Genetic analysis of coordinate flagellar and type III regulatory circuits in pathogenic bacteria

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Abstract

The bacterial flagellum represents one of the best understood molecular machines. Comprised of 40 parts that self-assemble into a true rotary engine, the biochemistry and genetics of these systems has revealed an unanticipated complexity. An essential component to assembly is the subset of parts that function as a protein secretory pump to ensure and discriminate that the correct number of protein subunits and their order of secretion is precisely regulated during assembly. Of further interest is the recognition of late that a number of important plant and animal pathogens use a related protein secretory pump fused to a membrane-spanning needle-like syringe by which a subset of toxins can be injected into target host cells. Together, the flagellar and virulence protein pumps are referred to as Type III Secretion Systems (TTSS). The archetype for TTSS systems has been the pathogenic members of the genus *Yersinia* which includes the organism responsible for bubonic plague, *Y. pestis*. Our interest in the *Yersinia* centers on the coordinate genetic regulation between flagellum biosynthesis and virulence TTSS expression. *Y. enterocolitica*, for example operates three TTSSs (motility, Ysa, and Yop), but each is expressed under defined mutually exclusive conditions. *Y. pestis* has lost the ability to assemble flagella (the genes are present on the chromosome) and expresses only the Yop system at 37^{o}C, mammalian temperature. Using a combination of microarray analysis, genetic fusions, and behaviors of specific engineered mutants, we demonstrate how environmental factors influence gene expression of these multigene families, where the influence is exerted within each system, and propose why segregating these systems is critical for the organism. Our model

further offers an explanation as to why an important subset of human pathogens has lost motility during their histories.

1 Introduction

The rotary engine comprising the bacterial flagellum has been studied as a model for organelle development and assembly. Approximately 30 genes are involved in coding for its individual parts with another 10 genes regulating expression and assembly and 10 more for sensory perception or chemotaxis. These 50 genes constitute roughly one percent of the *Escherichia coli* or *Salmonella typhimurium* genome, a modest but significant information investment.

The expression of flagellar genes is tightly controlled and regulated in a sequential genetic hierarchy mirroring organelle assembly from the inner membrane to the outer cell surface. As such, the sequence is initiated by the master control operon, *flhDC*, or Class I genes. FlhDC forms a protein tetramer to transcriptionally activate the set of genes comprising the flagellar basal body-hook complex, collectively referred to as Class II genes. This complex includes several ring structures housing the motor, rod proteins equivalent to a drive shaft, and the hook, which acts as a universal joint. Once this complex is assembled, a Class II flagellar-specific RNA polymerase sigma factor, FliA, activates the flagellar Class III genes which include chemotaxis receptors and relays, and the major flagellar component, flagellin, which forms the long filamentous propeller.

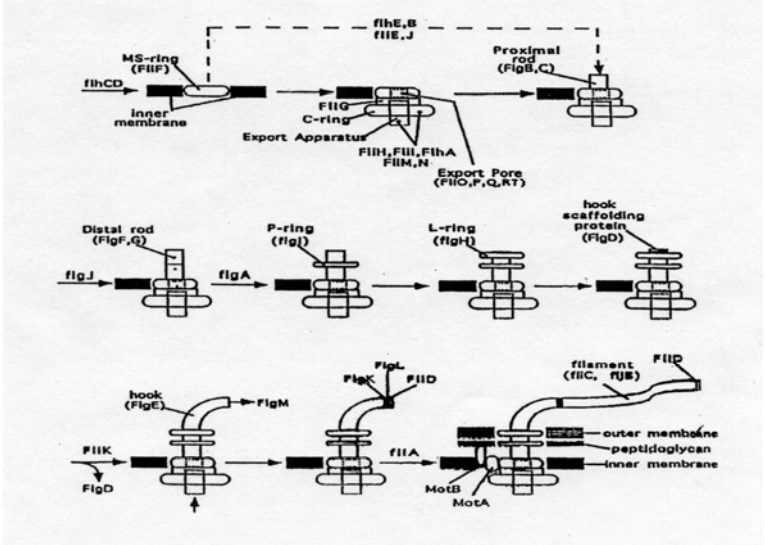


Figure 1. Flagellar gene regulatory cascade and assembly

Flagellin monomers are transported through the hollow core of the basal body hook structure and polymerize at the distal tip. This assembly process (see Figure 1) includes several key check points (feed back loops) that must be satisfied during the expression of each class of genes in the hierarchy or the succeeding sets of genes are transcriptionally arrested. Additionally, a protein complex of approximately 10 factors is positioned at the base of the flagellum to discriminate the order and number of basal body and flagellin components secreted through the flagellar core. This secretory complex is referred to as a type III secretory apparatus (for a review of flagellum synthesis see Macnab [1]).

