INTRODUCTION

Invasion into host epithelial cells in the intestine is a common step in the pathogenesis of the genera Shigella and Salmonella. To date, it has been established that Shigella and Salmonella utilize the invasion effectors, IpaBCD and SipBCD, respectively, which are secreted by the type III secretion machinery possessed by these bacteria. The regulatory circuits controlling expression of these effector proteins and the secretion machinery have been intensively analysed (for reviews, see Darwin & Miller, 1999; Dorman & Porter, 1998; Galan, 1996; Lucas & Lee, 2000). One of the key steps in these regulatory circuits is regulation of the synthesis of the first global activators, VirF in Shigella and HilA in Salmonella. We have reported that the expression level of Shigella sonnei virF is controlled in response to pH. This regulation is accomplished by a two-component regulatory system, cpxR–cpxA (Nakayama & Watanabe, 1995, 1998); the sensor kinase CpxA phosphorylates its cognate transcriptional regulator CpxR upon sensing the appropriate environmental signal. For the regulation of Salmonella hilA, many regulatory loci have been recently reported. The sirA gene product, which belongs to the response regulator family, activates hilA expression (Johnston et al., 1996). Later, BarA was reported as a sensor kinase for SirA (Altier et al., 2000a). Fis, a DNA nucleoid-associated protein, is also one of the activating factors for hilA (Wilson et al., 2001). Lucas et al. (2000) described fadD, which is involved in uptake and degradation of fatty acids, and a flagellar gene fliZ as positive regulatory loci, and the phoB–phoR two-component regulatory system as a negative regulatory locus for hilA. Attenuation of the virulence of the fliZ mutant in mice was confirmed by Iyoda et al. (2001). Two AraC/XylS-type transcriptional factors, SirA and HilD, seem to have more direct roles in the activation

Activation of hilA expression at low pH requires the signal sensor CpxA, but not the cognate response regulator CpxR, in Salmonella enterica serovar Typhimurium

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A two-component regulatory system, cpxR–cpxA, plays an important role in the pH-dependent regulation of virF, a global activator for virulence determinants including invasion genes, in Shigella sonnei. The authors examined whether the cpxR–cpxA homologues have some function in the expression of Salmonella enterica serovar Typhimurium invasion genes via the regulation of hilA, an activator for these genes. In a Salmonella cpxA mutant, the hilA expression level was reduced to less than 10% of that in the parent strain at pH 6.0. This mutant strain also showed undetectable synthesis of an invasion gene product, SipC, at pH 6.0 and reduced cell invasion capacity – as low as 20% of that of the parent. In this mutant, the reduction in hilA expression was much less marked at pH 8.0 than at pH 6.0 – no less than 50% of that in the parent, and no significant reduction was observed in either SipC synthesis or cell invasion rate, compared to the parent. Unexpectedly, a Salmonella cpxA mutant strain and the parent showed no apparent difference in all three characteristics described above at either pH. These results indicate that in Salmonella, the sensor kinase CpxA activates hilA, and consequently, invasion genes and cell invasion capacity at pH 6.0. At pH 8.0, however, CpxA does not seem to have a large role in activation of these factors. Further, the results show that this CpxA-mediated activation does not require its putative cognate response regulator, CpxR. This suggests that CpxA may interact with regulator(s) other than CpxR to achieve activation at low pH.
(or derepression) of hilA (Lucas & Lee, 2001; Schechter et al., 1999). The effect of csrA–csrB, genes for a protein–RNA complex, on hilA is largely, if not totally, mediated through the expression of hilC and hilD (Altier et al., 2000a, b). Furthermore, the phoP–phoQ two-component system (Pegues et al., 1995) and several other genes (Fahlen et al., 2000) were reported as regulatory factors with negative effects on hilA expression.

With this background, we assumed that some common regulatory locus or loci controlling Shigella virF and Salmonella hilA, and invasion genes of the two genera, might exist. Based on the effect of cpxR–cpxA on virF in Shigella, we investigated the effect of Salmonella cpxR–cpxA homologues on hilA expression.

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. SL1344 is a standard virulent strain of Salmonella enterica serovar Typhimurium. KK1501, an LT2 derivative defective in the three known restriction systems, was used as the host for gene disruption and transformation with DNA prepared in Escherichia coli. E. coli cpxR mutant SN1216 harbou sing pHW848, a lacZ fusion reporter plasmid for the Shigella virF gene, was used as the host in cloning of Salmonella cpxR by intergeneric complementation.

