tract to particular sites the three cell types that cooperate to generate an inflammatory allergic response.

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10. The percent of $CCR3^+$ T cells in peripheral blood varied in different donors from <0.2% to 8%. Three-color immunofluorescence analysis showed that a variable fraction (10 to 60%) of the $CCR3^+$ T cells express CD69. In all cases, however, the $CCR3^+$ cells account for only a fraction of all $CD69^+$ cells (*25*).
11. Peripheral blood mononuclear cells (PBMCs) were stained with monoclonal antibody 7B11 (anti-CCR3; IgG2a) (*9*). Positive and negative cells were sorted and expanded as polyclonal lines, using PHA (1 μg/ml; Wellcome, Buckingham, UK), human recombinant IL-2 (rIL-2; 500 U/ml) (*26*), and irradiated PBMC. After 14 days, the cells were washed and stimulated with plastic-bound anti-CD3 (monoclonal antibody TR66; IgG1) plus 10^{-7} M phorbol 12-myristate 13-acetate (PMA). Cytokine production was measured in the 24-hour culture supernatant by enzyme-linked immunosorbent assay (ELISA), using matched pairs of antibodies specific for IL-2, IL-4, IL-5, IL-10, tumor necrosis factor–α (TNF-α), TNF-β, and IFN-γ (PharMingen, San Diego, CA).
12. T cells were stimulated with 10^{-7} M PMA plus ionomycin (1 μg/ml) for 4 hours. Brefeldin A (10 μg/ml) was added during the last 2 hours. Cells were fixed with 2% paraformaldehyde, permeabilized with phosphate-buffered saline containing fetal bovine serum (1%) and saponin (0.5%) and stained with fluorescein isothiocyanate (FITC)–labeled anti–IFN-γ (IgG1) and phycoerythrin (PE)-labeled anti–IL-4 (IgG2b) monoclonal antibodies (Becton Dickinson, Mountain View, CA). In some experiments, the cells were stained, before permeabilization, with anti-CD4 (BL4, IgG2a, Immunotech, Marseille, France) or anti-CD8 (OKT8, IgG2a, American Type Culture Collection), then with biotin-labeled goat anti-mouse IgG2a (Southern Biotechnology, Birmingham, AL) and streptavidin-tricolor (Molecular Probes, Eugene, OR). Because T cell activation results in a rapid down-regulation of CCR3, it is not possible to assess CCR3 expression and intracellular cytokines simultaneously.
13. The difficulty in obtaining pure $CCR3^+$ T cell lines by direct sorting from peripheral blood may arise from the low level of CCR3 expression and the consequently high contamination by negative cells. A second sorting from partially enriched cell lines resulted in all cases in a substantial enrichment in $CCR3^+$ cells. Results comparable to those in Fig. 1 were obtained in sorted cell lines obtained from two healthy and three atopic individuals. In three out of five cases the CCR3-depleted cells produced lower concentrations of IL-4 and IL-5 than the unsorted cells. No clear differences were found in the production of IL-10, IFN-γ, IL-2, TNF-α, or TNF-β, or between healthy and atopic donors. A correlation between CCR3 expression and production of IL-4 but not IFN-γ was observed in 13 antigen-specific T cell clones (*14*).
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Fluorescence-Based Isolation of Bacterial Genes Expressed Within Host Cells

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A selection strategy was devised to identify bacterial genes preferentially expressed when a bacterium associates with its host cell. Fourteen *Salmonella typhimurium* genes, which were under the control of at least four independent regulatory circuits, were identified to be selectively induced in host macrophages. Four genes encode virulence factors, including a component of a type III secretory apparatus. This selection methodology should be generally applicable to the identification of genes from pathogenic organisms that are induced upon association with host cells or tissues.

Many bacterial pathogens survive in professional phagocytes by coordinately regulating the expression of a wide spectrum of genes (*1*). Because a microbe's ability to survive killing by phagocytes correlates with its ability to cause disease (*2*, *3*), the identification of genes that are preferentially transcribed in the intracellular environment of the host is central to our understanding of how pathogenic organisms mount a successful infection. So far, selections for in vivo–expressed genes (*4*-*6*) have been limited to bacterial pathogens with tractable genetic systems, because of the requirement for high frequencies of homologous recombination and extensive strain manipulation before gene selection (*4*, *6*).