Viewed as a whole, the flagellum is a true nanomachine of remarkable complexity in structure and assembly control. This macromolecular machine self-assembles and repairs, displays assembly control and processing, operates with two gears, is fueled by proton motive force, and the apparatus is 'hard-wired' to a sensory apparatus that functions on short term memory (chemotaxis). Rotor speeds for *E. coli* are estimated at 17,000 rpm but motors of some marine vibrios have been clocked upwards of 100,000 rpms [2].

The Gram negative genus *Yersinia* includes three human and animal pathogens, *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* (etiologic agent of bubonic plague). Although each species cause a unique disease in the mammalian host, they share the same basic strategy for infection. In part, this strategy relies on a commonly shared set of genes localized on a large plasmid, referred to as pYV (plasmid for *Yersinia* virulence). This common plasmid encodes at least 12 anti-host factors collectively referred to as Yops (for *Yersinia* outer proteins) that are now known to be injected directly into target host cells. All of the Yops are required for full virulence. The structure built to deliver these toxins into mammalian cells is likewise encoded by pYV (termed *ysc* and *lcr* genes). Expression of the Yop, Ysc, and Lcr genes is temperature-dependent. These genes are not transcribed at temperatures of $\leq 30^{o}$ C, but when cells encounter a temperature of 37^{o}C, reflective of mammalian body temperature, their expression is activated. Secretion and injection of Yops is triggered by contact of the delivery system apparatus with a mammalian cell. In vitro expression of *yop ysc* and *lcr* genes is induced at 37^{o}C but Yop secretion and increased production only occurs under limiting calcium ion concentration. In fact, in vitro, *Yersinia* shows calcium-dependent growth, a unique phenotype, and along with temperature, calcium limitation is a key environmental cue used by these organisms [3].

Temperature also affects several other suits of genes in these organisms. These include flagellar synthesis for *Y. enterocolitica*, and *Y. pseudotuberculosis* (*Y. pestis* is nonmotile), outer-membrane proteins, and surface proteins required for invasion of mammalian cells. Of these motility shows the opposite temperature requirements compared to Yop synthesis.

In the early 1990s, several observations intimated the reciprocal temperature requirements of flagellar biosynthesis and Yop expression were more than coincidental effects. In the dissection of the mechanism of temperature regulation, Rohde *et al*. [4] isolated several spontaneous mutants that showed loss of motility and Yop synthesis suggesting regulatory coordination of these

two disparate systems. Ramakrishnan *et .al*. [5], and Sanders *et. al*. [6] also found that the sequence of the *Caulobacter crescentus flhA* flagellar gene showed predicted sequence similarity to pYV-encoded LcrD. Soon thereafter another set of *Caulobacter* flagellar genes showed sequence similarity to additional *ysc* and *lcr* genes [7]. The common denominator for this association identified both sets of genes as required for protein secretion in each process, flagellar assembly or Yop secretion. It was this association that led to the recognition that virulence protein secretion and flagellar protein assembly involved similar mechanisms now classed as type III secretion systems III (TTSS). More recently, yet another TTSS was identified in *Y. enterocolitica*. The function of this system, designated Ysa, is not clearly defined. However it does contribute to virulence and is expressed in vitro under high salt/low temperature conditions; conditions that inhibit the expression of Yop and flagellar biosynthesis [8].

2. Rationale for coordinate segregation of *Yersinia* TTSSs.

Before the separate parallel nature of the flagellar and Yop systems was dissected, our laboratory proposed that the simplest explanation for temperature regulation of these systems was a direct overlap in function. Thus, the flagellum in our view could be used not only as a propulsion machine, but a dedicated highly efficient protein secretory machine for the Yops. Hence, the *Y. enterocolitica* and *Y. pseudotuberculosis* coordinate reciprocal regulation of motility and Yop secretion by temperature was due to alternative secretory function of the flagellar basal body dependent upon habitat.

