Molecular Characterization of the Prototrophic *Salmonella* Mutants Defective for Intraepithelial Replication

Akamol E. Suvarnapunya,¹ Daniel V. Zurawski,¹ Rebecca L. Guy,² and Murry A. Stein^{1*}

Departments of Microbiology & Molecular Genetics and Animal Sciences¹ and Department of Pharmacology,² University of Vermont, Burlington, Vermont 05405

Received 1 November 2002/Accepted 18 December 2002

Three MudJ prototrophs demonstrated that intracellular replication is a *Salmonella* virulence trait (K. Y. Leung and B. B. Finlay, Proc. Natl. Acad. Sci. USA, 88:11470–11474, 1991). *mutS* and *mutH* are disrupted in mutants 3-11 and 12-23, and *ssaQ* is disrupted in mutant 17-21. Further analysis revealed that loss of *Salmonella* pathogenicity island 2 function underlies the intracellular replication defect of 3-11 and 17-21.

Three prototrophic *Salmonella* MudJ mutants defective for intraepithelial replication (Rep[−]) were instrumental in establishing several significant and enduring concepts regarding *Salmonella* virulence (15). These concepts include the following: intracellular replication is critical for *Salmonella* disease, discrete molecular determinants distinct from housekeeping genes mediate intracellular replication, and changes to the host cell's endocytic system, typified by *Salmonella*-induced filament formation (Sif), are associated with wild-type intracellular replication (10, 15). While the Rep[−] mutants were all severely attenuated in mice and Sif negative (Sif[−]), the Sif[−] phenotype was found to be unlinked to the MudJ insertional mutations (24). To gain insight into the molecular basis for the Rep[−] phenotype and possible causes for the unlinked Sif[−] phenotype, we genetically characterized the three Rep[−] mutants identified by Leung and Finlay: 12-23, 3-11, and 17-21 (15). Strain 12-23 was originally found to be a rough lipopolysaccharide (LPS) variant refractile to P22 transduction (15). Subsequent analysis of 12-23 LPS revealed that a small amount of smooth LPS (9), known to serve as the P22 phage receptor, was present. Therefore, the MudJ-disrupted allele was backcrossed by P22 transduction (21) to a clean parental background, designated 12-23c. Inverse PCR (11) revealed that 12-23c contains a MudJ insertion within *mutH* (Table 1). This strain also produces wild-type smooth LPS (data not shown), and thus, the LPS defect of the original isolate did not directly result from MudJ insertion. *Salmonella mutH* encodes a component that functions along with MutS and MutL to mediate the long-patch DNA mismatch repair (MMR) system (8). While MutL and MutS are widely distributed among bacteria, MutH is found only in very close relatives of *Escherichia coli* and was selectively acquired by *Salmonella* (7).

Southern blot analysis (data not shown) and identification of MudJ lesions within strain 3-11 and backcrossed variants demonstrated that two independent insertions had occurred. As indicated in Table 2, one insertion occurred within *mutS* (strain 3-11c1), while the other was within *hisD* (strain 3-11c2). The junction of the *hisD*::MudJ insertion in 3-11c2 was identical to

that of the TT10289 *hisD*9953::MudJ donor strain (14) used to generate the mutant pool in the study by Leung and Finlay (15). Therefore, the *hisD*::MudJ allele was most likely introduced into 3-11 during MudJ mutagenesis, as commonly occurs when the recipient strain contains the histidine operon (14). In contrast, the *mutS*::MudJ insertion resulted from the desired random transposition event.

We reasoned that the *mutS*::MudJ mutation within 3-11c1 was probably the more relevant of the two insertions to the Rep[−] phenotype for several reasons. Introduction of *hisD*9953::MudJ does not result in a new phenotype since *Salmonella enterica* serovar Typhimurium SL1344 is already a histidine auxotroph (Table 1). Unlike 3-11 (15), SL1344 *hisD*9953::MudJ is fully virulent. In fact, this strain is used as a wild-type reference in assays for virulence (Table 2) (24). Finally, two of the three Rep[−] mutants contained MudJ within enzymes required for MMR (e.g., MutH and MutS), and this seemed to suggest a relationship between this repair system and the Rep[−] mutant phenotypes. Therefore, 3-11c1, and not 3-11c2, was characterized further.

