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Differentiate to thrive: lessons from the *Legionella pneumophila* life cycle

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Summary

When confronted by disparate environments, microbes routinely alter their physiology to tolerate or exploit local conditions. But some circumstances require more drastic remodelling of the bacterial cell, as sporulation by the *Bacillus* and *Streptomyces* species of soil bacteria vividly illustrates. Cellular differentiation is also crucial for pathogens, the challenge for which is to colonize one host, then be transmitted to the next. Using the Gram-negative *Legionella pneumophila* as a model intracellular pathogen, we describe how biogenesis of the replication vacuole is determined by the developmental state of the bacterium. Subsequently, when replicating bacteria have exhausted the nutrient supply, the pathogens couple their conversion to stationary phase physiology with expression of traits that promote transmission to a new host. The cellular differentiation of *L. pneumophila* is co-ordinated by a regulatory circuit that integrates several elements that are broadly conserved in the microbial world. The alarmone (p)ppGpp promotes transcription directed by the alternative sigma factors RpoS, FliA and, probably, RpoN, and also post-transcriptional control mediated by a two-component regulatory system, LetA/S (GacA/S), and an mRNA-binding protein, CsrA (RsmA). By applying knowledge of microbial differentiation in combination with tools to screen the complete genomes of pathogens, experiments can be designed to identify two distinct classes of virulence traits: factors that promote replication and those dedicated to transmission.

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'Adapt yourself to changing circumstances'

Even the simplest of organisms heed this Chinese proverb, as bacteria acclimate to fluctuations in their environments. Whether to make use of local energy sources or to tolerate a range of acidity, osmolarity or temperature, microbes adjust their physiology via sophisticated signal transduction pathways. Unicellular organisms can also respond to environmental cues by activating morphogenetic programmes during a life cycle, a commitment that we shall distinguish from metabolic regulation by referring to it as differentiation.

Conspicuous examples of microbial differentiation are the obligate intracellular pathogens, which must periodically abandon a cytoplasmic or vacuolar niche to face unpredictable circumstances during their transmission to a new host. For example, *Coxiella burnetii*, which causes Q fever, alternates between the replicative large cell variant and the environmental small cell variant, a form that is highly stress resistant (reviewed by Samuel *et al.*, 2003). Similarly, during its biphasic life cycle, the common sexually transmitted pathogen *Chlamydia trachomatis* changes between an intracellular replicative reticulate body and a resilient and infectious elementary body (reviewed by Hammerschlag, 2002). Because neither pathogen can be manipulated genetically, identification of the regulatory circuits that control their life cycles is a formidable challenge.

Insight into the molecular mechanisms of differentiation by pathogens has been obtained from studies of more tractable Gram-negative bacteria. The *Bordetella* species are extracellular respiratory pathogens that use a BvgA/S phosphorelay together with sigma factors that regulate both motility (Frl) and a type III secretion system (BtrS) to control expression of three sets of traits: those thought to promote colonization of the respiratory tract (Bvg$^+$ form), transmission to a new host (Bvg^i form) and survival in the environment (Bvg$^-$ form; reviewed by Cotter and Jones, 2003; S. Matoo and J. F. Miller, personal communication). As an experimental model for analysing a pathogen's reciprocal expression of replicative and transmissive traits and its impact on host cell biology, we focus here on the Gram-negative intracellular bacterium *Legionella pneumophila*.

Emergence from amoebae to human pathogen

Ubiquitous in aquatic environments, *L. pneumophila* endures a range of temperatures, osmolarity and other stresses, including ingestion by protozoa that feed on bacteria. Aerosolized microbes that are inhaled by humans can also replicate inside alveolar macrophages. When a robust host defence is absent, the progressive and potentially fatal pneumonia known as Legionnaires' disease develops (reviewed by Fields *et al*., 2002; Swanson and Hammer, 2000). However, as *L. pneumophila* do not spread from person to person, humans have been inconsequential to the evolution of its virulence. Rather, the ability of *L. pneumophila* to colonize alveolar macrophages probably reflects conservation of cell biological pathways between the professional phagocytes that patrol the lungs and those that inhabit ponds. Indeed, the microbe's life cycle in amoebae and macrophages is remarkably similar (Fields *et al*., 2002), bolstering the notion that the virulence strategy of *L. pneumophila* has been shaped by selective pressures in aquatic ecosystems.

Cycles of replication and transmission

Legionella *differentiation*

In natural and potable fresh water supplies, *L. pneumophila* probably resides within biofilm communities, where it falls prey to grazing amoebae. When ingested, the microbe can resist digestion and, instead, replicates profusely before killing its protozoan host and returning to the aquatic reservoir. As predicted for a microbe that transits between phagocytes and water, the *L. pneumophila* life cycle consists of at least two phases. In pioneering studies, Rowbotham (1986) infected amoebae and watched as the bacteria alternated between two morphologically distinct forms, changing motility, shape, surface and stores of energy-rich polymers. Subsequent analysis of *L. pneumophila* differentiation has been expedited by the discovery that many aspects of the pathogen's life cycle can be modelled in synchronous broth cultures, as judged by the growth phase-dependent expression of numerous traits and genes (Table 1). Identification of regulatory elements that govern the bacterial life cycle has also been advanced by the development of genetic tools to study *L. pneumophila*, including transposon mutagenesis, transformation via natural competence, conjugation and electroporation, recombinant green fluorescent protein (*gfp*) genes and the genome sequence. Accordingly, the working model put forward here assimilates data obtained from several independent morphological, genetic and molecular approaches.

