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CadC Is the Preferential Target of a Convergent Evolution Driving Enteroinvasive *Escherichia coli* toward a Lysine Decarboxylase-Defective Phenotype

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Enteroinvasive *E. coli* (EIEC), like *Shigella*, is the etiological agent of bacillary dysentery, a particularly severe syndrome in children in developing countries. All EIEC strains share with *Shigella* the inability to synthesize lysine decarboxylase (the LDC phenotype). The lack of this function is considered a pathoadaptive mutation whose emergence was necessary to obtain the full expression of invasiveness. Cadaverine, the product of lysine decarboxylation, is a small polyamine which interferes mainly with the inflammatory process induced by dysenteric bacteria. Genes coding for lysine decarboxylase and its transporter constitute a single operon (*cadBA*) and are expressed at low pH under the positive control of CadC. This regulator is an inner membrane protein that is able to sense pH variation and to respond by transcriptionally activating the *cadBA* genes. In this study we show that, unlike in *Shigella*, mutations affecting the *cad* locus in the EIEC strains we have analyzed are not followed by a novel gene arrangement and that the LCD$^-$ phenotype is dependent mainly on inactivation of the *cadC* gene. Introduction of a functional CadC restores cadaverine expression in all EIEC strains harboring either an IS*2* element or a defective *cadC* promoter. Comparative analysis between the *cad* regions of *S. flexneri* and EIEC suggests that the LDC$^-$ phenotype has been attained by different strategies within the *E. coli* species.

In bacteria the evolution toward pathogenic phenotypes revolves around two distinct mechanisms, the acquisition of additional genes encoding virulence determinants and the loss or modification of preexisting genetic material. The acquisition of multiple linked virulence traits by horizontal gene transfer is very important in triggering a virulence phenotype in a commensal organism, which can thus gain access to new host environments (11, 25). The new pathogen then reaches optimal fitness within the novel environment by adaptation of its genome through mutations in preexisting genes. These so-called pathoadaptive mutations improve survival within host tissues, increase the pathogenic potential of the bacteria, and drive the evolution of a microrganism toward a more pathogenic phenotype (26, 40).

In this context, evolution of *Escherichia coli* toward pathogenicity represents an interesting model for the enormous versatility of this microrganism, causing an impressive variety of different diseases (23). Acquisition of virulence genes by horizontal transfer has played an important role in the evolution of pathogenic *E. coli* strains. Virulence determinants have been acquired by *E. coli* cells as parts of plasmids, bacteriophages, transposons, or pathogenicity islands (13). Enteroinvasive *E. coli* (EIEC) strains are facultative intracellular pathogens able to enter epithelial cells of the colon, replicate within them, and move between adjacent cells with a mechanism similar to that of *Shigella*, the causative agent of bacillary dysentery (35, 36). In EIEC and *Shigella*, the critical event in the transition toward a pathogenic life-style probably relies on the acquisition of a large (220- to 260-kb) F-type plasmid (pINV) (35). This plasmid contains all the genes required for invasion and for intra- and intercellular spread, including their positive activators. Plasmid virulence genes are silenced outside the host by one of the major histone-like proteins, H-NS, which acts as a repressor on two positive activators (27). pINVs isolated from EIEC and *Shigella* strains share wide regions of high structural and functional homology (15).

Although EIEC strains may be developing the full *Shigella* phenotype, they do not have the full set of characters that define *Shigella* strains and are not included in any of the three *Shigella* clusters (16, 28, 29). EIEC strains generally correspond to bioserotypes found in a dozen *E. coli* serogroups, and recent molecular analyses confirm that they are widely distributed among *E. coli* phylogenetic groups (28, 30). Many EIEC strains have *Shigella*-like features: they can be Lac$^-$; nonmotile, low-level indole producing, and/or unable to produce gas during fermentation. Some have the same O antigen that is present in *Shigella*. In contrast to *Shigella*, they have a high metabolic activity since they still retain the ability to catabolize substrates widely utilized by *E. coli* (29, 39). Despite these differences, EIEC and *Shigella* share the inability to catabolize lysine due to the lack of lysine decarboxylase (LDC) activity. Whereas almost 90% of *E. coli* strains are LDC$^+$, all EIEC and *Shigella* strains are LDC$^-$ (18, 39). In *E. coli* the ability to use lysine as carbon source is determined by the products of the *cadBA* operon, encoding lysine decarboxylase (*cadA*) and a lysine-cadaverine antiporter (*cadB*), and is coinduced by low pH, anerobiosis, and lysine. Expression of the *cadBA* operon is dependent on the CadC positive activator, whose gene maps upstream of the operon and is transcribed independently from the same strand (20, 21, 42). Under noninducing conditions,

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TABLE 1. Bacterial strains used in this study

Strain and original designation[a]	Serotype	LDC[b]	Invasiveness[c]	Relevant features[d]	Country of origin or source (reference)[e]
EIEC					
HN280	O135	−	+	Lac$^-$, pINV (260 kb), pCRY (160 kb)	Somalia (24)
HN11	O135	−	−	Lac$^-$, pINV (260 kb), pCRY (160 kb)	Somalia (24)
HN13	O135	−	+	Lac$^-$, pINV (260 kb), pCRY (160 kb), pR (Cm^r Hg^r Sm^r Su^r Tc^r [100 kb])	Somalia (24)
4608	O143	−	+	Lac$^+$, pINV (250 kb)	WRAIR (35)
53638	O144	−	+	Lac$^-$, pINV (250 kb), pCRY (140 kb)	WRAIR (35)
13.80	O124	−	+	Lac$^-$ pINV (240 kb)	IPC (35)
6.81	O115	−	+	Lac$^-$ pINV (250 kb), pCRY (160 kb)	IPC (35)
S. flexneri					
SFZM50	1b	−	+	SRL, pINV (220 kb), pR (Su^r Sm^r [6.3 kb]), pCRY (4.1, 3.2, 2.7, 2.3, and 1.6 kb)	Somalia (5)
SFZM46	2a	−	+	SRL, pINV (220 kb), pR (Su^r Sm^r [6.3 kb]), pCRY (4.1 and 3.2 kb)	Somalia (5)
YSH6000	2a	−	+	SRL, pINV (230 kb), pCRY (4.1 and 3.2 kb)	Japan (37)
SFZM49	3a	−	+	SRL, pINV (220 kb), pCOL (5.2 kb, Col$^+$), pCRY (3.2 and 2.1 kb)	Somalia (5)
SFZM53	4	−	+	SRL, pINV (220 kb), pR (Su^r Sm^r [6.3 kb]), pCRY (7.0, 4.1, 3.2, 1.9, and 1.6 kb)	Somalia (5)
M90T	5	−	+	pINV (213 kb), pCRY (2.1 and 1.6 kb)	WRAIR (35)
SFZM43	6	−	+	SRL, pINV (220 kb), pR (Su^r [6 kb]), pCRY (4.1, 2.8, 2.7, and 1.9 kb)	Somalia (5)