This hypothesis was testable by the following predictions. First, nonmotile *Y. pestis* would also have to contain a set of flagellar genes, some of which would be expressed. Second, it predicted that both *Y. enterocolitica* and *Y. pseudotuberculosis* would maintain expression of a subset of flagellar genes even at the nonpermissive temperature (37^{o}C) for motility. Finally, it suggested that a subclass of mutants should be defective in both Yop secretion and motility. Using genetic fusion with a reporter gene (lacZ) and northern blot assays, it was determined that at 37^{o}C *Y. enterocolitica* continues to synthesize Class I and Class II flagellar genes. The loss of motility appears to be due to the immediate transcriptional arrest of Class III genes (25-30 fold repression) on exposure to 37^{o}C [9]. A subset set of flagellar gene transposon insertionally inactivated genes were mapped to flagellar genes and also fail to secrete Yops. Finally, *Y. pestis* does contain a complete set of flagellar genes, and using RT PCR, some are transcribed [10].

Whereas all these predictions turned out to be true, subsequent work in a number of laboratories showed that the TTSS and flagellum are in fact separate and parallel systems. Further, it was shown by Kubori and co-workers that the TTSS system of *Salmonella typhimurium* assembles into a 'needle-like' structure, remarkably similar to the basal body of the flagellum. This structure acts as a nanoinjector; its hollow core serves as a specific conduit to export virulence proteins [11]. These structures have since been isolated in a number of organisms including *Yersinia* [12].

Given that the TTSS of the flagellum and virulence systems are separate parallel structures, several factors suggest that the relationship and regulatory parameters of the flagellar and virulence TTSS are still intimately related. Further, as shown below, maintaining the segregational nature of their regulation may be critical for function. A key to this understanding was the observation that TTSS virulence proteins from disparate organisms could be cross recognized for secretion. Schneewind and co-workers [13] showed that TTSS proteins of plant pathogens were recognized and secreted by *Y. enterocolitica* and vice versa. This suggested that the secretory signal of TTS proteins was conserved across genera. Based on this observation, we came full circle back to our original hypothesis of dual function of the flagellar basal body structure. Expression of multiple TTSSs in the same organism by mutually exclusive environmental conditions prevents cross contamination of secreted proteins. That is to say, if flagellum biosynthesis were expressed simultaneously with the Yop TTSS, flagellin monomers may be exported out the 'needle-like' structure as well as the flagellar basal body and vice versa in regard to Yops. Efficiency of both systems would suffer as a consequence.

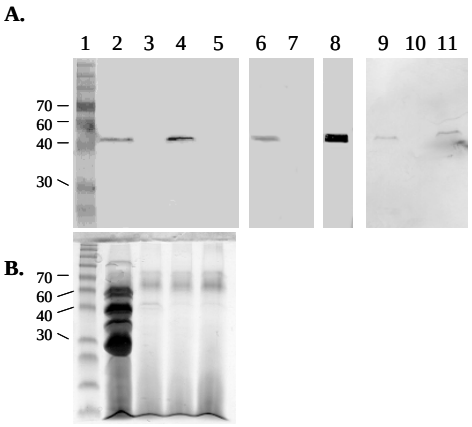
3. Type three secretory systems display promiscuity.

To test the above hypothesis, a flagellin and two Yop genes (*fleB* and, *yopD* and *yopM* respectively) were cloned and fused to the inducible p*tac* promoter. By placing these plasmid constructs in various mutant strains lacking either a flagellum TTSS (*flhD*-, *flhB*-) or Yop TTSS (*yscE*-, pYV-), expression and secretion of FleB, YopM, and YopD could be examined under conditions when these proteins are not normally expressed. For example, p*tac* induction of FleB in an *flhD*- strain at the 37^{o}C and limiting calcium ion concentration (conditions conducive to Yop expression and secretion) permit determination if FleB is exported outside the cell. Expression of FleB in the double mutant strain *flhD*- and pYV- or *ysc*E- serves as a negative control. As can be seen in Figure 2, it was found that FleB is indeed secreted by the Yop TTSS and even the Ysa TTSS (low temperature, high salt). Conversely, it has been shown that YopM is recognized by both the Ysa and flagellar TTSSs. The overall view of *Y. enterocolitica* type III segregation is illustrated in Figure 3.

