytogenes	Heat shock	48, 71
Pseudomonas aeruginosa	Iron, osmolarity	21, 22, 75
Salmonella typhimurium	Osmolarity, starvation, stress, pH, growth phase	12, 29, 34, 53
Shigella species	Temperature	1, 38, 49, 50
Staphylococcus aureus	Growth phase	42
Vibrio cholerae	Osmolarity, pH, temperature, amino acids, CO_2, iron	23, 35, 55, 62, 70
Yersinia species	Temperature, Ca^{2+}	6, 7, 16, 33

genes required for crown gall tumer formation (3, 10). Similarly, in *Pseudomonas syringae* certain phenolic β-glycosides in combination with mono- or disaccharides strongly induce the genes required for production of the phytotoxin syringomycin, although the regulatory genes involved in this response remain undefined (57). The phenolic compounds and saccharides mediating both of these responses are thought to be normal components of plant tissues but apparently accumulate within infected wounds, perhaps through the action of glycosidases and other host or microbial enzymes acting on plant cell walls. In *A. tumefaciens* a two-component regulatory system (2, 74) composed of the VirA membrane sensory protein and VirG transcriptional activator mediate this response to plant exudate compounds (10, 14). Interestingly, the synergistic coupling of the monosaccharide response and the phenolic response occurs in *A. tumefaciens* through the action of the *chvE* gene product, a periplasmic sugar-binding protein that interacts with the periplasmic domain of VirA (3, 14). Stimulated VirA phosphorylates itself and VirG, which in turn goes on to bind to and activate other *vir* promoters, including its own (43). Superimposed on this regulation is transcriptional control of *virG* by signals such as low phosphate and acidic pH, with the latter possibly linked to a heat shock stress response (80). This complex regulation gives us a glimpse of what might be expected as we go deeper into the analysis of regulatory systems controlling virulence in animal pathogens.

REGULATION OF VIRULENCE BY ENVIRONMENTAL CUES

Short of having host signature molecules directly control the expression of virulence factors, the next best means of control might be though environmental cues that signal the entry of the microbe into host tissues. Two such parameters, low iron concentration and elevated temperature, have occupied a predominant position in our thinking about this regulatory strategy. However, additional physical-chemical parameters such as osmolarity, pH, oxygen, CO_2, or ions besides iron could play a similar role. Alternatively, because many of these latter parameters vary widely within different tissues of the host, the pathogen may use these as signals to detect anatomical differences at either a gross (e.g., gut versus lung) or fine (e.g., intracellular versus extracellular) level.

Iron. The role for iron as a regulator of virulence expression was first established in 1936 through studies by Pappenheimer and Johnson on diphtheria cytotoxin production (59). In the decades that followed, the transcriptional regulation of several other cytotoxins has been linked to low iron concentration and analyzed genetically (13, 21, 38, 75). Recent studies have shown that in two of these cases the cytotoxin regulation is part of a global response to iron-limiting growth conditions that includes derepression of genes for the synthesis of siderophores and the transport of the Fe(III)-siderophore complexes. Because host tissues are rich in the iron-binding proteins transferrin and lactoferrin, microbes need to have these iron-scavenging systems to be successful pathogens (4, 17).

The phage-encoded genes for Shiga-like toxin type I (*slt* genes) or *E. coli* have been shown to be regulated by the resident chromosomal *fur* locus, which encodes an aporepressor that becomes active for DNA binding upon complexing with Fe(II) or certain other divalent cations (4, 13). An "iron box" operator sequence overlaps the promoter elements of the *slt* promoter as it does in other *fur*-regulated *E. coli* genes.

The molecular basis for regulation of diphtheria toxin production by iron was established with the cloning of the *C. diphtheriae dtxR* gene, which encodes a ca. 25-kDa iron-responsive repressor of the diphtheria toxin promoter (11). Although DtxR shares little primary amino acid sequence similarity with Fur (about 18 to 25%), it apparently plays the same role as Fur in the regulation of siderophore expression in *C. diphtheriae* (69).

