Transcriptional Regulation in *Yersinia*: an Update

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Abstract
In response to the ever-present need to adapt to environmental stress, bacteria have evolved complex (and often overlapping) regulatory networks that respond to various changes in growth conditions, including entry into the host. The expression of most bacterial virulence factors is regulated; thus the question of how bacteria orchestrate this process has become a recurrent research theme for every bacterial pathogen, and the three pathogenic *Yersinia* are no exception. The earliest studies of regulation in these species were prompted by the characterization of plasmid-encoded virulence determinants, and those conducted since have continued to focus on the principal aspects of virulence in these pathogens. Most *Yersinia* virulence factors are thermally regulated, and are active at either 28°C (the optimal growth temperature) or 37°C (the host temperature). However, regulation by this omnipresent thermal stimulus occurs through a wide variety of mechanisms, which generally act in conjunction with (or are modulated by) additional controls for other environmental cues such as pH, ion concentration, nutrient availability, osmolarity, oxygen tension and DNA damage. *Yersinia*'s recent entry into the genome sequencing era has given scientists the opportunity to study these regulators on a genome-wide basis. This has prompted the first attempts to establish links between the presence or absence of regulatory elements and the three pathogenic species' respective lifestyles and degrees of virulence.

Introduction
Compared to cells of multicellular organisms, microorganisms face a significant additional challenge: they encounter a wide array of sudden, intense and sometimes even life-threatening environmental changes, and must therefore rapidly modify their structure and metabolism accordingly. Although other mechanisms exist, these changes in bacterial physiology mainly occur by regulating the production of the appropriate structural proteins and enzymes. Adaptation of gene expression in response to such situations appears to be essential for bacterial survival, and thus the regulators involved in these processes should be treated as being as important as the effectors themselves. In bacterial pathogens like those of the *Yersinia* genus, most outside-to-inside stress-induced responses lead to changes in the expression of virulence factors. In fact, most of the known *Yersinia* virulence genes are regulated, and elements controlling their expression are thus also virulence factors.

All regulatory systems have a common purpose: to create an interface between the perception of one or several stimuli and to activate or repress expression of their cognate effectors. As we will see by reviewing what is known about *Yersinia*, the means used to regulate the production of a given bacterial factor range from very simple mechanisms (where the DNA-binding properties of the transcriptional regulator are directly altered by the stimulus) to extremely complex systems which sometimes require lengthy signal transduction cascades and/or simultaneous contribution of multiple regulators that may act at different levels – indeed all the way from initiation of gene transcription to protein turn-over. As in most bacteria, *Yersinia* regulatory networks are generally organized hierarchically, with a global regulatory system involving a master regulator such as a sigma factor or a histone-like proteins; and lower-level, downstream-acting secondary regulators that control only a subset of a regulon's genes in response to more specific stress situations. Regulatory circuits may interfere with each other, leading to the discovery of unexpectedly dense, overlapping regulons.

The present review is divided into two parts. The first aims to provide a comprehensive overview of the better-known regulatory systems in *Yersinia* (summarized in Fig. 1). The second part is dedicated to what we can (and cannot) learn from genomic analysis. The genomic sequences *Y. pestis* and *Y. pseudotuberculosis* have recently been released into public databases, and at least one *Y. enterocolitica* sequence will be available in the very near future. This prompts opportunities to search for potentially new genus– or species-specific regulators. What will *Yersinia* genome-wide analysis tell us about regulation?

Part one

The pre-genomic era: what we knew about *Yersinia* regulation

Transcriptional regulation of the pYV plasmid-borne antihost genes
The 70-kb pYV (Yersinia virulence) plasmid is found in all human pathogenic *Yersinia* strains and governs the synthesis of two major virulence factors: the first consists of the various Yop (Yersinia outer membrane protein) effector proteins (YopE, YopH, YopM, YopO/YpkA, YopP/YopJ, YopT) along with the type III secretion system (TTSS) subunits required for their delivery into the eukaryotic cytosol. Yops play a major role during

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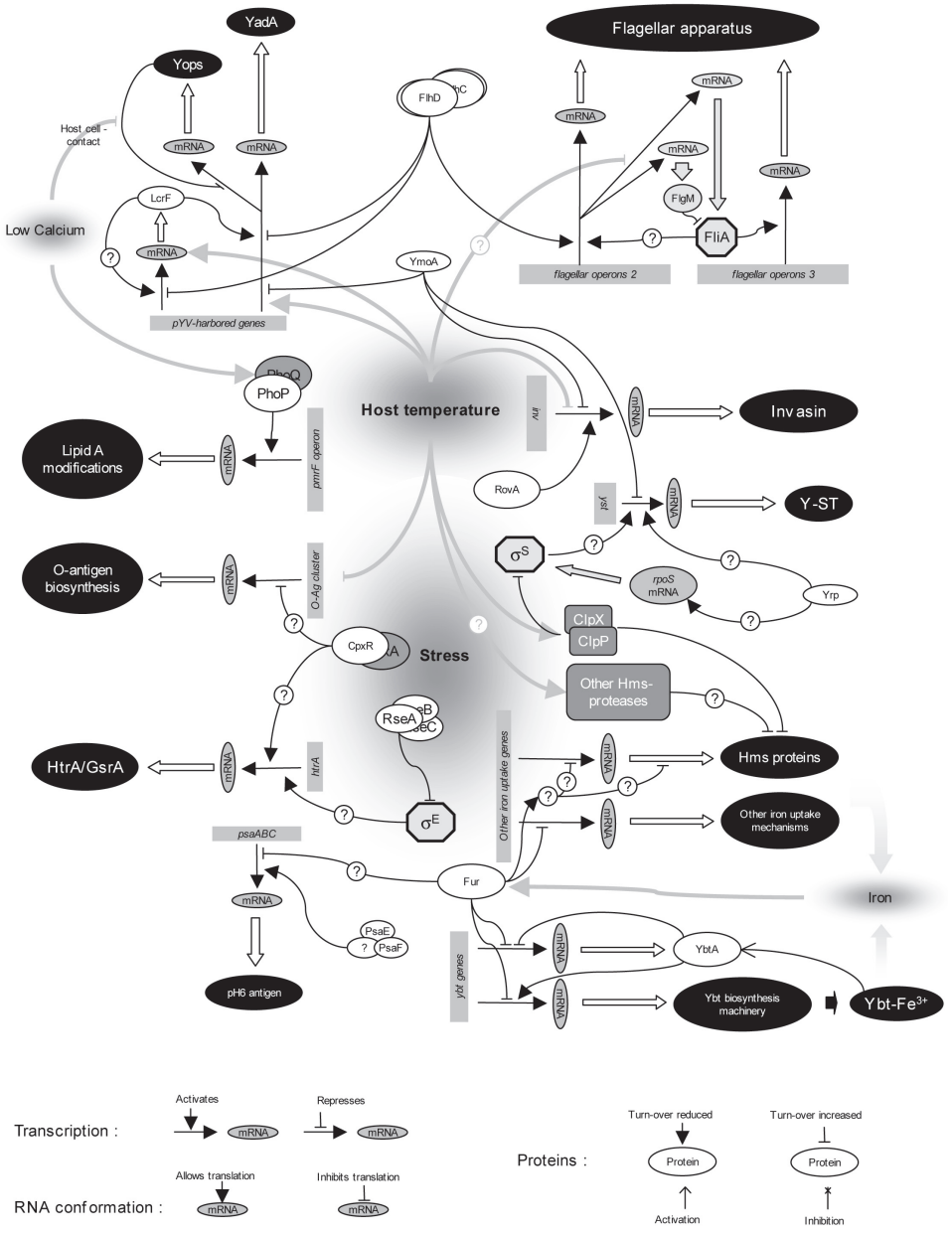

Fig. 1. Overview of regulation networks discussed in this chapter. Question marks have been added for regulation processes either yet uncharacterized or supposed to exist by similarity with other enterobacteria.

the course of *Yersinia* host infection by contributing to phagocyte resistance, triggering macrophage apoptosis and provoking disorders in cytokine release patterns. The second virulence determinant encoded by the pYV plasmid is YadA, which is principally known as a major adhesin involved in bacterial adherence to various eukaryotic extracellular matrix elements. YadA is also involved in resistance to the host's non-specific defences: it protects *Yersinia* from certain antimicrobial peptide classes synthesized by polymorhponuclear leukocytes (PMNs), and also interrupts formation of the Molecular Attack Complex (MAC). It is noteworthy that the *yadA* gene is inactivated in *Y. pestis* but not in the two other pathogenic species. A recent review of YadA has been published by El Tahir and Skurnik (2001).

The genes governing the synthesis of the pYV-encoded virulence factors belong to the same stimulon (the *yop* stimulon), which means they are all upregulated by the same environmental stimulus– an increase in temperature to 37°C upon entry into the mammalian host (Bölin *et al.*, 1988). Some members of the *yop* stimulon are also controlled by an additional stimulus: Ca^{2+} availability. This gene subset is often referred to as the LCRS (Low Calcium Response Stimulon) (Goguen *et al.*, 1984; Straley *et al.*, 1993). Transcriptional control of the Yop-encoding genes is a complex process, and provides

a good example of how non-related stimuli may be taken into account hierarchically by the regulatory system.

Temperature and the overall regulation of pYV-harboured virulence genes

The 8.1 kDa YmoA protein was first characterized by Cornelis *et al.* (1991). Transposon mutagenesis revealed *Y. entrocolitica ymoA* mutants which displayed unusually high *yop* and *yadA* transcription levels at 28°C, suggesting that this protein behaves as a global repressor. The YmoA protein is highly similar (82% identity) to the haemolysin expression modulating protein Hha from *Escherichia coli,* and (at least partial) restoration of haemolysin synthesis in an *hha* mutant by complementation *in trans* with *ymoA* showed that these two molecules share similar functions (Mikulskis and Cornelis, 1994; Balsalobre *et al*., 1996). In light of their amino acid composition, Hha and YmoA were predicted to be histone-like proteins that modulate gene expression through control of DNA topology. Like Hha, YmoA may interact with H-NS (another chromatin-associated protein known to play a role in the thermal modulation of virulence factor expression) and thus form a nucleoid-protein complex responsible for thermoregulation (Nieto *et al.,* 2002). In addition to its effect on pYV-borne genes, the YmoA histone is also involved in the silencing of *yst*, which encodes a thermostable toxin (see below).

However, studies carried out by Lambert de Rouvroit *et al.* (1992) with *yopH::cat* fusions showed that transcription of the *yop* genes is enhanced by temperature upshifts even after inactivation of *ymoA*, suggesting that YmoA modulates rather than regulates transcription of the *yadA* and *yop* genes, and that other means of thermoregulation might exist. Novobiocin is a compound that affects DNA superhelicity by interacting with gyrases. The fact that Yop genes were still expressed by wild type *Y. enterocolitica* at 30°C in the presence of sub-inhibitory levels of this drug (i.e., the *ymoA* mutant phenotype) argues in favour of mechanisms based on DNA conformation (Rohde *et al.,* 1994). Consistent with this hypothesis is the demonstration that thermo-induced Yop expression coincided with variations of pYV DNA supercoiling (Rohde *et al.,* 1994). Five years later, the same authors showed that these dramatic topological changes could be initiated by the melting of local DNA bends as a consequence of a temperature shift from 30°C to 37°C (Rohde *et al.,* 1999).

However, in contrast to the *ysc* genes involved in the synthesis of the Yop secretion apparatus itself, transcription of most *yop*s– as well as other pYV-borne genes such as *ylpA yadA, sycE* (Skurnik and Toivanen, 1992; Wattiau and Cornelis, 1993) and the *virC* operon (Michiels *et al*., 1991)– has been shown to require an additional regulator: LcrF (Yother *et al.,* 1986; Lambert de Rouvroit *et al.,* 1992). The 30.9-kDa *Y. enterocolitica* LcrF -VirF (Cornelis *et al*., 1989) transcriptional activator belongs to the AraC family: it contains two adjacent C-terminal Helix-Turn-Helix (H-T-H) DNA binding motifs. The *lcrF* gene is pYV-borne and, as with the other Yop and Ysc encoding genes, its transcription is induced at 37°C following changes in DNA superhelicity (Cornelis *et al.,* 1989). Additionally, translation of LcrF can be enhanced– at least in *Y. pestis*– by the melting of heat-unstable mRNA secondary structures at the Ribosome Binding Site (RBS) (Hoe and Goguen, 1993). The VirF-binding region deduced from footprinting assays is 13-bp long, with the following consensus sequence: TTTTaGYcTtTat (where nucleotides conserved in 60% or more of the sequences are in uppercase letters, and y indicates C or T) (Wattiau and Cornelis, 1994). Yop promoters are expressed constitutively by *ymoA virF* double mutants. Accordingly, *yop* expression has been found to be VirF-independent in *ymoA* mutants (Lambert de Rouvroit *et al.,* 1992). These observations suggest that the role of VirF may be to counteract the negative effects of YmoA. However, the fact that *yop* expression is still enhanced by temperature upshifts (Lambert de Rouvroit *et al*., 1992) suggests the involvement of other thermoregulation mechanisms.