For continuity, in vitro phenotypes of 12-23c and 3-11c1 MMR mutants are reported together. Both are strong hypermutators acquiring forward mutations imparting rifampin resistance at a rate over 3 orders of magnitude greater than that of their parent (Table 2). This is as expected for MMR loss-of-function mutants (8). Neither MMR mutant displayed significant replication deficiencies (Fig. 1A) in Madin-Darby canine kidney (MDCK) cells when a gentamicin protection assay (26) was performed as was detailed earlier (24). They also proliferated within RAW 264.7 macrophage (Fig. 1B) when methods that were described previously were used (11). While a slight increase in cefotaxime resistance was displayed (Table 2), the 2.7-fold increase in survival relative to their parent strain was less than the fivefold increase considered to portend an intraepithelial replication deficit (11, 15) (ST216 [Table 2]). Both MMR mutants make Sif at wild-type frequencies, as was reported before (11) and is confirmed in this study (Table 2).

Both 3-11 and 12-23 were found to be strongly attenuated for virulence (15), yet MMR is dispensable for *Salmonella* pathogenesis, since *mutS* mutants were fully virulent in mice (4, 26). It is therefore unlikely that the MMR lesions within 3-11 or 12-23 caused the attenuation of these Rep[−] mutants.

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Vermont, 95 Carrigan Dr., 118 Stafford Hall, Burlington, VT 05405-0068. Phone: (802) 656-5727. Fax: (802) 656-8749. E-mail: mastein@zoo.uvm.edu.

TABLE 1. Reagents used in this study

Strain or primer	Genotype	Source or reference	Comment
<i>E. coli</i> strains			
TOP10	<i>mcrA</i> Δ (<i>mcrCB-hsdSMR-mrr</i>) (ϕ 80 <i>lacZ</i> Δ M15)	Invitrogen	TA cloning vector
DH5 α	Δ <i>lacX74 deoR recA1 araD139 Δ (<i>ara-leu</i>)7679 <i>galU galK rpsL endA1 nupG supE44</i> Δ(<i>lac</i>)U169 <i>hsdR17 recA1 endA1</i></i>		Vector propagation
DH5 α λ pir	<i>gyrA96 thi-1 relA1 deoR</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>supE44</i> Δ (<i>lac</i>) U169 <i>hsdR17 recA1 endA1</i> <i>gyrA96 thi-1 relA1 deoR</i> (ϕ 80 <i>lacZ</i> Δ M15) λ pir		Propagation of pCVD442 allelic exchange vector
SM10 λ pir	<i>thi thr leu tonA lacY supE recA::RP4-2-Tc:Mu</i> Kan <i>pir</i> R6K		Transfer of pCVD442
Serovar Typhimurium strains			
SL1344	<i>hisG46</i>		Virulent wild type
ST218	<i>hisD9953::MudJ</i>	24	Virulent, Lac ⁺ , for CI determination
3–11	<i>mutS::MudJ, hisD::MudJ</i>	15	Rep [−] , Sif [−] , SPI2 [−] , avirulent
3–11c1	<i>mutS::MudJ</i>	Present study	Rep ⁺ , Sif ⁺
3–11c2	<i>hisD::MudJ</i>	Present study	Rep ⁺ , Sif ⁺
12–23	<i>mutH::MudJ</i>	15	Rep [−] , Sif [−] , rough LPS, avirulent
12–23c	<i>mutH::MudJ</i>	Present study	Rep ⁺ , Sif ⁺ , virulent
17–21	<i>ssaQ::MudJ</i>	15	Rep [−] , Sif [−] , SPI2 [−] , avirulent
17–21c	<i>ssaQ::MudJ</i>	Present study	Rep ⁺ , Sif ⁺ , SPI2 ⁺
ST216	Δ <i>ssaQ</i>	Present study	Rep [−] , Sif [−] , SPI2 [−] , avirulent
ST244	Δ <i>ssaV</i>	11	Rep [−] , Sif [−] , SPI2 [−]
69F3	<i>ssaP::MudJ</i>	11	Rep [−] , Sif [−]
ST319	Δ <i>sseB</i>	28	SPI2 [−] , Sif [−]
Primer pairs ^a			
Upstream oligonucleotide			
Q1: 5'-ATG GAA ACT TTG CTG GAG ATA ATC GC-3'			Amplified downstream portion of <i>ssaQ</i> deletion allele
Downstream oligonucleotide			
Q2- <i>PmeI</i> : 5'-GTTTAAAC TCA TTC GCT ATT CTT AAC ATA GAA TAT CTC-3'			
Upstream oligonucleotide			
Q3: 5'-GTTTAAAC GAT AAA CCT GAT AAG AAA AAT AAT ATG CGA AC-3'			Amplified upstream portion of <i>ssaQ</i> deletion allele
Downstream oligonucleotide			
Q4- <i>PmeI</i> : 5'-TCA TGA AAA GCT CTG TAC CAA TTG CG-3'			