[a] All the strains are designated by their original laboratory name.
[b] LDC, lysine decarboxylase. Lysine decarboxylase activity has been tested by the method of Meng and Bennet (20).
[c] Bacterial invasion of HeLa cells was measured as previously described (35).
[d] SRL, *Shigella* resistance locus (Ap^r Cm^r Sp^r Tc^r) (41); pINV, invasivity plasmid; pCRY, cryptic plasmid; pR antibiotic resistance plasmid; pCOL, colicin-producing plasmid. Cm, chloramphenicol, Hg, hygromycin, Sm, streptomycin, Su, sulfonamide; Tc, tetracycline; Ap, ampicillin; Sp, spectinomycin.
[e] WRAIR, Walter Reed Army Institute of Research; IPC, Institut Pasteur Collection.

H-NS represses the *cadBA* operon (38). While the lack of a functional *hns* gene also induces high and constitutive expression of the *cadBA* operon at basic pH, null mutations in *cadC* completely silence the operon (38, 42). CadC is an inner membrane protein with a carboxy-terminal periplasmic domain, responsible for sensing pH, and an amino-terminal cytoplasmatic domain, providing the DNA binding activity necessary for transcriptional activation (10).

Recent evidence shows that cadaverine, a polyamine resulting from decarboxylation of lysine, negatively interferes with the *Shigella* pathogenicity process. Cadaverine seems to induce the attenuation of enterotoxicity (18) and also to inhibit the migration of polymorphonuclear leukocytes (PMN) across the intestinal epithelial monolayers (12, 19). The *Shigella*-induced inflammatory response of the host is a "Trojan's horse" step in the invasive process, since PMN trafficking across intestinal epithelia opens the tight junctions between epithelial cells and thus facilitates the invasion of the colonic mucosa at basolateral sites (36). It has been proposed that silencing of the *cad* locus in *Shigella* represents an important pathoadaptive mutation necessary for increasing the pathogenic potential of bacteria in host tissues (9, 18, 40).

Here we report an analysis of the molecular rearrangements in the *cad* region of several EIEC strains belonging to different serotypes and isolated in different geographic areas. Our results show that the main strategy adopted for silencing the *cad* operon relies on mutations in *cadC* and that the arrangement of genes neighboring the *cad* locus is colinear to that of *E. coli* K-12. In most EIEC strains, the introduction of a functional *cadC* gene restores wild-type (wt) levels of lysine decarboxylase activity. Comparative analysis with respect to the *cad* region of *Shigella flexneri* shows that convergent evolution has operated independently to prevent the synthesis of cadaverine and suggests that the rearrangement observed in EIEC could be considered an evolutionary intermediate of a recombination process leading to the loss of the entire region.

MATERIALS AND METHODS

Bacterial strains and general procedures. The bacterial strains used are listed in Table 1. Growth media were Trypticase soy broth and Luria broth medium (34). The solid media contained 1.5% agar. Congo red was used at 0.01% in Trypticase soy agar to check for the Congo red binding (Crb) phenotype. When required, antibiotics were included at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 30 μg/ml; spectinomycin, 20 μg/ml; streptomycin, 100 μg/ml; sulfonamide, 600 μg/ml in M9 minimal medium; and tetracycline, 5 μg/ml. To quantify the expression of the *cadA* gene, strains were grown in modified Falkow lysine decarboxylase medium buffered at pH 5.5 with 100 mM morpholineethanesulfonic acid (MES). Bacterial invasion of HeLa cells was performed as previously described (8, 24) using strains grown at 37°C in Trypticase soy broth to an optical density at 600 nm (OD_{600}) of 0.6. *E. coli* K-12 MC4100 [F$^-$ *araD139* Δ(*argF-lac*)$_{U169}$ *rpsL150 relA flbB5301 deoC1 pstF25 rbsR*] (22) was used as the recipient strain for plasmids carrying gene fusions. β-Galactosidase assays were performed as previously described (22) on sodium dodecyl sulfate-chloroform-permeabilized cells grown in Luria broth supplemented with ampicillin. Units of β-galactosidase are calculated by the method of Miller (22), and results are the average of three independent experiments.

DNA manipulations. Total and plasmid DNA extraction, restriction digestion, electrophoresis, and purification of DNA fragments were carried out as described previously (3, 8). Southern hybridization was performed using α-^{32}P-labeled DNA probes essentially by the method of Sambrook and Russell (34). *cad*-specific probes were obtained by PCR amplification using MG1655 total DNA as template and the primer pairs A1 plus A2 (*cadA* probe) or B1 plus B3 (*cadB* probe). The IS*1* and IS2 probes were obtained from λ::IS*1* and λ::IS2, as previously described (7). PCR was routinely performed using *Taq* polymerase, or *ExTaq* (TaKaRa) for fragments larger than 4 kb. The primers used throughout this study are described in Table 2 and were designed mainly on the basis of the sequence of the *E. coli* K-12 MG1655 genome (4).

