

# Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*

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## Summary

For intracellular pathogens such as *Salmonellae*, *Mycobacteriae* and *Brucellae*, infection requires adaptation to the intracellular environment of the phagocytic cell. The transition from extracellular to intravacuolar environment has been expected to involve a global modulation of bacterial gene expression, but the precise events have been difficult to determine. We now report the complete transcriptional profile of intracellular *Salmonella enterica* sv. Typhimurium following macrophage infection. During replication in murine macrophage-like J774-A.1 cells, 919 of 4451 *S. Typhimurium* genes showed significant changes in transcription. The expression profile identified alterations in numerous virulence and SOS response genes and revealed unexpected findings concerning the biology of the *Salmonella*–macrophage interaction. We observed that intracellular *Salmonella* are not starved for amino acids or iron (Fe<sup>2+</sup>), and that the intravacuolar environment is low in phosphate and magnesium but high in potassium. *S. Typhimurium* appears to be using the Entner–Doudoroff pathway to use gluconate and related sugars as a carbon source within macrophages. Almost half the *in vivo*-regulated genes were of unknown function, suggesting that intracellular growth involves novel macrophage-associated functions. This is the first report that identifies the whole set of *in vivo*-regulated genes for any bacterial pathogen during infection of mammalian cells.

## Introduction

Infectious diseases are the result of a co-ordinated battle between pathogens and their host, in which professional

phagocytic cells provide a central innate defence barrier. Macrophages and dendritic cells have the ability to express antibacterial factors, such as reactive oxygen, nitrogen species (Mastroeni *et al.*, 2000; Vazquez-Torres *et al.*, 2000) and antibacterial peptides (Ernst *et al.*, 1999). These cells also communicate with the adaptive immune system through antigen presentation and cytokine release (Mastroeni, 2002). In spite of these defence mechanisms, the infection pathogenesis of several clinically important bacterial pathogens includes phases when the pathogen successfully proliferates inside macrophages (Baldwin *et al.*, 1993; Xu *et al.*, 1994; Richter-Dahlfors *et al.*, 1997). Many serovars of *Salmonella enterica* behave as facultative intracellular parasites, and *S. enterica* sv. Typhimurium has evolved to survive and replicate within host cells by deploying specific sets of genes (Lucas and Lee, 2000; Marcus *et al.*, 2000; Garcia-del Portillo, 2001).

The biology of the *S. Typhimurium*–macrophage interaction has been dissected by combining cellular, genetic and molecular approaches. The rapid formation of a modified phagolysosome, known as the *Salmonella*-containing vacuole (SCV), represents a central feature in the intracellular survival and growth of these bacteria (Mills and Finlay, 1998; Garcia-del Portillo, 2001). Compared with the classical phagolysosome, SCVs have reduced antibacterial activity and are less acidic, but are still limiting for certain nutrients such as aromatic amino acids and purine bases (Hoiseth and Stocker, 1981; Fields *et al.*, 1986; Alpuche Aranda *et al.*, 1992; Buchmeier and Libby, 1997; Gallois *et al.*, 2001; Garcia-del Portillo, 2001; Chakravorty *et al.*, 2002). Intracellular replication and the creation of the SCV is bacteria directed and dependent on the expression of specific bacterial virulence functions, particularly those coded within the *Salmonella* pathogenicity islands (SPIs), and the virulence plasmid pSLT (Gulig *et al.*, 1993; Beuzón *et al.*, 2000; Marcus *et al.*, 2000; Gallois *et al.*, 2001; Chakravorty *et al.*, 2002). Additionally, intracellular replication of *Salmonella* requires the presence of selected metabolic genes (Hoiseth and Stocker, 1981; Fields *et al.*, 1986). Despite the attempts to avoid the antibacterial activities generated by the macrophage, *Salmonella* does suffer damage to central bacterial components, as demonstrated by the requirements of stress responses, recombination and SOS systems for virulence (Buchmeier *et al.*, 1993; De Groote *et al.*, 1997). *S. Typhimurium* does not generally express factors

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specifically required for pathogenesis when grown *in vitro* and has a remarkable ability to control the expression of virulence genes in response to environmental change (Mahan *et al.*, 1993; Hensel *et al.*, 1995; Valdivia and Falkow, 1997; Deiwick *et al.*, 1999; Pfeifer *et al.*, 1999; Lucas and Lee, 2000; Knodler *et al.*, 2002). What has been missing from our understanding of *Salmonella* pathogenesis is a complete picture of the bacterial gene regulatory processes that occur during infection of host cells. Until now, *in vivo* expression studies have relied on the use of reporter gene fusions rather than the direct analysis of mRNA. DNA microarrays are now the tool of choice for studying global gene expression in bacteria, but it has not been possible to use this technology to study *in vivo* regulation of gene expression in bacterial pathogens. This has become the 'ultimate goal of whole-genome expression studies' but has remained 'technically formidable' (Schoolnik, 2002). We have developed a new method for extracting bacterial RNA from infected cells, and used DNA microarray technology to provide a global transcriptional profile of the response of *S. Typhimurium* during the intracellular growth phase of infection pathogenesis. The results reveal that almost a quarter of the genome shows changes in expression during infection of murine macrophages, and this brings new insights into the process of *Salmonella* infection. The new set of *in vivo*-regulated *Salmonella* genes reported here promises to aid the understanding of the macrophage as both a growth niche and a component of the host defence systems, and to further our insight into the biology of this key host-pathogen interaction.

## Results and discussion

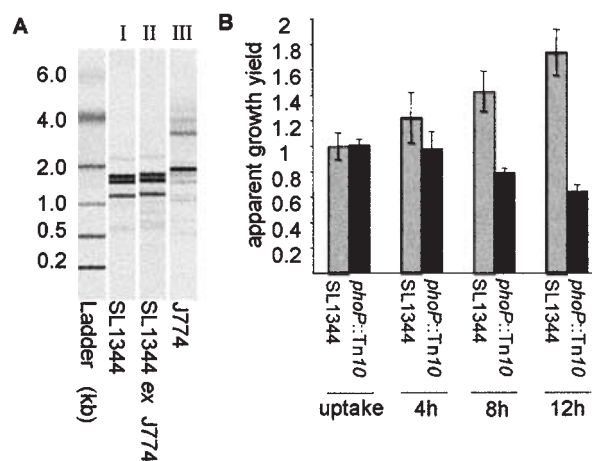
### Infection model and RNA isolation

We constructed a whole-genome *S. Typhimurium* microarray (Lucchini *et al.*, 2001; Thompson *et al.*, 2001) and screened for genes that showed changes in expression during bacterial intracellular growth in the SCV. In these experiments, J774-A.1 macrophage-like cells were infected with complement-opsonized *S. Typhimurium* SL1344 (De Groote *et al.*, 1997; Garvis *et al.*, 2001; Vazquez-Torres and Fang, 2001). The use of complement opsonization of non-invasive bacteria (Eriksson *et al.*, 2000) was chosen to maximize bacterial uptake, while minimizing the early induction of cytotoxicity (Monack *et al.*, 2001). J774-A.1 cells were used because they display a killing capacity that is comparable to BALB/c mouse peritoneal macrophages, but are much less efficient in limiting intracellular bacterial replication (Buchmeier and Libby, 1997). A prerequisite for the experiments was the development of a method to isolate bacterial RNA from infected macrophages. Our protocol involved two key fea-