Understanding the regulation of other cytotoxin and virulence factors by iron has been more difficult. In two recent examples, the transcription of *Pseudomonas aeruginosa* exotoxin A (75) and of the virulence-associated IrgA outer membrane protein of *Vibrio cholerae* (35) has been shown to be controlled by the positive regulatory genes *regAB* and *irgB*, respectively. Transcriptional control of the genes encoding these activators may occur through a cascade involving direct iron-dependent, Fur-like repression.

Temperature. The transition from ambient low temperature to body temperature has been correlated with dramatic changes in the expression of virulence determinants in several organisms. Mutational analysis of the effect of temperature on invasion in *Shigella flexneri* and on Pap pilus expression in *E. coli* has led to the identification of the same regulatory locus, designated *virR* (38, 49, 50) or *drdX* (37), respectively. Mutations in the same locus have been shown to affect the osmoregulation of *proU* (*osmZ*) (24, 25) and type I pilus phase variation (*pilG*) (44). It is now clear that all these mutations are alleles of the *hns* locus, encoding the nucleoid protein H-NS, which has histonelike properties (39). Recently, another histonelike protein has been impli-

cated in the regulation of alginate capsule synthesis in *P. aeruginosa* (19).

Three different theories have been proposed for how H-NS and other histonelike proteins (e.g., integration host factor) control gene expression: after binding DNA they cause either transcriptional silencing (37), changes in supercoiling (24, 25, 39), or DNA bending (66). Evidence for any of these mechanisms is mixed at best. For example, expression of the invasion phenotype of *Salmonella typhimurium* (and *proU* as a control) can be modulated by osmolarity, gyrase inhibitors, and a *topA* mutation (34). Similarly, another report suggests that alterations in supercoiling (seen either in the presence of low levels of novobiocin or in certain novobiocin-resistant mutants) can be correlated with the abnormal expression of *Yersinia enterocolitica* virulence genes at 25°C (33). In contrast, recent evidence suggests that supercoiling is not involved in osmoregulation of *proU* (64), and no evidence for supercoiling was found for the effect of *pilG* mutations on pilus phase variation (44). This rather controversal area is beyond the scope of this review. At this time it is clear that mutations in the *hns* promoter or structural gene can affect expression of many genes, but *hns* is not likely to be a dedicated regulator of virulence per se. Among the genes that may respond to temperature through effects on H-NS are genes that do play a more direct regulatory role in virulence.

One such gene is *virB*, located on the virulence plasmid of *S. flexneri* (1, 38, 78). Transcription of this gene is modulated by temperature, as is the transcription of a group of plasmid-encoded genes, called *ipa* or *inv* loci, involved in eukaryotic cell invasion (38). Transcription of *virB* requires an activator encoded by the *virF* locus, which in turn also responds to temperature. However, *ipa* and *inv* gene expression can be achieved even at low temperature by constitutively expressing *virB* via the *tac* promoter (78). Because this also circumvents the need for *virF*, it may be that thermoregulation proceeds as a cascade from *virR* to *virF* to *virB* and finally to the *inv* and *ipa* genes.

In *Yersinia* species a set of plasmid-encoded virulence genes called *vir* (58), *lcr* (6), or *yop* (7) loci are expressed only at 37°C in the absence of Ca^{2+}. The expression of several *vir* genes is dependent on the transcriptional activator encoded by the *Yersinia virF* gene (not to be confused with the *Shigella virF*), a 31-kDa protein that exhibits similarity to the AraC class of transcriptional activators (16). VirF is apparently the sensor of temperature inasmuch as it can perform temperature-dependent activation of *vir* gene fusions in an *E. coli* background (16). However, it remains to be determined whether an *E. coli* gene like *hns* could be controlling *Yersinia virF* transcription.