We have developed a selection methodology, on the basis of differential fluorescence induction (DFI) (*7*), for the rapid identification of bacterial genes induced upon association with host cells that would work independently of drug susceptibility and nutritional requirements. Green fluorescent protein (GFP) (*8*) was used as a selectable marker in conjuction with fluorescence-activated cell sorting. Host cells infected with a bacterium bearing a transcriptionally active *gfp* gene fusion were separated by a fluorescence-activated cell sorter (FACS) and lysed, and the bacteria recovered were then grown under ex vivo conditions. Bacteria were then isolated by FACS on the basis of low to no fluorescence in the absence of host cells. Because all screening and selection steps are per-

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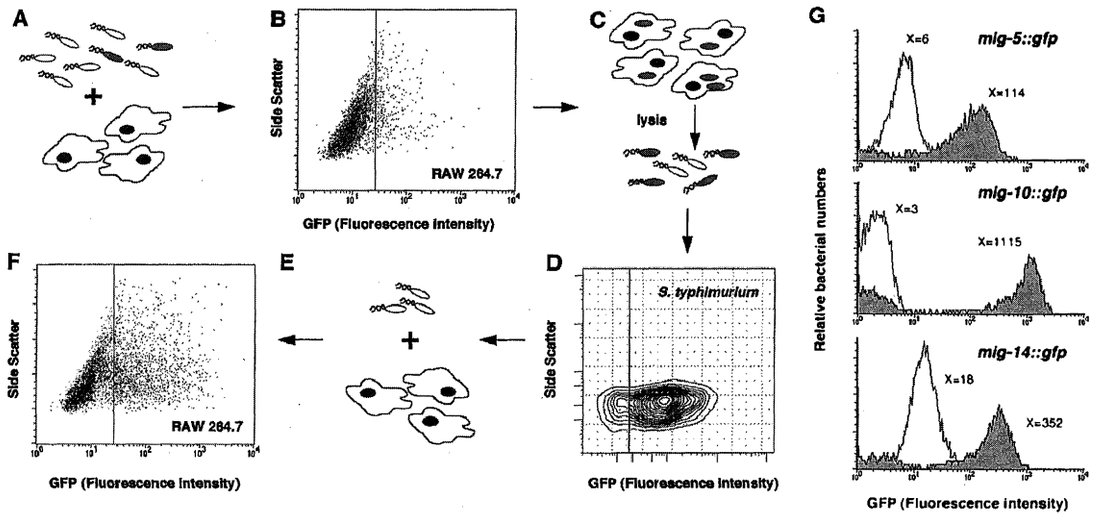

Fig. 1. Fluorescence-based selection for intracellularly induced genes. (**A**) A library of *S. typhimurium*–bearing plasmids with random DNA fragments inserted upstream of a promoterless *gfp* gene (*39*) was used to infect a monolayer of RAW 264.7 macrophage-like cells at a multiplicity of infection (MOI) of 5:1. Fluorescent bacteria are shaded. (**B**) After a 6-hour infection, RAW 264.7 cells were gently scraped from the tissue culture wells, resuspended in cold Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), and analyzed in a FACStar cytometer. Cells infected with a fluorescent bacterium were readily distinguished from uninfected cells or cells infected with *S. typhimurium*–bearing unproductive *gfp* gene fusions. (**C**) Fluorescent RAW 264.7 cells were sorted, lysed with 1% Triton X, and the recovered bacteria were plated on L-agar. Bacterial colonies were pooled and a sample grown in DMEM (supplemented with 10% FCS) at 37°C in 5% CO_2 for 6 hours. (**D**) FACS analysis of this population yielded bacteria with a wide range of fluorescence intensities. Bacteria with the lowest fluorescence (lower 15%) were sorted as described (*7*). (**E**) The bacteria with low fluorescence in the absenceof host cells were amplified on L agar and used to reinfect RAW 264.7 cells at an MOI of 0.5 to 1 for 6 hours. (**F**) Flow cytometric analysis showed enrichment for fluorescent infected cells as compared to infection with the initial library. The fluorescent cells were collected and the bacteria within recovered. (**G**) Independent clones exhibited varied levels of intracellular-dependent gene induction. Histograms show three different strains in which the relative fluorescence intensity of bacteria grown under ex vivo conditions (DMEM + 10% FCS) (solid line) and fluorescence of bacteria released from infected cells after 6 hours (shaded) were compared (*7*). Analysis and quantitation of fluorescence were performed with CellQuest software (Beckton Dickinson).

