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The Iron- and Temperature-Regulated *cjrBC* Genes of *Shigella* and Enteroinvasive *Escherichia coli* Strains Code for Colicin Js Uptake

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A cosmid library of DNA from colicin Js-sensitive enteroinvasive *Escherichia coli* (EIEC) strain O164 was made in colicin Js-resistant strain *E. coli* VCS257, and colicin Js-sensitive clones were identified. Sensitivity to colicin Js was associated with the carriage of a three-gene operon upstream of and partially overlapping *senB*. The open reading frames were designated *cjrABC* (for colicin Js receptor), coding for proteins of 291, 258, and 753 amino acids, respectively. Tn*7* insertions in any of them led to complete resistance to colicin Js. A near-consensus Fur box was found upstream of *cjrA*, suggesting regulation of the *cjr* operon by iron levels. CjrA protein was homologous to iron-regulated *Pseudomonas aeruginosa* protein PhuW, whose function is unknown; CjrB was homologous to the TonB protein from *Pseudomonas putida*; and CjrC was homologous to a putative outer membrane siderophore receptor from *Campylobacter jejuni*. Cloning experiments showed that the *cjrB* and *cjrC* genes are sufficient for colicin Js sensitivity. Uptake of colicin Js into sensitive bacteria was dependent on the ExbB protein but not on the *E. coli* K-12 TonB and TolA, -B, and -Q proteins. Sensitivity to colicin Js is positively regulated by temperature via the VirB protein and negatively controlled by the iron source through the Fur protein. Among EIEC strains, two types of colicin Js-sensitive phenotypes were identified that differed in sensitivity to colicin Js by 1 order of magnitude. The difference in sensitivity to colicin Js is not due to differences between the sequences of the CjrB and CjrC proteins.

Some strains of *Escherichia coli* and related members of the family *Enterobacteriaceae* produce antibacterial proteins called colicins (6, 34, 41). Colicins are active on sensitive strains of the same family and preferably on strains within the same species (32, 33). Besides colicin synthesis, colicinogenic strains code for immunity proteins that specifically inhibit the action of the colicin types they produce. Colicin synthesis is believed to provide producer bacteria a selective advantage over noncolicinogenic, sensitive strains (35). Genes for colicin synthesis are plasmid encoded, with the possible exception of bacteriocin 28b of *Serratia marcescens* (44).

Growth-inhibitory effects of colicins are limited to sensitive bacterial strains which possess specific outer membrane receptor proteins. Colicins bind to bacterial receptors whose primary function is often to facilitate the uptake of nutrients (e.g., vitamin B_{12}, ferric siderophores). In this respect, colicins resemble bacteriophages, which also appropriate receptor proteins to infect a sensitive bacterium. Several outer membrane receptors were identified as colicin receptors (e.g., BtuB, FepA, FhuA, Tsx, and OmpA). Some colicins show additional dependence on lipopolysaccharide molecules and outer membrane porins (12, 40). This complex dependence on outer membrane components is characteristic for colicins taken up by the Tol system. Ton-dependent colicins bind to individual outer membrane proteins, with the exception of colicins 5 and 10, which show dependence on both the Tsx and TolC outer membrane proteins (28, 29). Receptor binding is followed by translocation of the colicin molecule through the cell envelope by either the Ton or the Tol system (4, 43).

Colicin Js is a polypeptide toxin (molecular mass, 10.4 kDa) originally identified as a product of *Shigella sonnei* (1). Compared to other colicins, colicin Js has a unique antimicrobial spectrum, being active only on enteroinvasive serotypes of *E. coli* and *Shigella* strains able to produce a positive reaction in the Serény test, an experimental keratoconjunctivitis in rabbits or guinea pigs. Enteroinvasive *E. coli* (EIEC) serotypes not sensitive to colicin Js were reported to be negative in this enteroinvasiveness test (19).

Some *Shigella* and EIEC strains cause a bacillary dysentery characterized by bacterial invasion of the colonic and rectal mucosa. The enteroinvasiveness phenotype of these strains is associated with a 230-kb virulence plasmid coding for the majority of genes that contribute to the enteroinvasiveness phenotype (38). Entry of bacteria into host cells is followed by lysis of the internalized vacuole, bacterial growth in the cytoplasm, and infection of adjacent cells in the intestinal mucosa (37, 39). The enteroinvasiveness phenotype is regulated by the *virF-virB* regulatory cascade, which is expressed at 37°C and repressed at 30°C (11).

This communication describes the identification of EIEC genes coding for colicin Js sensitivity, iron and temperature regulation of these genes, and comparisons of the primary structures of the genes from two EIEC strains and one *Shigella* strain.

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MATERIALS AND METHODS

Media. Bacterial strains were grown at 37°C in TY medium containing (per liter) 8 g of Bacto Tryptone (Difco Laboratories), 5 g of yeast extract, and 5 g of

NaCl (pH 7). For selection and maintenance of plasmids, we added (per milliliter of liquid medium or 1.5% [wt/vol] TY agar) 25 μg of chloramphenicol, 100 μg of ampicillin, or 25 μg of kanamycin. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were used at 0.5 mM and 80 μg ml^{-1}, respectively. The iron-chelating compound 2,2′-dipyridyl was added to TY plates at 0.2 mM. *Campylobacter* strains were grown on Campylobacter CSM Selective Medium plates (Becton Dickinson Biosciences, Sparks, Md.).

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Colicin Js producer strain *S. sonnei* type 7, colicin Js-sensitive *S. sonnei* 17 (colicin type 6), and EIEC strain O164 were kindly provided by J. Šmarda, Brno, Czech Republic. Strains of the genera *Acinetobacter*, *Campylobacter*, *Enterobacter*, *Pasteurella*, *Klebsiella*, *Morganella*, *Pseudomonas*, *Salmonella*, *Serratia*, and *Shigella*, as well as EIEC and enteropathogenic *E. coli* strains, were from the strain collection of B. Murray, University of Texas Houston Medical School. *Mannheimia*, *Yersinia*, two *Pseudomonas* strains, and one *Pasteurella* strain were from the Czech Collection of Microorganisms, Brno, Czech Republic. *Vibrio cholerae* and some *Salmonella* strains were from the strain collection of this laboratory. The *E. coli*, EIEC, and *Shigella* strains used in this study are shown in Table 1. *Campylobacter* strains were grown at 42°C on TY or CSM plates under microaerophilic conditions generated by the CampyPak Microaerophilic System (Becton Dickinson Biosciences).