4. Practical implications

The potential for cross-recognition between type III exported proteins of different systems in the same cell carries several implications. First, these observations explain why segregation of these systems by specific environmental cues is necessary. For example, expression of a flagellum under host conditions would result in loss of polarized secretion of Yop proteins into target host cells. Additionally, display of flagellin to macrophages by direct injection via the Ysc secretin would countermand the anti-inflammatory strategy used by the *Yersinia*. Flagellin is a potent cytokine inducer [14]. Further, because flagellin expression is controlled by such high expression promoters, it also suggests that flagellin, if

expressed, may competitively interfere with virulence protein secretion. Indeed, this latter suggestion may explain why an important subset of major human



	Flagellum	Ysc
1. M.W. Std's		
2. *flh*D::Ω 37ºC - Ca^{2+}	-	+
3. *flh*D::Ω 37ºC + Ca^{2+}	-	-
4. *flh*D::Ω 25ºC (neg. control)	-	-
5. *flh*D::Ω 37ºC -pYV + Ca^{2+}	-	-
6. *flh*B::*lac*ZI 37ºC - Ca^{2+}	-	+
7. *flh*B::*lac*ZI 37ºC + Ca^{2+}	-	-
8. Wild-type 37ºC - Ca^{2+}	-	+

Figure 2. A. Western blot showing extracellular secretion of p*tac*-induced FleB. Lane designations are in the table to the right showing strain genotypes. These results show secretion of FleB occurs only when either the Yop or Ysa TTSS are expressed. B. Stained gel from western blot represented in lanes 1-5 showing Yop secretion.

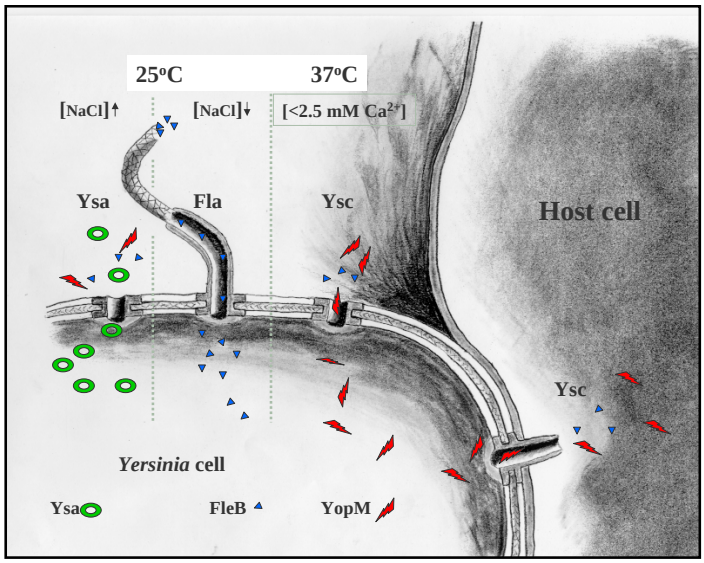


Figure 3: Schematic showing the three segregating environmental

conditions required for Ysa, flagellar
and Yop TTSS expression.

pathogens, including *Y. pestis*, *Shigella* spp., *Bordetella pertussis* and recent isolates of *E. coli* O157:H7, have lost flagellar biosynthetic capacity altogether, even though they have the requisite flagellar genes. Each of these species has mutations in the flagellar master control operon *flhDC*, shutting down the entire flagellar regulon. For example, *Y. pestis* has a single T insertion in *flhD* causing a frameshift mutation. Function of FlhD can be restored by a spontaneous 5 base pair insertion (Smith and Minnich, unpublished observation). Based on these results, we predicted that recent atypical virulent strains of *E. coli* O157:H- (nonmotile) would contain a similar genetic lesion. Indeed, this clonal isolate carriers a 12-base pair in-frame deletion in *flhC* [15]. Further analysis showed this deletion involves a critical amino acid residue for FlhC function. Repair of this lesion restores full motility and H7 antigen expression.

Examination of the *Y. pestis* flagellar system shows there are additional consequences in loss of motility (*flhD*$^-$ phenotype). DNA sequence analysis shows that at least three other deletions are present in flagellar genes. Loss of expression of *flhDC* in this organism renders downstream genes silent and susceptible to further decay and permanent loss due to the improbability of repairing 4 lesions sequentially. Pruss *et. al* [16]. showed that the *E. coli flhDC* operon regulates ca. 30 nonflagellar operons including genes involved with amino acid synthesis and growth under various environmental conditions. Using microarray analysis of *Y. enterocolitica* wild-type and *flhDC*$^-$ mutants, similar results have been observed. *Y. pestis* displays multiple vitamin and amino acid requirements, some of which may be due to loss of *flhC*.