^a Introduced *PmeI* site is underlined.

In fact, the secondary mutation resulting in the rough LPS of 12-23 rendered this strain serum sensitive, which is sufficient to account for its attenuation. Nevertheless, the contribution of the selectively acquired *MutH* MMR component to virulence has never been directly elucidated. 12-23c virulence was eval-

uated by determining competitive indices (CI). BALB/c mice were coinfectd with equal numbers of CFU of 12-23c and a Lac⁺ wild-type variant (ST218 [Table 2]). A total volume of 200 μ l of phosphate-buffered saline, containing 10⁶ CFU of both strains, was delivered orally by using an 18-gauge feeding

TABLE 2. Intracellular replication mutants^g

Strain ^a	Gene at <i>MudJ</i> junction ^b	Rif ^r ^c	Ctx ^r ^d	CI ^e	Sif formation ^f
3–11c1	<i>mutS</i> ; base 1749 of M18965	2.4×10^{-4}	2.7	ND	Wild type
12–23c	<i>mutH</i> ; base 568 of U16361	1.14×10^{-4}	2.7	0.80 ± 0.28	Wild type
17–21c	<i>ssaQ</i> ; base 7725 of YO9357	2.4×10^{-8}	1.3	ND	Wild type
ST216 (Δ <i>ssaQ</i>)	NA	ND	22.8	≤ 0.001	Negative

^a Mutant designations are those of Leung and Finlay (15) with the suffix “c” appended to represent variants backcrossed to parental SL1344 backgrounds.^b The base number that abuts the left arm of *MudJ* and the accession number is given.^c Spontaneous mutation frequency to rifampin (Rif) resistance (50 μ g/ml) was calculated as the total number of CFU per milliliter divided by the number of Rif^r CFU per milliliter. The mutation frequency shown for 17–21c is in the range reported for wild-type SL1344 (24). The data shown are from an experiment representative of four, and in each experiment, several independent cultures were examined.^d The increase in cefotaxime (Ctx) resistance relative to the parental strain is given (11). The data shown are representative of three experiments performed.^e The CI for the mutant strain indicated was calculated as follows: the number of mutants was divided by the number of Lac⁺ reference variants obtained from spleen homogenates from mice coinfectd 5 days earlier by the oral route. The values given are averages from 5 (ST216) or 12 (12–23c) different BALB/c mice with the error reflecting 1 standard deviation from the mean. When lactose differentiation was used, the lower limit of our assay was 0.001.^f The presence of Sif was determined within infected HeLa cells fluorescently stained for lysosomal membrane glycoproteins as previously described (11).^g NA, not applicable; ND, not determined.

FIG. 1. Intracellular proliferation of serovar Typhimurium, backcrossed, prototrophic replication mutants over 20 h within MDCK epithelial cells (A) and over 24 or 25.5 h in RAW 264.7 macrophages (B) as described previously (11). Error bars represent 1 standard deviation from the mean of triplicate time points. They are visible only if the error was larger than the area displaced by the data point symbol. Data shown are from a representative of three independent experiments.

needle. After 5 days, mice were killed and spleen homogenates were plated on media differential for lactose utilization (24a). As shown in Table 2, nearly identical numbers of CFU of 12-23c and the SL1344 parental strain were obtained. Therefore, the loss of MutH, like the loss of MutS (4, 26), does not diminish *Salmonella* virulence. This demonstrates that neither conserved nor *S. enterica*-specific MMR factors serve a role in systemic *Salmonella* virulence.

