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**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 *Hind*III 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.

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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 μ g/ml; chloramphenicol (Cm), 25 μ g/ml; ampicillin (Ap), 40 μ g/ml; and tetracycline (Tc), 12.5 μ g/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 $4,000 \times g$ 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 μ l 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 μ l of bovine pancreas RNase (5 μ g/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 I. Bacterial strains and plasmids used in this work

Strains and plasmid	Relevant characteristics	Reference and/ or Source
<i>S. flexneri</i>		
9268N	Serotype 1b, invasion plasmid+, cryptic plasmids, virulent	(24)
HW1002	A large plasmid-cured derivative of 9268N, avirulent	(24)
HW283	Serotype 2a, invasion plasmid+, cryptic plasmids, virulent	(35)
<i>S. sonnei</i>		
HW383	<i>nad</i> , pSS120, cryptic plasmids, virulent	(35)
<i>S. dysenteriae</i>		
HW257	invasion plasmid+, cryptic plasmids, virulent	(35)
<i>E. coli</i> K-12		
MC1061	<i>hsdR mcrB araD139 Δ(araABC-leu)7679 ΔlacX74 galU galK rpsL thi</i>	(23)
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (Δlac-proAB)</i> F' [<i>traD36 proAB⁺ lacI^q lacZΔM15</i>]	(23)
MB76	MC1061 carrying pHB76; Km ^r Ap ^r	this work

Table I continuing

Plasmid		
pACYC184	p15A replicon vector; Cm ^r Tc ^r	(36)
pSU19	pACYC184-derived vector carrying the same multicloning sites and <i>lacZ</i> with pUC19; Cm ^r	(37)
pHSG595	pSC101 replicon vector containing multicloning sites; Cm ^r	Hoshimoto-Gotoh (a gift)
pJK1142	pJK292 (an F plasmid derivative vector) carrying a 37-kb invasion region from virulence plasmid pSS120 of <i>S.sonnei</i> HW383; <i>invE</i> ⁺ , <i>virF</i> ⁻	(6)
pHB76	pJK1142 <i>ipaB</i> ::Tn3- <i>lac</i> , noninvasive; Km ^r , <i>invE</i> ⁺ , <i>virF</i> ⁻	(17)
pHB118	pJK1142 <i>ipaC</i> ::Tn3- <i>lac</i> , noninvasive; Km ^r , <i>invE</i> ⁺ , <i>virF</i> ⁻	(17)
pHB37	pJK1142 <i>invE</i> ::Tn3- <i>lac</i> ; Km ^r Ap ^r	(17)
pHW735	pJK282 (an F plasmid derived vector)+ a <i>Ban</i> HI fragment carrying most of <i>ipaB</i> ::Tn3- <i>lac</i> of pHB76; Ap ^r Km ^r , <i>invE</i> ⁻	(17)
pHW736	pJK280 (an F plasmid derived vector)+ a <i>Hind</i> III fragment carrying a region of <i>invE</i> ::Tn3- <i>lac</i> of pHB37; Ap ^r Km ^r	(17)
pHW741	pHSG595 + a 3.5-kb <i>Hind</i> III fragment containing <i>invE</i> ; Cm ^r , <i>invE</i> ⁺	(17)
pCHR82	A derivative of pCHR71 transposed by Tn5-TC1; Tc ^r	(26)
pHW771	pHSG595 + a 2-kb <i>Sma</i> I DNA fragment containing <i>virF</i> region; Cm ^r , <i>virF</i> ⁺	this work
pQ446	pACYC184 + a 11-kb <i>Sau</i> 3A-DNA fragment from chromosome of HW1002 strain; Cm ^r	this work
pQS613	A derivative of pQ446 deleting a 4.2-kb <i>Hind</i> III-DNA fragment; Cm ^r	this work
pQSH03	pSU19 + a 3.2-kb <i>Hind</i> III-DNA fragment of pQ446; Cm ^r	this work
pQSH02	pSU19 + a 2.6-kb <i>Hind</i> III-DNA fragment of pQ446; Cm ^r	this work
pQSS01	pSU19 + a 2-kb <i>Sal</i> I-DNA fragment of pQSH02; Cm ^r	this work
pQD01	A deletion mutant of pQSH02 constructed by unidirectional exonuclease III digestion; Cm ^r	this work
pQSK201	A derivative of pQSH02 deleting a 0.6-kb <i>Pst</i> I-DNA fragment + Km cassette; Cm ^r , Km ^r	this work