tures: (i) immediate stabilization of bacterial RNA, resulting in minimal degradation and only traces of contaminating eukaryotic RNA (Fig. 1A); and (ii) the use of a detergent to disrupt the eukaryotic cells that did not affect the integrity of the bacterial membrane. Bacterial RNA was extracted at 4 h, 8 h and 12 h after infection from the infected cells to determine the temporal order of expression of *S. Typhimurium* genes during infection. The extracted RNA was labelled and hybridized to the *S. Typhimurium* microarray. The time points were chosen to reflect the events that follow phagocytosis of *S. Typhimurium* according to the literature. In J774-A.1 cells, the initial host defence system, such as the oxidative burst, is activated at 1 h after infection (Goldman, 1990; Tsolis *et al.*, 1995a). The maturation of the SCV is completed by 4 h after phagocytosis, after which bacterial replication begins (Mills and Finlay, 1998; Garcia-del Portillo, 2001). This is followed by an induction of macrophage nitric oxide (NO) production, starting around 8 h after infection (Eriksson *et al.*, 2000). The net yield of SL1344 in our experiment increased from 4 h up to 12 h, in accordance with the literature cited above. In comparison, the isogenic *phoP*-deficient strain had decreased growth yields throughout the experiment (Fig. 1B).

### The global transcriptional profile

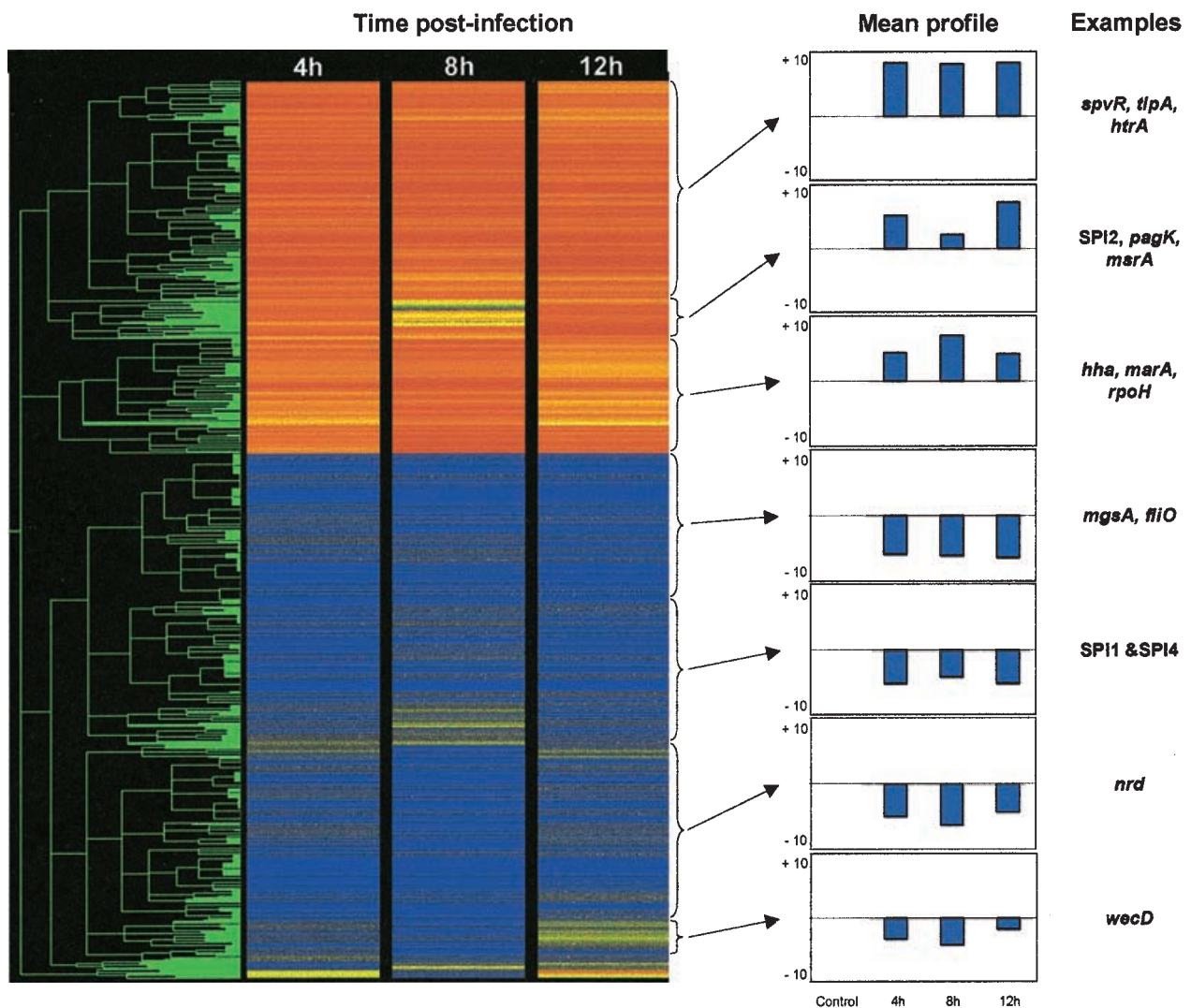
The gene expression profiles obtained from intracellular bacteria were compared with the results from opsonized bacteria grown in cell culture medium, and revealed that



**Fig. 1.** A. Size chromatographic separation of RNA. Total RNA was extracted from (I) *S. Typhimurium* grown in defined media *in vitro*, (II) *S. Typhimurium* from infected J774-A.1 cells and (III) J774-A.1 cells alone. B. Growth of *S. Typhimurium* SL1344 within J774-A.1 cells. Apparent growth yields are defined as the intracellular load/uptake (2 h). The values obtained from J774-A.1 cells infected with *S. Typhimurium* are shown in grey; cells infected with an isogenic *phoP::Tn10* mutant are in black. Data are given as means; error bars indicate standard deviation.

919 of 4451 *S. Typhimurium* coding sequences (CDS) showed changes in expression during infection of murine macrophages (Fig. 2; see *Supplementary material*, Tables S1 and S2). To illustrate the scale of changes, the *mgtB* magnesium transport gene was induced by 50-fold (Fig. 3), whereas the *sipC* invasion gene was downregulated by 50-fold. About 44% of the *in vivo*-regulated genes represented CDS of unknown function. Additionally, 19% of the genes on the transcriptionally dormant virulence plasmid pSLT were induced for expression. We confirmed that the expression profile did not simply reflect leakage of low levels of gentamicin into the SCV during infection by performing a control microarray experiment; growth-inhibitory but sublethal levels of gentamicin altered the

expression of only a few genes, and none of these showed similar patterns of up- or downregulation to the 919 *in vivo*-regulated genes (data not shown). We were keen to understand whether the *in vivo*-regulated genes simply reflected a change in growth phase upon infection. Therefore, we compared the expression profile seen at 4 h after infection with the expression profile obtained for bacteria grown to stationary phase *in vitro* (Fig. 4). We observed that a small proportion of the genes induced at 4 h after infection were also induced during stationary phase. However, the fact that the majority of the stationary phase-induced genes were not upregulated shows that intracellular bacteria are not in classical stationary phase.



