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Mig-14 is an inner membrane-associated protein that promotes *Salmonella typhimurium* resistance to CRAMP, survival within activated macrophages and persistent infection

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Summary

Salmonella enterica **serovar Typhimurium (*S. typhimurium*) infects a wide variety of mammalian hosts and in rodents causes a typhoid-like systemic disease involving replication of bacteria inside macrophages within reticuloendothelial tissues. Previous studies demonstrated that the *mig-14* and *virK* genes of *Salmonella enterica* are important in bacterial resistance to anti-microbial peptides and are necessary for continued replication of *S. typhimurium* in the liver and spleen of susceptible mice after orogastric inoculation. In this work we report that inflammatory signalling via interferon-gamma (IFN-γ) is crucial to controlling replication of *mig-14* mutant bacteria within the liver and spleen of mice after oral infection. Using a *Salmonella* persistence model recently developed in our laboratory, we further demonstrate that *mig-14* contributes to long-term persistence of *Salmonella* in the spleen and mesenteric lymph nodes of chronically infected mice. Both *mig-14* and *virK* contribute to the survival of *Salmonella* in macrophages treated with IFN-γ and are necessary for resistance to cathelin-related anti-microbial peptide (CRAMP), an anti-microbial peptide expressed at high levels in activated mouse macrophages. We also show that both Mig-14 and VirK inhibit the binding of CRAMP to *Salmonella*, and demonstrate that Mig-14 is an inner membrane-associated protein. We further demonstrate by transmission electron microscopy that the primary locus of CRAMP activity appears to be intracytoplasmic, rather than at the outer membrane, suggesting that Mig-14 may prevent the penetration of the inner membrane by CRAMP. Together, these data indicate an important role for *mig-14* in anti-microbial peptide resistance *in vivo*, and show that this resistance is important to the survival of *Salmonella* in systemic sites during both acute and persistent infection.**

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Introduction

Salmonella enterica serovar Typhimurium (*S. typhimurium*) is a Gram-negative facultative intracellular bacterium that can cause a diverse spectrum of disease from self-limiting gastroenteritis to typhoid fever that is often host specific. Experimental infection of rodents with *S. typhimurium* leads to a systemic typhoid-like disease that in many ways resembles human typhoid fever caused by *Salmonella enterica* serovar Typhi (*S. typhi*). Oral infection by *Salmonella enterica* is associated with bacterial attachment to and invasion of specialized terminally differentiated epithelial cells known as M cells in the Peyer's patches of the small intestine (Jones *et al*., 1994). Invasion leads to M cell destruction and the subsequent entry of bacteria into the underlying macrophages and dendritic cells that reside in the Peyer's patch and ordinarily act as sentinels of the innate immune response. After their establishment in the Peyer's patches, *Salmonella* subsequently spreads via the lymphatics and bloodstream to elements of the reticuloendothelial system (RES), the mesenteric lymph nodes (MLN), liver and spleen. In susceptible mouse strains, *Salmonella* replicates to high numbers within the RES; the inability of the host to control this replication is usually fatal.

Salmonella enterica serovar Typhimurium infections of laboratory mouse strains have been used successfully to study complex host–pathogen interactions. In the mouse, a significant component of the innate resistance or susceptibility to infections with *S. typhimurium* is controlled by the gene *Nramp1* (also called *Slc11a1*), which is expressed in cells of the monocyte/macrophage lineage and affects the capacity of the cells to control intracellular replication of microorganisms such as *Salmonella* that reside in the phagolysosome (Vidal *et al*., 1993). *Nramp1*

is involved in the control of the exponential growth of *S. typhimurium* in the reticuloendothelial organs during the early phase (week 1) of infection (Vidal *et al*., 1995). Susceptibility in mice is associated with a mutated *Nramp1* allele and in these mice *S. typhimurium* replicates to high numbers inside macrophages, sometimes leading to death of the host before the engagement of adaptive immunity.

The control of *Salmonella* clearance during the late phase of infection (3–4 weeks post infection) requires both $CD4^+$ and $CD8^+$ T lymphocytes. Oral infection of mice with *S. typhimurium* can lead to the rapid expansion of $CD4^+$ T cells within MLN that have elevated intracellular levels of the proinflammatory cytokines interleukin (IL)-2, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) (McSorley *et al*., 2002). Activated $CD4^+$ and $CD8^+$ T cells are classically thought to be the major producers of IFN-γ during the adaptive phase of host responses (Frucht *et al*., 2001). IFN-γ, IL-18 and TNF-α, along with bacterial components such as lipopolysaccharide (LPS), flagellin subunits, and bacterial cell wall components, bind surface receptors and trigger signalling pathways that lead to changes in gene expression that result in macrophage activation (Schroder *et al*., 2004). Activated macrophages secrete additional proinflammatory cytokines and chemokines, and upregulate expression of major histocompatibility complex (MHC) molecules on the cell surface, all of which lead to increased killing of invading microbes.

Activated macrophages restrict the replication of intracellular bacterial pathogens through mechanisms such as increased endosome trafficking to the lysosome, the cellular degradative compartment. Within the lysosome, the foreign organism is subjected to a variety of lytic enzymes, such as cathepsins, lipases and lysozyme, as well as to the presence of anti-microbial peptides, an acidified pH and highly oxidative conditions generated by the NADPH phagocyte oxidase that is recruited to the phagosome (Nathan and Hibbs, 1991; Rathman *et al*., 1996; Vazquez-Torres *et al*., 2000a). *Salmonella* that infect activated macrophages reside in vacuoles that exhibit an increased level of colocalization with lysosomes relative to *Salmonella* that infect unactivated macrophages (Ishibashi and Arai, 1990). Cathelin-related anti-microbial peptide (CRAMP) (Gallo *et al*., 1997) is expressed in murine macrophages and CRAMP expression and activity are significantly elevated in macrophages treated with IFN-γ (Rosenberger *et al*., 2004).

While professional phagocytes rapidly degrade non-pathogenic microbes via these anti-microbial mechanisms, pathogenic organisms often survive these host barriers to infection by resisting the lytic contents of the lysosome or preventing the fusion of early endosomes with lysosomal vesicles. For example, recent studies have demonstrated that *Salmonella* inhibits the recruitment of the NADPH phagocyte oxidase to the *Salmonella*-containing vacuole (SCV) via a mechanism that is dependent on the genes within *Salmonella* pathogenicity island 2 (SPI-2) (Vazquez-Torres *et al*., 2000b). SPI-2 encodes a type III secretion system (TTSS) that secretes bacterial effector molecules into the host cell (Cirillo *et al*., 1998; Hensel *et al*., 1998). Other work demonstrates that effector molecules secreted by the SPI-2 secretion system modify the *Salmonella* vacuolar membrane: in the absence of these effector proteins, the SCV rapidly disintegrates and the bacteria are killed within the macrophage cytosol by an unknown mechanism (Beuzon *et al*., 2000; 2002; Ruiz-Albert *et al*., 2002).

In a previous study aimed at better understanding how *S. typhimurium* survives within the vacuole of macrophages, our laboratory utilized a green fluorescent protein (GFP)-based promoter-trap screen to identify *Salmonella* genes that demonstrated increased expression within the intracellular environment (Valdivia and Falkow, 1997). One of these genes, a PhoP-regulated gene designated *mig-14*, is required for bacterial virulence during later stages (day 7) of an acute mouse infection. PhoP is the response regulator of the PhoP–PhoQ two-component regulatory system (Groisman *et al*., 1989). In *Salmonella*, PhoP regulates a large number of genes that are involved both in invasion of bacteria into host cells and in survival of *Salmonella* within macrophages (Miller and Mekalanos, 1990; Behlau and Miller, 1993; Bader *et al*., 2003). Although *mig-14* mutant bacteria can colonize the liver and spleen of mice identically to wild-type bacteria, they fail to replicate in these organs after day 5 post infection (Valdivia *et al*., 2000). This study also demonstrated that significantly higher levels of IFN-γ are present in sera of mice infected with *mig-14* relative to wild-type bacteria at day 7 post infection, despite the presence of lower numbers of mutant bacteria at this time.

More recent studies indicate that *mig-14* expression is upregulated in the presence of anti-microbial peptides and that *mig-14* is necessary for resistance to the anti-microbial peptides polymyxin B and protegrin-1 *in vitro* (Brodsky *et al*., 2002). *virK*, a gene that is adjacent to *mig-14* on the *S. typhimurium* chromosome (Baumler and Heffron, 1998; Valdivia *et al*., 2000), is also expressed in the macrophage vacuole and is necessary for maximal resistance to polymyxin B *in vitro* (Detweiler *et al*., 2003). Furthermore, a *virK* mutant is less virulent than wild-type bacteria as demonstrated by reduced levels of the *virK* mutant in spleen and liver of BALB/C mice after day 7 post infection and by decreased competitive indices relative to wild-type bacteria after 3 weeks in *Nramp1*$^{+/+}$ mice (Detweiler *et al*., 2003).

In this work we investigated the role of *mig-14* in resistance to IFN-γ-mediated inflammatory responses and in

persistent infection. We also examined the contribution of *mig-14* and *virK* to the ability of *S. typhimurium* to survive within activated macrophages and to resist killing by CRAMP.

Results

IFN-γ signalling limits replication of mig-14 *mutant bacteria in mouse infection*

Previous work demonstrated that mutation of *mig-14* leads to an inability of *S. typhimurium* to replicate within the spleen and liver of infected mice at later times post infection (Valdivia *et al*., 2000). Significantly higher amounts of serum IFN-γ were also present in *mig-14* mutant-infected mice compared with wild-type-infected mice on day 7 post infection (Valdivia *et al*., 2000). It has been reported that peak production of IFN-γ by $CD4^+$ and $CD8^+$ T cells occurs at day 7–14 post infection with vaccine strains of *S. typhi* (Lundin *et al*., 2002). We therefore hypothesized that the inability of *mig-14* mutant bacteria to replicate in RES organs at this time might result from the proinflammatory effects of IFN-γ and other cytokines secreted as a result of adaptive immune responses. We compared the infection of C57Bl/6J and isogenic IFN-$\gamma^{-/-}$ mice with wild-type (strain SL1344) (Hoiseth and Stocker, 1981) and isogenic *mig-14* mutant bacteria to examine the role of IFN-γ in controlling the replication of the *mig-14* mutant. As was previously reported with infection of BALB/c mice (Valdivia *et al*., 2000), we saw virtually no difference in the colony-forming units (cfu) of wild-type and *mig-14* mutant bacteria obtained from the spleen (Fig. 1A) and liver (data not shown) of C57Bl/6 mice at day 5 post infection. Also consistent with our previous observations, we saw significantly fewer *mig-14* mutant bacteria in these tissues at days 7 and 9 post infection than SL1344 bacteria (Fig. 1A), indicating that *mig-14* is required for replication in systemic tissues of wild-type mice at later times post infection.

