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CadA Negatively Regulates *Escherichia coli* O157:H7 Adherence and Intestinal Colonization▽†

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Adherence of pathogenic *Escherichia coli* strains to intestinal epithelia is essential for infection. For enterohemorrhagic *E. coli* (EHEC) serotype O157:H7, we have previously demonstrated that multiple factors govern this pathogen's adherence to HeLa cells (A. G. Torres and J. B. Kaper, Infect. Immun. 71:4985–4995, 2003). One of these factors is CadA, a lysine decarboxylase, and this protein has been proposed to negatively regulate virulence in several enteric pathogens. In the case of EHEC strains, CadA modulates expression of the intimin, an outer membrane adhesin involved in pathogenesis. Here, we inactivated *cadA* in O157:H7 strain 86-24 to investigate the role of this gene in EHEC adhesion to tissue-cultured monolayers, global gene expression patterns, and colonization of the infant rabbit intestine. The *cadA* mutant did not possess lysine decarboxylation activity and was hyperadherent to tissue-cultured cells. Adherence of the *cadA* mutant was nearly twofold greater than that of the wild type, and the adherence phenotype was independent of pH, lysine, or cadaverine in the media. Additionally, complementation of the *cadA* defect reduced adherence back to wild-type levels, and it was found that the mutation affected the expression of the intimin protein. Disruption of the *eae* gene (intimin-encoding gene) in the *cadA* mutant significantly reduced its adherence to tissue-cultured cells. However, adherence of the *cadA eae* double mutant was greater than that of an 86-24 *eae* mutant, suggesting that the enhanced adherence of the *cadA* mutant is not entirely attributable to enhanced expression of intimin in this background. Gene array analysis revealed that the *cadA* mutation significantly altered EHEC gene expression patterns; expression of 1,332 genes was downregulated and that of 132 genes was upregulated in the mutant compared to the wild-type strain. Interestingly, the gene expression variation shows an EHEC-biased gene alteration including intergenic regions. Two putative adhesins, flagella and F9 fimbria, were upregulated in the *cadA* mutant, suggestive of their association with adherence in the absence of the Cad regulatory mechanism. In the infant rabbit model, the *cadA* mutant outcompeted the wild-type strain in the ileum but not in the cecum or mid-colon, raising the possibility that CadA negatively regulates EHEC pathogenicity in a tissue-specific fashion.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are a group of food-borne enteric pathogens which include multiple serotypes associated with a broad spectrum of human illness ranging from uncomplicated diarrhea to hemorrhagic colitis and the hemolytic-uremic syndrome (reviewed in references 12 and 23). *E. coli* O157:H7 is the most common EHEC serotype associated with disease in North America. One key step in the pathogenesis of *E. coli* O157:H7 is its ability to colonize the intestine; however, this process is not completely understood in this organism because relatively little is known about those factors associated with adherence of this group of pathogens (reviewed in reference 37). Remarkably, the genome of *E. coli* O157:H7 contains multiple regions that encode putative adhesins not present in nonpathogenic K-12 strains and which may be important during colonization. It has been difficult to decipher EHEC adhesion mechanisms in vitro because these organisms do not adhere to tissue-cultured cell monolayers as well as other pathogenic *E. coli* strains (37). One reason for the relative lack of adherence of *E. coli* O157:H7 strains to tissue-cultured cells may be our current lack of understanding of in vitro conditions that promote expression of EHEC adherence factors (34). We hypothesized that there might be genes that repress the production of adherence factors during in vitro growth and screened a library of *E. coli* O157:H7 transposon mutants for hyperadherent strains (34). Several mutants exhibiting enhanced adherence to tissue-cultured cells were identified in this screen. One of these mutants contained an insertion in *cadA*, a gene that encodes lysine decarboxylase (LDC), and preliminary data suggested that CadA plays a role in EHEC adhesion (34).

The *cad* locus consists of three genes: *cadC*, *cadB*, and *cadA*.

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The *cadA* and *cadB* genes encode LDC and a lysine-cadaverine antiporter, respectively, and constitute the *cadBA* operon. The *cadC* gene is located upstream of *cadBA* and encodes a positive regulator for the expression of *cadBA*; CadC is thought to sense signals induced by external pH and lysine (reviewed in reference 31). A correlation between the inability to decarboxylate lysine and the isolation of clinically relevant EHEC and other Shiga toxin-producing *E. coli* (STEC) strains has been reported previously, although no direct link to adherence was identified (9, 10, 39, 41). A study by our group investigated the relationship between LDC activity and adherence to tissue-cultured cells in a phylogenetically characterized collection of diarrheagenic *E. coli* (DEC) strains (36). From the 80 DEC strains analyzed, 4 were LDC-negative strains corresponding to serotypes O111:NM, O111:H8, and O26:NM. Analysis of the *cad* locus indicated that this region was rearranged, and some of the genes were either missing or disrupted when compared to the *E. coli* K-12 *cad* region (36). Interestingly, the introduction of the *cad* locus on a plasmid to the strains lacking the LDC region reduced their adhesion compared to that of their corresponding wild-type LDC-negative strains. Finally, complementation studies showed that the products encoded in the *cad* operon affected the expression of intimin, a common adhesin expressed in STEC and enteropathogenic *E. coli* (EPEC) strains (36).

