IDENTIFICATION OF A SHIGELLA FLEXNERI criR GENE INCREASING ipa GENES EXPRESSION: A NOVEL MEMBER OF RESPONSE REGULATORS OF THE TWO-COMPONENT SIGNAL TRANSDUCTION FAMILY

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SUMMARY: A genetic locus named cri, which enhanced the expression of ipa genes, was cloned into Escherichia coli K-12 from Shigella flexneri 1b chromosomal DNA. Subcloning and Tn5-Tc1 transposon experiments showed that cri locus was located on a 2.6-kb HindIII fragment. Nucleotide sequence analysis of the region revealed at least three open reading frames (ORF), one of which, named criR, encoded a protein of 226 amino-acid residues and transcriptionally increased the ipaB expression. The deduced regulatory protein CriR shared a significant homology with bacterial transcriptional activators of the two-component signal transduction family. A homologue of the criR gene was present in genomic DNA of Shigella spp. and E. coli strains, and mapped at the 14.6-min region of E. coli K-12 chromosomal DNA. These results indicate that criR is a new member of response regulators.
INTRODUCTION

*Shigella* species is the causative agent of bacillary dysentery, invading to the human enteric epithelial cells (1). The invasion of *Shigella* is a multiple step process i.e., entering cells, lysing vacuoles, multiplying and moving therein and spreading to adjacent cells (2). All of *Shigella* species harbor a high-molecular-weight plasmid (invasion plasmid) encoding functions essential for the virulence (3). The function(s) of the invasion plasmid is interchangeable among four species of *Shigella* (4). For instance, a 37-kb region from such an invasion plasmid of *S. flexneri* restores the entry phenotype of a plasmid-cured *Shigella* strain (5), and a similar 37-kb segment from the invasion plasmid of *S. sonnei* confers the ability to enter LLC-MK2 cells on *E. coli* K-12 (6).

Genetic analysis of the region has shown that many closely related genes are required for the invasion of bacteria, including *ipa* (invasion plasmid antigen), *mxi* (membrane expression of invasion plasmid antigen), and *spa* (surface presentation of invasion plasmid antigen), which were located on the 37-kb segment. The *ipa* genes encode the IpaABCD proteins that are dominant antigens in the humoral response to shigellosis (7). Both the *mxi* and *spa* genes code for the secretion apparatus to release the Ipa proteins (8-12). Through transposon insertion and deletion mutagenesis, it has been demonstrated that the *ipaB*, *ipaC* and *ipaD* genes belong to the same transcriptional unit and are essential for bacterial entry into epithelial cells via directed phagocytosis (12-15).

Expression of the *ipaBCD* genes is positively regulated by *virF* and *invE* (*virB*) genes on the invasion plasmid (16,17). *virF* gene, about 50 kb apart from the *ipa* operon, encodes a 30-kDa polypeptide and *invE* located downstream of *ipaBCD* transcriptional unit encodes a 35-kDa protein. VirF is required to activate the transcription of *invE*, and InvE on its turn transcriptionally enhances the expression of *ipaBCD* genes (16,17).

The invasiveness of *Shigella* is affected by the temperature and osmolarity of environmental conditions under which bacteria grow (18,19). It has been demonstrated that *Shigella* is virulent when cultured at 37 C but avirulent when cultured at 30 C, which is mainly due to the temperature-dependent expression of *invE* gene (20). Transcription of *invE* gene is repressed by the binding of H-NS protein encoded by a chromosomal locus to the *invE* promoter region at 30 C (20,21). The invasion gene expression of *Shigella* is also regulated in response to osmolarity, with an increase in high osmolarity of growth condition. It has been demonstrated that the two-component regulatory system EnvZ-OmpR is involved
in the regulation of gene associated with virulence of Shigella (18). We reported that extracellular pH regulated the invasiveness of Shigella spp. At pH 6.0, invasiveness was repressed about 10-fold compared with that at pH 7.4 (22). Nakayama et al. (22) recently described that CpxA encoded by a chromosomal locus, which was originally reported as a gene regulating F donor activity, was implicated in the pH-dependent regulation of virF expression.

In this report, we describe a novel genetic locus of Shigella chromosome involved in ipaB gene expression of virulence plasmid, which is homologous to response regulators of the two-component signal transduction family.