From low calcium levels to yop *transcriptional regulation*

During infection, the *Yersinia* pYV-encoded type III secretion machinery is activated by a succession of environmental signals. The initial cue is a temperature upshift to 37°C (the host-entry signal), which promotes transcription of all Yop and Ysc proteins. Other possible signals encountered within the host may be required for the complete assembly of the secretion apparatus (Lee *et al*., 2001). The final signal that triggers the translocation of the Yops *in vivo* is contact with the host cell (Rosqvist *et al*., 1994; Sory and Cornelis, 1994; Pettersson *et al*., 1996). Biosynthesis and activation of the Yop machinery can be induced *in vitro* by incubating bacteria at 37°C in calcium-poor media (i.e., a calcium concentration lower than 80 μM). This experimentally induced response is often referred to as the Low Calcium Response (LCR). However, Ca^{2+} concentrations are dramatically lower inside the target cell cytoplasm than outside. Thus, as suggested by Lee *et al*. (2001), low calcium may also be the real triggering stimulus sensed by *Yersinia in vivo*. In the presence of millimolar calcium concentrations, the type III secretion apparatus remains blocked, and Yops are not secreted (Forsberg *et al*., 1991; Yother and Goguen, 1985). Furthermore, transcription of genes governing Yop synthesis is repressed (Cornelis *et al*., 1987; Forsberg and Wolf-Watz, 1988; Straley *et al*., 1993). Despite its name, LcrF alone cannot account for this downregulation, since some genes of its regulon, including YadA, are not elements of the LCR Stimulon (Skurnik and Toivanen, 1992). This clearly indicates that temperature and calcium concentrations regulate Yop expression via two independent systems.

How then does calcium regulate the LCR stimulon? Genes encoding the Yop effectors are only actively transcribed when their respective products are not present in high amounts in the bacterial cytoplasm. This is typically the case in calcium deprivation, when Yops are expelled from the bacterium via the Ysc secretion apparatus. Calcium-dependent blockage of Yop secretion depends on a set of at least three TTSS subunits: LcrG, the channel gatekeeper (Skrzypek and Straley, 1993), TyeA, required for polarized delivery of Yop effectors (Iriarte *et al.,* 1998) and YopN/LcrE, the cell contact sensor (Forsberg *et al*., 1991). Mutations inactivating any of these proteins will result in the massive leakage of the Yop effectors from the bacterium and the subsequent

derepression of the *yop* transcription, regardless of the calcium concentration (Boland *et al*., 1996; Cheng and Schneewind, 2000; Forsberg *et al*., 1991; Iriarte *et al*., 1998; Skryzpek and Straley, 1993; Yother and Goguen, 1985).

How does the amount of Yops regulate yop transcription? In *Y. pseudotuberculosis*, another subunit, LcrQ, is co-injected into the target cell along with the Yop effectors (Cambronne *et al*., 2000). In *Y. enterocolitica,* two LrcQ counterparts, called YscM1 and YscM2, have been identified (Stainier *et al*., 1997). LcrQ is a negative regulator of the LCR stimulon. Its accumulation in the bacterial cytoplasm, resulting from blockage of the secretion apparatus, is thought to be the first step in the LCR stimulon downregulation cascade (Pettersson *et al*., 1996). Other Yop subunits, in particular YopD and LcrH (Williams and Straley, 1998; Francis *et al*., 2001) have been shown to be involved in this process.

Regulation of *yop* transcription by this kind of negative feedback system allows advance synthesis and cytoplasmic storage of ready-to-use Yop molecules prior to cell contact. In the absence of such a system, neosynthesized effectors (i.e. produced upon contact with macrophages) would never be available on time to prevent phagocytosis.

Yops and flagella are both temperature-regulated in *Yersinia*. However, the former are produced at 37°C but not the latter, and the opposite situation is observed at 28°C. Interestingly, LcrD, of the Yop secretion machinery, shares some structural similarity with elements involved in the flagellar apparatus assembly. Identification *in silico* of a putative binding site upstream of *lcrD* suggested that σ^{28} (an alternative sigma factor also called FliA and which controls flagellum assembly– see below), may be involved in these temperature-induced, physiological modifications. Studies performed by Iriarte *et al.* (1995c) showed that FliA as such is not involved in Yop regulation. However, these results do not rule out the possible contribution of an as yet unknown, FliA-like, global regulator. In accordance with these observations and the regulation of other flagellar regulatory system, FlhDC may well play such a role, as discussed below.

Yst biosynthesis: the extreme complexity of its regulation by a sigma factor

Y. enterocolitica synthesises and secretes an enterotoxin (Pai and Mors, 1978; Delor *et al*., 1990) which affects the digestive tract of its mammalian hosts by causing an overproduction of cyclic GMP within the intestinal epithelial cells (Robins-Browne *et al*., 1979). In light of its significant similarity to the heat stable *E. coli* ST-I toxin, this chromosome-encoded molecule was originally called Y-ST but is currently referred to as Y-STa or Y-STb, depending on the subtype (Ramamurthy *et al*., 1997). Transcription of *yst*, the Y-ST-encoding gene, is growth phase-regulated and is influenced by environmental cues such as pH, osmolarity and temperature (Mikulskis *et al*., 1994). However, with the discovery of the sigma factor RpoS (also known as σ^S, σ^{38} or katF) as one of the *yst* regulators (Iriarte *et al*., 1995b), it has become increasingly evident that control of *yst* expression may be one of the most complex regulatory systems ever encountered in bacteria. RpoS regulation has been the subject of intense study, especially in *E. coli* and pseudomonads. The idea that *yst* can be expressed in a σ^S-dependent manner arose from the observation that Y-ST synthesis was initially considered as stationary phase-specific and that its promoter region contains strong, σ^S-recognized consensus motifs (Iriarte *et al*., 1995b). However, direct regulation of *yst* transcription by this sigma factor has not been experimentally demonstrated.

Although first described as a stationary phase-specific regulator, it is now recognized that RpoS's influence extends beyond the stationary phase-related response. In light of what is currently known, RpoS should rather be considered as a global stress-response regulator required for tolerance of a variety of potentially lethal conditions, such as hyperosmolarity, temperature shocks, oxidative and UV stresses, most of the time in conjunction with other regulatory systems. The extreme complexity of RpoS-based control also stems from the fact that its own expression is finely controlled at each possible regulation level: transcription, translation, and turnover of *rpoS* mRNA and RpoS protein (for a recent comprehensive review see Hengge-Aronis, 2002).

rpoS transcription may increase approximately 5- to 10-fold during the stationary phase (Lange and Hengge-Aronis, 1991). In *E. coli*, *rpoS* transcriptional upregulation has been shown to result (at least partially) from interferences with previously characterized regulatory networks. These include the catabolite repression system, in which the CRP-cAMP complex negatively regulates *rpoS* transcription (Lange and Hengge-Aronis*,* 1991; 1994), and the BarA two-component sensor-kinase- phosphotransferase system. In *E. coli*, the latter acts positively (Mukhopadhyay *et al*., 2000) but probably independently of its only known cognate response regulator, UvrY (also referred to as YecB). Accumulation of ppGpp (Lange *et al*., 1995) and polyphosphate (Shiba *et al*., 1997) may also trigger *rpoS* transcription, although the mechanisms by which these compounds induce transcription are poorly understood. Comparison between the *E. coli* and *Pseudomonas* models (reviewed in Venturi, 2003) strongly suggests the existence of core *rpoS* transcription regulators. However, some other regulators controling *rpoS* transcription appear to be optional with regards to the bacterial species, possibly as the result of the adaptation to divergent lifestyles. *rpoS* transcriptional regulation has not so far been studied in the *Yersinia* genus. Translation of *E. coli rpoS* mRNA is both osmolarity- and temperature-dependent. The most likely site of *rpoS* translation regulation is a very long mRNA leader sequence (approximately 560 bp between the transcript start and the AUG codon) suspected of forming defined secondary structures (Cunning *et al*., 1998). The switch of this transcript from an inactive to an active state may occur through stabilization of this region in a translationally competent conformation that provides access to the ribosome binding sites. As demonstrated by a series of studies performed in *E. coli*, this *cis*-regulation results from complex interplays between several *trans*-acting elements, some of which act positively, like the Hfq (HF-1) RNA-binding protein, the nucleoid HU protein and DsrA, a low temperature-induced small RNA (Brown

and Elliott, 1997; Lease *et al*., 1998). In contrast, other elements have been shown to exert an opposite effect. For example, the *oxyS* gene transcript may inhibit *rpoS* translation by binding HF-1 and sequestering this molecule from *rpoS* leader RNA (Zhang *et al*., 1998). The H-NS histone-like protein has also been reported to antagonize *rpoS* mRNA translation (Yamashino *et al*., 1995).

Recent studies conducted in *Y. enterocolitica* are consistent with this model: Yrp, the recently characterized HF-1 (Hfq) counterpart in *Yersinia* (also referred to as Ymr in certain databases) has been shown to control *yst* expression at the transcriptional level (Nakao *et al*., 1995). In line with these results, it was hypothesized that Yrp may exert its effect though the control of DNA topology (Nakao *et al*., 1995). With regard to the *E. coli* model, it is also possible that this control may occur though regulation of *rpoS*.

Reducing protein turnover by preventing proteolysis is a very efficient means of promoting regulator accumulation. According to studies performed in *E. coli*, RpoS has a very short half-life (less than 2 min) during the exponential phase (Lange *et al*., 1994), due to rapid degradation by the cytoplasmic ATP-dependent ClpXP protease complex; this is not the case during the stationary phase, where the half-life can achieve values of over 30 min (Schweder *et al*., 1996). The ClpXP-catalysed degradation of RpoS depends on a two-component system response regulator, RssB, also called MviA in *Salmonella* (Bearson *et al*., 1996) and referred to or identified as Hnr by the *Yersinia* genome annotation groups. Unlike most two-component systems, this regulator does not appear to directly regulate gene expression, but promotes σ^S degradation by directly and specifically interacting with a domain of the RpoS protein (Muffler *et al*., 1996; Pratt and Silhavy, 1996). Accordingly, RssB's regulation of proteolysis does not extend to any other ClpXP substrate. The RssB cognate sensor has not been characterized yet in any bacterial species, suggesting that RssB may be regulated by another two-component system sensor kinase.

In *Y. enterocolitica*, RpoS is not essential for virulence in the murine model (Badger and Miller, 1995), in contrast to what has been observed in *Salmonella* (Nickerson and Curtiss, 1997). However, it has been shown that RpoS is required (only at the 37°C host temperature) for adaptation to at least some of the environmental stresses mentioned above (Badger and Miller, 1995). In agreement with these findings, growth phase regulation of the *yst* gene was found to be thermo-dependent, and once again YmoA (the negative histone-like regulator of the yop regulon, see above) seems to play a critical role in this process (Mikulskis *et al*., 1994). It is noteworthy that RpoS does not regulate *yop* transcription (Iriarte *et al*., 1995b). Y-ST host tissue-specific expression (most likely in the ileum) may thus result principally from the complex interplay between the RpoS and YmoA global regulators. To date, the role of RpoS in the virulence of *Y. pestis* and *Y. pseudotuberculosis* has not been analyzed.

The Yersinia *pH6 antigen: regulated by a ToxRST-like system?*

In order to produce *E. coli* Pap-like fimbrial adhesins termed Psa, *Y. pseudotuberculosis* and *Y. pestis* must be grown at 34°C or higher, consistent with their contribution to virulence (Lindler *et al*., 1990; Yang *et al*., 1996). These fimbrial adhesins are termed "Psa" (pH Six Antigen) due to the fact that, in addition to the temperature requirement, maximal expression of these appendages is obtained at pH 6. Psa biosynthesis requires two neighbouring gene clusters. The first (*psaABC*) encodes the structural subunit (A), along with its chaperone (B) and membrane usher (C); whereas the second (*psaEF*) is required for the transcriptional regulation of *psaA* (and possibly other genes) (Lindler *et al*., 1990; Yang *et al*., 1996; Yang and Isberg, 1997). Two similar genetic clusters displaying identical organisation and functions have been identified in *Y. enterocolitica* and designated *myfABC* and *myfEF* (Iriarte and Cornelis, 1995a). The 24-kDa PsaE and 18.5-kDa PsaF proteins are respectively 52% and 54% identical to their *Y. enterocolitica* counterparts, MyfE and MyfF (Iriarte and Cornelis, 1995a). Both elements are constitutively expressed and are essential for *psaA/myfA* transcription in the three pathogenic *Yersinia species*: no *psaA/myfA* mRNA could be detected in cultures following mutation of either of these two elements, even during growth in highly permissive conditions, unless *psaA* was under control of a constitutive promoter (Yang and Isberg, 1997). Surprisingly, these positive regulatory elements do not exhibit any obvious DNA-binding motifs. However, based on topological predictions and the results of fusions with *phoA*, it has been hypothesized that PsaE/MyfE and PsaF/MyfF may be functionally similar to ToxR and ToxS respectively, i.e. two of the three elements required for transcriptional activation of the *V. cholereae tcp* (toxin co-regulated pili) operon, and that their regulation may be similar (Yang and Isberg, 1997). The ways in which temperature and pH may influence this regulatory system are still unknown. In addition, it has been recently reported that the *psaEF* operon is possibly regulated at the transcription level by Fur (Panina *et al*., 2001a; see below).

