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Induction of type III secretion in *Shigella flexneri* is associated with differential control of transcription of genes encoding secreted proteins

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***Shigella*, the etiological agent of human bacillary dysentery, invades the colonic epithelium where it induces an intense inflammatory response. Entry of *Shigella* into epithelial cells involves a type III secretion machinery, encoded by the *mxi* and *spa* operons, and the IpaA–D secreted proteins. In this study, we have identified secreted proteins of 46 and 60 kDa as the products of *virA* and *ipaH9.8*, respectively, the latter being a member of the *ipaH* multigene family. Inactivation of *virA* did not affect entry into epithelial cells. Using *lacZ* transcriptional fusions, we found that transcription of *virA* and four *ipaH* genes, but not that of the *ipaBCDA* and *mxi* operons, was markedly increased during growth in the presence of Congo red and in an *ipaD* mutant, two conditions in which secretion through the Mxi–Spa machinery is enhanced. Transcription of the *virA* and *ipaH* genes was also transiently activated upon entry into epithelial cells. These results suggest that transcription of the *virA* and *ipaH* genes is regulated by the type III secretion machinery and that a regulatory cascade differentially controls transcription of genes encoding secreted proteins, some of which, like *virA*, are not required for entry.**

Keywords: intracellular/invasion/pathogenesis/regulation/secretion

Introduction

Numerous Gram-negative bacteria that are pathogens for humans, animals or plants use homologous protein secretion machineries to secrete their virulence factors. The Sec-independent type III secretion pathway is involved in secretion of *Yersinia* anti-host proteins, *Salmonella* and *Shigella* spp. effectors of entry into epithelial cells, EPEC signal transducing proteins, *Pseudomonas aeruginosa* toxins and virulence factors of many plant pathogens, as well as in flagellum assembly of bacteria such as *Salmonella typhimurium* and *Bacillus subtilis* (reviewed in Van Gijsegem *et al.*, 1993; Mecsas and Strauss, 1996; Alfano and Collmer, 1997). Possible characteristic features of this secretion pathway include the fact that secretion is activated by contact of the bacterium with host cells (Ménard *et al.*, 1994; Watarai *et al.*, 1995; Zierler and Galán, 1995), that some of the secreted proteins are delivered into the cytoplasm of host cells (Rosqvist *et al.*, 1994; Sory and Cornelis, 1994; Wood *et al.*, 1996; Collazo and Galan, 1997) and that transcription of genes encoding secreted proteins is controlled by secretion of regulatory proteins (Hughes *et al.*, 1993; Pettersson *et al.*, 1996).

Members of the genus *Shigella* cause bacillary dysentery in humans by invading the colonic epithelial mucosa and inducing a strong inflammatory response (LaBrec *et al.*, 1964). *In vitro*, cell invasion involves two steps: entry and intercellular dissemination. Genes involved in both steps are carried on a 200 kb virulence plasmid (reviewed by Hale, 1991; Parsot, 1994). A 31 kb fragment of this plasmid is necessary and apparently sufficient for entry into epithelial cells (Maurelli *et al.*, 1985; Sasakawa *et al.*, 1988). This fragment is organized in two divergently transcribed regions which schematically encode secreted proteins, the IpaA–D proteins and a type III secretion system, the Mxi–Spa secretion apparatus. The first region contains eight genes, including *ipaBCDA*, which are transcribed from a promoter located upstream from *icsB*. The second region contains 20 genes, designated *ipg*, *mxi* and *spa*, which are clustered in large operons. Inactivation of *ipa*, *mxi* and *spa* genes leads to a non-invasive phenotype, due to either loss of effector proteins (Sasakawa *et al.*, 1989; Ménard *et al.*, 1993) or failure to secrete them (Andrews and Maurelli, 1992; Venkatesan *et al.*, 1992; Allaoui *et al.*, 1993b; Sasakawa *et al.*, 1993).

Only a small proportion of IpaA–D proteins is secreted by wild-type *Shigella* growing in laboratory media. Inactivation of *ipaD* enhances secretion of IpaA, IpaB, IpaC and ~15 other proteins (Ménard *et al.*, 1994; Parsot *et al.*, 1995). These latter proteins are absent or barely detectable in the medium of the wild-type strain unless Congo red, a dye that induces secretion (Bahrani *et al.*, 1997), is present in the culture medium (Parsot *et al.*, 1995). In this study, we have characterized secreted proteins of 46 and 60 kDa which are overproduced by a Δ*ipaBCDA* mutant. The 46 kDa protein was identified as the product of *virA*, a gene which previously had been characterized in an *Shigella flexneri* 2a strain (Uchiya *et al.*, 1995). The 60 kDa protein was identified as the product of *ipaH9.8*, a member of the *ipaH* multigene family that comprises five genes which are designated by the size of the *Hin*dIII fragment on which they are carried by the virulence plasmid (Hartman *et al.*, 1990; Venkatesan *et al.*, 1991). Using *lacZ* transcriptional fusions, we have investigated transcription of *virA*, of four members of the *ipaH* family and of the *ipaBCDA* and *mxi* operons. We present evidence that transcription of *virA* and of four *ipaH* genes, but not that of the *ipaBCDA* and *mxi* operons, is increased when secretion through the type III secretion machinery is enhanced in response to addition of Congo red to the growth medium and to inactivation of *ipaD*. In addition, transcription of *virA–lacZ* and *ipaH–lacZ* fusions was activated during entry of bacteria into epithelial cells.

Characterization of a *virA* mutant indicated that VirA, in contrast to IpaB, IpaC and IpaD, is not required for entry into epithelial cells, which suggests that the differential expression of secreted proteins might reflect differences in the function of these proteins during infection.

Results

Some secreted proteins are overproduced by constitutively secreting strains

Inactivation of either *ipaB* or *ipaD* and deletion of the *ipaB, C, D* and *A* genes lead to the secretion of ~15 proteins that associate in the extracellular medium (Parsot *et al.*, 1995). Aggregates containing proteins secreted by the Δ*ipaBCDA* mutant (SF635) were used to immunize mice, and the resulting antiserum was tested by Western blotting on extracts of whole cultures, bacterial pellets and culture supernatants of M90T (wild-type), SF622 (*ipaD*), SF635 (Δ*ipaBCDA*), SF634 (*ipaD mxiD*) and BS176 (a virulence plasmid-cured strain). The serum reacted most strongly with a 46 kDa protein; this protein was present in high amounts in extracts of *ipaD* and Δ*ipaBCDA* strains, was present in low amounts in extracts of wild-type and *ipaD mxiD* strains, and was not present in extracts of the virulence plasmid-cured strain (Figure 1). SDS–PAGE analysis and Coomassie Blue staining also revealed that a protein, or possibly a mixture of proteins, of ~60 kDa was present in higher amounts in extracts of the *ipaD* and Δ*ipaBCDA* strains than in extracts of the wild-type and *ipaD mxiD* strains (Figure 1). These results suggested that production of 46 and 60 kDa secreted proteins was increased in the constitutively secreting *ipaD* and Δ*ipaBCDA* strains compared with the wild-type and secretion-deficient *ipaD mxiD* strains.