MATERIALS AND METHODS

**Bacterial strains, plasmids and growth conditions:** The strains and plasmids used in this study are listed in Table I. Enteroinvasive E. coli (EIEC), enterotoxigenic E. coli (ETEC), enterohemorrhagic E. coli (EHEC), enteropathogenic E. coli (EPEC), E. coli B, E. coli C, Salmonella Typhimurium and Yersinia enterocolitica strains used for Southern hybridization were from our collection. Bacteria were routinely grown in nutrient broth or on agar-solidified plates of antibiotic medium 3 (Difco Laboratories, Detroit, MI). LB agar containing X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside) was used to detect β-galactosidase activity. Medium supplemented with 1.5% casamino acid (Difco) and 0.1% glucose was used when β-galactosidase activity was to be measured. Antibiotics and their concentrations were as follows: kanamycin (Km), 40 μg/ml; chloramphenicol (Cm), 25 μg/ml; ampicillin (Ap), 40 μg/ml; and tetracycline (Tc), 12.5 μg/ml.

**Preparation of chromosomal DNA from Shigella and E. coli:** Chromosomal DNA was purified as described (23). Bacteria from 100-ml cultures were collected and washed in 10 mM Tris-HCl (pH 8.0) by centrifugation at 4,000 × g for 10 min. The pellets were resuspended in a mixed solution containing 9.5 ml of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0), 0.5 ml of 10% sodium dodecylsulfate (SDS) and 50 μl of proteinase K (20 mg/ml). After 1 hr incubation at 37 C, 1.8 ml of 5 M NaCl, 1.5 ml of CTAB/NaCl (10% hexadecyltrimethyl ammonium bromide in 0.7 M NaCl) and 10 μl of bovine pancreas RNase (5 μg/ml) were added to the suspension, and the mixture was incubated further for 20 min at 65 C. The lysate was then centrifuged at 15,000 rpm for 15 min, and the supernatant was collected. The DNA was then repeatedly extracted with an equal volume of chloroform-phenol (1:1) followed by final extraction with an equal volume of chloroform/isoamyl alcohol (24:1). The DNA was subsequently precipitated with ethanol and the pellet was washed with 70% ethanol.
<table>
<thead>
<tr>
<th>Strains and plasmid</th>
<th>Relevant characteristics</th>
<th>Reference and/ or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. flexneri</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9268N</td>
<td>Serotype 1b, invasion plasmid+, cryptic plasmids, virulent</td>
<td>(24)</td>
</tr>
<tr>
<td>HW1002</td>
<td>A large plasmid-cured derivative of 9268N, avirulent</td>
<td>(24)</td>
</tr>
<tr>
<td>HW283</td>
<td>Serotype 2a, invasion plasmid+, cryptic plasmids, virulent</td>
<td>(35)</td>
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<td><strong>S. sonnei</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HW383</td>
<td><em>nad</em>, pSS120, cryptic plasmids, virulent</td>
<td>(35)</td>
</tr>
<tr>
<td><strong>S. dysenteriae</strong></td>
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<td></td>
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<td>HW257</td>
<td>invasion plasmid+, cryptic plasmids, virulent</td>
<td>(35)</td>
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<td><strong>E. coli K-12</strong></td>
<td></td>
<td></td>
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<tr>
<td>MC1061</td>
<td><em>hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi recA1 supE44 endA1 hisD17 gyrA96 relA1 thi (Δlac-proAB)</em></td>
<td>(23)</td>
</tr>
<tr>
<td>JM109</td>
<td><em>F</em>[<em>traD36 proAB</em> lacFΔlacZΔM15]</td>
<td>(23)</td>
</tr>
<tr>
<td>MB76</td>
<td>MC1061 carrying pHB76; Km&lt;sup&gt;+&lt;/sup&gt;Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>this work</td>
</tr>
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<td>Table I continuing</td>
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<tr>
<td><strong>Plasmid</strong></td>
<td><strong>Description</strong></td>
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</tr>
<tr>
<td>pACYC184 p15A replicon vector; Cm&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
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<td>pSU19 pACYC184-derived vector carrying the same multicloning sites and lacZ with pUC19; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>pSC101 pSC101 replicon vector containing multicloning sites; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
