Salmonella maintains the integrity of its intracellular vacuole through the action of SifA

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A method based on the Competitive Index was used to identify Salmonella typhimurium virulence gene interactions during systemic infections of mice. Analysis of mixed infections involving single and double mutant strains showed that OmpR, the type III secretion system of Salmonella pathogenicity island 2 (SPI-2) and SifA [required for the formation in epithelial cells of lysosomal glycoprotein (lgp)-containing structures, termed Sifs] are all involved in the same virulence function. sifA gene expression was induced after Salmonella entry into host cells and was dependent on the SPI-2 regulator ssaA. A sifA⁻ mutant strain had a replication defect in macrophages, similar to that of SPI-2 and ompR⁻ mutant strains. Whereas wild-type and SPI-2 mutant strains reside in vacuoles that progressively acquire lgps and the vacuolar ATPase, the majority of sifA⁻ bacteria lost their vacuolar membrane and were released into the host cell cytosol. We propose that the wild-type strain, through the action of SPI-2 effectors (including SpIC), diverts the Salmonella-containing vacuole from the endocytic pathway, and subsequent recruitment and maintenance of vacuolar ATPase/lgp-containing membranes that enclose replicating bacteria is mediated by translocation of SifA.

Keywords: Competitive Index/SPI-2/trafficking/type III secretion/vacuole

Introduction

Systemic infection of mice by Salmonella typhimurium is a useful and intensively studied model of typhoid fever. Following oral inoculation, bacteria can survive the acid pH of the stomach, adhere to and invade cells of the intestinal epithelium, acquire nutrients, survive in the blood, replicate within macrophages and induce cytotoxicity (Finlay, 1994; Richters-Dahlfors et al., 1997).

Numerous genes that contribute to S. typhimurium virulence have been identified in recent years (Groisman and Ochman, 1997). Although the biochemical functions of some of these genes have been elucidated, little is known about their regulation in vivo and how they interact during the infectious process. Many virulence genes are clustered together on ‘pathogenicity islands’ (PAIs), which appear to have been acquired by horizontal transfer from unknown sources. SPI-1 and SPI-2 are two Salmonella PAIs that encode structurally similar but functionally distinct type III secretion systems (TTSSs) which translocate virulence proteins from bacterial to host cells during the infectious cycle (Hueck, 1998). The SPI-1 encoded TTSS, called Inv/Spa, plays an important role in invasion of epithelial cells (Galán and Curtiss, 1989; Galán, 1996). Most of the genes associated with Inv/Spa are encoded within SPI-1 at 63 centosomes (cs) on the chromosome (Mills et al., 1995), but at least two of the secreted effector proteins are encoded elsewhere: the sopB gene is present on the SPI-5 pathogenicity island (Wood et al., 1998) and SopE is encoded by a temperate bacteriophage (Hardt et al., 1998).

SPI-2, located at 30 cs, encodes the second type III secretion system (Ochman et al., 1996; Shea et al., 1996). The SsaAB two-component regulatory system of SPI-2 is required for SPI-2 gene expression (Valdivia and Falkow, 1997; Cirillo et al., 1998; Deiwick et al., 1999). Recent work has shown that in cultured host cells, transcription of ssaA is modulated by OmpR, a two-component regulatory system protein which responds to changes in osmolarity, pH and temperature (Lee et al., 2000).

The SPI-2 secretion system plays a crucial role in systemic growth of Salmonella in its host (Hensel et al., 1995; Shea et al., 1996) and is required for bacterial proliferation in macrophages (Ochman et al., 1996; Cirillo et al., 1998; Hensel et al., 1998). Salmonella typhimurium replicates intracellularly within a vacuole that diverts from the normal phagocytic pathway (Méresse et al., 1999b). The Salmonella-containing vacuole (SCV) rapidly loses early endocytic markers such as EEA1 and the transferrin receptor (Steele-Mortimer et al., 1999) and acquires some lysosomal membrane glycoproteins (lgps), but does not interact extensively with either the early endocytic pathway or mature lysosomes (Garcia del Portillo and Finlay, 1995; Rathman et al., 1997). After uptake into macrophages, the SCV undergoes acidification to a pH between 4.0 and 5.0 (Rathman et al., 1996). SPI-2 mediated secretion can be induced in vitro by acidic conditions, suggesting that pH could be a physiological signal for SPI-2 secretion (Beuzón et al., 1999; Lee et al., 2000). Only one SPI-2 gene (spIC/ssaB) has been demonstrated to encode an effector protein. SpIC is reported to inhibit fusion of SCVs with lysosomes, homotypic fusion of endosomes and transferrin recycling (Uchiya et al., 1999). Two leucine-rich repeat proteins, SspH-1 and SspH-2, which are encoded outside SPI-2, have also been identified as targets of this secretion system (Miao et al., 1999),
although their functions remain unknown. This raises the possibility that, as for the Inv/Spa system, other effectors may be encoded elsewhere on the chromosome.

To identify virulence genes that interact during infection, we have developed a method based on the Competitive Index (C.I.), in which single and double mutant strains are analysed by mixed infections of mice. In this paper we describe the application of this approach to SPI-2, ompR and sifA of S.typhimurium. C.I. analysis indicates that these three loci interact during systemic infection. The sifA gene is required for the formation in epithelial cells of IgG-containing tubular membrane structures termed Sifs (Garcia del Portillo et al., 1993b; Stein et al., 1996). We show that expression of sifA is strongly induced after Salmonella enters host cells and this induction is dependent on srrA. Although Sifs are not detectable in infected macrophages, Sifa function is important in these cells because a sifA− mutant strain has a strong replication defect in macrophages. Several hours after uptake, the majority of sifA− mutant bacteria lose their vacuoles and are found in the host cell cytosol. We conclude that SifA has an important role in the maintenance of the vacuolar membrane surrounding wild-type bacterial cells.

Results

Analysis of virulence gene interactions in vivo

The method we have developed for identifying virulence gene interactions relies on the additive effect of mutations in virulence genes with different functions. Because Salmonella virulence is multifactorial, combination of mutations in genes with different functions results in strains with increased attenuation (Baumler et al., 1997; Shea et al., 1999).

The C.I. is a sensitive measure of the relative degree of virulence attenuation of a particular mutant in mixed infection with the wild-type strain. It is defined as the ratio of the mutant strain to the wild-type in the output divided by the ratio of the two strains in the input (Freter et al., 1981; Taylor et al., 1987).