Crude colicin preparations. Cells from the TY cultures of colicinogenic strains (producers of colicins Js, 5, 10, E2, and U) induced by mitomycin C (0.5 μg ml^{-1}; Sigma) were harvested, resuspended in distilled water, washed, and sonicated. The sonicates were used as crude colicins. *E. coli* LMG194, containing a plasmid (pDS83) with the *cja* gene under the control of the *lac* promoter, was induced at an optical density at 600 nm (OD_{600}) of 0.4 by addition of 1 mM IPTG and then cultivated for an additional 4 h at 37°C. Cells were subsequently harvested, washed, and lysed.

Determination of sensitivity to colicin Js and colicin activity assays. Colicin Js producer bacteria were inoculated onto agar plates with a single stab and subsequently grown for 48 h at 37°C. After that, plates were exposed to chloroform vapor for 30 min to lyse the producer bacteria and then overlaid with 100 μl of colicin Js-sensitive bacteria in 3 ml of 0.75% (wt/vol) TY agar. After overnight cultivation at 37°C, bacteria sensitive to colicin Js formed a zone of growth inhibition around the colicin Js producer.

Colicin Js activity was tested by spotting 10-fold dilutions of colicin-containing crude cell lysates on agar plates seeded by sensitive bacteria; TY agar plates were overlaid with 3 ml of 0.75% (wt/vol) TY agar with 100 μl of an overnight culture of indicator bacteria. Each experiment was performed at least three times, and the data reported are averages of three independent measurements. Colicin dilutions causing both clear zones (complete inhibition of sensitive bacteria) and turbid zones (any detectable form of growth inhibition) were used for description of colicin activity.

Tn*phoA* transposon mutagenesis. EIEC strain O164 was grown to an OD_{600} of 0.5 and mixed with λTn*phoA* at a multiplicity of infection equal to 1. Phages were allowed to attach to sensitive bacteria for 30 min at 30°C, and the bacteria were subsequently grown for 4 h at 30°C. Aliquots were plated on TY plates supplemented with kanamycin. Colicin Js-resistant colonies were isolated, and their resistance phenotype was tested with Tol-dependent colicin E2 and Ton-dependent colicin 5 or 10. Strain O164Tn10, which is resistant to colicin Js, was used for further characterization. Genomic DNA of EIEC strain O164Tn10 with a Tn*phoA* insertion was digested with *Bam*HI, cutting once inside Tn*phoA*, as well as in the flanking bacterial DNA. To allow PCR amplification, ends of digested DNA were ligated to each other and ligation products were used for subsequent PCR amplification with primers GW472 and GW473 by using priming sites on Tn*phoA* and directing DNA synthesis toward the outside of Tn*phoA*. The PCR product was cloned in pCR2.1 and sequenced, and the point of Tn*phoA* insertion was identified.

Construction of a genomic library of EIEC strain O164 in *E. coli* VCS257 and screening for colicin Js sensitivity. Genomic DNA of EIEC strain O164 was partially digested with restriction enzyme *Sau*3AI, and 25- to 50-kb fragments were isolated and ligated into cosmid pBeloBAC11. Two microliters of ligation solution was mixed with 25 μl of Gigapack III Gold Packaging Extract (Stratagene) and incubated at room temperature (22°C) for 2 h. Subsequently, 500 μl of autoclaved SM buffer (5.8 g of NaCl, 2.0 g of $MgSO_4 \cdot 7H_2O$, 50 ml of 1 M Tris-HCl [pH 7.5], and 5.0 ml of 2% [wt/vol] gelatin in 1 liter of deionized water) was added to tubes together with 20 μl of chloroform. The cosmid packaging reaction mixture was then diluted 10-fold, and 25 μl was mixed with 25 μl of VCS257 bacteria, resuspended in 10 mM $MgSO_4$ at an OD_{600} of 0.5, and cultivated at room temperature for 30 min. For expression of antibiotic resistance, 200 μl of TY medium was subsequently added and bacteria were cultivated for 1 h at 37°C, inoculated onto TY plates supplemented with chloramphenicol, and incubated overnight at 37°C. Individual colonies (more than 1,000) were picked, and their sensitivity to colicin Js was tested by streaking them across the region of a TY plate containing dried crude colicin Js. Strains showing inhibition of growth in the colicin Js-containing region were tested for colicin Js sensitivity by using a colicin activity assay.

Southern blot analyses. Chromosomal or plasmid DNA was digested with *Eco*RI or *Hin*dIII and electrophoresed on a 0.8% agarose gel. DNA was subsequently transferred to a nylon membrane by a standard capillary method. The probes used in Southern blot analyses were prepared by using a Gene Images CDP-Star detection module (Amersham Pharmacia Biotech), and the fluorescein-labeled probes were detected with anti-fluorescein–alkaline phosphatase conjugate. Blocking, hybridization, and detection of hybridized probe were performed in accordance with the manufacturer's recommendations.

Restriction analysis. Standard methods were used for DNA isolation, restriction endonuclease analysis, ligation, and transformation of plasmid DNA (36).

Recombinant DNA methods. Plasmids were isolated and cells were transformed by standard techniques. PCR products were cloned into vector pCR2.1 or pCR2.1-TOPO in accordance with the manufacturer's (Invitrogen) recommendations. Cloning in other vectors was done after restriction digestion of both plasmid (cosmid) DNA and target DNA. Insert DNA was sequenced by using the *Taq* Dye-deoxy Terminator method and a model 377 DNA sequencing system (Applied Biosystems, Foster City, Calif.). The PCR primers used in this study are shown in Table 2.

In vitro transposition. For cosmid mutagenesis, an in vitro Tn7 transposition system (GPS-1 Genome Priming System; New England Biolabs) was used in accordance with the manufacturer's recommendations.

Computer-assisted sequence analysis. Computer-assisted sequence analysis was performed by using programs in the Genetics Computer Group (Madison, Wis.) software package. ProtParam at ExPASy was used for calculations of theoretical polypeptide molecular weight, isoelectric point, etc. Signal sequence prediction (signalp) and protein localization (psort) programs were used.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this study have been deposited in the GenBank database under accession numbers AF283288 to AF283294.