In the model's simplest form, the *L. pneumophila* life cycle is composed of two reciprocal phases: replication and transmission (Fig. 1). When conditions are favourable

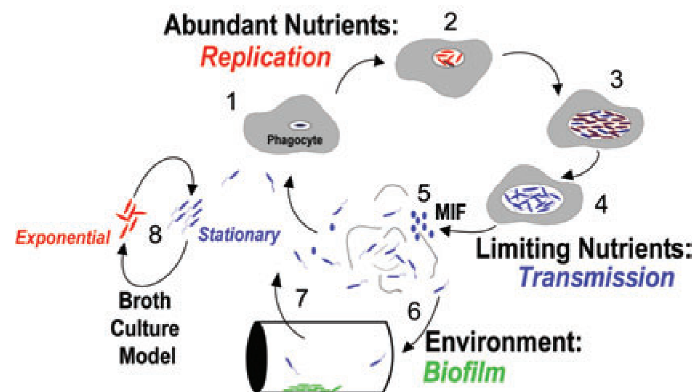

Fig. 1. The life cycle of *L. pneumophila*. Studies of broth and phagocyte laboratory cultures support the following model for the persistence of *L. pneumophila* in aquatic reservoirs.
1. Free-swimming transmissive *L. pneumophila* that are engulfed by phagocytic cells (amoebae or alveolar macrophages) establish vacuoles that provide protection from lysosomal digestion.
2. When nutrients and other conditions are favourable, intracellular bacteria repress transmission traits and activate pathways that promote replication.
3. As conditions in the replication compartment deteriorate, the progeny stop dividing and co-ordinately express traits that promote survival in the environment and transmission to a new phagocytic host.
4. After a prolonged period, the microbes may continue to develop into a mature intracellular form (MIF), a cell type that is highly resilient and infectious.
5. The phagocyte host is lysed, and the microbes are released into the aqueous environment.
6. *L. pneumophila* that do not immediately encounter a new phagocyte probably establish biofilms in both water systems and ponds, where they are resistant to biocidal agents.
7. When planktonic microbes encounter a new phagocyte, the cycle begins anew.
8. Microbes cultured in broth to either exponential or stationary phase display many attributes of the replicative and transmissive forms, respectively, that are observed in phagocyte cultures.

Table 1. Reciprocal expression of multiple traits by replicative and transmissive *L. pneumophila*.

Traits	Replicative phase	Transmissive phase	Regulators	References
Replication*[a]	+	–	+: CsrA, RpoS?	Bachman and Swanson (2001); Molofsky and Swanson (2003); Bachman and Swanson (2004a)
CsrA global repressor expression*[a]	+	–	–: LetA/S	Fettes *et al.* (2001); Molofsky and Swanson (2003)
(p)ppGpp accumulation	–	+	+: RelA, SpoT?	Hammer and Swanson (1999); Zusman *et al.* (2002)
Motility/ flagellar regulon*[a,b]	–	+	+: RelA, LetA/S, LetE, RpoS, RpoN/FleQ, FliA –: CsrA, FlaR, RpoS	Byrne and Swanson (1998); Fettes *et al.* (2001); Hammer *et al.* (2002); Heuner *et al.* (1999); Heuner *et al.* (2000); Lynch *et al.* (2003); Molofsky and Swanson (2003); Rowbotham (1986); Zusman *et al.* (2002); Bachman and Swanson (2004a,b); Jacobi *et al.* (2004)
Contact-dependent cytotoxicity*[a]	–	+	+: LetA/S, FliA –: CsrA	Alli *et al.* (2000); Byrne and Swanson (1998); Hammer *et al.* (2002); Molofsky and Swanson (2003)
Stress resistance	–	+	+: LetA/S, LetE –: CsrA	Bachman and Swanson (2001); Hales and Shuman (1999); Lynch *et al.* (2003); Molofsky and Swanson (2003); Bachman and Swanson (2004b)
Pigment production	–	+	+: LetA/S, LetE, RelA –: CsrA	Molofsky and Swanson (2003); Zusman *et al.* (2002); Bachman and Swanson (2004b); Fettes *et al.* (2001)
Beta-hydroxybutyrate storage granules*[b,c]	–	+	ND	Faulkner and Garduno (2002); Greub and Raoult (2003); Rowbotham (1986)
Small coccoid cells*[a,b,c]	–	+	+: LetA/S, LetE –: CsrA	Faulkner and Garduno (2002); Fettes *et al.* (2001); Molofsky and Swanson (2003); Rowbotham (1986); Bachman and Swanson (2004b)
Lysosome evasion*[a]	–	+	+: LetA/S, FliA –: CsrA	Byrne and Swanson (1998); Hammer *et al.* (2002); Joshi *et al.* (2001); Molofsky and Swanson (2003); Sturgill-Koszycki and Swanson (2000)
Endoplasmic reticulum recruitment*[a]	–	+	ND	A. O. Amer and M. Swanson (in preparation)
Sodium sensitivity*[a]	–	+	+: LetA/S, LetE, RpoS –: CsrA	Bachman and Swanson (2001); Byrne and Swanson (1998); Hammer *et al.* (2002); Molofsky and Swanson (2003); Bachman and Swanson (2004b)
RalF, LidA[d] or SidC expression – Dot/Icm substrates	Low	High	ND	Conover *et al.* (2003); Luo and Isberg (2004); Nagai *et al.* (2002)
Mip[d], DotO[d], DotH[d], MagA[c] expression –	Low	High	+: RpoS (MagA)	Watarai *et al.* (2001a); Wieland *et al.* (2002); Bachman and Swanson (2004a); Hiltz *et al.* (2004)

Traits regulated similarly by *L. pneumophila* cultured in both broth and macrophages[a], amoebae[b] or HeLa cells[c] are indicated by *. Traits only regulated during macrophage infection but not in broth culture are indicated with [d]. Regulators controlling various traits are indicated: – indicates repression; + indicates activation; ND indicates that experiments to identify regulators have not been done.