The hilA–lacZ translational fusion reporter plasmid pSN849 was constructed as follows. The DNA fragment extending from –483 to +560, coordinated to the transcriptional start site of hilA as +1, as determined by Schechter et al. (1999), was amplified by PCR. The PCR product was blunt-ended with Klenow fragment of DNA polymerase I, and introduced into the Smal site of pFR109, making the precise fusion of hilA to lacZ. The resultant plasmid was digested with SalI to excise the hilA–lacZ fusion gene, which was inserted into the SalI site of pHSG595. A plasmid clone in which the direction of the inserted fusion gene was opposite to that of the transcript from the vector was selected and named pSN849.

pSN902 was selected from a library of SalI-digested SL1344 DNA in pBR322, as a clone which complemented the phenotype of SN1216/pHW848. From pSN902, a 2·0 kb EcoRI–EcoRV fragment covering the Salmonella cpxA region was recloned into the EcoRI–EcoRV sites of pBR322. The resultant plasmid was named pSN455.

Salmonella cpxR mutant SN1457 and cpxA mutant SN1459 were constructed as follows. pSN902 was digested with SalI (for SN1457) or AcclIII (for SN1459), whose recognition sites were unique in this plasmid. The Tn903-derived Km-resistance gene cassette [1430 bp HaeIII fragment containing the aph(T) gene; Oka et al., 1981] was introduced into the SacI (for SN1457), or AcclIII (for SN1459) site, which resulted in disruption of cpxR or cpxA, respectively. The 7·4 kb SalI fragments containing cpxR::aph(T) or cpxA::aph(T) were excised. The purified fragments were recloned into the SalI site of the suicide vector pKH5002, which can replicate only in MS8, an RNase H deficient E. coli strain. The plasmid prepared from MS8 was introduced into KK1501, a restriction-deficient Salmonella strain. Amp' Km' transformants were selected as single-crossover strains and the absence of an episomal copy of the introduced plasmid was confirmed. The transformants were sensitive to Sm because a wild-type rpsL allele, which is dominant to the resistant type, is contained in pKH5002. After culture in drug-free medium, Sm' Km' variants were selected and checked for their loss of the Amp' phenotype, which showed segregation of pKH5002-derived sequences and recombination of cpxR::aph(T) or cpxA::aph(T) into the corresponding chromosomal gene. Each mutated locus on the chromosome was

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salmonella typhimurium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL1344</td>
<td>A standard virulent strain, hisG rpsL</td>
<td>SGSC*</td>
</tr>
<tr>
<td>KK1501</td>
<td>LT2, hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 iIV452 rpsL120 xyl–E. coli Klenow fragment</td>
<td>Kutsukake et al. (1994)</td>
</tr>
<tr>
<td>SN1457</td>
<td>SL1344, cpxR::aph(T), Km'</td>
<td>This study</td>
</tr>
<tr>
<td>SN1459</td>
<td>SL1344, cpxA::aph(T), Km'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli K-12</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS8</td>
<td>MCI061, mha::cat, Cm'</td>
<td>Ohmori et al. (1995)</td>
</tr>
<tr>
<td>SN1216</td>
<td>MCI061, cpxR::aph(T), Km'</td>
<td>Nakayama &amp; Watanabe (1998)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFR109</td>
<td>pMB1 replicon lacZ translational fusion vector</td>
<td>Shapira et al. (1983)</td>
</tr>
<tr>
<td>pHSG595</td>
<td>pSC101 replicon cloning vector</td>
<td>Takeshita et al. (1987)</td>
</tr>
<tr>
<td>pBR322</td>
<td>pMB1 replicon cloning vector</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pSN849</td>
<td>hilA–lacZ fusion gene cloned into pHSG595</td>
<td>This study</td>
</tr>
<tr>
<td>pSN902</td>
<td>6 kb SalI fragment containing S. typhimurium cpxP, cpxR, cpxA genes inserted into the SalI site of pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pSN455</td>
<td>2 kb EcoRI–EcoRV fragment containing S. typhimurium cpxA gene inserted into the EcoRI–EcoRV sites of pBR322</td>
<td>This study</td>
</tr>
<tr>
<td>pKH5002</td>
<td>Cloning vector for gene disruption</td>
<td>Ohmori et al. (1995)</td>
</tr>
</tbody>
</table>

*Salmonella Genetic Stock Center.*
transferred from the double-crossover strain into the virulent strain SL1344 by following the standard procedure of P22-mediated generalized transduction (Schmieder, 1972) and Km\(^r\) transductants were selected. The chromosomal construct in each disruptant strain was confirmed by Southern hybridization analysis (data not shown). The cpxR mutation site in SN1457 was rather close to the 5' terminus of the gene and this mutant did not show any apparent phenotypic difference from the parent (see Results). Hence, we further confirmed whether SN1457 still produced functional CpxR by Western analysis with polyclonal antiserum raised against CpxR. In this analysis, we could not detect the CpxR band in the lysate of SN1457, whereas in the lysates of SL1344 and SN1459, we could. This is a strong evidence that cpxR gene is truly disrupted in SN1457 (data not shown).

