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MINI-REVIEW

Hyperstructure interactions influence the virulence of the type 3 secretion system in yersiniae and other bacteria

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Abstract A paradigm shift in our thinking about the intricacies of the host–parasite interaction is required that considers bacterial structures and their relationship to bacterial pathogenesis. It has been proposed that interactions between extended macromolecular assemblies, termed hyperstructures (which include multiprotein complexes), determine bacterial phenotypes. In particular, it has been proposed that hyperstructures can alter virulence. Two such hyperstructures have been characterized in both pathogenic and nonpathogenic bacteria. Present within a number of both human and plant Gram-negative pathogens is the type 3 secretion system (T3SS) injectisome which in some bacteria serves to inject toxic effector proteins directly into targeted host cells resulting in their paralysis and eventual death (but which in other bacteria prevents the death of the host). The injectisome itself comprises multiple protein subunits, which are all essential for its function. The degradosome is another multiprotein complex thought to be involved in cooperative RNA decay and processing of mRNA transcripts and has been very well characterized in nonpathogenic *Escherichia coli*. Recently, experimental evidence has suggested that a degradosome exists in the yersiniae as well and that its interactions within the pathogens modulate their virulence. Here, we explore the possibility that certain interactions between hyperstructures, like the T3SS and the degradosome, can ultimately influence the virulence potential of the pathogen based upon the physical locations of hyperstructures within the cell.

Keywords Molecular assembly · Degradation · Disease · Membrane · RNA · Enolase

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Introduction

Bacteria were once believed to be unstructured and the physiological processes within them, like RNA decay, were thought to occur randomly. However, it is now clear that bacterial cells are highly structured and that these processes are highly ordered, well organized, and, as in the case of the cell cycle, synchronized. This structuring takes the form of a wide variety of molecular machines and cytoskeletal and "enzoskeletal" filaments and assemblies. It includes proteins being inserted posttranslationally into membranes, dynamic groupings of mRNA and nascent protein into membranes resulting from co-translational insertion (Herskovits et al. 2001; Iost and Dreyfus 1995; Miller et al. 1970; Vos-Scheperkeuter and Witholt 1982), and dynamic groupings of genes (including both reading frames and regulatory sequences in the DNA) plus nascent mRNA plus nascent proteins during the coupling of transcription, translation, and insertion of proteins into membranes (transertion) (Binenbaum et al. 1999; Gowrishankar and Harinarayanan 2004; Lynch and Wang 1993; Norris 1995). This structuring also includes the microcompartments formed as a result of the coupling of transcription, translation, and assembly in the cytoplasm (transembly) as in the case of the foci for ribosome production (Berger et al. 2010; Cabrera and Jin 2003; Norris 2011; Woldringh and Nanninga 1985). Such super-structured complexes exist at a higher organizational level in the cell than their constituent molecules. These

objects have been termed *hyperstructures*, and it has been proposed that interaction between them could partly determine the phenotype of the cell (Norris et al. 2007a). Because the number of hyperstructures is much less than that of the individual macromolecules that constitute them, the number of combinations between hyperstructures is much less than that between macromolecules. This would have the advantage of helping solve one of biology's fundamental, combinatorial problems, namely, how to reduce the hyperastronomical number of different phenotypes apparently available to cells from the combinations of many different macromolecules (Kaufmann 1993); for example, the number of phenotypes arising from the $2^{4,000}$ on–off combinations of the 4,000 or so genes in certain bacteria is likely to be much higher than the number of phenotypes arising from the 2^{100} on–off combinations of a 100 or so hyperstructures (Norris et al. 2012). Moreover, the existence of the higher order level of hyperstructures would allow the bacterium to reduce background noise from the lower level of macromolecules (for example, from those macromolecules that are not part of a hyperstructure) and hence facilitate the emergence of a coherent, meaningful pattern at the higher level of the cell itself (Lemke 2000; Salthe 1985), thus producing the desired phenotype. Viewed in this light, the challenge for modern biology is to understand how hyperstructures interact.

A speculative exploration of such interactions has been undertaken in the case of the regulation of the cell cycle (Norris 2011; Norris et al. 2002; Norris and Fishov 2001; Rocha et al. 2003). Here, we extend this exploration to virulence. We consider the case of the agent responsible for bubonic plague, *Yersinia pestis* (YP) and its closely related relative, *Yersinia pseudotuberculosis* (YPT). These Gram-negative bacteria can construct a type III secretion system (T3SS) capable of injecting cytotoxic effector proteins, termed yersinial outer proteins (Yops), directly into the cytosol of a targeted host cell (Fig. 1). The T3SS has been found in many bacteria that interact with eukaryotes while proteins such as RNase E, RNA polymerase, and ribosomal proteins are found in all bacteria. It is therefore likely that the yersiniae possess the full range of hyperstructures involved in protein synthesis, metabolism, protein secretion, and RNA turnover in model organisms such as *Escherichia coli*, *Bacillus subtilis*, and *Caulobacter crescentus*. Rather than consider the ways an individual hyperstructure integrates information, we reflect here on interactions between hyperstructures in the yersiniae, particularly between the T3SS, the degradosome, and putative proteolytic, secretion, polyphosphate-based, and EF-Tu hyperstructures. We propose that these interactions depend on transertion and transembly which act by instructing molecular "messenger boys" and by locating hyperstructures near to or far from one another.

Model of interactive hyperstructures resulting in the modulation of bacterial virulence

The model is based on several assumptions and comprises several propositions:

1. The T3SS hyperstructure comprises the T3SS injectisome (comprised of 27 *Yersinia* secretion proteins—Yscs), the Ysc-encoding transcripts (plus the *ysc* genes themselves), as well as the full-length T3SS injectisome substrate transcripts and the *yop* genes that encode the aforementioned Yops, all of which may undergo coupled transcription–translation–insertion (transertion).
2. T3SS-dependent virulence results from the dialogue between hyperstructures that include the degradosome, the EF-Tu hyperstructure, and polyphosphate-containing hyperstructures.
3. This speculative model is based on the use by hyperstructures of "messenger boys" and on the relative positions of the hyperstructures. The "messenger boys" include calcium and lipids while the positioning of hyperstructures depends in part on the processes of transembly and transertion such that, for example, the nascent components of the degradosome attach it to a larger transembly hyperstructure, thereby furthering or preventing contact between the degradosome and its target mRNAs within other coupled transcription–translation hyperstructures.