RESULTS

Identification of the region conferring colicin Js sensitivity. A colicin Js-sensitive strain, EIEC strain O164, was subjected to Tn*phoA* transposon mutagenesis, and colicin Js-resistant colonies were isolated. Strains tolerant to colicin Js, defective in translocation of colicin Js (e.g., *exbB* mutants), were excluded by testing for sensitivity to colicins 5 and 10. A strain specifically resistant to colicin Js was identified. The point of the Tn*phoA* insertion was identified by DNA sequencing of plasmid pDS144 (see Materials and Methods and Table 1). The insertion was localized 26 nucleotides upstream of the start codon of *senB* (23). The *senB* gene codes for the TieB product, which may have some role in enterotoxin production by EIEC strains. However, cloning of *senB* into pCR2.1 (pDS154) was not associated with acquisition of colicin Js sensitivity.

Because the gene (or genes) for the colicin Js receptor could be near *senB*, another approach was undertaken. A cosmid library of DNA from sensitive strain *E. coli* O164 was made in resistant strain *E. coli* VCS257, and eight colicin Js-sensitive clones were identified. In all of them, the *senB* gene was detected. The restriction map of one cosmid, pDS150, with a 40-kb insert from EIEC strain O164 in pBeloBAC11 conferring sensitivity to colicin Js is shown in Fig. 1. Cosmid DNA of pDS150 was subcloned into the pBluescript SK(+) plasmid by using *Eco*RI and *Pst*I restriction sites, resulting in plasmids pDS173, pDS174, pDS175, pDS177, pDS179, pDS180, and pDS186 (Fig. 1). The ends of the inserts were sequenced.

The colicin Js receptor coding region is flanked upstream

TABLE 1. Bacterial strains, plasmids, and bacteriophage used in this study

Strain, plasmid, cosmid, or bacteriophage	Genotype and/or phenotype	Source or reference
Escherichia coli		
K-12 strains		
5K	*hsdR lacZ rpsL ser thi thr*	G. Schrempf
VCS257	Derivative of DP50 *supF*	Stratagene
LMG194	K-12 Δ*lacX74 galE thi rpsL* Δ*phoA* (*Pvu*II) Δ*ara714 leu*::Tn*10*	Invitrogen
189 BM	pColE2-P9	B. Stocker
BN4020	*thr araC* Δ(*argF-lac*) *tsx* qsr′ *glnV44 fur-1*::Tn*5 galK* λ⁻ *rac-0 hisG4 rfbD1 mgl rpoS rpsL kdgK xylA mtl argE thi*	CGSC[a]
A592	*tolA fhuA21 lacY1 thi leuB6 supE44 thr-1*	45
TPS13	GM1 *tolQ*	43
A593	*tolB fhuA21 lacY1 thi leuB6 supE44 thr-1*	45
GUC6	*thr-1 leuB6 fhuA21 lacY1 glnV44*(AS) λ⁻ e14⁻ *tonB50 rfbD1 glpR200 thi-1*	18
GUC41	*thr-1 leuB6 fhuA21 lacY1 glnV44*(AS) λ⁻ *rfbD1 exbB41 thi-1 met-96*	18
BL21	F⁻ *hsdS gal*	42
EIEC strains		
O164		J. Šmarda
O143		B. Murray
O164Tn10	Colicin Js-resistant strain with Tn*phoA* insertion	This work
Shigella sonnei	Colicinotype 7: colicin Js producer	1
Shigella sonnei 17	Colicinotype 6: $E6^{im}$ B^{res} M^{res}: colicin Js indicator	1
Shigella flexneri #1	Colicin Js sensitive	B. Murray
Cosmids		
pBeloBAC11	Cosmid vector; F⁻ replicon; Cm^r	H. Shizuya
pDS146	pBeloBAC11 with 40-kb insert from EIEC O164 coding for colicin Js sensitivity	This work
pDS150	pBeloBAC11 with 40-kb insert from EIEC O164 coding for colicin Js sensitivity	This work
pDS153	pBeloBAC11 with 40-kb insert from EIEC O164 coding for colicin Js sensitivity	This work
pDS199	pDS150 with Tn*7* insertion in *cjrC* gene	This work
pDS202	pDS150 with Tn*7* insertion 24 bp upstream from Fur box	This work
pDS219	pDS150 with Tn*7* insertion 9 bp downstream from Fur box	This work
Plasmids		
pBSSK(+)	Phage T7 gene *10* promoter	Stratagene
pCR2.1	Cloning vector for *Taq* polymerase-amplified PCR products	Invitrogen
pCR2.1-TOPO	Cloning vector for *Taq* polymerase-amplified PCR products	Invitrogen
pPD101	Phage T7 gene *10* promoter; *ori* from pSC101	10
pHP10	pBCKS+ carrying *cta cti ctl*	28
pHP20	pBCKS+ carrying *cfa cfi cfl*	29
pDS1	pBCSK+ carrying pColU in *Cla*I restriction site	40
pDS83	pCR2.1 with *cja* in Js3S21-Js3S19 DNA fragment of pColJs	This work
pDS173	pBSSK(+) with 9-kb *Eco*RI fragment from pDS150	This work
pDS174	pBSSK(+) with 14-kb *Eco*RI fragment from pDS150	This work
pDS175	pBSSK(+) with 5.5-kb *Pst*I fragment from pDS150	This work
pDS177	pBSSK(+) with 2-kb *Eco*RI fragment from pDS150	This work
pDS179	pBSSK(+) with 1-kb *Pst*I fragment from pDS150	This work
pDS180	pBSSK(+) with 4.5-kb *Pst*I fragment from pDS150	This work
pDS186	pBSSK(+) with 4-kb *Eco*RI fragment from pDS150	This work
pDS144	pCR2.1 with PCR product of GW472 and GW473 primers amplified after *Bam*HI digestion and religation of EIEC O164Tn10 chromosomal DNA	This work
pDS154	pCR2.1 with *senB* gene (*senB* amplified with SenBU and SenBL primers)	This work
pDS213	pCR2.1-TOPO with *cjrAB* genes (*cjrAB* amplified with PhuWU and TonBL primers)	This work
pDS215	pPD101 with *cjrC* in *Hin*dIII (*cjrC* amplified with HasRUH and HasRLH primers)	This work
pDS221	pDS213 with 0.3-kb intragenic deletion in *cjrB*	This work
pDS222	pDS213 with 0.1-kb intragenic deletion in *cjrA*	This work
pDS253	pCR2.1-TOPO with *virB* amplified with VirBU and VirBL primers from EIEC strain O143	This work
pDS282	pCR2.1-TOPO with *tonB* gene (*tonB* amplified with TonBUEC and TonBLEC primers)	This work
Bacteriophage Tn*phoA*	λ Tn*phoA* derivative *c*I857 *P*am80	22