In contrast, in IFN-$\gamma^{-/-}$ mice at day 5 post infection, the levels of SL1344 were 10-fold higher than the amount of bacteria present at day 9 post infection of C57Bl/6 mice (Fig. 1B), consistent with observations demonstrating a clear role of IFN-γ in controlling replication of intracellular bacterial pathogens during infection (Nauciel and Espinasse-Maes, 1992; Flynn *et al*., 1993). At day 7 post infection, both *mig-14* mutant-infected and SL1344-infected mice had extremely high levels of bacteria present in the tissues, and the infectious burdens of both wild-type and *mig-14* mutant bacteria were identical (Fig. 1B). These data indicate that IFN-γ signalling is crucial for controlling the replication of *mig-14* mutant bacteria. However, we found that at day 5 post infection, the levels of *mig-14* mutant bacteria in the tissues of mice that had not died were significantly lower than those of SL1344 (Fig. 1B), indicating the possibility that an IFN-γ-independent mechanism controls the replication of *mig-14* bacteria in these mice initially, but cannot contain the infection by these mutant bacteria in the absence of IFN-γ.

A significant number of IFN-$\gamma^{-/-}$ mice infected with either SL1344 or *mig-14* mutant bacteria died or had to be euthanized because of the overwhelming replication of bacteria before the organs could be analysed for cfu counts. The survival curves of the SL1344- or *mig-14*-infected mice were virtually superimposable (Fig. 1C), indicating that in the absence of IFN-γ, *mig-14* mutant bacteria rapidly replicate in systemic organs and kill infected mice indistinguishably from wild-type bacteria. This is in contrast to wild-type mice, which show significantly greater levels of survival when infected with *mig-14* mutant bacteria relative to SL1344. To ensure that the rapid replication of *mig-14* bacteria within tissues of IFN-$\gamma^{-/-}$ mice was not a general effect of immune dysfunction, we infected both C57Bl/6 and IFN-$\gamma^{-/-}$ mice with *S. typhimurium* containing a mutation in a gene encoding a structural component of the SPI-2 TTSS. The SPI-2 secretion system is necessary for *Salmonella* replication in macrophages and is essential for virulence in mice (Cirillo *et al*., 1998; Hensel *et al*., 1998). Studies have demonstrated that even IFN-γ-deficient mice restrict replication of a SPI-2 mutant (Raupach *et al*., 2003). We observed extremely low levels of SPI-2 mutant bacteria in the organs of IFN-$\gamma^{-/-}$ mice at this infectious dose even at day 7 post infection (geometric mean of bacterial cfu per gram of spleen = 367), indicating that IFN-$\gamma^{-/-}$ mice do indeed control replication of some mutant bacteria even though they cannot control the replication of the *mig-14* mutant.

mig-14 *contributes to* Salmonella *persistence in host tissues*

Mice that are naturally resistant to infection with *Salmonella* due to the presence of the wild-type *Nramp* (also called *Slc11a*) gene product can be persistently infected with wild-type *S. typhimurium* (Monack *et al*., 2004). Persistently infected mice can harbour bacteria within organs of the RES for up to 1 year post infection and periodically shed bacteria in their stool. Treating persistently infected mice with neutralizing antibody against IFN-γ led to rapid replication of *Salmonella* in MLN and Peyer's patches, and slightly lower replication of bacteria in spleen and liver (Monack *et al*., 2004). This suggested that IFN-γ controls replication of *Salmonella* within systemic sites even at later stages of persistent infection. As IFN-γ controls bacterial replication in the acute and persistent infection and is necessary for clearance of *mig-14* mutant bacteria in susceptible mice, we examined the potential role of *mig-14* in *Salmonella* persistence.

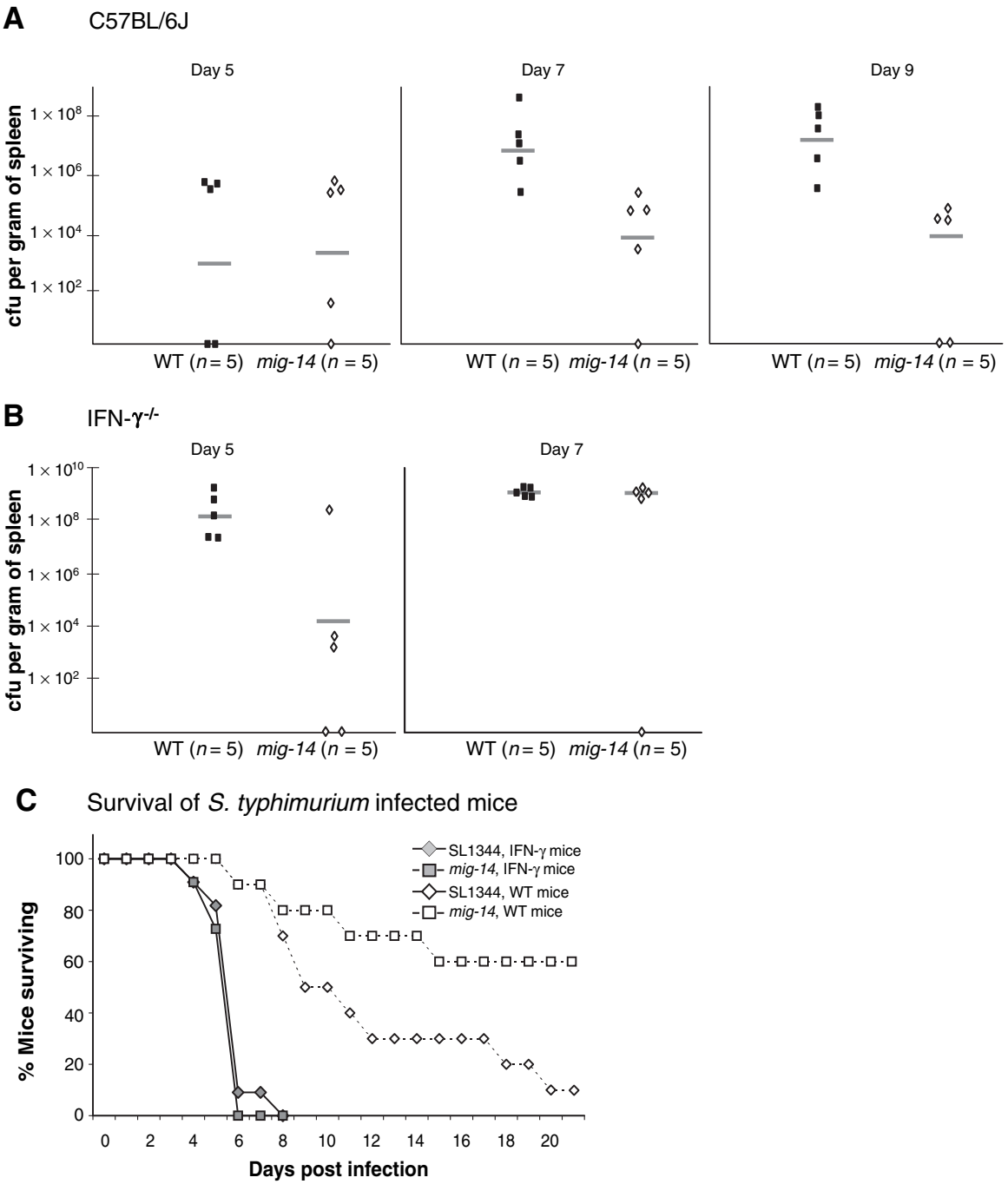

Fig. 1. Bacterial load in spleen of wild-type (WT) and isogenic IFN-$\gamma^{-/-}$ mice infected with WT or *mig-14* mutant *S. typhimurium*.
A. Bacterial colony forming units (cfu) present in spleen of C57BL/6J mice infected with either SL1344 (WT) or *mig-14* mutant bacteria at days 5, 7 and 9 after oral inoculation of 5×10^5 bacteria. At day 5 the bacterial loads are approximately equal, but at days 7 and 9 significantly more WT bacteria are present per gram of spleen ($P = 0.00794$, Mann–Whitney *U*-test).
B. Bacterial cfu present in spleen of isogenic IFN-$\gamma^{-/-}$ mice infected with either WT or *mig-14* mutant bacteria at days 5 and 7 post infection. Too many IFN-$\gamma^{-/-}$ mice died before day 9 to recover data from this time point. At day 5 more WT bacteria than *mig-14* mutant bacteria are present in the spleen of IFN-$\gamma^{-/-}$ mice, although the numbers are not quite statistically significant ($P = 0.055$, Mann–Whitney *U*-test). By day 7 the numbers of WT or *mig-14* mutant bacteria present are virtually equivalent ($P = 0.31$, Mann–Whitney *U*-test). Each data point is representative of one infected mouse. Grey bars are geometric means.
C. Survival curve of C57BL/6J (wild-type) and isogenic IFN-$\gamma^{-/-}$ mice infected with either SL1344 (wild-type) or *mig-14* mutant bacteria. IFN-$\gamma^{-/-}$ mice infected with either wild-type or *mig-14* mutant bacteria are killed rapidly with very similar kinetics, and virtually all mice are dead within the first week after infection. Wild-type mice survive significantly longer, with SL1344-infected mice showing lower levels of survival and being killed more rapidly than *mig-14* mutant-infected mice. For these experiments a number of mice had to be euthanized for humane reasons.

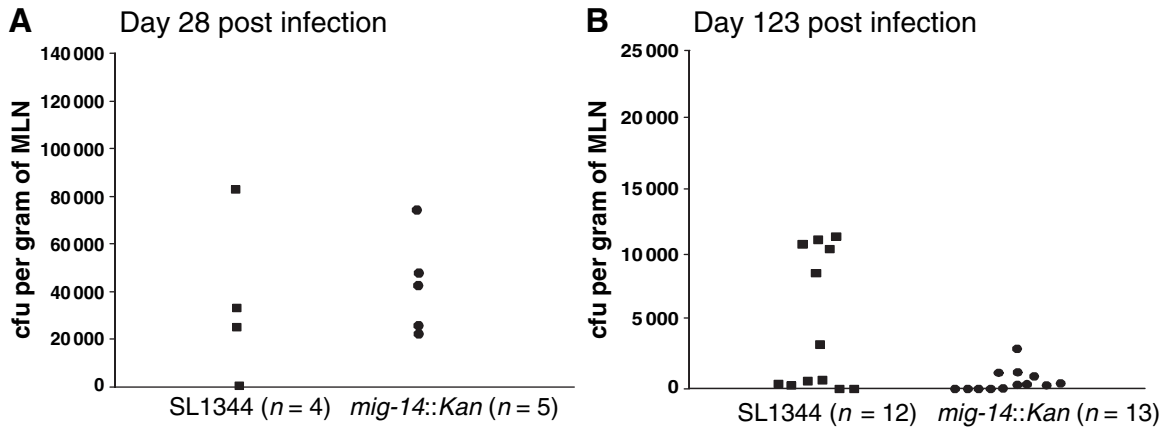

Fig. 2. Bacterial load in mesenteric lymph nodes of chronically infected mice.
A. 28 days after oral infection of 129XiSv/J mice with wild-type (SL1344) or *mig-14* mutant *S. typhimurium*, bacterial load of the two strains in mesenteric lymph nodes was approximately equivalent.
B. 123 days after oral infection, wild-type bacteria still persist in mesenteric lymph nodes whereas *mig-14*-infected mice have significantly reduced bacterial burdens ($P = 0.0492$, Mann–Whitney *U*-test). Geometric mean of SL1344 cfu present in tissues is 1585, whereas geometric mean of *mig-14* cfu is 384 (see Table 1).