To establish if mutations in two different virulence genes have additive effects on virulence attenuation, we determined C.I. values for double mutant versus single mutant strains. In the case of two genes that contribute equally to the same virulence function (by encoding essential components of a macromolecular structure, for example), strains carrying mutations in either single gene would be expected to have the same level of attenuation as a strain carrying both mutations. Therefore, the C.I. of either single mutant versus the double mutant should be 1.0 (Figure 1, upper panel). In the case of two genes (a and b) with independent functions, the virulence attenuation of a double mutant strain will reflect the additive effect of both mutations (Figure 1, lower panel). The C.I. of a double mutant strain versus an a− strain will be equivalent to the C.I. of a b− strain versus the wild-type strain. Similarly, the C.I. of a double mutant strain versus a b− strain will be equivalent to the C.I. of an a− strain versus the wild-type strain, and all comparisons will have values of <1.0.

We have demonstrated previously that the C.I. of a purD− mutant strain of S.typhimurium (a purine auxotroph) versus the wild-type strain is not statistically different from the C.I. of a purD−, ssaV− double mutant strain versus an ssaV− single mutant (Shea et al., 1999), and this result was confirmed as a control for this study (Table I). The ssaV gene is thought to encode an inner membrane component of the SPI-2 secreton (Hensel et al., 1997), and is required for the secretion of SseB, which is probably a translocon component (Beuzón et al., 1999). When subjected to the analysis indicated in Figure 1, the C.I.s (Table I) confirm that purD and ssaV have totally unrelated functions, whereas sseB and ssaV are equally necessary for SPI-2 function.

SPI-2 and ompR interact in vivo

The OmpR/EnvZ two-component system responds to a variety of physicochemical changes in the environment (Heyde and Portalier, 1987; Thomas and Booth, 1992). Strains carrying mutations in ompR/envZ are highly attenuated in systemic infection of mice but mutations in genes previously known to be regulated by OmpR could not account for its virulence defect (Dorman et al., 1989; Chatfield et al., 1991). However, recently it has been shown that srrA transcription is regulated by OmpR in infected host cells (Lee et al., 2000). Furthermore, we have found that a mutation in ompR reduces SseB levels during bacterial growth in host cells and certain laboratory media (results not shown). It was therefore of interest to determine if ompR interacts with the SPI-2 TTSS genes in vivo.
Table I. Competitive Index analysis of *S.*typhimurium mutants

<table>
<thead>
<tr>
<th>Mixed infection</th>
<th>C.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
</tr>
<tr>
<td>wt versus ssaV-</td>
<td>0.006</td>
</tr>
<tr>
<td>wt versus purD</td>
<td>0.0005</td>
</tr>
<tr>
<td>ssaV- versus ssaV-, purD-</td>
<td>0.0038</td>
</tr>
<tr>
<td>purD- versus ssaV-, purD-</td>
<td>0.019</td>
</tr>
<tr>
<td>wt versus ssaV-, purD-</td>
<td>0.00006</td>
</tr>
<tr>
<td>wt versus sseB-</td>
<td>0.016</td>
</tr>
<tr>
<td>ssaV- versus ssaV-, sseB-</td>
<td>1.2</td>
</tr>
<tr>
<td>sseB- versus ssaV-, sseB-</td>
<td>0.82</td>
</tr>
<tr>
<td>wt versus ssaV-, sseB-</td>
<td>0.014</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Strains used in the mixed infection experiments are referred to by the relevant genotype and are described in Table II. The ssaV- strain used was marked with either Km or Cm resistance cassettes to distinguish it from the competing strain.

\textsuperscript{b}The C.I. was calculated as the output ratio of mutant or double mutant to wild-type or single mutant bacteria, divided by the input ratio. The C.I.s shown are the means of three independent infections of mice.

\textsuperscript{c}Mice were inoculated i.p. with a mixture of two strains comprising \( \sim 5 \times 10^8 \) c.f.u. of each strain. Mouse spleens were harvested after 48 h for enumeration of bacterial c.f.u. The different strains used were differentiated on the basis of antibiotic sensitivities. The C.I.s of double mutant versus single mutant strains were analysed by Student’s t-test.

\textsuperscript{d}Not significantly different from 1.0 (\( p < 0.05 \)).

The C.I. of an ompR\textsuperscript{-} mutant strain versus the wild-type strain following intraperitoneal (i.p.) inoculation confirmed that its level of attenuation was similar to that of a typical SPI-2 mutant strain (Table I). To investigate possible interactions between ompR and SPI-2 genes in vivo, a double mutant strain was constructed carrying mutations in ompR and ssaV. The C.I. of this strain versus either single mutant strain was not significantly different from 1.0. This result implies that the virulence functions of the two genes are related, and the in vitro regulation of SPI-2 genes by ompR (Lee et al., 2000) also occurs in vivo.

SPI-2 and sifA interact in vivo

The sifA gene is located within the potABCD operon on the *Salmonella* chromosome, and was probably acquired by horizontal transfer from an unknown source (Stein et al., 1996). Strains carrying mutations in sifA, ompR or envZ are all defective for the formation of Sifs (Garcia-del Portillo et al., 1993b; Stein et al., 1996; Mills et al., 1998). sifA\textsuperscript{-} mutant strains are reported to be attenuated in virulence following oral inoculation of mice, although they do not have a replication defect in epithelial cells (Stein et al., 1996). In competition experiments, the virulence of a strain carrying a transposon insertion in sifA was found to be attenuated as an sseB\textsuperscript{-} mutant following i.p. inoculation of mice, and this defect was complemented by introduction of the wild-type sifA allele on a plasmid (Table I). A sifA\textsuperscript{-} mutation was therefore introduced to the ssaV\textsuperscript{-} mutant strain and single and double mutant strains were analysed by C.I. The double mutant strain was no more attenuated than either single mutant (Table I). Therefore, the proteins encoded by these genes appear to be involved in the same important virulence function of *S.*typhimurium during systemic infection.