Evidence for hyperstructures

Coupled transcription–translation hyperstructures

Currently, there is a plethora of evidence supporting the existence of transcription–translation hyperstructures (for a review, see Norris et al. 2007b). Some of this evidence includes the fact that the mRNA remains next to the transcribed gene for the five genes studied in *C. crescentus* and for *lacZ* in *E. coli* (Llopis et al. 2010). Since not only the nascent mRNA but also the full-length mRNA often remain in the vicinity of the gene, translation must also occur there as well on account of spatial availability; however, recent evidence has revealed that certain subsets of transcripts may go to various regions of the cell (e.g., the membrane) where they are then translated (Nevo-Dinur et al. 2011). That said, the percentage of the cell's overall transcript load that is localized prior to translation during various environmental responses rather than translated in the nucleoid region remains unclear. In the more canonical view of coupled transcription–translation, this geographical clustering of gene, transcript, and translational machinery strongly suggests that prokaryotic transcription and translation occur

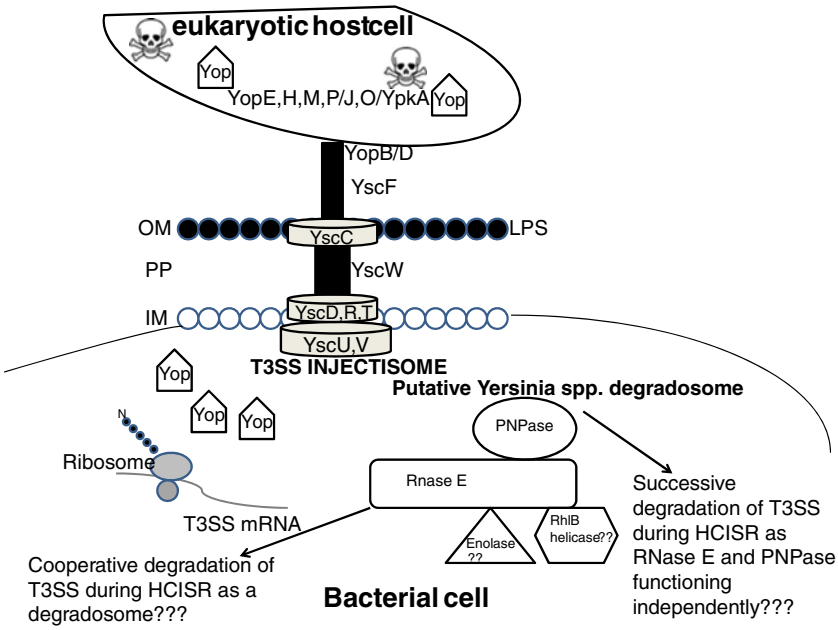

Fig. 1 T3SS and degradosome hyperstructure interactions. The T3SS injectisome hyperstructure is the primary virulence-associated machinery of the yersiniae that enables the direct delivery/injection of toxic Yop effector proteins into the targeted host cell. PNPase has been shown to play a role in the optimum functioning of the yersinial T3SS (Rosenzweig et al. 2007; Rosenzweig et al. 2005) in a manner independent of its catalytic activity. This suggested that PNPase could be interacting with RNase E in a degradosome-dependent manner. The Δ*pnp* mutants exhibited de-repressed T3SS transcription and protein production (Rosenzweig et al. 2007) suggesting that PNPase works to degrade T3SS transcripts during the host cell-induced stress response (HCISR). Since the PNPase requirement for T3SS function was independent of its catalytic activity, a truncated RNase E, unable to form a degradosome, was used to characterize degradosome involvement in the HCISR and T3SS function and appeared to also be required for optimal T3SS function. Therefore, the current model envisions PNPase potentially trapping mRNA transcripts with its S1 RNA-binding domain and subsequently feeding trapped transcripts into the RNase E catalytic core for degradation in a degradosome-dependent fashion. What remains unclear is whether enolase and RhlB helicase also associate with the yersinial degradosome and whether they contribute to its function

primarily within a hyperstructure for each gene or, in some cases, operon or cluster. Such hyperstructures may be more complicated when they synthesize or degrade the constituents of another type of hyperstructure. In such cases, a large macromolecular machine may be created and—perhaps maintained—as part of a still larger hyperstructure with a dynamic, transertion, or transembly part (Cabin-Flaman et al. 2005). This is likely to be true for the flagellum in *C. crescentus* insofar as the flagellin transcripts are located together (Llopis et al. 2010).

The T3SS hyperstructure

Virulence hyperstructures, like certain other hyperstructures such as the flagellar hyperstructure (Cabin-Flaman et al. 2005), can go through both equilibrium and non-equilibrium periods while undergoing developmental programming (i.e., the genetic program induced by various stresses) including synthesis/birth, maturation, and finally decay/death. T3SSs are macromolecular machines that are generally considered to consist of the structural components, secreted proteins (including pore-forming translocators and effectors), chaperones, and cytoplasmic regulators (for a review, see Deane et al. 2010). The injectisome machine at the heart of the T3SS comprises a basal body that spans both bacterial membranes and an extracellular needle that protrudes from the bacterial surface. The basal body has rotational symmetry and is made up of several rings of integral membrane and periplasmic proteins (within which lie the rod proteins). During assembly, the components of the transmembrane rings are handled by the Sec machinery while later components are exported by the machine itself. The needle, which projects from the basal body, is formed from the polymerization of a single, small needle subunit, YscF. Both the basal body and needle contain a channel through which proteins are believed to be secreted. At the anterior, extracellular end of the needle is a tip structure made of several copies of the tip protein.