formed by a FACS, the biases inherent to manual screening are avoided.

Salmonella typhimurium survives in phagocytes (*2*) and causes human gastroenteritis and murine typhoid fever (*9*). We applied DFI to identify *S. typhimurium* genes that are induced in phagocytic cells (Fig. 1). To analyze bacterial clones that were isolated in the selection scheme, we compared the fluorescence intensity of individual bacteria grown in tissue culture media with the fluorescence intensity of the same bacteria clone after release from infected cells (Fig. 1G). Intracellular-dependent induction of the *gfp* gene fusions isolated was further confirmed by fluorescence microscopy (Fig. 2). Approximately a third to a half of all *S. typhimurium* that were recovered after one enrichment cycle contained a *gfp* gene fusion with host cell–dependent activity.

Fourteen promoters with intracellular-dependent activity were identified (Table 1). The genes downstream of these promoters were isolated by recombinational cloning (*10*), sequenced, and characterized. Eight of these macrophage-inducible genes (*mig*s) have either been described or have close homologs with known functions in other bacterial species. Two *mig*s encode known virulence factors. The *him*A gene (*mig-23*) encodes subunit A of integration host factor (IHF), which is involved in DNA replication, recombination, and gene regulation (*11*), and is preferentially expressed in vivo (*4*). The *mig-10* gene product is homologous to a family of bacterial virulence proteins (YscF, MxiH, and PrgI) that are essential components of virulence-associated type III secretion systems present in several bacterial pathogens (*12–14*). Sequence analysis of the loci associated with *mig-10* indicated that this open reading frame (ORF) is present within the *Salmonella* pathogenicity island 2 (SPI-2), which is necessary for *S. typhimurium* survival in BALB/c mice (*15*). For consistency, *mig-10* will be referred to as *ssaH* (*16*). The *mig-1* gene encompasses the intergenic region between the gene encoding the galactose repressor (*galR*) and a close homolog of the *Escherichia coli aas* gene that encodes acylacylglycerolphosphoethanolamine acyltransferase, which is involved in phospholipid recycling and possibly membrane repair (*7*, *17*). *mig-2* (*pagA/ugd*) encodes Ugd, which is necessary for growth in a low-magnesium environment, is homologous to a *Streptococcus pneumoneae* gene product involved in capsule biosynthesis (*18*), and is transcriptionally active inside macrophages (*7*, *19*). The *mig-4* gene product is a homolog of the *E. coli* periplasmic phosphate transport protein PhoS (*20*), the induction of which may represent *S. typhimurium*'s