SPI-2 mutant strains fail to induce Sifs

The genetic evidence linking SPI-2 and sifA (Table I) led us to ask if SPI-2 mutant strains could induce the formation of Sifs in epithelial cells. *Salmonella typhi-

murium* strains carrying a plasmid constitutively expressing green fluorescent protein (GFP) were used to infect HEK293 cells, and these were subsequently stained with an anti-LAMP-1 antibody to reveal Sifs (Garcia-del Portillo et al., 1993b) (Figure 2). At 7 h post-invasion, >70% of cells infected with wild-type bacteria contained Sifs, whereas Sifs were detected in 1% or less of cells infected with strains carrying mutations in either ssaV, ssaV\textsuperscript{-} or ompR. Approximately 1% of cells infected with either sseB\textsuperscript{-} or sifA\textsuperscript{-} mutant strains contained Sifs, and the introduction of plasmids carrying functional genes to the respective mutant strains restored Sif formation to ≥40% of infected cells. Similar results to those shown in Figure 2 were obtained when infected cells were examined 16 h after bacterial invasion, which shows that the onset of Sif formation is not simply delayed in SPI-2 mutants. It has been reported that *Salmonella* strains with non-auxotrophic mutations affecting replication inside host cells are incapable of inducing Sif formation (Garcia-del Portillo et al., 1993a). Therefore, the intracellular replication defect of SPI-2 and ompR\textsuperscript{-} mutant strains (Figure 4; Lee et al., 2000) could account for the lack of Sifs. A phoP\textsuperscript{-} mutant strain was used to investigate this possibility. phoP/Q exerts regulatory control over at least 40 virulence genes of *Salmonella* (Miller and Meiklejohn, 1990), is required for bacterial replication in macrophages, but functions independently of SPI-2 in host cells (Valdivia and Falkow, 1997; results not shown). The phoP\textsuperscript{-} strain was nevertheless capable of inducing Sifs in ≥40% of infected cells (Figure 2G). Therefore, we conclude that the failure of SPI-2 and ompR\textsuperscript{-} mutants to induce Sifs is not simply a consequence of their replication defect, but is specifically connected with the function of these genes.

sifA expression requires the SPI-2 regulator ssrA

SPI-2 gene expression is induced following uptake of bacteria into host cells (Cirillo et al., 1998). The expression of some genes can be detected after 1 h (Uchiya et al., 1999), and maximal expression is achieved after 6 h (Cirillo et al., 1998). This expression is dependent on the SPI-2 two-component regulatory system SsrA/B (Cirillo et al., 1998; Hensel et al., 1998). In view of the phenotypic similarities between SPI-2 and sifA\textsuperscript{-} mutant strains, it was of interest to know if their patterns of gene expression are similar. To analyse this, a plasmid was constructed carrying a sifA::gfp reporter fusion, transferred into different *S.*typhimurium strains, and expression of the fusion protein was examined during bacterial growth in HEK293 cells. Wild-type bacterial cells expressed sifA::gfp
only when they were intracellular (Figure 3, top panel). Approximately 2% of bacterial cells expressed sifA::gfp as early as 1 h post-invasion. The percentage of bacterial cells expressing the fusion increased gradually thereafter, with ~90% of cells positive for GFP by 6 h post-invasion (results not shown). These kinetics are similar to those reported for SPI-2 (ssaH::gfp) expression (Cirillo et al., 1998). No sifA::gfp expression was detected in the ssaA− mutant strain (Figure 3, middle panel). In ompR+ mutant strains, the number of cells expressing sifA::gfp was ~50% that of wild-type cells, and the overall level of expression was lower than that seen in wild-type cells, consistent with the effect of an ompR+ mutation on SPI-2 ssaH gene expression (Lee et al., 2000). As expected, the level of sifA::gfp expression in a phoP− mutant strain was comparable to that of the wild-type strain (Figure 3).

A sifA mutant strain has a replication defect in macrophages
As most SPI-2 mutants have a replication defect inside host cells, and sifA− mutants are attenuated in the systemic phase of infection, a sifA− mutant strain was tested for replication in macrophage-like RAW cells. The sifA− mutant strain was found to have a replication defect comparable to that of a strain carrying a mutation in ssaV (Figure 4). This result was surprising, since sifA− strains are reported to be proficient for replication in HeLa cells (Stein et al., 1996). The introduction of the plasmid carrying a functional sifA gene to the sifA− strain restored replication to wild-type levels (Figure 4E). This demonstrates that the replication defect is not due to a polar effect of the integrated transposon on the downstream portC gene, or an unrelated mutation. In these assays, the ompR− mutant strain also displayed a replication defect, in agreement with a recent report (Lee et al., 2000).

Association of LAMP-1 with SCVs in RAW macrophages
Since sifA has an important role in intra-macrophage replication of S.typhimurium we investigated the distribution of LAMP-1 in RAW macrophages infected with different S.typhimurium strains. At different time points following bacterial uptake, cells were fixed and stained with an antibody against LAMP-1, then examined by confocal immunofluorescence microscopy. No structures resembling Sifs were observed at any time point in macrophages infected with wild-type bacteria. At 2 h post-uptake, LAMP-1 was associated with ~80% of wild-type, ssaV− or sifA− strains (Figure 5A, upper panel; Figure 5C). However, at 10 h post-uptake, LAMP-1 was present on >50% of vacuoles containing wild-type, and >70% of vacuoles containing ssaV− bacteria, whereas <10% of the sifA− bacteria were associated with this marker (Figure 5A, lower panel; Figure 5B). A time-course experiment

Fig. 2. Sif formation by S.typhimurium mutant strains in HeLa cells. Confocal immunofluorescence analysis of Sif formation in cells infected with: 12023 (A and B), sseB− (C), sseB+ sseB− (D), sifA− (E) and sifA− prfA− (F) strains. Cells were fixed at 7 h post-infection and stained with mouse anti-LAMP-1 and TRSC-conjugated donkey anti-mouse antibodies. In (A–E) the bacterial strains carried a plasmid expressing gfp constitutively. In (F), bacterial cells were detected by goat anti-Salmonella and FITC-conjugated donkey anti-goat antibodies. sseB− and sifA− mutant strains fail to induce Sifs (C and E). This defect can be complemented by the introduction of plasmids carrying the respective wild-type alleles (D and F). Bars correspond to 4 μm; the scale in (C–F) is equivalent to that of (A). (G) represents typical results from one of three experiments where cells (n = 50) infected by each S.typhimurium strain were evaluated for Sif formation at 7 h post-invasion. Values are given as percentage of infected cells containing Sifs.
showed that the decline in LAMP-1 association with the sifA− strain began ~5 h post-uptake (Figure 5C). We conclude from these results that the maintenance of LAMP-1 on vacuoles containing wild-type bacteria is dependent on the sifA gene.