**Fig. 2.** Expression profile of *S. Typhimurium* genes that show altered mRNA levels during infection. The cluster diagram shows the expression profile of *S. Typhimurium* growing within J774-A.1 cells for 4 h, 8 h and 12 h relative to control bacteria. Each horizontal line represents one gene; red indicates a minimum twofold increase in expression, yellow indicates no change, and blue indicates a minimum twofold decrease in expression. Selected expression profiles and examples of genes expressed with similar patterns are shown graphically. The vertical axis shows the fold change in expression for each gene on a logarithmic scale.



and the genes encoding the SPI2 effectors were strongly induced (Fig. 3). Similarly, the *mgtBC*, *cigR*, *marR*, *slsA* and *fidL* genes of SPI3 and the *pipABD* genes of SPI5 were induced at 4 h after infection (Fig. 3). A number of the induced SPI2, SPI3 and SPI5 genes are essential for intracellular survival and growth of *Salmonella* (Shea *et al.*, 1996; Lucas and Lee, 2000; Marcus *et al.*, 2000; Garcia-del Portillo, 2001). The manganese acquisition genes *sitABCD* of SPI1 were initially repressed, followed by a derepression at 12 h (Kehres *et al.*, 2002). In addition, the *pagCK* genes were induced during later stages of the infection. Conversely, reduced expression was observed at all time points for most of the invasion-associated SPI1 genes (including genetically unlinked SPI1 effector protein genes), the SPI4 genes and *pipC* encoded by SPI5 (Fig. 3). These microarray data agreed with the predicted regulation of pathogenicity islands within host cells (Ochman *et al.*, 1996; Hensel *et al.*, 1998; Smith *et al.*, 1998; Deiwick *et al.*, 1999; Heithoff *et al.*, 1999; Pfeifer *et al.*, 1999; Beuzón *et al.*, 2000; Lucas and Lee, 2000; Marcus *et al.*, 2000; Garcia-del Portillo, 2001; Knodler *et al.*, 2002).

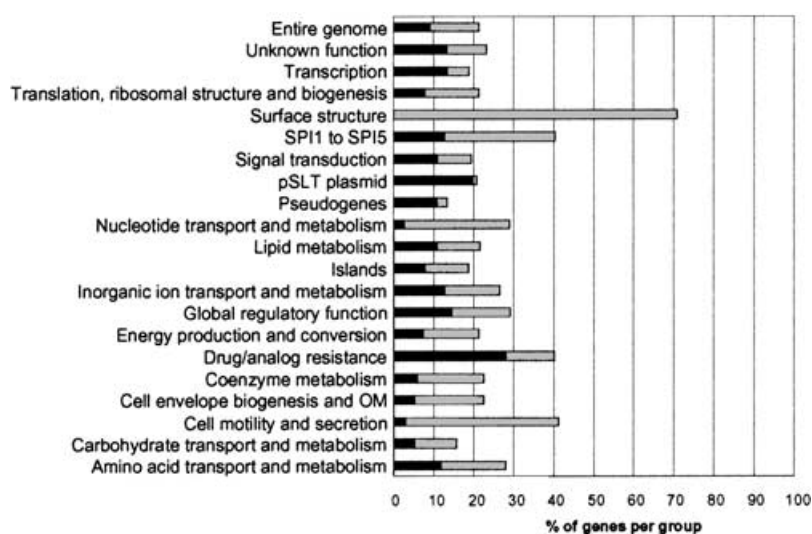
The lipopolysaccharide (LPS) is a constituent of the outer membrane of Gram-negative bacteria and, apart from being an essential structural component, these glycolipids are also well-defined virulence factors. The structure and decoration of the LPS is strictly regulated, and it has been reported that the composition of the LPS of *S. Typhimurium* is modulated *in vivo* (Garcia-del Portillo *et al.*, 1997; Guo *et al.*, 1997). We found three main gene clusters coding for LPS synthesis that showed reduced expression; the *wecDE*, *waa* (*rfa*) and *rfb* genes were downregulated at 4 h (Fig. 2), in agreement with Maurer *et al.* (2000).

#### The virulence plasmid

*Salmonella Typhimurium* carries a 94 kb virulence-associated plasmid (pSLT). Most of the 108 CDS encoded by pSLT are not strongly expressed *in vitro* (Koski *et al.*, 1992) and are of unknown function (McClelland *et al.*, 2001). The microarray analysis revealed that, during intracellular bacterial growth, 20 out of 108 pSLT genes were induced, and one was repressed for expression (Fig. 5). Three of the induced loci have a defined or proposed virulence-associated function (Gulig *et al.*, 1993; Rhen *et al.*, 1993; Baumlér *et al.*, 1996; Hurme *et al.*, 1997). The best characterized is the *spvRABCD* gene cluster, which is known to be induced for expression in intracellular bacteria and required for intracellular replication and full virulence (Gulig *et al.*, 1993; Heithoff *et al.*, 1999). During intracellular growth, expression of *spvRA* was induced at 4 h after infection (Figs 2 and 3), and induction of *spvBC* was observed at later time points. These observations agree with previous studies regarding *spv* induction in J774-A.1 cells (Rhen *et al.*, 1993). In addition, the temperature-inducible regulatory gene *tlpA* was significantly induced (Fig. 2; Hurme *et al.*, 1997), as were the *pef* genes coding for virulence-associated fimbriae (Baumlér *et al.*, 1996). The intracellular induction profile of pSLT suggests that there are new inducing conditions present in the SCV that remain to be identified.

#### Regulatory gene expression

Regulation of bacterial gene expression is mediated by proteins that act globally or at specific DNA regions to activate or repress transcription or modulate DNA topology. We observed that most global regulatory genes of *Salmonella* did not show changes at the level of transcription during macrophage infection.



**Fig. 5.** Expression changes of genes belonging to functional groups and pathogenicity islands (McClelland *et al.*, 2001; Tatusov *et al.*, 2001). The bars show the percentage of genes belonging to each group that are altered for expression inside J774-A.1 cells at any time point during infection. The grey bars indicate the proportion of genes that are downregulated, and the black bars represent the proportion of upregulated genes for each group.

A number of DNA-binding proteins are responsible for the structure of the bacterial nucleoid. The binding of these nucleoid-associated proteins alters DNA topology and influences transcription, giving these proteins distinct regulatory roles (Azam and Ishihama, 1999). We found that the genes coding for the nucleoid-associated proteins H-NS, StpA, Fis and Lrp were downregulated *in vivo*. In contrast, we detected induction of the *hha* gene during macrophage infection (Fig. 2). The Hha protein is able to bind to DNA and to the H-NS protein, and has been reported to repress SPI1 (Fahlen *et al.*, 2001); our data suggest that Hha plays a role in regulating *Salmonella* gene expression *in vivo*.