Conventionally, the T3SS is considered to be constructed and to operate as follows. First, the membrane rings are formed: YscR, YscS, YscT, YscU, and YscV assemble into a secretion complex in the inner membrane (Diepold et al. 2011), and independently of this, YscC and YscW assemble into a ring in the outer membrane (Ross and Plano 2011); then, YscJ and YscD form a ring that links the secretion complex to the outer membrane ring (Schraidt et al. 2010);

these are then joined by the "early" substrates such as the proteins YscI and YscF that make up the rod and needle, respectively (Buttner 2012). Once the needle has been made, the tip proteins are exported. Secretion is blocked until contact with a host cell membrane is made and low intracellular calcium levels are detected (i.e., less than the 1.2 mM Ca^{2+} levels present in the blood); the T3SS then exports two types of proteins: the translocator Yops that form a pore in the target membrane and the six effector Yops that travel through the needle conduit/cytoplasmic bridge into the host cell (Deane et al. 2010). Although the distribution of and the exact basal level of T3SS hyperstructures residing on the surface of the yersiniae at any given time are not known, it is believed that after the T3SS becomes fully induced through its two-tiered sensing system of the mammalian body temperature (37 °C) and low intracellular levels of calcium, many functional T3SS injectisomes become inserted in the bacterial membrane. How stable the T3SS injectisomes are once formed and how long residual T3SS hyperstructures are maintained on the bacterial surface following inducing environmental conditions remain unknown. However, given the fierce battle for survival in various environments and limiting nutritional resources, it is unlikely that the yersiniae would invest much metabolic energy in the maintenance of multiple virulence hyperstructures that were no longer needed, beyond the several basal injectisomes needed for anticipation of a rapid environmental change when re-introduced into a mammalian host.

Protein production and secretion are not always coupled. With the exception of YopN and YopQ (Goss et al. 2004), most T3SS protein substrates, with the help of their respective chaperone proteins, are believed to be stored in the bacterial cytoplasm in a secretion-competent state (Page and Parsot 2002; Wilharm et al. 2004) in which the protein is tightly folded (except for the chaperone-bound N-terminus) to be unfolded later by YscN during export (Akeda and Galan 2005). The precise localization of these secretion-competent Yops is unknown; however, they are likely to be found congregating near the injectisome machinery to expedite their secretion when the system becomes fully induced. Secretion of the Yops may also occur partly via transertion or via co-translational insertion using a signal within the mRNA (Anderson and Schneewind 1999) although the high rate of secretion within the first few minutes of infection argues against this being sufficient (Enninga et al. 2005) (for other references, see Buttner 2012). Note that the possible production of the Yops via coupled transcription–translation may be separated in time and space from the actual secretion of the Yops.

Obviously, the full picture of an operational/induced T3SS hyperstructure is more complicated than presented above and probably entails the processes of transertion and transembly of both the genes encoding the T3SS constituents (as in the case of flagella, see above) and those encoding the secreted proteins. Such dynamic attachment of these genes to the machine under assembly means that these genes should be in the vicinity of the machine to ensure the greatest efficiency. Reciprocally, the position of these genes, with respect to the rest of the cell, should determine the position of the entire hyperstructure. The resulting question is what determines the position of the gene within the cell (see below).

The degradosome

The bacterial RNA degradosome is believed to be central to normal RNA processing and degradation. However, the precise physiological role played by the multiprotein complex remains elusive. *E. coli* mutants deficient for degradosome assembly are viable but are defective in RNA processing and degradation (Carpousis 2007). In *E. coli*, the degradosome is a multiprotein cytoskeletal structure that includes Ribonuclease E (RNase E), RNA helicase B (RhlB), polynucleotide phosphorylase (PNPase), and enolase. RNase E is an essential endoribonuclease that plays a key role in the maturation of stable RNA and the degradation of messenger RNA; PNPase is a phosphorolytic 3′-exonuclease; RhlB is a DEAD-box RNA helicase that facilitates mRNA degradation by RNase E and PNPase. The interaction of RhlB with the RNase E degradosomal component is necessary for RhlB activity in vitro (Vanzo et al. 1998) (for other references, see Khemici et al. 2008). Enolase is a glycolytic enzyme that catalyzes conversion of 2-phosphoglycerate into phosphoenolpyruvate, and it is active in both a degradosome-dependent and independent fashion (Carpousis 2007; Chandran and Luisi 2006).

RNase E can directly interact with membrane phospholipids in vitro (Khemici et al. 2008), and the degradosome itself lies close to the cytoplasmic membrane of *E. coli* as a helical structure (Miczak et al. 1991; Singh et al. 2009; Taghbalout and Rothfield 2007). Association of the degradosome with the membrane is likely of importance insofar as cells that express only the RNase E fragment lacking the cytoskeletal localization domain have several mRNAs with an increased half-life and a defective processing of rRNA and tRNA (Ow et al. 2000). The degradosome is also associated with the chromosome in *C. crescentus* (Llopis et al. 2010), and given the different distributions of RNase E in control cells and in those depleted in mRNA, it has been proposed that mRNA substrate availability has some influence on the cellular distribution of RNase E (Llopis et al. 2010). In short, the picture now emerging is that the organization of the degradosome and its associations with the membrane and chromosome ultimately affect RNA processing and degradation of some transcripts; however, whether

the degradosome is specifically required for a particular stress response still remains unclear.

The polyphosphate hyperstructure

Polyphosphate plays a central role in the life of cells and, in particular, in virulence. In a wide range of pathogens, for example, mutants in polyphosphate kinase 1 were defective in motility, biofilm formation, response to stringencies, and tolerance to heat, acid, and desiccation (Rao et al. 2009); these pathogens included the salmonellae, *Shigella* sp., *Vibrio cholerae*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, and *Neisseria meningitidis*. Additionally, polyphosphate is clearly important in the life of YPT (Buzoleva et al. 2006).

