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/secreción

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 Gijssegem 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 Galán, 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 ics, 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 ΔipaBCDA 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 HindIII 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 ΔipaBCDA 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 (ΔipaBCDA), 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 ΔipaBCDA 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 ΔipaBCDA 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 ΔipaBCDA 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 ΔipaBCDA 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-T-S-N-I-T-N-H-E and those of two internal peptides as N-terminal sequence of the protein was identified as M-Q-

Fig. 1. Secretion of proteins by various Shigella strains. Cultures of M90T (wild-type), BS176 (the virulence plasmid-cured strain) and the ipaD (SF622), ΔipaBCDA (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 ΔipaBCDA 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.

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 immediately 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.

**Characterization of the gene encoding a 60 kDa secreted protein**

The 60 kDa proteins which were secreted in high amount by the ΔipaBCDA strain were transferred onto a PVDF
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 Mₚ 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M90T</td>
<td>Wild type</td>
<td>Sansonetti et al. (1985)</td>
</tr>
<tr>
<td>M90T-Sm</td>
<td>spontaneous Sm8 derivative of M90T</td>
<td>Alloua et al. (1992)</td>
</tr>
<tr>
<td>BS176</td>
<td>plasmidless derivative of M90T</td>
<td>Sansonetti et al. (1985)</td>
</tr>
<tr>
<td>SF132</td>
<td>icsB–lacZ</td>
<td>Alloua et al. (1992)</td>
</tr>
<tr>
<td>SF134</td>
<td>ipgD–lacZ</td>
<td>Alloua et al. (1993a)</td>
</tr>
<tr>
<td>SF401</td>
<td>mxiD</td>
<td>Alloua et al. (1993b)</td>
</tr>
<tr>
<td>SF403</td>
<td>mxiD–lacZ</td>
<td>Alloua et al. (1993b)</td>
</tr>
<tr>
<td>SF626</td>
<td>ipaD</td>
<td>Ménard et al. (1993)</td>
</tr>
<tr>
<td>SF623</td>
<td>ipaD–lacZ in M90T-Sm</td>
<td>Ménard et al. (1993)</td>
</tr>
<tr>
<td>SF624</td>
<td>ipaD–lacZ in SF622 (ipaD)</td>
<td>Ménard et al. (1994)</td>
</tr>
<tr>
<td>SF634</td>
<td>ipaD mxiD</td>
<td>Ménard et al. (1994)</td>
</tr>
<tr>
<td>SF635</td>
<td>βipaBCDA</td>
<td>Parsot et al. (1995)</td>
</tr>
<tr>
<td>SF803</td>
<td>icsB–lacZ in SF622 (ipaD)</td>
<td>this work</td>
</tr>
<tr>
<td>SF806</td>
<td>ipaD–lacZ in SF622 (ipaD)</td>
<td>this work</td>
</tr>
<tr>
<td>SF808</td>
<td>mxiD–lacZ in SF622 (ipaD)</td>
<td>this work</td>
</tr>
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<td>SF1001</td>
<td>virA–lacZ in M90T-Sm (virA+)</td>
<td>this work</td>
</tr>
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<td>SF1002</td>
<td>virA–lacZ in SF622 (virA+)</td>
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<td>SF1003</td>
<td>virA–lacZ in M90T-Sm (virA+)</td>
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<td>ipaH4.3–lacZ in M90T-Sm</td>
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<td>SF1012</td>
<td>virA–lacZ in SF401 (mxiD)</td>
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Table II. Expression of lacZ transcriptional fusions by bacteria growing in vitro

<table>
<thead>
<tr>
<th>Fusion</th>
<th>β-Galactosidase activity (Miller units)a</th>
<th>ipaD+</th>
<th>ipaD−</th>
<th>Ratio b icpA+</th>
<th>Ratio icpA−</th>
</tr>
</thead>
<tbody>
<tr>
<td>virA–lacZ</td>
<td>16</td>
<td>280</td>
<td>17</td>
<td>280</td>
<td>17</td>
</tr>
<tr>
<td>virA–lacZ mxiD</td>
<td>17</td>
<td>NA</td>
<td>NA</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>ipaH9.8–lacZ</td>
<td>28</td>
<td>580</td>
<td>21</td>
<td>325</td>
<td>12</td>
</tr>
<tr>
<td>ipaH7.8–lacZ</td>
<td>20</td>
<td>NA</td>
<td>NA</td>
<td>235</td>
<td>12</td>
</tr>
<tr>
<td>ipaH4.5–lacZ</td>
<td>31</td>
<td>360</td>
<td>12</td>
<td>110</td>
<td>3.5</td>
</tr>
<tr>
<td>ipaH1.4–lacZ</td>
<td>53</td>
<td>280</td>
<td>5.3</td>
<td>270</td>
<td>5.1</td>
</tr>
<tr>
<td>ipaA–lacZ</td>
<td>485</td>
<td>510</td>
<td>1.1</td>
<td>390</td>
<td>0.8</td>
</tr>
<tr>
<td>icsB–lacZ</td>
<td>290</td>
<td>305</td>
<td>1.1</td>
<td>285</td>
<td>1.0</td>
</tr>
<tr>
<td>mxiD–lacZ</td>
<td>275</td>
<td>260</td>
<td>0.9</td>
<td>320</td>
<td>1.2</td>
</tr>
<tr>
<td>ipgD–lacZ</td>
<td>450</td>
<td>400</td>
<td>0.9</td>
<td>475</td>
<td>1.1</td>
</tr>
</tbody>
</table>