**Trafficking of S.typhimurium strains in epithelial cells**

To determine if the apparent absence of lgp in the vicinity of the sifA− mutant cells was restricted to LAMP-1 and to macrophages, we examined the localization of another lgp, LIMP-1, also known as LAMP-3 or CD63 antigen, in infected epithelial cells. We found that LIMP-1 is a constituent of Sifs in cells infected with wild-type bacteria (Figure 6, upper panel). Furthermore, whereas LIMP-1 was present in >75% of vacuoles containing wild-type bacteria and >90% of vacuoles containing ssuV− bacteria, <25% of sifA− cells were associated with this marker (Figure 6). Similar results were obtained in HeLa cells using antibodies against LAMP-1 (Figure 7, upper panel) and LAMP-2 (not shown). LIMP-1, LAMP-1 and LAMP-2 share similar lysosomal targeting sequences, and are likely to follow the same trafficking pathway (Hunziker and Geuze, 1996). We therefore examined the acquisition of the vacuolar ATPase (vATPase), which is responsible for the acidification of endocytic vacuoles (Sato and Toyama, 1994), since its origin, function and targeting process are different to those of these lgps (Hunziker and Geuze, 1996). Interestingly, vATPase was also found to be a component of Sifs (Figure 7, lower panel), was present on vacuoles containing wild-type and
Fig. 5. The lysosomal membrane protein LAMP-1 is not associated with sifA− mutant bacteria 10 h post-uptake in RAW macrophages. Confocal microscopic analysis was carried out on macrophages infected with wild-type (12023), ssaV− or sifA− strains constitutively expressing GFP, at 2 h (upper panels (A)) or 10 h (lower panels (A)) post-uptake. LAMP-1 was detected using a rabbit polyclonal and TRSC-conjugated donkey anti-rabbit secondary antibodies. Scale bar corresponds to 4 μm. The percentage of bacteria (n=50) co-localizing with LAMP-1 was determined for these and two other bacterial strains 10 h after uptake. The percentage of bacteria (n=50) co-localizing with LAMP-1 at different time points was determined for 12023 (open squares) and sifA− mutant (open circles) strains (C). Results shown in (B) are the means ± SE of three independent experiments. For the sifA−, psifA strain, bacteria were detected with goat anti-Salmonella and FITC-conjugated donkey anti-goat antibodies.
**ssaV** bacteria, but was only detected at a low frequency in association with **sifA** bacteria (Figure 7, lower panel).

**Maintenance of the vacuolar membrane enclosing wild-type S.typhimurium is dependent on sifA**

The lack of association of lgp and vATPase with **sifA** mutant bacteria suggested that either vacuoles containing **sifA** bacteria are non-fusogenic, as is the case for vacuoles containing some other intracellular pathogens, such as *Legionella* spp. (Sinai and Joiner, 1997), or that these bacteria are no longer enclosed by a membrane. The presence of a vacuolar membrane around **sifA** bacteria was investigated by testing the accessibility of intracellular *S.typhimurium* strains to anti-LPS antibody in RAW cells treated with the pore-forming toxin streptolysin-O from *Streptococcus pyogenes*. The rationale of this experiment is that, after plasma membrane permeabilization, bacteria present in the macrophage cytosol would be accessible to the antibody added to the extracellular medium whereas bacteria protected by a vacuole would not. Infected cells were treated with streptolysin-O, fixed and stained with antibodies in the absence of detergent. At 8 h post-uptake most wild-type and **ssaV** bacteria were protected from the anti-LPS antibody. In contrast, >55% of **sifA** bacteria were decorated with the anti-LPS antibody, indicating their presence in the cytosol (Figure 8A).

Examination of intracellular bacteria under the electron microscope confirmed the presence of cytosolic **sifA** bacteria (Figure 8D), whereas wild-type, **ssaV** and **sifA**, *psifA* cells were always associated with a vacuolar membrane (Figure 8B, C and E). These results show that maintenance of a lgp/vATPase-containing membrane around wild-type bacteria requires the **sifA** gene.

**SifA is similar to secreted proteins from Salmonella**

The results described above show that the maintenance of the lgp/vATPase-containing membrane of the SCV is dependent on SifA, which in turn requires the SPI-2 TTSS regulator, **ssrA**. The C.I. analysis also suggests that **sifA** and **ssaV** are functionally related (Table I). One obvious explanation of these results is that SifA is a SPI-2 effector protein. BLAST and FASTA searches using the predicted amino acid sequence of **sifA** revealed that it has weak but significant similarity at the N-terminal region to three *Salmonella* proteins: *SspH-2*, *SspH-1* and *SipP* (Figure 9A). *SspH-1* and *SspH-2* are preferentially translocated into the host cell cytosol by the SPI-1 and SPI-2 TTSS, respectively (Miao et al., 1999), but their function is unknown. The **sipP** gene is present on a pathogenicity island and, like *SspH-1* and *SspH-2*, shares similarity to other proteins secreted by TTSSs (Tsolis et al., 1999). However, unlike all these proteins, SifA does not contain leucine-rich repeats. Its region of similarity is confined to approximately the first 100 amino acids of

**Fig. 6.** LIMP-1 is a component of Sifs and is not associated with **sifA** mutant bacteria 10 h after invasion of HeLa cells. Confocal microscopic analysis was carried out on cells infected with wild-type (12023), **ssaV** or **sifA** strains constitutively expressing GFP. LIMP-1 was detected using a mouse mAb and TRIC-conjugated donkey anti-mouse secondary antibodies. Low magnification images show that LIMP-1 is a component of Sifs in cells infected with wild-type bacteria (upper panel). Higher magnification images show LIMP-1-containing SCVs for wild-type and **ssaV** mutant bacteria, but weak or no association of LIMP-1 with **sifA** mutant cells. Scale bars correspond to 4 μm. The percentage of bacteria (n = 50) co-localizing with LIMP-1 was determined for these and two other bacterial strains. Results shown are the means ± SE of three independent experiments. For the **sifA**; *psifA* strain, bacteria were detected with goat anti-*Salmonella* followed by FITC-conjugated donkey anti-goat antibodies.
SspH-2, SspH-1 and SlrP; over this region, SifA is most similar (30% identical) to SspH-2, and has lower similarity to SlrP and SspH-1 (Figure 9A). The N-terminal regions of SspH-2 and SspH-1 are sufficient for their secretion and translocation (Miao et al., 1999), suggesting that SifA may be a secreted protein.