Plasmid construction. Plasmids pCC280, pCC681, pCC53, pCC90, and pCA681 were generated by cloning *Pfu* amplicons into pGem-T (Promega) as specified by the manufacturer. For pCC280, pCC681, pCC53, and pCC90, the amplicons were obtained with primer pair B2 plus C2 from genomic DNA of strains HN280, 6.81, 53638, and M90T, respectively. For pCA681, the amplicon was obtained with the oligonucleotide pair A1 plus B2R using genomic DNA of EIEC 681. Plasmid DNA from two independent clones was then used as tem-

TABLE 2. Nucleotide sequences of primers used for PCR analysis of the *cadBA* operon

Primer name	Nucleotide sequence	Target sequence
A1	5′-CTT CCC TTG TAC GAG CTA ATT-3′	*cadA*
A2	5′-CCTGGA GAT ATG ACT ATG AAC-3′	*cadA*
B1	5′-CCA ACA CAT GGG ACA AAA TGA-3′	*cadB*
B2	5′-GCC AGG TTA CCA ATC CAG-3′	*cadB*
B2R	5′-CTG GAT TGG TAA CCT GGC-3′	*cadB*
B3	5′-GTT GGT ATA ATA TGT TGC GGC-3′	*cadB*
C1	5′-GTC ATG CAC CAT AAA GGT GG-3′	*cadC*
C2	5′-CGC TTG CTC ATG CAA AGA C-3′	Intergenic *cadC-pheU*
I2	5′-GTG GCC GTG AAA GAA AGC-3′	IS*2* element
Y1	5′-GGA AGG TCA TGG ATC AGG-3′	*yjd1*
Y2	5′-AAT ATC CGC CAA GCG CAG-3′	*ytfA*
I1	5′-CAT ACG CGT AAA ACA GCC-3′	IS*1* element
LC1	5′-CGT AAC GAA TTC ATA AAG CAC AAC-3′	Upstream of *cadC*
LC2	5′-CAA CGG GAT CCA CAG GTT GTT GCA T-3′	*cadC*

plate for sequencing with the appropriate end-labeled primer (34). For complementation assays, we constructed plasmid pCC55, containing the *cadC* gene and its transcriptional and translational signals, by cloning a *Pfu* amplicon, obtained from of *E. coli* MG1655 with oligonucleotides B2 and C2, into the *Eco*RI site of the low-copy-number vector pACYC184 (34). Plasmids pLacC55 and pLacC53 were constructed by cloning the amplicons obtained with the oligonucleotide pair LC1 plus LC2, carrying the *cadC* promoter region and the first four codons of the *cadC* gene present in MG1655 and EIEC 53638, upstream of the *'lacZYA* genes of the translational fusion vector pRS414 (22).

Cadaverine excretion assay. The amount of secreted cadaverine was assayed in the supernatants of strains grown in Falkow modified medium to an OD_{600} of 0.5 to 0.7 unit as described previously (20). Briefly, 10 mM 2′,4′,6′-trinitrobenzylsulfonic acid (TNBS) was added and allowed to react (43°C for 5 min) with cadaverine, lysine, and trace amounts of other amines and amino acids. The colored product, *N*,*N*′-bistrinitrophenylcadaverine (TNP-cadaverine), was separated from *N*,*N*′-bistrinitrophenyllysine by extraction with 2 ml of toluene. The OD_{340} of the extract was read. Appropriate medium blanks were used to verify that trace amounts of other amines and amino acids in the supernatant did not interfere with the cadaverine measurement. Standard curves show that the assay is linear between $OD_{340} = 0.1$ and $OD_{340} = 1.0$, with the latter corresponding to 250 μM cadaverine.

Nucleotide sequence accession number. DNA sequence data were compared to known nucleotide and protein sequences using the BLAST Server (National Center for Biotechnology Information, Bethesda, Md.). The sequences of the *cadBC* regions of EIEC HN280, EIEC 6.81, EIEC 53638, and *S. flexneri* M90T have been deposited at GenBank under accession numbers AY225450, AY225451, AY319764, and AY225452, respectively, and the sequences of the *cadBA* operon of EIEC 6.81 have been deposited under accession number AY319765.

RESULTS

Genetic variability of the *cadBA* operon in EIEC. EIEC is not a homogenous group. EIEC strains differ with respect to serotype, plasmid content, and biochemical features. Moreover, recent studies of the genetic relationships between pathogenic and commensal *E. coli* strains by multilocus enzyme electrophoresis or ribosomal DNA restriction fragment length polymorphism confirm the presence of EIEC among different clusters of *E. coli* species (28, 30). Despite this diversity, these strains are all defective in the synthesis of lysine decarboxylase (18, 39). Since no data are available to explain the origin of this phenotype, we studied the genetic organization of the *cad* region in several EIEC strains and its relationship to that of *S. flexneri.*

Some EIEC and *S. flexneri* strains used in this study were isolated previously during a 2-year epidemiological survey of diarrheal diseases in children from Somalia (5, 6, 24), while others were obtained from the Reference Centers of the Pasteur Institute and of the Walter Reed Army Institute of Research (35). The EIEC strains were isolated in different geographic areas, belong to different serotypes, and display different plasmid contents, but they are all positive in invasivity assays. Moreover, no EIEC strains produce lysine decarboxylase (LCD^- phenotype) or cadaverine, even under inducing conditions (Table 1).

To detect molecular rearrangements in the *cad* operon leading to the LCD^- phenotype, we first analyzed EIEC strains for the presence of the *cadA* gene by PCR amplification using primers (A1 and A2 [Table 2]) flanking the complete gene. As reported in Table 3, EIEC strains generate a DNA fragment with the same size (2.2 kb) as that of the *E. coli* K-12 control strain MG1655 (4). Only EIEC 13.80 lacks the *cadA* product. The presence of a *cad* region is also confirmed by the appearance of a *cadA*-specific 2.4-kb *Pst*I fragment in Southern blots of genomic digests from all EIEC strains except 13.80 (data not shown). Taken together, these results suggest that despite their inability to produce cadaverine, most of the EIEC strains assayed contain the *cadA* gene.