**Human cell line.** The human embryonic intestinal 407 epithelial cell line INT40 was obtained from the American Type Culture Collection.

**Media.** LB medium containing 100 mM NaCl and buffered with 0-1 M sodium phosphate (pH 6-0 or 8-0) was used for bacterial growth. In some experiments, such as DNA preparation, non-buffered LB was used. Antibiotics were added to the media when necessary at the following concentrations in μg ml\(^{-1}\): Amp, 100; Km, 10; Km, 40; Sm, 150.

**Preparation and manipulation of DNA.** This was done essentially as described by Maniatis et al. (1982).

**β-Galactosidase assay.** β-Galactosidase activity was determined essentially as described by Miller (1972). Bacterial cultures were grown at 37 °C in 1-5 ml LB medium containing 100 mM NaCl and 0-1 M sodium phosphate buffer (pH 6-0 or 8-0) for 3 h with slow shaking, to an OD\(_{600}\) of approximately 0-7. The cells were harvested and suspended in 1-5 ml Z buffer (0-1 M sodium phosphate, pH 7-0, 10 mM KC1, 1 mM MgSO\(_4\), 50 mM 2-mercaptoethanol). After appropriate dilution with Z buffer, the samples were used for assay. All assays were performed at least five times. Activities are expressed as the mean Miller units ± standard deviation.

**Analysis of the synthesis of the SipC protein.** Bacterial cultures were grown at 37 °C in 3 ml LB medium containing 100 mM NaCl and 0-1 M sodium phosphate buffer (pH 6-0 or 8-0) for 3-5-4-5 h with slow shaking, to an OD\(_{600}\) of approximately 1-3. The bacterial cells were harvested, rinsed with phosphate-buffered saline (PBS), suspended in 50 mM Tris/HCl (pH 8-0), 2% SDS solution, and lysed by boiling for 5 min. The protein concentration of the supernatant was determined with a BCA protein assay kit (Pierce). Equal amounts of the proteins were analysed by SDS-PAGE (8-16% gradient gel) and the separated proteins were electrophoretically transferred to an Immobilon-P\(^{TM}\) membrane (Millipore). For blocking, the blot was incubated with 1% BSA in TST solution (100 mM Tris/HCl pH 8-0, 150 mM NaCl, 0-05% Tween 80) at room temperature for 30 min. To detect SipC protein, the blot was incubated with rabbit anti-SipC antiserum at a dilution of 1:1000 in TST solution at room temperature for 30 min. The rabbit anti-SipC antiserum was described previously (Iyoda et al., 2001), and was a kind gift from K. Hirose. After incubation with the primary antibody, the blot was incubated with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Promega) at a dilution of 1:7500 in TST solution at room temperature for 30 min. The blot was then developed with nitro blue tetrazolium (NBT; Promega) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Promega) in alkaline phosphatase buffer (100 mM Tris/HCl pH 9-5, 100 mM NaCl, 5 mM MgCl\(_2\)). For quantitative analysis, the blot was scanned by a densitometer to quantify the intensity of the band using the Quantity One software (pdi). The results are expressed as the mean value of three independent experiments ± standard deviation; the intensity of the band in SL1344 grown at pH 6-0 was set to 100.

**Cell invasion assay.** The invasion assay was performed essentially as described previously (Huang et al., 1998). INT407 cells were cultured in minimal essential medium (Eagle MEM; Gibco) with heat-inactivated 10% fetal calf serum (Gibco) and 2 mM l-glutamine under 5% CO\(_2\) in 75 cm\(^2\) tissue culture flasks at 37 °C. Monolayers were trypsinized and washed, and one third of the monolayer was inoculated into fresh cuture medium. Confluently grown cells were used in invasion assays. Bacterial cultures grown at 37 °C with slow shaking in 5 ml LB medium containing 100 mM NaCl and 0-1 M sodium phosphate buffer (pH 6-0 or 8-0) to an OD\(_{600}\) of approximately 0-7 were harvested by centrifugation and suspended in PBS. The 25 μl suspension containing ~5 × 10\(^{6}\) c.f.u. was added to the confluent INT407 cell monolayers in 24-well tissue culture plates (Sarstedt). Each well, as counted by haemocytometer, contained ~5 × 10\(^5\) INT407 cells in 1 ml medium; i.e. an m.o.i. of about 10. The bacterial inoculum was not centrifuged to initiate contact with the cells. The infected monolayers were incubated in 5% CO\(_2\) at 37 °C for 1 h to allow bacterial invasion, and washed three times with 1 ml minimal essential medium balanced salt solution (EBSS; Gibco). The infected cells were then incubated for an additional 2 h in 1 ml Eagle MEM containing gentamicin (100 μg ml\(^{-1}\)) to kill the remaining extracellular bacteria. The infected monolayers were washed as described above and lysed with 1 ml PBS containing 0-1% Triton X-100 by shaking for 15 min. The released bacteria were diluted appropriately with PBS and plated on agar plates. Invasion efficiency was calculated as (number of internalized bacteria at the end of the assay/starting inoculum) × 100. All assays were conducted in triplicate and repeated independently three times. Results are expressed as the mean ± standard deviation.