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)[g]	Position
GW472	CGTGATATTGCTGAAGAGCTTGG	4141–4163[a]
GW473	CGAATAGCCTCTCCACCCAAG	3526–3506[a]
HasRLH	AAAGT*AAGCTT*TCAGGTGGATCGATAGCCATG	4275–4244[b]
HasRUH	TATTT*AAGCTT*TATCCAAGGAAATTTATGAACGTTATAAAA	1899–2028[b]
Js3S19	GGTATAGCTTAGGCAGGATTA	2967–2947[c]
Js3S21	CAGGAGATTATATGATTTCTC	2575–2596[c]
PhuWU	GCGGACCCCATAATATCGGCTGGTAAA	36–62[b]
SenBL	GTTC*AGATCT*TCACGACTTTTTCAAATCAATGCT	1399–1376[d]
SenBU	GTGG*AGATCT*ATGGATATTTGGCGGGGACATTCG	125–148[d]
TonBL	TTACTCCTTCAACTTAAAGGTAATCGG	1845–1819[b]
TonBU	TAAA*GAATTC*ATGATGAATATTCTCCACTTCCCACAG	1069–1095[b]
TonBLEC	GC*GGATCC*TTACTGAATTTCGGTGGTGCC	1056–1036[e]
TonBUEC	GC*GGATCC*ATGATTATGACTTCAATGACC	322–342[e]
VirBL	TTATGAAGACGATAGATGGCG	1189–1169[f]
VirBU	ATGGTGGATTTGTGCAACGAC	260–280[f]

[a] Numbers indicate positions on synthetic transposon Tn*phoA* (22).
[b] Numbers correspond to positions in the *cjrABC* coding region (accession no. AF283288).
[c] Numbers correspond to positions in pColJs (accession no. AF282884).
[d] Numbers indicate positions in the sequence with accession no. Z54195 (23).
[e] Numbers show positions in the sequence with accession no. K00431 (31).
[f] Numbers correspond to positions in the GenBank sequence with accession no. X14340 (2).
[g] Nucleotides in italics correspond to introduced *Bam*HI, *Bgl*II, and *Hin*dIII restriction sites.

with DNA showing sequence similarity to *E. coli* insertion sequences IS*91* and IS*1294* and to *Shigella boydii* IS*1SB*. Downstream from the *cjr* operon is located the *senB* gene, DNA similar to *S. sonnei* IS*629* and *E. coli* IS*1294*, and DNA homologous to enteropathogenic *E. coli* adherence factor plasmid pB171. Regions similar to DNA sequences at 0.5 and 0.7 min of the genome of *E. coli* strain MG1655 were found 25 and 15 kb upstream of the *cjr* operon, respectively. To test whether *cjr* genes are present on the chromosome or virulence plasmid of sensitive bacteria, we isolated chromosomal and plasmid DNAs from EIEC strain O143 and performed Southern blot analysis with three different probes. The *tonB*, *virB*, and *cjrB* probes were prepared by PCR amplification from a single colony of EIEC strain O143 with the TonBUEC-TonBLEC, VirBU-VirBL, and TonBU-TonBL primer pairs (Table 2), respectively. The *tonB* gene was identified in *E. coli* chromosomal DNA (31), while the *virB* gene was shown to be encoded by a virulence plasmid present in *Shigella* and EIEC strains (2). The *tonB* probe hybridized only with chromosomal DNA, while the *virB* and *cjrB* genes hybridized with both plasmid and chromosomal DNAs. Plasmid DNA was thus likely coisolated during the preparation of chromosomal DNA. The identical hybridization patterns of the *virB* and *cjrB* genes suggested that these genes were colocalized in the large virulence plasmid.

Tn*7* in vitro transposition was used for a more detailed analysis of pDS150 cosmid DNA. Tn*7* insertion mutations resistant to Js were found over a region of more than 4 kb upstream of the *senB* gene (Fig. 2). DNA sequencing revealed three open reading frames designated *cjrABC* (for colicin Js receptor; accession number AF283288) and coding for putative proteins of 291, 258, and 753 amino acids, respectively (Fig. 2). Upstream of *cjrA*, a sequence similar to the 19-bp consensus Fur box (5′ GATAATGATAATCATTATC 3′) was found, suggesting regulation of the *cjr* operon by the Fur protein (9).

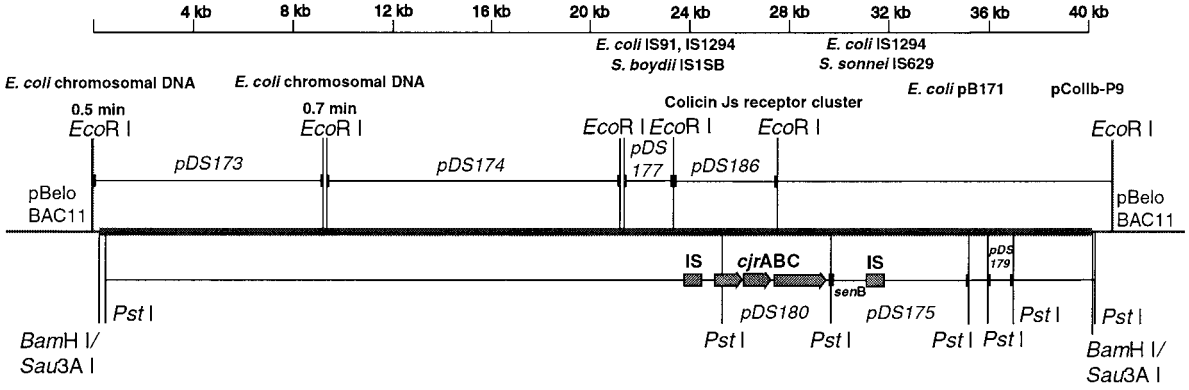

FIG. 1. Restriction map of the 40-kb DNA insert of pDS150. Restriction sites for *Eco*RI and *Pst*I are indicated. *Eco*RI and *Pst*I fragments were subcloned, and the ends of inserts were sequenced. Hits with respect to *E. coli* chromosomal DNA, *E. coli* and *Shigella* IS sequences, and *E. coli* plasmids pB171 and pColIb-P9 are indicated. Subcloned DNA fragments of pDS150 in pBSSK(+) are shown (pDS173-175, pDS177, pDS179-180, and pDS186). The *cjrABC* operon, together with the downstream *senB* gene, is flanked by IS-like sequences.