We then analyzed the same EIEC strains for the presence of the *cadB* gene. First, we amplified the *cadB* region using primers (B1 and B3) flanking the complete *cadB* gene. We obtained a PCR product of the same size (1.7 kb [Table 3]) as the one from the control strain. Also in this case, no product was observed using EIEC 13.80 genomic DNA as template. Unlike the Southern analysis with the *cadA* probe, hybridization of *Pst*I-digested genomic DNA with a *cadB* probe revealed a certain variability among our EIEC strains (Fig. 1). Only in EIEC 53638 did the restriction fragments containing the *cadB* gene have the same size as in *E. coli* K-12: a 2.4-kb *Pst*I fragment (containing almost the entire *cadA* gene and part of the *cadB* gene) and a 7.3-kb *Pst*I fragment (containing most of the *cadB* gene). Surprisingly, in four strains (HN280, HN11, HN13, and 4608-58), the 7.3-kb *Pst*I fragment was replaced by an 8.6-kb fragment, suggesting the acquisition of insertion sequences or the presence of internal duplications. In agreement with the results obtained by PCR analysis with *cadB* primers, it is logical to expect that the rearrangement is located outside the *cadB* region and therefore within the *cadC* gene or its upstream region.

Detection of IS elements in the *cadC* gene. To find which kinds of molecular alterations have caused the loss of lysine

TABLE 3. Analysis of regions containing the *cad* operon in EIEC

Strain	Size (kb) of PCR product (primers)				
	yjdI cadB (Y1-B2R)	*cadA* (A1-A2)	*cadB* (B1-B3)	*cadB cadC* (B2-C2)	*cadC* IS*2* (C1-I2)
MG1655[a]	7.9	2.2	1.7	2.5	ND
HN280	7.9	2.2	1.7	3.8	0.8
HN11	7.9	2.2	1.7	3.8	0.8
HN13	7.9	2.2	1.7	3.8	0.8
4608–58	7.9	2.2	1.7	3.8	0.8
53638	7.9	2.2	1.7	2.5	ND
1380	NP[b]	NP	NP	NP	ND
6–81	7.9	2.2	1.7	1.9	ND

[a] *E. coli* K-12, used as control.
[b] np, no PCR product.
[c] ND, not done.

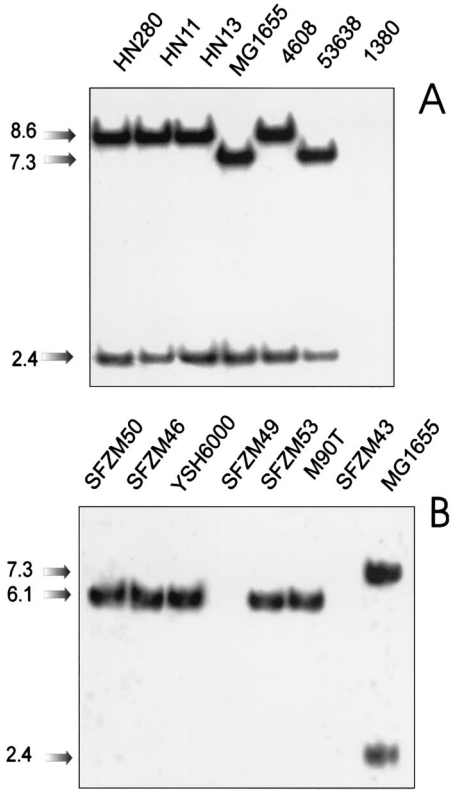

FIG. 1. Southern blot analysis of EIEC (A) and *S. flexneri* (B) digested with *Pst*I. Total DNAs were digested with *Pst*I, separated by electrophoresis on an 0.8% agarose gel, blotted onto nitrocellulose, and then probed with a specific *cadB* α-P^{32}-labeled probe. Total DNA of *E. coli* K-12 MG1655 was used as control. In EIEC 6.81, a large fragment of undigested DNA appears with the *cadA* and *cadB* probes (data not shown), indicating in vivo modifications that prevent restriction digestion by *Pst*I. The arrows show the position of the restriction fragment containing the *cadB* sequences. Fragment sizes are in kilobases.

decarboxylase activity in EIEC strains despite the presence of apparently integer *cadA* and *cadB* genes, we performed PCR analysis with a primer pair (B2 plus C2) spanning from within *cadB* to the intergenic region between *cadC* and *pheU*. While no product was observed when EIEC 13.80 genomic DNA was used as template, only total DNA from EIEC 53638 generated an amplicon with the same size (2.5 kb) as the control strain, MG1655. As expected, amplification performed using DNA from strains HN280, HN11, HN13, and 4608 gave a product 1.3 kb larger than that in the control strain (3.8 kb instead of 2.5 kb [Table 3]). This result is in agreement with the Southern analysis based on the *cadB* probe, where a 8.6-kb *Pst*I fragment appeared instead of the control 7.3-kb fragment. To investigate the basis of this genetic variation, we cloned the 3.8-kb amplicon obtained from EIEC strain HN280 into pGem-T. Sequence analysis of the resulting plasmid (pCC280) revealed that an IS*2* element (about 1.3 kb) is inserted into the *cadC* gene, 431 bp downstream the translational start site (Fig. 2). At the insertion junction, we detected a duplication of a AGGTGG sequence, present as direct repeats flanking IS*2* elements (17). The presence of IS*2* homologous sequences in the strains carrying the *cadC* region on a 8.6-kb *Pst*I fragment (Fig. 1) was confirmed by Southern blot analysis with an IS*2*-specific probe (data not shown). The location and orientation of the IS*2* element were then confirmed by PCR analysis with a primer pair (C1 and I2) internal to the *cadC* gene and to the IS*2* sequence, respectively (Table 3; Fig. 2).