One possibility is that polyphosphate forms its own hyperstructure. In many bacteria, it can be found in the form of particles or volutin granules (Rao et al. 2009), perhaps existing as such in the yersiniae. Such granules resemble the eukaryotic acidocalcisome, an organelle involved specifically in the storage and metabolism of cellular polyphosphate (Docampo et al. 2005; Seufferheld et al. 2008). Polyphosphate kinase, PPK, catalyzes the reversible polymerization of the gamma-phosphate of ATP into polyphosphate, and in *P. aeruginosa*, the synthesis of polyphosphate by PPK1 leads to the formation of filaments suspected to contain an actin-like protein insofar as the process is inhibited by the addition of phalloidin (Rao et al. 2009). Another possibility (see below) is that polyphosphate forms an integral part of other hyperstructures such as the degradosome, given the presence in the degradosome of PPK (Blum et al. 1997).

The EF-Tu and proteolytic hyperstructures

Elongation factor EF-Tu provides the ribosome with aminoacylated tRNAs during the elongation step of translation. EF-Tu/GDP is recycled by the guanine nucleotide exchange factor EF-Ts. EF-Tu has long been suspected of being an actin homologue (for references, see Norris et al. 2007b). Its cytoskeletal nature became clear with the discovery that it forms protofilaments and networks in vivo and that polysomes are attached to these protofilaments (Mayer 2006). Subsequently, in *B. subtilis*, EF-Tu was found to form a helical pattern that had a strong influence on the distribution of another hyperstructure, that of the actin-like protein MreB (Defeu Soufo et al. 2010).

Additionally, proteases may be confined to a proteolytic hyperstructure. In *B. subtilis*, the Clp proteases and related proteins form hyperstructures (Kirstein et al. 2008), while in *C. crescentus*, the assembly of the ClpXP protease into a hyperstructure in the poles is required for the degradation of the CtrA "master regulator" (Iniesta and Shapiro 2008). In fact, in YP, ClpXP and Lon proteases have been shown to promote T3SS de-repression through the cooperative degradation of YmoA, which forms heterodimers with H-NS to affect supercoiling (Madrid et al. 2007), thereby providing a direct link between a proteolytic hyperstructure and T3SS function (Jackson et al. 2004).

Integration at the level of a single hyperstructure

The criterion of having to belong to a hyperstructure to affect the phenotype is one solution to reducing the hyperastronomical number of combinations of molecules and macromolecules that might otherwise affect it. Here we illustrate this idea in the case of the RNA degradosome.

Degradosomes of various compositions in different bacteria may form in different environmental conditions (Lawal et al. 2011; Mackie et al. 2008) and can communicate information about intracellular and extracellular conditions to other hyperstructures, including virulence hyperstructures like the T3SS, which depends on temperature and calcium levels. In addition to its four core constituents, the degradosome can also contain the heat shock proteins GroEL and DnaK (Miczak et al. 1996) and polyphosphate kinase, PPK, which catalyzes the reversible polymerization of the gamma-phosphate of ATP into polyphosphate (Blum et al. 1997). (With respect to the presence of PPK, it should be noted that in *Mycobacterium bovis* BCG the degradosome contains polyphosphate/ATP-NAD kinase (Kovacs et al. 2005)). RNase E can degrade specific mRNAs by forming a complex with the RNA-binding protein Hfq and either of the small regulatory RNAs, SgrS, in the case of phosphosugar stress, or RyhB, in the case of iron depletion (Morita et al. 2005; Prevost et al. 2011). RNase E may also be able to form a "cold shock" RNA degradosome that contains CsdA, another RNA helicase (Prud'homme-Genereux et al. 2004); it may, therefore, be significant that PNPase is a cold shock protein. RNase E is now known to interact physically with ribosomal protein L4 (Singh et al. 2009) and with the regulatory proteins RraA (Gorna et al. 2010) and RraB (Gao et al. 2006).

What does it all mean? In *E. coli*, microarray analysis of mutants affected in the four core components of the degradosome revealed the increased abundance of 119 mRNAs, and it was found that the decay of some mRNAs in vivo depends on the action of the assembled degradosome while the decay of other mRNAs depends on the action of the degradosome proteins independent of the degradosome (Bernstein et al. 2004). Assembly of a variant of the degradosome with Hfq and SgrS requires the same region of RNase E as for the assembly of the canonical RNA degradosome, consistent with the restructuring of the degradosome by interactions with different partners. Indeed, the

present consensus is that "degradosome remodelling may well be a mechanism for the differential regulation of RNA cleavages and adaptation to various stress conditions in *E. coli*" (Gao et al. 2006) and that the "multiple components of the RNA degradosome [may exist] in a dynamic, energy-dependent equilibrium" (Gorna et al. 2010). In the photosynthetic bacterium *Rhodobacter capsulatus*, the RNase E-based degradosome is remodeled in response to oxidative stress (Jager et al. 2004). In *E. coli*, the L4-RNase E interaction affects 65 mRNAs encoding the stress response proteins HslO, Lon, CstA, YjiY, and YaeL, as well as proteins involved in carbohydrate and amino acid metabolism and transport, in transcription and translation, and in DNA and RNA synthesis. Stresses such as elevated temperature or amino acid starvation lead, via the nucleolytic attack of ribosomal RNA, to ribosome disassembly and to the Lon degradation of the free r-proteins to provide extra amino acids for new functions. It has, therefore, been proposed that free r-proteins, which include L4, inhibit the RNase E-mediated decay of stress-related mRNA to help in the adaptation to adverse environments (Singh et al. 2009). Finally, there is another minor constituent of the degradosome whose significance should not be overlooked: PPK. In vitro, polyphosphate inhibits the degradosome, and it is thought that degradosome-associated PPK removes inhibitory polyphosphate and NDPs and regenerates ATP (Blum et al. 1997).