Iron homeostasis systems: global and specific regulation

Iron – an essential cofactor for many enzymatic processes – plays a vital role in most living species. Iron sources and availability vary from one environment to another: this metallic ion exists as insoluble ferric (Fe^{3+}) iron hydroxides in aerobic conditions, soluble ferrous iron (Fe^{2+}) in anaerobic environments or complexed with iron-binding molecules (siderophores) within the host (reviewed in Weinberg, 1978). Thus, bacterial pathogens display a wide arsenal of uptake systems adapted to these various iron sources, some of which are encoded by pathogenicity islands. Although numerous, these mechanisms are not as redundant as they first appear, and some experimental evidence encourages the belief that to ensure optimal iron uptake, bacteria preferentially activate (via specific regulatory systems) the most appropriate mechanism for their environment. In contrast, iron overload can be deleterious for the bacterial cell, leading to the accumulation of strongly oxidizing hydroxyl radicals that damage DNA and provoke cell death (Halliwell and Gutteridge, 1984). Hence, all iron uptake mechanisms are ultimately repressed by iron. This global

downregulation involves the Fur repressor, a regulator with an unexpectedly wide potential sphere of influence.

Fur: more than just an iron uptake regulator

Fur (for Ferric Uptake Regulator) plays a central role (Staggs and Perry, 1991; 1992; Staggs *et al*., 1994) in directly or indirectly regulating the expression of most of the genes involved in iron metabolism (for general reviews, see Crosa, 1997; Crosa and and Walsh 2002; Escolar *et al*., 1999), although separate, Fur-independent iron regulatory system may exist in *Yersinia*. The 17-kDa Fur protein is a Fe^{2+}-dependent transcriptional repressor. When cytoplasmic Fe^{2+} is in excess, two overlapping dimers of Fur binds to the operator sequence ("Fur boxes" or "iron boxes") of iron-repressible (*irp*) genes, including *fur* itself. In contrast, in the absence of this micronutrient, the Fe(II)-free Fur aporepressor is released from DNA, leading to gene derepression. Fur exhibits two major differences from classical substrate-binding repressors, and thus it has been speculated that Fur may be more than just a transcriptional regulator. Firstly, the amount of Fur is more than a hundred times higher than typical repressor levels: in *E. coli*, around 5,000 copies of Fur per cell may be achieved in normal growing conditions, and up to 10,000 following oxidative stress (Zheng *et al*., 1999). Secondly, the 19-bp Fur box is unusually long, i.e. 7 bp more than the box recognized by classical regulators containing helix-turn-helix motifs (Harrison *et al*., 1990). The 19-bp minimal consensus sequence (5'-GATAATGATAATCATTATC-3') has been shown to consist of a 5'-GATAAT-3' hexamer tandem repeat followed by a third hexamer in the opposite orientation (F-F-x-R configuration) (De Lorenzo *et al*., 1987). Some Fur boxes may contain additional hexamers, but these motifs make minor contributions to the Fur-DNA interaction (Escolar *et al*., 1998; 1999). Each dimer binds a 13 nucleotide-spanning region on opposite faces of the helix, with fewer phosphate contacts than observed for classical regulators (Baichoo and Helmann, 2002).

Based on experimental and/or computational evidence, all the iron scavenging systems in *Yersinia* studied to date have been shown to be Fur-controlled: the Hmu/Hem (Thompson *et al*., 1999) and *Serratia*-like Has haemophore-dependent heme acquisition machineries (Rossi *et al*., 2001), the siderophore-dependent yersiniabactin (Ybt) inorganic iron transport system, the YfeABCD iron and manganese uptake system (Bearden *et al*., 1998; Bearden and Perry, 1999), and the *yfuABC* operon-encoded iron transporters (Saken *et al*., 2000).

In the last few years, genome-wide computational analyses and the use of biochemical and genetic techniques have revealed a number of potentially Fur-controlled genes, showing that the initial size of the Fur regulon was probably underestimated. In other bacteria, a systematic search for Fur-controlled genes has revealed that this molecule may also regulate physiological functions that go beyond iron uptake. In *E. coli*, for example, Fur has been shown to directly control the expression of SodA and SodB, two oxidative stress-combative superoxide dismutases, and *fur* has also been shown to be controlled by *oxyS* in this species, demonstrating that Fur also contributes to protection against oxidative damage and mutagenesis (Zhang *et al*., 1998). Fur may also exert (mainly indirect) negative or positive control of a broad range of cellular processes, such as acid shock and redox-stress responses, chemotaxis, metabolic pathways (e.g. glycolysis and TCA cycle) and the production of toxins and virulence factors (McHugh *et al.,* 2003). In *Y. pestis*, Fur is suspected of directly controlling expression of the pH6 antigen at the transcriptional level (Panina *et al*., 2001). Given this recent information, it is tempting to consider Fur as a global regulator, rather than just an iron uptake-specific transcription factor, that controls other aspects of bacterial metabolism besides extracellular iron availability.

How to favour use of the most appropriate iron uptake system: the ybtA lesson

Upon iron starvation, the three pathogenic *Yersinia* species release a high-affinity iron-binding compound called yersiniabactin (Ybt), which captures ferric iron from the environment. The resulting iron-siderophore complex is then transported back in to the bacterial cytosol in a TonB-dependent manner via a specific surface receptor, termed FyuA in *Y.enterocolitica* and Psn in the two other pathogenic species. *psn/fyuA* and the other genes required for yersiniabactin biosynthesis and secretion are located on a 36 to 43- kb chromosomal region within the unstable *pgm* locus. This region is known as the High-Pathogenicity Island (HPI) and is required for full virulence in highly pathogenic isolates of *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. It has been shown to be essential during the early stages of infection in the mouse model. Like most other iron uptake systems in *Yersinia*, it is Fur-controlled (see Carniel, 2001 for a review).

As with other siderophores, like pyochelin and pyoverdin produced by pseudomonads (Crosa, 1997), yersiniabactin plays a regulatory role by enhancing its own synthesis along with that of its Psn/FyuA receptor. This activation occurs via binding to an HPI-encoded 36–kDa transcriptional activator, YbtA (Fetherston *et al*., 1996), a member of the AraC/XylS family which contain two adjacent C-terminal Helix-Turn-Helix (H-T-H) DNA binding motifs. Like AraC and PchR (the latter being the YbtA homologue for the uptake and synthesis of pyochelin in *P. aeruginosa*), YbtA inhibits transcription of its own gene. However, unlike these two regulators, YbtA does not seem to act as a repressor in the absence of its cognate ligand. In addition to *ybtA* itself, YbtA-binding DNA inverted repeats have been identified immediately upstream from the –35 box of *psn/fyuA*, the *ybtPQXS* operon and the Ybt biosynthesis gene *irp2*. (Fetherston *et al*., 1996; Bearden *et al*., 1997; Fetherston *et al*., 1999). YbtA is thought to bind to these sequences as a dimer and to positively regulate their transcription. Conversely, it has also been shown that the *psn/fyuA*, *ybtA, irp2* and *ybtPQXS* operons are repressed by Fur (Carniel *et al*., 1992; Gehring *et al*., 1998; Fetherston *et al*., 1996; Staggs *et al*., 1994; Panina *et al*., 2001).

Although its free form is thought to exhibit residual activity, YbtA must bind yersiniabactin to be fully active (Fetherston *et al*., 1996). In the bacterial cytosol, the only yersiniabactin source comes from the Ybt-Fe^{3+} recovered from the surrounding medium. Thus, the Ybt iron-uptake

system will be maximally active when Fe^{3+} is available in the surrounding medium and there is no cytoplasmic iron overload, but will be less active in the presence of iron sources other than Fe^{3+}. In other words, by this kind of positive feedback, the yersiniabactin system will be most efficient when best suited to the iron source.

Yersinia *iron uptake systems: are they differentially expressed?*

In pseudomonads, production of exogenous siderophore receptors is selectively upregulated by cognate ligands in the environment, mostly via processes requiring extracellular sigma factors (Poole *et al*., 2003). It has been suggested that the *Y. pestis* Yfe and Ybt systems function during different stages of the infectious process in bubonic plague (Bearden and Perry, 1999). Thus, the various iron uptake systems available in one given *Yersinia* cell may be differentially regulated according to the iron source available in the environment, with preferential expression of the most suitable systems at the expense of the others. In an attempt to verify this hypothesis, Jacobi *et al.* (2001) used translational fusions with reporter genes to monitor the expression of *yfuA* and *hemR* (encoding the Fe^{3+}-Yersiniabactin and haem receptors respectively) in *Y. enterocolitica* during its course of infection in the murine model. Expression of these two genes was found to fluctuate from one organ to another, with the highest expression levels in the peritoneal cavity and the lowest in the intestinal lumen and liver. However, because identical variations were observed for both *yfuA* and *hemR,* these genes may be coordinately regulated. Nevertheless, the anticipated environment-driven, differential expression of *Yersinia* iron-uptake systems still awaits direct experimental confirmation.

Haemin storage: its unusual regulation by temperature and iron

The haemin absorption system, also known as the haemin storage system or Hms (Carniel *et al*., 1989; Fetherston *et al*., 1992), is essential for the flea-mediated transmisison of *Y. pestis* but does not contribute to the pathogenesis of bubonic plague in mammals (Lillard *et al*., 1999). At least two haemin storage loci (*hmsHFRS* and *hmsT*) enable *Yersinia pestis* to accumulate haemin, ferric iron and Congo Red in a process that is only active at temperatures lower than 34°C (Perry *et al*., 1990; Lillard *et al*., 1999). Interestingly, as recently shown by Perry *et al*. (2004), thermal control of the Hms+ phenotype is unlikely to depend on transcriptional regulation for at least two reasons. Firstly, levels of mRNA transcribed from both loci do not change significantly with growth temperature. Secondly, levels of the HmsH, F, R and S proteins seem to result from independent regulation processes – whereas HmsH, and HmsR protein levels appear to decrease upon temperature upshifts, levels of HmsF and HmsS do not change, even though their genes belong to the same polycistronic unit. This second observation also suggests that translational control is unlikely (this is their hypothesis, it has not yet been demonstrated). Temperature-regulated expression of the Hms^+ phenotype may rather depend on differential lability of the Hms proteins to proteases (Perry *et al*., 2004). Except in the case of HmsT, which is degraded by the Lon, ClpXP, and/or ClpAP complexes at 37°C, candidate proteases have not been identified yet.

Earlier studies had shown that the Fur repressor was an essential actor in the regulation of the Hms phenotype, since *fur* mutants were found to be constitutively Hms+, (Hms^c, i.e. formation of red colonies even at 37°C on CR agar) (Staggs *et al*., 1994). The detection of a putative upstream Fur binding site for *hmsT* but not for *hmsHFRS* strongly suggested that the Hms phenotype could be regulated by Fur through the expression of HmsT (Jones *et al*., 1999). Given the recent finding that *hmsT* transcription is not regulated by temperature (Perry *et al*., 2004), the mechanism for Fur control of the Hms+ phenotype still needs to be elucidated.

Flagellar biosynthesis: a highly hierarchical regulatory system

Y. enterocolitica and *Y. pseudotuberculosis* are flagellated and motile, whereas *Y. pestis* is not. Type III flagella are critical (at least in *Y. enterocolitica*) for swarming motility, migration and adherence to host cells (Young *et al*., 2000). However, in *Y. enterocolitica*, flagella are synthesized at 30°C or below but not at the host temperature, strongly suggesting that their contribution may not last beyond the early stages of infection. Biosynthesis of the bacterial flagellum requires more than 40 genes, including those necessary for flagellar rotation and those encoding the chemosensory apparatus. *Yersinia* flagellar operons (also called motility operons) display significant homologies and similar arrangements to those of *E. coli* and *Salmonella*, and even though there is often no direct experimental evidence, it is commonly presumed that flagellar synthesis in *Yersinia* is identical in most aspects to the paradigm established from studies in these two species. Flagellar operons (sometimes referred to as flagellar regulons) fall into three classes which are expressed in a hierarchical manner: expression of the class 2 operons required for basal rod and hook assembly depends on products encoded by the unique class 1 operon, *flhDC* (also known as the master operon). In turn, transcription of the class 3 genes required for i) biogenesis of the filament and motor torque generator, and ii) motility and chemotaxis regulation, depends on the complete and correct assembly of the class 2 subunits: mutants lacking these components do not express the subunits needed for the later stages. In addition to assembly monitoring, expression of motility genes may be further regulated in response to environmental signals. The key transcriptional regulators involved in this complex processes are the products of the master flhD/flhC regulatory operon and the FliA/FlgM sigma/anti-sigma factors (Chilcott and Hughes, 2000; Shapiro, 1995, for review).

The *flhDC* operon (the sole class I operon) is at the top of the regulatory cascade and is thus required for expression of all the class 2 and class 3 genes in the flagellar regulon as well as its own transcription (see below). Mutations within *flhDC* completely abolish swimming and swarming motilities. FlhD and FlhC form a heterotetrameric (C2D2) complex in which FlhC may act as an allosteric activator of FlhD, the DNA-binding subunit (Campos and Matsumura, 2001). Very recent reports strongly suggest that this regulatory complex is not only a

motility-specific activator (as initially thought) but probably also a global regulator. In *E. coli*, the heterotetrameric complex has been shown to regulate flagellum-unrelated physiological functions, such as membrane transport, respiration, sugar metabolism and other enzymatic processes (Pruss *et al*., 2001; 2003). In pathogens like *Proteus mirabilis* and the insect pathogen *Xenorhabdus nematophilu*s, amongst others, it may control various virulence-associated phenotypes including invasion and production of proteases, haemolysins and phospholipases (Fraser *et al*., 2002; Givaudan *et al*., 2000).