As standard BLAST or FASTA database searches are not always sufficient to detect subtle sequence relationships, we used a multiple sequence alignment of the N-terminal regions of SifA, SspH-1, SspH-2 and SlrP (from Trp31 to Glu106 of the SifA amino acid sequence) to build a profile that was then used to search the non-redundant sequence database (NRDB). This method allows more sensitive database searches to be carried out, by highlighting the critical residues within a set of aligned sequences. The search using this profile revealed similarity to a portion of only one protein: the N-terminal end of Salmonella dublin SopD, with an E value of 0.0559 (indicating that the similarity is likely to be significant) (Figure 9A). SopD is translocated into HeLa cells via the Inv/Spa TTSS, its N-terminal region being sufficient to direct translocation (Jones et al., 1998).

**Infection of HeLa cells expressing SifA with a sifA- mutant strain restores Sif formation**

To investigate whether SifA is secreted into the host cell cytosol, three SifA-adenylate cyclase (CyaA) fusion proteins were constructed. The CyaA reporter system has...
Escherichia coli cyaA\textsuperscript{-} mutant strain (Glaser et al., 1988). These constructs were transferred into wild-type, \textit{ssaV}\textsuperscript{+} or \textit{sifA}\textsuperscript{-} strains. These were then used to infect RAW macrophages, which were assayed for CyaA activity at different time points (Miao et al., 1999). No accumulation of cAMP (reflecting CyaA activity) was detected at 6 or 8 h post-uptake for any of the strains, despite their efficient uptake by macrophages (results not shown). This suggests that either SifA is itself not translocated into the host cell cytosol, or that following translocation, its conformation or interaction with a receptor prevents determination of CyaA activity of the fusion protein.

In view of the strong circumstantial evidence favouring an effector function for SifA, HeLa cells were transiently transfected with plasmids encoding either SifA::GFP, or the GFP as a control, then infected with different \textit{S.typhimurium} strains. Confocal imaging showed that the GFP was localized both in the cytoplasm and the nucleus. In contrast, the SifA::GFP fusion protein was associated mainly with membranes, vacuoles containing wild-type or \textit{sifA}\textsuperscript{-} strains, and Sifs. In addition, formation of Sifs was restored in SifA::GFP expressing cells infected with the \textit{sifA}\textsuperscript{-} mutant strain (Figure 9B). Collectively these results suggest that SifA is translocated on to the cytosolic face of the vacuolar membrane where it mediates membrane fusion events.

**Maintenance of the vacuolar membrane enclosing SPI-2 mutant strains is not dependent on sifA**

We have established that \textit{sifA} expression requires the SPI-2 regulator \textit{ssrA}, and that SifA is required for the maintenance of the vacuolar membrane enclosing wild-type bacteria. If, as suggested by our results, SifA is secreted by the SPI-2 TTSS, an \textit{ssaV}\textsuperscript{+} mutant strain [which lacks a functional secreton (Beuzón et al., 1999)] would be defective in SifA function and might also be expected to be released into the host cell cytosol. However, \textit{ssaV}\textsuperscript{+} mutant bacteria were found within vacuoles in macrophages (Figure 8C). If SifA is not secreted by the SPI-2 TTSS, SifA could account for the maintenance of a vacuolar membrane surrounding \textit{ssaV}\textsuperscript{+} bacteria. In this case, bacterial cells carrying both \textit{sifA}\textsuperscript{-} and \textit{ssaV}\textsuperscript{+} mutations would be released into the cytosol. However, vacuoles containing these double mutant bacteria were associated with LAMP-1 in macrophages, and with LIMP-1, LAMP-1, LAMP-2 and \textit{vATPase} in HeLa cells (Figures 5 and 6 and results not shown). Furthermore, since \textit{sifA} expression is \textit{ssrA} dependent (Figure 3), an \textit{ssrA}\textsuperscript{-} strain would also be expected to be found in the host cell cytosol. Like \textit{ssaV}\textsuperscript{+} and \textit{ssaV}\textsuperscript{-}, \textit{sifA}\textsuperscript{-} double mutant bacteria, \textit{ssrA}\textsuperscript{-} cells were associated with LAMP-1 in macrophages (Figure 5). Therefore, \textit{sifA} is not required for maintenance of the vacuolar membrane enclosing \textit{ssaV}\textsuperscript{+} or \textit{ssrA}\textsuperscript{-} bacteria, and the recruitment of \textit{lpg}\textit{vATPase}-containing membrane to these cells must reflect a different process to that observed with the wild-type strain. The simplest explanation for these results is that the SPI-2 TTSS translocates other effectors which prevent the SCV from interacting with the endocytic pathway, prior to the action of SifA. An \textit{ssaV}\textsuperscript{-} strain cannot secrete any effectors, and is enclosed by a vacuole by interactions with the endocytic pathway (Figure 10).
Fig. 9. (A) Multiple alignment of the N-terminal region of SifA with other Salmonella proteins. The alignment was generated by ClustalW (http://www2.ebi.ac.uk/clustalw/) and Multalin (http://www.toulouse.inra.fr/multalin.html) and edited by hand. (B) Formation of Sifs is restored in sifA-mutant-infected cells expressing SifA::GFP. HeLa cells transiently expressing SifA::GFP (upper panels) or GFP as a control (lower panels) were infected with wild-type (12023) or sifA- strains, and fixed with paraformaldehyde at 8 h post-infection. LAMP-1 and LPS were detected using TRSC- and Cy5-conjugated donkey anti-rabbit and anti-mouse secondary antibodies, respectively. Three-colour confocal microscopic analysis was performed. Scale bar represents 10 μm. Expression of SifA::GFP in HeLa cells restores the presence of a LAMP-1-enriched vacuole around sifA-bacteria. SifA-GFP is localized on SCVs and Sifs (arrows in the top panels).