for replication, traits that promote transmission are repressed, and the intracellular bacteria multiply (Fig. 2A). As vacuolar nutrients become limiting, the progeny differentiate into the transmissive phase, repressing multiplication while expressing a number of traits that are believed to equip *L. pneumophila* to escape from its spent host cell, survive as a planktonic cell and re-establish a replicative niche within a new phagocyte (Fig. 2B). In particular, as broth cultures enter the stationary phase, the bacteria coordinately express: (i) an inducer of phagocyte necrosis; (ii) motility; (iii) resistance to the stresses of UV light, heat, osmotic pressure and nutrient limitation; and (iv) factors to evade degradation within phagocyte lysosomes (Table 1 and references therein). We define these phenotypes as transmission traits to emphasize that none is expressed by replicating bacteria and all are thought to promote spread of the pathogen from its protective vacuole in one host to that in another. After successfully assembling another intracellular niche, *L. pneumophila* reverts to the replicative form, beginning the cycle anew.

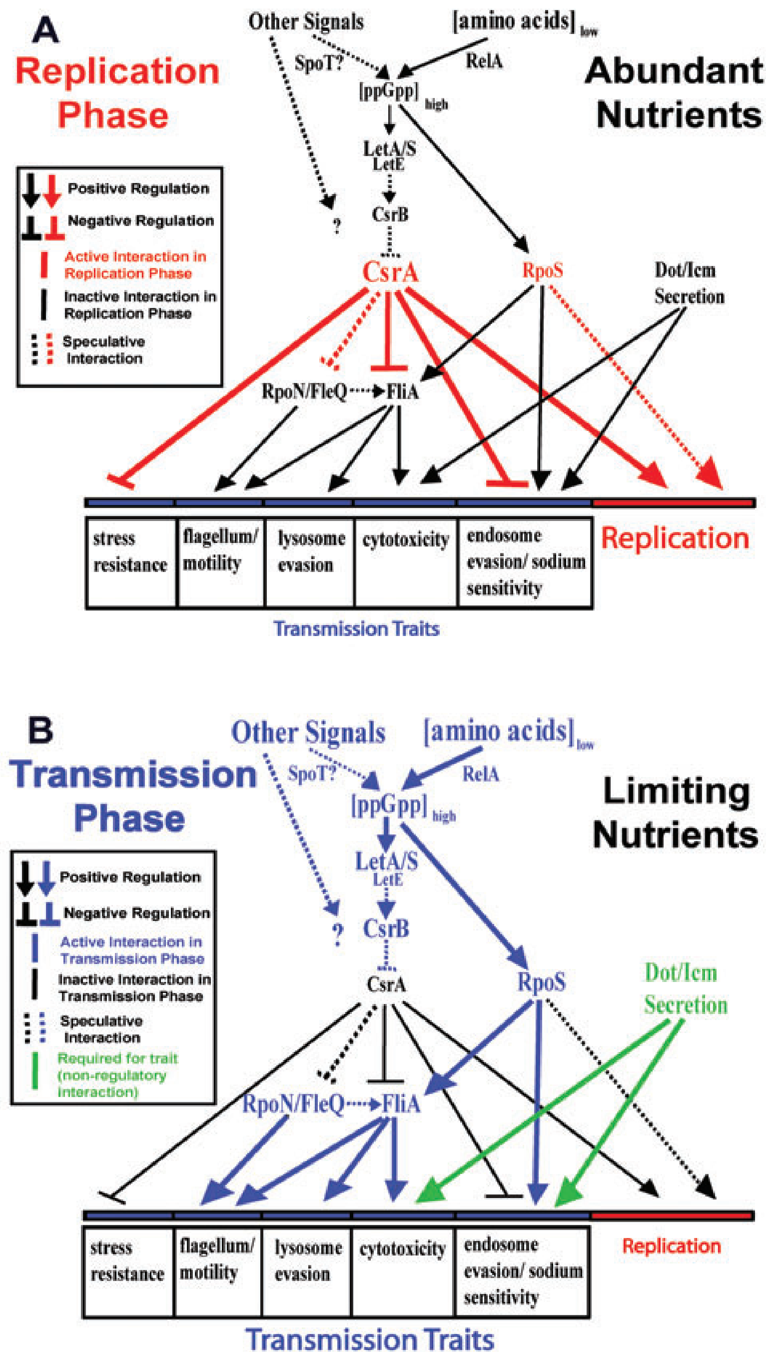

Fig. 2. Reciprocal expression of the replicative and transmissive phenotypes is co-ordinated by (p)ppGpp levels and an integrated network of alternative sigma factors and a post-transcriptional control regulatory system. A model deduced from genetic studies of regulatory interactions that may be direct or indirect is shown.
A. During the replicative phase, nutrients are abundant, and the post-transcriptional regulator CsrA represses transmission traits and promotes replication. The alternative sigma factor RpoS also stimulates intracellular replication and osmotic resistance and inhibits FliA-dependent traits. CsrA inhibits flagellar production and the co-regulated traits of lysosomal evasion and induction of phagocyte necrosis (cytotoxicity) by repressing the flagellar sigma factor FliA (σ^{28}) directly or indirectly or perhaps by regulating the putative master flagellar regulators RpoN (σ^{54}) and/or FleQ. CsrA also represses resistance to several environmental stresses, sodium sensitivity and endosomal evasion.
B. During the transmissive phase, a poor amino acid supply may trigger the ribosomal-associated enzyme RelA to produce the alarmone (p)ppGpp. Alternative intracellular signals may stimulate the SpoT hydrolase/synthase to produce the alarmone. (p)ppGpp stimulates the two-component system LetA/S, the enhancer protein LetE, the alternative sigma factor RpoS and perhaps the putative master flagellar regulator RpoN, which together promote the expression of transmission traits. Active LetA may induce the production of a *csrB*-like regulatory RNA to sequester CsrA and relieve CsrA post-transcriptional repression, whereas RpoS, FliA and RpoN probably activate transcription of the transmission regulon genes. In macrophage culture, undefined signal(s) can bypass both RelA and LetA to induce transmission. FliA-dependent and -independent pathways are induced that promote escape, motility, resilience and infection of a new phagocyte host. Type IV Dot/Icm secretion is required for cytotoxicity as well as evasion of the endocytic network, probably by directly exporting the effectors of virulence, whereas RpoS induces expression of the FliA regulon and traits that confer endosomal evasion and sodium sensitivity.