**Enzymes.** Restriction and modifying enzymes were obtained from Takara Shuzo, or from TOYOBO, and used as recommended by the manufacturers.

## RESULTS

**Cloning of cpxR–cpxA homologues from Salmonella enterica serovar Typhimurium**

As we previously reported, an E. coli cpxR mutant cannot express the cloned Shigella virF\(^{-}\) gene (Nakayama & Watanabe, 1998). In fact, the E. coli cpxR mutant SN1216 harbouring the virF\(^{-}\)-lacZ plasmid pHW848 does not show the blue colour phenotype on X-Gal plates. We expected that the Salmonella cpxR homologue could be cloned from the total DNA library of Salmonella by the complementation method using SN1216/pHW848.

First, we confirmed that a cpxR homologue of Salmonella enterica serovar Typhimurium strain SL1344 was present on a 6 kb SalI fragment of the chromosomal DNA by Southern analysis using the cpxR gene of E. coli as the probe (data not shown). The total DNA from SL1344 was digested completely with SalI and introduced into the SalI site of the vector pBR322. The bulk DNA library was introduced into SN1216/pHW848, and blue colour transformants were selected on X-Gal plates. All three colonies analysed contained a plasmid having the expected 6 kb insert in the pBR322 SalI site. This plasmid was named pSN902. Nucleotide sequence analysis of the inserted fragment confirmed the presence of cpxP–cpxR–cpxA homologues. The nucleotide sequence of the 3358 bp region that covers cpxP, cpxR and cpxA corresponded to nucleotide
The thick arrows at the bottom indicate the inserted fragments containing the *Salmonella* cpxP–cpxR–cpxA region. The disruption sites with the Km-resistance cassette in SN1457 and SN1459 are indicated by triangles. The open boxes indicate the reading frame of each gene and the cpxP–cpxR–cpxA operon. The disruption sites with the Km-resistance cassette in SN1457 and SN1459 are indicated by triangles. The thick arrows at the bottom indicate the inserted fragments in pSN902 and pSN455. Drug-resistance cassettes are not drawn to scale. Abbreviations: Ac, AccI; E, EcoRI; Ev, EcoRV; S, SalI; Sa, SacI.

**Fig. 1.** Schematic diagram of the organization of the cpxP–cpxR–cpxA region in *Salmonella enterica* serovar Typhimurium SL1344 showing the mutations in SN1457 (cpxR) and SN1459 (cpxA), and plasmids pSN902 and pSN455. The horizontal line in the upper part of the figure represents a 6 kb SalI fragment containing the *Salmonella* cpxP–cpxR–cpxA region. Open boxes indicate the reading frame of each gene. The arrows at the top show the directions of the cpxP gene and the cpxR–cpxA operon. The disruption sites with the Km-resistance cassette in SN1457 and SN1459 are indicated by triangles. The thick arrows at the bottom indicate the inserted fragments in pSN902 and pSN455. Drug-resistance cassettes are not drawn to scale. Abbreviations: Ac, AccI; E, EcoRI; Ev, EcoRV; S, SalI; Sa, SacI.

numbers 4 268 086 to 4 271 443 of *Salmonella typhimurium* strain SGSC1412 (McClelland et al., 2001; http://genome.wustl.edu/projects/bacterial/styphimurium/). We did not find any difference in nucleotide sequences between SL1344 and SGSC1412 within this region. The order of the three genes, including the direction of transcription, was identical to that in the *E. coli* cpxP–cpxR–cpxA region. The genes are shown schematically in Fig. 1. We also observed typical ρ-independent terminator sequences just downstream of cpxP and cpxA as reported in the corresponding region of *E. coli* (Danese & Silhavy, 1998; McClelland et al., 2001; Weber & Silverman, 1988). The deduced amino acid sequences of these gene products were highly homologous to those of their *E. coli* counterparts. CpxP had 88 % identity to *E. coli* CpxP. CpxR and CpxA showed 97 % and 96 % identity to the respective *E. coli* counterparts (Danese & Silhavy, 1998; Dong et al., 1993; McClelland et al., 2001; Weber & Silverman, 1988). This is consistent with the fact that we could easily clone this region by the complementation method described above.