This operon has also been designated *flhDC* in *Yersinia*, because of its high degree of identity to the master operons of other Gram-negative bacteria and because of its similar contributions to regulation of flagellar biosynthesis (Young *et al*., 1999a). As in some other enterobacterial pathogens, the operon has been shown to modulate the expression of other virulence factors, either (i) associated with flagella synthesis, like YplA, a phospholipase that requires the type III flagellum to be secreted (Young *et al*., 1999b; Young and Young, 2002) and is considered to form part of the flagellar regulon; or (ii) unrelated to flagella synthesis, like the Yops and their secretion apparatus (Bleves *et al*., 2002). Recent results demonstrate that transcription of the flagellar master operon is also environmentally controlled. In *Salmonella and E. coli*, it has been shown that expression of the *flhDC* operon is controlled by a wide array of regulatory systems, including the catabolite repression cascade via cAMP-CRP, the histone-like H-NS protein, and at least two signal transduction systems (also referred to as two-component systems): OmpR-EnvZ (Shin and Park, 1995) and RscC-RscB-RscA-YojN (Francez-Charlot *et al*., 2003). Additionally, flagellar biosynthesis has been seen to depend on cell density via two pairs of LuxR/I-type quorum sensing homologues (Atkinson *et al*., 1999).

FliA and FlgM

FliA, also known as sigma 28 (σ^{28}), is required for the master regulon-dependent expression of most class 3 operons, although at least two of them (*flg*KL and *fli*DS) may also be directly activated by FlhD-FlhC (Bartlett *et al*., 1988). FliA-dependent operons are only expressed upon complete and correct assembly of the class 2 gene products (Hughes *et al*., 1993). How then can the bacterial cell sense completion of the hook-basal body intermediate structure? The exact mechanism of this phenomenon remained obscure until FlgM, the FliA-cognate anti-sigma factor, was characterized (Ohnishi *et al*., 1992). As already shown in several enterobacteria, *flgM* mutants exhibit high transcription levels of the class 3 operons. Conversely, cytosolic accumulation of FlgM leads to class 3 gene silencing (Hughes *et al*., 1993). Thus, completion of the flagellar apparatus requires low FlgM levels. FlgM is normally secreted from the bacterial cell upon assembly of functional hooks and basal rods. If these latter structures are not functional for any reason, the anti-σ^{28} factor accumulates within the cytoplasm, blocking the later steps of flagellar synthesis by inactivating FliA (Gillen and Hughes, 1991; Hughes *et al*., 1993). *fliA* and *flgM* have been recently characterized in *Y. enterocolitica* and shown (by functional complementation) to exhibit similar properties with regard to counterparts previously studied in other enterobacterial species (Kapatral *et al*., 1996). Interestingly, neither *fliA* nor *flgM* (most likely because FliA might control the expression of at least some class 2 genes) is transcriptionally active at 37°C and, consequently, most class 3 operons remain silent (Kapatral and Minnich, 1995; Kapatral *et al*., 1996), arguing for the presence of flagella only during the very first steps of host infection. However, unlike the FlhC-FlhD complex, it is currently believed that FliA has no impact on the expression of virulence factors other than those associated with flagella– including the flagella themselves and YplA (Schmiel *et al*., 2000)– and is not involved in the temperature-sensitive regulation of the pYV-harboured genes (Iriarte *et al*., 1995c). Despite high structural similarity among enterobacterial flagella, slight differences exist: The fact that flagella are expressed at 37° in *Salmonella* but not in *Yersinia* is probably the best example. In agreement with this observation is the finding that a *Y. enterocolitica flgM* mutant is fully virulent but *Salmonella flgM* mutants display attenuated virulence, probably due to the abnormal expression level of FliC flagellin (Schmitt *et al*., 1994; 1996).

Chemotaxis regulation

Swimming behaviour of bacteria such as *E. coli* and *Salmonella* depends on the direction of flagellar rotation: the flagellar apparatus fluctuates between clockwise rotation (causing jerky movements known as tumbling motility) and counter-clockwise rotation (associated with straight swimming). The signal transduction system that mediates bacterial chemotaxis allows cells to modify the frequency of transition between these two states as a function of the environmental conditions (for review, see Macnab, 1996). In most cases, the absence of environmental input signals is sensed at the periplasmic level by specific, inner-membrane anchored receptors and is then transmitted to the chemotaxis regulation apparatus by the activation (via methylation) of membrane-spanning molecules called MCPs (for Methyl-accepting Chemotaxis Proteins). MCPs are often associated with several receptors, and may thus function as specific intermediates for several environmental cues. In some cases, they may be directly regulated by the environmental stimulus itself. In *E. coli*, the cytoplasmic chemotaxis regulation apparatus *per se* is composed of 6 subunits: CheA, CheB, CheR CheW, CheY and CheZ. Each MCP may assemble with two of these subunits (CheA and CheW), and activation occurs through conformational changes in this ternary complex (Gegner *et al*., 1992; Ninfa *et al*., 1991). CheA functions as a histidine-kinase which, once activated, phosphorylates CheY. In turn, this latter subunit binds to FliM, which belongs to the flagellar motor/switch complex (consisting of the three proteins FliG, FliM, and FliN), forcing a rotation change from counter-clockwise to clockwise and thus causing tumbling motility. Conversely, the sensing of an attractant stimulus will abrogate signal transduction and facilitate swimming behaviour. The high switch frequency is facilitated by controlling the methylation of MCPs by CheR (positive) and CheB (negative) and the dephosphorylation of CheY by CheZ (Hess *et al*., 1988). A complete chemotaxis system, including the six Che signal

transduction proteins, 8 MCP and the three flagellar motor/switch complex subunits was found in the three pathogenic *Yersinia* species (Deng *et al*., 2002; Hinchliffe *et al*., 2003; and results obtained from *Y. enterocolitica* genome BLAST searches – http://www.sanger.ac.uk). All six Che subunit-encoding genes share 70 to 90% identity with their respective *E. coli* and *Salmonella* counterparts. In constrast, FliG, FliM, and FliN amino-acid sequences were found to be much less conserved. Whether and how this evolution of the flagellar motor/switch complex might impact *Yersinia* chemotaxis remains to be assessed.

F1 antigen regulation

Expression of the *Y. pestis* antiphagocytic capsule, also known as F1 antigen (for review, see Perry and Fetherston, 1997), requires four genes harboured by the *Y. pestis*-specific 110kb pFra virulence plasmid. The first three are clustered in the *caf* operon and encode the molecular chaperone (Caf1M), the outer membrane anchor (Caf1A) and the F1 structural subunit (Caf1). The fourth gene, encoding the Caf1R AraC family regulatory protein, is located just upstream of, but in opposite orientation, to the *caf* operon, suggesting that both the regulator-encoding gene and the *caf* operon may have a common operating region. Expression of the *caf* operon is thermally sensitive, as evidenced by an increase in Caf1A levels following a temperature shift from 28°C to 37°C (Karlyshev *et al*., 1992). Caf1R is apparently required for this process. However, whether induction by temperature occurs through Caf1R has not yet been investigated. Recently, it has been proposed that Caf1A may be associated with a *Y. pestis*-specific galactolipid (Feodorova and Devdariani, 2001). This moiety can also be extracted from pFra-less strains, suggesting that its biosynthesis depends on as yet unidentified, chromosome-harboured genes. Like CafA1, higher amounts of this galactolipid are recovered from bacteria grown at 37°C than at 28°C, raising the possibility that synthesis of these two capsular compounds may be similarly regulated.

The pleiotropic PhoP–PhoQ regulatory system

Calcium (Ca^{2+}) and magnesium (Mg^{2+}) are essential for stabilizing the negatively-charged lipopolysaccharide (LPS) in the outer membrane. Hence, reduced availability of these two cations may be considered as a source of stress for the bacterial cell. The maintenance of LPS integrity under these conditions requires the PhoP–PhoQ two-component system (Groisman *et al*., 1997; Guo *et al*., 1997). PhoP–PhoQ responds not only to external Mg^{2+} and Ca^{2+} ion concentrations but also to Mn^{2+} (García Véscovi *et al*., 1996; Soncini *et al*., 1996). As in most two-component systems, activation of PhoP requires autophosphorylation of PhoQ upon Ca^{2+}/Mg^{2+} deprivation and subsequent phosphoryl transfer to PhoP. The phospho-PhoP response regulator switches from an inactive to active state, and its binding to DNA then promotes both transcription of PhoP-activated genes (*pag*s) – including the *phoPQ* operon itself (Soncini *et al*., 1995) – and repression of PhoP-repressed genes (*prg*s). Conversely, when bound to Mg^{2+} and/or Ca^{2+}, PhoQ is able to inactivate phospho-PhoP by dephosphorylation. As with almost all two-component systems, the means by which the sensor recognizes the response regulator is still poorly understood.

In *Salmonella enterica* serovar Typhimurium, PhoP–PhoQ has been shown to control the transcription of a wide array of unlinked genes that contribute to various modifications of cellular physiology. It became rapidly apparent that some of these were essential for survival inside the host, since *Salmonella phoP* mutants were found to show highly attenuated virulence in mice (Miller *et al*., 1989; Galán and Curtiss, 1989). In this species, the most intensively studied PhoP–PhoQ controlled phenotype is the ability to modify the lipid A moiety of LPS by performing at least two substitutions: one with palmitate (catalysed by a palmitoyl transferase termed PagP (Bishop *et al*., 2000) and a second with 4-amino-arabinose, through the upregulation of the 7-ORF *pmrHFIGKLM* operon, also referred to as *pmrF* (Gunn *et al*., 1998). This latter modification contributes to a decrease in the net negative charge of LPS, thus promoting stabilization of the outer membrane during calcium and magnesium starvation (Groisman *et al*., 1997). Both modifications have been shown to promote *Salmonella*'s resistance to a broad range of cationic antimicrobial peptides (essential components of the innate immune response) and facilitate the pathogen's survival within acidified macrophage phagosomes (Guo *et al*., 1998; Baker *et al*., 1999).

Operons encoding *Salmonella* PhoP and PhoQ orthologues have been identified in the three pathogenic *Yersinia* species. Recent studies have shown that the *Y. pestis* PhoP–PhoQ system is involved in infection of mice challenged by subcutaneous injection: although a *Y. pestis phoP* mutant was still virulent, its LD50 increased by 75 fold. PhoP was also shown to contribute to intra-macrophage survival– though to a lesser extent than in *Salmonella*, since *Yersiniae* mostly remain extracellular during infection (Oyston *et al*., 2000; Grabenstein *et al*., 2004). Accordingly, tolerance of the three pathogenic *Yersinia* species to antimicrobial peptides was found to depend (at least partially) on elements of the PhoP–PhoQ regulons, such as the *pmrF* operon (Marceau *et al*., 2004). In *Y. pestis*, PhoP–PhoQ may also control the production of an alternative lipo-oligosaccharide (LOS) form containing terminal galactose instead of heptose (Hitchen *et al*. 2002).

Over the past decade, the central role of the PhoP–PhoQ two-component system in virulence regulation has become increasingly clear in many bacterial pathogens. In *Salmonella*, more than forty genes have been shown to fall into the PhoP regulon (Miller and Mekalanos, 1990), and the first results from 2D protein gel analyses strongly suggest the existence of a regulon (including PhoP-activated and -repressed genes) of at least equal size in *Y. pestis* (Oyston *et al*., 2000). A subset of this regulon may be further regulated by temperature.

RpoE: regulation of virulence by outer membrane stress-response systems

Extracytoplasmic function (ECF) sigma factors (a subgroup of the σ^{70} family) regulate a range of physiological processes, including envelope homeostasis, folding, assembly and degradation of Outer Membrane Proteins (OMPs), in response to envelope-damaging environmental

stresses (for general reviews of ECF sigma factors and their regulation, see Helmann, 2002; Raivio and Silhavy, 2001).

In contrast to the general heat shock transcription factor σ^{32} which directly senses the misfolding of cytoplasmic proteins, the activity of the ECF sigma factor depends on one or several other signal transducing proteins called anti-sigma factors. The term anti-sigma arose from the fact that, in non-inducing conditions, these molecules complex with their cognate sigma factor and maintain it in an inactive form (for review, see Helmann, 1999). Of these envelope stress responsive systems, σ^E (also referred to as RpoE) and its cognate anti-sigma factors are, by far, the most intensively studied. In *E. coli*, the activity of σ^E is tightly controlled by two negative regulators encoded by the *rpoErseABC* operon (Missiakas and Raina., 1997). The first of these (RseA, a regulator of σ^E), spans the inner membrane and is referred to as the anti-sigma factor itself, in light of its cytoplasmic N terminal σ^E-binding domain. RseA is probably necessary and sufficient for downregulating the activity of σ^E. The second regulator (RseB) also exerts a negative effect on σ^E, possibly upon sensing misfolded OMPs. However, its periplasmic location and its affinity for the C-terminal periplasmic domain of RseA suggest that this molecule is more likely to stabilize the σ^E-RseA complex than regulate σ^E by distinct means. RseC is encoded by the last gene of the *rpoErseABC* operon and regulates σ^E activity in a positive manner. The respective roles and modes of action of RcsB and RcsC remain to be clarified.