Discussion
In this paper we describe a general method for identifying virulence gene interactions in vivo based on the C.I. The technique involves direct comparisons between single and double mutant strains rather than combinations of different single mutants and the wild-type strain. This approach allows the relative attenuation of two mutations to be assessed directly in the same animal, avoiding host-to-host variation.

In the present work, we have used C.I. analysis to show that ompR, SPI-2 genes and sifA interact during systemic
infection of mice. A functional relationship between these loci was suggested by the finding that \textit{ompR} mutations fail to induce Sif formation in epithelial cells (Mills \textit{et al.}, 1998), and the recent demonstration that OmpR regulates \textit{ssrA} expression during infection of cultured macrophages (Lee \textit{et al.}, 2000). Our C.I. analysis indicates that \textit{in vivo}, the attenuated virulence of an \textit{ompR} mutant can be attributed entirely to its interaction with SPI-2 genes, and vice versa. This was surprising in view of the finding that a mutation in \textit{ompR} delayed but did not eliminate induction of a SPI-2 \textit{ssaH} gene in cultured macrophages (Lee \textit{et al.}, 2000). It is possible that OmpR exerts tighter control over SPI-2 function \textit{in vivo} than in cultured cells; alternatively, the partial effect of an \textit{ompR} mutation over SPI-2 gene expression may be sufficient to reduce expression below a functionally effective threshold. OmpR regulation of SPI-2 gene expression is therefore analogous to that of PhoP/Q over SPI-1 and SPI-3 genes (Pegues \textit{et al.}, 1995; Blanc-Potard and Groisman, 1997); both regulators are present in the non-pathogenic relative \textit{E.coli}, but have extended their regulatory range in \textit{Salmonella} to encompass virulence genes on PAs.

In infected epithelial cells, \textit{sifA} induces the formation of dramatic tubular membrane structures (Sifs), that are enriched in lysosomal membrane glycoproteins. However, the physiological relevance of these structures is not clear, despite the fact that the \textit{sifA} gene is known to be important for virulence in mice (Stein \textit{et al.}, 1996). The C.I. tests confirm the importance of the \textit{sifA} gene in \textit{S.typhimurium} virulence, and suggest that \textit{sifA} and SPI-2 functions are intimately connected \textit{in vivo}. Since attenuated SPI-2 mutant strains fail to induce Sifs, and \textit{sifA} expression is dependent on \textit{ssrA}, this also explains the observation that \textit{ompR} mutants fail to make Sifs (Mills \textit{et al.}, 1998). The systemic \textit{in vivo} attenuation of the \textit{sifA} mutant strain can be explained by its intramacrophage replication defect, which is similar to that of SPI-2 mutants. Consistent with this, the onset of \textit{sifA} gene expression in cultured cells corresponds to that of SPI-2 genes (Cirillo \textit{et al.}, 1998).

Several lines of evidence suggest that SifA is a SPI-2 effector protein. First, the C.I. analysis implies that both loci are involved in the same function. Secondly, although \textit{sifA} is regulated by \textit{ssrA}, it is not present in SPI-2, which contains all the genes known to be required for secretion and translocation of SPI-2 effectors. Thirdly, \textit{sifA} has a significantly lower G+C content than the average for the \textit{S.typhimurium} chromosome, is flanked by repeat sequences, and is a virulence determinant specific to \textit{Salmonella}, all of which suggest that it was acquired by horizontal transfer (Stein \textit{et al.}, 1996). A gene for an effector protein of the Inv/Spa TTSS of \textit{Salmonella} was also acquired independently by horizontal transfer, which has led to the suggestion that this may allow bacterial pathogens to fine-tune their TTSSs (Hardt \textit{et al.}, 1998). Fourthly, SifA has weak but significant similarity to other \textit{Salmonella} secreted proteins, and this is restricted to the regions reported to be sufficient for secretion and translocation. Finally, the recruitment of the SifA::GFP fusion protein to the SCV and on Sifs when expressed in host
cells, and its ability to rescue Sif formation in cells infected with the sifA- mutant strain suggests that it is normally translocated from wild-type bacteria to the cytosolic face of the vacuole. The localization of SifA to host cell membranes is consistent with the hypothesis that it mediates membrane fusion events (see below). If SifA binds to a receptor in the vacuolar membrane this might also explain the failure to demonstrate translocation using SifA::Cya fusions, since this could interfere with CyaA activity of this fusion protein.

Our results show that the maintenance of a lgp/vATPase-containing membrane around wild-type but not SPI-2 mutant strains of *S. typhimurium* requires a functional sifA gene. We present a model (Figure 10) to account for the intracellular phenotypes of different mutant strains. The vacuole containing wild-type bacteria, through the action of the SPI-2 TTSS effector SpaC (Uchiya et al., 1999) and possibly other SPI-2 effectors, does not interact with late endosomes and lysosomes, and SifA actively recruits and maintains the lgp/vATPase-containing membrane. It is known that acquisition of lgps by the SCV in epithelial cells occurs by recruitment of pre-existing lgp-containing vesicles in a rab7-dependent manner, without direct interaction with lysosomes (Méresse et al., 1999a). Through the action of unknown SPI-2 effectors, wild-type *Salmonella* also inhibits trafficking of the NADPH oxidase to the SCV (Vázquez-Torres et al., 2000). These processes create an environment conducive to bacterial replication. Strains containing mutations in ssaV or sraA do not have a functional SPI-2 secretion system, and the vacuoles acquire lgps and vATPase through interactions with late endosomes and lysosomes. A strain carrying a mutation in sifA retains the ability to translocate SpaC and other effectors, which prevent interactions with late stages of the endocytic pathway. However, because this strain cannot recruit or maintain lgp/vATPase-containing membrane through the action of SifA, the vacuole cannot be sustained, and the bacterial cell is released into the cytoplasm. For intracellular pathogens to replicate but remain within a membrane-bound vacuole, there must be a progressive net increase in the surface area of the vacular membrane. SifA is therefore likely to control directed membrane fusion events, and the lack of SifA would lead to insufficient membrane acquisition to enclose the replicating bacterial cells, which would become exposed to the cytosol.