**Table 2.** Effects of cpxR and cpxA mutations on hilA expression

<table>
<thead>
<tr>
<th>Strain (effector plasmid)</th>
<th>Expression level of hilA monitored by pSN849 (β-galactosidase activity, Miller units)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At pH 6·0</td>
</tr>
<tr>
<td>SL1344 (none)</td>
<td>1099 ± 255</td>
</tr>
<tr>
<td>SN1457 (none)</td>
<td>1000 ± 282</td>
</tr>
<tr>
<td>SN1459 (none)</td>
<td>79 ± 29</td>
</tr>
<tr>
<td>SN1459 (pSN455)</td>
<td>921 ± 79</td>
</tr>
</tbody>
</table>

*Mean ± SD.

**hilA expression in cpx mutants**

The reading frames of cpxR and cpxA of pSN902 were disrupted by a Km-resistance cassette, and recloned into the suicide vector pKHK002. Disrupted strains, SN1457 [cpxR::aph(T)] and SN1459 [cpxA::aph(T)], were then obtained by allelic exchange between the disrupted gene copy cloned on pKHK002 and the *Salmonella* SL1344 chromosome (see Fig. 1 and Methods).

Plasmid pSN849, containing the hilA–lacZ translational fusion gene, was used as a reporter plasmid for monitoring hilA expression. Based on previous reports of pH-dependent regulation of *Shigella virF* (Nakayama & Watanabe, 1995, 1998) and of hilA (Bajaj et al., 1996), we monitored hilA expression at pH 6·0 and pH 8·0 (Table 2). Unlike the regulation of *Shigella virF*, hilA expression was not regulated by pH in the SL1344 background in our monitoring system (Table 2), which was inconsistent with a previous report demonstrating alkaline-pH-dependent activation of hilA expression (Bajaj et al., 1996). This apparent discrepancy seems to be due to the difference of the buffer used to adjust the pH of the media between the two studies (see Discussion).

The cpxA mutation in SN1459 did affect hilA expression. At pH 8·0, the level of hilA expression was about 50 % of that in the parent SL1344. At pH 6·0, the effect of the mutation was much greater: the level of hilA expression was less than 10 % of that in the parent (Table 2). This strongly suggested that cpxA is required for activation of hilA, especially at pH 6·0.

pSN455, a plasmid supplying CpxA only, was constructed and used for the complementation test. The introduction
of pSN455 into SN1459 restored hilA expression to a level comparable to that in SL1344 at both pHs (Table 2), while the introduction of pBR322, the vector for pSN455, had no activating effect on hilA expression in SN1459 (data not shown). This indicated that cpxA was indeed responsible for hilA activation.

On the other hand, the cpxR mutant SN1457 showed no significant difference in the level of hilA expression compared to SL1344 at either pH (Table 2). Accordingly, we concluded that cpxR is not required for the activation of hilA. This was unexpected, because cpxR and cpxA are located contiguously on the genome, and therefore their products were assumed to be a cognate pair of response regulator and sensor kinase which act in a concerted manner to regulate target gene(s). These results indicate that CpxA controls hilA activation via some pathway independent of CpxR.

**Analysis of SipC in cpx mutants**

It has been well established that HilA activates the expression of genes for the SPI-1 (*Salmonella* pathogenicity island 1)-encoded type III secretion machinery, which is required for secretion of effector molecules SipBCD for invasion (Bajaj *et al.*, 1995; Darwin & Miller, 1999; Eichelberg & Galan, 1999; Johnston *et al.*, 1996). HilA also activates the expression of the sipBCD genes per se, and this regulatory pathway works through the activation of a secondary activator, invF (Eichelberg & Galan, 1999). Overall, the decreased level of expression of hilA is thought to lower both the synthesis and the secretion of SipBCD. We therefore compared by Western blot analysis the amount of SipC in whole-cell lysates of the parent SL1344, or mutants SN1457 and SN1459, grown at pH 6-0 and at pH 8-0. The intensity of the SipC band was scanned with a densitometer and specified as a value. The relative amounts of SipC protein detected are shown in Fig. 2. In this experiment, we found that the amount of SipC product in SL1344 was slightly lower at pH 8-0 than at pH 6-0 (Fig. 2). This was inconsistent with a previous report demonstrating alkaline-pH-dependent activation of sipC (sspC, in the report) expression (Bajaj *et al.*, 1996). However, our data are consistent with those of hilA expression in SL1344 as presented in Table 2. SN1457 did not show significant alteration of the amount of SipC in the lysate compared to SL1344, at either pH (Fig. 2). This was consistent with the result that hilA expression levels in SL1344 and SN1457 were comparable, as described above.