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<tr>
<td>pJK1142 pJK292 (an F plasmid derivative vector) carrying a 37-kb invasion region from virulence plasmid pSS120 of S. sonnei HW383; inv&lt;sup&gt;E&lt;/sup&gt;+, vir&lt;sup&gt;F&lt;/sup&gt;−</td>
<td>(6)</td>
<td></td>
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<td>pHB76 pJK1142 ipaB::Tn3-lac, noninvasive; Km&lt;sup&gt;r&lt;/sup&gt; inv&lt;sup&gt;E&lt;/sup&gt;+ , vir&lt;sup&gt;F&lt;/sup&gt;−</td>
<td>(17)</td>
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<td>pPH735 pJK1142 ipaC::Tn3-lac, noninvasiev; Km&lt;sup&gt;r&lt;/sup&gt; inv&lt;sup&gt;E&lt;/sup&gt;+, vir&lt;sup&gt;F&lt;/sup&gt;−</td>
<td>(17)</td>
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<tr>
<td>pHB76 pJK1142 invE::Tn3-lac; Km&lt;sup&gt;r&lt;/sup&gt;Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(17)</td>
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<td>pHW735 pJK282 (an F plasmid derived vector)+ a BamHI fragment carrying most of ipaB::Tn3-lac of pH76; Ap&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt; inv&lt;sup&gt;E&lt;/sup&gt;−</td>
<td>(17)</td>
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<td>pHW736 pJK280 (an F plasmid derived vector)+ a HindIII fragment carrying a region of invE::Tn3-lac of pH37; Ap&lt;sup&gt;r&lt;/sup&gt; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(17)</td>
<td></td>
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<tr>
<td>pHW741 pHSG595 + a 3.5-kb HindIII fragment containing inv&lt;sup&gt;E&lt;/sup&gt;; Cm&lt;sup&gt;r&lt;/sup&gt;, inv&lt;sup&gt;E&lt;/sup&gt;+</td>
<td>(17)</td>
<td></td>
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<td>pCHR82 A derivative of pCHR71 transposed by Tn5-TC1; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(26)</td>
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<td>pHW771 pHSG595 + a 2-kb Smal DNA fragment containing vir&lt;sup&gt;F&lt;/sup&gt; region; Cm&lt;sup&gt;r&lt;/sup&gt;, vir&lt;sup&gt;F&lt;/sup&gt;+</td>
<td>this work</td>
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<td>pQ446 pACYC184 + a 11-kb Sau3A-DNA fragment from chromosome of HW1002 strain; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
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<td>pQS613 A derivative of pQ446 deleting a 4.2-kb HindIII-DNA fragment; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
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<tr>
<td>pQSH03 pSU19 + a 3.2-kb HindIII-DNA fragment of pQ446; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
<td></td>
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<tr>
<td>pQSH02 pSU19 + a 2.6-kb HindIII-DNA fragment of pQ446; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
<td></td>
</tr>
<tr>
<td>pQSS01 pSU19 + a 2-kb SauI-DNA fragment of pQSH02; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
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<td>pQD01 A deletion mutant of pQSH02 constructed by unidirectional exonuclease III digestion; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
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<td>pQSK201 A derivative of pQSH02 deleting a 0.6-kb PstI-DNA fragment + Km cassette; Cm&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>this work</td>
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</table>
Chromosomal DNA was dissolved in TE buffer to a final concentration of 0.5 mg/ml. DNA prepared in this manner was used for the construction of a genomic library and Southern blot analysis.

Construction of Shigella genomic library: A chromosomal DNA library was constructed by the standard procedures from *S. flexneri* 1b HW1002 cured of the large virulence plasmid (24). High molecular weight HW1002 chromosomal DNA was isolated and partially digested with *Sua3A*. The digested DNA was fractionated on a 10 to 40% continuous sucrose gradient to enrich fragments of around 10 kb. These fragments were then ligated by using T4 ligase into the BamHI site of pACYC184. The ligated DNA was then transformed into *E. coli* MC1061 carrying pHB76 (*ipaB::lac*). Deep blue colonies producing β-galactosidase on the LB agar plates were picked and the plasmid DNA was purified by cesium chloride density gradient centrifugation.