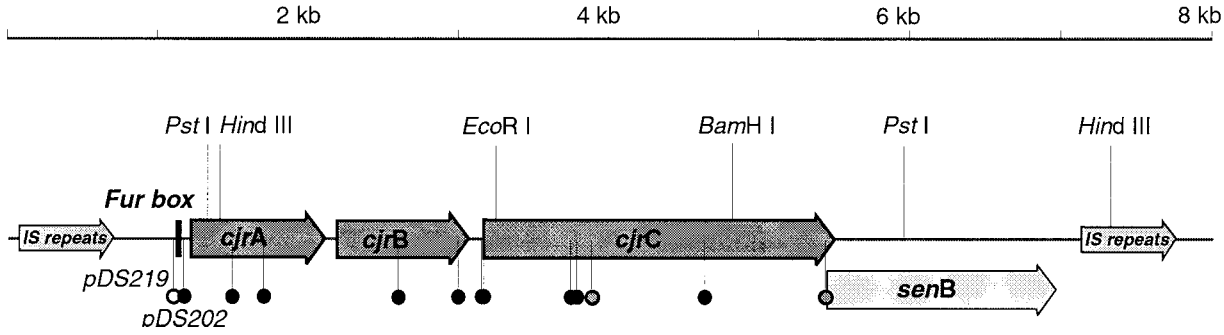

FIG. 2. Genetic organization of the *cjrABC* gene operon. Black (gray) circles indicate Tn*7* (Tn*phoA*) insertions leading to resistance to colicin Js, while the open circle represents a Tn*7* insertion not affecting sensitivity to colicin Js. Restriction sites for *Eco*RI, *Hin*dIII, and *Pst*I are indicated. Insertion of Tn*7* into pDS150 24 bp upstream of the Fur box (pDS219) did not interfere with colicin Js sensitivity, but insertion of Tn*7* 9 bp downstream from the Fur box (pDS202) resulted in complete loss of colicin Js sensitivity. Downstream from the *cjr* genes is located *senB*. Note that the *cjr* and *senB* genes are flanked by IS-like sequences.

All *cjr* genes had the same polarity, with no overlaps between them. The *cjrC* gene partially overlapped the *senB* gene, explaining the phenotype of the original Tn*phoA* insertion 26 bp upstream from the *senB* start codon. Insertion of Tn*7* into each gene, as well as an insertion 9 bp downstream of the Fur box, was associated with complete loss of sensitivity to colicin Js. However, a Tn*7* insertion 24 bp upstream of the Fur box did not interfere with colicin Js sensitivity, localizing the *cjr* promoter sequence downstream from this insertion point. The DNA sequence of the *cjrABC* genes showed no significant homologies to sequences deposited in the GenBank database, but homology was found at the amino acid level (Table 3): the CjrA protein was most homologous to the *Pseudomonas aeruginosa* iron-regulated PhuW protein, whose function is unknown; CjrB was homologous to the TonB protein from *Pseudomonas putida*; and CjrC was homologous to the putative outer membrane siderophore receptor protein from *Campylobacter jejuni.* Sequence similarities of CjrABC proteins to known protein sequences are shown in Table 3. For CjrA, an N-terminal lipoprotein signal sequence (signalp) and an inner-outer membrane localization (psort) were predicted. The CjrB protein was proposed to be localized in the periplasm (psort) with an N-terminal anchor (signalp). CjrC was predicted to be an outer membrane protein (psort).

Cloning of *cjr* genes. To confirm the role of the *cjr* genes as colicin Js receptor determinants and to identify the gene(s) coding for the Js receptor, *cjr* genes were subcloned into two separate compatible plasmids. Since a role for downstream gene *cjrC* in colicin Js sensitivity was more probable than one for *cjrA* and *cjrB*, *cjrC* was cloned in a separate plasmid, pPD101, resulting in pDS215, while the *cjrAB* genes were cloned in pCR2.1-TOPO (pDS213). Clones containing *cjrC* or *cjrAB* alone did not acquire sensitivity to colicin Js. However, *E. coli* BL21 with pDS213 and pDS215 was fully sensitive to colicin Js, indicating that the *cjrC* gene must be present with *cjrA*, *cjrB*, or both. Deletions in pDS213 were prepared in *cjrA* (pDS222; 0.1-kb intragenic deletion of *cjrA*) or *cjrB* (pDS221; 0.3-kb intragenic deletion *cjrB*) and tested with *cjrC*. The results are summarized in Table 4 and show that the *cjrB* and *cjrC* genes are sufficient for colicin Js sensitivity.

Uptake of colicin Js by sensitive bacteria. To test whether colicin Js is taken up by the Ton or Tol system, we examined the sensitivities of different *E. coli* mutants to colicin Js. Because of the unique antimicrobial spectrum of colicin Js, *E. coli*

TABLE 3. Sequences producing significant alignments with *cjrABC* by BLASTx

Gene	BLASTx hit	Accession no.	Organism	Expect value	Reference(s)
cjrA	Hypothetical protein PhuW	AF055999	*Pseudomonas aeruginosa*	8e-54	24
	Putative lipoprotein	AL139074	*Campylobacter jejuni*	4e-28	27
	Putative protein	AE000742	*Aquifex aeolicus*	5e-04	8
cjrB	TonB protein	X70139	*Pseudomonas putida*	4e-16	3
	Possible TonB transport protein	AL139074	*Campylobacter jejuni*	2e-14	27
	TonB protein	AF070473	*Pasteurella multocida*	2e-06	Unpublished
	TonB-like protein	AF119047	*Vibrio parahaemolyticus*	9e-05	25
	TonB protein	U04996	*Haemophilus influenzae*	5e-04	21
cjrC	Putative outer membrane siderophore receptor	AL139074	*Campylobacter jejuni*	e-129	27
	Putative outer membrane substrate binding protein	AL162756	*Neisseria meningitidis*	6e-62	26
	Probable TonB-dependent receptor HI1217 precursor	U32801	*Haemophilus influenzae*	3e-48	16
	Heme receptor HasR	AF127223	*Pseudomonas aeruginosa*	6e-43	Unpublished
	HasA receptor protein	AB023289	*Pseudomonas fluorescens*	1e-36	20
	HasR	Y08983	*Serratia marcescens*	1e-34	17

TABLE 4. Sensitivity of *E. coli* BL21 carrying plasmids to colicin Js

Plasmid(s)	Gene(s) introduced	Colicin Js sensitivity
None		R[b]
pDS213	*cjrAB*	R
pDS215	*cjrC*	R
pDS213, pDS215	*cjrABC*	4[a]
pDS221, pDS215	*cjrAC*	R
pDS222, pDS215	*cjrBC*	4

[a] The number indicates the highest colicin dilution still active on *E. coli* BL21 bacteria; e.g., 4 = 1:10^4 dilution.
[b] R, resistant.

mutants were transformed with pDS146, a cosmid with a DNA fragment from EIEC strain O164 that confers sensitivity to colicin Js. The results are summarized in Table 5. An *exbB* mutant was insensitive to colicin Js, while *tonB* mutants and all tested *tol* mutants were sensitive to an extent similar to that of control strain *E. coli* 5K(pDS146). Introduction of the *E. coli* K-12-derived *tonB* gene (cloned on plasmid pDS282) into strain *E. coli* GUC6 *tonB*(pDS146) resulted in a 10-fold decrease in colicin Js sensitivity (Table 5). This fact is consistent with decreased iron starvation as a result of complementation of *tonB* function.