Amplification of EIEC 6.81 genomic DNA with primers B2 and C2 gave a shorter amplicon (1.9 kb) than that of the control strain, MG1655 (2.5 kb [Table 3]), suggesting that this strain has suffered a deletion upstream of the *cadB* locus. The 1.9-kb amplicon from EIEC 6.81 was then cloned into pGem-T, yielding pCC681. Sequence analysis reveals that in this case an IS*1* element is inserted into the *cadC* gene (Fig. 2). Thus, unlike the IS*2* insertion in the *cadC* gene of the EIEC strains described above, the IS*1* insertion has promoted a severe deletion (1,385 bp) at the 5′ end of the *cadC* gene.

These results suggest that in 5 of 7 EIEC strains examined, the lack of lysine decarboxylase activity depends on IS*1* or IS*2* insertional inactivation of the *cadC* gene. EIEC strain 13.80 seems to harbour a wide deletion encompassing the entire *cad* region, since we never observed homology to *cad* probes (Fig. 1; Table 3). In contrast, in EIEC 53638 (Fig. 1 and 2; Table 3), the organization of the *cad* region is similar to that of *E. coli* K-12. The most likely explanation for the absence of lysine decarboxylase activity in this strain includes the possibility of point mutations or small deletions within *cad* genes. Alternatively, the absence of lysine decarboxylase activity may be due to mutations in other (albeit yet unknown) regulators mapping outside the *cad* region.

To verify whether, in EIEC strains conserving the *cadBA* operon, the LDC^- phenotype is determined only by the absence of CadC, we transformed these strains with a plasmid (pCC55) harboring a functional *cadC* gene. Introduction of pCC55 into the four EIEC *cadC*::IS*2* strains (HN280, HN11, HN13, and 4608-58 [Fig. 2]) restores the expression of the *cadBA* operon, as confirmed by the excretion of a large amount of cadaverine into the medium (Fig. 3). Also in the EIEC strain with an apparently wt *cad* region (EIEC 53638), we observed complementation by pCC55 (Fig. 3). Interestingly, sequence analysis of the *cadC* locus of strain 53638 revealed the presence in the promoter region of a transversion (A to C) positioned in the −10 box (5′-AATTCT-′3). To verify the promoter activity of *cadC*, we cloned the *cad* promoter region (−341 to +40) from MG1655 and EIEC 53638 in the promotor probe vector pRS414, obtaining pLacC55 and pLacC53, respectively. Compared to the wt strain, β-galactosidase expression of the *cadC-lacZ* hybrid gene was almost silenced under the control of the *cadC* promoter of 53638 (5 MU with pFlacC53 versus 400 MU with pLacC55 in the MC4100 background), suggesting that in this strain the LCD^- phenotype was obtained through the inactivation of the *cadC* promoter.

Since the presence of a functional *cadC* regulator is not able to restore cadaverine secretion in EIEC 6.81 (which carries a severe *cadC* rearrangement due to an IS*1* insertion and an IS*1*-mediated deletion), we suppose that in this strain the LDC^- phenotype also stems from a mutation in the *cadBA* operon. In fact, sequence analysis of this operon confirms that in this strain the *cadA* gene is also inactivated by several missense mutations in the coding sequence.

Analysis of the region flanking the *cadA* gene. The results obtained by PCR, Southern blotting, and sequence analysis indicate that in all EIEC strains (with the single exception of

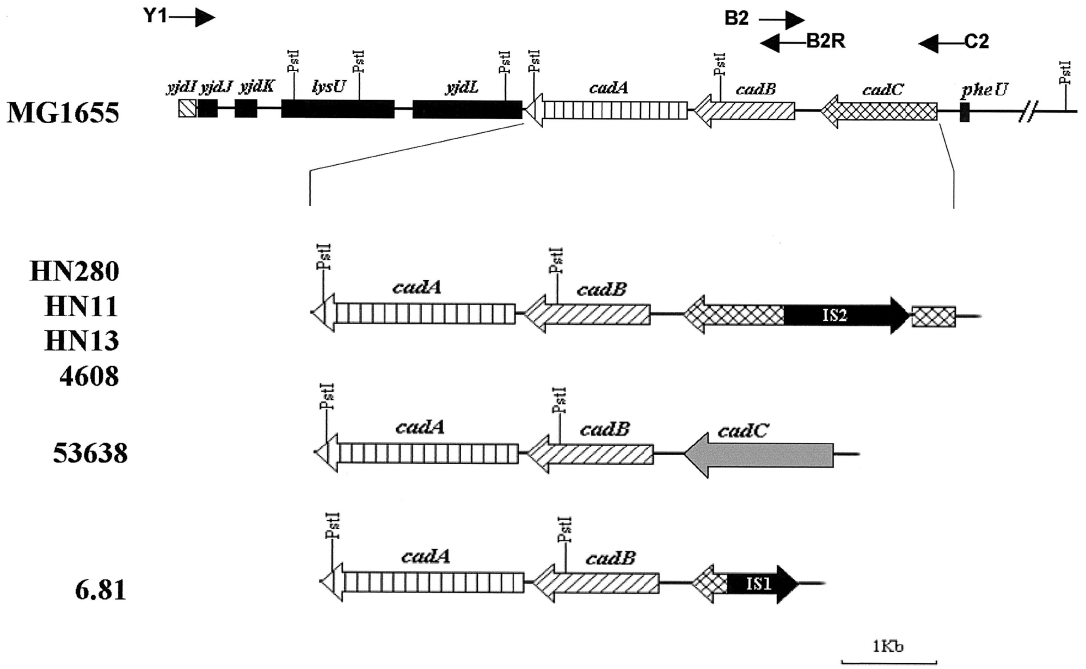

FIG. 2. Comparative analysis of the *cad* operon of EIEC and *E. coli* K-12. Arrows indicate the orientation of the IS sequences and of the *cad* open reading frames. The locations of the primers used are shown below the appropriate region. The positions of the *Pst*I restriction sites were derived from PCR amplicons and confimed by sequence analysis. Sequences of *E. coli* K-12 MG1655 were as reported previously (4).