Construction of deletion mutants: Preparation and manipulation of plasmid DNA were carried out essentially as described (23). pQ446 was partially digested with *HindIII*, self-digested and transformed into JM109. Resultant transformants were analyzed for the presence of *HindIII* fragments. pQ615, pQ612, and pQ613 lost some of *HindIII* fragments from pQ446 (Fig. 3). A 3.2-kb DNA fragment was extracted from agarose gel loaded with *HindIII*-partially digested pQ613 DNA and ligated into a *HindIII* site of pSU19. A plasmid, named pQSH3, carrying contiguous 2.6- and 0.6-kb *HindIII* fragments was selected (Fig. 3). pQSH02 was a derivative of pQSH03 losing a 0.6-kb *HindIII* fragment. pQSS01 was constructed by ligating a 2-kb *SalII* fragment (one site of *SalII* is from the insert and the other site from the vector; see Fig. 3) of pQSH02 into a *SalII* site of pSU19. pQD01 was a derivative of deletion mutants of pQSH02 constructed with a Deletion kit of Takara (see DNA sequencing). pQSK201 was constructed by deleting a 0.6-kb *PstI* fragment of pQSH02 and inserting the kanamycin-resistance cassette with a *PstI* linker (Pharmacia Biochemicals, Milwaukee, WI).

β-galactosidase assay: β-galactosidase was assayed as described (25). Bacteria were cultured with shaking at 37°C in 2 ml of medium until *A*600 of 0.5. The bacteria were collected by centrifugation and suspended in 2 ml of Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptopethanol). Five hundred microliters of the bacteria suspension was mixed with 20 μl of 0.05% sodium dodecyl sulfate (SDS) and 20 μl of CHCl3 for 10 s and vortexed. The mixture was incubated for 10 min at 28°C. The reaction was started by adding 100 μl of o-nitrophenyl-β-D-galactoside (4 mg/ml OPNPG, Wako Junyaku Corp., Osaka). After 10 min at 28°C, the reaction was stopped by addition of 250 μl of 1 M Na2CO3. The reaction mixtures were centrifuged to remove bacterial cells. Absorbances at 420 nm and 550 nm were measured. β-galactosidase activity (units) was calculated by the formula: \{(A_{420}) - 1.75 \times (A_{550})\} \times 1,000 / \{\text{reaction minutes} \times \text{volume of culture used in the assay (ml)} \times (A_{600})\}. 

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**Analysis with transposon Tn5-Tcl:** pQSH03 (Cmr; chloramphenicol resistance) was transformed into *E. coli* MC1061 which had been transferred with pCHR82 carrying the Tn5-Tcl (Tcr; tetracycline resistance) (26). Transformants which are non-permissive to pCHR82 replication were grown at 42°C, and colonies resistant to both tetracycline and chloramphenicol were selected. Plasmid DNAs were prepared from the TcrCmr bacteria and transformed into MC1061, again. TcrCmr colonies are considered to carry pQSH03 plasmid inserted by Tn5-Tcl. Insertion sites of Tn5-Tcl on pQSH03 were determined by routine molecular genetical manipulations (23).

**Western blot (immunoblot) analysis:** The whole bacterial cell extracts were run on SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride filter as described previously (17). Hundred-fold diluted serum from a monkey orally challenged with virulent *S. flexneri* 1b and a rabbit immunized with purified InvE protein (details will be described elsewhere) were used to identify virulence plasmid-encoded proteins and InvE protein, respectively. Horse radish peroxidase-conjugated anti-monkey or rabbit immunoglobulin G was used for detection of the bound antibody.

**Preparation of DNA probe and Southern hybridization:** The probe DNA fragment was extracted from a 0.8% agarose gel by the procedure described (27). The chromosomal DNA preparation from *Shigella* spp., *E. coli* strains or others was digested with appropriate restriction endonuclease and an about 5-10 μg aliquot was electrophoresed on 0.8% agarose gel. The DNA was subsequently transferred to nylon filter membrane by the method of Southern (23). A 0.6-kb PstI fragment (Fig. 3) containing a criR region was labeled with an ECL-labeling system (Amersham International, Buckinghamshire, UK). After pre-hybridization, the filters were hybridized with the probe for 14-16 hr at 42°C in hybridization solution. The unbound label was removed by washing twice each for 10 min at 55 or 37°C in either a high stringency solution (0.1×SSC, 0.4% SDS) or a low stringency solution (0.5×SSC, 0.4% SDS) and subsequently twice for 5 min at room temperature in solution B (20×SSC). The filter was treated with an ECL gene detection system (Amersham), and exposed to Hyper-ECL film at room temperature with a film cassette for appropriate time.