The intra-macrophage replication defect displayed by the sifA- mutant strain is comparable to the phenotype of *S. dublin* or *E. coli* K-12 strains carrying the hly gene of *Listeria monocytogenes*. These bacteria can also escape to the macrophage cytosol by secreting listeriolysin, but they fail to replicate (Gentschev et al., 1995). This suggests that, unlike other intracellular pathogens such as *Listeria* which can replicate in the macrophage cytosol (de Chastellier and Berche, 1994), *Salmonella* cannot exploit this environment for growth. To our knowledge, SifA is the first example of a bacterial protein responsible for the maintenance of a membrane-bound vacuole. Specificity of vesicular trafficking in the eukaryotic cell is conferred by the integral membrane vSNAPE and tSNAPE proteins (Rothman and Wieland, 1996), and is modulated by rab GTPases (Schimmoller et al., 1998). In view of the involvement of the rab7 GTPase in the biogenesis of SCVs (Méresse et al., 1999a) and its presence on Sifs (S. Méresse and J.-P. Gorvel, unpublished observations) it is possible that SifA acts as an activator of this membrane fusion machinery, thereby ensuring a continuous flow of membrane towards the SCV.

**Materials and methods**

**Bacterial strains and growth conditions**

The bacterial strains used in this study are listed in Table II. H3P6 carries a mini-Tn5 insertion poster that was mapped between nucleotides 44 and 139 of the sifA open reading frame (ORF: 1012 nucleotides in length) and was identified but not further characterized in the original STN screen of *S. typhimurium* (Hensel et al., 1995). The mutation was transduced by phase P22 into strain 12023 prior to use in this study. Double mutant strains were also constructed by P22-mediated transductions, carried out as described previously (Davis et al., 1980). Bacteria were grown in Luria–Bertani (LB) medium supplemented with ampicillin (50 µg/ml), kanamycin (50 µg/ml), tetracycline (25 µg/ml) or chloramphenicol (50 µg/ml for plasmid-containing strains, 10 µg/ml for chromosomal integrants) as appropriate. CJD359 and CS105 were kindly provided by C. Dorman and S. Miller, respectively.

**Plasmids**

Plasmid pPvV2.1, carrying gfpmut3A under the control of a constitutive promoter, was introduced into bacterial strains for fluorescence visualization where indicated (Valdivia and Falkow, 1997). pD812 is a derivative of pFPV25, a vector carrying promoterless gfpmut3A (Valdivia and Falkow, 1997) containing a translational fusion of a fragment of sifA to the gfp gene. A fragment including 160 bp of the sifA promoter region and the sequence encoding the N-terminal 270 amino acids of SifA was amplified by PCR from 12023s genomic DNA using SIFA2 (5'-GGATCCGAGCTATACTGCGGGAGCTC-3') and SIFA2 (5'-CATATGGTTC- TATCCGCTATGAAACCTCTTTC-3') primers. The 1 kb PCR product, containing terminal BamHI and NdeI sites, was digested and ligated into pFPV25, generating pD812. pJ5B is a derivative of pACYC184 (Chang and Cohen, 1978) carrying the sifA gene under the control of a constitutive promoter. A DNA fragment including the complete ORF of sifA and its ribosomal binding site (rbs) was amplified by PCR from 12023s genomic DNA using SIFN-B (5'-GGATCCCT- TATCCGCTATAGGTGAG-3') and SIFN2 (5'-CTCGAGGTTGAC- GTCTAGAAGAG-3') primers. The –1 kb PCR product, containing terminal BamHI and XhoI sites, was digested and ligated into pACYC184 EcoRV-SaII sites inside the 'Ter' gene generating pJ5B. All the constructions were verified by DNA sequencing.

**Mouse mixed infections**

Female BALB/c mice (20–25 g) were used for all infection studies and were inoculated i.p. with a 0.2 ml volume of bacterial cells suspended in physiological saline. To prepare the inocula bacteria were grown overnight at 37°C in LB broth with shaking (150 r.p.m.) and then used to inoculate fresh medium (1:100) and grown under the same conditions for 2–3 h until an OD550 of 0.35–0.6 was reached. Cultures were then diluted in physiological saline to a concentration of 2.5 × 10^8 bacteria per strain and mixed before the infection. The c.f.u. were enumerated by plating a dilution series of the inoculum, using antibiotics to distinguish between the strains. To confirm that the presence of the antibiotic did not result in lower bacterial recovery, strains carrying antibiotic resistance were plated on LB and LB with the appropriate antibiotic. Mice were killed at 48 h post-inoculation by carbon dioxide inhalation. The spleens were removed and bacteria recovered and enumerated after plating a dilution series on to LB agar and LB agar with the appropriate antibiotics (Shea et al., 1999).

**Statistical analysis**

Each C.I. value is the mean of three independent mice infections. Student's t-test was used to analyse the C.I.s of single mutant strains versus double mutant strains (i.e. a versus a b) with two null hypotheses: mean C.I. is significantly different from 1.0 and mean C.I. is significantly different from the C.I. of the single mutant versus the wild-type strain (wt versus b). Probabilities (p) of <0.05 were considered significant.
Table II. Strains used in this study

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<th>Name</th>
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<td>this study</td>
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<td>pho-P-102::Tn10/Cm in 12023s</td>
<td>Millier et al. (1989)</td>
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<td>ompR-1009::Tn10 in SL1344</td>
<td>Dorman et al. (1989)</td>
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</tr>
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Sequence analysis

Sequence database similarity searches were carried out by Blast2 (http://www.ncbi.nlm.nih.gov/BLAST/), WU-Blast2 and FastA3 (http://www2.cki.ac.uk/blast2/ and FastA3/) (Pearson and Lipman, 1988; Altschul et al., 1997). All the searches used derived amino acid sequences.

The multiple alignment shown in Figure 9A was generated by ClustalW (http://www2.cki.ac.uk/clustalw/) and Multalin (http://www.toulouse.inra.fr/multalin.html) (Corpet, 1988; Thompson et al., 1994). The sequences included can be found under the SWALL accession numbers Q56061 (SidA), Q9XDN9 (SspH-1), AFA00615 (SspH-2) and Q9XCV2 (StrP). A ClustalW multiple alignment of the region of interest was used to generate a profile by ProfileWeight. This profile was then used to search the NRDB using the program ProfileSearch. Both programs are available at the Biocelerator Web server (http://shag.emb- heidelberg.de:8000/Bisc/).