We challenged 129Xi/SvJ by oral infection with 1×10^7 cfu of SL1344 or *mig-14* mutant bacteria. At various times post infection, mice were sacrificed and the bacterial load present in each tissue was examined. At day 28 post infection, there was no statistically significant difference between mice infected with any of the bacterial strains (Fig. 2A), as was the case for all of the time points before day 28 (data not shown). As persistently infected mice carry the highest bacterial burdens in the MLN (Monack *et al*., 2004), we focused our analysis of these mice on the spleen and MLN. In the MLN at day 123 post infection, five *mig-14*-infected mice had cleared the bacteria, whereas only two SL1344-infected mice had cleared the infection (Fig. 2B). Of the mice that had not cleared the infection, the MLN of *mig-14*-infected mice contained significantly lower amounts of bacteria compared with SL1344-infected mice, although there was not a substantial difference in the number of mice that had bacteria present in the MLN (Fig. 2B and Table 1). In the spleen, we observed that although the bacterial counts were uniformly lower than in the MLN, there was not a significant difference in colony counts between mutant- and wild-type-infected mice. However, significantly fewer mice still harboured *mig-14* bacteria in the spleen compared with the spleens of SL1344-infected mice (Table 1). These data suggested that *mig-14* contributes to long-term persistent infection. As the majority of persisting *S. typhimurium* reside within macrophages in the RES (Monack *et al*., 2004), we examined whether the decrease in the ability of *mig-14* mutant bacteria to persist in mice correlated with a decrease in bacterial survival within macrophages.

mig-14 *promotes* Salmonella *replication in activated macrophages*

The observation that control of *mig-14* mutant bacterial replication in the spleen and liver subsequent to day 5 post infection was dependent on IFN-γ suggested that host inflammatory responses could be important for controlling replication of *mig-14* mutant bacteria. Macrophages activated by IFN-γ upregulate a variety of effector functions including production of reactive oxygen and nitrogen species (Ding *et al*., 1988; Xie *et al*., 1993), increased expression of anti-microbial peptides (Rosenberger *et al*., 2004) and increased trafficking of phagocytosed material to the lysosome (Ishibashi and Arai, 1990). We therefore examined the ability of *mig-14* mutant bacteria to survive and replicate in either unactivated or activated cells of the RAW 264.7 macrophage-like cell line.

mig-14 mutant bacteria replicated in unactivated RAW 264.7 macrophages indistinguishably from SL1344 wild-type bacteria. Both SL1344 and *mig-14* mutant bacteria

Table 1. Colonization of mesenteric lymph nodes (MLN) and spleen in persistently infected mice.

Strain (*n*) mean	% MLN colonized	Geometric mean	% Spleen colonized	Geometric mean
SL1344 (12)	83.3	1585.2	83.3	149.9
mig-14 (13)	61.5	383.6	38.5	163.7

showed an approximately 10- to 15-fold increase in cfu over the infection time-course (Fig. 3A). *virK* is located immediately upstream of *mig-14* on the *S. enterica* genome, and shares a number of characteristics with *mig-14*, including PhoP-dependent regulation and a role in resistance to anti-microbial peptide killing (Detweiler *et al*., 2003). We therefore examined the ability of *virK* mutant bacteria to survive and replicate within host macrophages and found that, like *mig-14*, *virK* mutant bacteria replicated to the same level as wild-type bacteria in unactivated macrophages (Fig. 3A). In contrast to both *mig-14* and *virK* mutants, mutation of *phoP* caused complete loss of *Salmonella*'s ability to replicate within unactivated macrophages, consistent with previous studies demonstrating the requirement of *phoP* for intracellular replication (Fig. 3A) (Fields *et al*., 1989; Miller *et al*., 1989).

When RAW 264.7 cells were activated before bacterial infection, we recovered approximately 10-fold more bacteria at the 2 h time point compared with unactivated macrophages, indicating that activated macrophages are more phagocytic (compare Fig. 3A and B). This was not surprising considering that before infection bacteria were opsonized in 50% normal mouse serum and activated macrophages are known to upregulate the surface expression of complement receptors and Fc receptors (Schroder *et al*., 2004). However, we observed only a two- to threefold increase in bacterial cfu of the SL1344 strain from 2 to 24 h in the activated macrophages (Fig. 3B), consistent with observations that activated macrophages limit intracellular replication of *Salmonella* (Rosenberger and Finlay, 2002). As expected, mutation of *phoP* resulted in marked reduction of bacterial survival in activated macrophages as well. Both *mig-14* and *virK* mutant strains showed significant reduction in numbers of bacteria recovered at 24 h post infection relative to the wild-type SL1344 strain (Fig. 3B). In order to confirm these results in primary macrophages, we next examined the survival of *mig-14* and *virK* mutant bacteria within bone marrow-derived macrophages (BMDM).

Primary macrophages control bacterial replication better than cultured macrophage cell lines (Buchmeier and Heffron, 1989). Consistent with these results, we observed only slight replication of wild-type SL1344 at 8 h post infection, and a reduced bacterial cfu at 24 h post infection even in unactivated BMDM (Fig. 3C). Consistent with published results with IFN-γ-treated macrophages, activating BMDM with IFN-γ before infection resulted in reduced levels of SL1344 replication at 8 h post infection and even further reduction at 24 h post infection compared with untreated BMDM (Fig. 3D). In both unactivated and activated BMDM, however, *mig-14* and *virK* mutant bacteria had reduced survival relative to wild-type bacteria (Fig. 3C and D). This difference was the greatest at 8 h and was larger in IFN-γ-treated than in untreated BMDM (although more statistically significant in untreated BMDM). The *mig-14 virK* double mutant appeared to have slightly decreased survival relative to the single mutants in both activated and unactivated primary macrophages; however, this difference was not statistically significant.

mig-14 *and* virK *are required for resistance to the murine anti-microbial peptide CRAMP*

Recently published studies demonstrated that a mouse homologue of the human cathelicidin LL-37, designated CRAMP (Gallo *et al*., 1997), is expressed in murine macrophages and colocalizes with the *SCV* (Rosenberger *et al*., 2004). These studies also demonstrated a role for *phoP* in resistance to the effects of CRAMP. We had previously observed that *mig-14* and *virK* mutant bacteria are more susceptible to killing by several different anti-microbial peptides *in vitro* (Brodsky *et al*., 2002). We therefore examined whether *mig-14* and *virK*, both of which are PhoP-regulated genes, might be necessary for *S. typhimurium* resistance to CRAMP. We found that both *mig-14* and *virK* mutants were significantly more sensitive to killing by CRAMP than the wild-type SL1344 strain when the bacteria were grown in nutrient-limiting acidic media before the anti-microbial peptide killing assay (Fig. 4). Mutation of *virK* resulted in even greater sensitivity to killing by CRAMP than *mig-14*, and the *mig-14 virK* double mutant was significantly more sensitive than either single mutant (Fig. 4), suggesting that *mig-14* and *virK* promote anti-microbial peptide resistance by different pathways. The PhoP–PhoQ two-component system is absolutely required for resistance to anti-microbial peptides and regulates many genes involved in modification of the bacterial envelope, in terms of both protein expression and lipid composition (Guo *et al*., 1997; Gunn *et al*., 1998; Guina *et al*., 2000). Consistent with these observations, we observed complete killing of the *phoP* mutant even at the lowest concentration of CRAMP.

Mig-14 is an inner membrane-associated protein

In order to address the mechanism by which *mig-14* and *virK* might contribute to anti-microbial peptide resistance, we isolated outer and inner membranes from SL1344, *mig-14* and *virK* mutant bacteria grown in low Mg^{2+} medium which induces *mig-14* and *virK* expression. Both outer and inner membrane proteins have been implicated in resistance to anti-microbial peptides (Parra-Lopez *et al*., 1993; 1994; Guina *et al*., 2000), suggesting the possibility that the *mig-14* or *virK* genes might be membrane proteins or play a role in expression of bacterial membrane proteins. We analysed equal amounts of total membrane proteins by one-dimensional (1D) SDS-PAGE.