Sensitivities of indicator strains to colicin Js at different temperatures. Expression of genes specific for EIEC and *Shigella* strains was shown to be temperature regulated (11). The enteroinvasiveness phenotype is observed at 37°C but not at 30°C. We tested the sensitivities of colicin Js indicator strains to colicin Js at different temperatures. The results are summarized in Table 6. *S. sonnei* 17 was most sensitive to colicin Js at 37°C, less sensitive at 30°C, and even less sensitive at 22°C. The decrease in colicin Js sensitivity was most obvious in the decrease in the dilution causing complete growth inhibition of the indicator bacteria (clear zones of growth inhibition). At 22°C, no clear zones were formed. Similar results were obtained with EIEC strain O164, where the decrease in sensitivity was even greater. EIEC strain O143 showed results similar to those of EIEC strain O164, with lower initial sensitivity to colicin Js. On the other hand, the sensitivity of *E. coli* 5K to colicin U was not changed when it was grown at 37 or at 22°C. *E. coli* 5K(pDS146) showed the same sensitivity to colicin Js at all of the temperatures tested, indicating that the uptake of colicin Js was not regulated in this non-EIEC strain and that the growth-inhibitory activity is not affected by temperature.

TABLE 5. Sensitivities of *tol, tonB*, and *exbB* mutants to colicin Js

Strain	Relevant genotype	Colicin Js active dilution
5K(pDS146)		1 (4)[a]
A592(pDS146)	*tolA*	2 (4)
TPS13(pDS146)	*tolQ*	2 (4)
A593(pDS146)	*tolB*	2 (4)
GUC6(pDS146)	*tonB*	3 (4)
GUC41(pDS146)	*exbB*	T[b]
GUC6(pDS146)(pDS282)	*tonB*$^+$	2 (3)

[a] The numbers indicate the highest colicin dilution that resulted in a clear zone of growth inhibition and the last dilution that resulted in turbid zones (in parentheses) on a lawn of sensitive bacteria; e.g., 4 = 10^4.
[b] T, tolerant.

TABLE 6. Sensitivities of indicator strains to colicins Js and U at different temperatures

Strain[b]	Colicin Js activity on indicator bacteria at:			Colicin U activity on indicator bacteria at:	
	37°C	30°C	22°C	37°C	22°C
Shigella sonnei 17	2 (4)[a]	1 (4)	— (3)	NT[c]	NT
EIEC O164	1 (4)	— (3)	— (2)	— (—)	— (1)
EIEC O143	0 (3)	— (2)	— (1)	NT	NT
E. coli 5K(pDS146)	1 (3)	1 (3)	1 (3)	NT	NT
E. coli 5K	NT	NT	NT	2 (3)	2 (3)

[a] The numbers indicate the highest colicin dilution that resulted in a clear zone of growth inhibition and the last dilution that resulted in turbid zones (in parentheses) on a lawn of sensitive bacteria; e.g., 4 = 10^4. —, no growth inhibition.
[b] *E. coli* K-12 strain 5K was used as a colicin U indicator strain or transformed with a cosmid conferring sensitivity to colicin Js (pDS146) to indicate colicin Js activity.
[c] NT, not tested.

Role of the *virB* gene in regulation of sensitivity to colicin Js. Regulation of colicin Js sensitivity by temperature was not observed in the *E. coli* 5K strain with the *cjr* operon on cosmid pDS146. Since many of the genes responsible for the enteroinvasiveness phenotype were shown to be regulated by the VirB protein (11), we tested the sensitivities of strains carrying cloned *virB* to colicin Js at different temperatures. By using PCR amplification, we cloned *virB* from EIEC strain O143(pDS253) (Tables 1 and 2). When cloned in the pCR2.1-TOPO plasmid, the *virB* gene was oriented under the control of the T7 promoter. Although the EIEC strains did not contain T7 RNA polymerase, "background" transcription of the *virB* genes in this high-copy-number plasmid was sufficient to produce increased colicin Js sensitivity. The EIEC O143 strain grown at 25°C was at least 2 orders of magnitude less sensitive to colicin Js than when it was grown at 37°C. The same strain with the *virB* gene in plasmid pDS253 grown at 25°C was as sensitive to colicin Js as EIEC strain O143 grown at 37°C. No effect of pDS253 was observed for EIEC strain O143 grown at 37°C. The sensitivity to colicin Js was thus regulated by the *virB* gene, and the regulatory effect of cloned *virB* was detectable only when bacteria were grown at room temperature. At this temperature, the VirF-VirB regulatory cascade of enteroinvasive bacteria was shown to be turned off (11). Sequencing of the *virB* gene from EIEC strain O143 (accession no. AF283290) revealed a DNA sequence identical to that previously described for *virB* from *Shigella flexneri* (2).

To more closely characterize the promoter region of the *cjr* genes, we tested the increase in sensitivity to colicin Js after the introduction of *virB* in pDS253 into *E. coli* 5K carrying pDS146 or pDS219. pDS219 is the pDS150 cosmid with a Tn*7* insertion 24 bp upstream from the Fur box. The results are shown in Table 7. Introduction of the *virB* gene on pDS253 increased the sensitivity of strains with cosmid pDS146 by 1 order of magnitude. In contrast, the sensitivity of strain 5K(pDS219) remained the same with or without pDS253. These results indicate that the site of interaction between the *cjr* promoter and the VirB protein was in the region of the Tn*7* insertion in pDS219 or further upstream.