Characterization of the gene encoding the 46 kDa secreted protein

The 46 kDa protein secreted by the Δ*ipaBCDA* mutant was transferred onto a PVDF membrane and subjected to Edman degradation and proteolysis by endolysin. The N-terminal sequence of the protein was identified as M-Q-T-S-N-I-T-N-H-E and those of two internal peptides as I-I-T-F-G-I-Y-S-P-H-E-T-L-A and V-H-T-I-T-A-P-V-S-G-N. Oligonucleotides based on the N-terminal sequence and one internal peptide were used to screen, by Southern blotting, a set of overlapping cosmids representing the entire virulence plasmid. Both probes hybridized to a 6.4 kb *Hin*dIII fragment of cosmid pCos3 (data not shown) which was then cloned into pUC19 to give rise to pBD3 (Figure 2). *Escherichia coli* derivatives harboring this plasmid produced a 46 kDa protein that was recognized by the serum raised against the mixture of secreted proteins (data not shown), thereby indicating that the entire gene had been cloned.

Subcloning experiments and Southern blot analysis of recombinant plasmids using oligonucleotides as probes allowed us to localize the gene encoding the 46 kDa protein on a 3.2 kb *Hin*cII–*Hin*dIII fragment located upstream from *icsA* (Bernardini *et al.*, 1989; Lett *et al.*, 1989). Sequence analysis revealed an open reading frame (ORF) starting 487 bp upstream from the *icsA* translation start codon and oriented in the opposite direction. Amino acid sequences deduced from positions 43–71, 159–200 and 442–473 of the ORF were identical to those determined for the N-terminal end and the two internal peptides of the secreted protein. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the accession number AF047364. The deduced sequence of the 46 kDa protein was identical to that of VirA, a secreted protein encoded by the virulence plasmid of an *S.flexneri* strain of serotype 2a (Uchiya *et al.*, 1995) and, therefore, the corresponding gene of *S.flexneri* 5 was designated *virA*. No other ORFs were detected immediatly upstream or downstream from *virA*. Restriction analysis of overlapping cosmids indicated that *virA* was located ~10 kb downstream from the *spa* operon (Venkatesan *et al.*, 1992; Sasakawa *et al.*, 1993) on the virulence plasmid pWR100.

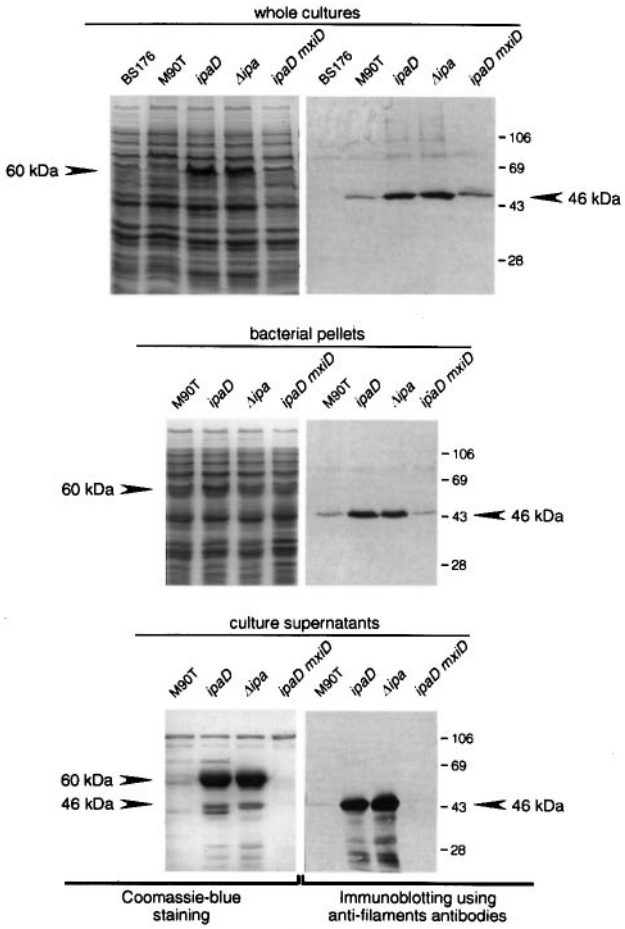

Fig. 1. Secretion of proteins by various *Shigella* strains. Cultures of M90T (wild-type), BS176 (the virulence plasmid-cured strain) and the *ipaD* (SF622), Δ*ipaBCDA* (SF635) and *ipaD mxiD* (SF634) mutants were used to prepare either whole culture extracts, by adding Laemmli sample buffer directly to the cultures, or bacterial pellets and culture supernatants, by centrifugation of the cultures. Proteins present in culture supernatants were concentrated 10 times by TCA precipitation. Samples were separated by SDS–PAGE and analyzed by either Coomassie Blue staining or immunoblotting using an antiserum raised against aggregates recovered from the medium of the Δ*ipaBCDA* mutant. Numbers indicate the position and the size (in kDa) of standard proteins and arrows indicate the position of the 60 and 46 kDa proteins.

Characterization of the gene encoding a 60 kDa secreted protein

The 60 kDa proteins which were secreted in high amount by the Δ*ipaBCDA* strain were transferred onto a PVDF