strain 13.80), the *cad* region is indeed present and shows colinearity with the *E. coli* K-12 genome extending beyond the *pheU* gene, as indicated by the *Pst*I digestion patterns (Fig. 1). Also in EIEC 1380, despite the complete loss of the *cad* genes, PCR-based analysis with oligonucleotides internal to the neighboring genes indicates that colinearity with the *E. coli* K-12 chromosome is reestablished upstream of *yjdF* and downstream of *dsbD* (data not shown). To verify whether the region flanking *cadA* is also conserved in EIEC strains, we performed PCR analysis with a primer pair (B2R and Y1) corresponding to sequences internal to the *cadB* and *yjdI* genes (Fig. 2). Since the amplicon obtained has the same size (7.9 kb [Table 3]) and the same *Pst*I restriction pattern (data not shown) as the MG1655 control, we conclude that in our EIEC strains (except EIEC 13.80), the region adjacent to *cadA* is also colinear with the *E. coli* K-12 chromosome. This is schematically illustrated in Fig. 2. These results, together with the results of the Southern blot analysis of the region adjacent the *cadC* gene, strongly suggest that in the majority of EIEC strains the inactivation of the *cad* region is caused only by localized insertions or deletions (mainly in the *cadC* gene).

Comparative analysis of the *cad* region in EIEC and *S. flexneri*. Data reported above indicate that in EIEC the lack of lysine decarboxylase activity has been attained by different mutational strategies, including point mutations, insertions, and large deletions. Although *S. flexneri*, the most prevalent *Shigella* species causing bacillary dysentery, has high homology to EIEC in terms of plasmid and chromosomal sequences, as well as of pathogenicity processes (15, 29, 36), only a single mutational strategy has been reported (9) to account for the LDC^- phenotype. To ascertain the possible contribution of convergent evolution to this phenotype, we analyzed seven *S. flexneri* strains (at least one for each of the serotypes 1 to 6 and representative of the two *S. flexneri* clusters I and III [Table 1]) isolated from different geographic areas (the United States, Japan, and Africa). While we did not obtain amplicons corresponding to *cadA* and *cadB* sequences by PCR analysis (Table 4), we observed *cadB* hybridization signals in all strains except

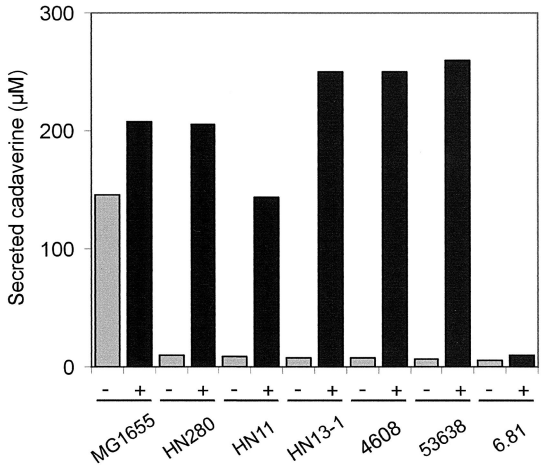

FIG. 3. Complementation assay with a functional *cadC* gene. Cells with (+) or without (−) pCC55, a recombinant plasmid carrying a wt *cadC* gene, were grown anaerobically in modified Falkow lysine decarboxylase medium at pH 5.5. The amount of cadaverine excreted into the medium was determined by measuring the OD_{340} of TNP-cadaverine and is expressed as micromoles of cadaverine (OD_{340} = 1 corresponds to 250 μmol). Values are the average of three independent experiments. For this assay, we used HN13-1, a tetracycline-sensitive mutant of HN13 previously isolated in our laboratory (24).

TABLE 4. Analysis of the *cad* locus in *S. flexneri*

Strain	Size (kb) of PCR product (primers)					
	ytfa cadB (Y2-B2)	*cadA* (A1-A2)	*cadB* (B1-B3)	*cadB cadC* (B2-C2)	*cadB* IS*1* (B2-I1)	IS*1 cadB* (I1-B2R)
MG1655[a]	ND[c]	2.1	1.7	2.5	ND	ND
SFZM50	4.7	NP	NP	1.2	0.8	2.0
SFZM46	4.7	NP	NP	1.2	0.8	2.0
YSH6000	4.7	NP	NP	1.2	0.8	2.0
SFZM49	NP[b]	NP	NP	NP	ND	ND
SFZM53	4.7	NP	NP	1.2	0.8	2.0
M90T	4.7	NP	NP	1.2	0.8	2.0
SFZM43	NP	NP	NP	NP	ND	ND

[a] *E. coli* K-12, used as control.
[b] NP, no PCR product.
[c] ND, not done.

SFZM49 and SFZM43. As shown in Fig. 1, the presence of sequences homologous to the *cadB* gene only in a single *Pst*I fragment (6.1 kb), as opposed to the 2.4- and 7.3-kb *E. coli* K-12 *Pst*I control fragments, indicates that genetic rearrangements causing deletions have altered the *S. flexneri cad* region. Following PCR amplification with oligonucleotides B2 and C2, all strains conserving remnants of the *cad* locus displayed the same 1.2-kb product (instead of a 2.5-kb fragment [Table 4]).

The 1.2-kb fragment generated using *S. flexneri* M90T DNA as template was cloned into pGem-T to obtain pCC90. Sequence analysis of pCC90 revealed that an IS*1* insertion sequence was present upstream of the *cadB* gene and that *cadC* was completely deleted. All *S. flexneri* strains containing remnants of the *cad* genes were similar to M90T (Fig. 4), as confirmed by PCR analysis with oligonucleotide pairs I1 and B2 (Table 4). The recently published genome sequences of two *S. flexneri* serotype 2a strains, Sf301 (14) and 2457T (43), indicate that both strains display a rearrangement similar to the one we observed in the M90T strain (Fig. 4).