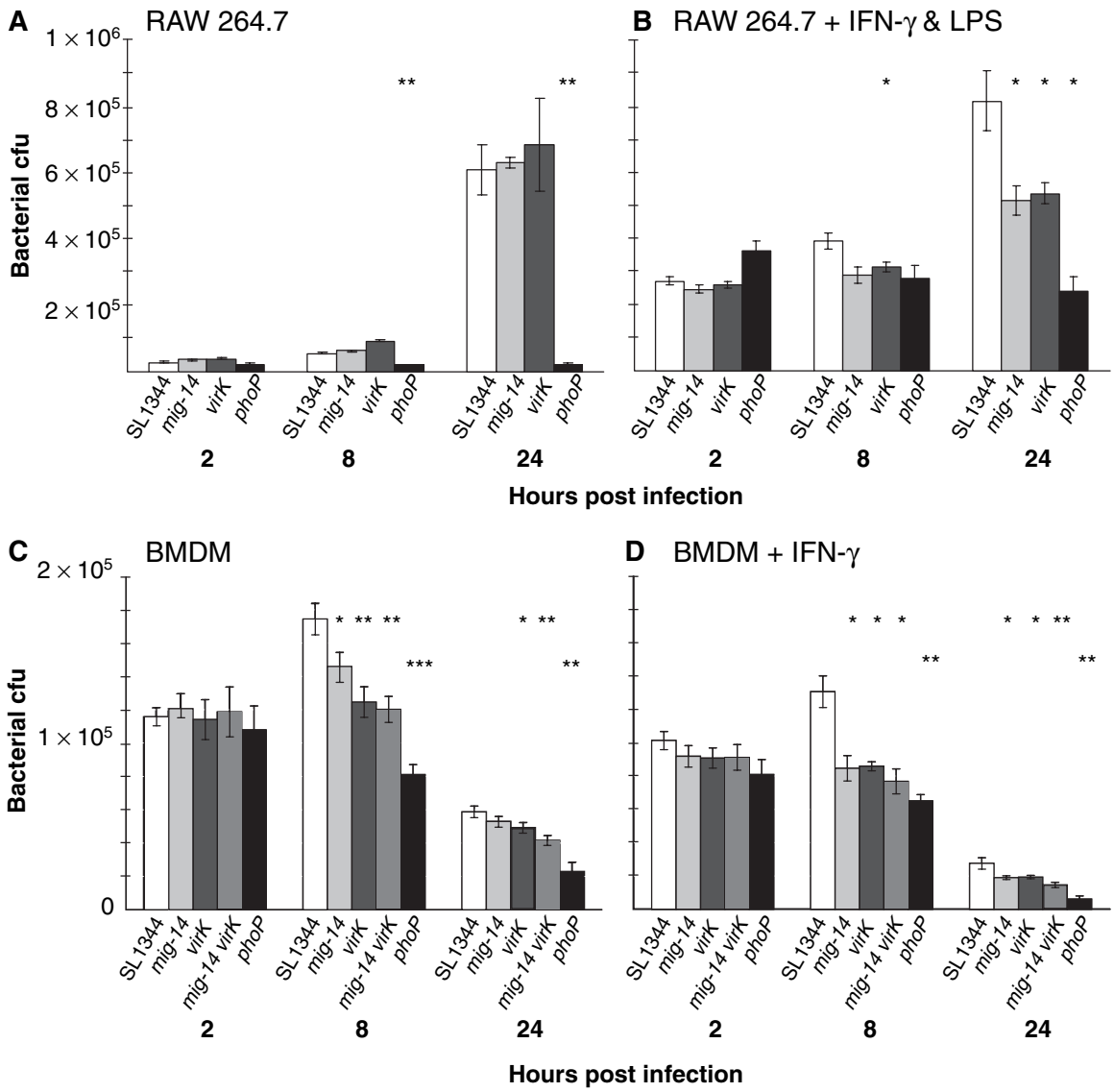

Fig. 3. Replication of *S. typhimurium* in activated and unactivated macrophage-like cell line and in primary macrophages.
A. RAW 264.7 cells were infected with SL1344, *mig-14*, *virK* or *phoP* mutant bacteria. Bacterial cfu were determined at 2, 8 and 24 h post infection. No statistically significant differences were observed between wild-type, *mig-14* or *virK* strains. There is a 10- to 15-fold increase in cfu of these strains from 2 to 24 h. No increase in cfu is seen for the *phoP* mutant. Data are representative of at least three independent experiments. $^{**}P \leq 0.01$ (Student's unpaired *t*-test).
B. RAW 264.7 cells were activated by treatment with IFN-γ and LPS 18–24 h before infection. SL1344 strain shows a two- to threefold increase in cfu, whereas the *phoP* mutant shows no increase or a slight decrease. *mig-14* and *virK* mutants demonstrate reduced replication or survival at 24 h in activated cells. Data are representative of three independent experiments. $^{*}P \leq 0.05$ (Student's unpaired *t*-test).
C. Primary bone marrow-derived macrophages (BMDM) were infected with same bacterial strains as in (A) and (B) with the addition of *mig 14 virK* double mutant strain. Bacterial cfu were determined at 2, 8 and 24 h post infection. At 8 h post infection there is a statistically significant difference between SL1344 and *mig-14*, and a slightly greater difference between SL1344 and *virK*. *mig-14 virK* double mutant bacteria do not appear to have greatly decreased ability to survive relative to either single-mutant strain. By 24 h there are reduced cfu for all bacterial strains with the greatest reduction observed for the *phoP* strain. Data are representative of three independent experiments. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.005$ (Student's unpaired *t*-test).
D. BMDM were seeded in 24-well dishes and activated with IFN-γ for 18–24 h before infection with the same bacterial strains as in (C). At 8 h there is a marked difference in the ability of SL1344 bacteria to survive relative to the survival of the mutant strains. As with the unactivated BMDM, at 24 h there is a significant reduction in the surviving bacteria across all the strains, with the greatest loss of cfu sustained by the *phoP* strain. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ (Student's unpaired *t*-test).

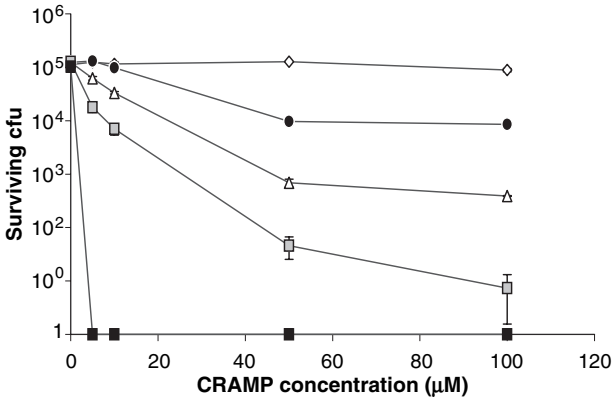

Fig. 4. Sensitivity of *S. typhimurium* strains to killing by cathelin-related anti-microbial peptide (CRAMP). Approximately 2×10^5 SL1344 (◇), *mig-14* (●), *virK* (△), *mig-14 virK* (□) and *phoP* (■) bacteria were treated with indicated concentrations of CRAMP for 60 min as described in *Experimental procedures*. Under these conditions SL1344 bacteria can survive the presence of up to 100 μM CRAMP. In contrast, there is a one, two and nearly four order-of-magnitude reduction in viable counts of *mig-14*, *virK* and *mig-14 virK* double mutant bacteria at 100 μM peptide concentration. No viable *phoP* mutant bacteria were recovered even at 5 μm peptide concentration, highlighting the extreme sensitivity of *phoP* mutant bacteria to anti-microbial peptides.

A protein of approximately 35 kDa was absent from the inner membrane fraction of the *mig-14* mutant that was visible in inner membranes of both SL1344 and *virK* mutant bacteria (Fig. 5A). The predicted size of Mig-14 is 35.3 kDa, suggesting that this missing protein could be Mig-14. To test this possibility, we isolated membrane fractions from SL1344 bacteria carrying a low-copy plasmid containing the *mig-14* promoter region and coding sequence. Inner membrane fractions from the *mig-14* overexpressing strain showed significantly greater expression of this 35 kDa protein (Fig. 5B). In contrast to outer membrane fractions from SL1344, *mig-14* and *virK* mutant bacteria, outer membranes isolated from the pMig-14 strain showed detectable levels of this 35 kDa protein as well. In order to confirm that this protein was indeed Mig-14, we determined its identity by in-gel tryptic digest and peptide sequencing. Mig-14 was identified as the 35 kDa protein in both inner and outer membrane fractions (Fig. S1).

In order to test the purity of the inner and outer membrane fractions that we isolated, we transferred the outer and inner membrane fractions to nitrocellulose after 1D SDS-PAGE, and immunoblotted with anti-sera against the Imp protein (Braun and Silhavy, 2002). The Imp protein is an 83 kDa outer membrane protein, and should therefore be absent from the inner membrane. This anti-sera also cross-reacts with a 55 kDa inner membrane protein of unknown function (Braun and Silhavy, 2002) and therefore allowed us to determine the purity of outer and inner membrane fractions. We observed that the 55 kDa protein was absent from the outer membrane fractions, and that Imp was absent from inner membranes (Fig. 5C), indicat-

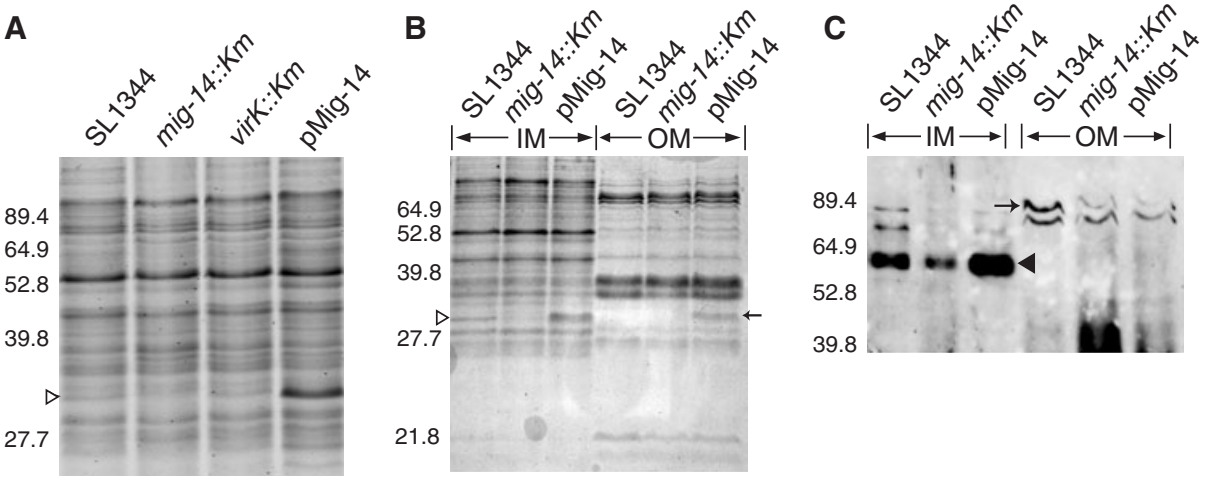

Fig. 5. Membrane fractions of *mig-14* mutant and *mig-14* overexpressing *S. typhimurium*.
A. Inner membrane fractions were isolated from SL1344, *mig-14* mutant (*mig-14*::*Km*), *virK* mutant (*virK*::*Km*) and SL1344 containing a low-copy *mig-14*-expressing plasmid (SL1344 pMig-14). Bacteria were grown in low Mg^{2+} medium to induce expression of Mig-14 protein. A 35 kDa protein is present in the inner membrane of SL1344 and *virK* mutant bacteria that is absent from the inner membrane fraction of the *mig-14* mutant. A protein of the same size is expressed at significantly higher levels in the inner membrane fraction of SL1344 pMig-14 bacteria. Open arrowhead indicates location of 35 kDa protein.
B. Outer membrane fractions of SL1344 and *mig-14* mutant bacteria are indistinguishable. However, a 35 kDa protein is present in the outer membrane fraction of SL1344 pMig-14. Open arrowhead and arrow indicate location of 35 kDa protein.
C. Anti-Imp immunoblot of inner and outer membrane fractions shown in (B). Anti-Imp serum detects an 83 kDa outer membrane protein (Imp) and cross-reacts with a 55 kDa inner membrane protein of unknown function. Black arrowhead indicates location of 55 kDa protein; arrow indicates location of Imp.

ing that there was minimal cross-contamination between the two sets of membranes.

mig-14 *and* virK *inhibit association of CRAMP with bacterial cell*

To further investigate the role of *mig-14* and *virK* in antimicrobial peptide resistance by *S. typhimurium*, we conjugated synthetic CRAMP to fluoresceine isothiocyanate (FITC) and analysed the association of FITC-CRAMP with different *S. typhimurium* strains by flow cytometry. SL1344, *mig-14*, *virK* and *phoP* mutant bacteria expressing a rapidly maturing DsRed protein (Sorensen *et al*., 2003) were incubated with FITC-CRAMP for 30 min in a low osmolarity buffer before fluorescence activated cell sorting (FACS) analysis. Intact bacteria were identified based on side-scatter and level of DsRed fluorescence (Fig. 6A and B). The amount of FITC associated with this population was then quantified for each bacterial strain. The side-scatter versus DsRed dot plots for SL1344 either in the absence of FITC-CRAMP (Fig. 6A) or in the presence of FITC-CRAMP (Fig. 6B) are representative of the dot plots for the other bacterial strains in the absence or presence of peptide.