Thermoregulation is also apparent in *Bordetella pertussis*, for which a set of chromosomal genes encoding virulence determinants has been shown to be coordinately regulated by the products of the *bvgA* and *bvgS* genes (51, 52, 65, 67, 72). The *bvgA* and *bvgS* genes encode a two-component regulatory system that responds to modulators such as temperature, SO_4, and nicotinate (52, 65, 67, 72). While the temperature effect varies among strains (51), the isolation of constitutive mutations that affect the response to both temperature and other modulators argues convincingly that temperature is an environmental signal sensed by this system (45). The role of other modulators, such as SO_4 and nicotinate, in the response is unclear, but these might mimic a signal within the intracellular milieu (47). BvgA and BvgS are also unique among virulence regulatory proteins in that their sensory response seems to be fully functional in *E. coli*, at least with the *fha* and *bvg* promoters (52, 65). Interestingly, BvgA and BvgS are not sufficient to regulate other virulence genes in *B. pertussis*, and evidence has been presented implicating a 23-kDa protein as a possible downstream regulator in a *bvgAS*-initiated cascade (40).

It should be noted that not all virulence genes are optimally expressed at high temperature. For example, *inv* (41), an invasion gene of *Yersinia pseudotuberculosis*, is expressed optimally at low temperature, as are virulence genes belonging to the ToxR regulon of *V. cholerae* (see below) (60). It should be considered that for organisms that seldom exist free living in the environment, virulence gene expression may have simply been optimized for growth at 37°C (perhaps through the action of proteins like H-NS) and therefore the observed temperature regulation may have little relevance to the normal biology of the organism.

Calcium. In addition to temperature, calcium plays a role in the regulation of virulence genes in all species of *Yersinia*. At 37°C, growth is dependent on millimolar levels of calcium but virulence gene expression occurs only in its absence (6, 7, 21, 28, 30, 58). Thus, a paradox exists that suggests that yersinae cannot both divide and express virulence determinants in the host if these in vitro environmental conditions reflect the in vivo case. There are two possibilities: (i) there may be a low-calcium environment that contains a host factor which stimulates growth at 37°C or (ii) there may be a high-calcium environment that contains a host factor which stimulates virulence gene expression. The intracellular milieu of host cells may provide one of these two environments, given that yersiniae are quite capable of surviving inside host cells (28, 30). Alternatively, the intracellular environment may induce virulence gene expression but only for use in subsequent rounds of growth in the high-calcium, extracellular environment (30).

Progress is being made on defining the genes involved in the low-calcium response. Recently, two groups have concluded that *lcrH* encodes a product that represses *yop* transcription in the absence of calcium unless the *lcrV* product is present (6, 7, 63). The *lcrV* gene encodes the V antigen, a potential virulence determinant which together with other *yop* gene products such as *yopN* may assemble a calcium and/or host factor sensory apparatus in the bacterial outer membrane (32).

Osmolarity. Osmolarity has been noted as an environmental signal controlling virulence in several organisms. In *V. cholerae*, the expression of cholera toxin, Tcp pili, and other virulence determinants is affected by osmolarity, with an optimum expression occurring in the physiologic range of host tissues (55). This effect appears to be mediated at least in part through the *toxR* gene product, given that ToxR-PhoA fusion proteins appear to be constitutively active even in the presence of high salt concentrations (56). The *toxR* gene product is a transmembrane protein that binds DNA, and together with the membrane protein encoded by the *toxS* locus, it is capable of transcriptional activation of the *ctx* promoter (22, 56). The transmembrane structures of ToxR and ToxT may be involved in their sensory response to physical changes in the membrane or periplasm brought on by variations in medium osmolarity. ToxR controls the expression of a large group of genes but does so indirectly by controlling the transcription of a second regulator, the product of the *toxT* gene (23). ToxR-regulated genes also respond to changes in pH of the medium, and this effect precedes *toxT* expression in the regulatory cascade and therefore may be ToxR mediated (23).

The expression of the virulence-associated alginate cap-

sule of *P. aeruginosa* is affected in some strains by osmolarity as well as other environmental parameters that may reflect the altered environment inside the salty cystic fibrosis lung. The capsule biosynthesis genes are regulated by AlgR and AlgB, proteins with homology to other two-component regulatory systems, but current evidence suggests that neither of these proteins is the direct sensor of osmotic signals (18, 20). A histonelike protein, AlgP, has been proposed to help the activation of AlgR-responsive promoters by bending DNA (19), and this protein may play a role in osmoregulation of alginate expression.