To test for structural changes in the region flanking the *cadB* remnant of our *S. flexneri* strains, we performed a PCR analysis with primer Y2, internal to the *ytfA* gene, and primer B2R (Table 4). The amplicon obtained has the same size (4.7 kb) and the same *Pst*I restriction pattern as that of *S. flexneri* 2457 T and Sf301. By PCR analysis with the oligonucleotide pair I1 plus B2R, we were able to detect an additional IS*1* element (Fig. 4), located as in the previously described serotype 2a strains (9, 14, 43). Nevertheless, as illustrated in Fig. 1, the complete absence of hybridization signals from any of the *cad* probes in SFZM49 (serotype 3, cluster I) and in SFZM43 (serotype 6, cluster III) indicates that besides the rearrangement reported in Fig. 4, the LCD$^-$ phenotype in *S. flexneri* can also be obtained by deletion of the entire *cad* region. A PCR-based analysis was performed to verify whether the linear arrangement of the genes flanking the *cad* locus was similar to that described in *S. flexneri* 2a (9, 14, 43). The results obtained indicate that in these strains the colinearity with *S. flexneri* 2a is maintained, since the *ytfA* and *dsbD* genes are conserved in the same chromosome location upstream and downstream, respectively, of the *cad* disrupted locus (data not shown).

DISCUSSION

Enteric bacteria like *E. coli*, *Salmonella*, and *Shigella* experience dramatic pH fluctuations in nature, particularly during pathogenesis. To face the extremely low pH of the stomach, as well the presence of volatile fatty acids in the intestine, these neutralophilic bacteria have evolved complex, acid-inducible survival strategies. The low pH induces a programmed molecular response which, among other things, activates the transcription of several amino acid decarboxylases acting as pH-homoestatic systems (2). In *Salmonella* and *E. coli*, the three major amino acid decarboxylase systems operate on glutamate, arginine, and lysine and are able to increase the pH by consuming a proton during decarboxylation and then exchanging the decarboxylation end product for a new substrate via a membrane-bound antiporter (2). For the *cad* operon, it has been shown that the acid-induced synthesis of cadaverine from lysine by CadA and its subsequent excretion through the lysine-cadaverine antiporter CadB lead to neutralization of the external pH, thus protecting the cell from acidification (20, 42). Moreover, in vivo experiments have revealed that in *E. coli* and in *S. enterica* serovar Typhimurium the induction of the *cadBA* operon by low pH raises the pH and provides a survival advantage over *cad* mutants (2).

Although the *cad* system may be important during bacterial transit through the intestinal tract, in *Shigella* and EIEC such a system has been completely lost (9, 18, 39). Recently, a detailed analysis of the inflammatory process induced by *Shigella* has revealed that cadaverine, the end product of pH-induced lysine decarboxylation, attenuates the ability of the bacterium to induce the migration of PMN across model intestinal epithelia (19). The migration of PMN is a critical step in *Shigella*

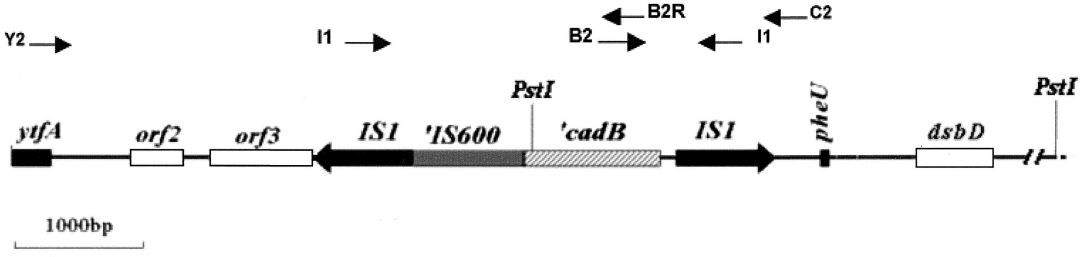

FIG. 4. Structure of the genetic region containing remnants of the *cad* operon in *S. flexneri*. The genetic organization of the *cad* locus in *S. flexneri* SFZM46, SFZM50, YSH6000, SFZM53, and M90T is shown schematically. The same organization is displayed by *S. flexneri* serotype 2a Sf301 (Genbank no. AE005674 [14]) and 2457T (GenBank no. AE014073; [43]). Arrows indicate the orientation of IS sequences. The location of the primers used is shown below the appropriate region. Symbols are the same as in Fig. 2.

pathogenicity process since it facilitates bacterial invasion of the basolateral membrane domain (36). Cadaverine seems to reduce the ability of *S. flexneri* to lyse the phagocytic vacuole, thus preventing the bacterium from interacting with the cytoskeleton. On the basis of this observation, it has been postulated that loss of the ability to disseminate intra- and intercellularly perturbs signaling pathways that are essential to the promotion of PMN transepithelial migration (12).

It has been suggested that evolution from *E. coli* ancestors to pathogenic lineages expressing the *Shigella* phenotype has occurred several times and is the result of acquisition of the main virulence traits through horizontal gene transfer followed by the occurrence of so-called pathoadaptive mutations, i.e., mutations affecting resident genes, which, if expressed, would lower the pathogenic potential (11, 16, 25, 29, 40). Although EIEC strains share essential steps of the pathogenicity pathway with *Shigella* (23, 35), their metabolic activity is nevertheless more closely related to that of the commensal *E. coli* strains (39). It is still an open question whether EIEC strains are potential precursors of *Shigella* or a distinct form, albeit still better adapted to life outside the host (16, 29).