Antibodies and reagents

The mouse monoclonal antibodies (mAbs) anti-LAMP-1 H3A4 and anti-CD63 HSC6 developed by J.T.August and J.E.K.Hildreth were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa, IA), and were used at a dilution of 1:2000. Anti-LAMP-1 mouse mAb CD3 was obtained from Dr Minoru Fukuda (The Burnam Institute, La Jolla Cancer Research Foundation, La Jolla, CA), and was used at a dilution of 1:1000, and anti-vATPase mouse mAb OSW2 was obtained from Dr Satoshi B.Sato (Kyoto University, Kyoto, Japan) and was used at a dilution of 1:2000. Anti-LAMP-1 rabbit polyclonal antibody 156 against the 11-residue cytoplasmic domain of LAMP-1 has been described previously (Steele-Mortimer et al., 1999), and was used at a dilution of 1:1000. Anti-Salmonella goat polyclonal antibody CSA-1 was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD) and was used at a dilution of 1:400. Anti-Salmonella LPS mouse mAb IE5 was purchased from Biodiagnose International (Kennebunk, ME) and was used at a dilution of 1:10000. Texas Red sulfonfyl chloride (TRSC)-, cyamine 5 (Cy5)- and fluorescein isothio-cyanate (FITC)-conjugated donkey anti-mouse, anti-rabbit and anti-goat antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and used at a dilution of 1:400.

Cell culture

RAW 264.7 cells were obtained from ECACC (ECACC 91062702). HeLa (clone HTA1) cells were kindly provided by Dr H.Bujard (Heidelberg, Germany). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37°C in 5% CO2.

Bacterial infection of HeLa cells

HeLa cells were seeded on to glass coverslips (12 mm diameter) in 24-well plates at a density of 5 × 104 cells per well, 24 h before infection. Bacteria were incubated for 16 h at 37°C with shaking, diluted 1:33 in fresh LB broth and incubated in the same conditions for 3.5 h. The cultures were diluted in Earle’s buffered salt solution (EBSs) pH 7.4 and added to the HeLa cells at a multiplicity of infection (m.o.i.) of ~100-1.

The infection was allowed to proceed for 15 min at 37°C in 5% CO2. The monolayers were washed once with DMEM containing FCS and 100 μg/ml gentamicin and incubated in this medium for 1 h, after which the gentamicin concentration was decreased to 16 μg/ml.

Bacterial infection of macrophages and survival assays

Macrophages were seeded at a density of 4 × 104 cells per well in 24-well tissue culture plates, 24 h before use. Bacteria were cultured at 37°C with shaking until they reached an OD600 of 2.0. The cultures were diluted to an OD600 of 1.0 and opsonized in DMEM containing FCS and 10% normal mouse serum for 20 min. Bacteria were added to the monolayers at an m.o.i. ~100-1, centrifuged at 170 g for 5 min at room temperature and incubated for 25 min at 37°C in 5% CO2. The macrophages were washed once with DMEM containing FCS and 100 μg/ml gentamicin and incubated in this medium for 1 h. The medium was replaced with DMEM containing FCS and 16 μg/ml gentamicin for the remainder of the experiment. For enumeration of intracellular bacteria, macrophages were washed three times with phosphate-buffered saline (PBS), lysed with 0.1% Triton X-100 for 10 min and a dilution series was plated on to LB agar.

Immunofluorescence and electron microscopy

For immunofluorescence, cell monolayers were fixed in 3% glutaraldehyde prepared in 0.1 M cacodylate buffer pH 7.3. Fixation was for 1–2 h at room temperature, after which the cells were washed in fresh buffer before post-fixing in 1% osmium tetroxide in the same buffer. The cells were entranced in agar (Ryder and MacKenzie, 1981), dehydrated through a graded series of alcohols and embedded in Araldite epoxy resin. Ultrathin sections were cut on a diamond knife and stained in alcoholic uranyl acetate and lead citrate before examination in a transmission electron microscope operated at 75 kV.

Streptolysin-O permeabilization of RAW cells

Streptolysin-O was obtained from Dr S.Bhakdi (Mainz, Germany). RAW cells were grown on coverslips, infected for 8 h with different strains, then rapidly washed with ice-cold ICT buffer (50 mM HEPES–KOH pH 7.1, 4 mM MgCl2, 10 mM EGTA, 8.4 mM CaCl2, 78 mM KCl, 1 mM DTT, 1 mg/ml BSA). They were then incubated for 5 min at 4°C with 2 μg/ml streptolysin-O in ICT buffer, extensively washed, incubated for 5 min at 37°C in the same buffer and immediately fixed in 3.7% paraformaldehyde. Coverslips were treated for immunofluorescence by double labelling as described above but in the absence of detergent.
Mouse anti-Salmonella LPS was used to detect bacteria present in the cytosol. Antibody raised against the LAMP-1 cytoplasmic domain was used as control for plasma membrane permeabilization.

Expression of SiaA–GFP in HeLa cells
The full siaA ORF was amplified from S.typhimurium genomic DNA by PCR using the following primers: 5’-AAAAAGAATTCACCATC-GCCGATTCTACGATGGAAAGT-3’ and 5’-AAAAACCCGGGTCA-AAAACAACATAAACACGGCCG-3’. The PCR product was subcloned into the unique EcoRI and Xmal sites of pEGF-C1 vector (Clontech Laboratories) into the same reading frame as GFP, generating pSiaA:绿色荧光蛋白(绿色荧光蛋白) Vectors encoding GFP or SiaA:绿色荧光蛋白 were used to transfect HeLa cells with FuGene 6 (Boehringer Mannheim) following the manufacturer’s instructions. Cells were further incubated for 24–48 h and infected with bacterial strains as described. The expressed SiaA::绿色荧光蛋白 fusion protein was checked by western blot analysis using an anti-GFP mouse mAb JL-8 (Clontech) to be of the expected size (583 amino acid residues, 66 kDa).

Acknowledgements
We thank Agnès Ullmann for providing an antibody against adenylate cyclase, Antoine Danchin for E.coli strain TP61pVUC1, and Christoph Tang and Herb Arst for valuable suggestions. We are very grateful to Colin Gleseson for practical assistance. This project was supported by grants from the MRC (UK) to D.W.H., and from CNRS and INSERM to S.M. C.B. was supported by an EMBO Fellowship.

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Received April 10, 2000; revised and accepted May 10, 2000