The lysates of SN1459 and SN1459/pBR322 grown at pH 6-0 contained no detectable SipC (Fig. 2). This was expected because hilA expression, which is required for sipC expression per se (Eichelberg & Galan, 1999), was greatly reduced in SN1459 at low pH (see above). However, the effect was much greater than we expected. This suggested that the expression level of hilA observed at pH 6-0 was not high enough for SipC synthesis. This complete loss of SipC synthesis was restored to almost the wild-type level by introduction of pSN455, a plasmid which contains cpxA (Fig. 2). This result indicated that the complementation of SipC expression was due to the functional recovery of CpxA, and thereby hilA expression.

After growth at pH 8-0, the cpxA mutant SN1459 showed a comparable amount of SipC synthesis to that seen in SL1344 and SN1457 (Fig. 2). This suggested that the expression level of hilA observed at pH 8-0 was high enough for the expression of sipC in SN1459.

Thus, these results indicate that cpxA is an essential activator for sipC at pH 6-0, and this activation is via hilA expression under this condition. At the same time, these results suggest that cpxA is not necessarily required for sipC expression at pH 8-0.

We confirmed that secretion of SipC into the supernatant of SN1459 was not observed after growth at pH 6-0. On the other hand, when grown in pH 8-0, as expected, it secreted an amount of SipC comparable to that secreted by SL1344. SN1457 showed no significant alteration in the amount of secreted SipC compared to SL1344 at either pH (data not shown).

![Fig. 2. Detection and quantification of SipC in cpx mutants by Western analysis. Whole-cell extract samples were prepared from SL1344, SN1457, SN1459, SN1459/pSN455, and SN1459/pBR322. Samples with an equal amount of total protein were separated by SDS-PAGE followed by Western blot analysis. The intensity of the SipC band was quantified from the Western blots as described in Methods. Mean values of three independent experiments are represented by bars as percentages of the control (SL1344 grown at pH 6-0). Vertical lines show standard deviations. Filled and open bars are for cultures of the indicated strains grown at pH 6-0 and at pH 8-0, respectively.](http://mic.sgmjournals.org)
Table 3. Percentage (mean ± SD) of inoculated bacteria internalized into INT407 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>At pH 6·0</th>
<th>At pH 8·0</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL1344</td>
<td>14·3 ± 2·5 %</td>
<td>16·7 ± 4·4 %</td>
</tr>
<tr>
<td>SN1457</td>
<td>19·9 ± 3·8 %</td>
<td>23·7 ± 4·8 %</td>
</tr>
<tr>
<td>SN1459</td>
<td>2·8 ± 0·57 %</td>
<td>21·1 ± 4·4 %</td>
</tr>
</tbody>
</table>

Cell invasion phenotype of cpx mutants
From the observed loss of SipC in the whole-cell lysate and in the supernatant of SN1459 grown at pH 6·0, we predicted that this mutant would be defective in its invasion into host cells after growth at this pH. This was indeed the case. The results of the invasion assay into INT407 cells at an m.o.i. of about 10 are shown in Table 3. SN1459 grown at pH 6·0 showed a decreased invasion rate, only about 20 % of that of the parent SL1344. However, when SN1459 was grown at pH 8·0, no reduction in invasion rate was observed. As expected, SN1457 did not show any reduction of invasion efficiency at either pH. Overall, the results obtained with the cpx mutants described above were completely consistent with variation in the synthesis and secretion of SipC. We thus concluded that cpxA-mediated activation of hilA at pH 6·0 has an important role in the phenotype of cell invasion.

DISCUSSION
In this study, we investigated the effects of cpxR and cpxA on the expression of hilA, and consequently of the invasion genes in Salmonella enterica serovar Typhimurium. This investigation was based on the fact that cpxR–cpxA homologues are very important for the pH-dependent regulation of virF expression in Shigella sonnei (Nakayama & Watanabe, 1995, 1998).