Hyperstructure interactions determine virulence

How exactly do the above changes in the composition and structure of the degradosome take effect in the context of a highly structured cell? Furthermore, how do interactions between hyperstructures determine not only the fate of hyperstructures but also their very nature? These interactions may take on many forms (Norris et al. 2007a). Here, however, we will consider just two: molecular messengers and the locations of genes.

Communication between hyperstructures—messenger boys

In the hyperstructure approach, inorganic ions, small molecules, and macromolecules are not the controllers of the phenotype but rather the messenger boys instructed by hyperstructures to communicate with other hyperstructures (Norris et al. 2007a). In the following section, we explore this proposal with respect to polyphosphate and emphasize that the question is not whether polyphosphate is important in the life of YP (as is clearly the case for YPT; Buzoleva et al. 2006) but whether polyphosphate is involved in the dialogue between hyperstructures regarding virulence. There are several ways this might occur. First, polyphosphate might form its own hyperstructure. In many bacteria, it can be found in the form of particles or volutin granules (Rao et al. 2009), but in the yersiniae, it does not appear to have been reported as either present or absent. Such granules resemble the eukaryotic acidocalcisome, an organelle involved specifically in the storage and metabolism of cellular polyphosphate (Docampo et al. 2005; Seufferheld et al. 2008). In *P. aeruginosa*, the synthesis of polyphosphate by PPK1 leads to the formation of filaments that might be suspected to contain an actin-like protein insofar as the process is inhibited by the addition of phalloidin (Rao et al. 2009). Second, polyphosphate might form an integral part of another hyperstructure such as the degradosome, given the presence of PPK within it. In either case, the polyphosphate could allow virulence-related communication via ions, lipids, or proteins.

The ion most likely to be involved in communication here is calcium since (1) calcium is intimately involved with the eukaryotic acidocalcisome and with polyphosphate in general (for example, polyphosphate and polyhydroxybutyrate form a calcium channel in both eukaryotes and prokaryotes; Das et al. 1997), and (2) calcium is involved in the virulence of YP (Fowler et al. 2009). An intriguing possibility is that calcium communication between hyperstructures results from the condensation of counterions onto the linear polymers that constitute these structures. This would occur at a critical value of the charge density of the polymer and would resemble a phase transition (Manning 1969). In such condensation, a counterion such as calcium is delocalized along the polymers, diffuses along the so-called near regions, and can help bring polymers together (Ray and Manning 2000). Such polymers include DNA and polyphosphate and those formed by actin and tubulin that condense counterions in vitro (see Ripoll et al. 2004 and references within). It may, therefore, be significant that *ppk* mutants of several bacterial species are unable to adapt to many stresses, including high concentrations of calcium (Seufferheld et al. 2008), particularly since ATP levels and intracellular levels of free calcium are intimately related. Low levels of ATP lead to high levels of calcium in *E. coli*, and changes in intracellular levels of free calcium regulate over 100 genes in *E. coli*, including metabolic enzymes such as enolase (Naseem et al. 2009); it has therefore been speculated that calcium may regulate ATP levels through glycolysis and/or the Krebs cycle.

What of lipids? The nature—and perhaps the very existence—of transertion hyperstructures depends on the nascent proteins binding to the lipids for which they have an affinity (Binenbaum et al. 1999). Competition between hyperstructures for lipids might, therefore, help determine which hyperstructures survive. Cardiolipin is present in YPT only when grown at 37 °C, the temperature at which T3SS virulence factors are expressed (Salamah and Ali 1995). Cardiolipin has a strong affinity for calcium, and this

again raises the possibility that ion condensation might be involved, particularly since condensation occurs readily on charged planar surfaces (Manning 2007).

In the case of proteins as messenger boys, basic histone-like proteins that bind to nucleic acids are promising candidates. The HU proteins of *E. coli* and *P. aeruginosa* bind more strongly to polyphosphate than to linear double-stranded DNA, and the affinities of the HU proteins for polyphosphate differ with respect to the chain length; this has led to the proposal that the specificity of interaction of polyphosphate with these basic proteins may determine the structure and function of the bacterial chromosome (Rao et al. 2009). Consistent with this, the nucleoid undergoes a major compaction in *ppk1* mutants of *P. aeruginosa* and genes involved in the type III secretion system are down-regulated (Fraley et al. 2007; Rao et al. 2009). Although HU remains to be characterized in the yersiniae, the YmoA, histone-like protein has been studied (see above for its putative relationship with the proteolytic hyperstructure) but its interaction with polyphosphate remains to be investigated. The global transcriptional silencer, H-NS, is believed to repress horizontally acquired genes until conditions are right for expression of virulence (Lucchini et al. 2006; Navarre et al. 2006). This DNA binding protein oligomerizes to form hyperstructures that bring together different regions of the chromosome (Wang et al. 2011). H-NS hyperstructures may be the target of other proteins. For example, Fis may compete with H-NS at rRNA gene promoters (Schneider et al. 2003) and HU may compete with H-NS to open up H-NS-condensed or bridged regions (van Noort et al. 2004). H-NS is important in the virulence of yersiniae and, for example, the temperature-dependent expression of the invasin virulence factor in YPT is silenced by H-NS (Heroven et al. 2004). Intriguingly, in the light of a possible role for ion condensation, divalent ions affect the interactions between H-NS and DNA in a temperature-dependent fashion (Liu et al. 2010).

By binding to DNA, RNA, and proteins, noncoding RNAs are well suited for a role as the glue holding one or more hyperstructures together (Norris et al., in preparation). A corollary of this speculation is that these RNAs could then play a central role in communicating between hyperstructures whereby, for example, the disassembly of one hyperstructure frees noncoding RNAs to participate in the assembly of another hyperstructure.