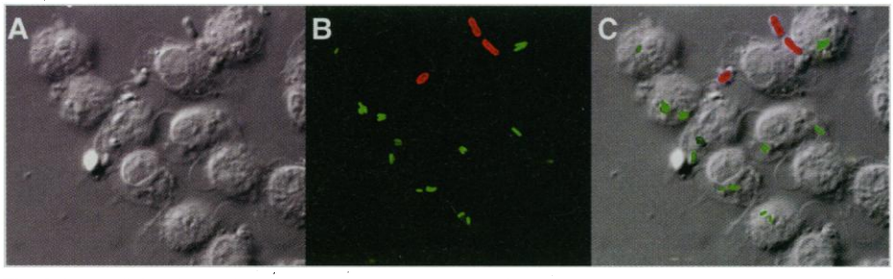

Fig. 2. Intracellular-dependent bacterial gene expression. *Salmonella typhimurium* bearing an *ssaH::gfp* fusion (*mig-10*) were used to infect RAW 264.7 cells for 4 hours. (**A** and **C**) Differential interference contrast (DIC) image showing the relative topology of bacterial association with the host cell. External bacteria were immunostained with a polyclonal antisera to *Salmonella* followed by a second stage phycoerythrin conjugate (red). (**B**) Only internalized bacteria show active synthesis of the GFP tag (green). Fluorescence images were acquired and processed on an Applied Precision Deltavision Deconvolution System. (C) The fluorescence and DIC images were superimposed with Adobe Photoshop software.

Table 1. Characterization of macrophage-inducible genes (*mig*). DNA fragments (0.2 to 1.2 kb) with intracellular-dependent activity trapped by differential fluorescence enrichments (Fig. 1) were sequenced and compared with the available DNA sequence databases at the National Center for Biotechnology Information (NCBI). Putative functions were assigned either when a particular gene had been previously described in *S. typhimurium* or when the function of a close homolog (>50% amino acid identity) had already been determined. For promoter regions with no significant homology to previously described genes or with homology to genes with unknown function, flanking DNA was isolated by recombinational cloning (*10*) and sequenced. Five ORFs downstream of macrophage-inducible promoters were inactivated either by insertion of a kanamycin resistance gene flanked by transcriptional terminators (ΩKn) or by insertion of the suicide vector pGP704 (Amp^r) into coding regions (*27*). ND, not determined.

Construct	Homology	Fold induction in macrophages*	Protein features or putative function	Regulation†	Role in virulence‡		
						Ref.	CI
mig-1§	*aas*	16.4	Phospholipid recycling	OmpR/EnvZ (*40*)	–	–	ND
mig-2	*pagA/ugd*	16.6	Capsule biosynthesis	PhoP/PhoQ	No	(*29*)	ND
mig-3	–	31.1	Phage-derived genes	PhoP/PhoQ	–	–	ND
mig-4	*phoS*	9.4	Phosphate transport	PhoB/PhoR (*20*)	No	This study	1.7 ± 1.2
mig-5	–	24.1	Virulence plasmid lipoprotein	PhoP/PhoQ	Yes	This study	0.15 ± 0.01
mig-7	*yjbA (orf o156)*	15.2	Inner-membrane protein	PhoP/PhoQ	No	This study	0.77 ± 0.5
mig-10	*ssaH*	442.9	Type III secretion	SsrA/SsrB	Yes	(*15, 16*)	<0.001
mig-13	*orf f198*	8.0	Transmembrane protein	–	No	This study	1.6 ± 0.25
mig-14	–	22.4	–	PhoP/PhoQ	Yes	This study	0.05 ± 0.02
mig-20‖	–	12.6	–	–	–	–	ND
mig-23	*himA*	14.9	Gene regulation	–	Yes	(*4*)	ND
mig-26	*exc(traT)*	9.4	Plasmid exclusion protein	PhoP/PhoQ	Serum resistance	(*21*)	ND
mig-29	*hslU*	23.7	Stress-inducible protease	PhoP/PhoQ	–	–	ND
mig-30	–	11.1	–	PhoP/PhoQ	–	–	ND

**mig* activity inside cells was determined as described in Fig. 1G. †Macrophage induction of the various *mig::gfp* gene fusions was tested in wild-type, PhoP$^-$, and OmpR$^-$ *S. typhimurium* backgrounds. In addition, the expression of *mig-10::gfp* was tested in a *S. typhimurium* background bearing a miniTn5Km insertion in either *ssaR* or *ssrB* (*15*). ‡The role in virulence of the various *mig* genes was determined either from previous work (Ref.) or from information derived from insertionally inactivated *mig* (*28*). The SPI-II mutant P3F4 (*15*) was included as a control. §*mig-1* represents a DNA fragment spanning the intergenic region between *galR* and *aas* and is present ~400 bp upstream of the *aas* start codon. ‖*mig-20* could not be isolated by recombinational cloning.

response to phosphate deprivation within the host cell. The *mig-26* gene encodes the exclusion protein (TraT) of the *S. typhimurium* virulence plasmid, which is partially responsible for the serum resistance phenotype of *S. typhimurium* (*21*). The *mig-29* gene product is a homolog of the *E. coli* heat shock–inducible proteasome component Hslu (*22*) and represents an example of a stress response protein that is synthesized during cell infection.