**Preparation of RNA and Northern hybridization:** The bacteria grown in L-broth at 37°C were harvested at an A600 of 0.5 and resuspended in 3 ml of acetate/SDS buffer (0.02 M sodium acetate, pH 4.5; 0.5% SDS and 1 mM EDTA) (28). After adding 3 ml of redistilled phenol (equilibrated with 0.02 M sodium acetate, pH 4.5), the mixture was incubated at 60°C for 5 min with gentle shaking. After centrifugation, the aqueous phase was re-extracted with phenol/sodium acetate. The RNA dissolved in acetate/SDS buffer was precipitated by adding three volumes of ethanol. The final precipitate was dissolved in 0.5 ml of 0.5% SDS. The RNA concentration was estimated from A260. Twenty micrograms of total RNA was electrophoresed in 1.0% formaldehyde-agarose gel and then transferred to nylon membrane (29). A 2.4-kb EcoRI fragment of the 37-kb invasion region
(pJK1142) containing ipaB and ipaC genes was labeled with $^{32}$P-dCTP and used as a probe under highly stringent hybridization condition. RNA isolated from E. coli K-12 was used as a negative control.

**Mapping on the chromosome:** Gene Mapping Membrane (Takara) containing 467 clones originally constructed by Kohara et al. (30) was used for locating the cloned genes on the E. coli K-12 chromosome. The membrane was hybridized with the 0.6-kb PstI-DNA fragment of pQSH03 (Fig. 3) by using an ECL hybridization kit (Amersham). The filter was washed in 0.1 × SSC, 0.4% SDS and processed with an ECL gene detection system (see Southern blot).

**DNA sequencing:** Deletion mutants of the plasmid pQSH02 were constructed by unidirectional exonuclease III digestion after cleaving with XbaI and SacI (Deletion kit, Takara). Double stranded DNA of the deletion mutants was prepared for sequencing by alkaline lysis and used as templates for Taq polymerase reaction. Sequencing reactions were performed as described in instructions of the Taq Dye-primer. Sequencing kit (Applied Biosysye, USA). Sequencing was performed on an Applied Biosysye 373A DNA Sequencer using 6% polyacrylamide gel containing 20% (w/v) urea and 1 × TBE buffer.

**Protein database and nucleotide sequence accession:** Homology of proteins was searched by using the databases of SWISS-PROT and GeneBank with Blast soft. Nucleotide and amino acid sequence data reported in this paper have been deposited in the DDBJ/EMBL/GeneBank nucleotide sequence databases under accession number U29654.

**RESULTS**

**Molecular Cloning of a Positive Regulator for the Expression of ipaB Gene**

We tried to isolate regulatory gene(s) of the Shigella chromosome increasing the expression of the ipa genes. To do this, we used translational fusion of ipaB gene with the structure gene of $\beta$-galactosidase, (ipaB::Tn3-lac) (17). Plasmid pHB76 carrying ipB::Tn3-lac was transferred into MC1061 to obtain a strain MB76. Sau3A-partially digested S. flexneri 1b HW1002 (invasion plasmid-less strain) DNA was cloned into a BamHI site of pACYC184 to construct a genomic library, which was transferred to MB76. Colonies were screened on L-agar containing X-gal, Km, and Cm. Parent MB76 colonies are pale blue on the L-plate, and colonies with a recombinant plasmid carrying a gene increasing the expression of ipaB are expected to be dark blue. We found two dark blue colonies out of 800 transformants. The recombinant plasmids purified from these two colonies
were transformed into MB76 and MC1061. Resultant transformants in MB76 but not in MC1061 showed dark blue colonies again. Restriction enzyme analysis of the recombinant plasmids showed that the two DNA clones carried 11-kb (named pQ446) and 4.3-kb inserts (named pQ445), respectively. These inserts did not have a common restriction site, indicating that the two clones were not overlapping. Plasmid carrying 11-kb insert (pQ446) was chosen for further investigation, because the pQ446 raised the ipaB expression more prominently. The increase of ipaC gene expression by pQ446 was also shown by the use of ipaC::Tn3-lac fusion, pHB118 as well as that of ipaB (Table II). The enhancement of ipaBC gene expression by pQ446 was not so high as that by virF but was significant (Table II). Western blotting analysis by using anti-IpaB monoclonal antibody and anti-IpaBCD serum provided further evidence showing that regulatory gene(s) on pQ446 increased the expression of not only IpaB (Fig. 1) but also IpaCD proteins.
Table II. Enhancement of *ipa* gene expression by pQ446