The expression of an invasion gene of *S. typhimurium*, *invA*, has been reported to be induced by media of high osmolarity (34). Agents and mutations that affect DNA supercoiling were shown to affect the expression of *invA* but not in a fashion totally consistent with supercoiling being solely responsible for the effect. A mutation in *ompR*, a gene which together with *envZ* encodes an osmoresponsive two-component regulatory system (2), had no effect on expression of *invA*. However, an *ompR* mutation was found to affect virulence of *S. typhimurium*, an effect that was only partially explained by loss of OmpF and OmpC (15).

In contrast, OmpR and EnvZ may play a role in the control of virulence and in the osmoregulation of invasion genes (*vir* genes) in *S. flexneri* (8). An *envZ* mutation was found to decrease expression of a *vir-lacZ* fusion but not to significantly alter its derepression by high osmolarity, while a deletion of both *envZ* and *ompR* completely abolished the expression of the fusion. These data suggest that EnvZ is not the only osmosensor for *Shigella vir* genes and argue either that OmpR receives osmoregulatory signals by crosstalk from a heterologous sensor (2, 74) or that the envelope changes brought on by *ompR* or *envZ* mutations has in some way affected the function of a distinct osmoregulatory system controlling *vir* gene expression.

Anaerobiosis. Like osmolarity, anaerobiosis can also alter supercoiling (24, 39) and has been shown to induce the invasion phenotype in *S. typhimurium* (26, 46, 68). An earlier finding of induction of invasion by interaction with host cells (31) may therefore be related to reduced oxygen tension when bacteria are adhered to eukaryotic cell surfaces or may simply be an artifact of the experimental design (46). In addition to reduced levels of oxygen, the metabolic activity of eukaryotic cells should lead to elevated levels of CO_2. It is therefore interesting that elevated CO_2 levels induce the expression of genes involved in capsule and toxin synthesis in *Bacillus anthracis* (5) and in enterotoxin production by *V. cholerae* (70).

Stress. There is accumulating evidence that stress at the level of starvation, acidic pH, and heat shock may control the expression of some virulence genes. The two-component regulatory system PhoP-PhoQ controls the expression of genes required for survival of *S. typhimurium* inside macrophages (29, 53). This regulatory system appears to respond to carbon and nitrogen starvation as well as to acidic pH, suggesting that PhoPQ may be responsive to environmental conditions inside phagolysosomes (53). Constitutive *phoPQ* mutants are also attenuated, suggesting that virulence depends on balanced regulation or on the expression of PhoP-repressed genes (54).

The expression of listeriolysin, a protein involved in intracellular virulence of *Listeria monocytogenes*, is induced by heat shock and oxidative stress (71). Similarly, *S. typhimurium* expresses heat shock proteins upon invasion of eukaryotic cells (12). These results together with immunological data (81) suggest that a stress response may be a component of many bacterial infections. However, it is interesting that in one case, induction of a heat shock response actually results in reduced expression of the ToxR virulence regulator (60). This response may correspond to an early phase in the infection cycle when surviving acidic pH, anoxia, and starvation is more important than the expression of virulence determinants.

Catabolite repression. Carbon sources can affect expression of virulence factors in many systems and could do so by alterations in growth rate, metabolic pools, pH, etc. (9, 21). However, there is evidence that in *E. coli* cyclic AMP and cyclic AMP receptor proteins are involved in glucose-mediated catabolite repression of Pap piliation (36) and heat-stable toxin expression (73). Perhaps the presence of glucose is used by the organism to detect the transition from the mucosal surface to the bloodstream. Alternatively, glucose may serve to down regulate virulence gene expression and thus balance the degree of infection (and perhaps disease) with the availability of an optimal host-derived substrate (see "no harm, no foul," below).