Six of the *mig* gene products showed either no significant homology to previously identified bacterial genes or homology to gene products of unknown function. The *mig-3* gene is present within an ORF with homology to phage tail-fiber assembly proteins and likely represents a promoter within an integrated phage. Although virulence determinants may be acquired by horizontal gene transfer (*14*), we have no evidence that any virulence genes were transduced within these phage sequences. The *mig-30::gfp* fusion is present at the 3′ end of a gene encoding a homolog of an *E. coli* putative transmembrane protein (YjsH) (*23*). However, the polarity of transcription is in the opposite orientation to that of *yjsH* and would therefore generate an antisense transcript that could down-regulate this protein's synthesis during intracellular infection (*24*). The *mig-5*, *mig-7*, *mig-13*, and *mig-14* genes encode either small, highly hydrophobic proteins or contain motifs characteristic of bacterial lipoprotein-specific signal sequences. The *mig-5* gene maps to the *S. typhimurium* virulence plasmid and is located ~2 kb upstream of the *spv* virulence operon, which is required by *Salmonella* sp. to cause systemic disease (*25*, *26*). The *mig-14* gene product has an internal stretch of amino acids that is significantly homologous (30% amino acid identity) to members of the LysR family of transcriptional activators. This observation suggests a possible role for *mig-14* in the regulation of bacterial gene expression in the intracellular environment.

To establish the role in virulence of these macrophage-inducible proteins, we disrupted five of the unknown *mig* genes by insertion of either an ΩKn (Kan^r) element or plasmid pGP704 (Amp^r) into each ORF (*27*). These mutants were tested in competition assays against the wild-type *S. typhimurium* for the ability to colonize the spleens of BALB/c mice and for survival in the macrophage-like cell line RAW 264.7. Insertions in *mig-5* and *mig-14* resulted in a decrease in spleen colonization for each mutant strain (Table 1) (*28*). However, none of the mutant strains tested showed any growth defect in RAW 264.7 cells. These results, coupled with the roles of the previously reported virulence determinants *himA* and *ssaH*, suggest that genes induced in the intracellular environment, although not essential for survival in tissue culture models of infection, are important for bacterial survival within the host.

A key regulator of *S. typhimurium* gene expression inside host cells is the two-component regulatory system PhoP/PhoQ (*29*).

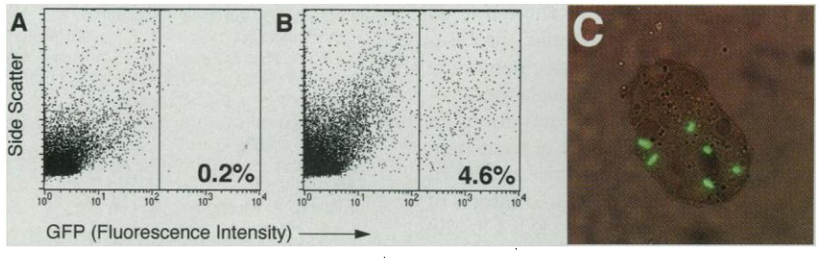

Fig. 3. *mig* gene expression within infected spleenocytes. FACS analysis of spleen homogenates from (**A**) *S. typhimurium*– or (**B**) *S. typhimurium* (*mig-5::gfp*)–infected BALB/c mice. (**C**) A transmitted light and fluorescence image of sorted fluorescent cells from (B) as imaged through a long-pass fluorescein-rhodamine filter (Chroma Technologies). BALB/c mice were injected intraperitoneally with 10^5 *S. typhimurium* bearing various *mig-gfp* fusions. After 3 days the spleens were removed and gently homogenized in phosphate-buffered saline supplemented with 10% FCS. The cell suspension was analyzed in a FACScalibur cytometer (Beckton Dickinson) and in a Nikon epifluorescence microscope.