In a wide range of living organisms, the σ^E-dependent stress response includes the synthesis of a periplasmic chaperone/heat shock serine protease called DegP and also known as HtrA, for high temperature requirement A. Inactivation of *htrA* leads to a decrease in tolerance to high temperatures (i.e. exceeding 39°C) and osmotic and oxidative stresses. Additionally, pathogens such as *Brucella*, *Salmonella*, and *Legionella*, also need this enzyme for survival within macrophage phagosomes (for review, see Pallen and Wren, 1997; Pedersen *et al*., 2001). The 49.5-kDa GsrA (global stress requirement) protein is the *Yersinia* counterpart of HtrA (Wren *et al*., 1995; Yamamoto *et al*, 1996). *Y. enterocolitica gsrA* mutants display similar virulence phenotypes as those observed for *Brucella* and *Salmonella* (Li *et al*., 1996; Elzer *et al*., 1996; Chatfield *et al*., 1992). Contrasting with this result, a *Y. pestis htrA* mutant was attenuated and exhibited increased sensitivity to oxidative stress, but to a much lesser extent than seen for mutants of the three above-mentioned species (Williams *et al*., 2000). HtrA/GsrA is most likely present in *Y.pseudotuberculosis*, but how it contributes to these phenotypes has not been reported so far. Transcription levels of *gsrA* and *rpoE* were found to be significantly increased following pathogen uptake by macrophages, suggesting that GsrA is induced (probably by σ^E as judged by the presence of a specific binding motif) in response to stresses encountered within phagosomes (Yamamoto *et al*., 1996; 1997). The discovery of the three anti-sigma orthologues in *Yersinia* suggest that GsrA and HtrA homologues found in other bacterial species may have identical regulation processes.

In *E. coli*, the HtrA-mediated protective response also depends on an overlapping regulation mechanism involving the CpxA-CpxR two-component system (Missiakas and Raina, 1997). CpxA-CpxR has been described for its ability (among others) to monitor and, if necessay, downregulate the synthesis of P-pili, as a consequence of an excess of misfolded Pap subunits (Jones *et al*., 1997; Hung *et al*., 2001). The CpxA sensor and its cognate regulator have been identified in the three pathogenic *Yersinia* species. However, whether they contribute identically to this physiological process has not been demonstrated.

O-Antigen synthesis: also regulated by a stress response system

O-Antigen, the outermost part of the LPS, is required for the full virulence of *Y. pseudotuberculosis* and *Y. enterocolitica*, in contrast to *Y. pestis*, which is unable to produce this moiety. In *Yersinia*, expression of the O-Antigen is repressed at 37°C (Al-hendy *et al*., 1991; Bengoechea *et al*., 2002). Although this fact has been known for over ten years, the first steps towards characterization of the mechanisms behind *Yersinia* O-antigen biosynthesis thermoregulation cascade were only initiated very recently. Studies performed with *Y. enterocolitica* serotype O:8 indicate that control of O-antigen synthesis involves finely balanced regulatory mechanisms at both the transcriptional and post-transcriptional levels, and is also associated with stress-response systems (Bengoechea *et al*., 2002). Promoter cloning experiments reveal that a promoter, termed P*wb1*, drives the transcription of most of the O-antigen biosynthetic genes; whereas a second promoter, named P*wb2*, controls the last two genes of the O-antigen gene cluster, *gne* and *wzz*. The activity of both promoters is temperature regulated and is repressed at 37°C. A molecular analysis of the loci involved in O-antigen regulation demonstrated that Wzz avalability and the activity of the thermo-inducible RosAB system (Bengoechea *et al*., 2000) are key elements in the regulatory network. RosA and RosB are an inner membrane efflux pump and a K+ antiporter respectively, i.e. functions which argue against direct regulation based on interaction with DNA. This inconsistency could be explained by a mechanism whereby RosA and RosB indirectly repress the transcription of *gne* and *wzz* by modifying H+ and K+ intracellular levels, similarly to what has been observed for transcription of their own genes.

Even though overexpression of *wzz* causes an increase in O-antigen expression at 37°C, this upregulation does not result from the activation of P*wb1* and P*wb2*. This observation initially prompted researchers to suppose that Wzz played its regulatory role at a post-transcriptional level, probably by interfering with the O-antigen assembly and export machinery. However, it has since been shown that Wzz downregulates the transcription of *rosA* and *rosB*. In other words, the less Wzz is produced, the more transcription of its gene is repressed (Bengoechea *et al*., 2002). This regulatory circuit may thus explain the almost complete abolishment of O-antigen synthesis at 37°C.

How Wzz downregulates *rosA* and *rosB* transcription is still unknown. However, recent data suggest that this could occur via the envelope stress-combative CpxA-

CpxR two component system, with CpxA possibly sensing outer-membrane disorders resulting from an excess of Wzz. The detection of potential binding motifs in the P*wb1*, P*wb2* and *rosAB* promoter region may suggest direct transcriptional regulation by CpxR, the CpxA cognate response regulator (Bengoechea *et al*., 2002).

Regulation of invasin expression.

Invasin – produced only by the two enteropathogenic species – is one of the most studied *Yersinia* virulence factors, along with the Yops. However, in contrast to the Yops, little was known about the regulation of the invasin gene (*inv*) until recently. Invasin expression is thermoregulated (Isberg *et al*., 1988) but differs from the other host temperature-induced *Yersinia* adhesins (like YadA, Ail or the newly characterized YAPI encoded type IV pilus) in being poorly expressed at 37°C, pH8. A *Y. enterocolitica inv* mutant displays delayed Peyer's patch-colonization, but no change in LD_{50} for mice (Pepe and Miller, 1993). It was therefore initially proposed that this adhesin contributed only to an acceleration of the early stages of host infection. However, two pieces of evidence suggested that regulation of *inv* transcription during infection may well depend on stimuli other than temperature (Pepe *et al*., 1994). Firstly, invasin can still be detected in murine Peyer's patches two days after oral challenge with *Y. enterocolitica*. Secondly, its expression can be modified *in vitro* in response to several environmental cues other than temperature that may be encountered in the host, such as mildly acidic pH, nutrient availability, growth phase and oxidizing and osmotic stresses. In view of these environmental cues, it was initially thought that the sigma factor RpoS might be a key regulator of invasin expression. However, experimental evidence has ruled out this preliminary hypothesis (Badger *et al*., 1995).

The transcriptional regulator RovA (regulator of virulence) was characterized in *Y. enterocolitica* and *Y. pseudotuberculosis* by two different groups using opposite approaches (Revell *et al*., 2000; Nagel *et al*., 2001). It was shown to be essential for production of high invasin levels *in vitro* in both species. RovA belongs to the MarR family (which mostly contains non-specific, antibiotic resistance regulators), and is comparable in size (with around 75% identity at the amino acid level) to the *Salmonella typhimurium* pleiotropic transcriptional regulator SlyA. Potential RovA/SlyA orthologues have also been identified in a wide range of bacterial species. Based on recent structural studies performed with SlyA, RovA may contain a winged-helix DNA-binding domain (i.e. two helixes separated by a glycine-rich hinge region) and may function as a dimer (Wu *et al*., 2003). In *Y. pseudotuberculosis*, it has been proposed that RovA binds with unequal affinities to two similar palindromic motifs within the *inv* promoter region and that it positively regulates transcription of this gene in response to low temperature, mild acidic pH, and growth in stationary phase. This environment-dependent activation is thought to occur mainly through post-transcriptional control of RovA biosynthesis (Nagel *et al*., 2001). It can be enhanced by auto-activation, since RovA also promotes the transcription of its own gene. In contrast to the results obtained in the invasin studies, the *Y. enterocolitica rovA* mutant displays a 70 to 500-fold increase in the LD_{50} mice (depending on the mouse lineage) when compared to its wild type counterpart (Revell *et al*., 2000; Dube *et al*., 2003). RovA may thus be a pleiotropic regulator and, control one or several as yet unknown virulence factors in addition to invasin. Interestingly, such a dramatic virulence decrease was not observed when mice where challenged by routes bypassing the Peyer's patches (Dube *et al*., 2003). This strongly suggests that these yet unkonwn members of the RovA regulon may be required to improve survival within these tissues.

Other studies suggest that other *inv* regulators exist: Tn*5* insertions in *sspA* or *uvrC* caused a significant decrease in invasin expression, whereas inactivation of these two genes had the opposite effect on flagellin transcription – strongly suggesting the existence of mechanisms regulating both *inv* expression and flagella biosynthesis (Badger *et al*., 1998). Recently, expression of *slyA* has been shown to be PhoP-controlled in *Salmonella typhimurium* (Norte *et al*., 2003).

Part two

The post-genomic era: what might we learn from *Yersinia* genome sequences?

The field of molecular biology has changed dramatically over the last ten years. In particular, advances in DNA sequencing have provided data with ever-increasing accuracy and speed. Genome sequence analyses of several *Yersinia pestis* strains (CO92 – biovar Orientalis- and KIM -biovar Medievalis- are the first ones) and *Y. pseudotuberculosis* strain IP32953 have recently been completed (Parkhill *et al*, 2001; Deng *et al*., 2002; Chain *et al*., 2004), and the release of the and *Y. enterocolitica* sequence is imminent. Within the next decade, genome-wide analyses and derived experimental techniques will undoubtedly provide important clues to the two following fundamental and recurrent questions: how do pathogenic Yersiniae cause disease, and how did *Y. pestis* diverge so rapidly from *Y. pseudotuberculosis* (switching from an environmental enteropathogenic lifestyle to a host-dependent, septicaemic lifestyle in less than 20,000 years)? Whole genome sequences and associated annotation are prerequisites for the assessment of genome-wide transcript profiling, and also offer unprecedented opportunities for opening up new fields of investigation in gene regulation. This will enable research strategies to move from conventional regulator hunting (i.e. starting from the effector genes and trying to characterize their regulators) towards systematic searching for the cognate target regulons on the basis of each identified transcriptional regulator. There is no doubt that comparison of the huge amount of new data being generated by the use of these new technologies with our current physiological knowledge will lead to a wealth of discoveries – providing new insights into how pathogenic *Yersinia* regulate the expression of their virulence gene arsenal and how these mechanisms may differ from those in other pathogenic bacteria.

At this point in time, what can we already learn in terms of regulation from the currently available *Yersinia* genome sequences, from comparison with the genomes

of other enterobacteria and from genomic divergences within the pathogenic *Yersinia* themselves?

Yersinia *regulators: an overview*

Genome-wide screening in *Y. pestis* has revealed the presence of approximately 250 transcriptional regulators (including sigma and anti-sigma factors, two component systems and histone-like molecules), which is probably slightly less than the estimated number of regulators in *E. coli* K12 (reviewed by Perez-Rueda and Collado-Vides, 2000). Not surprisingly, less than half of them (the 79 listed in Table 1) have an assignable function, based on either experimental evidence and/or high similarity (i.e. >50% identity over the length of the whole molecule: the high stringency of this criteria being justified by the fact that some non-orthologue regulators exhibit around 30% baseline identity due to the presence of highly conserved domains). The remaining regulatory elements (except for obvious phage-related transcriptional regulators YPO0878, YPO1904, YPO2785, YPO2823, YPO3485, YPO3612 and YPO4031) are listed in Table 2 and fall into two categories: firstly, those which have been previously identified in other bacterial species but have an as yet uncharacterised function (interestingly, several of these display the highest similarities with regulators found in *Photorhabdus luminescens*, an insect pathogen); and secondly, those identified as transcriptional regulators because they contain canonical DNA binding motifs but are not highly similar to regulators currently found in protein databases. Among these latter molecules, some have no known counterparts other than in *Yersinia*, and will therefore be of great interest for deciphering potentially new aspects of *Yersinia*-specific physiology.

Sigma and anti-sigma factors

The bacterial DNA-dependent, RNA polymerase contains five core enzyme structural subunits that associate with sigma factors to provide transcription specificity. In *E. coli*, seven such molecular species have been identified and extensively studied: σ^D (sigma 70), the four alternative sigma factors (σ^N, σ^S, σ^H and σ^F, also referred to as FliA), the extracellular function regulating (ECF) σ^E, and FecI (a fur-repressed regulator of ferric citrate (Fec) transport system (Angerer *et al*., 1995). All these factors except FecI have been identified in *Y. pseudotuberculosis* and in the two *Y. pestis* genomes. However, there are no extra sigma factors compared to *E. coli*, although two copies of FliA have been identified, consistent with the existence of two distinct flagellar apparatuses. In addition to the FliA and σ^E cognate anti-sigma factors (FlgM and RseA respectively), eight sigma factor modulating proteins have been identified in *Y. pestis*: one for σ^D, another possibly modulating at least one of the two FliAs, two for σ^E (discussed above) and three possible σ^N modulators (one of which is a pseudogene in *Y. pestis* but not in *Y. pseudotuberculosis*). One last element (YPO3571/y0142/YPTB3515) has also been considered as a sigma factor regulator by the *Yersinia* genome annotation teams, in light of its weak homology with RsbV, an agonist of the σ^B alternative sigma factor that controls the general stress response in Gram-positive bacteria, but its role and potential cognate sigma factor remain unknown.