Collectively, it seems that both the Rep⁻ and Sif⁻ phenotypes and the murine attenuation displayed by 3-11 and 12-23 (10, 15) were the result of mutations other than the MudJ insertions. In the case of 3-11, we identified a defect that can account for all the mutant phenotypes displayed by 3-11 (below).

The 17-21c mutant was disrupted within *ssaQ*, located in the structural II region (Fig. 2A) of the *Salmonella* pathogenicity island 2 (SPI2) (20, 23). SPI2 encodes a type III secretion system (TTSS) required for intramacrophage survival (5, 12, 20), intraepithelial replication (5, 11), and Sif formation (1, 2, 11). The *ssaQ*::MudJ insertion occurred near the beginning of the *ssaQ* open reading frame at base position +100 disrupting the V34 codon of the 322-amino-acid protein. SsaQ shares significant similarity with the C terminus of YscQ of the *Yersinia* TTSS (13). As depicted in Fig. 2B, there is now a family

of TTSS proteins that share this C-terminal surface presentation of antigens (SpoA; Pfam 01052) motif. The archetype is FliN, which is part of the flagellar C ring (17) located within the cytoplasmic membrane. SpoA proteins of virulence-associated TTSS are components of a surface-exposed (e.g., references 6 and 16) adapter complex predicted to allow the translocator to interact with the secretion apparatus (22). Therefore, we anticipate that SsaQ is also exported to the bacterial surface and is directly involved in effector traffic, but this theory awaits experimental demonstration.

As reported previously (24), we found that 17-21c still generates Sif at a wild-type frequency (Table 2). 17-21c also replicates within epithelial cells based on by its wild-type cefotaxime sensitivity (Table 2) and its proliferation within MDCK cells (Fig. 1A). However, a consequence of the MudJ insertion is revealed as a partial defect when 17-21c resides within the RAW 264.7 macrophage (Fig. 1B). 17-21c does not proliferate like wild-type strains, but a net reduction in bacterial numbers does not occur as it does with SPI2 loss-of-function mutants. For example, 69F3 (*ssaP*::MudJ) (11) is reduced by 75% within the macrophage when the earliest and latest time points are compared (Fig. 1B).

The wild-type Sif and intraepithelial replication phenotypes displayed by 17-21c contrast with the Sif⁻ (2, 11) and Rep⁻

FIG. 2. (A) Schematic diagram of the SPI2 virulence locus. The inverted triangle depicts the locations of the *MudJ* insertion within 17-21c. The internal deletion of *ssaQ* in ST216 is schematically shown below the SPI2 diagram. (B) Alignment of the C-terminal portion of SsaQ with components of TTSS proteins possessing an SpoA motif. Proteins aligned are as follows: SsaQ (GenBank accession no. P74860), serovar Typhimurium FliN (GenBank accession no. P26419), serovar Typhimurium SPI1-encoded SpaO (GenBank accession no. AAC43938.1), *Yersinia enterocolitica* plasmid-encoded YscQ (GenBank accession no. AAD16827), *Shigella flexneri* plasmid-encoded Spa33 (GenBank accession no. 49846), and enteropathogenic *E. coli* SepQ (GenBank accession no. AAC38386.1).

phenotypes displayed by previously characterized SPI2 mutants (5, 11). Therefore, either SsaQ is not essential for SPI2 function or the particular *ssaQ*::*MudJ* lesion allows production of a truncated SsaQ able to mediate most SPI2 phenotypes. To discriminate between these two possible reasons for the nearly wild-type behavior of 17-21c, a *ΔssaQ* strain was generated. As detailed previously (11), an internal deletion allele was generated by joining regions upstream and downstream of *ssaQ* with *PmeI* sites introduced during PCR amplification of these regions. Oligonucleotides Q1 and Q2-*PmeI* amplified the upstream region, and oligonucleotides Q3-*PmeI* and Q4 amplified the downstream region (Table 1). The resultant deletion allele was used for positive-selection allelic exchange (11). The resulting *ΔssaQ* strain (ST216) has an in-frame, internal deletion that removes nearly the entire *ssaQ* open reading frame (Fig. 2A; Table 1).