aActivities are the means of at least three independent experiments. Standard deviations are within 25% of the reported values.
bActivity present in ipaD+ strains versus activity present in ipaD− strains.
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 icsBC, ipaA–, ipaD–, 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
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 wild-type 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 (open symbols) and mxiD (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.

![Graph showing β-galactosidase activity over time](image)

**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 strain, whereas no transcriptional activity was observed between addition of Congo red and activation of 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.

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

<table>
<thead>
<tr>
<th>Fusion</th>
<th>β-Galactosidase activity (Miller units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vitro 60 min of infection</td>
</tr>
<tr>
<td>ipgD–lacZ</td>
<td>450</td>
</tr>
<tr>
<td>virA–lacZ</td>
<td>16</td>
</tr>
<tr>
<td>ipaH9.8–lacZ</td>
<td>28</td>
</tr>
<tr>
<td>ipaH7.8–lacZ</td>
<td>20</td>
</tr>
<tr>
<td>ipaH4.5–lacZ</td>
<td>31</td>
</tr>
<tr>
<td>ipaH11.4–lacZ</td>
<td>53</td>
</tr>
</tbody>
</table>

aActivities are the means of at least three independent experiments. Standard deviations are within 25% of the reported values.
bActivity present after 60 min of infection versus activity present in bacteria grown in vitro.
cActivity 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 (Miller units) present after 60 min of infection versus activity present in bacteria grown 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 ipgD–lacZ fusion, chosen as a representative of genes which were expressed constitutively in vitro, the β-galactosidase activity present within intracellular bacteria increased with the time of incubation, which was consistent with the increased transcription of these genes (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...
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 *ipgD–lacZ* fusions had not been transcribed between 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. Inoculated 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; Bahrami 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 (Bahrami 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 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 σ28 requires the integrity of the basal-hook body complex which constitutes an export apparatus related to type III secretion machinery. Secretion of the anti-sigma factor FlgM allows transcription of the flagellin gene by a σ28-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 ability 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.
Signal for secretion

YopM: KF1NP01VSMTFQPLQKPLHSNSLTEMPVEAENVKSXET (38)
IpaH9.8: MFLJNNFSLPQFNSFYINTGTAD (25)

Signal for translocation

YopM: YNYAWSEWERNAPPNGEQEREMAVSVLRDLCZDROAHDELLENLNLG (82)
IpaH9.8: YFSAOQNKMDQALP-GEERDEAUVSLKELINNSDELRDLRLNL (67)

First repeated motifs

YopM: LSLPILPHELKLSLSSACNS- LTLHELPEFLSDDLVDDNH (122)
IpaH9.8: LSLPILDPILPAQ2TLLNKSYYK LTLHELPEFVTLLKLYSAANH (108)

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.

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(Exercise 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 Shigella flexneri strains were performed according to standard methods. Nucleotide sequences were determined by the dideoxy chain termination procedure on alkaline-denatured plasmid DNA.

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

Plasmid pLAC4 (Figure 2) was constructed by cloning a 1.5 kb XbaI–EcoRI fragment, containing the 5' part of icsA, the icsA–virA intergenic region and the 5' part of virA, into the Smal 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 (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 HindIII 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 (Δapa) 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) trichloroacetic acid (TCA). Electrophoresis in 10% SDS–PAGE was performed as described (Laemmli, 1970). After electrophoresis, proteins were either stained with Coomasie Brilliant Blue or transferred to a nitrocellulose membrane. Immunoblotting procedures were carried out with mouse polyclonal antibodies, mouse monoclonal anti-IpaB antibodies (Bärzu et al., 1993) and rabbit polyclonal anti-lcsA 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 against 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-oxa-1,3-
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