Two-component systems: trying to reassemble the puzzle

In bacteria, the most rapid and efficient means of transcriptional adaptation to extracellular signals occur through sophisticated and powerful systems based on phospho-transfers between conserved transmitter domains of (generally transmembrane) molecules that sense the input signal and the receiver regions of cytoplasmic, regulatory elements which exhibit DNA-binding properties in most cases. These sensor kinases and response regulators are generally arranged in cognate pairs, referred to as two-component systems (TCSs). In *E. coli* K12, 62 TCS-subunits have been identified by genome-wide scanning. 32 are response regulators and 23 are canonical sensor kinases with one histidine kinase (HK) domain; the remaining seven are also sensory kinases but ones that display more complex structures, i.e., containing additional phosphotransfer (HPt) or response regulator (RR) modules, or both (Mizuno, 1997). At least 26 sensors (including 7 hybrid molecular species) and 29 molecular species harboring response-regulator modules have been identified in *Y. pestis* CO92, resulting in 24 possible complete TCSs. More than half of these (16 sensors and 20 response regulators) are highly similar (i.e. >60% identity at the amino acid level, for the same reasons as mentioned above: non-orthologue TCS subunits can exhibit around 40% identity) to elements of previously well-documented systems in other bacteria; they are thus expected to have identical functions in *Yersinia*. On this basis and building on the fact that two partner subunit-encoding genes are often closely linked, 13 of these TCSs can be reconstructed *in silico* with a fair degree of confidence (Table 3a). Four other systems (UhpB-UhpA, RcsC-RcsB/A, PmrB-PmrA and ZraS-ZraR) may be assembled according to the same criteria, although in these cases either the sensor or the regulator may exhibit more marked differences (<60% identity) from their putative counterparts in protein databases (Table 3b). In some cases, functional assignation may be further facilitated by the detection of additional peptide signatures at consistent positions. The presence of a conserved ExxxE motif (reported as binding Fe^{3+}) in PmrB (Wosten *et al*., 2000) perfectly illustrates this point. ZraS and ZraR (previously referred to as HydH and HydG) were found only in *Y. pseudotuberculosis*[1] and in partially sequenced strains of the *Y. pestis* biovar Antiqua (Radnedge *et al*., 2002). It is noteworthy that *Yersinia* ZraS differs from its *E. coli* counterpart by an insertion of over one hundred amino acids containing a putative PAS domain, which is found in many TCS sensor subunits (for review, see Taylor and Zhulin, 1999). Two other systems (listed in Table 3e) may be deduced from further computational analyses. The first consists of the NarX sensor and the NarP regulator. In other enterobacteria, these elements are part of distinct TCSs (NarX-NarL and NarQ-NarP), both of which reportedly contribute to regulation of nitrate/nitrite metabolism (Stewart, 1994). The fact that NarQ and NarL are absent from the *Yersinia* genomes and that NarX can cross-activate NarP in *E. coli* strongly

Table 1. CO92 transcriptional regulators with assigned functions

CO92 ID	Name	Regulated function	Possible regulon found in *Yersinia*	Function in Yersinia?	Relevant DB entry
YPO0002	AsnC	Amino acid metabolism	*asnA, gidA*	By sim.	P03809
YPO0046	Ttk	Resistance to antibiotics and detergents?	??	By sim.	P06969
YPO0072	Ada	Bifunctional: regulatory/DNA repair	??	By sim.	P06134
YPO0108	CytR	Catabolizing enzymes	*deoCA*BD, udp, cdd, nupC, nupG**…	By sim.	P06964
YPO0114	MetJ	Met and Sam Biosynthesis	*met* regulon	By sim.	P08338
YPO0120	GlpR	Glycerol-3-phosphate metabolism	*glpEGR*	By sim.	P09392
YPO0123	MalT	Maltose regulon.	Maltose regulon	By sim.	P06993
YPO0175	Crp	Catabolic repression	Pleitropic regulation	By sim.	P06170
YPO0236	ZntR	Zn(II)-responsive regulator	*zntA*	By sim.	P36676
YPO0314	LexA	SOS system	SOS regulon	By sim.	P03033
YPO0315	Zur?	Zinc uptake	*znuACB*	By sim.	P32692
YPO0332	RhaS	L-rhamnose metabolism	*rha* genes	By sim.	P27029
YPO0333	RhaR	L-rhamnose metabolism	*rha* genes	By sim.	P09378
YPO0373	**Yrp**	***Yersinia* multiple regulator**	***yst*, in *Y. enterocolitica***	**Nakao *et al*., 1995**	**P25521**
YPO0444	NadR	Transcriptional regulator NadR	*nadA*, *nadB*, *pncB*	By sim.	P24518
YPO0453	TrpR	Trp operon repressor	*trp* operon	By sim.	P03032
YPO0471	NhaR	Na+/H+ antiporter system	*nha*A	By sim.	P10087
YPO0535	LeuO	LysR-family transcriptional regulator LeuO	*leuABCD*	By sim.	P46924
YPO0543	FruR	Putative fructose repressor	*fruAKB*	By sim.	P21168
YPO0576	ExuR	Hexuronate utilization repressor	*exuT*, *uxaCA, uxuR*, *uxuA* and *uxuB*	By sim.	P42608
YPO0795	GalR	Galactose operon repressor	gal regulon (*mgl*)	By sim.	P03024
YPO0797	LysR	Diaminopimelate decarboxylase.	*lysA*	By sim.	P03030
YPO0985	**YspR**	**Quorum-sensing regulator**	**Flagellar regulon**	**Atkinson *et al*., 1999**	**O87971**
YPO1029	GcvA	Glycine cleavage (activator)	*gcv* operon	By sim.	P32064
YPO1167	BetI	Choline-glycine betaine pathway	*betABT*	By sim.	P17446
YPO1279	ExuR	Sugar interconversion regulator	*uxuR, uxuA and uxuB*	By sim.	P39161
YPO1301	**PsaE**	**Psa type pili regulatory protein**	***psaABC***	**Yang *et al*., 1997.**	**P31524**
YPO1308	**RscR**	**Possible role in virulence**	**See Nelson *et al*. 2001**	**Young and Miller, 1997**	AAK81923
YPO1322	DeoR	Nucleotide and deoxynucleotide catabolism	*deoCA*BD, udp, cdd, nupC, nupG**…	By sim.	P06217
YPO1375	Lrp	Mediates a global response to leucine.	*ilvIH* operon and others	By sim.	P19494
YPO1642	CscR	Sucrose utilization	*csc* operon (not found)	By sim.	P40715
(YPO1662)	**FlhD**	**Flagellum biosynthesis**	**Flagellar regulon**	**Young *et al*., 1999a**	**P11164**
YPO1663	**FlhC**	**Flagellum biosynthesis**	**Flagellar regulon**	**Young *et al*., 1999a**	**P11165**
YPO1714	KdgR	Pectinolysis and pectinase secretion.	*kdgK*, *kdgT*	By sim.	P37728
YPO1760	HpcR	Homoprotocatechuate degradation	*hpaBC* and *hpaGEDFHI*	By sim.	Q07095
YPO1857	WrbA	Possible Trp repressor binding protein	*trp* operon	By sim.	P30849
YPO1912	**YbtA**	**Yersiniabactin synthesis**	**Ybt biosynthesis genes**	**Fetherston *et al*., 1996.**	**T17438**
YPO1973	HutC	Histidine utilization	*hut* operon	By sim.	P22773
YPO2065	HexR	Hex regulon repressor.	*zwf*, *eda*,*glp*…	By sim.	P46118
YPO2144	FadR	Fatty acid metabolism.	*fadA, fadB, fadD*,*fadL* and *fadE*	By sim.	P09371
YPO2175	**Hns**	**Pleiotropic**	**Pleitropic regulation**	**Bertin *et al*., 2001**	**P08936**
YPO2219	CysB	Biosynthesis of L-cysteine	*cys* regulon	By sim.	P06613
YPO2258	AraC	Arabinose operon regulatory protein	Arabinose operon	By sim.	P07642
YPO2268	Mlc	Glucose uptake or glycolysis	?	By sim.	P50456
YPO2300	Fnr	Fumarate and nitrate reduction	Global regulation of over 100 genes	By sim.	P03019
YPO2344	TyrR	Aromatic amino acid biosynthesis	8 operons in *E.coli*	By sim.	P07604

Table 1. Continued

CO92 ID	Name	Regulated function	Possible regulon found in *Yersinia*	Function in Yersinia?	Relevant DB entry
YPO2352	**PspF**	**Phage shock protein F**	***pspA*, *B*, *C*, *E***	**Darwin and Miller, 2001**	**P37344**
YPO2374	**RovA**	**Inv and virulence regulation**	***inv* + yet uncharacterized**	**Nagel *et al*., 2001**	**P55740**
YPO2387	PurR	Purine metabolism	*pur* genes	By sim.	P15039
YPO2445	**YfeE**	**Inorganic iron transport**	***yfeABCD***	**Bearden *et al*., 1999**	**Q56956**
YPO2457	**YpeR**	**Quorum-sensing**	**Flagellar regulon**	**Atkinson *et al*., 1999**	**O87971**
YPO2556	PecT	Pectinase gene expression	Pectate lyase genes and others	By sim.	P52662
YPO2625	NagC	Uptake and degradation of GlcN and GlcNac	*nagE, A, B*	By sim.	P15301
YPO2634	**Fur**	**Iron uptake**	**Global regulation**	**Staggs *et al*., 1991**	**P06975**
YPO2681	ChbR	Possible diacetylchitobiose repressor	previously annotated as *celA, B, C*	By sim.	P17410
YPO3063	GcvR	Glycine cleavage (repressor)	*gcv* operon	By sim.	P23483
YPO3085	CueR	Copper efflux regulator	*ybaR*?	By sim.	P23483
YPO3131	AcrR	Multidrug efflux pump	*acrAB* operon repressor	By sim.	P34000
YPO3138	**YmoA**	**Pleiotropic**	**Pleitropic regulation**	**Cornelis *et al*., 1991**	**P27720**
YPO3143	GlnK	Nitrogen assimilation	*glnA*	By sim.	P38504
YPO3266	EmrR	Drug resistance	*emr* operon	By sim.	P24201
YPO3346	ArsR	Arsenical resistance	*arsB*?	By sim. – see * (table legend)	P15905
YPO3396	SfsA	Sugar fermentation stimulation	*mal* genes	By sim.	P18273
YPO3420	PdhR	Pyruvate dehydrogenase complex	*aceEF* and *lpdA*	By sim.	P06957
YPO3456	PhnF	Carbon-phosphorus bond cleavage	*phn* operon	By sim.	P16684
YPO3517	ArgR	Arginine biosynthesis	*carAB* operon	By sim.	P15282
YPO3561	**SspA**	**Survival during starvation**	Invasin/motility – global response	**Badger *et al*., 1998**	**P05838**
YPO3695	Rnk	Nucleoside diphosphate kinase activity	*ndk*??	By sim.	P40679
YPO3698	TreR	Trehalose utilization	*treBC* operon	By sim.	P36673
(YPO3723)	IclR	Glyoxylate bypass	*aceBAK* operon	By sim. – inactive	P16528
YPO3759	BirA	Biotin synthesis	*bioA, bioBFCD…*	By sim.	P06709
YPO3770	RfaH	K antigen and lipopolysaccharide	Hemolysin?	By sim.	P26614
YPO3789	MetR	Methionine biosynthesis	*met A, E, H*	By sim.	P19797
YPO3889	IlvY	Isoleucine-valine biosynthetic pathway	*ilvGMEDA*	By sim.	P05827
YPO3904	HfdR	Control of the flagellar master operon	*flhDC*	By sim.	Q8ZAA7
YPO3915	OxyR	Oxydative stress	Catalases, glutathione-reductases	By sim.	P71318
YPO3955	GntR	Gluconate utilization	*gntRK, edd, eda*	By sim.	P46860
YPO4034	XylR	Xylose transport and metabolism	*xylAB* and *xylFG*	By sim.	P37390
YPO4066	MtlR	Mannitol utilization	*mtlD* and manitol operon	By sim.	P36563

Includes transcriptional regulators (including histone-like proteins) with putatively assignable functions identified in *Y. pestis* CO92. Unless mentioned (written in bold characters and with relevant reference to experimental work), the function and target regulons have been deduced by similarity (by sim.). Putative pseudogenes are in parentheses.
* A pYV plasmid-harbored arsenic resistance operon (with an ArsR-like transcriptional repressor) was characterized by Neyt *et al*. (1997) in low-virulence *Y. enterocolitica* strains.

suggests that these apparent orphans may work together as cognate partners in *Yersinia*. The second system is constituted by the YehT and YehU orthologues. In *E. coli*, genes encoding the sensor kinase and response regulator reside next to each other, and probably belong to the same monocistronic unit. Although *yehT* and *yehU* are found at separate locations on the *Yersinia* chromosomes, these two regulatory elements may also be cognate partners by analogy with the ArcB-ArcA, NarQ-NarP, RscC-RscB-RscA-YojN and BarA-UvrY two component systems in *E. coli*. The functions of all currently identified *Yersinia* TCSs and the possible cross regulations are represented in Fig. 2. Readers should bear in mind that with the exception of PhoP–PhoQ, all the depicted relationships are theoretical, and – even though highly probable – have not been verified by experimental evidence. The three last putative TCSs (and all the orphan subunits, except for RssB) are probably the most interesting regulatory TCS elements, since they have no obvious counterparts in *E. coli*. Although this must be checked experimentally, the first TCS may be a potential virulence regulation system in *Yersina*. It is encoded by two *ssrA* (*spiR*) and *ssrB*-like tandem genes that have already been reported to belong to a *Salmonella*-like pathogenicity island (PI)