<table>
<thead>
<tr>
<th>Resident Tn3-lac fusion plasmid in MC1061</th>
<th>β-galactosidase activity (U/ml) in the present of incoming plasmid*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no plasmid</td>
</tr>
<tr>
<td>pHB76 (ipaB::Tn3-lac)</td>
<td>44</td>
</tr>
<tr>
<td>pHB118 (ipaC::Tn3-lac)</td>
<td>63</td>
</tr>
<tr>
<td>pHW735 (ipaB::Tn3-lac, invE&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>164</td>
</tr>
<tr>
<td>pHW736 (invE::Tn3-lac)</td>
<td>87</td>
</tr>
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</table>

*Values represent the average of three separate assays. Errors were within 20% of the values.

(data not shown). We named the genetic determinant(s) on pQ446 *cri* (chromosome-coded positive regulator for *ipaBCD* expression).

**Cri Locus Increasing the Expression of *ipaB* at Transcriptional Level**

The *ipaBCD* genes are transcribed mainly as one transcriptional unit (13,31). In order to find whether *cri* increases the expression of *ipa* genes at the transcriptional level, we performed Northern blot analysis of total RNAs prepared from *E. coli* K-12 MC1061 carrying pJK1142 alone or MC1061 carrying pJK1142 plus pQ446 (*cri*). MC1061 itself and MC1061 carrying pJK1142 and pHW771 (virF) were used negative and positive controls, respectively. Two bands hybridized with an *ipaBC* probe were detected in RNAs prepared from strains carrying both pJK1142 and pQ446 or both pJK1142 and pHW771 (Fig. 2, lanes 3 and 4). The sizes of the two bands were 3.3-kb and 2.4-kb long corresponding to *ipaBC*- and *ipaB*-specific mRNA, respectively, as shown by virF-invE (virB) regulators (13). Although MC1061 carrying pJK1142 expressed undetectable amount of the *ipaBC* transcripts, introduction of pQ446 into the bacteria resulted in a significant increase of the transcripts but a less increase than that of pHW771 (Fig.
Fig. 2. Enhancement of ipaBC transcript by cri locus. Twenty micrograms of total RNA from each strain was separated on 1% agarose gel, transferred to nylon membrane filter and hybridized with a 2.4-kb EcoRI fragment containing ipaBC genes. The filter was exposed to X-ray films for 48 hr. Lane 1, MC1061; lane 2, MC1061 carrying pJK1142; lane 3, MC1061 carrying both pJK1142 and pQ446; and lane 4, MC1061 carrying both pJK1142 and pHW771. The molecular size (kb) is indicated on the right.

2, lanes 2, 3 and 4), which was consistent with the results of translational fusion. These results indicated that cri locus increased the expression of ipa genes at the transcriptional level.

Positive Regulation of pQ446 not Mediated by invE

InvE, a positive regulator necessary for the expression of the ipaBCD genes was located on the 37-kb segment (pJK1142) of the virulence plasmid of S. sonnei (17). In order to find whether the positive regulation by cri was mediated by invE or not, pHW735 (ipaB::Tn3-lac, invE) and pHW736 (invE::Tn3-lac) (17) were used as monitor plasmids for the expression of ipaB and invE, respectively. As shown in Table II, the ipaB expression without invE increased more than 10-fold in the presence of cri locus, while the expression of invE (pHW736) was not in-
duced by the introduction of cri locus. The result of immunoblot with anti-InvE serum provided further evidence showing that the cri gene had little effect on the expression of invE (data not shown). The results suggested that the cri locus increased the expression of ipaB not through the invE expression.