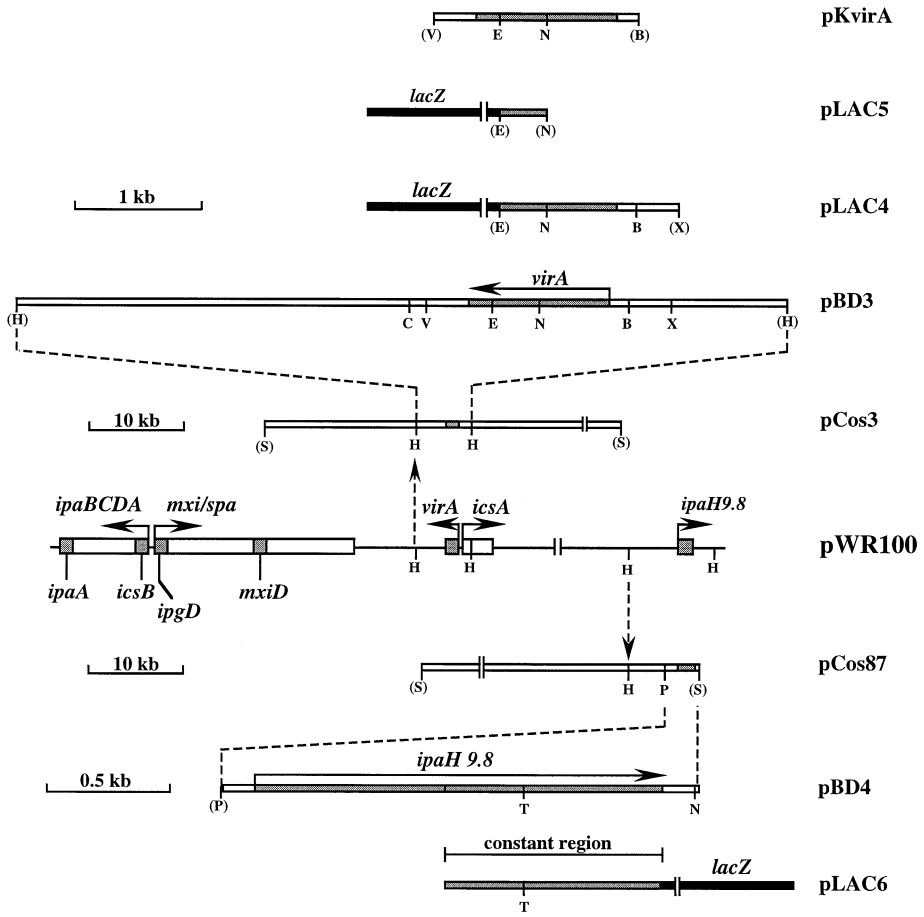

Fig. 2. Structure of plasmids carrying *virA* and *ipaH9.8*. A schematic genetic map of a portion of the virulence plasmid pWR100 is shown in the center, along with the position of some relevant restriction sites. Symbols used for restriction sites are: B, *Bsp*EI; C, *Hinc*II; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; P, *Hpa*I; S, *Sau*3AI; T, *Stu*I; V, *Bbv*I; X, *Xba*I. The DNA corresponding to *virA* and *ipaH9.8* is shown by shaded bars and the *lacZ* gene by a solid bar. Arrows indicate the orientation of transcription of the genes. Restriction sites of the virulence plasmid that were used for cloning are indicated in brackets.

membrane and the lower part of the band was used for N-terminal sequence determination and proteolysis by endolysin. Analysis of the N-terminal sequence indicated that the sample contained two proteins; the sequence of the major species was determined as M-L-P-I-N-N-N-F-S-L-P-Q. The sequence of an internal peptide was determined as Y-E-M-L-E-N-E-Y-P-Q-R-V-A-D-R, which was almost identical to a fragment of the constant region of members of the IpaH family. IpaH proteins are characterized by a constant C-terminal region of ~300 residues which is preceded by a variable N-terminal region composed of repetitive motifs (Hartman *et al.*, 1990; Venkatesan *et al.*, 1991). The N-terminal sequence of the 60 kDa secreted protein was different from those deduced from the 5′ end of *ipaH7.8*, *ipaH4.5*, *ipaH2.5* and *ipaH1.4* (Hartman *et al.*, 1990; Venkatesan *et al.*, 1991), which suggested that this protein might correspond to the fifth IpaH protein, IpaH9.8, whose gene had not been sequenced yet.

Southern blot analysis using a probe derived from the constant region of *ipaH* genes indicated that *ipaH9.8* was present in cosmid pCos87 (data not shown). Deletion derivatives of pCos87 were constructed to give rise to pBD4 (Figure 2), whose 2.4 kb insert was entirely sequenced. The amino acid sequences deduced from positions 40–75 and 1477–1521 of the ORF identified by sequence analysis were identical to those of the N-terminal end and of the internal peptide of the 60 kDa secreted protein. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the accession number AF047365. The *ipaH9.8* gene encodes a 545 residue protein with a deduced M_r of 61 886. No ORFs were identified upstream or downstream from *ipaH9.8*. Restriction analysis of overlapping cosmids indicated that *ipaH9.8* was located 45 kb downstream from the *spa* operon.

Inactivation of ipaD increases transcription of virA and ipaH genes

Western blot analysis indicated that a higher amount of VirA was produced by the *ipaD* mutant than by the wild-type strain (Figure 1). To investigate *virA* transcription, we constructed a *virA–lacZ* transcriptional fusion by cloning the *icsA–virA* intergenic region and the 5′ part of *virA* upstream from the *lacZ* gene in a suicide vector. The recombinant plasmid pLAC4 (Figure 2) was integrated at the *virA* locus of the virulence plasmid harbored by the wild-type and *ipaD* strains to construct SF1001 (*virA–lacZ ipaD*$^+$) and SF1002 (*virA–lacZ ipaD*$^-$) (Table I). Expression of the *virA–lacZ* fusion was 17 times higher in the *ipaD*$^-$ strain as compared with the *ipaD*$^+$ strain (Table II), indicating that the increased production of VirA

Table I. *Shigella* strains

Strain	Genotype	Reference
M90T	Wild type	Sansonetti *et al.* (1985)
M90T-Sm	spontaneous Sm^R derivative of M90T	Allaoui *et al.* (1992)
BS176	plasmidless derivative of M90T	Sansonetti *et al.* (1985)
SF132	*icsB–lacZ* in M90T-Sm	Allaoui *et al.* (1992)
SF134	*ipgD–lacZ* in M90T-Sm	Allaoui *et al.* (1993a)
SF401	*mxiD*	Allaoui *et al.* (1993b)
SF403	*mxiD–lacZ* in M90T-Sm	Allaoui *et al.* (1993b)
SF622	*ipaD*	Ménard *et al.* (1993)
SF623	*ipaA–lacZ* in M90T-Sm	Ménard *et al.* (1993)
SF624	*ipaA–lacZ* in SF622 (*ipaD*)	Ménard *et al.* (1993)
SF634	*ipaD mxiD*	Ménard *et al.* (1994)
SF635	Δ*ipaBCDA*	Parsot *et al.* (1995)
SF803	*icsB–lacZ* in SF622 (*ipaD*)	this work
SF806	*ipgD–lacZ* in SF622 (*ipaD*)	this work
SF808	*mxiD–lacZ* in SF622 (*ipaD*)	this work
SF1001	*virA–lacZ* in M90T-Sm ($virA^+$)	this work
SF1002	*virA–lacZ* in SF622 ($virA^+$)	this work
SF1003	*virA–lacZ* in M90T-Sm ($virA^-$)	this work
SF1004	*virA–lacZ* in SF622 ($virA^-$)	this work
SF1005	*ipaH9.8–lacZ* in M90T-Sm	this work
SF1006	*ipaH9.8–lacZ* in SF622 (*ipaD*)	this work
SF1007	*ipaH7.8–lacZ* in M90T-Sm	this work
SF1008	*ipaH4.5–lacZ* in M90T-Sm	this work
SF1009	*ipaH4.5–lacZ* in SF622 (*ipaD*)	this work
SF1010	*ipaH1.4–lacZ* in M90T-Sm	this work
SF1011	*ipaH1.4–lacZ* in SF622 (*ipaD*)	this work
SF1012	*virA–lacZ* in SF401 (*mxiD*)	this work