Location of hyperstructures

What processes are responsible for the location—and co-location—of hyperstructures? One process is probably related to the position of the genes involved. Both the nature of a plasmid (Ho et al. 2002) and the position of a gene on the chromosome/plasmid (Wiggins et al. 2010) can help determine the location of a hyperstructure within the cell. Plasmids appear to have specific intracellular addresses since, for example, the three plasmids, F, P1, and RK2, form foci in different locations within *E. coli* (Ho et al. 2002). In the case of the chromosome, a spatial organization of genetic loci exists in *E. coli* (Llopis et al. 2010; Niki et al. 2000; Wang et al. 2006), while in *C. crescentus*, 112 individual genetic loci were found to occupy spatial locations that corresponded to their linear order on the chromosome (Viollier et al. 2004).

What is the physical nature of the underlying mechanism (s)? Transertion, which would tether an expressed gene to a proteolipid domain, and transembly, which would tether an expressed gene to a macromolecular assembly, are obvious candidates. mRNA may also play a direct role; indeed, certain mRNAs contain information sufficient for them to be correctly positioned within the cell without the need for their translation (Anderson and Schneewind 1997; Nevo-Dinur et al. 2011). In particular, it was found that the sequence encoding the first two transmembrane helices of a membrane-bound sugar permease, BglF, is sufficient for membrane targeting of the mRNA and that the sequence encoding the N-terminal RNA-binding domain of a transcription factor, BglG, is sufficient for mRNA polar targeting. This may mean that both nascent mRNA and nascent protein can direct a hyperstructure to the right location (Nevo-Dinur et al. 2011). A strong argument has been made that the spatial location of genes in slow growing *E. coli*, prior to the initiation of chromosome segregation, is dominated by strong intranucleoid interactions (Wiggins et al. 2010). Such interactions could result from transertion and transembly creating hyperstructures (Rocha et al. 2003) but could also result from several other factors. These factors include the distribution of binding sites for proteins—and in particular for transcription factors—along the chromosome (Junier et al. 2010).

In the case of plasmids, the highly conserved ParA family of partitioning systems plays a major role in the location of the DNA insofar as plasmids in *par*$^-$ mutants are highly mobile (Adachi et al. 2006; Derman et al. 2008; Yao et al. 2007). This does not necessarily mean that different incompatibility groups of plasmids become mixed in such mutants since immiscibility and mobility are different properties. Another factor in the positioning of plasmids or, more exactly, making clusters of them immiscible, may be their liquid crystalline state. This state has been shown to characterize multicopy plasmids (Reich et al. 1994). The immiscibility of cholesteric liquid crystals due to different cholesteric pitches has been proposed to contribute to the separation of daughter chromosomes during replication (Bouligand and Norris 2001). One question here is how hyperstructure dynamics might influence a location mechanism based on cholesteric pitch. A possible answer is via a competition for inorganic ions, such as magnesium or calcium, or for organic ones, such as spermidine or spermine; in vitro evidence

for this is, for example, the fact that the state of the cholesteric phase is affected differently by the nature of the cation (Catte et al. 2004; Raspaud et al. 2005) and by its concentration (Stanley et al. 2005). In this context, we note that the binding of SopB, part of the partitioning system of the F plasmid, reduces its negative supercoiling and decreases its mobility (Biek and Strings 1995).

What then of yersiniae? In YP and several other yersiniae, virulence genes are carried on the low calcium response (LCR) plasmid. This raises the possibility of a central role for calcium as a counterion in the condensation of this plasmid (see above). Specific regions of the chromosome may also be devoted to virulence genes as in the case of *Yersinia enterocolitica* Biovar 1B which carries the Ysa pathogenicity island, encoding a T3SS, called the plasticity zone that contains numerous genes implicated in virulence (Matsumoto and Young 2009).

How might location determine operation? In many cases, mRNA levels are poorly correlated with protein levels (Taniguchi et al. 2010). The location of a hyperstructure might be expected to affect the availability of the translation machinery and hence the functioning of the hyperstructure. In other words, the proximity to the EF-Tu hyperstructure might affect the extent to which mRNA is translated. One logical location for the EF-Tu hyperstructure during fast growth, when much of the synthetic machinery is devoted to making ribosomes, would be the location of the ribosomal hyperstructures which become apparent in *E. coli* at high growth rates (Berger et al. 2010; Cabrera and Jin 2003); such a location might physically separate the EF-Tu hyperstructure from the T3SS hyperstructure, for example, that is only operational in slow or no growth conditions.

Another way that the location of hyperstructures may be important in the phenotype is via the destruction of constituents that fail to join or to stay with the hyperstructure. The idea is well accepted that the position of the degradosome may be a factor in the degradation of RNA, particularly since target RNAs are now known to have specific locations even when expressed from plasmids (Khemici et al. 2008; Llopis et al. 2010; Taghbalout and Rothfield 2007; Valencia-Burton et al. 2009). In a sense, there is a similar idea with respect to proteolysis insofar as it has long been thought that enzymes in a complex may be stable because they are protected from proteases while those that are free may be unstable because they are exposed to them (Miller 1996). Such ideas can be readily extended into the dynamics of hyperstructures like those of the degradosome and the proteolytic hyperstructure that themselves occupy specific locations. Does their location affect their functioning and do their constituents affect that location? It has been suggested that the location of "RNase E and other enzymes to the inner cytoplasmic membrane [means] that RNA processing and degradation is also compartmentalized and that a class of transcripts, which remains to be identified, is processed or degraded on the inner cytoplasmic membrane" (Khemici et al. 2008). So the T3SS and degradosome are either held together or held apart. A factor here to consider is transembly. Consider, for example, transembly into a glycolytic hyperstructure and into the degradosome of a component like enolase (for a recent example of an enzoskeletal hyperstructure, see Cabeen and Jacobs-Wagner 2010, and for other references, see Norris et al. 2007b); the enolase in the degradosome amounts to some 5–10 % of cellular enolase (comparatively, for PNPase the figure is 10–20 %) where it is active (for references, see Bernstein et al. 2004); hence, simultaneous transembly into the two hyperstructures might allow the degradosome to degrade glycolytic mRNAs and would explain the involvement of enolase in such degradation. A similar rationale would explain the presence of the ribosomal protein L4 in the degradosome (Singh et al. 2009) since this might bring the degradosome in contact with ribosomal hyperstructures. Such contact might provide a mechanism to determine which mRNAs are translated and which degraded. Furthermore, it might even help explain the importance of an intact degradosome in the degradation of the T3SS mRNA in YPT (Yang et al. 2008).