To determine how many *mig*s were under the control of PhoP/PhoQ, we conjugated plasmids bearing *mig::gfp* fusions into a *S. typhimurium* strain bearing a *phoP*::Tn10 insertion (*30*). Eight of the 14 *mig*s required PhoP/PhoQ for induction in macrophages (Table 1), including two *mig*s (*mig-5* and *mig-14*) important for *S. typhimurium* virulence in BALB/c mice. Thus, the PhoP/PhoQ two-component system is a major regulator of macrophage-inducible genes important in *Salmonella* sp. virulence as previously noted (*19*, *29*, *31*, *32*). Because *S. typhimurium* can invade and replicate in a wide variety of cell types (*9*), we tested *mig* induction in RAW 264.7 macrophages, activated RAW 264.7 macrophages (10 U of interferon-γ and 1.5 ng/ml lipopolysaccharide), the epithelial cell line Hep-2 , HeLa fibroblasts, and FSDC dendritic cells (*33*). The level of intracellular *mig::gfp* expression was indistinguishable among these different cell types. From these results, we conclude that *mig*s respond to common intracellular cues rather than those unique to macrophages. Our finding that PhoP/PhoQ-dependent genes are not macrophage specific is consistent with a previous report of a PhoP/PhoQ-dependent gene that is induced in polarized epithelial cells (*34*). The macrophage-inducible virulence factor (*ssaH*) present within SPI-2 did not require PhoP/PhoQ for intracellular activity. *ssaH* is part of an operon that includes other essential components of the type III secretion apparatus such as *ssaJ* (*16*). This suggests that the regulation of the synthesis of components of this secretory apparatus may occur through a different mechanism, possibly the putative two-component regulatory system (SsrA/SsrB) that is also present within SPI-2 (*15*, *35*). Therefore, we tested an *ssaH::gfp* fusion in *S. typhimurium* backgrounds bearing a miniTn5Km insertion in either *ssrA* or *ssrB* (*15*). Indeed, disruption of either the sensor or regulator components resulted in a loss of *ssaH::gfp* induction within RAW 264.7 cells.

Type III secretion systems in pathogenic bacteria are also called contact-dependent secretory systems because of their role in the translocation of bacterial proteins across the eukaryotic plasma membrane upon cell contact (*36*). Although the virulence defects associated with mutations in components of the secretion apparatus in SPI-2 have been described, little is known about the molecular function and targets of SPI-2 gene products (*15*, *16*, *35*). On the basis of our results, it seems plausible that a secretory apparatus assembled within the host cells may function in translocating bacterial proteins across the vacuolar membrane.

To establish whether *mig::gfp* fusions were expressed within infected animal cells, we injected BALB/c mice intraperitoneally with 10^5 *S. typhimurium* bearing different *mig::gfp* fusions. Three days after infection, the spleens and livers were homogenized and analyzed by fluorescence microscopy and flow cytometry (Fig 3). All *mig::gfp* fusions were active within infected spleenocytes and hepatocytes. In heavily colonized animals, as much as 5% of all spleenocytes were infected with one or more fluorescent organisms. The ability to track infected cells in an animal host should extend the utility of this selection technology to enable the identification of host cell subsets that are preferentially targeted by a pathogen.

Although many intracellular-induced bacterial genes (for example, *spv* and those contained in SPI-2) do not seem to have a clear role in survival in tissue culture models of infection, they are essential for virulence in animals (*15*, *25*). This suggests that intracellular induction of key bacterial virulence determinants is required for systemic infection. By tracking the expression of these genes in infected animals, we might be able to further define when and where virulence factors are expressed. New generations of spectrally distinct fluorescent protein reporters (*37*) may soon permit the simultaneous monitoring of multiple bacterial genes in response to the host environment.