by the *ipaD* mutant was due to an increased transcription of *virA*. To determine whether VirA was involved in the regulation of the *virA* promoter, a DNA fragment internal to *virA* was cloned upstream from the *lacZ* gene in a suicide vector, and the recombinant plasmid pLAC5 (Figure 2) was integrated at the *virA* locus of the wild-type and *ipaD* strains to construct strains SF1003 (*virA–lacZ ipaD*$^+$ *virA*$^-$) and SF1004 (*virA–lacZ ipaD*$^-$ *virA*$^-$). Inactivation of *virA* had no effect on transcription of the *virA–lacZ* fusion in either the *ipaD*$^+$ or *ipaD*$^-$ backgrounds (data not shown), indicating that *virA* was not auto-regulated.

To analyze transcription of the various *ipaH* genes, the constant region of *ipaH9.8* was cloned upstream from the *lacZ* gene in a suicide plasmid, and the recombinant plasmid pLAC6 (Figure 2) was transferred by conjugation into the wild-type and *ipaD* strains. Transconjugants were screened by Southern blotting to identify the *ipaH* gene into which the plasmid was integrated. This allowed us to construct *ipaH9.8–lacZ*, *ipaH4.5–lacZ* and *ipaH1.4–lacZ* fusions in both the wild-type and *ipaD* strains, as well as an *ipaH7.8–lacZ* fusion in the wild-type strain. Expression of *ipaH9.8–lacZ*, *ipaH4.5–lacZ* and *ipaH1.4–lacZ* fusions was low in derivatives of the wild-type strain and was increased 5–20 times in derivatives of the *ipaD* mutant (Table II).

To investigate transcription of genes of the entry region, we used *lacZ* transcriptional fusions in *icsB* and *ipaA*, which are the first and last genes of the *ipaBCDA* operon, respectively, and in *ipgD* and *mxiD*, which are the first and twelfth genes of the *mxi* operon, respectively (Figure 2). These fusions were constructed in both the wild-type and *ipaD* strains. Integration of the suicide plasmids used to construct these fusions did not affect the secretion phenotype of recombinant strains (data not shown). For each fusion, similar amounts of β-galactosidase were present in derivatives of the wild-type and *ipaD* strains, indicating that transcription of these genes was not affected by inactivation of *ipaD* (Table II), which was consistent with the observation that production of IpaB and IpaC was not affected in an *ipaD* mutant (Ménard *et al.*, 1993, 1994).

Table II. Expression of *lacZ* transcriptional fusions by bacteria growing *in vitro*

Fusion	β-Galactosidase activity (Miller units)[a]				
	ipaD$^+$	*ipaD*$^-$	Ratio I[b]	*ipaD*$^+$+CR	Ratio II[c]
virA–lacZ	16	280	17	280	17
virA–lacZ mxiD	17	NA	NA	18	1.1
ipaH9.8–lacZ	28	580	21	325	12
ipaH7.8–lacZ	20	NA	NA	235	12
ipaH4.5–lacZ	31	360	12	110	3.5
ipaH1.4–lacZ	53	280	5.3	270	5.1
ipaA–lacZ	485	510	1.1	390	0.8
icsB–lacZ	290	305	1.1	285	1.0
mxiD–lacZ	275	260	0.9	320	1.2
ipgD–lacZ	450	400	0.9	475	1.1

[a]Activities are the means of at least three independent experiments. Standard deviations are within 25% of the reported values.
[b]Activity present in *ipaD*$^-$ strains versus activity present in *ipaD*$^+$ strains.
[c]Activity present in derivatives of the *ipaD*$^+$ strain grown in the presence of Congo red versus activity present in the same strains grown in the absence of Congo red.
NA: not applicable.

Congo red increases transcription of virA and ipaH genes

Secretion of IpaB and IpaC is enhanced when bacteria grow in the presence of Congo red (Parsot *et al.*, 1995). To investigate the effect of Congo red on *virA* transcription, we assayed the β-galactosidase activity in strain SF1001 (*virA–lacZ ipaD*$^+$) after growth in the presence of various concentrations of Congo red. Transcription of the *virA–lacZ* fusion was low at concentrations of dye up to 20 μg/ml and then increased with the concentration of the dye to reach a plateau at ~100 μg/ml of Congo red (Table II; data not shown). Likewise, ~3–12 times more β-galactosidase activity was present in strains carrying *ipaH9.8–*, *ipaH7.8–*, *ipaH4.5–* and *ipaH1.4–lacZ* fusions after growth in the presence of 100 μg/ml of Congo red (Table II). In contrast, transcription of *icsB–*, *ipaA–*, *ipgD–* and *mxiD–lacZ* fusions was not affected by the presence of Congo red in the growth medium (Table II), which was consistent with the observation that the amount of IpaB and IpaC was not affected by the presence of Congo red in the growth medium (Parsot *et al.*, 1995).

Secretion is required for activation of virA transcription

To determine whether regulation of *virA* transcription was dependent on the type III secretion machinery, we compared the β-galactosidase activities produced by the *virA–lacZ* fusion in derivatives of wild-type and *mxiD* strains during growth in the presence of Congo red and the production of VirA in *ipaD* and *mxiD ipaD* strains. The presence of Congo red in the growth medium of the

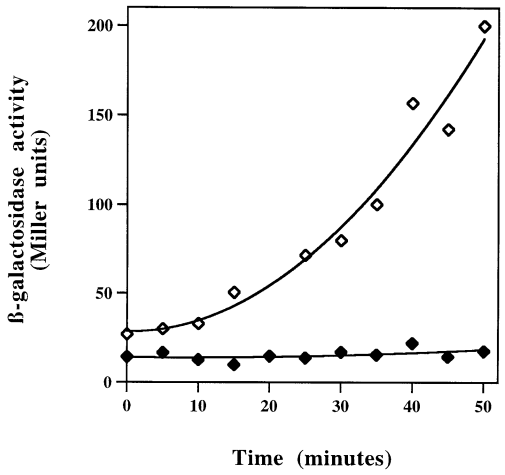

Fig. 3. Transcription of the *virA–lacZ* fusion upon addition of Congo red to the growth medium. Congo red (100 μg/ml) was added to the growth medium during the exponential phase of growth of derivatives of the wild-type (open symbols) and *mixD* (closed symbols) strains carrying the *virA–lacZ* fusion. Samples were then collected at 5 min intervals and assayed for β-galactosidase activity. For both strains, no increase in β-galactosidase activity was detected in the absence of Congo red.

derivative of the *mxiD* strain carrying the *virA–lacZ* fusion did not lead to an increase in β-galactosidase activity (Table II), and lesser amounts of VirA were present in the *ipaD mxiD* strain as compared with the *ipaD* strain (Figure 1). This indicated that activation of the *virA* promoter in response to Congo red and inactivation of *ipaD* required the integrity of the secretion machinery.