Table 2. Putative CO92 transcriptional regulators with unknown functions

CO92 ID	Size	Family	% id	Overlap (aa)	Closest species	Specific comments
YPO3913	211	TetR	96	206	*E. coli* K12	YijC
YPO3545	297	LysR	89	294	*E. coli* K12	YhaJ
YPO2807	297	LysR	84	297	*E.coli* CFT073	Possible Xanthosine operon or exotoxin regulation
YPO3146	153	AsnC	83	153	*P. luminescens*	Possible LRP-like transcriptional regulator
(YPO0414)	306	SorC	81	306	*P. luminescens*	Regulation of sugar utilization, (sorbose?) – Inactive in CO92
YPO3683	303	LysR	80	301	*E. coli* K12	
YPO2283	305	LacI	79	305	*P. luminescens*	
YPO2568	344	LacI	78	344	*E.coli* CFT073	
YPO1929	294	LysR	78	292	*S. enteritidis*	Possible regulator of pathogenicity island genes
YPO2685	175	-	72	175	*E. coli* K12	Involved DNA replication, possible transcription factor as well
YPO2497	313	LysR	74	299	*E. coli* K12	
YPO2388	310	LysR	70	304	*E. coli* K12	YdbH
YPO3211	304	ROK	69	299	*E. coli* K12	YajF – doubtfull: alternatively, possible sugar kinase
YPO3348	319	DeoR	68	316	*P. multocida*	
YPO3017	292	RpiR	69	284	*S. typhimurium* LT2	
YPO0010	229	GntR	67	229	*E. coli* K12	
YPO3651	224	GntR	68	219	*B. fungorum*	
YPO0341	191	TetR	65	191	*P. luminescens*	
YPO0669	303	LysR	66	293	*R. solanacearum*	
YPO2150	301	LysR	64	300	*B. parapertussis*	
YPO1938	256	DeoR	64	252	*E. coli* (plasmid)	
YPO2169	286	LysR	62	286	*P. luminescens*	
YPO3223	133	-	62	133	*S. typhimurium* LT2	Refered to as Crl – regulon (curli) not found on chromosome
YPO0799	302	LysR	63	291	*P. syringae*	
YPO2926	279	RpiR	60	279	*E. coli* K12	
YPO2880	345	XRE	59	345	*S. typhimurium*	
YPO3310	314	DeoR	59	314	*P. syringae*	
YPO0679	297	AraC	61	285	*E. coli* O157:H7	
YPO0841	408	-	61	382	*P. multocida*	Possible arylsulfatase regulator
YPO2979	292	LysR	58	281	*S. typhimurium*	
(YPO2267)	304	LysR	59	287	*E. coli* K12	Possible *als* operon regulator – Inactive in CO92
YPO1960	473	GntR	56	467	*P. luminescens*	Possible pyridoxal-phosphate dependent enzyme.
YPO0846	360	LacI	54	359	*E.coli* CFT073	
YPO1651	149	AsnC	56	142	*S. meliloti*	
YPO2324	318	DeoR	54	313	*B. fungorum*	
YPO0758	331	LacI	53	331	*V. parahaemolyticus*	
YPO0883	132	XRE	56	124	*P. luminescens*	Possibly phage-related
YPO0401	291	AraC	53	287	*E. coli* K12	
YPO1237	270	DeoR	55	256	*P. luminescens*	Possibly involved in sugar metabolism
YPO2845	501	GntR	54	472	*B. parapertussis*	
YPO2378	199	TetR	51	196	*E. coli* O157:H7	
(YPO2449)	194	LuxR	50	194	*P. luminescens*	Inactive in CO92
YPO2537	330	LacI	50	330	*S. typhimurium* LT2	
YPO1503	289	LysR	51	282	*E. coli* K12	
(YPO3840)	221	TetR	54	200	*P. luminescens*	Inactive in CO92
(YPO1728)	338	LacI	49	336	*E. coli* K12	Raffinose utilization? – Inactive in CO92
YPO1934	320	LysR	52	299	*B. parapertussis*	
(YPO1671)	338	LacI	49	333	*E. coli* K12	Inactive in CO92

Table 2. Continued

CO92 ID	Size	Family	% id	Overlap (aa)	Closest species	Specific comments
YPO0165	328	LacI	48	328	*V. vulnificus*	
YPO3978	375	-	46	375	*V. vulnificus*	Possible sugar diacid utilization regulator
YPO0084	411	LysR	47	394	*P. putida*	
YPO0831	258	DeoR	46	252	*P. luminescens*	Possible transcriptional regulator of *aga* operon
YPO1253	246	RpiR	46	240	*B. halodurans*	
YPO2762	261	AraC	48	243	*V. parahaemolyticus*	
YPO0849	357	LacI	43	353	*E. coli* K12	LacI?
YPO0631	318	LysR	43	307	*B. pertussis*	
YPO1810	325	DeoR	41	303	*S. meliloti*	
YPO1737	128	AraC	50	97	*P. vulgaris*	Possible regulator of blood coagulation
YPO1169	297	LysR	39	287	*S. typhimurium*	
YPO1890	265	GntR	41	233	*C. crescentus*	
YPO0611	328	LacI	38	308	*L. innocua*	
YPO3259	277	RpiR	35	277	*B. fungorum*	
YPO3327	269	DeoR	35	264	*B. halodurans*	
YPO0260	259	AraC	35	246	*C. violaceum*	LcrF-like N-term domain – PI harbored
YPO2458	308	LysR	34	299	*C. crescentus*	
YPO2498	334	LacI	35	295	*M. morganii*	
YPO2243	297	AraC	33	265	*P. luminescens*	
YPO2036	384	RpiR	31	364	*A. tumefaciens*	
YPO0276	327	LysR	31	305	*R. solanacearum*	
YPO1837	291	AraC	29	258	*S. typhimurium*	
YPO2478	346	LacI	27	314	*E. coli* K12	
YPO3228	303	LysR	28	252	*P. luminescens*	*Yersinia* specific?
YPO0720	88	-	30	68	*V. parahaemolyticus*	Possible FlgM-like anti-sigma factor
YPO3682	288	LysR	26	232	*P. luminescens*	
YPO3619	292	AraC	23	263	*P. aeruginosa* PA01	
YPO0804	219	-	41	94	*V. vulnificus*	*Yersinia* specific?
YPO0736	348	-	26	189	*L. anguillarum*	*Yersinia* specific? Possible Response Regulator (doubtfull)
YPO2593	205	LuxR	51	52	*E. coli* K12	*Yersinia* specific?
YPO2955	200	LuxR	43	55	*S. coelicolor*	*Yersinia* specific?
YPO2337	279	MerR	35	67	*C. tetani*	*Yersinia* specific?

encoding a putative type III secretion system (Deiwick *et al*., 1999; Garmendia *et al*., 2003). The two other systems, YPO2997–2998 and YPO3008–YPO3009 (Table 3d), have no counterparts in the currently available databases. Their function(s) and role(s) in virulence and regulation are thus certainly worth investigating. Interestingly, as for ZraR–ZraS, the latter of these two TCS could not be found in the *Y. enterocolitica* strain 8081 genome using BLAST searches [(1)].

Of the five orphan subunits, only YPO0712 has a predictable function. This regulator is similar (51% identity across 70% of the protein) to the *P. aeruginosa* FleR flagellar regulator. The immediate vicinity of genes encoding a flagellar apparatus pleads for a similar contribution in *Yersinia*. However, that fact that YPO0712 appears to be truncated at its N-terminus (i.e. the region supposedly encoding the response regulator domain) strongly suggests that it is inactive.

Lastly, four TCS subunits with similarity to UhpB, YojN, BaeS, and EvgS have also been predicted to be inactive in CO92 (Table 3, shown in parentheses) due to frameshifts or IS insertions in their coding sequences (Parkhill *et al*., 2001). With the exception of EvgS, these elements were also predicted to be inactive in *Y. pestis* KIM also (Deng *et al*., 2002). In contrast, it has been assumed that all four proteins are functional proteins in the fully-sequenced *Y. pseudotuberculosis* strain [(1)]. How this may impact the physiological evolution of *Y. pestis* will be discussed below.

Did evolution of the regulators contribute to emergence of Y. pestis*?*

Y. pestis and *Y. pseudotuberculosis* are genetically very close but are responsible for very different diseases in

[(1)] Determined by BLAST searches run on the *Y. pseudotuberculosis* and *Y. enterocolitica* genome sequence data available at the websites:
http://bbrp.llnl.gov/bbrp/bin/y.pseudotuberculosis_blast
http://www.sanger.ac.uk/cgi-bin/blast/submitblast/y_enterocolitica

Table 3. Two component systems predicted in *Yersinia*, according to the currently available literature

Locus	CO92	Gene	Modules	Function	DB entry	Relevant references [1]
3a: > 60% aminoacid identity and similar genetic organization						
1	YPO0022	NtrC	RR	Nitrogen assimilation (sigma 54-dependent)	P06713	Ninfa *et al*., 1995
	YPO0023	NtrB	HK		P06712	
2	YPO0073	CpxA	HK	Protein misfolding	P08336	DiGiuseppe *et al*., 2003
	YPO0074	CpxR	RR		P16244	
3	YPO0136	OmpR	RR	Osmotic regulation	P03025	Forst *et al*., 1994
	YPO0137	EnvZ	HK		P24242	
4a	YPO0458	ArcA	RR	Aerobic respiration control	P03026	Luchi *et al*., 1993
4b	YPO3555	ArcB	HK, Hpt		P22763	
5	YPO0896	CreB	RR	Catabolic regulation.	P08368	Wanner, 1996
	YPO0895	CreC	HK		P08401	
6	YPO1633	PhoQ	HK	Ca^{2+}/Mg^{2+} metabolism Virulence	P23837	Oyston *et al*., 2000
	YPO1634	PhoP	RR		P23836	
7a	YPO1666	CheA	Hpt, HK	Chemotaxis regulation	P07363	Djordjevic *et al*., 1998
	YPO1667	CheW	HK		P07365	
7b	YPO1680	CheY	RR		P06143	
8a	YPO3381	BarA	HK, RR, Hpt	Hydrogen peroxide sensitivity	P26607	Pernestig *et al*., 2001
8b	YPO1865	UvrY	RR		P07027	
9	YPO2308	RstA	RR	Possibly involved in stress response.	P52108	Hirakawa *et al*., 2003
	YPO2309	RstB	HK		P18392	
10	YPO2688	KdpE	RR	Low turgor pressure-dependent potassium transport	P21866	Walderhaug *et al*., 1992
	YPO2689	KdpD	HK		P21865	
11	(YPO2851)	BaeS	HK	Resistance to extracellular stresses	P30847	Raffa *et al*., 2002
	YPO2853	BaeR	RR		P30846	
12	YPO2914	YfhA	RR	sigma 54-dependent regulation	P21712	–
	YPO2916	YfhK	HK		P52101	
13	YPO3204	PhoR	HK	Control of phosphate regulon	P08400	Tommassen *et al*., 1982
	YPO3205	PhoB	RR		P08402	
3b: <60% aminoacid identity for one of the TCS subunits but similar genetic organization						
14a	YPO1217	RcsC	HK, RR	Control of exopolysaccharide biosynthesis ?	P14376	Stout *et al*., 1990
14b	YPO1218	RcsB	RR		P14374	
	(YPO1219)	YojN	HK, HPt		P39838	
14c	YPO2449	RcsA	RR		P14374	
15	(YPO4008)	UhpB	SK	Sugar phosphate transport	P09835	Island *et al*., 1992
	YPO4012	UhpA	RR		P10940	
16	YPO3507	PmrA	RR	Possibly involved in Fe^{3+}-induced regulation	P30843	Roland *et al*., 1993
	YPO3508	PmrB	SK		P30844	
17	see text	ZraR	RR	Response to zinc and lead.	P14375	Leonhartsberger *et al*., 2001
		ZraS	SK		P14377	
3c: <60% aminoacid identity for both TCS subunits but similar genetic organization						
18	YPO0255	SsrB	RR	Possible pathogenicity island regulating system	AE0700	Garmendia *et al*., 2003
	YPO0256	SsrA	HK, RR		AD0700	
19a	(YPO1923) [2]	EvgS	HK, RR, Hpt	Possible regulator of virulence in response to diverse environmental signals	P30855	Masuda *et al*., 2002
19b	YPO1925	EvgA/FimZ	RR		P21502	
20	YPO2000	CopS?	HK	Copper resistance	P76339	Mills *et al*., 1993
	YPO2001	CopR	RR		P77380	

Table 3. Continued

Locus	CO92	Gene	Modules	Function	DB entry	Relevant references [1]
3d: unknown						
21	YPO2997	-	HK	Unknown function	-	-
	YPO2998	-	RR		-	
22	YPO3008	-	SK	Unknown function.	-	-
	YPO3009	-	RR		-	
3e: Possible TCSs reconstructed in silico						
23a	YPO1959	NarX	HK	Nitrate-nitrite metabolism	P10956	Rabin *et al*., 1993
23b	YPO3041	NarP	RR		P31802	
24a	YPO3943	YehU	HK	Unknown function.	P33357	Hirakawa *et al*., 2003
24b	YPO3287	YehT	RR		P33356	
3f: Orphans						
25	YPO3965	CvgSY?	HK, RR	Unknown function	-	-
26	YPO3958	-	RR	Unknown function	-	-
27	YPO2173	RssB	RR	RpoS regulator (MviA, Hnr)	P37055	Bearson *et al*., 1996
28	YPO0712	FleR	RR	Sigma 54-dependent flagellar regulatory protein (partial)	P17899	Ritchings *et al*., 1995
29	YPO1576	-	RR	Unknown function	-	-

Pseudogenes in CO92 are shown between parentheses.
[1] not necessarily princeps publication
[2] Pseudogene in CO92, but not in KIM.

terms of severity. Compared to its *Y. pseudotuberculosis* ancestor, *Y. pestis* displays a restricted ability to grow outside the host but, on the other hand, has become highly pathogenic. According to recent analyses, this change in lifestyle took place very recently on the evolutionary time scale – 20,000 years ago at most (Achtman, *et al*., 1999). The switch from a bimodal (environment + host) to a host-restricted lifestyle is the easiest to explain because it is consistent with the ongoing reductive evolution of the *Y. pestis* genome: this latter phenomenon probably constitutes the most salient information that has emerged from both CO92 and KIM genome analyses. One can reasonably expect the inactivation of certain regulators to greatly accelerate this evolutionary process, since the silencing of regulons is likely to be as dramatic as complete gene-block deletions. On the other hand, inactivation of transcriptional repressors leading to gene overexpression may account (at least partially) for an increase in pathogenicity. However, the issue of how regulator inactivation may have directed the evolution of *Y. pestis* physiology will never be clear until the regulons have been comprehensively characterised.