We monitored hilA expression, SipC synthesis and cell invasion at pH 6·0 and pH 8·0. In the course of these studies, we found that these characteristics were not activated in an alkaline-pH-dependent manner in the SL1344 background in our monitoring system. This was inconsistent with the previous report by Bajaj et al. (1996), which showed that the expression of hilA, and consequently of sipC (sspC, in the report) was subject to alkaline-pH-dependent activation. Our preliminary investigation suggested that the difference in the culture media might be the reason for this discrepancy: we used LB supplemented with 0·1 M sodium phosphate buffer for pH adjustment, while they used LB buffered with 0·1 M Tris/maleate. When we monitored hilA expression levels in cultures grown in LB containing 0·1 M Tris/maleate buffer, the apparent alkaline-pH-dependent activation of hilA was reproduced. However, this does not seem to be the effect of pH itself, because both the sodium phosphate and Tris/maleate buffers maintained the pH of the media well during culturing (data not shown). Importantly, perhaps, the hilA expression level after growth in LB containing 0·1 M Tris/maleate was greatly reduced at pH 6·0, but not significantly reduced at pH 8·0, compared to that in LB containing 0·1 M sodium phosphate pH 6·0 or 8·0 (data not shown). Therefore, we now hypothesize that, in LB containing 0·1 M Tris/maleate pH 6·0, some specific environmental factor other than pH might lead to the hilA repression, and consequently, to the apparent hilA activation at alkaline pH reported in the previous study. It was also probable that the concentration of O2, which also affects hilA expression (Bajaj et al., 1996), was significantly higher in our culture medium than that in the previous study, because in our assay, bacterial cultures were slowly shaken. A higher O2 concentration would lead to some repression of hilA expression, which may have masked the reported alkaline-pH-dependent hilA activation in our experiments. We therefore repeated the monitoring of hilA expression level with completely static cultures. This experiment confirmed that the static cultures showed higher levels of hilA expression than shaken cultures at both pHs. However, this culture condition did not resolve the discrepancy between our study and the previous report by Bajaj et al. (1996): a static culture grown in LB containing 0·1 M sodium phosphate did not show a pH-dependent regulation of hilA expression (data not shown). These findings suggest that the apparent inconsistency was due to some critical difference in the culture conditions, probably the buffers used for pH adjustment, and that hilA and sipC were not subject to alkaline-pH-dependent activation in our experimental conditions.

In the analysis of SipC synthesis, we found that the amount of product in SL1344 was slightly lower at pH 8·0 than at pH 6·0 (Fig. 2). We do not have a clear explanation for this slight reduction of SipC synthesis in SL1344 at alkaline pH, nor do we know whether this reduction was truly significant. We do not think that this slight reduction has biological significance, since it was not reflected in the cell invasion rate (Table 3).

We report here that cpxA was fully required for hilA activation at pH 6·0. This cpxA-mediated activation of hilA at low pH seems to be biologically significant because the consequent expression and secretion of SipC, and the efficient cell invasion phenotype, at pH 6·0 also depended upon the presence of cpxA. The complete loss of SipC synthesis in the cpxA mutant grown at pH 6·0 (Fig. 2) may imply a functional significance of CpxA at pH 6·0. Although pH 6·0 per se is not a natural environmental condition in the host intestinal niche where Salmonella spp. invade, bacteria may encounter some in vivo conditions or stimuli that require cpxA-mediated activation. In vitro culture at pH 6·0 might mimic such conditions or stimuli. If this scenario is correct, the phenotype of the cpxA mutant at pH 6·0 means that this mutant is unable to produce and secrete invasion effectors when they are required. Further examination will be needed to confirm the necessity of cpxA in natural invasion.
We have demonstrated the involvement of \textit{cpxA} in the regulation of \textit{Salmonella} invasion genes, through activation of the central regulator gene, \textit{hilA}. We have previously reported that \textit{Shigella cpxR–cpxA} has an important role in the pH-dependent regulation of the invasion genes via the expression of the central activator gene \textit{virF} (Nakayama & Watanabe, 1995, 1998). These results imply that these two genera share not only the apparatus for cell invasion, but also a part of the regulatory mechanism: the involvement of a signal sensor \textit{cpxA} in the regulation of the invasion genes. On the other hand, there are some differences between the two genera. In \textit{Shigella}, inactivation of \textit{cpxR} results in the complete loss of \textit{virF} expression regardless of pH (Nakayama & Watanabe, 1998), whereas in \textit{Salmonella}, as reported in this study, \textit{cpxR} has no significant effect on \textit{hilA} expression. Inactivation of \textit{cpxA} in \textit{Shigella} results in alteration of pH-dependent regulation of \textit{virF}, but never abolishes its expression (Nakayama & Watanabe, 1995). On the other hand, in \textit{Salmonella}, \textit{cpxA} mutation leads to almost complete loss of \textit{hilA} expression at pH 6–0 and to a moderate reduction at pH 8–0. These findings suggest that the regulatory circuits for invasion genes in \textit{Shigella} and \textit{Salmonella} have evolved divergently and only \textit{cpxA} remains as the common important factor.

The phenotype of SN1457 means not only that \textit{cpxR} has no significant effect on \textit{hilA} expression, but also that our \textit{cpxR} disruption does not cause a polar effect on \textit{cpxA}. Perhaps the insertion of the cassette in SN1457 artificially provided an active promoter for \textit{cpxA}. Alternatively, the \textit{Salmonella cpxR–cpxA} operon might have a native internal promoter for \textit{cpxA}. We cannot exclude the latter possibility at present, since, to our knowledge, a detailed transcription map of the operon has never been reported.