5. Philosophical implications.

To paraphrase the original rendition of the Department of Energy's Genomes to Life web site, 'the molecular machines present in the simplest cells, produced by evolution, dwarf the engineering feats of the 20th century'. The dissection of the complexity and sophistication of simple machines like the bacterial flagellum are indeed a testimony to the power of modern molecular biological techniques. Yet, the elegant structural properties, efficiency, and the highly controlled genetic programming to produce these machines was neither anticipated nor predicted. The potential applications of this knowledge are legion and have spawned a new discipline focused on nanotechnology.

In light of this new information, some scientists have questioned whether the mechanism of mutation, natural selection, and time are sufficient to account for the origin of such machines. Behe [17] has proffered the concept of irreducible complexity using the flagellum as a paradigmatic example. It is this very concept that has been the bread and butter of molecular geneticists allowing them to identify genes in any given system by loss of function. Behe argues that natural selection and random mutation cannot produce the irreducibly complex bacterial flagellar motor with its ca. forty separate protein parts, since the motor

confers no functional advantage on the cell unless all the parts are present. Natural select can preserve the motor once it has been assembled, but it cannot detect anything to preserve until the motor has been assembled and performs a function. If there is no function, there is nothing to select. Given that the flagellum requires ca. 50 genes to function, how did these arise? Contrary to popular belief, we have no detailed account for the evolution of any molecular machine. The data from *Y. pestis* presented here seems to indicate that loss of one constituent in the system leads to the gradual loss of others. For progression to work, each gene product must maintain some function as it is adapted to another.

To counter this argument, particularly as it applies to the flagellum, others have used the TTSS. Since the secretory system that forms part of the flagellar mechanism can also function separately, Miller [18, 19] has argued that natural selection could have "co-opted" the functional parts from the TTTS and other earlier simple systems to produce the flagellar motor. And, indeed, the TTSS contains eight-ten proteins that are also found in the forty protein bacterial flagellar motor. Miller thus regards the virulence secretory pump of the *Yersinia* Yop system as a Darwinian intermediate, case closed.

This argument seems only superficially plausible in light of some of the findings presented in this paper. First, if anything, TTSSs generate more complications than solutions to this question. As shown here, possessing multiple TTSSs causes interference. If not segregated one or both systems are lost. Additionally, the other thirty proteins in the flagellar motor (that are not present in the TTSS) are unique to the motor and are not found in any other living system. From whence, then, were these protein parts co-opted? Also, even if all the protein parts were somehow available to make a flagellar motor during the evolution of life, the parts would need to be assembled in the correct temporal sequence similar to the way an automobile is assembled in factory. Yet, to choreograph the assembly of the parts of the flagellar motor, present-day bacteria need an elaborate system of genetic instructions as well as many other protein machines to time the expression of those assembly instructions. Arguably, this system is itself irreducibly complex. In any case, the co-option argument tacitly presupposes the need for the very thing it seeks to explain—a functionally interdependent system of proteins. Finally, phylogenetic analyses of the gene sequences [20] suggest that flagellar motor proteins arose first and those of the pump came later. In other words, if anything, the pump evolved from the motor, not the motor from the pump.

Molecular machines display a key signature or hallmark of design, namely, irreducible complexity. In all irreducibly complex systems in which the cause of the system is known by experience or observation, intelligent design or engineering played a role the origin of the system. Given that neither standard neo-Darwinism, nor co-option has adequately accounted for the origin of these machines, or the appearance of design that they manifest, one might now consider the design hypothesis as the best explanation for the origin of irreducibly complex systems in living organisms. That we have encountered systems that tax our own capacities as design engineers, justifiably lead us to

question whether these systems are the product of undirected, un-purposed, chance and necessity. Indeed, in any other context we would immediately recognize such systems as the product of very intelligent engineering. Although some may argue this is a merely an argument from ignorance, we regard it as an inference to the best explanation [21, 22], given what we *know* about the powers of intelligent as opposed to strictly natural or material causes.

We know that intelligent designers can and do produce irreducibly complex systems. We find such systems within living organisms. We have good reason to think that these systems defy the creative capacity of the selection/mutation mechanism. The real problem may not be determining the best explanation of the origin of the flagellum. Rather it may be amending the methodological strictures that prevent consideration of the most natural and rational conclusion—albeit one with discomfiting philosophical implications.

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