To investigate kinetics of activation of the *virA* promoter, Congo red (100 μg/ml) was added to the growth medium during the exponential phase of growth of derivatives of the wild-type and *mxiD* strains carrying the *virA–lacZ* fusion. Samples were then collected at 5 min intervals and assayed for β-galactosidase activity. An increase in the β-galactosidase specific activity was detected 10 min after addition of the dye to the medium of the derivative of the wild-type strain, whereas no transcriptional activation of the *virA–lacZ* fusion was detected in the derivative of the *mxiD* mutant (Figure 3). The 10 min lag time observed between addition of Congo red and activation of *virA* transcription in the derivative of the wild-type strain was similar to that observed for induction of IpaB and IpaC secretion by Congo red (Bahrani *et al.*, 1997).

These results differentiated the *virA* and *ipaH* genes, the transcription of which was increased after growth in the presence of Congo red or by inactivation of *ipaD*, from the genes of the entry region, the transcription of which apparently was constitutive with respect to these parameters. Moreover, this suggested that transcription of the *virA* and *ipaH* genes was regulated by the Mxi–Spa secretion machinery, since (i) conditions leading to an enhanced transcription of these genes were the same as those known to increase secretion through the Mxi–Spa secretion machinery, and (ii) in these conditions, the secretion machinery was required for the enhanced transcription of the *virA–lacZ* fusion and for the enhanced production of the VirA protein.

Table III. Expression of *lacZ* transcriptional fusions by intracellular bacteria

Fusion	β-Galactosidase activity (Miller units)[a]				
	In vitro	60 min of infection	Ratio I[b]	150 min of infection	Ratio II[c]
ipgD–lacZ	450	490	1.1	465	1.1
virA–lacZ	16	280	18	49	5.7
ipaH9.8–lacZ	28	350	13	49	7.1
ipaH7.8–lacZ	20	590	30	150	3.9
ipaH4.5–lacZ	31	280	9.0	21	13.3
ipaH1.4–lacZ	53	300	5.7	49	6.1

[a]Activities are the means of at least three independent experiments. Standard deviations are within 25% of the reported values.
[b]Activity present after 60 min of infection versus activity present in bacteria grown *in vitro*.
[c]Activity present after 60 min of infection versus activity present after 150 min of infection.

Transcription of virA– and ipaH–lacZ fusions upon entry and during intracellular multiplication

To investigate *virA* and *ipaH* transcription during infection of epithelial cells, we measured the β-galactosidase activity that was present in bacteria shortly after entry into epithelial cells. Cells were infected for 30 min to allow entry and then treated with gentamicin for 30 min to kill extracellular bacteria. Infected cells were then washed to remove killed bacteria and lysed, and intracellular bacteria were recovered by centrifugation. The number of intracellular bacteria was determined by plating, and the β-galactosidase activity present in these bacteria was assayed by using 4-methyl-umbelliferyl-β-D-galactoside (MUG) as a substrate. The specific activity was first expressed in units of fluorescence per bacterium and then converted into Miller units. For the strain carrying the *ipgD–lacZ* fusion, chosen as a representative of genes which were expressed constitutively *in vitro*, the β-galactosidase activity present within intracellular bacteria recovered after 60 min of infection was similar to that found after growth in laboratory medium (Table III). This confirmed that, following gentamicin treatment, washes of infected cells were sufficient to remove killed extracellular bacteria which, otherwise, could have contributed to the total β-galactosidase activity without being numbered by plating. For strains carrying the *virA–* and *ipaH–lacZ* fusions, the β-galactosidase activity was 6–30 times higher in intracellular bacteria than in bacteria grown *in vitro* (Table III). This indicated that transcription of *virA*, *ipaH9.8*, *ipaH7.8*, *ipaH4.5* and *ipaH1.4* had been induced upon entry or shortly thereafter.

To investigate *virA* transcription during growth in the intracellular compartment, infected cells were lysed after various periods of incubation in the presence of gentamicin, and intracellular bacteria were counted by plating and assayed for β-galactosidase activity. The number of intracellular bacteria carrying the *virA–lacZ* fusion increased with the time of incubation, which was consistent with their intracellular multiplication (Figure 4). In contrast, the specific β-galactosidase activity present in these bacteria decreased steadily; the slope of the decrease in β-galactosidase specific activity was similar to that of the increase in the number of intracellular bacteria, suggesting

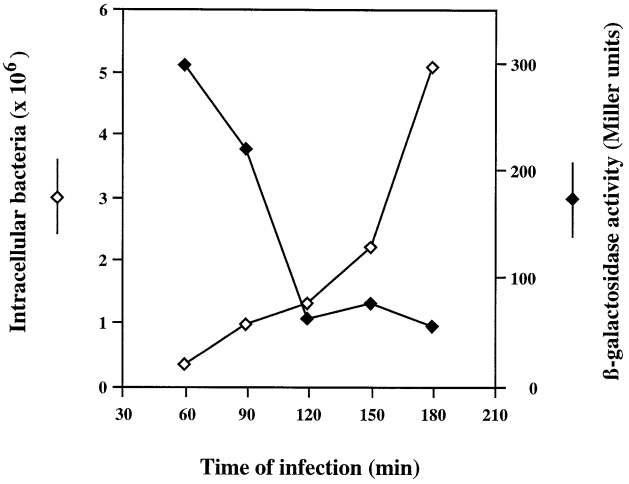

Fig. 4. Transcription of the *virA–lacZ* fusion by intracellular bacteria. Intracellular bacteria recovered after various times of infection of HeLa cells by SF1001 (*virA–lacZ)* were counted by plating (open symbols) and used to assay β-galactosidase activity (closed symbols).