A larger screen to determine whether the lack of LDC activity was a widespread phenotype in different serogroups of *E. coli* was recently performed by Jores et al. (11). This group screened 212 intestinal *E. coli* strains for their LDC activity and then determined whether the LDC-negative strains displayed structural or regulatory alterations of the *cad* locus. The study identified 25 LDC-negative strains belonging to the STEC, EPEC, and enterotoxigenic *E. coli* (ETEC) pathotypes. Twenty-one of those strains did not react with either *cadA* or *cadB* probes (11). Complementation studies and adherence assays on tissue-cultured cells indicate overall that mutations in the *cad* operon are present in different categories of pathogenic *E. coli* and that the Cad regulon modulates adherence of EHEC O157:H7 and other LDC-negative strains from different pathotypes.

To explore the role of CadA as a regulator of EHEC O157:H7 adhesion to tissue-cultured cells and colonization of the infant rabbit intestine, we constructed a *cadA* deletion mutant and showed that CadA is a novel regulator of EHEC O157:H7 adhesion, mediating expression of intimin and other putative adhesins. Furthermore, the *cadA* mutant outcompeted the wild-type strain in the infant rabbit ileum, suggesting that CadA negatively regulates EHEC pathogenicity in a tissue-specific fashion.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The following bacterial strains were used in this study. Strain 86-24 is a prototype STEC O157:H7 isolate (Sm^r Nal^r) (30). Strain 86-24 *eae* (Δ10 Sm^r) was a gift from A. D. O'Brien, and P9C12D4 (*cadA*::Tn*p* Sm^r Km^r) was described previously (36). Strains RCV02 (86-24 *cadA*::*cat* Sm^r Cm^r), and RCV03 (Δ*eae cadA*::*cat* Sm^r Cm^r) were constructed as described below. Strains were routinely grown in Luria-Bertani (LB) broth or on L agar at 37°C (17). The construction of plasmid pCadABC, a pBR322 derivative containing the *cad* locus, has been described previously (36). Plasmids pVS50Z and pVS24Z, pRS551 derivatives containing the *eae* and *tir* promoter regions, respectively, were obtained from V. Sperandio (29). Plasmids were introduced into *E. coli* by electroporation as described by Dower et al. (5). When indicated, the bacterial strains were grown in Dulbecco's minimal Eagle's medium (DMEM) (Gibco/Invitrogen) at 37°C. Antibiotics were added to media at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 30 μg/ml; and streptomycin, 100 μg/ml.

Construction of *E. coli* O157:H7 *cadA* and *cadA eae* mutants. Disruption of the *cadA* gene was performed in the chromosome of EHEC strains 86-24 and 86-24 *eae* by a marker exchange as described by Datsenko and Wanner (4). Primer pair 5λCADA (5′-CCCTTCTACACGGCCACCGCTCATACCGCATTTACCTTC GTAAATCATATGAATATCCTCCTTAG-3′) and 3λCADA (5′-ATCCACTT TGACTCCGCGTGGGTGCCTTACACCAACTTCTCACCGGTGTAGGC TGGAGCTGCTTCG-3′) were used to amplify the *cat* cassette from plasmid pKD3 along with 45 nucleotides on each side of the cassette corresponding to the sequence of the *cadA* gene. The purified PCR product was introduced by electroporation into strains 86-24 and 86-24 *eae* containing the lambda red recombinase plasmid pKM201 (a gift from Kenan C. Murphy), and the transformed bacterial cells were plated and grown on L agar containing streptomycin and chloramphenicol at 37°C. Colonies resistant to chloramphenicol and streptomycin were tested for ampicillin sensitivity. The presence of the disrupted *cadA* gene in strains RCV02 and RCV03 was confirmed by PCR with primers 5CADA (5′-CCTCTAGAATACTGCGGATACCACTG-3′) and 3CADA (5′-CCGGAT CCGGACCAGTCGTTCTC-3′). In this *cadA*::*cat* construct, the *cat* cassette is inserted at nucleotide 1061 of the *cadA* gene.

β-Galactosidase assays and statistical analysis. The *E. coli* strains containing the *eae* or *tir* promoter-*lacZ* fusions (pVS50Z and pVS24Z, respectively) or the promoterless vector pRS551 were grown with shaking at 37°C in LB broth and then diluted 1:100 in fresh DMEM and grown to mid-exponential phase (optical density at 600 nm of ~0.5 to 0.6). Cultures were diluted 1:10 in Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol) and were assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate as previously described (21). The results of the β-galactosidase assays were analyzed with a paired Student's *t* test.