The *ΔssaQ* strain was severely attenuated in mice as indicated by its CI (Table 2) and was completely unable to generate Sif within HeLa epithelial cells (Table 2). It is also defective for intraepithelial replication, as demonstrated by a 22.8-fold increase in cefotaxime resistance (Table 2) and a

single doubling in MDCK cells, while 17-21c undergoes nearly three doublings over the same time course (Fig. 1A). The *ΔssaQ* strain also displays an intracellular survival deficiency within RAW 264.7, with nearly 75% of the internalized bacteria killed after 24 h (Fig. 1B). Thus, SsaQ is essential for SPI2 function, and it is likely that a truncated SsaQ variant is produced by 17-21c, which is sufficient for nearly normal SPI2 function. We speculate that an internal restart occurs within the *ssaQ*::*MudJ* allele, since a polar effect is not exerted upon the downstream *ssaU* (Suvarnapunya and Stein, unpublished data) and *ssaR* (2) genes that encode proteins essential for SPI2 function.

The implication of the SPI2 regulon by the *ssaQ*::*MudJ* insertion, albeit insufficient to abolish SPI2-mediated phenotypes such as Sif formation (11), prompted us to evaluate SPI2 function within the Rep⁻ mutants. To this end, we assessed SPI2 function by the export of the SPI2 translocon component, SseB (e.g., reference 19) to the bacterial surface. Equal numbers of whole bacteria were extracted with *n*-hexadecane. This selectively removes exported SseB by using methodologies that were detailed recently (28). As shown in Fig. 3, 17-21 and 3-11

FIG. 3. Immunoblot analysis of SPI2 TTSS function. Bacteria were grown in MgM at pH 5.0 and identical numbers were extracted with *n*-hexadecane to selectively remove the SseB translocon component exported to the bacterial surface by SPI2. Proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10.5% Tris-tricine) gels and were immunoblotted with anti-SseB mouse polyclonal antibody as detailed elsewhere (28). SL1344 is the wild-type parent. Controls for nonspecificity are Δ ssaV (ST244), deficient for SPI2 secretion, and Δ sseB (ST319). The apparent molecular mass of standards is given at right.

are SPI2 secretion mutants. The Δ ssaQ strain also fails to export SseB to the bacterial surface. However, 17-21c does export SseB, confirming that the particular *ssaQ::MudJ* lesion within 17-21c does not abolish SPI2 function. In fact, we consistently observed that *n*-hexadecane extracts from 17-21c contain more SseB than was obtained from the parent. This may suggest that certain alterations in SsaQ, as documented for the related FliN (17), may also influence the length of type III assembled organelles.

In contrast to the other original Rep⁻ mutants, 12-23 appears to possess a functional SPI2 secretion system at least to the point of SseB export. However, SPI2 mutations have been identified that abolish SPI2 translocation function without interfering with SseB export (19). It is unlikely that 12-23 is defective for production of SifA, which is also required for Sif formation (24). This is because the associated hyperreplication phenotype displayed by SifA loss-of-function mutants (24) is not displayed by 12-23 (data not shown). Alternatively, we have recently identified several *Salmonella* loci distinct from SPI2 and SifA that also result in an abolition of Sif formation (Suvarnapunya and Stein, unpublished). It is therefore conceivable that 12-23 sustained a lesion within one of these loci. We also cannot exclude the possibility that the unidentified secondary lesion that rendered 12-23 rough may also underlie the Sif⁻ and/or Rep⁻ phenotype. Additional studies are required should the cause for the 12-23 Rep⁻ Sif⁻ phenotypes be elucidated.

The molecular characterization of the prototrophic replication mutants of Leung and Finlay (15) yields the unanticipated conclusion that mutations secondary to the *MudJ* insertion underlie phenotypes attributed to the original isolates. The means by which unlinked, secondary mutations were acquired by 12-23 and 3-11 is readily suggested by their strong hypermutability combined with the potent selection exerted during cefotaxime enrichment. However, the possibility that a point mutation was acquired prior to *MudJ* insertion cannot be eliminated.