Alternative sigma factors play a crucial role in responses to environmental stress by enabling transcription of appropriate subsets of genes. Surprisingly, we observed only a modest (twofold) increase in the gene coding for the virulence-associated sigma factor RpoS, perhaps reflecting post-transcriptional regulation (Cunning and Elliott, 1999); however, it is possible that insufficient RpoS function could be compensated by other sigma factors during intracellular bacterial growth. For example, the genes coding for RpoE and RpoH were both upregulated for expression, as was the RpoE-dependent *htrA* gene, which encodes a stress-induced protease. The importance of HtrA during infection has been confirmed by experiments showing that deletion of *htrA* leads to reduced replication within macrophages and hence attenuation of virulence (Chatfield *et al.*, 1992). RpoE is known to have some functional overlap with RpoS and is also required for full virulence of *Salmonella* (Humphreys *et al.*, 1999).

One of the master regulators of intracellular *Salmonella* gene expression is the PhoPQ two-component system. PhoPQ responds to the environmental concentration of Ca<sup>2+</sup> and/or Mg<sup>2+</sup>, and directly or indirectly affects the expression of several known virulence functions (Ernst *et al.*, 1999; Groisman, 2001). Expression of the *phoP* gene was not increased in intracellular bacteria, although we did observe the expected PhoPQ-regulated control of PhoP-activated genes (*pag*; examples include *mgtBC* and genes encoded by SPI2) and PhoP-repressed genes (*prg*; examples include genes encoded by SPI1). Therefore, PhoPQ regulation was functional in our experiment, as confirmed by the ablation of intracellular growth of a *phoP* mutant of SL1344 observed in our infection model (Fig. 1B). It has been reported that PhoP activates *phoPQ* during logarithmic growth (Soncini *et al.*, 1995), but the situation may be more complex *in vivo*. Perhaps the lack of *phoP* induction reflects the unique physiological state of intracellular bacteria. In summary, PhoP does play an essential role in intracellular replication, but we did not detect an increased amount of *phoP* transcript.

The two global regulator genes that showed the strongest *in vivo* repression were *cya* (coding for the enzyme adenylate cyclase, which generates cAMP) and *crp* (coding for cAMP receptor protein CRP). The cAMP–CRP complex regulates the starvation stress response, and deletion of either *cya* or *crp* causes attenuation of virulence (Curtiss and Kelly, 1987). The cAMP–CRP complex activates many catabolite-repressed systems for carbohydrate utilization and regulates some virulence genes. In the LT2 background, the pSLT-encoded *spv* operon is repressed by the cAMP–CRP complex (O'Byrne and Dorman, 1994), and we have now confirmed that *spv* is induced *in vivo* (Fig. 3). As the cAMP–CRP complex was thought to be induced upon starvation, this represents an apparent paradox. Such a response to the cAMP–CRP complex is also seen in *Vibrio cholerae*, in which the expression of cholera toxin and toxin co-regulated pilus is repressed by the cAMP–CRP complex (Skorupski and Taylor, 1997). Our finding that *cya/crp* were strongly downregulated within the host environment shows that *cya* and *crp* belong to the group of genes that is required for virulence, and are either not induced or are repressed for expression inside macrophages.

#### *Effects of free radical production; superoxide and NO*

Innate defence responses of the phagocytic cell involve reactive oxygen and nitrogen species. Macrophages express two major enzymes involved in free radical production: NADPH phagocyte oxidase (Phox), which produces superoxide, and inducible nitric oxide synthase (iNOS), which produces NO (Mastroeni *et al.*, 2000; Vazquez-Torres *et al.*, 2000). These free radical molecules damage key bacterial components by oxidizing nucleotides, nitrosylating proteins and damaging membranes. The pivotal role of reactive oxygen and nitrogen species in salmonellosis has been confirmed *in vivo* using single and double knock-out mice lacking Phox and/or iNOS (Mastroeni *et al.*, 2000; Vazquez-Torres *et al.*, 2000; Vazquez-Torres and Fang, 2001). Strategies used by *Salmonella* to avoid reactive substances involve the enzymatic destruction of radicals or interference with the localization of Phox or iNOS.

As mentioned above, SPI2 effector genes were upregulated for expression *in vivo*, consistent with the role of these effector proteins in limiting the fusion of Phox and iNOS with the SCV to separate bacteria from toxic free radicals spatially (Gallois *et al.*, 2001; Chakravorty *et al.*, 2002). To determine whether *Salmonella* showed an oxidative stress response during macrophage infection, we compared the intracellular transcription profile of *Salmonella* with the whole-genome expression profile obtained from *Escherichia coli* treated with hydrogen peroxide *in vitro* (Zheng *et al.*, 2001). From a list of the 30 *E. coli*

genes most profoundly induced by oxidative stress, we found only six of these to be induced in intracellular *Salmonella*. These included *soxS*, *ibpA*, *ycfR*, *trxC*, *ibpB* and *sbp*. The fact that the majority of the genes in the oxidative stress regulon were not upregulated probably reflected the rapid induction of SPI2 genes after *Salmonella* infection (Fig. 3). Nevertheless, these data show that *S. Typhimurium* experiences some degree of oxidative stress during growth in J774-A.1 cells.

The inducible systems that protect against oxidative stress are either OxyR or SoxRS regulated and can be switched on by hydrogen peroxide and superoxide respectively (Carmel-Harel and Storz, 2000). The *Salmonella*-mediated detoxification of superoxide is a two-step process; the first step involves conversion of superoxide to hydrogen peroxide by superoxide dismutases, and the second step is a catalase-mediated destruction of hydrogen peroxide. Induction of the first step for breakdown of superoxide is shown by the increased expression of *sodB*, which codes for iron superoxide dismutase. In addition, we detected an induction of *sodC* (at 12 h), which encodes SodCI on the Gifsy-2 prophage, but not of the *sodC* gene, which encodes SodCII. These data fit with the recent report of the role of Cu, Zn-superoxide dismutases in infection; SodCI, but not SodCII, is known to be induced in macrophages and required for virulence in mice (Uzzau *et al.*, 2002). However, we did not detect increased transcription of the *kat* genes, which encode catalases. The consequent accumulation of hydrogen peroxide will activate the regulator OxyR, which would be predicted to lead to the transcriptional activation of genes such as those involved in the thioredoxin and glutaredoxin systems. The role of these systems is to restore protein function by reducing oxidized residues (Aslund and Beckwith, 1999). The two pathways exhibit redundancy, but some protein substrates are specific for one or the other. We found induction of the OxyR-dependent *trxC* gene, encoding thioredoxin-2. However, other OxyR-regulated genes were not induced, and the glutaredoxin pathway was downregulated. One of the enzymes specifically reduced by the thioredoxin pathway is methionine sulphoxide reductase (*msrA*; Aslund and Beckwith, 1999). MsrA has been implicated in the virulence of several pathogens (Wizemann *et al.*, 1996; St John *et al.*, 2001), and we note that the pattern of induction of *msrA* resembled that of SPI2. These data indicate that *Salmonella* experiences an intracellular oxidative stress in J774-A.1 cells, and that *Salmonella* favours the thioredoxin pathway over the glutaredoxin pathway for maintenance of redox potential and for repairing damaged proteins when inside macrophages.