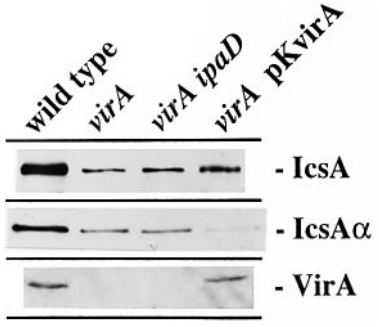

Fig. 5. Production of IcsA and secretion of IcsAα by wild-type and *virA* strains. Bacterial pellets (lanes IcsA and VirA) and culture supernatants (lane IcsAα) of the wild-type strain, the *virA* and *virA ipaD* mutants, and the *virA* mutant harboring plasmid pKvirA were separated by centrifugation and samples were analyzed by SDS–PAGE and immunoblotting with antibodies raised against IcsA or aggregated proteins from the Δ*ipaBCDA* strain.

that the decrease in specific activity was due to bacterial multiplication. Similarly, the β-galactosidase activity present in bacteria carrying the various *ipaH–lacZ* fusions was 6–13 times lower after 150 min of infection as compared with the activity present after 60 min of infection (Table III). These results suggested that the *virA*– and *ipaH–lacZ* fusions had not been transcribed between 60 and 150 min of infection. In contrast, for the strain carrying the *ipgD–lacZ* fusion, similar amounts of β-galactosidase were present after 60 and 150 min of infection (Table III), suggesting that the intracellular compartment had no effect on *ipgD* transcription.

Phenotypic characterization of a virA mutant

The presence of *virA* on the virulence plasmid, the regulation of its transcription by the type III secretion machinery, and previous results obtained with a *virA* mutant of *S.flexneri* 2a (Uchiya *et al.*, 1995) suggested that VirA might be involved in *Shigella* virulence. To investigate the role of VirA, the *virA* gene of the wild-type strain was inactivated by integration of a suicide plasmid containing a *virA* internal fragment (Figure 2). Phenotypic characterization of the *virA* mutant was performed using both animal models of infection and cultured cell lines. Infection of rabbit ligated ileal loops revealed no difference between the mutant and the wild-type strains using such criteria as the volume of exudate, the intensities of ulceration and destruction of the villi, and the number of polymorphonuclear neutrophils accumulating in the mucosa (data not shown). In contrast, inoculation of guinea pig conjunctival sac (Sereny test) revealed an attenuation of the virulence of the *virA* mutant; whereas the wild-type strain provoked a frank keratoconjunctivitis within 48 h of infection, the *virA* mutant elicited a mild keratoconjunctivitis that was detectable only after 72 h of infection. The phenotype of the *virA* mutant harboring the plasmid pKvirA (Figure 2) was similar to that of the wild-type strain, which indicated that the attenuation of the virulence observed with the *virA* mutant was indeed due to the lack of VirA.

Inactivation of *virA* affected neither the production and secretion of IpaA–D proteins (data not shown) nor the efficiency of entry into epithelial cells, as estimated by the gentamicin resistance assay after infection of HeLa cells, by the number of plaques formed after infection of Caco-2 cell monolayers and by the contact hemolytic activity on sheep erythrocytes (data not shown). In contrast, the *virA* mutant exhibited a reduced ability to disseminate from cell to cell: the plaques formed on a Caco-2 cell monolayer were three times smaller with the mutant than with the wild-type. Infected Hela cells were also analyzed by immunofluorescence microscopy, after labeling of F-actin and bacteria, and by electron microscopy: cells infected by the mutant had fewer protrusions and contained more bacteria (data not shown). Immunofluorescence microscopy analysis using antibodies raised against IcsA, the outer membrane protein required for the movement of intracellular bacteria (Makino *et al.*, 1986; Bernardini *et al.*, 1989), indicated that both the number of labeled bacteria and the intensity of the IcsA labeling were decreased in the *virA* mutant as compared with the wild-type (data not shown). Western blot analysis of whole cell extracts and culture supernatants of wild-type and *virA* strains indicated that the amounts of both IcsA and IcsAα, the secreted form of IcsA, were markedly decreased in the *virA* mutant as compared with the wild-type strain (Figure 5). Introduction of the plasmid pKvirA into the *virA* mutant did not complement the mutant for the production of IcsA nor for the phenotype observed in the plaque assay, although this strain produced an amount of VirA similar to that of the wild-type (Figure 5). Uchiya *et al.* (1995) had observed that insertion of a transposon into the *virA* gene of an *S.flexneri* 2a strain led to a decrease in *icsA* transcription and that this effect was not complemented by a plasmid carrying a wild-type copy of *virA*. This suggests that inactivation of *virA* by integration of a suicide plasmid or a transposon has a *cis*-acting effect on the *icsA* promoter.

Discussion

Production of most bacterial virulence factors is tightly regulated in response to environmental signals. Both the temperature and the osmolarity of the growth medium modulate transcription of genes involved in entry of

Shigella into epithelial cells (Maurelli *et al.*, 1984; Bernardini *et al.*, 1990). In addition, contact of *Shigella* with epithelial cells (Ménard *et al.*, 1994; Watarai *et al.*, 1995) and exposure of bacteria to Congo red (Sankaran *et al.*, 1989; Parsot *et al.*, 1995; Bahrani *et al.*, 1997) or bile salts (Pope *et al.*, 1995) activate secretion of IpaB and IpaC. In the present study, we have identified a set of genes, including *virA* and four members of the *ipaH* family, the transcription of which appears to be activated in response to an increased secretion through the type III secretion machinery.

We used *lacZ* fusions to investigate transcription of *virA*, *ipaH9.8*, *ipaH7.8*, *ipaH4.5* and *ipaH1.4*, as well as that of operons located in the entry region. Transcription of genes of the entry region was high in derivatives of the wild-type strain and was not increased in derivatives of the *ipaD* mutant or after growth in the presence of Congo red. These results indicate that: (i) the increased secretion observed with the wild-type strain growing in the presence of Congo red and with the *ipaD* mutant is not due to an increased transcription of the *mxi* operon; (ii) transcription of *mxi* and *ipaBCDA* operons is the same whether the secretion machinery is poorly active (in the wild-type strain) or deregulated (by addition of Congo red or inactivation of *ipaD*). This transcriptional analysis and previous Western blot analysis, which indicated that similar amounts of IpaB and IpaC were present in wild-type, *ipaD* and *mxiD* strains (Allaoui *et al.*, 1993b; Ménard *et al.*, 1993), suggest that expression of genes of the entry region is not controlled by the secretion machinery. In contrast, transcription of the *virA* and *ipaH* genes was low in derivatives of the wild-type strain and was increased during growth in the presence of Congo red and in derivatives of the *ipaD* mutant. These results, together with the low production of VirA in the *ipaD mxiD* mutant and the low transcription of *virA* in the *mxiD* mutant growing in the presence of Congo red, indicate that the secretion machinery is involved in the control mechanism of the *virA* promoter and suggest that transcription of *virA* and of four copies of the *ipaH* family is enhanced in response to an active secretion through the type III apparatus. Activation of the transcription of the *virA–lacZ* fusion in response to addition of Congo red to the growth medium occurred within 10 min, which was consistent with the kinetics of secretion of IpaB and IpaC in response to Congo red (Bahrani *et al.*, 1997).