Although the experimental support for the current model of the pathogen's life cycle was initially obtained by studies of exponential and stationary phase broth cultures, the reciprocal expression of numerous replicative and transmissive traits by *L. pneumophila* has been confirmed by analysing the pathogen's life cycle in macrophages, amoebae and HeLa cells (Table 1). For example, when a stationary phase inoculum of cytotoxic, sodium-sensitive, flagellated and motile cells is incubated with macrophages, the microbes that are ingested subsequently suppress each of these traits during the replication period; as the primary infection period ends with macrophage lysis, the progeny then induce the expression of all four traits (Byrne and Swanson, 1998; Alli *et al*., 2000).

Likewise, four *L. pneumophila* proteins known to promote bacterial entry are expressed during the invasion and exit period, but not during the replication phase in macrophages; these proteins are FlaA, Mip, DotO and DotH (Cianciotto and Fields, 1992; Hammer and Swanson, 1999; Dietrich *et al*., 2001; Watarai *et al*., 2001a; Wieland *et al*., 2002). Conversely, the repressor of transmission traits, CsrA, exhibits the opposite pattern of expression: its promoter is active exclusively when intracellular bacteria are multiplying (Molofsky and Swanson, 2003). Remarkably, even the trait that is a hallmark of *L. pneumophila* virulence, lysosome evasion, is inactive during the intracellular replication period (Sturgill-Koszycki and Swanson, 2000; Joshi *et al*., 2001), as discussed in detail below.

Advantages and limitations of the broth model

Compared with phagocyte models, synchronous broth cultures offer several technical advantages for studies of *L. pneumophila* differentiation. With relative ease and economy, large quantities of pure populations of replicative or transmissive bacteria cultured in defined medium can be obtained. Moreover, the samples are free from eukaryotic protein, DNA and RNA. Accordingly, broth cultures are an attractive system for studies of *L. pneumophila* differentiation by modern molecular approaches such as DNA microarrays, proteomics and real-time polymerase chain reaction (PCR). Broth- and agar-grown microbes are also amenable to classical genetic screens, an approach that has already identified several of the activators and repressors of *L. pneumophila* differentiation discussed below.

Although studies of broth-grown microbes have accurately predicted many of the pathogen's behaviours in macrophage or amoebae models (Table 1), more complex experimental systems have revealed additional attributes that are not observed in broth cultures. For example, to replicate to large numbers in macrophages, *L. pneumophila* requires an acidic pH, whereas exponential phase broth cultures are acid sensitive (Sturgill-Koszycki and Swanson, 2000). The bacterial progeny that emerge from amoebae are more infectious than cells harvested from agar (Cirillo *et al*., 1999). Even more striking is the phenotype of *L. pneumophila* after prolonged culture in HeLa epithelial cells. In this setting, the pathogen differentiates into a thick-walled, pleomorphic, highly resilient and infectious mature intracellular form (MIF) (Faulkner and Garduno, 2002; Garduno *et al*., 2002), a cell type also observed in amoebae and clinical specimens (Greub and Raoult, 2003). Although this developmental form shares several traits with stationary phase broth-grown *L. pneumophila*, MIFs appear to be hyperinfectious and -resistant to environmental stress. Analogous to the small cell variant of *C. burnetii*, the spore-like MIF is postulated to be the transmissive form of *L. pneumophila* in nature (Garduno *et al*., 2002). Therefore, broth-grown stationary phase microbes probably represent an intermediate stage of a developmental pathway that culminates in the MIF transmissive cell type (Fig. 1). A different habitat in which *L. pneumophila* survive for extended periods are biofilms, complex microbial communities that pose a significant hazard to potable water supply systems, especially in hospitals (reviewed by Fields *et al*., 2002). How closely the bacterial forms that develop in either broth or eukaryotic cell culture resemble the cell types that persist in biofilms is a critical open question.

Developmental state determines fate in phagocytes

To survive ingestion by a phagocyte, *L. pneumophila* avoids immediate delivery to digestive lysosomes (Horwitz, 1983a,b). This hallmark of the species' virulence dramatically illustrates how microbial differentiation impinges on the host cell response and determines the outcome of the encounter. When transmissive, stationary phase *L. pneumophila* are phagocytosed, they immediately occupy a spacious cholesterol-rich compartment that does not fuse with lysosomes (Joshi *et al*., 2001; Watarai *et al*., 2001b). If *L. pneumophila* are genetically locked in the transmissive form, the bacteria infect macrophages efficiently, but persist for days without replicating (Molofsky and Swanson, 2003). In contrast, when macrophages are fed *L. pneumophila* that are in the replicative state, the bacteria are delivered to the endocytic network and swiftly killed. In particular, *L. pneumophila* fail to evade the destructive lysosomes when the transmission regulon is inactive, because the bacteria are in the exponential growth phase, lack an activator of transmission or constitutively express a repressor of transmission (Byrne and Swanson, 1998; Joshi *et al*., 2001; Molofsky and Swanson,

2003; regulators depicted in Fig. 2 and described in detail below).