Another protein implicated in maintaining redox potential is the flavohaemoglobin HmpA. Expression of *hmpA* is repressed by Fur in *Salmonella*, making it responsive

to iron concentration. The effect of iron availability on *hmpA* expression is negated by macrophage-derived defence factors; NO is believed to mimic iron depletion by destroying the Fe–Fur complexes leading to derepression, even in the presence of iron (Crawford and Goldberg, 1998). We observed a drastic repression of *hmpA* expression at 4 h after infection, followed by induction at 8 h. This induction coincides with the induction of NO synthesis in J774-A.1 cells (Eriksson *et al.*, 2000). As the array data show that intracellular *Salmonella* are not starved for iron (see below), induction of *hmpA* must reflect exposure of the bacteria to NO. This would fit with the recent observation that inactivation of the *S. Typhimurium* HmpA protein results in decreased resistance to NO and reduced intracellular bacterial yields in the macrophage (Stevanin *et al.*, 2002). Furthermore, we found that other genes coding for flavin-containing oxidoreductases were upregulated for expression during infection (STM2839–2841). The *E. coli* homologues of these genes (*norRVW*) have recently been demonstrated to metabolize NO (Gardner *et al.*, 2002). These data offer clues to a mechanism for *in vivo* detoxification of NO by *S. Typhimurium*.

#### The SOS response

Free radicals are potent inducers of bacterial SOS responses *in vitro* (Buchmeier *et al.*, 1993; Khil and Camerini-Otero, 2002; Motohashi and Saito, 2002). Our microarray analyses show a moderate induction of the SOS response, exemplified by the genes *recA*, *umuCD*, *uvrABY*, *sulA* and *mutH*. Induction of the SOS response would normally be linked to a cessation of bacterial replication caused by the SulA protein (Clerch *et al.*, 1996). However, the repressor protein for *sulA*, encoded by *lexA*, showed a similar induction of expression, suggesting a balancing role for LexA in the activation and suppression of the SOS response. At the same time, we detected strong induction of the gene encoding DinI. DinI interferes with RecA-induced cleavage of LexA and hence functions to switch off the SOS response (Yasuda *et al.*, 2001). Thus, for intracellular *Salmonella*, the SOS response appears to be expressed at a level that facilitates both bacterial replication and DNA repair. One consequence of the replication of damaged DNA would be an increased mutation rate, which has been reported to occur *in vivo* (Björkman *et al.*, 2000).

#### Phagosomal acidity

Sudden exposure of *S. Typhimurium* to low pH *in vitro* induces the acid tolerance response (ATR), which allows bacteria to survive further decreases in pH as well as additional stress conditions. This induction is dependent on global gene regulators, such as OmpR, PhoP and

RpoS (Audia *et al.*, 2001). The pH in the SCV of J774-A.1 cells is estimated to decrease from 5.5 to 4.9 between 2 h and 6 h after infection, making the SCV a moderately acidic compartment (Alpuche-Aranda *et al.*, 1992). We screened the microarray expression data for genes that are known to be acid inducible and discovered upregulation of the cadaverine transporter gene *cadB* (Park *et al.*, 1996) and the activators of arginine decarboxylase, *cysB* and *adiY* (Lin *et al.*, 1996). We observed induction of the genes *marAB* and *emrAB*, which suggests that the low pH of the SCV caused an accumulation of weak acids by *Salmonella* (Slonczewski *et al.*, 1987; Lomovskaya *et al.*, 1995). Taken together, these data confirm the acidity of the SCV. However, we did not observe significant increases in ATR regulators such as *ompR*, *phoP* or *rpoS*. We conclude that either the regulation occurs post-translationally or the decrease in intravacuolar pH is not sudden enough, or acidic enough, to induce a typical ATR.

#### Salmonella metabolism

The observation that many auxotrophic mutants of *Salmonella* fail to grow intracellularly and are attenuated for virulence has led to the suggestion that intracellular bacteria rely on *de novo* synthesis of selected macromolecular precursors once inside host cells (Hoiseh and Stocker, 1981; Fields *et al.*, 1986). We analysed the expression profiles of a number of functional categories of genes involved in *de novo* synthesis or in the transport of metabolites. Generally, these were not regulated at the level of gene expression, the exception being the *aro* genes, which were downregulated up to 10-fold during intracellular growth. This was surprising considering that aromatic amino acid synthesis is essential for virulence (Hoiseh and Stocker, 1981). The intracellular bacteria appeared to experience some nutritional stress around 12 h; the expression of several stringently regulated amino acid biosynthetic operons was slightly upregulated, and the majority of ribosomal proteins were repressed to a small degree. The genes coding for components of transcription and translation were not altered for expression. In contrast, genes coding for purine and pyrimidine synthesis, as well as ribonucleotide reductases (*nrd*; Fig. 2), were downregulated, which could be connected to the slow replication rate observed in macrophages (Garcia-del Portillo, 2001).

We also analysed the expression levels of genes involved in sugar catabolism to gain insight into the carbon sources that were available intracellularly. *S. Typhimurium* has three catabolic pathways for intermediary sugar metabolism: the Embden–Meyerhof, the pentose phosphate and the Entner–Doudoroff pathways (Fraenkel, 1996). The genes involved in the first two pathways were repressed for expression by up to 85-fold. In contrast, the

genes coding for the degradative enzymes of the Entner–Doudoroff pathway were not generally altered for expression. However, the genes coding for the transport of gluconate, glucuronate/galacturonate and galactonate permeases (*gntT*; STM3134; *dgoT*) were upregulated, as were genes involved in the interconversion of these sugars to pyruvate and glyceraldehyde-3-phosphate (*dgoK* and *dgoA*). These data suggest that gluconate and related carbohydrates may be a principal source of carbon for growth of intracellular bacteria. The Entner–Doudoroff pathway may also be acting as a rich source of NADPH for use in biosynthetic pathways and redox cycling (Fraenkel, 1996).