In the absence of FITC-CRAMP, virtually all bacteria showed very low background levels of FITC fluorescence (Fig. 6C). A control FITC-linked peptide in which five of the lysine residues of CRAMP were changed to alanine also showed background levels of FITC labelling (Fig. 6D). This indicated that staining of bacteria with FITC-CRAMP does not result from non-specific binding of FITC to bacteria, but that fully functional CRAMP is required for this binding. In the presence of FITC-CRAMP, SL1344 bacteria showed a bimodal distribution of staining, with approximately 50% of the population at an intermediate level of staining, and 10.5% demonstrating a very high level of staining (Fig. 6E). Even in the

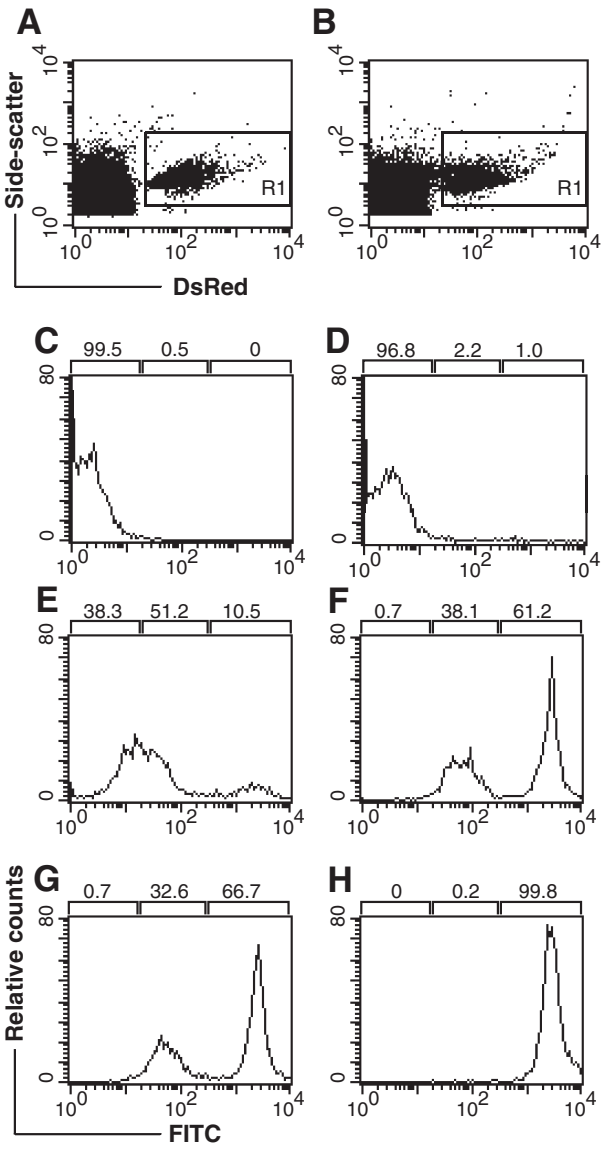

Fig. 6. Flow cytometric analysis of CRAMP binding to *S. typhimurium*. SL1344 (WT), *mig-14*, *virK* and *phoP* mutant bacteria were transformed with DsRed-expressing plasmid. Bacteria were treated with FITC-CRAMP and degree of fluorescence associated with bacteria was quantified by FACS analysis. All plots are representative of four independent experiments.
A. DsRed fluorescence versus side-scatter of SL1344 bacteria in the absence of FITC-CRAMP. Intact DsRed-positive bacteria are contained within gate designated R1. Representative of DsRed versus side-scatter plots for other bacterial strains in the absence of FITC-CRAMP.
B. DsRed fluorescence versus side-scatter of SL1344 bacteria in the presence of FITC-CRAMP. Gate R1 is same as in (A). Representative of dot plots for other bacterial strains in the presence of FITC-CRAMP. Histograms C-H were generated by analysing FITC fluorescence of R1-gated population. A total of 5000 events in R1 were analysed for each histogram.
C. Histogram of FITC fluorescence intensity of SL1344 bacteria from R1 in (A). Virtually all bacteria demonstrate background levels of fluorescence.
D. Histogram of FITC fluorescence intensity of *phoP* mutant bacteria treated with control FITC-CRAMP5A in which five lysine residues were mutated to alanine. This peptide has a neutral net charge and does not kill bacteria. Although the *phoP* mutant is highly sensitive to killing by CRAMP, 97% of *phoP* bacteria show background levels of staining with FITC-CRAMP5A.
E. Histogram of FITC fluorescence intensity of SL1344 treated with FITC-CRAMP. Thirty-eight per cent of bacteria stain at background levels, 51% of bacteria show intermediate fluorescence, 10.5% demonstrate high levels of FITC fluorescence.
F. FITC fluorescence intensity of *mig-14* mutant bacteria in the presence of FITC-CRAMP. Bacteria show a bimodal distribution with 38% of bacteria at an intermediate level of fluorescence and 61% of bacteria at maximal fluorescence intensity.
G. FITC fluorescence intensity of *virK* mutant bacteria in the presence of FITC-CRAMP. Distribution is similar to (F) with a slightly higher percentage of bacteria in the high-staining population and correspondingly lower percentage of bacteria in the intermediate-staining population.
H. FITC staining of *phoP* mutant bacteria in the presence of FITC-CRAMP. Virtually all *phoP* mutant bacteria demonstrate high FITC staining.

presence of peptide, however, nearly 40% of wild-type bacteria showed background levels of fluorescence.

In comparison to SL1344, all the mutants demonstrated a significant shift towards higher staining (Fig. 6F–H). The *mig-14* and *virK* mutants also demonstrated a bimodal distribution, with a significantly greater proportion in the high-staining population (61% and 67% respectively) compared with 10.5% of SL1344 (Fig. 6F and G). In contrast to SL1344, neither the *mig-14* nor *virK* mutants had any bacteria in the low-staining population. The *phoP* mutant showed only a single peak with virtually the entire population exhibiting a high level of FITC staining (Fig. 6H). These percentages correlate well with the degree of sensitivity of these mutant bacteria to killing by CRAMP. One possible reason for this could be that after bacteria are killed they simply bind more peptide in a non-specific manner. We therefore incubated heat-killed bacteria with FITC-CRAMP to determine whether dead bacteria non-specifically bind fluorescent peptide. While in general heat-killed bacteria bound more FITC-CRAMP than live bacteria, SL1344 nonetheless exhibited the lowest level of staining, *phoP* mutant bacteria showed the highest amounts of staining, and *mig-14* and *virK* mutants stained with intermediate levels of peptide (data not shown). This suggests that the staining of bacteria by FITC-CRAMP does not solely result from the fact that the bacteria are first killed by CRAMP before binding fluorescent peptide and that the presence of *mig-14* and *virK* gene products prevents association of CRAMP with the bacterial cell.

We additionally examined the association of FITC-CRAMP with several other bacterial strains: *trkA*, *sapAF* and SL1344 pMig-14. *trkA* and *sapAF* contribute to bacterial resistance to killing by protamine, another anti-microbial peptide. Both loci encode inner membrane proteins – *trkA* encodes a sodium channel (Parra-Lopez *et al*., 1994), while the *sapAF* locus shows homology to ATP-binding cassette peptide transport complexes (Parra-Lopez *et al*., 1993). We observed that the *trkA* mutant also showed a slightly higher degree of FITC-CRAMP staining (Fig. S2E) although not as high as either the *mig-14* or *virK* mutant. The *sapAF* mutant strain, which deletes the entire *sapAF* locus, showed the same degree of FITC-CRAMP staining as SL1344 (Fig. S2F), indicating that the increased association of *mig-14* and *virK* mutants with FITC-CRAMP is specific to these particular mutations. The SL1344 pMig strain actually showed a higher degree of FITC-CRAMP association than SL1344, although not as high as the *mig-14* mutant (Fig. S2D). These data, along with the mislocalization of Mig-14 to the outer membrane in SL1344 pMig, are consistent with previous observations that only a single-copy *mig-14* plasmid is able to complement the virulence of the *mig-14* mutant in mice (Valdivia *et al*., 2000).

CRAMP treatment disrupts bacterial cytoplasm

In order to understand how an inner membrane protein might be involved in preventing binding of an anti-microbial peptide to the bacterial surface, we examined the effect of CRAMP treatment on *Salmonella* by transmission electron microscopy. In contrast to bacteria treated with the anti-microbial peptide polymyxin B, treatment of bacteria with CRAMP did not result in the typical disruptions to outer membrane structure, and membrane blebbing that has been previously described (Koike *et al*., 1969; Lounatmaa *et al*., 1976; and Fig. 7B). Even *phoP* mutant bacteria, which are hypersusceptible to anti-microbial peptides, develop the classic outer membrane projections with relatively little disruption of the cytoplasmic contents after incubation with polymyxin B (Fig. 7D). Rather, we observed that treatment of bacteria with concentrations of CRAMP (10 μM) well below the amount necessary to see significant levels of killing (see Fig. 4) led to a marked disruption of the cytoplasmic contents of the bacterial cell, characterized by a condensation of the cytoplasmic material in some areas of the cell and the complete absence of cytoplasm in other areas (Fig. 7A).

Discussion

The outcome of bacterial infection is determined by the balance between bacterial virulence factors and host immune responses, in which proinflammatory cytokines such as IFN-γ, TNF-α, IL-12 and IL-18 play a critical role (Nauciel, 1990; Mastroeni *et al*., 1998; 1999). IFN-γ and TNF-α are crucial in controlling replication of many different intracellular pathogens, including *S. typhimurium* and *Mycobacterium tuberculosis* (Nauciel and Espinasse-Maes, 1992; Flynn *et al*., 1993). Macrophages, in which these intracellular pathogens survive and replicate, respond to the presence of IFN-γ and TNF-α by entering an activated state that is characterized by increased generation of reactive nitrogen and oxygen species (Nathan and Hibbs, 1991), increased production of anti-microbial peptides (Hiemstra *et al*., 1993; Rosenberger *et al*., 2004) and secretion of other proinflammatory cytokines and chemokines. Activated macrophages are thus more microbicidal than their unactivated counterparts, and are a critical component of host responses against infection. While the mechanisms by which *Salmonella* breaches epithelial barriers and survives within macrophages are beginning to be understood, very little is known about how pathogenic bacteria such as *Salmonella* survive and persist in the face of robust inflammatory responses.