The existence of proteolytic hyperstructures at particular locations in some bacteria (see above) is consistent with this being a factor in protein turnover. Indeed, it has been suggested that substrate specificity could be determined by the spatial and temporal organization of proteases (Simmons et al. 2008). There is also the possibility of a spatial role in proteolysis for polyphosphate as a hyperstructure or as part of a hyperstructure. This is not unreasonable because in *E. coli* the binding of polyphosphate to the Lon protease increases its activity 20 times (Kuroda et al. 2001). What then of the T3SS? In the following section, we invoke degradative hyperstructures in an attempt to explain a number of results obtained with the yersiniae.

Hyperstructure-based interpretation of T3SS results

Enolase (a degradosome constituent) was recently identified as a virulence-associated gene in an emerging Gram-negative pathogen *Aeromonas hydrophila* (which also possesses a T3SS) wherein it was upregulated during a murine infection (Sha et al. 2003). Furthermore, enolase was shown to bind to human plasminogen facilitating its conversion to plasmin which when bound to the bacterial surface proved more resistant to its anti-plasmin inhibitor (Sha et al. 2009). In addition to enolase, several ribonucleases have been implicated in bacterial virulence; however, PNPase has been given much attention for its modulation of virulence in multiple pathogens (potentially in a degradosome-dependent fashion)

including the yersiniae (Lawal et al. 2011). With regards to YP and YPT, PNPase was shown to enhance bacterial virulence and T3SS function despite being catalytically inactive and instead required its S1 RNA-binding domain. Furthermore, S1 RNA-binding domains expressed independently of their derived proteins were able to functionally complement Δ*pnp* YP and YPT mutants (Rosenzweig et al. 2007; Rosenzweig et al. 2005). Presently, we are not certain whether these S1 RNA-binding domains interact with the degradosome directly; however, PNPase's interaction/assembly within the degradosome was shown to be independent of its S1 RNA-binding domain (Stickney et al. 2005), and the specific physiological roles played by the S1 domains resulting in complementation of the Δ*pnp*-mutants remains unclear. Curiously, the Δ*pnp* YP and YPT mutants, although reduced in their virulence potential, exhibited de-repressed T3SS transcript levels as well as concomitantly elevated T3SS-associated protein levels (Rosenzweig et al. 2007). One evident possibility is that the role of PNPase in the T3SS is not directly catalytic but rather structural. For example, interaction with PNPase might be needed for the activity of another enzyme that itself is needed for T3SS functioning. Since PNPase is part of an enzoskeletal hyperstructure, the degradosome, Yang et al. looked at the involvement of a major component of the degradosome, RNase E, with the T3SS and with PNPase (Yang et al. 2008). As it turns out, the functioning of the T3SS was inhibited by production of a truncated RNase E that is catalytically active but that interferes with the assembly of the degradosome (Yang et al. 2008). In the language of hyperstructure dynamics, hyperstructure interactions with a PNPase–RNase E degradosome appear to be needed for the T3SS to function correctly. Whether the numerous constituents of the degradosomes found in other bacteria play a role in the functioning of the degradosome in YPT remains unknown.

The important question here is, "What are these hyperstructure interactions?" Do they involve, for example, locations? First, consider the action of the degradosome on its own constituent-encoding transcript levels. It was found that the RNase E-encoding mRNA, *rne*, was higher in the Δ*pnp* mutant than in the wild-type, as might be expected if the degradosome were either no longer formed or no longer operational close to the *rne* gene (Yang et al. 2008). This explanation would explain the finding in both YPT and *E. coli* that RNase E's "auto-regulatory activity could be inhibited by expressing a carboxyl-terminally truncated RNase E variant that conferred a dominant-negative phenotype." Indeed, then, the *rne* transcript is not degraded if the degradosome is not formed properly or not operational near the *rne* gene. The interpretation in our model is that normal degradation of the *rne* transcript requires a close proximity of *pnp* and *rne* genes to the degradosome hyperstructure as provided by transembly. What about the reciprocal situation regarding *pnp* transcript levels measured in the YPT strain expressing the truncated RNase E? Unfortunately, these data were not reported. Protein levels were, however, determined with respect to the isogenic wild-type strain. RNase E appeared unchanged while PNPase was lower. This is difficult to explain using concepts of unstructured bacteria and tightly correlated levels of mRNA and proteins (a naive expectation would be that the levels of mRNA and protein of both RNase E and PNPase should be high). One type of hyperstructure-based explanation is that the levels of both *rne* and *pnp* mRNAs are higher in the absence or altered location of the degradosome but the translated products are in different places with respect to proteolytic hyperstructures and, therefore, undergo different fates. In other words, in the absence of the degradosome, RNase E is made at the membrane far from a proteolytic hyperstructure while PNPase is made in the cytoplasm next to a proteolytic hyperstructure which degrades it; the level of PNPase is therefore low even though the level of its mRNA is high. In this classical use of molecular biology, the truncated RNase E was encoded by the pBAD plasmid under the control of arabinose while the reporter plasmids used to measure RNase E activity consisted of the 5′-untranslated region of *rne* (which in *E. coli* is subject to cleavage by RNase E) fused to either *lacZ* or *kan*. Based on ideas concerning spatial organization of hyperstructures, several questions arise including: Where is this truncate made? Does it cause the degradosome to assemble somewhere else or to not assemble at all? Does it mean that degradation becomes uncontrolled? Where are the reporter plasmids located relative to the hyperstructure(s)? Is *rne-lacZ* part of (a) hyperstructure(s) and, if so, which one(s) (see Legent and Norris 2009)? What exactly are they reporting? Are they reporting local RNase E activity and if so where?