Because of the different sensitivities of EIEC strains O164 and O143, we also sequenced the *cjr* promoter region in EIEC strain O143. DNA sequencing revealed identical sequences in

TABLE 7. Role of *virB* in Js sensitivity

Strain	Relevant genotype	Colicin Js activity on indicator bacteria with:	
		No plasmid	pDS253
E. coli 5K(pDS146)	*cjr* genes on cosmid pBeloBAC11	1 (3)[a]	2 (4)
E. coli 5K(pDS219)	Tn*7* insertion in promoter of *cjr* on pBeloBAC11	1 (3)	1 (3)

[a] The numbers indicate the highest colicin dilution that resulted in a clear zone of growth inhibition and the last dilution that resulted in turbid zones (in parentheses) on a lawn of sensitive bacteria; e.g., $3 = 10^3$.

both strains for more than 300 bp upstream from the *cjrA* start codon (data not shown). The different sensitivities of these strains to colicin Js were thus not due to differences in promoter structure.

Sensitivity of *E. coli* strains to colicin Js under iron-depleted conditions. To test the hypothesis that *cjr* genes are regulated by the Fur protein, we determined the sensitivities of bacteria to colicin Js under standard and iron-depleted conditions (Table 8). The presence of the iron-binding compound dipyridyl increased the colicin Js sensitivity of both EIEC strains, despite their different sensitivities to colicin Js in the presence of iron. Under iron-limited conditions, they reached the same sensitivity. The same sensitivity to colicin Js under iron-depleted conditions was observed also for *E. coli* strains BL21 and 5K transformed with cosmids with the *cjr* operon (pDS146 and pDS153, respectively). The difference between the sensitivities of EIEC strains O164 and O143 to colicin Js was thus likely due to a difference between the basal levels of iron regulation of the *cjr* operon in these strains. Increased colicin Js sensitivity was observed for *E. coli* BN4020 *fur*(pDS146) under standard conditions (Table 8), indicating negative transcriptional regulation of the *cjr* operon by the Fur protein.

Sensitivities of different gram-negative pathogenic bacterial strains to colicin Js. Since *cjrBC* showed homology to protein products from other bacteria, we tested the sensitivities of some gram-negative bacteria to colicin Js (Table 9). Despite the similarity of CjrBC proteins to gene products of other bacteria, no bacterial strains other than EIEC and *Shigella* strains were found to be sensitive to Js. The action of colicin Js seems to be specific for EIEC and *Shigella* strains, resembling the narrow inhibition spectrum of other colicins. Three out of six EIEC strains sensitive to colicin Js were as highly sensitive to colicin Js as EIEC strain O164; the other three were less sensitive to colicin Js (as sensitive as EIEC strain O143). EIEC strains thus appear to have two types of colicin Js sensitivity phenotype (data not shown).

TABLE 8. Sensitivities of *E. coli* strains to colicin Js under iron-depleted conditions

Strain	Plasmid	Colicin Js sensitivity in TY	Colicin Js sensitivity in TYD[a]
EIEC O143		0 (3)[b]	3(4)
EIEC O164		1 (4)	3(4)
E. coli 5K	pDS146	1 (3)	3(4)
E. coli BL21	pDS153	1 (3)	3(4)
E. coli BN4020	pDS146	3 (4)	NT[c]

[a] TYD is TY with 0.2 mM dipyridyl.
[b] The numbers indicate the highest colicin dilution that resulted in a clear zone of growth inhibition and the last dilution that resulted in turbid zones (in parentheses).
[c] NT, not tested.

Sequence homology of CjrB and CjrC from two EIEC strains and *S. flexneri*. The *cjrBC* genes from EIEC strain O143 and *S. flexneri* #1 were PCR amplified by using the TonBU-TonBL and HasRU-HasRL primer pairs (Table 2), respectively. The PCR products were subsequently sequenced, and the sequences were compared to the *cjrBC* sequence from EIEC strain O164. Since PCR primers recognizing the first 27 and last 24 nucleotides of *cjrB* were used for *cjrB* amplification from bacterial DNA, minor changes in primer binding sequences would not be detected. The *cjrB* gene from EIEC strain O143 (accession no. AF283291) differed from *cjrB* of EIEC strain O164 by one base pair, which does not change the amino acid sequence of CjrB. *cjrB* from *S. flexneri* #1 (accession no. AF283292) differed from the *cjrB* gene from EIEC strain O164 by two base pairs, but this does not change any of the amino acids of CjrB. In the case of *cjrC*, primers recognizing the first 15 nucleotides and primers binding downstream of *cjrC* were used for PCR amplification. The *cjrC* gene from EIEC strain O143 (accession no. AF283293) was completely identical to *cjrC* of EIEC strain O164. *cjrC* from *S. flexneri* #1 (accession no. AF283294) differed from *cjrC* of EIEC strain O164 by two nucleotides, changing one amino acid (T734 to S734). Since the CjrB and CjrC proteins from EIEC strains O164 and O143 were identical, the difference between the sensitivities of the two strains to colicin Js was not due to differences between their *cjrCB* genes.

DISCUSSION

Independent approaches identified the region involved in colicin Js sensitivity near and upstream from the *senB* gene. *senB* was previously identified in a cosmid clone with DNA from EIEC strain EI34 (23). The *senA* gene, located on the same cosmid, was shown to be encoded on the large virulence plasmid in EIEC and *Shigella* strains (23). Although the distance between the *senA* and *senB* genes is not known, a polar effect on *senA* of a Tn*phoA* insertion into the *senB* gene was proposed (23). However, we did not find the *senA* gene in an 8-kb region downstream from *senB* (data not shown). This might reflect a more than 8-kb distance between the *sen* genes, or it might be explained as a result of EIEC strain differences. The hybridization pattern of the *cjrB* gene was found to be the same as that of the plasmid-encoded *virB* gene and different from that of the chromosome-encoded *tonB* gene. These data are consistent with the localization of the *cjr* operon on the large virulence plasmid of this enteroinvasive strain. The *cjr* genes were found to be flanked on both ends by direct repeats and sequences producing significant similarity to bacterial insertion sequences, suggesting DNA rearrangements near the *cjr* genes.