In the case of 17-21, possible causes for its SPI2 secretion deficit (Fig. 3) are harder to tender, especially with the finding that the actual *ssaQ::MudJ* lesion is not sufficient to prevent SPI2 export of SseB (Fig. 3). Nevertheless, it appears that either a spontaneous secondary mutation occurred within SPI2 or that the original *MudJ* insertion exerted a SPI2 loss-of-function effect in a manner not retained when backcrossed.

This study confirms and extends several previous findings. The virulence characterization of 12-23c verified (4, 27) that MMR is dispensable for *Salmonella* virulence, despite the contribution of MMR to the virulence of other intracellular pathogens (18). Nevertheless, DNA damage does occur during the course of *Salmonella* infection. The pleiotropic DNA repair system regulator, RecA, is required for *Salmonella* intramacrophage proliferation and for full virulence (3), and the base excision repair system may also facilitate repair of macrophage-mediated damage to DNA (25).

The in vitro phenotypes (10, 15) (Fig. 1 and 3) and in vivo behavior (15) of 3-11 and 17-21 are consistent with those ultimately attributed to SPI2 mutants (1, 2, 5, 11, 12, 20, 23). While it cannot be excluded that additional secondary mutations unrelated to SPI2 are present in these Rep⁻ mutants, their behavior in our hands is indistinguishable from that of targeted SPI2 deletion or insertion mutants (11, 25, 27). In retrospect, it appears that the identification and descriptions of these particular Rep⁻ mutants were the first characterization of SPI2 function.

The present finding that cefotaxime selected for hypermutable strains also highlights an important consideration when evaluating mutants obtained after multiple enrichment steps, as occurs in cefotaxime selection assays. The realization that point mutants may be obtained in this manner could be a useful strategy for molecular analysis via second-site suppressor screens.

We thank C. T. Parker and C. Fredericks for their contributions to identifying the *MudJ* insertion sites and L. Gonias, P. Fives-Taylor, B. B. Finlay, and K. P. Mintz for critical reading of the manuscript. A.E.S. and D.V.Z. contributed equally to this study.

REFERENCES

1. Beuzón, C. R., S. Méresse, K. E. Unsworth, J. Ruiz-Albert, S. Garvis, S. R. Waterman, T. A. Ryder, E. Boucrot, and D. W. Holden. 2000. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J.* **19**:3235–3249.
2. Brumell, J. H., C. M. Rosenberger, T. G. Gotto, S. L. Marcus, and B. B. Finlay. 2001. SifA permits survival and replication of *Salmonella typhimurium* in murine macrophages. *Cell. Microbiol.* **3**:75–84.
3. Buchmeier, N. A., C. J. Lipps, M. Y. So, and F. Heffron. 1993. Recombination-deficient mutants of *Salmonella typhimurium* are avirulent and sensitive to the oxidative burst of macrophages. *Mol. Microbiol.* **7**:933–936.
4. Campoy, S., A. M. Perez de Rozas, J. Barbe, and I. Badiola. 2000. Virulence and mutation rates of *Salmonella typhimurium* strains with increased mutagenic strength in a mouse model. *FEMS Microbiol. Lett.* **187**:145–150.
5. Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow. 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol. Microbiol.* **30**:175–188.
6. Collazo, C. M., and J. E. Galán. 1996. Requirement for exported proteins in secretion through the invasion-associated type III system of *Salmonella typhimurium*. *Infect. Immun.* **64**:3524–3531.
7. Eisen, J. A., and P. C. Hanawalt. 1999. A phylogenomic study of DNA repair genes, proteins, and processes. *Mutat. Res.* **435**:171–213.
8. Friedberg, E. C., G. C. Walker, and W. Siede. 1995. Mismatch repair, p. 367–405. In E. C. Friedberg, G. C. Walker, and W. Siede (ed.), *DNA repair and mutagenesis*. ASM Press, Washington, D.C.
9. Garcia-del Portillo, F., M. A. Stein, and B. B. Finlay. 1997. Release of lipopolysaccharide from intracellular compartments containing *Salmonella typhimurium* to vesicles of the host epithelial cell. *Infect. Immun.* **65**:24–34.
10. Garcia-del Portillo, F., M. B. Zwick, K. Y. Leung, and B. B. Finlay. 1993. Intracellular replication of *Salmonella* within epithelial cells is associated with filamentous structures containing lysosomal membrane glycoproteins. *Infect. Agents Dis.* **2**:227–231.
11. Guy, R. L., L. A. Gonias, and M. A. Stein. 2000. Aggregation of host endosomes by *Salmonella* requires SPI2 translocation of SseFG and involves SpvR and the *fms-aroE* intragenic region. *Mol. Microbiol.* **37**:1417–1435.
12. Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden. 1998. Genes

- encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol. Microbiol.* **30**:163–174.
13. Hensel, M., J. E. Shea, B. Raupach, D. Monack, S. Falkow, C. Gleeson, T. Kubo, and D. W. Holden. 1997. Functional analysis of *ssaJ* and the *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* Pathogenicity Island 2. *Mol. Microbiol.* **24**:155–167.
 14. Hughes, K. T., and J. R. Roth. 1988. Transitory cis complementation: a method for providing transposition functions to defective transposons. *Genetics* **119**:9–12.
 15. Leung, K. Y., and B. B. Finlay. 1991. Intracellular replication is essential for the virulence of *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **88**: 11470–11474.
 16. Li, J., H. Ochman, E. A. Groisman, E. F. Boyd, F. Solomon, K. Nelson, and R. K. Selander. 1995. Relationship between evolutionary rate and cellular location among the Inv/Spa invasion proteins of *Salmonella enterica*. *Proc. Natl. Acad. Sci. USA* **92**:7252–7256.
 17. Makishima, S., K. Komoriya, S. Yamaguchi, and S. I. Aizawa. 2001. Length of the flagellar hook and the capacity of the type III export apparatus. *Science* **291**:2411–2413.
 18. Merino, D., H. Reglier-Poupet, P. Berche, A. Charbit, and The European *Listeria* Genome Consortium. 2002. A hypermutator phenotype attenuates the virulence of *Listeria monocytogenes* in a mouse model. *Mol. Microbiol.* **44**:877–887.
 19. Nikolaus, T., J. Deiwick, C. Rappl, J. A. Freeman, W. Schroder, S. I. Miller, and M. Hensel. 2001. SseBCD proteins are secreted by the type III secretion system of *Salmonella* pathogenicity island 2 and function as a translocon. *J. Bacteriol.* **183**:6036–6045.
 20. Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman. 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc. Natl. Acad. Sci. USA* **93**:7800–7804.
 21. Schmieger, H. 1972. Phage P22-mutants with increased or decreased transduction abilities. *Mol. Gen. Genet.* **119**:75–88.
 22. Schuch, R., and A. T. Maurelli. 2001. Spa33, a cell surface-associated subunit of the Mxi-Spa type III secretory pathway of *Shigella flexneri*, regulates Ipa protein traffic. *Infect. Immun.* **69**:2180–2189.
 23. Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden. 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **93**:2593–2597.
 24. Stein, M. A., K. Y. Leung, M. Zwick, F. Garcia-del Portillo, and B. B. Finlay. 1996. Identification of a *Salmonella* virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. *Mol. Microbiol.* **20**:151–164.
 25. Suvarnapunya, A. E., H. A. D. Lagassé, and M. A. Stein. The role of DNA base excision repair in the pathogenesis of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.*, in press.
 26. Tang, P., V. Foubister, M. G. Pucciarelli, and B. B. Finlay. 1993. Methods to study bacterial invasion. *J. Microbiol. Methods* **18**:227–240.
 27. Zahrt, T. C., N. Buchmeier, and S. Maloy. 1999. Effect of *mutS* and *recD* mutations on *Salmonella* virulence. *Infect. Immun.* **67**:6168–6172.
 28. Zurawski, D. V., and M. A. Stein. 2003. SseA acts as the chaperone for the SseB component of the *Salmonella* Pathogenicity Island 2 translocon. *Mol. Microbiol.* **47**:1341–1351.

Editor: A. D. O'Brien