Finally, production of the dominant, inhibitory truncate of RNase E inhibited the T3SS functioning despite this inhibition being due to a reduction in the secretion of YopE but not in the levels of YopE protein. Possible hyperstructure-based explanations for this include the effect of an altered or absent degradosome on (1) other hyperstructures such as the Sec hyperstructure or on enzymatic ones such as those putatively responsible for glycolysis, (2) the transembly process needed to make a functional T3SS machine and allows its constituents to be made in the wrong place and order, (3) a polyphosphate hyperstructure needed to supply the energy for secretion of YopE, (4) the transertion needed for YopE to be made next to the T3SS so allowing YopE to be made in the wrong place, and (5) the production of messengers such as the regulatory RNAs that are higher in *Salmonella enterica* strains that either lack

PNPase or express a truncated RNase E (Viegas et al. 2007)—and that are diminished in yersiniae mutants.

Predictions

"Messenger boys"

The levels of messengers, which include calcium, lipids, regulatory proteins, and RNA, should be altered by modifications to the structure and location of hyperstructures rather than just to the enzymatic activity of their constituents, as must happen in the extreme case of EF-Tu, where mutations that abolish formation of the hyperstructure—but that leave its activity intact—result in lysis (Mayer 2006).

Locations

The location of transertion and transembly hyperstructures should be changed by the position of the genes on the genome that encode their constituent proteins, by succeeding in selecting new constituents of the hyperstructure, by interfering with transcription or translation. The *lac* operon has been the paradigm for genetic regulation and a Lac hyperstructure might prove to be a similar paradigm for the study of transertion hyperstructures (Kennell and Riezman 1977; Llopis et al. 2010; Norris et al. 2007a) while the ribosomal hyperstructure would be an excellent candidate for the study of transembly hyperstructures (Berger et al. 2010; Cabrera and Jin 2003). In the case of other types of hyperstructure, such as the degradosome or the T3SS, an important proviso here is that nascent proteins be inserted into the hyperstructure at some stage. Another proviso is that the mature hyperstructure be dynamic so that transertion and transembly can still play a role in its location. Indeed, it turns out that inhibiting transcription alters the location of the degradosome as revealed by an RNase E–GFP fusion (Llopis et al. 2010).

Ion condensation

Probes based on the light emitted by aequorin have been used to measure intracellular free calcium in different compartments of *E. coli* (Jones et al. 2002). Such probes might be fused to proteins such as PPK, EF-Tu, H-NS, and certain ribosomal subunits. Depending on the topology of the fusion protein, the probe might measure either condensed calcium in the near region or decondensed calcium leaving the near region. Hyperstructures containing polyphosphate should reveal ion condensation, providing fusions can be made to the protein constituents of these hyperstructures. Finally, calcium condensation on the LCR plasmid could maintain its repressed state and be detectable via fusions of aequorin to histone-like proteins while decondensation from the LCR plasmid leading to a redistribution of calcium to the cytoplasm might be detectable straightforwardly via cytoplasmic aequorin.

Proximity

Relocating the EF-Tu hyperstructure so that it cannot interact readily with the nascent T3SS hyperstructure at the transertion stage should prevent assembly or functioning. Similar relocations of proteolytic hyperstructures and of the degradosome should also affect the T3SS. Such relocating might be achieved by changing the nature of the constituents of these hyperstructures or the position of the genes encoding them (see above). For example, ribosomal protein L4 might be altered so that it either no longer joins the degradosome or binds it tighter.

Conclusion

To address the challenge of determining the nature of interactions at the hyperstructure level in bacteria, there are several candidates on which one might focus (for references, see Norris et al. 2007a). These include DNA supercoiling (Travers and Muskhelishvili 2005), water structure (Vacha et al. 2009; Wiggins 2008), a wide variety of collective oscillations (for example, Matsuhashi et al. 1995; Pelling et al. 2004), ion condensation (Manning 1969), and molecular messengers and locations. We have chosen here to focus on messengers and locations. In the new paradigm of the *hyperstructured* cell, the former are treated as "messenger boys" instructed by hyperstructures while the latter are treated as the locations of the hyperstructures relative to one another. Previously, we have examined the possibility that interactions at the hyperstructure level regulate the cell cycle (Norris 2011; Norris et al. 2002; Norris and Fishov 2001; Rocha et al. 2003). In this paper, we have examined the possibility that interactions between hyperstructures regulate virulence in YP/YPT, and we have shown that such interactions may explain findings on the relationships between RNase E, PNPase, the T3SS, and virulence that would otherwise be difficult to reconcile. That said, our model is far from adequate as, for example, a lack of information means we neglect the likely contribution of the chemotaxis, the PTS, and flagella hyperstructures to hyperstructure interactions (Cabin-Flaman et al. 2005; Lopian et al. 2010; Page and Parsot 2002). Such information may come, of course, from further work on virulence and on the structure of the bacterial cell. It may also come from two-hybrid studies where the concept of a hyperstructure level provides a new interpretation for proteins that have two or more roles in different processes. More specifically,

proteins may be acting as "messenger boys" between hyperstructures or as factors in locating hyperstructures.

If hyperstructure dynamics does indeed determine virulence, the manipulation of such dynamics could have therapeutic implications. This might be achieved by supplying cells with hybrid metabolites, or *hybolites*, made by a pair-wise, covalently linked combination of the thousands of small molecules involved in metabolism. Some of these hybolites would be substrates for two, very different, hyperstructures and might lead to two normally separate hyperstructures being located together or lead to hyperstructures communicating via the "wrong" messenger boys. Such messenger boys include calcium. The ultimate goal of such an approach would be to turn a pathogenic Mr. Hyde into an inoffensive Dr. Jekyll (Legent and Norris 2009).

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