We previously observed that *S. typhimurium* lacking the *mig-14* or *virK* genes are more sensitive to killing by anti-microbial peptides *in vitro* (Brodsky *et al*., 2002; Detweiler *et al*., 2003). These genes are adjacent to each other on

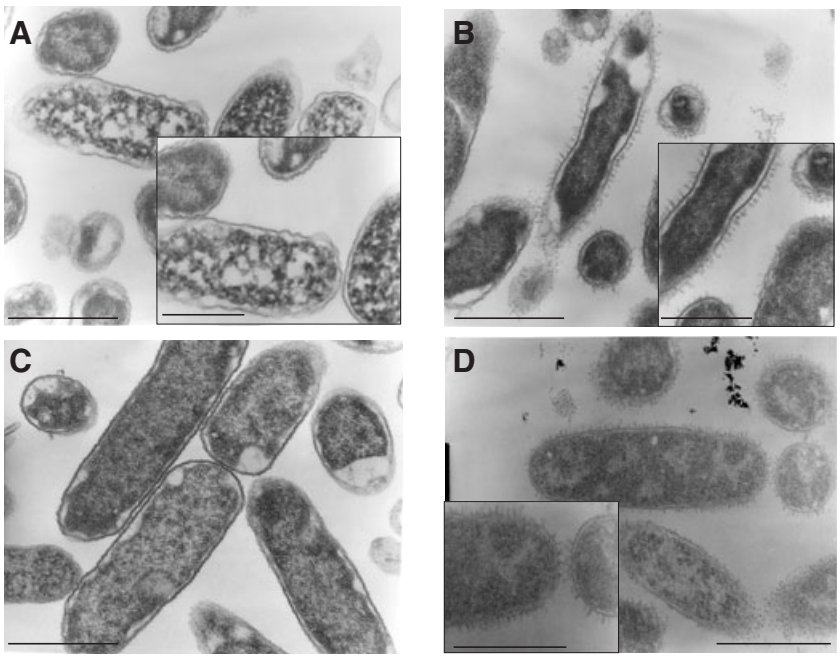

Fig. 7. Transmission electron micrographs of CRAMP- and polymyxin B-treated *S. typhimurium*.
A. *mig-14* mutant *S. typhimurium* treated with 10 μM CRAMP.
B. *mig-14* mutant *S. typhimurium* treated with 5 μg ml^{-1} polymyxin B.
C. *mig-14* mutant *S. typhimurium* untreated.
D. *phoP* mutant bacteria treated with 5 μg ml^{-1} polymyxin B.
Magnification = 28 000×, bar = 1.08 μM. Inset magnification = 45 000×, bar = 0.6 μM. SL1344 had substantially the same morphologies as *mig-14* or *phoP* under the same conditions, although in most cases perturbation of cellular structures was less dramatic.

the chromosome, regulated by the PhoP–PhoQ two-component system, and are transcriptionally upregulated in the macrophage vacuole and in the presence of anti-microbial peptides in the media (Valdivia and Falkow, 1997; Brodsky *et al*., 2002; I.E. Brodsky and S. Falkow, unpublished results). *mig-14* or *virK* mutant bacteria can colonize both the liver and the spleen after oral infection, but their replication is attenuated after day 5 post infection (Valdivia *et al*., 2000; Detweiler *et al*., 2003).

In order to further study the potential correlation of anti-microbial peptide resistance of *S. typhimurium* with pathogenesis, we performed additional infections with *mig-14* mutant bacteria. As *mig-14* mutant bacteria can colonize the deeper tissues of the RES, *mig-14* presumably is not required for resisting innate host defences such as defensins secreted by Paneth cells in the small intestine, or for initial survival within phagocytes present in the Peyer's patches. In contrast, mutation of *phoP* or SPI-2 TTSS machinery genes leads to drastic reduction in the ability of bacteria to colonize systemic tissues (Galan and Curtiss, 1989a; Beuzon *et al*., 2001), which also correlates with the inability of these mutant bacteria to replicate even within unactivated macrophages (Fields *et al*., 1989; Miller *et al*., 1989; Cirillo *et al*., 1998; Hensel *et al*., 1998). The timing of the *mig-14* mutant replication defect (days 5–7) suggested that adaptive immune responses might be involved in controlling its replication. As *mig-14* mutant bacteria are more sensitive to anti-microbial peptide killing, and activated macrophages upregulate expression of anti-microbial peptides, we hypothesized that *mig-14* might promote resistance to immune responses that increase expression of macrophage anti-microbial peptides. Consistent with this hypothesis, we observed that although *mig-14* mutant bacteria cannot replicate in systemic tissues of wild-type mice, they replicate rapidly in the spleen and liver of isogenic IFN-$\gamma^{-/-}$ mice, and achieve the same bacterial load as wild-type bacteria by day 7 post infection (Fig. 1A and B). These data suggested that *mig-14* plays a role in resistance of *Salmonella* to IFN-γ-dependent host responses. Interestingly, IFN-$\gamma^{-/-}$ mice still limit the replication of *mig-14* mutant bacteria at day 5 post infection, suggesting that IFN-γ-independent mechanisms may operate initially to control replication of *mig-14* mutant bacteria.

A hallmark of *S. typhi* infection of humans is that a low percentage of infected individuals become chronic lifelong carriers and periodically shed infectious organisms into the environment. While these individuals comprise only 1–6% of the total infected population (Hoffman *et al*., 1975; Levine *et al*., 1982), they act as a reservoir that enables bacteria to persist within human populations. It has been suggested that the coevolution of host-adapted *Salmonella* serovars with their hosts has paradoxically led to increased virulence because of the correlation between increased bacterial invasiveness and long-term persistence (Baumler *et al*., 1998). Bacterial genes that are necessary for persistence are therefore likely to be essential for the evolutionary success of microorganisms that are carried for long periods, indeed even for a lifetime of a specific host. Recent work in our laboratory demonstrated that 129Xi/SvJ mice harbour *S. typhimurium* within systemic tissues and shed bacteria in their faeces for at least 1 year after oral infection (Monack *et al*., 2004). Treatment of chronically infected mice with anti-IFN-γ neu-

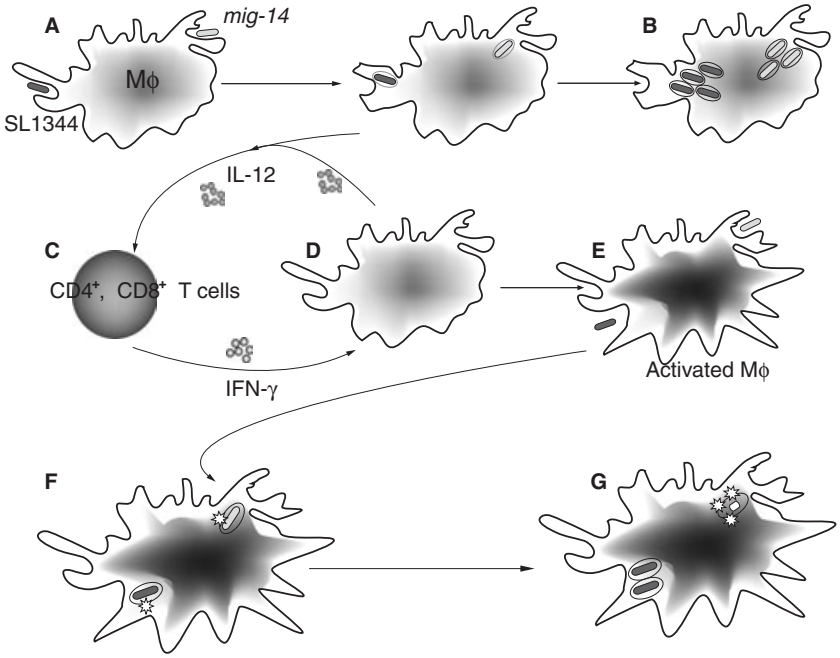

Fig. 8. Role of *mig-14* in resistance of *S. typhimurium* to anti-microbial defences of activated macrophages. Invasion of macrophages within Peyer's patches by *Salmonella* (A) leads to survival and replication of either wild-type (SL1344) or *mig-14* mutant bacteria in the infected cells (B). Presumably these cells transport *Salmonella* to deeper tissues such as the mesenteric lymph nodes, liver and spleen. However, macrophages that come in contact with bacteria and bacterial components such as LPS secrete proinflammatory cytokines including IL-12, which signals activation of $CD4^+$ and $CD8^+$ T lymphocytes (C). Activated T lyphocytes secrete IFN-γ, among other cytokines, which in turn activates macrophages resulting in increased secretion of IL-12 and upregulation of macrophage microbicidal functions (D and E). Peak IFN-γ levels are detected in serum of infected hosts at days 7–14 post infection with *Salmonella* (Lundin *et al*., 2002), coincident with the time at which *mig-14* mutant bacteria begin to have defects in replicating in systemic sites. Because it takes several days to generate adaptive responses and to accumulate appropriate circulating levels of cytokines, during initial stages of infection most bacteria encounter macrophages that have not yet been activated. Later during infection (days 5–7), most macrophages that bacteria encounter are likely to have been activated. Although both SL1344 and *mig-14* mutant bacteria find themselves in a more bactericidal environment within activated macrophages (F), SL1344 resists the effect of anti-microbial peptides such as CRAMP, whereas *mig-14* mutant bacteria cannot (G).

tralizing antibody led to rapid bacterial replication within the lymph nodes and Peyer's patches and led to significant increase in shedding even in the presence of normal adaptive immune responses to infecting bacteria (Monack *et al*., 2004). These data indicated that IFN-γ not only plays a critical role during initial stages of acute infection, but also controls bacterial replication during persistent infection.

As *mig-14* is important for resistance to IFN-γ-mediated host responses during acute infection, we examined whether *mig-14* contributes to persistent infection as well. We observed that although *mig-14* mutant bacteria can establish a persistent infection, long-term persistence of *mig-14* mutant bacteria is measurably attenuated. It is surprising that there were any *mig-14* mutant bacteria present at all in tissues after this length of time, given their attenuation in activated macrophages. However, it is important to note that the *in vivo* context contains many cell types other than just macrophages, and may therefore contain a cell type that enables some *mig-14* mutant bacteria to survive. It would be of interest to determine whether *mig-14* mutants do indeed occupy a different cellular niche from the corresponding wild-type bacteria. Recent studies demonstrated the presence of wild-type *Salmonella* within a specific subset of splenic macrophages, and demonstrated that SPI-2 was important for bacterial replication in these cells *in vivo* (Salcedo *et al*., 2001). However, whether a particular alternative niche might exist for bacterial mutants with defects in intracellular replication has not been investigated.