Like exponential phase cells obtained from broth cultures, *L. pneumophila* that have begun to replicate in macrophages are also delivered to acidic lysosomes. When macrophages are infected with stationary phase *L. pneumophila*, the bacteria persist without multiplying for several hours in a vacuole that appears to be completely separate from endosomal traffic (Horwitz, 1983a,b; Joshi *et al.*, 2001). However, once replication vacuoles contain six or more bacteria, exclusion of the late endosomal and lysosomal protein LAMP-1 is rare (Sturgill-Koszycki and Swanson, 2000). During the next 10–15 h period, the pathogen replication vacuole continues to accumulate lysosomal markers. Yet, it is evident that the intracellular *L. pneumophila* are acclimated to this harsh environment, as the bacteria multiply profusely in an acidic vacuole (Sturgill-Koszycki and Swanson, 2000). Thus, transmissive cells pause phagosome maturation, then the bacteria differentiate into an acid-resistant replicative form that exploits phagosome–lysosome fusion to multiply to large numbers.

The model in which *L. pneumophila* downregulates virulence factors that arrest phagosome maturation during its intracellular replication period is further substantiated by molecular genetic studies of the Dot/Icm type IV secretion system (reviewed by Sexton and Vogel, 2002). The pathogen requires Dot/Icm function to enter cholesterol-rich spacious vacuoles, avoid immediate delivery to the endosomal pathway, associate with endoplasmic reticulum and establish its replicative vacuole (Berger *et al.*, 1994; Swanson and Isberg, 1995; Watarai *et al.*, 2001b). Nevertheless, *dotA* mutants can replicate to large numbers within macrophages when their initial trafficking defect is bypassed experimentally, either by inducing transient expression of *dotA* from a heterologous promoter or by forcing co-habitation in a vacuole with a wild-type transmissive microbe (Roy *et al.*, 1998; Coers *et al.*, 1999). As *dotA* is predicted to encode an integral component of the Dot/Icm apparatus (Roy *et al.*, 1998), the data indicate that, although type IV secretion is necessary to block immediate fusion with endosomes, it is dispensable during the period of bacterial replication. Likewise, two other Dot/Icm-dependent traits that promote replication vacuole biogenesis, namely recruitment of vesicles from the endoplasmic reticulum and activation of caspase 3, are vital only during the first 30 min of infection (Kagan and Roy, 2002; Molmeret *et al.*, 2004). In addition to these studies of replication vacuole biogenesis, results of multiple independent genetic and cell biological experiments support the model that the transmissive and replicative states are reciprocal (Table 1). While expressing transmission properties, *L. pneumophila* do not replicate; conversely, replicating cells do not express transmission traits, including factors that block their delivery to lysosomes.

The host signals that trigger differentiation of transmissive *L. pneumophila* to the replicative form are not known, but the switch occurs before its delivery to lysosomes. First, vacuoles that harbour as many as four bacterial progeny typically lack the late endosomal/lysosomal protein LAMP-1; not until vacuoles harbour more than five microbes do most contain appreciable LAMP-1. Secondly, when acidification and maturation of phagosomes is inhibited by treating macrophages with the proton pump inhibitor bafilomycin, *L. pneumophila* multiplication is inhibited, but the bacteria do not arrest at the single-cell stage (Sturgill-Koszycki and Swanson, 2000). Therefore, although an acidic lysosomal network promotes robust microbial growth, a signal other than acidic pH triggers differentiation to the replicative form. Perhaps after differentiation, during the first cycles of pathogen multiplication, virulence factors that block phagosome maturation become dilute or unstable; consequently, the paused vacuole merges with the lysosomal compartment, a rich source of both nutrients and vacuolar membrane. Alternately, replicative phase *L. pneumophila* could actively alter gene expression to promote lysosomal fusion.

Like *L. pneumophila*, the intracellular pathogens *C. burnetii* and certain *Leishmania* spp. practice a similar ‘pregnant pause’ strategy to thrive in macrophages. All three pathogens alternate between an infectious stationary phase cell type that initially blocks phagosome maturation and an intracellular form that replicates in acidic lysosomes (reviewed by Swanson and Fernandez-Moreira, 2002). The reciprocal expression of transmission and replication traits is a logical strategy for intracellular pathogens to limit costly energy expenditures. When nutrients are plentiful in the replication niche, transmission phase virulence structures are neither required nor built. Conversely, when conditions are not favourable for growth, the biochemical pathways that promote replication are superfluous and not expressed.

Although the biogenesis of the *L. pneumophila* replication vacuole has been described in some detail, numerous important questions remain. By what mechanism does *L. pneumophila* arrest phagosome maturation? How are vesicles from the endoplasmic reticulum recruited to the phagosomal membrane? Does the endoplasmic reticulum contribute to bacterial survival? What intracellular cues trigger differentiation of *L. pneumophila* to the replicative form? What is the composition of the vacuole in which the microbe begins to replicate? Knowledge of the regulatory circuitry that controls microbial differentiation can provide experimental tools to investigate the macrophage cell biology that determines the outcome of an *L. pneumophila* infection.

Coupling transmission to the stationary phase

To escape deteriorating conditions in its replication niche, *L. pneumophila* co-ordinately activates traits necessary to exit a spent host, survive environmental stress and parasitize its next host, while repressing traits dedicated to intracellular multiplication. Knowledge of the environmental cues and components of the regulatory circuitry that co-ordinate this developmental programme has been obtained from genetic and molecular studies of the *L. pneumophila* life cycle in both broth and phagocyte experimental models.