Like *Shigella*, EIEC strains lose the ability to use lysine as a carbon source. In this work, we have analyzed the molecular rearrangements in the *cad* operon which determine the silencing of the lysine decarboxylase activity in a group of EIEC strains and have compared them to those present in several *S. flexneri* strains. Our results show that in EIEC, the major event triggering the loss of the LDC^+ phenotype is the transposition of IS*1* or IS*2* elements into the *cadC* locus (Fig. 2). This gene encodes a regulator essential for the activation of the *cadBA* operon, and its insertional inactivation results in the complete silencing of *cadBA* expression (10, 21, 42). All the EIEC strains we have examined from Eastern Africa harbor an IS*2* sequence exactly in the same position in *cadC*. The lack of lysine decarboxylase activity depends only on this insertion, since expression in *trans* of a functional CadC regulator restores cadaverine secretion to the *E. coli* K-12 standard level (Fig. 3). Also, EIEC 4608, a strain which differs in serotype, geographic origin, plasmid profile, and lactose utilization from the other EIEC *cadC*::IS*2* strains, contains an IS*2* element at exactly the same location (Fig. 2). Inactivation of *cadC* seems to be a common strategy for silencing *cadA*, since another strain (53638 [Table 1]) carrying a mutation in the *cadC* promoter (Fig. 1) is complemented by the introduction of a wt *cadC* gene (Fig. 3). Furthermore, the fact that *cadC* is the preferential target of a convergent evolution driving the bacterium toward the LCD^- phenotype is confirmed by the presence in EIEC strain 6.81 of an IS*1* element which, unlike the IS*2* insertion, has provoked a partial deletion of *cadC* (Fig. 2). A possible explanation of the inactivation of the *cadC* gene derives from evidence indicating that the CadC regulator may play a direct or indirect role in the control of other genes or operons involved in the adaptation of the bacterium to the host environment. In this respect, it has been recently reported (32, 33) that the mere expression of the CadC protein, even in the absence of cadaverine, leads to a reduction in the amount of both OmpF and OmpC porins, thus altering the permeability of the outer membrane.

An IS*1* sequence is always inserted in *S. flexneri* between the *cadB* remnant and *pheU*, and the rearrangements induced by this element probably account for the complete deletion of *cadC* observed in *S. flexneri* (Fig. 4). In this context, EIEC 6.81 may represent the "missing link" in the evolutionary transition toward the genetic organization of most *S. flexneri* strains, where rearrangements are more severe. In fact, in EIEC 6.81 the *cadBA* operon is still present although it is inactivated by a frameshift mutation in the *cadB* gene (Fig. 3), while in *S. flexneri* it is almost completely deleted and has been replaced by IS*1* and IS*600* insertions. Moreover, it is worth stressing that unlike EIEC, *S. flexneri* contains only remnants of *cadB* and has completely lost colinearity with the *E. coli* K-12 genome in the region downstream of the *cadB* remnant (Fig. 4). Comparative analysis of different serotypes of *S. flexneri* (Fig. 1 and 4) suggests that in *Shigella* the *cad* operon has undergone a multistep rearrangement, involving not only the insertion of IS elements but also independent deletion events resulting in the lack of the entire *cadA* and *cadC* genes. Furthermore, the absence of *cad* hybridization signals in one EIEC strain (13.80) and in two *S. flexneri* strains (SFZM43 and SFZM49) (Fig. 1) may represent the final result of several progressive steps leading to complete lack of the *cad* locus.

IS sequences play a crucial role in bacterial evolution, either directly, by modifying gene expression through deletions or insertions, or indirectly, by promoting homologous recombination (17). In *Shigella*, the lack of motility has been correlated with insertion of IS*1* primarily into the master operon *flhDC* (1). Also in this case, the insertion of IS*1* elements, differing in number and insertion position among the different *Shigella* species, has determined the appearance of progressive genetic defects, resulting in the complete loss of flagella. Moreover, in *Shigella*, multiple IS elements often interrupt genes encoding thin aggregative fimbriae or curli, triggering genetic rearrangements resulting in deletions with different extensions (31). The recent availability of the *S. flexneri* 2a Sf301 and 2457T genome sequences further reveals how critical the role of IS elements might be in chromosome dynamics, since these elements are found in astonishingly large amounts compared to those in the commensal *E. coli* strains (14, 43).

The phylogenetic relationships of *Shigella* strains to each other and to *E. coli* strains have been analyzed by sequencing eight housekeeping genes in four separate regions around the chromosome (16, 28, 29). These data show that the majority of *Shigella* strains fall into three main clusters within *E. coli* and that EIEC strains, as well as *S. sonnei* strains and some *S. dysenteriae* strains (D1, D8 and D10), are outside these clusters. Our results show that the majority of *S. flexneri* strains we have analyzed have undergone the same modification in the *cad* operon, in excellent agreement with that described for *S. flexneri* 2a 2457T (9, 43) and Sf301 (14). Nevertheless, the complete absence of any remnants of the *cad* genes in two *S. flexneri* strains (Fig. 1) indicates that it is difficult to find a correlation between a specific kind of rearrangement and a specific serotype and/or cluster. The disparity of the rearrangements in the EIEC *cad* region leading to loss of lysine decarboxylase activity provides good evidence of the heterogeneity present in this group of pathogenic *E. coli* strains (28, 30) and supports the idea that the lack of lysine decarboxylase activity is accomplished through diverse strategies within the *E. coli* species.

Our results suggest that each time a commensal *E. coli* strain acquires the invasive plasmid, it encounters a strong selective pressure for silencing the lysine decarboxylase gene. In the

transition toward pathogenicity, the bacterium generally loses some traits that are important for survival in the environment but redundant for the life inside the host. The loss of several catabolite pathways widely present in *E. coli*, as well as the loss of flagella, exemplifies how *Shigella* sheds functions that are not crucial for survival inside eucaryotic cells (11, 16, 26, 29, 40). In this case, the same product, cadaverine, which could be useful for buffering low pH in the stomach, is also detrimental for the pathogenicity process (2, 12, 18, 19, 33). In this context, the pathoadaptive mutations responsible for the silencing of *cadA* could be the result of choosing the lesser of two evils.

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