Around 150 genes are thought to be pseudogenes, due mainly to IS insertions, frameshift or nonsense mutations, in *Y. pestis* but not in *Y. pseudotuberculosis*. Fourteen encode transcriptional regulators, and inactivation of at least seven of these could have effects on bacterial phenotype. Of these seven regulators, three are parts of TCS sensors with high similarity to *E. coli* BaeS, UphB and YojN, respectively. In view of the situation in *E. coli*, the *Yersinia* BaeR-BaeS-like TCS may act as an envelope stress adaptive system, distinct from those controlled by sigma E and the CpxR-CpxA TCS: in other words, it is probably an as yet uncharacterised stress response system. In *E. coli*, YojN is a phosphotransfer intermediate associated with RcsC-RcsB, a TCS required for colanic acid biosynthesis assumed to play a role in the remodeling of the bacterial surface (Ferrieres and Clarke, 2003). Again, the inactivation of YojN observed in the CO92 and KIM strains may argue for the evolution of *Y. pestis* from an environmental lifestyle towards a strictly parasitic role. Evidence for changes in the regulation of certain metabolic pathways is also consistent with this evolutionary scenario. Firstly, inactivation in *Y. pestis* of the third TCS mutated subunit (UphB) probably causes downregulation of genes involved in the uptake and metabolism of hexose phosphates. Secondly, frameshifts in *sorC* and *rafR* homologues (YP00414 and YPO1728) may possibly lead to deficiencies in utilization of sorbose and raffinose. Thirdly (and potentially of greater impact) is the inactivation of YPO3583 (an YhbH-like σ^{54} modulator), which probably results in dramatically altered regulation of the σ^{N}-dependent genes in the two species (for review on σ^{N}, see Reitzer and Schneider, 2001).

How, then, might evolution of the regulator pool have contributed to the increase of *Y. pestis* pathogenicity? The recurrent questions concerning the determinism of and reasons for the very rapid evolution of a contrasting virulence phenotype in *Y. pestis* can only be partially explained by the acquisition of the pPCP1 (9.6 Kb) and pMT1 (102 Kb) plasmid-harboured, virulence determinant-encoding genes. Acquisition of chromosomal fragments of exogenous origin (predicted to have been a frequent occurrence) can hardly account for this dramatic evolution either, since it is thought that all the known fragments were acquired prior to *Y. pestis* speciation (Hinchliffe *et al*., 2003). An additional, simple explanation may be provided by the selective deregulation of genes common

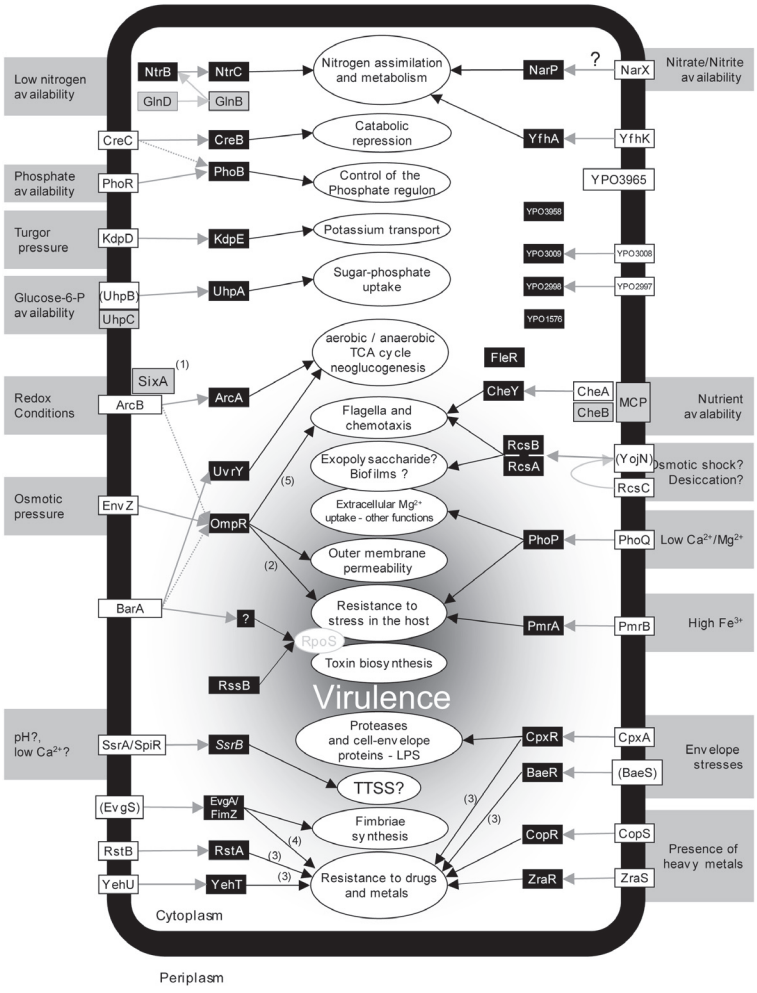

Fig. 2. Putative two-component systems of *Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica.* Sensors and regulators are represented in white and black boxes respectively along with their their possible functions and input signals (when known) deduced by similarity to regulation networks studied in other enterobacteria. Elements which are parts of the signaling pathways but are not TCS subunits *per se* are represented in gray. Sensors with names in parentheses are thought to be inactive in CO92. For clarity, only the major direct regulations (plain arrows) or cross-regulations (dotted arrows) between these systems are represented. For the same reason, transcriptional regulation of two-component systems by other two-component systems are not included. Additional references: Ogino *et al*., 1998; Brzostek *et al*. 2003; Hirakawa *et al*. 2003; Masuda and Church, 2002; Shin and Park, 1995.

to both species. This will be discussed below through one example. Conversely, gene silencing through the inactivation of regulators may also be of great importance, particularly in the case of physiological functions that are necessary for the *Y. pseudotuberculosis* lifecycle but not for that of *Y. pestis* in a similar manner to what has been demonstrated previously with other pathogens (Parish *et al*., 2003). At least one repressor inactivation may have promoted virulence in *Y. pestis*. FlhD is encoded by the flagellar master operon (*flhDC*). As mentioned in the first part of this review, transcription of this polycistronic unit is essential for initiating transcription of most flagellar subunits and their subsequent assembly into a motile organelle. In both the sequenced *Y. pestis* strains, FlhD was found to be inactivated by a nonsense mutation leading to the production of a truncated protein (60% of its wild-type length). Inactivation of this regulator in *Y. enterocolitica* led to partial derepression of Yop expression (Bleves *et al*., 2002). Besides loss of flagella, expression of these molecules at higher levels may confer a selective advantage to *Y. pestis* during its course of infection. By analogy with other bacterial pathogens, the FlhDC complex may co-ordinately regulate the expression of other as yet unknown virulence factors (Pruss *et al*. 2001; 2003). Hence, it is worth investigating the impact of FlhD inactivation on *Y. pestis*.

Conclusion: the limitations and pitfalls of in silico *analysis*
Attempts to explain phenotypic differences between *Y. pestis* and *Y. pseudotuberculosis* by the evolution of their regulators, based on comparing a limited amount of genomic sequences, must never be considered as more than a starting point for experimental investigations. This is true for several reasons, the most evident of which is that strain-to-strain differences may exist within a given species, as illustrated below by two examples. For example, the ZraS-ZraR TCS, although found in *Y. pestis* strain 32953 and at least some biovar Antiqua strains, was apparently lost in both CO92 and KIM strains. Similarly, EvgS (one of the four TCS sensor subunits) is not active in CO92 but is predicted to be functional in the sequenced KIM strain. A second major reason is that predicting

function in terms of similarity is often very speculative. By anticipating the role of a given regulator based on what is known about its role in other bacterial species, one assumes that the element controls a similar regulon and that its inactivation has the same physiological consequences, which in fact depend on the presence or integrity of other potential co-regulatory systems (i.e. independent, overlapping or cross-regulatory systems). One striking example is the role of YPO3223, designated as *crl* by the two *Y. pestis* annotation teams in light of the 62.1% identity of its product with the *E. coli* curlin genes transcriptional activatory protein. Unexpectedly, none of the genomes of the three pathogenic *Yersinia* species harbour genes (csgA and B-like) coding for such appendages[1]. One may then suppose that either these genes have been eliminated or that YPO3223 may serve other purposes. Similarly, the presence of the Rsc-YojN TCS suggests the synthesis of exopolysaccharide in *Yersinia*. However, nothing is known about this antigen in *Yersinia*, and some genes thought to be essential for its biosynthesis have not been detected by *Y. pestis* genome annotation or by BLAST searches of the genomes of the two other pathogenic species. In other, even more complex cases, target regulons have been identified but are seen to differ among the three species, despite the fact that the regulators share > 90% identity at the peptide level. The comparative analysis of the respective roles of the CtrA response regulator in *Caulobacter crescentus* and *Brucella abortus* reported by Bellefontaine *et al*. (2002) perfectly illustrates the possibility of regulation network plasticities when comparing one bacterial species to another.

Computer-assisted identification of operating regions in sequenced bacterial genomes may be useful in refining prediction of co-regulated genes and thus the regulated physiological function. Although this is extremely difficult most of the time due to the low degree of sequence conservation in prokaryotic operating regions, some apparently successful attempts (based on the simultaneous analysis of several gamma proteobacterial genomes) have been reported already for regulons of highly structurally conserved regulators, including Fur. In *Y. pestis*, the presence of upstream Fur-boxes has lead to the identification of several previously characterized Fur-controlled operons (including, very surprisingly, the *psaEF* operon that is discussed above), as well as potentially new operons (Panina *et al*, 2001a). Similar studies have also been performed in *Y. pestis* with other regulators, such those of aromatic amino acid, ribose, arabinose, and xylose metabolism (Laikova *et al*., 2001; Panina *et al*., 2001b).

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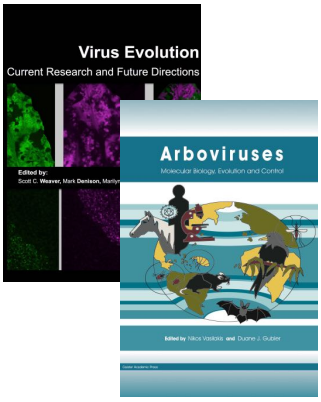

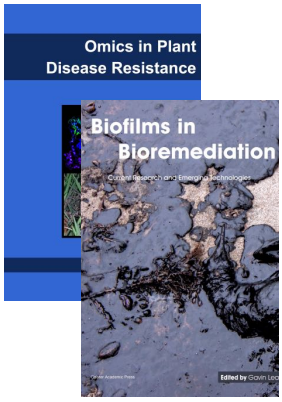

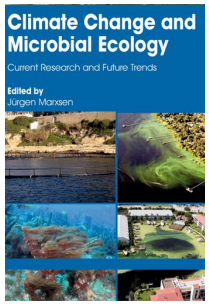

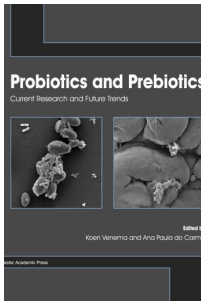

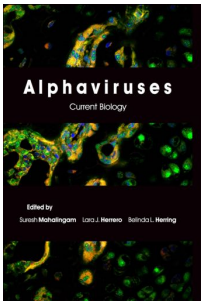

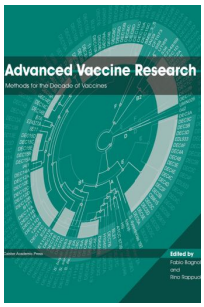

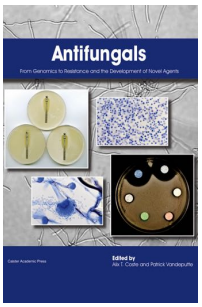

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