#### Ion transport

The presence and absence of divalent cations have an impact on bacterial sensing and gene responses. We analysed the expression of ion transporters to establish which inorganic molecules were limiting in the SCV (Lucas and Lee, 2000; Groisman, 2001). As has been reported previously, the *mgtBC* genes involved in  $Mg^{2+}$  transport were highly upregulated at 4 h after infection in J774-A.1 cells (Smith *et al.*, 1998). Given our current understanding of the inducibility of the  $Mg^{2+}$  transport system, we can infer that the level of  $Mg^{2+}$  in the SCV is limiting (Snavelly *et al.*, 1989; Tao *et al.*, 1998). Because the microarray data are expressed in relation to the control sample grown in RPMI medium, which has a reference concentration of  $Mg^{2+}$  of 405  $\mu M$ , we can predict the  $Mg^{2+}$  concentration in the SCV to be significantly lower. The *pst* genes involved in inorganic phosphate transport were strongly upregulated 4 h after infection. At the same time, phosphate starvation-responsive loci, such as the *phoBR* genes, were substantially upregulated (Lucas and Lee, 2000). Comparison of these data with the literature suggests that phosphate levels in the SCV are limiting, and this is supported by the fact that SPI2 is induced under low phosphate conditions (Deiwick *et al.*, 1999). It is possible that many of the changes observed, such as decreased expression of genes involved in DNA replication, LPS synthesis or decreased replication in macrophages, could be a consequence of this phosphate starvation. We found that the genes encoding the defined transport systems for potassium were not induced, suggesting that high levels of potassium are present in the SCV, as has been reported for the phagocytic vacuole of neutrophils (Reeves *et al.*, 2002). We conclude that  $Mg^{2+}$  limitation, phosphate limitation and high levels of potassium are important signals for intracellular *Salmonella*.

Bacteria possess systems to sense iron and to regulate the expression of proteins involved in iron acquisition, storage and utilization. In the presence of high iron con-

centrations, the Fur protein binds to  $\text{Fe}^{2+}$  and represses the expression of genes involved in iron uptake in *Salmonella* (Tsolis *et al.*, 1995b). The Fur-dependent *entABCE* and *iroDEN* genes were highly downregulated during intracellular growth, suggesting that the SCV of J774-A.1 cells contain more than 5–10  $\mu\text{M}$   $\text{Fe}^{2+}$  (Tsolis *et al.*, 1995b; Heithoff *et al.*, 1999). An alternative iron-responsive mechanism involves the PmrAB two-component system, which is induced by high levels of  $\text{Fe}^{3+}$  (Wösten *et al.*, 2000). Our data show that expression of the PmrAB-dependent *pmrA* (*basR*), *pmrB* (*basS*), *pmrD*, *pmrF* (*pbgP*) and *ugd* genes is downregulated intracellularly, reaching a minimum at 8 h after infection. This suggests that repression by Fur is occurring *in vivo*, and that the SCV is rich in  $\text{Fe}^{2+}$  but does not contain significant levels of  $\text{Fe}^{3+}$ . The high  $\text{Fe}^{2+}$  content might reflect the fact that J774-A.1 cells are derived from an Nramp1<sup>-</sup> (BALB/c) background and are hence potentially defective in intracellular iron translocation (Wyllie *et al.*, 2002). Our finding that genes involved in iron acquisition are not induced during J774-A.1 macrophage infection is consistent with the report that *Salmonella* mutants defective in the *ent* gene were fully virulent after intraperitoneal infection of mice (Benjamin *et al.*, 1985).

#### Resistance to antibacterial peptides

Phagocytic cells produce vacuolar antibacterial peptides active against *Salmonellae*, and these have been found in murine macrophage cell lines such as RAW264.7 and J774-A.1 (Hiemstra *et al.*, 1993; 1999). Several loci have been described in *S. Typhimurium* that confer resistance to the peptide antibiotic polymyxin and/or antibacterial peptides. These include the *sap* (Parra-Lopez *et al.*, 1994), *pmr* (Wösten *et al.*, 2000), *phoPQ* (Groisman *et al.*, 1997; Groisman, 2001) and *pgtE* genes (Guina *et al.*, 2000), implying that intracellular *Salmonella* encounter antibacterial peptides *in vivo* and need to express resistance factors to survive. The PhoP/Q two-component system functions as a master regulator for many of these resistance responses, including the PmrAB regulatory system (Groisman, 2001). The microarray analyses showed a clear induction of the *pgtE* gene, an outer membrane protease that is involved in resistance to antibacterial peptides (Guina *et al.*, 2000). The expression of *pgtE* is not directly dependent on PhoPQ, but the translocation of PgtE to the outer membrane does require PhoPQ activity (Guina *et al.*, 2000). As described previously, the microarray data showed repression of the PmrAB-regulated *pmrF* gene. Collectively, these data show that PhoP/Q regulation is active, and that PmrAB regulation is inactive. Furthermore, these data agree with the suggestion that *pmrAB*-mediated peptide tolerance is not required within the parenteral space but is important

during the intestinal phase of the infection when free iron concentrations are thought to be higher (Gunn *et al.*, 2000). We propose that expression of the *pgtE* protease protects intravacuolar bacteria against antibacterial peptides in the SCV.

#### Flagella, fimbriae and chemotaxis

*Salmonella* produce flagella and fimbriae, which facilitate the movement of bacterial cells against a nutrient gradient and adhesion respectively. We discovered that the genes coding for the flagellar machinery, as well as many genes involved in chemotaxis, were strongly downregulated for expression during macrophage infection (Figs 2 and 5). Apart from the obvious conclusion that motility is not required for intracellular proliferation, it is interesting to note that flagellin is the ligand for the proinflammatory Toll-like receptor 5 (Hayashi *et al.*, 2001), suggesting that the downregulation of flagella synthesis could reduce the innate response to *Salmonella*.

Fimbriae and their respective adhesins mediate adhesion to various surfaces, including mammalian cells. The four major fimbrial operons, *fim*, *lpf*, *pef* and *agf*, all contribute to virulence, but mutants lacking only one of these show little change in virulence (van der Velden *et al.*, 1998). We found a drastic reduction in the expression of *fimA*, encoding the major subunit for type 1 fimbriae, indicating that these are not expressed within macrophages. In contrast, the *csg* operon, which codes for the AgfA fimbrial fibres, was induced for expression, as was the *pef* operon (mentioned above). In summary, the flagella and the type 1 fimbriae were downregulated for expression during infection, which may reflect an attempt to limit immune recognition by modulating the surface structures of *Salmonella*.

#### Oxygen status of the SCV

We examined the expression profiles of genes known to be regulated by the level of oxygen to determine the conditions within the SCV. The genes encoding the microaerophilic cytochrome *bd* oxidase were downregulated for expression. We did not see upregulation of FNR or ArcA, the two major regulators responding to anaerobic conditions, or other anaerobically induced genes (Gunsalus and Park, 1994). In contrast, enzymes known to be expressed only in aerobically growing cells, such as the succinate dehydrogenase operon, were induced. From these data, we conclude that oxygen is freely available in the SCV.

Aerobic respiration drives active transport of protons ( $\text{H}^+$ ) to the extracellular environment, generating the  $\text{H}^+$  gradient used to fuel transport into the bacterium and to

provide energy for vital ATP synthesis. As discussed above, the *in vivo* expression profile of *S. Typhimurium* suggested that aerobic respiration was occurring, but we also observed that the genes encoding the components of ATP synthase were repressed by as much as 10-fold. Under acidic conditions, respiration can occur without stoichiometric generation of ATP, and individual components of the respiratory chain are then expressed at a level that balances the rate of proton leakage (Nicholls and Ferguson, 1992). If this was the case, we hypothesize that the observed decrease in flagella expression *in vivo* would allow *Salmonella* to block proton influx through both the H<sup>+</sup>/ATPase and the flagellar base structure. This idea is supported by the recently observed decrease in flagellin protein expression by *Salmonella* at low pH *in vitro* (Adams *et al.*, 2001).