The finding that \textit{cpxR} is not required for \textit{hilA} activation was rather unexpected. A sensor kinase and response regulator set encoded contiguously on a genome generally function in a concerted manner to regulate the expression of target gene(s). However, \textit{cpxA} and \textit{cpxR} of \textit{Salmonella} did not work cooperatively in the regulation of \textit{hilA}. How then could \textit{cpxA} activate \textit{hilA}? We postulate the presence of response regulator(s) other than \textit{CpxR} which cooperate with \textit{CpxA}. We expected that such a putative response regulator might be identified through the selection of multi-copy suppressors rescuing the phenotype of SN1459 at pH 6–0. In this experiment, we identified several loci including \textit{hilC}, whose product is an AraC/XylS-type transcription activator for \textit{hilA} (Lucas & Lee, 2001; Schechter et al., 1999). However, we could not obtain any candidate for a response regulator with this strategy. This may be due to the full dependence of the activation of the putative response regulator upon \textit{CpxA} at pH 6–0, regardless of its overproduction.

To date, a two-component regulatory system, \textit{barA–sirA}, has been identified as a positive regulator for \textit{hilA} (Altier et al., 2000a; Johnston et al., 1996). Although \textit{BarA} is believed to be the cognate sensor of the regulator \textit{SirA} (Altier \textit{et al.}, 2000a; Johnston \textit{et al.}, 1996), we postulate that \textit{CpxA} is also a fairly probable candidate for the partner sensor of \textit{SirA}, especially at pH 6–0. It is possible that \textit{SirA} has the capacity to interact with both sensors, exchanging partner sensors dependent on the change of environmental conditions. This hypothesis would explain our failure to identify the response regulator paired with \textit{CpxA} by searching for multi-copy suppressors at pH 6–0. Identification of the regulator interacting with \textit{CpxA}, including the possibility stated above, would be another future goal.

Assuming that a putative response regulator paired with \textit{CpxA} exists, the specific mechanism of the \textit{hilA} activation by \textit{CpxA} is not known at present. However, our preliminary investigations suggest that this pathway functions, at least in part, if not totally, through activation of \textit{hilC} and \textit{hilD}, whose products are both AraC/XylS-type transcription activators for \textit{hilA} (Lucas & Lee, 2001; Schechter \textit{et al.}, 1999) (unpublished results). It would therefore be possible for us to monitor the expression levels of \textit{hilC} and \textit{hilD} for analysis of this pathway.

We have indicated in this paper that \textit{cpxA} but not \textit{cpxR} is involved in the regulation of \textit{hilA} in \textit{Salmonella enterica} serovar Typhimurium. Of course, we do not exclude the possibility that this sensor–regulator set controls other target gene(s) in a concerted manner. One strong candidate for such a target may be \textit{htrA}, because in \textit{E. coli} it has been well established that the expression of a periplasmic protease gene, \textit{degP}, an \textit{htrA} homologue of \textit{E. coli}, is activated by the \textit{cpxR–cpxA} two-component system (Danese \textit{et al.}, 1995). Following this example, we assume that \textit{htrA} is also subject to regulation by the \textit{cpxR–cpxA} system in \textit{Salmonella}.

Leclerc \textit{et al.} (1998) reported that the \textit{cpxA} mutation in \textit{Salmonella enterica} serovar Typhi greatly reduced the invasion capacity. Following the above example, and without direct evidence, they presumed that the observed effect was due to reduced activity of the cognate response regulator, \textit{CpxR}, which caused a reduction of \textit{HtrA} and would then lead to decreased invasion capacity. However, we think this scenario is unlikely. \textit{htrA} is reported to be involved in intra-macrophage survival and virulence in mice of \textit{Salmonella enterica} serovar Typhimurium (Johnson \textit{et al.}, 1991); however, its involvement in the cell invasion step has never been described. Furthermore, we observed that inactivation of \textit{cpxR} of \textit{Salmonella enterica} serovar Typhimurium had no detectable effect on the invasion capacity. The \textit{cpxR} of \textit{Salmonella enterica} serovar Typhi may also have no effect on cell invasion.

Overall, our results indicate that in the regulatory pathways of invasion genes in \textit{Shigella} and \textit{Salmonella}, a common factor, \textit{CpxA}, is involved through the central regulators, \textit{Shigella virF} and \textit{Salmonella hilA}. This might be important in the evolution of the genera \textit{Shigella} and \textit{Salmonella}, and might present an opportunity to create
novel and common methods to control the pathogenesis of these two bacterial genera.

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REFERENCES


coded by a cloned gene fused to lacZ gene sequences encoding an enzymatically active carboxy-terminal portion of β-galactosidase. 

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