REGULATION WITHOUT A CAUSE

There are many regulatory systems that control the expression of virulence properties but do not have a clear environmental signal to which they respond. For example, in *Neisseria gonorrhoeae* pilus gene expression is controlled by a two-component regulatory system, but its sensory function has not been established (76). In *P. aeruginosa* the pilus and flagellum genes are controlled by *rpoN*, which encodes an alternate sigma factor that is involved in nitrogen metabolism in this organism (79). Yet nitrogen does not seem to be involved in the regulation of pilus or flagellum expression (79). Finally, in two different gram-positive organisms the expression of virulence determinants has been found to be dependent on two-component regulatory systems, but again no clear-cut environmental signal has been linked to these systems (42, 61). These and many other examples tell us that although constitutive expression of virulence properties may be apparent under laboratory conditions, underlying regulatory systems may still exist and play some unknown role in the regulation within host tissues.

STRATEGIES FOR THE REGULATION OF VIRULENCE PROPERTIES

Examination of the environmental signals controlling virulence is just one step in the process of deducing the underlying strategies that microbes have adopted to become successful commensal and pathogenic organisms. In an effort to theorize (and thus stimulate thought and discussion in this area), let me propose five strategies for virulence gene expression and for the sake of fun and fantasy label them with the names of basketball game plans or tactics.

The "no harm, no foul" strategy. The virulence genes are derepressed by a restrictive host environmental condition that can be relieved through the action of the virulence factor. A balance is sought in this regulatory loop, in which the microbe gets what it needs and the host is not severely harmed in exchange. If the scales tip, either the microbe is eliminated or the host faces an aggressive microbial response with resultant disease. Possible example: iron regulation of cytotoxins. If the toxin can successfully release iron by minor damage to host tissues, then the bacterium will respond by decreased toxin expression. Such a strategy will increase the frequency of infection compared with disease

and helps explain why seroconversion rates typically run much higher than incidence of disease for many pathogens. Prediction: constitutive expression should increase virulence.

The "intentional foul" strategy. In this case the expression of virulence genes is either constitutive to begin with or fully derepressed by an invariant host environmental cue. A battle ensues between the stimulated defensive host immune response and the pathogen's rapid aggressive multiplication. Possible example: invasion gene expression in *Shigella* species. Predictions: these diseases should show a high disease-to-infection ratio, and constitutive expression should not adversely affect virulence.

The "transition game" strategy. The virulence genes are expressed in a developmental cycle-like fashion. Thus, a virulence property is expressed not because it is needed in the environment in which it is induced but because its expression allows for efficient transition into a new environment. Possible example: expression of serum resistance and antiphagocytic properties by intracellular yersinae. Prediction: constitutive expression may decrease fitness in the previously inducing environment but increase fitness in a noninducing environment.

The "full-court press" strategy. This is an all-out attack that seeks to open up a weakness in the defenses and then take advantage of it. In this system the expression of virulence genes may be controlled by the same regulatory gene but is organized in at least two groups that respond to signals in a reciprocal pattern. Thus, different virulence factors are expressed at different sites within the host and act synergistically in the overall infection. Possible example: the PhoP-regulated virulence determinants of *S. typhimurium*. Prediction: constitutive expression of one group of virulence genes would cause attenuation by adversely affecting the expression of the other, oppositely regulated group of genes.

The "four corners" strategy. The infecting microbe plays a stalling game in order to prolong its interaction with the host. The expression of the virulence factors is set up to be less than optimal in animal tissues, and the ensuing infection and immune response to it are slow in developing. Possible example: normal flora in a noncompromised host. Prediction: a constitutive mutant may be capable of more rapid and extensive infection but less capable of long-term persistence than the wild-type strain.

CONCLUSION

Ultimately, to understand why virulence genes are regulated the way they are, we must know the whens and the wheres of virulence gene expression. This will depend on the development of techniques and approaches for identifying which genes are expressed in vivo during the infection cycle. This together with a more detailed understanding of the mode of action and role in infection of virulence determinants should lead to a full molecular description of pathogenesis.

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