An increase in the ADP:ATP ratio is a direct consequence of the downregulation of ATP synthesis. An important consequence of changing the ADP:ATP ratio is an effect on DNA supercoiling; when ATP levels are reduced, the ability of DNA gyrase to generate negative supercoils in DNA is inhibited (Drlica, 1992). The level of DNA supercoiling in *S. Typhimurium* has been reported to become more relaxed during growth in J774-A.1 cells (Marshall *et al.*, 2000). Consequently, changes in the level of DNA supercoiling may be modulating gene expression intracellularly and could provide a mechanism for global *in vivo* gene regulation.

#### *FUN* genes

Genomic sequence analyses of eubacteria have revealed a high proportion of genes of unknown function (FUN; Hinton, 1997). Our definition of FUN genes includes those with a putative function that has only been assigned by homology to other genomes, without biological evidence. The *in vivo* expression profile revealed that 408 FUN genes showed altered expression during growth in macrophages. This set of *in vivo*-regulated FUN genes should lead to the identification of new virulence-associated functions for *Salmonella* and could prove to be the most important legacy of this work.

We determined whether the *in vivo*-regulated *Salmonella* FUN genes were more likely to be conserved between several facultative intracellular pathogens (various *S. enterica* serovars, *Mycobacterium tuberculosis* and *Brucella melitensis*). The rationale behind this was that many of these conserved FUN genes might be directly involved in adaptation to the intracellular environment or might reflect conserved virulence genes. We found no link between interspecies conservation and *in vivo* regulation.

We identified two FUN regions in the genome sequence of *S. Typhimurium* LT2 that shared structural features with pathogenicity islands (McClelland *et al.*, 2001) and were

induced for expression during infection. One island, which was upregulated in macrophages, contains STM0854–0859 and includes a putative hydrogenase, a putative electron transport system and a transcriptional regulator, which deserves further investigation.

The analysis of FUN genes is rapidly advanced by searching for patterns of expression that resemble well-characterized virulence genes or members of defined regulons. This assignment of function 'guilt by association' has proved extremely powerful in other systems (Eisen *et al.*, 1998). We notice that the putative islet (STM3117–3120) was strongly upregulated within macrophages and showed the same pattern of expression as SPI2 in our experiment, raising the possibility that the STM3117–3120 genes will prove to play an important role in the pathogenesis of *S. Typhimurium*. Homologues of these genes belong to SPI6 of *S. enterica* sv. Enteritidis and are required for macrophage infection (GenBank AF376036).

We noticed that STM3117 is a putative glutathione-dependent glyoxalase, which may have a role in neutralizing methylglyoxal, a compound that is lethal to *Salmonella* (Ferguson *et al.*, 1998). Other genes involved in detoxifying methylglyoxal are also induced in the SCV, exemplified by the four- to eightfold increase in *kefB* gene expression after infection. The KefB potassium efflux system of *E. coli* is known to be induced by S-lactoylglutathione, an intermediate in the glyoxalase detoxification pathway, and acts to reduce intracellular pH, which protects against methylglyoxal toxicity (Ferguson *et al.*, 1998). At the same time, the methylglyoxal synthase gene (*mgsA*), which converts dihydroxyacetone phosphate to methylglyoxal, is strongly downregulated. These data suggest that macrophages may accumulate methylglyoxal in the SCV in an attempt to kill *Salmonella*.

#### Concluding remarks

This is the first report on global bacterial gene expression from intracellular bacteria and offers a new approach for the understanding of infectious disease. Our experimental strategy has established a novel method for extracting RNA from bacteria that have been replicating within host cells. The availability of bacterial RNA from infected cells will also facilitate traditional approaches to gene expression analysis, such as Northern blots. Analysis of the microarray data identified well-characterized families of virulence-associated genes, as well as 408 FUN genes that are *in vivo* regulated. The discovery that such a high proportion of the genome was *in vivo* regulated and that half these genes were of unknown function is particularly striking (Fig. 5). The exciting findings from our study include the observation that intracellular *Salmonella* are not starved for amino acids or iron (Fe<sup>2+</sup>), and that the SCV environment is low in phosphate and high in potas-

sium. We also suggest that *Salmonella* use gluconate as a carbon source during intracellular growth.

Acidification of the SCV is reported to be essential for intracellular replication of *Salmonella* in RAW 264.7 cells (Rathman *et al.*, 1996) and *Francisella tularensis* in primary macrophages (Fortier *et al.*, 1995). The need for a low-pH environment for intracellular replication within the SCV has been linked to a directed uptake of nutrients or iron. As mentioned earlier, the microarray data indicated that the bacteria had adapted to an acidic environment in J774-A.1 cells and were using galactonate or gluconate as carbon source. In this context, we observed that STM0860, coding for a putative H<sup>+</sup> gluconate symporter, was upregulated in intracellular SL1344 bacteria. This finding, coupled with the observed downregulation of flagellar machinery and H<sup>+</sup>/ATPase expression, suggests that, after macrophage infection, *Salmonella* relies upon the proton gradient to aid import of its primary carbon source, while downregulating other proton channels to prevent excessive proton influx and to maintain the intracellular pH required for active growth.

Extensive genetic screens and selections for *in vivo*-induced genes have identified a number of *Salmonella* virulence genes that become induced for expression within the host or host cells. These approaches include *in vivo* expression technology (IVET), signature-tagged mutagenesis and differential fluorescence induction (Mahan *et al.*, 1993; Hensel *et al.*, 1995; Valdivia and Falkow, 1997; Pfeifer *et al.*, 1999). Our work describes the first measurement of mRNA levels of bacterial genes on a whole-genome level during intracellular infection. The microarray data showed the induction of many genes that have been reported to be macrophage-inducible, such as *mgfBC* and the SPI2 locus (Hensel *et al.*, 1995; Smith *et al.*, 1998; Heithoff *et al.*, 1999; Pfeifer *et al.*, 1999; Benzòn *et al.*, 2000). However, some of our data are not consistent with common expectations regarding gene induction in intracellular bacteria. It is apparent that genes required for intracellular replication may not necessarily be induced in this environment, their presence and steady-state expression being sufficient for growth. Secondly, the microarray data may have revealed some limitations of reporter gene fusions. Although reporter gene-based expression data are often in agreement with that from microarray analyses (Hellauer *et al.*, 2001), there are reports of discrepancies. One study demonstrated that three different reporter genes could give very different patterns of expression for a single promoter if these were sensitive to DNA topology (Forsberg *et al.*, 1994). Pessi *et al.* (2001) pointed out that *lacZ* fusions would not reflect post-transcriptional gene regulation, which relied upon mRNA degradation. Additionally, Warner and Lolkema (2002) showed that the expression of beta-galactosidase varied according to growth phase, suggesting that appar-

ent induction could simply reflect increased growth rates under certain conditions. These factors suggest that reporter gene expression data cannot always be relied upon. One significant limitation of the reporter gene-based IVET system is that, unlike microarray analysis, it can only identify genes that show increased and not reduced expression. Taken together, the approaches based on *in vivo* selection, reporter gene fusions and microarray analyses do define common *in vivo*-regulated genes, and it is apparent that no single method will cover this pool of genes entirely.