In fact, significantly fewer ($P = 0.0492$, Mann–Whitney *U*-test) *mig-14* mutant bacteria were present in the MLN relative to SL1344-infected mice at day 123 post infection, although there was not a significant difference in the percentage of mice whose lymph nodes were colonized (see Table 1). In the spleen there was no difference in bacterial cfu present between *mig-14* mutant and SL1344 bacteria, although nearly half of the *mig-14*-infected mice that were colonized in the MLN contained no bacteria in the spleen. This suggests that the capacity of *mig-14* mutant bacteria to survive in the MLN is compromised, but if the bacteria overcome this barrier, *mig-14* is less important to their survival in the spleen. In this same context it is noteworthy that when persistently infected mice were treated with

neutralizing anti-IFN-γ antibodies, the greatest amount of bacterial replication was seen in the MLN and Peyer's patches, with only a relatively modest increase in bacterial numbers in the spleen (Monack *et al*., 2004). Whether this reflects the presence of different cytokine microenvironments within these tissues or the presence of tissue-specific anti-microbial factors or macrophage subtypes is currently under investigation. A recent study indicates that the spleen may indeed be a more permissive environment for *Salmonella* than the MLN (Cheminay *et al*., 2004), although the basis for this is currently unknown.

In order to be successful, pathogenic bacteria have developed a variety of mechanisms to resist or avoid both innate and adaptive arms of host immune responses (Rosenberger and Finlay, 2003). Thus, wild-type *S. typhimurium* can survive and replicate within systemic tissues despite the presence of inflammatory cytokines (Young *et al*., 2002). Studies have begun to elucidate some of the mechanisms by which *Salmonella* breaches epithelial barriers in the small intestine (Galan and Curtiss, 1989b; Jones *et al*., 1994), and subsequently survives in host leukocytes (Fields *et al*., 1986; Miller *et al*., 1990; Cirillo *et al*., 1998; Hensel *et al*., 1998). Our work demonstrates that *mig-14* contributes to bacterial survival in the face of host inflammatory responses and promotes both acute and persistent bacterial infection.

The involvement of *mig-14* in this aspect of *S. typhimurium* pathogenesis appears to depend on the requirement of *mig-14* for bacterial survival specifically in activated macrophages. In the absence of either *mig-14* or *virK*, *S. typhimurium* shows decreased survival in IFN-γ-treated RAW 264.7-cultured macrophage-like cells and in IFN-γ-treated primary BMDM. In RAW 264.7 cells, this decrease in survival depends on macrophage activation, as we saw no difference in the ability of *mig-14* or *virK* mutant bacteria and SL1344 to replicate in unactivated RAW 264.7 cells. In BMDM, *mig-14* and *virK* mutant bacteria demonstrate reduced survival relative to SL1344 in both activated and unactivated cells, but the difference is greater in activated cells.

Anti-microbial peptides, which are produced in large quantities by injured epithelial tissues as well as by activated leukocytes, comprise a diverse family of small amphipathic molecules that can kill microbes by a variety of mechanisms (Zasloff, 2002). Anti-microbial peptides that have activity against *Salmonella* were previously purified from macrophages pretreated with IFN-γ (Hiemstra *et al*., 1993). More recent investigation demonstrated that the CRAMP of mice, first identified by sequence homology to the human peptide LL-37 (Gallo *et al*., 1997), is expressed in murine macrophages, colocalizes with the *SCV*, and has significantly higher activity and expression in macrophages activated by IFN-γ (Rosenberger *et al*., 2004). The observation that *mig-14* and *virK* mutant bacteria are more sensitive to CRAMP killing suggests that these genes contribute to the survival of *S. typhimurium* within activated macrophages by promoting resistance to CRAMP. Alternatively (or additionally), *mig-14* and *virK* could promote resistance to other anti-microbial components such as oxidative intermediates or lysosomal proteases.

While the mechanisms by which *mig-14* and *virK* contribute to anti-microbial peptide resistance are not completely clear, both *mig-14* and *virK* mutant bacteria bind significantly higher amounts of FITC-labelled CRAMP than isogenic wild-type bacteria as determined by flow cytometry. Studies with radiolabelled anti-microbial peptides have shown that sensitivity of different bacterial strains to killing by anti-microbial peptides correlates with the degree of peptide binding (Vaara *et al*., 1979). More recently, flow cytometry has been used to examine binding of fluorescently labelled peptides to bacteria (Schmidtchen *et al*., 2002); however, to our knowledge this is the first study that quantifies differential binding of anti-microbial peptides to bacterial mutants by FACS. Our data suggest that the Mig-14 and VirK proteins prevent association of anti-microbial peptides with the bacterial cell.

One mechanism by which this might occur is alteration of the bacterial envelope, either in the structure of LPS or in membrane protein expression (Gunn *et al*., 1998; Guina *et al*., 2000). Such changes contribute to anti-microbial peptide resistance by a variety of mechanisms. We observed that a protein of the appropriate size is absent from inner membranes of *mig-14* mutant bacteria, present in SL1344 and expressed at higher levels in bacteria containing *mig-14* on a multicopy plasmid (pMig); we confirmed that this differentially present protein is indeed Mig-14 through tryptic digestion and peptide sequencing. In the *mig-14* plasmid containing strain, Mig-14 is also present at low levels in the outer membrane fraction, although it was not detected in the outer membrane from wild-type bacteria (Fig. 5B). The predicted sequence of Mig-14 does not contain an obvious hydrophobic transmembrane domain or a signal sequence which would be associated with an integral inner membrane protein. It may therefore be a periplasmic protein that associates with the inner membrane fraction. We analysed periplasmic shock fluid from SL1344, *mig-14* and pMig bacteria by SDS-PAGE, but in contrast to inner membrane fractions, we did not detect any differences between the protein profiles of the periplasmic contents of the three strains (data not shown). Some periplasmic proteins are not released from the periplasm by standard methods (Ames, 1994), leaving open the possibility that Mig-14 may be a periplasmic protein that is more tightly associated with the inner membrane. We are currently investigating these possibilities.

Our transmission electron microscopy studies indicated that whereas polymyxin B acts primarily by disrupting the outer membrane, the primary site of CRAMP activity is the cytoplasm. This appears to be similar to the anti-microbial peptide buforin, which rapidly enters the bacterial cytoplasm at concentrations well below its minimun inhibitory concentration (MIC) (Park *et al*., 1998). These data together with the inner membrane localization of Mig-14 suggest the possibility that Mig-14 acts by preventing penetration of CRAMP into the cytoplasm. Mig-14 may bind CRAMP directly at the inner membrane, thereby preventing access to the cytoplasm, or Mig-14 may associate with a protein complex that binds or translocates CRAMP out of the cytoplasm. These data are also consistent with the observation that *mig-14* mutant bacteria are more sensitive to CRAMP than to polymyxin B [10-fold killing compared with SL1344 (Fig. 4) versus four- to fivefold killing compared with SL1344 under the same conditions] (Brodsky *et al*., 2002).

Our data that *mig-14* and *virK* play a role in resistance to the killing mechanisms of CRAMP provide a functional link between *S. typhimurium* resistance to anti-microbial peptides of activated macrophages and the ability to maintain both acute and persistent systemic infection. Other studies have suggested that *Salmonella* that are more sensitive to killing by polymyxin B are attenuated for replication in systemic sites after oral infection (Gunn *et al*., 2000). Anti-microbial peptides are classically believed to act during early stages of infection to prevent or control microbial colonization of peripheral tissues such as the skin or gastrointestinal tract (Hancock and Scott, 2000; Zasloff, 2002). Recent work, however, demonstrates that anti-microbial peptides can recruit and activate immature T cells and dendritic cells, indicating that anti-microbial peptides function as innate modulators of adaptive immune responses (Yang *et al*., 2000; 2002; Biragyn *et al*., 2002). Anti-microbial peptide expression is in turn upregulated by the proinflammatory cytokines IFN-γ and TNF-α, indicating that cross-talk exists between the innate and adaptive arms of the immune system. Pathogenic bacteria whose lifestyle involves systemic persistent disease are likely to have acquired specialized virulence factors to resist this aspect of host defence.

Cathelin-related anti-microbial peptide protects mice from invasive skin infection caused by group A *Streptococcus* (Nizet *et al*., 2001). Our work suggests a possible role for CRAMP as a protective mechanism during systemic infection by *S. typhimurium*. Some pathogenic bacteria, including *Streptococcus pyogenes*, express proteases that cleave and inactivate the human homologue of CRAMP, LL-37 (Schmidtchen *et al*., 2002). Interestingly, like *Salmonella*, these bacteria are capable of causing both acute and chronic persistent disease presumably despite the presence of inflammation and a robust adaptive immune response (Hansson and Faergemann, 1995). Resistance to killing by anti-microbial peptides may thus contribute to the ability of pathogenic bacteria to cause persistent infections in eukaryotic hosts. Our observation that *mig-14* mutant bacteria are attenuated in their ability to persist in systemic tissues is consistent with this hypothesis.

Selective pressures that occur during interactions of pathogenic organisms with their hosts drive the evolution of virulence factors on the part of pathogens and defence mechanisms of host organisms. Although *virK* and *mig-14* are adjacent genes believed to have been horizontally acquired by *Salmonella* during evolution, they are not both universally present in all subspecies of *S. enterica* (Baumler and Heffron, 1998; Valdivia *et al*., 2000). Both genes are present in all subspecies I serovars, which contain the vast majority of *Salmonella* species that infect warm-blooded animal hosts and include *S. typhimurium* and *S. typhi*. However, *mig-14* is present in subspecies IIIa and IIIb, while *virK* is absent. The converse is true in the case of subspecies II. Both are absent from *Salmonella bongori* as well as from *S. enterica* subspecies IV. Subspecies IV and *S. bongori* are highly host range limited and primarily infect cold-blooded animals. In the course of their evolution, *S. enterica* of subspecies I have experienced a dramatic expansion of host range and acquired the capacity to infect a wide variety of warm-blooded animals, including birds, rodents, primates and livestock (Baumler *et al*., 1998). It is intriguing that *mig-14* and *virK* both appear to be present only in subspecies I, which exhibit the broadest host-range of the *Salmonella* subspecies, particularly given the apparent role of *mig-14* and *virK* in later stages of disease. The presence of both *mig-14* and *virK* in the ancestor of the *S. enterica* subspecies I lineage may have enabled these serovars to cope with mammalian immune responses not previously encountered by *Salmonella*.

In this work, we demonstrate that the *mig-14* and *virK* genes of *S. typhimurium* contribute to bacterial resistance to the murine anti-microbial peptide CRAMP, and that this resistance correlates with bacterial survival in macrophages activated by IFN-γ. We further show that *mig-14* mutant bacteria replicate rapidly in systemic tissues of IFN-γ knockout mice. While other studies have isolated bacterial mutants that will cause persistent infection in an otherwise acute disease model (Clements *et al*., 2002), we demonstrate here that *mig-14* mutant bacteria are attenuated in long-term persistence, suggesting that resistance to host inflammatory responses is important not only in initiating but also in maintaining persistent infection. It is reasonable to suspect that *Salmonella* might require additional virulence factors to survive the anti-microbial environment generated by the cross-talk between innate and adaptive arms of the host response.

mig-14 and *virK* appear to be such virulence factors, and may represent a class of virulence determinants that serve this function. We suggest a model for the role of *mig-14* in surviving inflammatory responses of activated macrophages (Fig. 8).