Alarms

In broth cultures, *L. pneumophila* accumulate (p)ppGpp as they exit the exponential growth phase, the amino acid supply is limited, or the expression of *relA* is induced (Fig. 2; Hammer and Swanson, 1999). Subsequently, the bacteria stop replicating and differentiate into the transmissive form (Fig. 2; Hammer and Swanson, 1999). By analogy with the *Escherichia coli* stringent response, when *L. pneumophila* that are replicating in macrophages exhaust the amino acid supply, the enzyme RelA is predicted to be activated. In response to uncharged tRNAs, the ribosome-associated RelA synthase converts GTP to (p)ppGpp. In *L. pneumophila* broth cultures, this second messenger then co-ordinates entry into stationary phase with expression of traits thought to promote transmission of the progeny to a new host (Fig. 2B; Hammer and Swanson, 1999). Genetic data also support the model in which RelA activity is dedicated to the transmissive phase and is dispensable during the replication period. *L. pneumophila relA* mutants replicate efficiently inside amoebae and macrophages but, when cultured to stationary phase, they fail to accumulate detectable (p)ppGpp and express some transmission traits poorly, including motility and pigment (Zusman *et al*., 2002). Nevertheless, the impact of the stringent response on differentiation of intracellular microbes has not been established. For example, (p)ppGpp accumulation during the pathogen's life cycle in phagocytes has not been measured, and the ability of *relA* mutants to express many of the transmission traits when cultured in either broth or phagocytes has not been examined. The broadly conserved stringent response pathway appears to be monitored by a wide array of pathogens, including *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* (Primm *et al*., 2000; Chatterji and Kumar Ojha, 2001; Okada *et al*., 2002; Taylor *et al*., 2002; Haralalka *et al*., 2003). By coupling expression of transmission traits to (p)ppGpp accumulation, pathogens can respond to metabolic stress by seeking more fertile territory.

In addition to the stringent-like response, additional signals and/or regulators appear to control *L. pneumophila* differentiation both *in vitro* and in macrophages. For example, the transmission trait defects of *relA* mutants are milder than those of other regulatory mutants (*letA* and *rpoS*; discussed below) or replicative microbes obtained from exponential phase cultures of wild-type *L. pneumophila* (Hammer and Swanson, 1999; Zusman *et al*., 2002). Accordingly, factors other than RelA probably trigger microbial differentiation. One candidate that has not been examined is the SpoT (p)ppGpp hydrolase/synthase, an enzyme that also generates (p)ppGpp in *E. coli* and appears to be essential for viability of *L. pneumophila* (Zusman *et al*., 2002). Furthermore, bacteria that lack either RelA or the transmission activator LetA appear to spread in macrophage monolayers as efficiently as wild-type microbes, as judged by the rate of increase in cfu throughout a 72 h infection, a period composed of secondary and tertiary infections (Hammer *et al*., 2002; Zusman *et al*., 2002). Therefore, when *L. pneumophila* are crowded in a replicative vacuole, certain transmission traits may be induced by signal(s) that can bypass both RelA and LetA. As predicted by this model, when compared with their broth counterparts, *letA* mutants harvested from macrophages are more infectious (B. Byrne and M. Swanson, unpublished). Although genome searches suggest that *L. pneumophila* lacks a classical quorum-sensing mechanism, it is plausible that an unorthodox form of quorum sensing induces transmission traits. Alternatively, each replicative *L. pneumophila* cell may respond independently to other cues in the lysosomal vacuole to activate the transmission programme. As timely differentiation is paramount for intracellular parasites, it is likely that *L. pneumophila* uses multiple redundant pathways to monitor (p)ppGpp levels and other local parameters to judge whether to divide or escape. Because methods to interfere with microbial differentiation could be exploited to prevent or treat infection, identification of alternative signal(s) of differentiation is one imperative of future research.

Sigma factors activate the transmission programme

To respond to the (p)ppGpp alarmone and differentiate into the transmissive state, *L. pneumophila* requires a number of alternative sigma factors (Fig. 2). The stationary phase factor RpoS (σ^S/σ^{38}), the flagellar regulator FliA (σ^{28}) and the alternative sigma factor RpoN (σ^{54}) have been determined genetically to be activators of particular transmission traits (Hales and Shuman, 1999; Bachman and Swanson, 2001; Hammer *et al*., 2002; Heuner *et al*., 2002; Heuner and Steinert, 2003; Jacobi *et al*., 2004). Studies to determine how *L. pneumophila* differentiation

is co-ordinated by this cohort of alternative sigma factors have been hampered by a lack of knowledge of the effector genes of transmission. Therefore, transcriptional control of the flagellar regulon has been the primary focus of research (for a recent review, see Heuner and Steinert, 2003).

Although the mechanism remains to be examined in *L. pneumophila*, biochemical and genetic studies of *E. coli* by Nystrom and colleagues indicate that (p)ppGpp acts as a global regulator of transcription by biasing the competition among sigma factors for binding to the RNA core polymerase (Farewell *et al*., 1998; Jishage *et al*., 2002; Laurie *et al*., 2003). In particular, (p)ppGpp appears to destabilize the interaction of the predominant vegetative sigma factor, σ^D (σ^{70}), with RNA core polymerase. As a consequence, an alternative sigma factor can replace σ^D in the core and recruit the enzyme to its cohort of promoters. Accordingly, (p)ppGpp accumulation is predicted to increase the amount of RpoS protein and also the activity of both RpoS and RpoN (Jishage *et al*., 2002). The prediction that *L. pneumophila* RpoS competes with other sigma factors for binding to RNA core polymerase is consistent with the phenotype of bacteria that lack or overexpress *rpoS.* For example, when present in multiple copies, *rpoS* inhibits the expression of three *fliA*-dependent transmission traits in primary murine macrophages (Bachman and Swanson, 2004a) and inhibits intracellular growth in *Acanthamoeba castellanii* (Hales and Shuman, 1999). How (p)ppGpp couples expression of RpoS-dependent stationary phase traits with FliA-dependent transmission traits can be analysed biochemically once genes that encode effectors of the *L. pneumophila* transmissive state are identified.