*ORF2 (criR) Responsible for cri Activity*

Deletion and subcloning experiments showed that the gene, cri, was located on the 2.6- and 0.6-kb contiguous HindIII fragments (3.2 kb) of pQ446 (the left end of their HindIII sites is derived from vector pACYC184, see Fig. 3). The 3.2-kb HindIII fragments cloned into pSU19, pQSH03, was used for the transposon mutagenesis with Tn5-Tcl. Four Tn5-Tcl insertions that eliminated the ability to enhance the ipa expression were mapped in the middle of the 2.6-kb HindIII fragment (Fig. 3). Then, we sequenced the 2.6-kb HindIII fragment of pQSH02. Three open reading frames were identified in the region (Fig. 3). Transcription of ORF1 and ORF2 proceeded in the same direction, and that of ORF3 in the opposite direction. The insertion sites of the four Tn5-Tcl were within ORF3 in the opposite direction. The insertions sites of the four Tn5-Tcl were within ORF1 and ORF2. In order to find which ORFs were involved in the cri activity, we constructed deletion mutants of pQSH02 and examined their activities. A deletion mutant of ORF1, pQSS01, and a deletion mutant of ORF3, pQD01, still retained the regulatory activity of cri (Fig. 3). However, pQSK201, which has a deletion of 0.6 kb PstI fragment in ORF2, lost the cri activity (Fig. 3). The results indicated that the ORF2 was responsible for the regulatory activity. Then, we named ORF2 criR.

The orientation of transcription of ORF1 and ORF2 (criR) in the plasmids used (pQSH03, pQSH02 and pQSS01, etc.) was the same as that of lacZ gene of the pSU19 vector. When the inserts of pQSH02 and pQSS01 were ligated at the opposite orientation, the resultant plasmids lost the cri activity (data not shown). We found the ribosome-binding domain but not apparent -10 or -35 promoter region upstream of ORF2. Further, ORF1 was truncated at the N-terminal in the 2.6-kb HindIII region sequenced (data not shown). These results suggested that ORF1 and ORF2 (criR) are transcribed as one transcriptional unit from the promoter of the vector (e.g., in the plasmid pQSH03).
CheY  MAHRILIVDDAAFMRMIMIKDILVK.NGFEVVAEAENGQAQAVEKYKEHS  
CriR  MTAPLTLLIVEDETPLAEMHAEYIRHIPGFSQILLAGNLAQARMIERF  
                  50
PilR  MSRQKALIVDDEPDIRELLEITLGRMK..LDTRSARNVKEARELLAREP

CheY  PDLVTMDITMPEDGITALKEIKQIDADAQARIIMCSAMGQSSMVIDAIQAG  
CriR  PGLILLDNYLPDGRGNNLLHELVQAHYPGDVFTTTAASDMETVSEAVRCG  
                  100
PilR  FDLCLTDMLRPDSLDDLTVQYIQQRHP0TPVAMITAYGSLDTAIQALKAG

CheY  AKDFIVKPFQAD.RVLEAINKTLN  
CriR  VFDYLIKPIAYE.RLGQTLTR  
                  120
PilR  AFDFLTTPVDFDFRRELVATAL
Homology of CriR with Response Regulators of the Two-component Signal Transduction System Family

The criR gene consisted of 678-bp nucleotides and was thought to be translated into 226 amino acids residues with a molecular weight of 25,452. Homology search between the deduced CriR amino acid sequence and other protein sequences in data bases revealed that CriR shares a striking homology to the N-terminal region with bacterial transcriptional activators of the two-component signal transduction system family. Figure 4 shows the amino acid residue comparison of CriR with other regulatory components from the N-terminus to around 120th amino acid residues. Sequence alignments between CriR and each response regulator domains showed 20 to 30% identical amino acids at corresponding positions. The residues that correspond to Asp-12, Asp-57, and Lys-107 in the CriR sequence were conserved among all response regulators previously reported (32). Asp-57 is presumably a site of phosphorylation by the cognate sensory element as shown in other response regulators (32).

Fig. 3. Map of various deletion mutants and their cri activities. The restriction map with five enzymes is shown. The column on the right side shows the activity of β-galactosidase, after the deletion mutants were transformed into MB76: (+), positive for cri activity; (−), negative for cri activity (values of β-galactosidase activity were similar to those shown in Table II). The arrows shown under pQSH03 indicate the sites of Tn5 insertions that resulted in the loss of cri activity. PstI-DNA fragment (solid bar) shown above pQSH03 was a probe for Southern blot. Abbreviations: B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SalI.