Experimental procedures

Media and culture conditions

Bacterial strains used are listed in Table 2. Bacteria were grown overnight at 37°C with aeration in the N-minimal medium of Nelson and Kennedy containing 10 mM $MgCl_2$ (Nelson and Kennedy, 1971) with modifications as previously described (Snavely *et al*., 1991) except where otherwise indicated. The pH of the medium was buffered with either 100 mM Tris-HCl pH 7.4, or 10 mM 2-(*N*-morpholino) ethane sulphonic acid (MES) pH 5.0. Mutations were made according to the lamda *red* recombinase method as previously described (Datsenko and Wanner, 2000), using primers available at http://www.cmgm.stanford.edu/falkow/whatwedo/wanner/. All marked mutations were moved into the SL1344 background by transduction with P22HT by standard methods (Maloy *et al*., 1996).

Cell culture conditions and media

RAW 264.7 macrophages (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS). Approximately 2×10^5 cells were seeded per well in 24-well dishes and allowed to adhere overnight. In order to activate cells, media was removed and fresh media containing 100 U ml^{-1} recombinant IFN-γ (US Biologicals) and 50 ng ml^{-1} LPS (Sigma) were added. Cells were maintained in IFN-γ and LPS-containing medium for 18–24 h before infection. Cells that were not activated before infection were infected the day after seeding into 24-well dishes.

Mouse infection

Mice were orally infected with either 1×10^7 cfu (129Xi/SvJ) or 5×10^5 cfu (C57Bl/6J and isogenic IFN-$\gamma^{-/-}$) of bacteria. Mice were deprived of food for 14–16 h before infection. In the case of acute infection in the C57Bl/6J and IFN-$\gamma^{-/-}$ mice, infected animals were sacrificed at days 5, 7 and 9 post infection and organs isolated and homogenized. Serial dilutions of homogenized tissue were made in PBS and plated on selective agar to determine the cfu present per gram of tissue. In the case of persistent infection of the 129Xi/SvJ mice, animals were sacrificed at days 3, 7, 16, 28, 93 and 123 post infection. As with the acutely infected mice, organs were isolated, homogenized and plated to determine the bacterial cfu present.

Preparation of bone marrow-derived macrophages

The legs from 4- to 5-week-old C57BL/6 mice (Jackson Laboratories) were dissected and the bone marrow was flushed out. Monocytes were isolated by flotation on a Histopaque gradient (Sigma) and maintained at 37°C, 5% CO_2 in DMEM (Gibco) containing 10% heat-inactivated FBS (Gibco), supplemented with 30% L929 cell supernatant, 50 μg ml^{-1} penicillin and 50 μg ml^{-1} streptomycin for 6 days. The adherent cells were then washed briefly with ice-cold PBS and gently harvested using rubber scrapers. Cells were seeded at 2×10^5 cells per well in 24-well dishes and activated with IFN-γ as described for the RAW 264.7 cells or not activated before infection.

Bacterial infection and gentamicin protection assay

Bacteria were grown in LB at 37°C on a rotating wheel overnight. Bacteria were diluted 1:10 into PBS and quantified in a spectrophotometer (Ultrospec2000, Pharmacia) at 600 nm based on the calculation that a reading of 1 is equivalent to 1.2×10^9 bacteria per millilitre of culture. Bacteria were opsonized in 50% normal mouse serum for 30 min at 37°C on a rotating wheel, washed vigorously twice with PBS and diluted into DMEM containing 10% FBS. Cells were infected at a multiplicity of infection (moi) of 10 bacteria per cell in a total volume of 200 μl. Immediately after addition of bacteria, culture dishes were centrifuged at 500 r.p.m. for 5 min in a table-top centrifuge (Beckman Instruments). Dishes were placed in 37°C humidified incubator containing 5% CO_2 for an additional 30 min. The medium was aspirated, the cells were washed with PBS and fresh DMEM containing 100 μg ml^{-1} gentamicin was added for 90 min. The medium was then removed, cells were washed and either lysed in PBS containing 1% Triton X-100 or maintained in medium containing 10 μg ml^{-1} gentamicin for the indicated times. The 2 h time point was harvested 90 min after adding the 100 μg ml^{-1} gentamicin. The 8 h time point was harvested 6 h after the 2 h time point, and the 24 h time point was harvested 16 h later.

Table 2. Bacterial strains used in this study.

Strain or plasmid	Description	Source or reference
SL1344	*rpsL hisG xyl*	Hoiseth and Stocker (1981)
SL1344 pMig-14	pACYC184-*mig-14*	This work
RVY-5	SL1344 *mig-14*::*Kan*	Valdivia *et al.* (2000)
virK::*Kan*	SL1344 *virK*::*Kan*	Detweiler *et al.* (2003)
mig-14::*Kan virK*::*Cm*	SL1344 *mig-14*::*Kan virK*::*Cm*	This work
phoP::Tn*10*	SL1344 *phoP*::Tn*10Tet*^{r}	Rathman *et al.* (1996)

CRAMP sensitivity assay

A synthetic peptide corresponding to the 38-amino-acid sequence of CRAMP-2 described previously (Gallo *et al*., 1997) was synthesized at the Stanford Protein and Nucleic Acid core facility by Fmoc chemistry using a Symphony/Multiplex TM automated peptide synthesizer. A more detailed description is available at http://www.cmgm.stanford.edu/panfac/pepsyn/index.html. Synthesized peptides were analysed using reverse-phase HPLC and mass spectrometry. The CRAMP sensitivity assay was performed as described (Groisman *et al*., 1997; Brodsky *et al*., 2002). Briefly, bacterial strains were inoculated into modified N-minimal medium at pH 7.4 containing 10 mM $MgCl_2$. Stationary-phase cultures were harvested after overnight growth and washed three times in N-minimal medium pH 7.4 without $MgCl_2$. Strains were then diluted 1:100 into N-minimal media pH 5.0 containing 10 μM $MgCl_2$. Bacteria were incubated for 3 h at 37°C with aeration. Bacteria were harvested and quantified by measuring the culture absorbance at 600 nm. Bacteria were washed once with a modified low-osmolarity medium (N-minimal medium without Tris or $MgCl_2$). Approximately 2×10^5 bacteria in a volume of 50 μl were inoculated into each well of a 96-well dish, and 150 μl of CRAMP diluted in low-osmolarity medium were added to each well. Peptide was diluted to yield final concentrations of 0, 5, 10, 50 or 100 μM. Triplicate wells were assayed with each bacterial strain and peptide concentration. The plate was incubated on a platform shaker at 37°C for 60 min. Serial dilutions from each well were made in PBS and plated on selective agar. Per cent survival was normalized to the cfu obtained by incubation of bacteria in the absence of peptide.

FITC-CRAMP binding assay

FITC-CRAMP was synthesized by the Stanford protein and nucleic acid core facility as described above. Bacterial strains expressing the DsRed protein (Sorensen *et al*., 2003) from the strong *rpsM* promoter were treated as described for the CRAMP sensitivity assay and incubated with 10 μM FITC-CRAMP for 30 min before analysis by flow cytometry with a FACSCalibur (Becton Dickinson) using CellQuest software. Intact bacteria were identified by gating on the DsRed-positive population in the appropriate side-scatter range, and the amount of FITC label associated with this population was quantified.

Isolation and analysis of bacterial membranes

Bacterial membranes were isolated by differential density centrifugation as previously described (Nikaido, 1994). Briefly, bacteria were grown overnight in N-minimal medium pH 7.4 containing 10 mM $MgCl_2$. Bacteria were washed and grown in low-magnesium N-minimal medium with vigorous aeration until they reached an OD_{600} of 0.4–0.6. Cultures were harvested, washed once with ice-cold 10 mM Hepes pH 7.4 and stored at −80°C. The bacterial pellet was resuspended in ice-cold 10 mM Hepes pH 7.4 with 1 mM PMSF, 10 mg ml^{-1} DNase and RNase. Bacteria were lysed in a French pressure cell at 16 000 psi and spun at 1000 *g* to remove unlysed cells. Supernatants were spun for 2 h at 100 000 *g*. Membrane pellets were homogenized in 20% sucrose in 10 mM Hepes pH 7.4 with a 22-guage needle. Membranes were then overlayed onto a 53%–70% sucrose step gradient and spun for 12–16 h at 100 000 *g*. Inner membranes were isolated from the 20%–53% interface, while the denser outer membranes were isolated from the 53%–70% interface. Membranes were snap-frozen and stored at −80°C. Total protein content of the membranes was measured by BCA protein assay (Pierce) and 60 μg of total protein per well was analysed by SDS-PAGE (12.5% poly acrylamide gel) and Coomassie staining. Protein bands were visualized after destaining with a Li-Cor scanner at 700 nM (Odyssey Systems). Immunoblotting was performed with anti-Imp antiserum (Braun and Silhavy, 2002) on inner and outer membrane fractions after SDS-PAGE (10% polyacrylamide gel) and transfer of proteins to nitrocellulose according to standard methods.

Peptide sequencing

Sequencing was performed by Integrated Analytical Solutions (Burlingame, CA, USA) according to the following procedures. Bands of interest at approximately 35 kDa were excised with a razor blade along with an equivalent size piece from a blank section of the gel. The excised bands were cut into approximately 1 mm pieces and digested in-gel with proteomics grade trypsin using standard procedures. These procedures included de-staining, reduction with dithiothreitol, alkylation with iodoacetamide and digestion at 10 ng μl^{-1} trypsin. The acetonitrile in the samples containing the extracted tryptic peptides was removed by incubation at 37°C. Samples were manually loaded onto a Peptide CapTrap (Michrom Bioresources), washed, then placed in line with a capillary LC-MS/MS system. The MS/MS spectra from each sample analysis were searched against the NCBI salmonella database via Mascot™ using up to two missed cleavages, a peptide mass tolerance of ±100 p.p.m and an MS/MS ion tolerance of ±0.1 Da.

Electron microscopy

Bacteria were grown overnight in N-minimal medium, pH 7.4, 10 mM $MgCl_2$, washed, diluted 1:40 into pH 5.0, 10 μM $MgCl_2$, and incubated with shaking for 3 h. Bacteria were then washed with low-salt buffer as described above and incubated for 45 min with either 10 μM CRAMP, 5 μg ml^{-1} polymyxin B, or left untreated. Bacteria were then prepared for electron microscopy as follows: bacteria were fixed with 2.5% glutataldehyde in 0.1 M sodium cacodylate, pH 7.4 for 2 h, post-fixed with 1% osmium tetroxide, and *en bloc* stained in 0.5% uranyl acetate overnight. Bacteria were dehydrated in a graded ethanol series, then embedded in Epoxy. Thin section were stained with 2% uranyl acetate and 1% lead citrate before examination with a Philips CM-12 transmission electron microscope.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4444/mmi4444sm.htm

Fig. S1. Sequence of Mig-14 protein indicating peptides identified by mass spectrometry.

Fig. S2. FITC-CRAMP binding to additional *S. typhimurium* strains.

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