Legionella pneumophila requires the flagellar sigma factor FliA not only to synthesize the flagellar filament and for motility (Fettes *et al*., 2001; Heuner *et al*., 2002), but also to express contact-dependent cytotoxicity, lysosome evasion in macrophages and replication in the social amoebae *Dictyostelium discoideum* (Hammer *et al*., 2002; Heuner *et al*., 2002; L. M. Shetron-Rama and M. S. Swanson, unpublished). Therefore, the FliA sigma factor (σ^{28}) of *L. pneumophila* may activate promoters of the flagellar regulon as well as other virulence effector genes (Hammer *et al*., 2002; Heuner *et al*., 2002; Molofsky and Swanson, 2003), a pattern also observed in *Salmonella enterica* (Eichelberg and Galan, 2000). An alternative mechanism that links motility to the expression of additional virulence traits is illustrated by *Vibrio cholerae*, which responds to flagellar motion and sodium gradients in a complex manner to alter expression of the ToxT virulence regulon (Hase and Mekalanos, 1999). Whatever the mechanism, identification of the cohort of genes regulated by FliA together with analysis of representative flagellar development mutants can provide insight to how *L. pneumophila* escapes from one host, then blocks phagosome maturation in the next.

Post-transcriptional repression of the transmission regulon

Whereas the alternative sigma factors RpoS, FliA and RpoN govern transcription initiation to induce transmission traits, post-transcriptional regulation of this class of mRNAs is likely to be controlled by the two-component regulatory system LetA/S. Originally identified in an *L. pneumophila* screen for mutants defective for flagellar synthesis, LetA/S, together with the novel protein LetE, induces a large panel of transmissive traits in response to the alarmone (p)ppGpp (Hammer *et al*., 2002; Lynch *et al*., 2003; Bachman and Swanson, 2004b). Several diverse bacterial species also rely on homologues of LetA/S, called ExpA/S, GacA/S, UvrY/BarA, VarA/S and SirA/BarA, to express a variety of extracellular virulence factors and to modulate carbon pathways when conditions deteriorate (reviewed by Heeb and Haas, 2001). Whether (p)ppGpp activates the membrane-bound sensor kinase LetS is not known but, in homologous systems, an active LetS sensor kinase phosphorylates the LetA response regulator to change gene expression. Consistent with the regulatory hierarchies delineated in *E. coli* and other microbes, genetic data indicate that the major, if not sole, function of activated LetA is to relieve repression by the global regulatory RNA-binding protein CsrA (RsmA).

CsrA belongs to a highly conserved family of global regulators that typically control stationary phase traits post-transcriptionally (reviewed by Romeo, 1998). In *E. coli*, CsrA binds near the ribosomal binding site of the *glgC* and *cstA* mRNA transcripts, preventing their translation and promoting premature degradation (Liu and Romeo, 1997; Dubey *et al*., 2003). CsrA can also stabilize transcripts, including those of the master flagellar regulatory locus *flhDC* (Wei *et al*., 2001). Microarray analysis revealed that the CsrA of *Salmonella enterica* co-ordinately controls a host of metabolic pathways as well as virulence traits encoded by the SPI-1 pathogenicity island (Lawhon *et al*., 2003). In *E. coli*, CsrA activity is inhibited when the repressor is bound by either of the two untranslated regulatory RNAs known as *csrB* and *csrC*, which are induced by UvrY/BarA (LetA/S homologues). In *L. pneumophila*, every transmission trait that has been examined is repressed by CsrA (Table 1, Fig. 2B). Moreover, genetic inactivation of the repressor *csrA* bypasses the requirement for the *letA* inducer of the transmission phenotype (Fig. 2B; Molofsky and Swanson, 2003). Accordingly, by analogy with other Gram-negative bacteria, it is likely that LetA/S in *L. pneumophila* induces as yet unidentified *csrB*-like regulatory RNA(s) that bind and inhibit CsrA activity when nutrients are limiting, thereby inducing virulence

traits and perhaps metabolic pathways that promote transmission of *L. pneumophila*.

A subset of the CsrA-repressed traits requires the flagellar sigma factor FliA for its transcription (Fig. 2B). Specifically, CsrA represses and FliA activates the transmissive phase traits of motility: contact-dependent cytotoxicity and immediate evasion of lysosomes (Table 1; Fettes *et al*., 2001; Hammer *et al*., 2002; Heuner *et al*., 2002; Molofsky and Swanson, 2003). Accordingly, CsrA is predicted to inhibit either *fliA* mRNA stability or translation directly or *fliA* message production indirectly. An efficient mechanism for CsrA to control multiple transmissive traits would be to repress the master switch of the flagellar regulon in *L. pneumophila*, presumably RpoN and/or FleQ (Fettes *et al*., 2001; Heuner and Steinert, 2003; Jacobi *et al*., 2004), but this possibility has not been examined. As CsrA represses multiple phenotypes linked to *L. pneumophila* virulence, identification of the mRNA species that it targets is a viable approach to delineating the molecular mechanisms of *L. pneumophila* pathogenesis.

Dot/Icm regulation

The type IV secretion system remains the best characterized virulence factor of *L. pneumophila*; accordingly, its substrates and transcriptional regulation have been the focus of considerable investigative effort. Encoded by the defective in organelle transport/intracellular multiplication loci of *L. pneumophila*, the apparatus is postulated to secrete the virulence effectors that immediately isolate the pathogen vacuole from the endocytic pathway (reviewed by Sexton and Vogel, 2002). In addition to its effects on macrophage cell biology, the Dot/Icm complex translocates protein–plasmid DNA complexes between bacteria (Vogel *et al*., 1998).