Similar amounts of β-galactosidase were present in bacteria carrying the *ipgD–lacZ* fusion prior to and after 60 min of infection. In contrast, the amount of β-galactosidase present in bacteria carrying *virA* and *ipaH–lacZ* fusions was ~10 times higher after 60 min of infection than prior to infection. Due to the period of incubation in the presence of gentamicin which is required to eliminate extracellular bacteria, we could not investigate whether *virA* and *ipaH* transcription was activated upon contact with or shortly after entry into epithelial cells. Only low amounts of β-galactosidase were present in bacteria carrying *virA–* and *ipaH–lacZ* fusions after 150 min of infection, which suggests that the *virA* and *ipaH* genes had not been transcribed between 60 and 150 min of infection. Since there is a correlation between *virA* and *ipaH* transcription and active secretion, these results suggest that secretion might not be active when bacteria are multiplying in the cytoplasm of HeLa cells. Alternatively, signals other than secretion might affect transcription of the *virA* and *ipaH* genes negatively in the intracellular compartment.

The mechanism involved in the transcriptional control of the *virA* and *ipaH* genes in response to active secretion is not known yet. The low transcription of *virA* by the *virA* mutant indicates that virA is not autoregulated and the low production of VirA by the *ipaD mxiD* mutant suggests that IpaD is not the effector of the regulation of the *virA* promoter. When the secretion apparatus is inactive, a negative regulator might accumulate in the cytoplasm and repress *virA* and *ipaH* transcription. Secretion of this regulator, due to the lack of IpaD or in response to external inducers, would decrease its cytoplasmic concentration, thereby leading to the transcriptional activation of its target promoters. Secretion of a negative regulator as a mechanism for the control of gene expression has been documented in *Salmonella* and *Yersinia*. In *S.typhimurium*, transcription of the flagellin gene by an RNA polymerase containing the alternate sigma factor σ^{28} requires the integrity of the basal-hook body complex which constitutes an export apparatus related to type III secretion machineries. Secretion of the anti-sigma factor FlgM allows transcription of the flagellin gene by a σ^{28}-containing RNA polymerase, thus coupling flagellin expression to flagellar assembly (Hughes *et al.*, 1993; Kutsukake *et al.*, 1994). In *Yersinia*, expression of the *yop* genes is down-regulated when Yop secretion is compromised (Cornelis *et al.*, 1987), and secretion of LcrQ via the type III secretion apparatus has been proposed to lead to the transcriptional activation of *yop* promoters by a mechanism which has not yet been characterized (Pettersson *et al.*, 1996).

The phenotype of a *virA* mutant in the Sereny test indicated that VirA is involved in virulence but its specific role could not be identified using *in vitro* tests. VirA does not appear to be required for entry, which is consistent with the fact that *virA* is located outside the entry region and that it is weakly expressed prior to infection of target cells. Uchiya *et al.* (1995) reported that inactivation of *virA* in *S.flexneri* 2a resulted in a 5-fold decrease in the ability to enter epithelial cells. This discrepancy might result from differences in the cell lines used for the assay or in the *Shigella* strains studied. In both cases, *virA* mutants produced a decreased amount of IcsA (VirG), which might be responsible for the reduced abilility of the mutants to spread from cell to cell. However, the reduced amount of IcsA does not appear to be responsible for the phenotype of the *virA* mutant in the Sereny test since the virulence of the mutant was restored by a recombinant plasmid.

Clues to the role of VirA and IpaH proteins might be found in the timing of their expression, in the fact that they are secreted by a type III secretion machinery and in sequence similarities between these and other proteins. YopM is a 41 kDa protein of *Yersinia* that is secreted by a type III secretion machinery (Leung and Straley, 1989), translocated into target cells (Boland *et al.*, 1996) and whose expression is regulated by the secretion machinery (Pettersson *et al.*, 1996). In addition to the repeated motifs that constitute the greater part of YopM (Leung and Straley, 1989) and the N-terminal half of IpaH proteins (Venkatesan *et al.*, 1991), the N-terminal regions of YopM and IpaH9.8 share extensive sequence similarities

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Signal for secretion
YopM    : MFINPRNVSNTFLQEPLRHSSNLTEMPVEAENVKSKTE (38)
IpaH9.8:              MLPINNNFSLPQNSFYNTISGTYAD (25)

Signal for translocation
YopM    : YYNAWSEWERNAPPGNGEQREMAVSRLRDCLDRQAHELELNNLG (82)
IpaH9.8: YFSAWDKWEKQALP--GEERDEAVSRLKECLINNSDELRLDRLN (67)

First repeated motifs
YopM    : LSSLPELPPHLESLVASCNS- LTELPELPQSLKSLLVDNNN (122)
IpaH9.8: LSSLPDNLPAQITLLNVSYKQ LTNLPELPVTLKKLYSASNK (108)
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Fig. 6. Comparison of the N-terminal sequences of IpaH9.8 and YopM. The regions of YopM that have been proposed as signals for secretion and translocation (Boland *et al.*, 1996) are indicated above the alignment of the N-terminal sequences of YopM (Leung and Straley, 1989) and IpaH9.8 (this work). Conserved amino acid residues are indicated in bold. Only the first two repeated motifs of YopM and IpaH9.8, which contain 13 and five repeated motifs, respectively, are shown.

(Figure 6). The N-terminal region of YopM has been proposed to carry the signals which are responsible for secretion and translocation of the protein (Boland *et al.*, 1996). These sequence similarities suggest that IpaH9.8 might be translocated by *Shigella* into epithelial cells, as described for YopM by *Yersinia*. Differences in the transcriptional regulation of genes encoding proteins secreted by the type III secretion machinery of *Shigella* are likely to reflect differences in the functional role of these secreted proteins during infection.

Materials and methods

Bacterial strains and growth media

All *S.flexneri* strains used in this study (Table I) are derivatives of the wild-type strain M90T (Sansonetti *et al.*, 1982). Bacteria were grown in Luria–Bertani (LB) medium or tryptic soy (TCS) broth. Antibiotics were used at the following concentrations: ampicillin 100 μg/ml; kanamycin 30 μg/ml; and streptomycin 100 μg/ml. Congo red (Serva, Heidelberg, Germany) was used to induce secretion by bacteria growing in LB medium.

Construction of plasmids and strains

DNA analysis, polymerase chain reaction (PCR), plasmid construction and transformation of *Escherichia coli* and *S.flexneri* strains were performed according to standard methods. Nucleotide sequences were determined by the dideoxy chain termination procedure on alkaline-denaturated plasmid DNA.