The selection methodology described here represents a tool with which to dissect the genetic basis of the interactions between two or more organisms. The only genetic requirements are that the test organism be able to maintain an episomal element and express a functional *gfp*. The technology should be widely applicable to the study of gene induction by bacterial, fungal, viral, and protozoan pathogens or to the study of symbiosis and gene regulation in complex microbial communities.

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10. A recombinational strategy was used to isolate loci downstream of each of the macrophage-inducible promoters characterized. Each plasmid bearing a *mig::gfp* fusion was conjugated into the *S. typhimurium* strain SL4702R (*polA rpsL*). This strain does not support the replication of ColE1 plasmids and thus the pFPV plasmid integrates by homologous recombination. Total DNA for each integrant was isolated, and 10 μg of DNA was digested either with Hind III or Sph I. The digested DNA was religated and used to transform *E. coli* strain DH12S. Plasmid DNA was isolated from Amp^r colonies, digested with restriction enzymes, and compared with predicted *S. typhimurium* chromosomal DNA fragments from DNA blot hybridizations. The captured DNA downstream of each *mig* promoter was sequenced by subcloning restriction enzyme–digested DNA fragments into the sequencing vector pBK-CMV (Stratagene) and by primer walking. ORFs, deduced amino acid sequence, and protein motifs were determined with programs from the Wisconsin GCG package. The sequences described have been deposited with GenBank (accession numbers AF020804-AF020812).
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24. The *mig-30::gfp* fusion was isolated as a 385–base pair (bp) DNA fragment. No ORFs were detected upstream of the *gfp* juncture, suggesting that the macrophage-induced transcript formed is antisense to *yjsH* and not the result of readthrough from an upstream gene.
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27. ORFs and associated DNA downstream of each cloned *mig* were inserted into the allelic exchange vector pRTP-1. Where appropriate restriction sites were available, an Ω Kan^r element was inserted to disrupt the ORF. Alternatively, an internal fragment from the ORF was amplified by polymerase chain reaction and inserted into the suicide vector pGP704 (Amp^r). These constructs were mobilized into SL1343R (*rpsL*), followed by selection of the appropriate antibiotic resistance marker. Gene disruptions by integration at the homologous site were confirmed by DNA blot hybridization (*38*) and mobilized into SL1344 by P22HT phage transduction. These mutations are likely to have polar effects on genes downstream of the disrupted ORFs, and thus *mig* insertional mutants represent mutations in potential *mig* operons.
28. Competition experiments between wild type *S. typhimurium* and *mig*-ΩKn mutants were performed to determine the virulence of each mutant strain. Eight-week-old female BALB/c mice were injected intraperitoneally with a 1:1 mix (total of 10^3 organisms) of wild-type and mutant *S. typhimurium* strain. After 6 days, the animals were killed, and the colony-forming units present in the spleen were determined. We determined the test strain's competitive index (CI) by calculating the ratio of mutant strain to wild-type present in the spleens. Virulence defects were scored by the ability of *mig* mutants to compete with wild-type *S. typhimurium*. A CI of 1.0 represents equal competitive advantage of the two strains to colonize the spleens of BALB/c mice. As a control, a known attenuated *S. typhimurium*

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39. *S. typhimurium* strain SL1344 total DNA was isolated, partially digested with Sau 3A, and size-fractionated on an agarose gel (0.4 to 1.6 kb). These DNA fragments were inserted at the calf intestinal alkaline phosphatase (CIAP)-treated Bam HI site of the promoter trap vector pFPV25 (*7*), creating gene fusions to a promoterless *gfp*mut3 gene (*8*). This multicopy plasmid (<50 copies per cell) was used to increase the range of fluorescence obtained from various *gfp* fusions. Similar results have been obtained with the use of single-copy *gfp* fusions to strongly induced promoters (*7*) and *lacZ* fusions (*4*, *19*). Eight pools of plasmids containing DNA inserts (3000 to 5000 thousand independent inserts per pool) were used to transform SL1344 and maintained as separate pools during selections.
40. R. H. Valdivia, M. B. Rathman, S. Falkow, unpublished data.
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