It is becoming increasingly clear that the mechanisms that govern virulence in a wide variety of bacteria, including both animal and plant pathogens, share common features; therefore, many of the findings described here will be applicable to a broad range of pathogens. Additional microarray experiments performed in parallel with *S. Typhimurium* strain SR-11 grown intracellularly in J774-A.1 cells revealed similar gene expression profiles to SL1344 (data not shown), indicating that the trends described here are likely to prove relevant to the *S. Typhimurium* species in general. We suggest that, although bacterial growth in cultured cells requires only a subset of gene functions, the intracellular environment induces the full repertoire of transcriptional responses required for successful pathogenesis, perhaps to prepare the pathogen for future challenges and for transmission. Until now, the study of bacterial gene expression *in vivo* has necessitated a focus on individual virulence genes. This identification of the *in vivo* transcriptome of *Salmonella*, coupled with other studies on the response of mammalian cells to infection, should allow us to move beyond the reductionist approach that has been prevalent in recent decades towards an integrated understanding of pathogenesis.

## Experimental procedures

### Bacterial strains

The strains used in this study were *S. Typhimurium* SL1344 obtained from Dr C. Lee (Hoiseth and Stocker, 1981) and an isogenic *phoP*::Tn10 mutant (Fields *et al.*, 1989).

### Cell culture and infection model

J774-A.1 cells (ATCC TIB67) were grown in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine (2 mM final concentration; Gibco) and HEPES (10 mM final concentration; Gibco) as described previously (Eriksson *et al.*, 2000). For each extraction of *Salmonella* RNA, a total of 10<sup>8</sup> J774-A.1 cells was seeded in 6-well cell culture plates (Costar; 120 wells in total) and infected with complement-opsonized *S. Typhimurium* SL1344 at a multiplicity of infection (MOI) of 100:1 (bacteria:cells) (De Groote *et al.*, 1997; Eriksson *et al.*, 2000; Garvis *et al.*, 2001). To minimize SPI1 expression, bacteria were grown overnight

on Luria broth plates at 37°C and suspended in phosphate-buffered saline (PBS) before opsonization. To increase the uptake of *Salmonella*, plates were centrifuged at 1000 *g* for 5 min, and this was defined as time 0 h. After 1 h of phagocytosis, extracellular bacteria were killed with 30 µg ml<sup>-1</sup> gentamicin. The media was replaced after 1 h with medium containing 5 µg ml<sup>-1</sup> gentamicin. Incubations were continued for as long as indicated (i.e. for a further 2 h, 6 h or 10 h). To estimate the amount of intracellular bacteria at each time point, cells were lysed under hypotonic conditions, and samples were taken for viable counts (Eriksson *et al.*, 2000).

#### RNA extraction

At each time point, infected macrophages were lysed on ice for 30 min in 0.1% SDS, 1% acidic phenol, 19% ethanol in water. The phenol-ethanol mixture acted to stabilize all bacterial RNA (Tedin and Blasi, 1996). For each time point, *Salmonella* were isolated from 120 wells of infected J774-A.1 cells and pooled. Pellets were collected by centrifugation, and RNA was prepared using the Promega SV total RNA purification kit. Bacterial RNA was purified further by phenol-chloroform extraction. Approximately 10<sup>9</sup> bacterial cells were isolated from each time point, and these yielded ≈ 50 µg of total bacterial RNA. Control RNA from *in vitro*-grown bacteria was obtained by growing complement-opsonized SL1344 statically at 37°C to mid-log phase in complete cell culture medium under 5% CO<sub>2</sub>. These conditions mimicked those used for the cell infection experiments. RNA was stabilized and isolated as described above. It should be noted that the choice of control bacteria for this experiment plays a profound role in the definition of the set of *in vivo*-regulated genes. The growth of control bacteria in cell culture media, as well as opsonization, will have had significant effects on global gene expression. However, we consider this to be a more relevant comparator than simply using *Salmonella* grown in nutrient laboratory media. Control eukaryotic RNA was isolated as described in the Promega SV total RNA purification kit handbook. Size chromatography of RNA was done with an Agilent 2100 Bioanalyser.

#### Microarray procedures

DNA microarray analysis of gene expression was performed essentially as described previously (Clements *et al.*, 2002), except that the arrays were printed on Corning CMT-GAPS™-coated slides.

#### Probe preparation and scanning

The labelling method for experimental RNA samples was chosen according to the amount of RNA that was available. Small amounts of RNA (up to 5 µg) were first reverse transcribed into cDNA and subsequently labelled by random priming, increasing labelling efficiency as a result of enhanced incorporation of fluorescent nucleotides by the Klenow enzyme. Larger amounts of RNA were labelled during reverse transcription. We have demonstrated that these two RNA labelling methods yielded comparable results (data not shown). For labelling protocols,

see <http://www.ifr.bbsrc.ac.uk/Safety/Microarrays/#Protocols>. Fluorescently labelled genomic DNA was used as a reference channel in each experiment. After hybridization, slides were scanned with a GenePix 4000 A scanner (Axon Instruments). Fluorescent spot and local background intensities were quantified using GENEPIX PRO software (Axon Instruments). All hybridizations were repeated at least three times, and the range of data for each spot is indicated in *Supplementary material*, Table S1.

#### Data analysis

Spots showing a reference signal lower than background plus two standard deviations or obvious blemishes were excluded from subsequent analyses. Local background was subtracted from spot signals, and fluorescence ratios were calculated. To compensate for unequal dye incorporation or any effect of the amount of template, data centring was performed by bringing the median natural logarithm of the ratios for each group of spots printed by the same pin to zero. The complete data set is available as *Supplementary material*. Data that passed the quality controls were analysed using GENE-SPRING™ 4.2 software (Silicon Genetics). Significance of the centred data at *P* = 0.005 was determined using a parametric-based statistical test adjusting the individual *P*-value with the Benjamini and Hochberg false discovery rate multiple test correction. A total of 1075 genes passed the significance filter in at least one time point. Of those, 919 genes showed more than twofold expression changes. Hierarchical clustering of gene expression profiles was performed for the 919 gene subset using the Pearson correlation (Fig. 2).

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#### Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/mole/mole3313/mmi3313sm.htm>

**Table S1.** The *in vivo* gene expression profile of *S. Typhimurium* (4451 gene data set in MS EXCEL 2000 format). Ratios of sample to reference DNA are the average of at least three replicate hybridizations after data centring. Standard deviations are shown for each gene.

**Table S2.** List of 919 *in vivo*-regulated genes that passed the statistical significance filter and showed more than twofold

change in expression at one or more time points. Standard deviations are shown for each gene.

**Table S3.** Data taken from Table S1, organized into functional groups and pathogenicity islands, as depicted in Fig. 5.

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