Whether *L. pneumophila* express a functional type IV secretion apparatus in both the replicative and transmissive phase has not been established definitively. The efficiency of plasmid transfer by pure cultures of exponential and stationary phase *L. pneumophila* has not been reported, although it appears that bacteria replicating on solid agar fail to conjugate plasmid DNA (Segal and Shuman, 1998). A series of studies using *lacZ* translational fusions demonstrated that, of nine *dot/icm* loci examined, the products of five were more abundant in the stationary phase of broth cultures but, in each case, the effect was modest (Gal-Mor *et al*., 2002). Of these, only *icmP* expression was reduced by mutation of *relA*, *rpoS* or *letA* (Zusman *et al*., 2002; Gal-Mor and Segal, 2003a). Whether the two- to threefold increase in expression of a subset of the *dot/icm* genes that occurs when *L. pneumophila* enter the stationary phase in broth contributes to the dramatic concomitant increase in lysosome evasion has not been tested. Also, the effect of LetA on transcription and translation of particular *dot*/*icm* genes warrants more detailed study, as another group who analysed total RNA instead of reporter constructs concluded that LetA is a strong inducer of *dotA* expression (Lynch *et al*., 2003). A second activator, the periplasmic stress two-component response regulator CpxR, strongly induces expression of *icmR*, a non-structural gene of the secretion apparatus, and more modestly activates the *dotA*/*icmV* and *icmW*/*X* operons in broth cultures (Gal-Mor and Segal, 2003b). However, as *cpxR* mutants replicate efficiently in macrophages and amoebae (Gal-Mor and Segal, 2003b), *L. pneumophila* must encode another mechanism to generate sufficient *dot*/*icm* expression to establish its replication niche. Biochemical studies of the Dot/Icm complex throughout the life cycle in broth and phagocyte culture are needed to determine whether regulated synthesis or assembly of the secretion system accounts for the dramatically different intracellular fates of replicative and transmissive *L. pneumophila.*

One possibility is that the type IV secretion apparatus is synthesized constitutively, but the effector substrates are expressed solely during the transmission phase. Proteins likely to be translocated by the Dot/Icm system have been identified by a variety of genetic strategies, and the expression pattern of the proteins and the ability of the corresponding mutants to establish replication vacuoles has been examined. As predicted for virulence factors that govern phagosome maturation, the RalF, LidA and SidC proteins are each induced when *L. pneumophila* is cultured to the stationary phase in broth. Furthermore, when cultured in macrophages, all three proteins are translocated to the phagosomal membrane by a *dotA*-dependent process (Nagai and Roy, 2001; Nagai *et al*., 2002; Conover *et al*., 2003; Luo and Isberg, 2004). Nevertheless, none of these translocated proteins is required by the pathogen either to evade the endosomal network or to replicate in macrophages. Either the RalF, LidA and SidC proteins are redundant or they contribute to some other aspect of the pathogen's life cycle. The model of the genetic circuitry that controls the *L. pneumophila* life cycle (Fig. 2) can provide a conceptual framework for the design of experimental strategies to identify virulence factors that directly alter membrane traffic in phagocytes, which are predicted to be targets of FliA, CsrA and RpoS regulation.

Impact of differentiation on experimental design

Appreciation that the expression of transmission and replication traits by intracellular pathogens is probably reciprocal and also genetically separable can facilitate the design and interpretation of classical and molecular genetic analyses to identify their virulence mechanisms. Typically, experimentalists focus on those virulence determinants that have a measurable impact on replication, as

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judged by the slope of intracellular growth curve plots. Given the reciprocal pattern of the transmission and replication phases, this strategy risks discounting determinants that promote immediate bacterial adherence, entry and lysosome evasion, traits that are likely to be vital to the establishment of infection in human hosts. For example, *L. pneumophila* that lack the LetA/S or FliA positive activators, the Hsp60 or RtxA surface proteins or the FlaA flagellar filament protein have discernable phenotypes when initial host–pathogen interactions are quantified. Nevertheless, the minority of each of the mutant microbes that adhere and survive phagocytosis proceeds to replicate at wild-type rates (Garduno *et al*., 1998; Cirillo *et al*., 2001; Dietrich *et al*., 2001; Hammer *et al*., 2002; Heuner *et al*., 2002; Lynch *et al*., 2003). Therefore, unless early events are quantified, the contribution of this class of transmission factors to *L. pneumophila* pathogenesis cannot be appreciated. On the other hand, certain transmission defects do prevent subsequent intracellular growth: stationary phase *dotA* and *dotB* mutants of *L. pneumophila* fail to establish replication vacuoles and, instead, persist without replicating in a non-toxic endosomal compartment (Joshi *et al*., 2001). By designing specific assays to measure either transmission or replication, both classes of virulence factors can be identified.

In the post-genomic era, a comprehensive analysis of microbial adaptation to a particular environment can be achieved by applying powerful molecular genetic methods such as microarrays, proteomics, *in vitro* expression technology and signature-tagged mutagenesis. As *L. pneumophila* differentiates in response to nutrient levels, insight into the physiological pathways that distinguish its transmissive and replicative stages can be obtained by comparing synchronous exponential and stationary phase populations obtained from chemically defined medium. In contrast, the heterogeneity of cell populations harvested from agar will obscure the microbe's biphasic design. Whether candidate loci are indeed dedicated to one phase of the pathogen's life cycle can then be confirmed by more laborious analysis of phagocyte cultures. By incorporating knowledge of microbial differentiation into experimental design, the physiological patterns obtained by modern genomic methods can be brought into sharp focus to illuminate the mechanisms of microbial pathogenesis.

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