Fig. 4. Amino-acid residue comparison of CriR with other representative response regulators, CheY (Bacillus subtilis) and PilR (Pseudomonas aeruginosa) from data bases. N-terminal amino acid residues of each protein were aligned. Marks (| and :) show identical and similar amino acid residues corresponding to CriR, respectively. Asp-12, Asp-57, and Lys-107 (boxed with dots) in the CriR sequence are highly conserved residues among all response regulators previously reported. Asp corresponding to Asp-57 is known to be phosphorylated by the protein kinase of cognate sensor in other signal transduction components (32).
Mapping cri Locus on the Chromosome

To locate the cri gene on the chromosome DNA, we used a Gene Mapping Membrane (Takara), containing a set of phage clones of the entire E. coli chromosome DNA (33). Hybridization of the 0.6-kb PstI-DNA fragment containing criR gene with the Gene Mapping Membrane showed a homology with 3G5 and 1G6 phage clones. The two clones carried an overlapping region. The overlapping region hybridized with the probe was mapped at 14.6 min on the chromosome of E. coli K-12 (30). The comparison of the restriction pattern among 3G5, 1G6 and the insertion fragment of pQ446 showed that the region containing the cri locus in S. flexneri 1b was very similar to the 14.6-min region of E. coli K-12 (data not shown).
Conservation of the cri gene among Shigella spp. and E. coli

We searched for a cri homology by Southern hybridization with HindIII-digested chromosomal DNA of Shigella spp., EIEC, ETEC, EPEC, EHEC and E. coli K-12. The DNA probe was the 0.6-kb PstI fragment used in the above experiment. A single 3.4-kb HindIII fragment of Shigella and E. coli chromosomal DNAs hybridized with the probe (Fig. 5), and so did a 5.4-kb fragment and a 8.3-kb fragment respectively of S. dysenteriae and EPEC strains. No hybridization signals were detected in S. Typhimurium or Y. enterocolitica under the high stringent conditions (data not shown).

DISCUSSION

We searched for genes which increased the expression of ipa genes from the S. flexneri 1b genomic library. One such gene, named criR, increased transcriptionally the expression of ipaBCD without intervention of InvE, a positive regulator identified in the invasion plasmid. Its homologue was present at 14.6-min map position of E. coli K-12. Recently, Tobe et al. (34) cloned a gene, vacB, being involved in expression of the ipa genes at the post-transcriptional level; it was located downstream of the purA gene (95 min) in E. coli K-12. Thus, the cri locus is distinct from the vacB locus and represents a novel gene involved in the enhancement of ipa genes expression.

A significant homology in the N-terminal region was found between Shigella CriR and other bacterial transcriptional regulators belonging to the two-component signal transduction regulatory system family. The residues that corresponded to Asp-12, Asp-57 and Lys-107 in the CriR amino acid residues tend to be conserved among all response regulators. The amino acid residue corresponding to Asp-57 is known to be phosphorylated by the kinase activity of a cognate sensor, of which phosphorylation is important for stimulation of the response regulator's activity (32). Among response regulators, NR1 and NifA bind to sites upstream of the regulated promoters and phosphorylation of NR1 is required for activation of transcription. As CriR is a homologue of response regulators and increased the transcription of ipa genes, it will be one possibility to think that phosphorylated CriR directly binds to a site upstream of ipa genes promoter and enhances the transcription.
The criR activity for ipaB transcription is independent of that of virF-invE because criR could increase ipaB expression without virF or invE. However, ipaB expression in the presence of virF-invE was much higher than that in criR only. We found also similar sized-transcripts for ipa locus both in the presence of only criR and in the addition of virF-invE. It may be possible to assume that CriR directly acts in the promoter region of ipa genes and that InvE increased by virF function modulates the action of CriR. To certify this hypothesis, it will be necessary to isolate a null mutant of criR in Shigella or E. coli and examine the expression of ipaB genes in that mutant.

CriR is a member of response regulators. It is reasonable to suppose that its cognate sensor gene exists upstream of criR because genes belonging to the two-component signal transduction system generally exist as contiguous genes and one transcription unit (32). We found an open reading frame (ORF1), whose 5' end was truncated in our clone, at the upstream of criR. Homology search of ORF1 indicates that its C-terminal amino-acid sequences are significantly homologous to a member of signal sensors (unpublished data). ORF1 and criR seem to be a new member of the two-component signal transduction family. Physiological function of ORF1 (criS)-criR and characteristics of the environmental stimuli for criS-criR remain to be elucidated in the future.

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