Tn*7* in vitro mutagenesis of cosmid DNA revealed three *cjr* genes in a 4-kb region. Insertion of Tn*7* into any of these genes or into the promoter region upstream of *cjrA* resulted in complete loss of sensitivity to colicin Js. Since cloning of *cjr* genes showed that *cjrB* and *cjrC* are sufficient to mediate colicin Js sensitivity, insertion of Tn*7* into *cjrA* likely led to colicin Js resistance due to a polar effect. CjrA was homologous to an iron-regulated hypo-

TABLE 9. Sensitivities of different gram-negative pathogenic bacterial strains to colicin Js

Organism	No. of strains	
	Investigated	Sensitive to Js
Acinetobacter sp.	10	
Campylobacter jejuni	10	
EIEC	17	6
Enterobacter sp.	19	
EPEC[a]	10	
Klebsiella sp.	17	
Mannheimia (*Pasteurella*) *haemolytica*	2	
Morganella sp.	4	
Pasteurella multocida	2	
Pseudomonas aeruginosa	2	
Pseudomonas putida	1	
Salmonella enterica serovar Typhi	2	
Salmonella enterica serovar Typhimurium	5	
Salmonella sp.	5	
Serratia sp.	7	
Shigella flexneri	5	3
Vibrio cholerae	1	
Yersinia enterocolitica	1	

[a] EPEC, entropathogenic *E. coli*.

thetical protein from *P. aeruginosa.* The function of CjrA protein remains unknown. The presence of an N-terminal lipoprotein signal sequence in CjrA suggests that the protein is exported from the cell cytoplasm. Based on sequence predictions, CjrA might be an inner membrane lipoprotein.

CjrB showed similarities to TonB proteins from some gram-negative pathogens, and in accordance with this, the CjrB protein was predicted to be a periplasmic protein anchored to the inner membrane. Colicin Js uptake requires at least one component of the Ton system, protein ExbB. Since *E. coli tonB* strains with *cjrBC* genes are fully sensitive to colicin Js, CjrB appears to be an EIEC-specific TonB protein homolog.

CjrC is similar to outer membrane receptors involved in siderophore or heme binding by gram-negative bacteria. Based on sequence similarities and sequence prediction, CjrC appears to be an outer membrane receptor for colicin Js. Ton-dependent colicins (group B) were shown to have a pentapeptide sequence near the N terminus called the TonB box. This sequence was proposed to be responsible for interaction with the TonB protein and for Ton-dependent translocation through the cell envelope. The TonB box of colicin B is a DTMVV sequence, and its introduction into the colicin U molecule resulted in TonB-dependent uptake of colicin U (30). Similar sequences were also identified near the N termini of TonB-dependent outer membrane proteins, and their function in the receptor protein-TonB interaction was described (5, 7). The absence of the TonB box in either the colicin Js polypeptide or the CjrC protein might be explained by their functional *tonB* independence. Different amino acid residues of colicin Js and CjrC might be involved in the interaction with CjrB. However, no significant homology was found within colicin Js and the N terminus of CjrC.

The sensitivity of EIEC and *Shigella* strains to colicin Js is temperature dependent, being greater when the bacteria are cultured at 37°C. The invasion phenotype of EIEC and *Shigella* strains is also temperature regulated, being expressed only when the bacteria are cultivated at 37°C and disappearing at 30°C. The invasion genes located on a large virulence plasmid are regulated by the products of the *virF* and *virB* genes (2). The *virB* gene is activated by the *virF* gene product, and the VirB protein positively regulates the transcription of the majority of invasion genes (11). *E. coli* strains with cosmid DNA conferring colicin Js sensitivity did not show this type of temperature-dependent sensitivity, indicating that the regulatory component is specific for EIEC and *Shigella* strains. Indeed, introduction of the *virB* gene expressed from the plasmid promoter into EIEC strain O143 increased the sensitivity of this strain to colicin Js at room temperature to the same extent as culturing of the bacteria at 37°C. Moreover, colicin Js sensitivity was increased by *virB* even for *E. coli* strains with *cjr* genes on a cosmid. The positive regulation of the *cjr* operon by the *virF-virB* regulatory cascade might suggest the involvement of *cjr* genes in the invasiveness phenotype. The effect of VirB in the transcriptional activation of *cjr* genes was blocked by the insertion of Tn7 24 bp upstream from the Fur box. Since the VirB protein is believed to be a DNA binding protein that activates promoters by directly binding to them (11), the VirB binding site(s) may be upstream from the Fur box.

In addition to temperature regulation, transcription of *cjr* genes is negatively regulated by the level of available iron. This regulation is mediated by the Fur protein. The Fur binding site before the *cjr* genes differed by four nucleotides from the consensus Fur binding palindromic sequence (9). More recently, the Fur consensus box was reinterpreted as a combination of three repeats of the 5′NAT(A/T)AT3′ motif (13). In this case, the *cjr* Fur box differs by three nucleotides from the Fur consensus sequence. The iron-Fur-regulated genes code for iron acquisition systems and, in many bacterial pathogens, for bacterial toxins or other virulence factors, e.g., shigella enterotoxin 1 (15). Moreover, many other genes were found to be regulated by the Fur protein, e.g., genes involved in acid shock response, defense against oxygen radicals, metabolic pathways, etc. (14). The function of the *cjr* operon, despite the role in colicin Js uptake, is unknown. The iron and temperature regulation of the *cjrABC* genes suggests the involvement of these genes in iron metabolism and/or in the enteroinvasiveness phenotype. However, genes with functions other than iron metabolism may be regulated by the Fur protein (14) and some VirB-regulated genes might be dispensable for the invasiveness phenotype.

The *cjrB* and *cjrC* genes from two different serotypes of EIEC strains and from *S. flexneri* are very similar to each other, differing by one or two base pairs per gene. The *cjrB* genes in all three strains and *cjrC* in two EIEC strains code for identical proteins, while *cjrC* from *S. flexneri* #1 differs by one amino acid residue. Both the *cjrB* and *cjrC* genes from EIEC strains are more related to each other than to *cjrBC* from *Shigella*.

Among the 120 strains of 16 species of gram-negative bacteria, only EIEC and *Shigella* strains were found to be sensitive to colicin Js. The presence of the *cjr* operon is thus likely to be specific for bacteria with the enteroinvasiveness phenotype. The high specificity of the lethal action of colicin Js, which is restricted to EIEC and *Shigella* strains, resembles the narrow antimicrobial spectra described for other colicins (41). Among the sensitive strains, two types of colicin Js sensitivity phenotype, differing in sensitivity to colicin Js by 1 order of magnitude, were found. EIEC strains O164 and O143, which differ in

colicin Js sensitivity, code for identical CjrB and CjrC proteins and have identical sequences in the promoter regions. The different levels of sensitivity of the two strains to colicin Js might be due to differences in the regulation of *cjr* genes and/or other differences between the EIEC strains.

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