Overlapping cosmids representing the entire virulence plasmid previously were constructed by inserting 40 kb fragments of pWR100 into the vector pJB8 (Maurelli *et al.*, 1985).

Plasmid pLAC4 (Figure 2) was constructed by cloning a 1.5 kb *Xba*I–*Eco*RI fragment, containing the 5′ part of *icsA*, the *icsA–virA* intergenic region and the 5′ part of *virA*, into the *Sma*I site located upstream from the *lacZ* reporter gene in the suicide plasmid pLAC1 that confers resistance to ampicillin (Allaoui *et al.*, 1992). pLAC4 was then transferred by conjugation into M90T-Sm and SF622 (*ipaD2*). Since pLAC4 does not replicate in *S.flexneri*, the Ap^r clones arose through homologous recombination between the identical sequences carried by the virulence plasmid pWR100 and the recombinant plasmid pLAC4. The recombinant strains, in which the *lacZ* reporter gene was placed under the control of the *virA* promoter, contained a wild-type copy of the *virA* gene located downstream from the integrated plasmid. Plasmid pLAC5 (Figure 2) was constructed by deleting a *Nde*I–*Eco*RI fragment from pLAC4 and thus contains a 380 bp fragment, internal to the *virA* gene. Integration of pLAC5 into the *virA* locus of pWR100 also placed the *lacZ* gene under the control of the *virA* promoter but, unlike that of pLAC4, led to the disruption of the *virA* gene. Southern analysis confirmed the structure of the pWR100 derivatives carrying the *virA–lacZ* transcriptional fusion; the strains were designated SF1001 (*virA–lacZ* $virA^+$ $ipaD^+$), SF1002 (*virA–lacZ* $virA^+ipaD^-$), SF1003 (*virA–lacZ* $virA^-$ $ipaD^+$) and SF1004 (*virA–lacZ* $virA^-$ $ipaD^-$). SF1003 constituted the *virA* mutant strain used for phenotypic evaluation. The plasmid pKvirA (Figure 2) was obtained by cloning a *Bsp*EI–*Bbv*I fragment that contains the entire *virA* gene into pK19 (Pridmore, 1987).

The constant region of *ipaH9.8* was amplified using the PCR technique, and the PCR product was cloned between the *Kpn*I and *Xba*I sites that are located upstream from the *lacZ* gene in the suicide vector pLAC2 (Allaoui *et al.*, 1993a) to construct pLAC6 (Figure 2). pLAC6 was then transferred by conjugation into M90T-Sm (wild-type) and SF622 (*ipaD*). Since pLAC6 carried the constant region of *ipaH*, integration of the suicide plasmid could occur into any of the five *ipaH* genes carried on the virulence plasmid. In each case, the *lacZ* reporter gene is placed under the control of the promoter of the *ipaH* gene into which the plasmid is integrated. Transconjugants were screened by Southern blot analysis of their virulence plasmid digested by *Hin*dIII using a probe from the *ipaH* constant region. The strains were designated SF1005 (*ipaH9.8–lacZ* $ipaD^+$), SF1006 (*ipaH9.8–lacZ* $ipaD^-$), SF1007 (*ipaH7.8–lacZ* $ipaD^+$), SF1008 (*ipaH4.5–lacZ* $ipaD^+$), SF1009 (*ipaH4.5–lacZ* $ipaD^-$), SF1010 (*ipaH1.4–lacZ* $ipaD^+$) and SF1011 (*ipaH1.4–lacZ* $ipaD^-$). The *ipaH2.5–lacZ* fusion in the wild-type background as well as the *ipaH7.8–lacZ* and *ipaH2.5–lacZ* fusions in the *ipaD* background were not obtained.

Protein analysis

Aggregated proteins were collected from the culture medium of SF635 (Δ*ipa*) and solubilized in 0.1% SDS. Mice were injected twice with this preparation, with a 1 week interval. Sera were collected on the fourth week, pooled and absorbed on sonicated extracts of BS176.

Bacteria in the exponential phase of growth were harvested by centrifugation at 14 000 *g* for 10 min. Crude extracts were obtained from the bacterial pellet, and proteins present in the culture supernatant were precipitated by the addition of 1/10 (v/v) trichloracetic acid (TCA). Electrophoresis in 10% SDS–PAGE was performed as described (Laemmli, 1970). After electrophoresis, proteins were either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane. Immunoblotting procedures were carried out with mouse polyclonal anti-filaments antibodies, mouse monoclonal anti-IpaB antibodies (Bârzu *et al.*, 1993) and rabbit polyclonal anti-IcsA antibodies (Goldberg *et al.*, 1993). Horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit antibodies were used as secondary antibodies and visualized by enhanced chemiluminescence. The N-terminal sequence of VirA and IpaH9.8 and that of internal peptides, which were obtained by endolysin digestion and purified by chromatography, were determined by the Edman degradation procedure. Labeling of bacteria grown *in vitro* to early–mid exponential phase was performed as previously described (Goldberg *et al.*, 1993), using a rabbit polyclonal antiserum raised against IcsA.

β-Galactosidase assays

The β-galactosidase activity present in bacteria growing in laboratory media was assayed by using the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described (Platt *et al.*, 1972). The β-galactosidase activity present in intracellular bacteria was assayed by using the substrate MUG as described (Klarsfeld *et al.*, 1994). Fluorescence was measured by using a Dynatec apparatus, with 365 nm excitation and 450 nm emission wavelengths. Activities were computed as fluorescence units per h per bacterium; four fluorescence units were equivalent to one Miller unit and all results are presented in Miller units.

Cell culture and virulence assays

Culture and infection of HeLa and Caco-2 cells were performed as previously described (Sansonetti *et al.*, 1986; Mounier *et al.*, 1992). Zones of F-actin accumulation within HeLa cells were visualized by fluorescence microscopy after staining cells with 7-nitrobenz-2-oxy-1,3-

diazole (NBD)–phalloidin (Molecular Probes, Junction City, OR) for 20 min. For transmission electron microscopy, infected cells were treated as described (Tilney and Portnoy, 1989) and observed using a Phillips CM12 electron microscope. Intercellular dissemination capacity was evaluated using the plaque assay on Caco-2 cells (Oaks *et al.* 1985). Contact hemolytic activity was detected as previously described (Sansonetti *et al.*, 1986). *In vivo* evaluation of the virulence was done using the Sereny test (Sereny, 1957) and rabbit ileal loop infection (Sansonetti *et al.*, 1995). Animal experiments were performed according to the recommendations of the Service Vétérinaire de la Santé et de la Protection Animales of the Ministère de l'Agriculture et de la Forêt (authorization 01754).

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