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 *Sua*3A. 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 *Bam*HI 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 *Hind*III, self-digested and transformed into JM109. Resultant transformants were analyzed for the presence of *Hind*III fragments. pQ615, pQ612, and pQ613 lost some of *Hind*III fragments from pQ446 (Fig. 3). A 3.2-kb DNA fragment was extracted from agarose gel loaded with *Hind*III-partially digested pQ613 DNA and ligated into a *Hind*III site of pSU19. A plasmid, named pQSH3, carrying contiguous 2.6- and 0.6-kb *Hind*III fragments was selected (Fig. 3). pQSH02 was a derivative of pQSH03 losing a 0.6-kb *Hind*III fragment. pQSS01 was constructed by ligating a 2-kb *Sal*I fragment (one site of *Sal*I is from the insert and the other site from the vector; see Fig. 3) of pQSH02 into a *Sal*I 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 *Pst*I fragment of pQSH02 and inserting the kanamycin-resistance cassette with a *Pst*I 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 Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 50 mM β -mercaptoethanol). Five hundred microliters of the bacteria suspension was mixed with 20 μl of 0.05% sodium dodecyl sulfate (SDS) and 20 μl of CHCl_3 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 Na_2CO_3 . 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: $\{(A_{420}) - 1.75 \times (A_{550})\} \times 1,000 / \{\text{reaction minutes} \times \text{volume of culture used in the assay (ml)} \times (A_{600})\}$.

Analysis with transposon Tn5-Tcl: pQSH03 (Cm^r; chloramphenicol resistance) was transformed into *E. coli* MC1061 which had been transferred with pCHR82 carrying the Tn5-Tcl (Tc^r; 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 Tc^rCm^r bacteria and transformed into MC1061, again. Tc^rCm^r 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 *Pst*I 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 *Eco*RI 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 *Pst*I-DNA fragment of pQSH03 (Fig. 3) by using an ECL hybridization kit (Amersham). The filter was washed in $0.1\times$ 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 *Xba*I and *Sac*I (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 Biosystems, USA). Sequencing was performed on an Applied Biosystems 373A DNA Sequencer using 6% polyacrylamide gel containing 20% (w/v) urea and $1\times$ 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 β -galactosidase, (*ipaB*::Tn3-*lac*) (17). Plasmid pHB76 carrying *ipB*::Tn3-*lac* was transferred into MC1061 to obtain a strain MB76. *Sau*3A-partially digested *S. flexneri* 1b HW1002 (invasion plasmid-less strain) DNA was cloned into a *Bam*HI 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

Fig. 1. Enhancement of IpaBCD protein expression by pQ446. Each bacterial suspension was adjusted to an A_{600} of 0.5. Whole extracts were run on 13% SDS-polyacrylamide gel, and immunoblot analysis was performed with monoclonal anti-IpaB antibody. MC1061 strain carrying pJK1142 and pHW771 (lane 1); pJK1142 and pQ446 (lane 2); pJK1142 and pQ445 (lane 3); and pJK1142 (lane 4). The molecular size (kDa) is indicated on the right.

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

Resident Tn3- <i>lac</i> fusion plasmid in MC1061	β -galactosidase activity (U/ml) in the present of incoming plasmid*		
	no plasmid	pQ446	pHW771(<i>virF</i>)
pHB76 (<i>ipaB</i> ::Tn3- <i>lac</i>)	44	645	3927
pHB118 (<i>ipaC</i> ::Tn3- <i>lac</i>)	63	765	4106
pHW735 (<i>ipaB</i> ::Tn3- <i>lac</i> , <i>invE</i> ⁻)	164	2048	159
pHW736 (<i>invE</i> ::Tn3- <i>lac</i>)	87	76	1864

*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 a *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 *Hind*III fragments (3.2 kb) of pQ446 (the left end of their *Hind*III sites is derived from vector pACYC184, see Fig. 3). The 3.2-kb *Hind*III 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 *Hind*III fragment (Fig. 3). Then, we sequenced the 2.6-kb *Hind*III 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 insertaion 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 *Pst*I 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 *Hind*III 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).

Fig. 3

*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. *Pst*I-DNA fragment (solid bar) shown above pQSH03 was a probe for Southern blot. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sa*II.

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).

Fig. 5. Hybridization analysis of genomic DNA from *Shigella* spp. and *E. coli* strains with a *Pst*I-DNA (*criR*) fragment of pQ446. Chromosomal DNAs were digested with *Hind*III and hybridized under the high-stringency conditions with the probe. Chromosomal DNAs: lane 1, *S. dysenteriae* 1 HW257; 2, *S. flexneri* 2a HW283; 3, *S. flexneri* 1b 9268N; 4, *S. sonnei* HW383; 5, EIEC (O124 serotype); 6, MC1061; 7, ETEC (LT-producing strain); 8, EPEC (O142 serotype); 9, EHEC (O157, Vero toxin-producing strain); 10, *E. coli* B; 11, *E. coli* C.

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 *Pst*I-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 *Hind*III-digested chromosomal DNA of *Shigella* spp., EIEC, ETEC, EPEC, EHEC and *E. coli* K-12. The DNA probe was the 0.6-kb *Pst*I fragment used in the above experiment. A single 3.4-kb *Hind*III 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, NR_I and NifA bind to sites upstream of the regulated promoters and phosphorylation of NR_I 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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