LDC assay. To determine the LDC activity, strains were grown in Moeller decarboxylase broth with lysine (10g/liter [BD BBL, Fisher Scientific]). A control culture made in the same medium but without L-lysine was included with each set of samples tested. Glycerol was added to the top of the inoculated medium to create an anaerobic environment, and the tubes were incubated at 37°C. The LDC activity results (a positive test if the control tube is acidic [yellow] and the LDC-positive tube is alkaline [purple]) were read after incubation for 24 or 48 h.

Bacterial adhesion to epithelial cells. For both qualitative and quantitative adhesion assays, *E. coli* strains were evaluated for their ability to adhere to HeLa cell monolayers by our standard protocol as previously described (32). Briefly, the strains were grown in LB broth overnight at 37°C and added in quadruplicate to tissue-cultured cells replenished with fresh DMEM at a concentration of 1×10^7 bacteria per well for 3 h at 37°C. One set of tissue-cultured cells were then washed, fixed, and stained with Giemsa solution for microscopic evaluation and the other three sets of cells were washed, lysed with 0.1% Triton X-100 in phosphate-buffered saline (PBS [pH 7.4]), and plated on L agar plates for quantification of the numbers of EHEC cells. To determine the effects of pH, cadaverine (1,5-pentanediamine dihydrochloride), and L-lysine on the adherence of the different EHEC strains, bacteria and HeLa cells were incubated for 3 h in DMEM (unadjusted pH); in DMEM at pH 7.4 by buffering with MOPS (morpholinepropanesulfonic acid) at a concentration of 0.1 M; in the presence 500 μM of cadaverine (Sigma-Aldrich); and in the presence of 0.5, 5, and 50 mM L-lysine (Sigma-Aldrich). Adherence data were expressed as CFU per ml of the bacterial inoculum recovered from triplicate wells, after subtracting bacteria attached to plastic wells, and the means were gathered from two independent experiments. Statistical differences were expressed as *P* values as determined by a *t* test analysis.

Preparation of whole-cell lysates, heat-extracted proteins, and Western blot assays. Bacterial cultures were grown overnight at 37°C in DMEM or LB broth and aliquoted into portions of approximately 4.0×10^8 (whole cells) or 4.0×10^9 (heat extracted) bacteria. The whole cells were harvested by centrifugation at $12,000 \times g$ for 5 min at room temperature, washed in 1 ml of 1× PBS (pH 7.4), centrifuged, resuspended in 100 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) solubilization buffer, and lysed at 100°C for 10 min. In the case of heat-extracted proteins, bacteria were collected and then harvested by centrifugation at $3,000 \times g$ for 10 min, resuspended in 160 μl of PBS, and incubated at 60°C for 30 min. The samples were then pelleted by centrifugation at $3,000 \times g$ for 10 min, and the supernatant was transferred to a fresh tube, 40 μl of 5× SDS sample buffer was added, and samples were boiled at 100°C for 5 min. Proteins were separated on 10% SDS-PAGE minigels (14) and transferred to Immobilon-P (polyvinylidene difluoride) membranes (Millipore) using a Trans-Blot SD transfer cell (Bio-Rad) at 15 V for 22 min. The

TABLE 1. Genes and primers used in quantitative real-time RT-PCR of *E. coli* O157:H7

Gene	Primer
flgB	
Forward	5′-ACCTCAACGCAACACATTCCGG-3′
Reverse	5′-TGTTATCGGCAAACTGGGTGCG-3′
cadA	
Forward	5′-CGGCAGCATGTTTTTCACACGC-3′
Reverse	5′-AAAAGACGGCACCATGAGCGAC-3′
flgE	
Forward	5′-TCCATGCAGCAAAATACCGGCG-3′
Reverse	5′-ATGCCAGACCTTCGTTGTTGGC-3′
Z2200 (F9 fimbriae)	
Forward	5′-AGTGGTAAATGCCGCTTGTGCG-3′
Reverse	5′-ATATTGAAGCCAACGGCGGAGC-3′
purD	
Forward	5′-TCATGCGCAGCATAATCGGCTG-3′
Reverse	5′-TTCATCAGCGCACGATGGAACG-3′
Z4969 (*lpfB1*)	
Forward	5′-TGCGAACGGCAATCTGCAAAAC-3′
Reverse	5′-TGTGAATATTACGCCCGGCCTG-3′
Z3601	
Forward	5′-TTTTCTGTTCGATACCCGCGCC-3′
Reverse	5′-AACGCGTCTTCTACCAACAGCG-3′
gapA	
Forward	5′-GAGCCCTTCCCAGAACATCATCCC GTC-3′
Reverse	5′-CGCCATACCAGTCAGTTTGC-3′

membranes were blocked with a solution containing PBS (pH 7.4)–0.5% Triton X-100 and 5% nonfat milk. Incubations with anti-intimin (1:2,000 dilution), anti-OmpA (1:10,000 dilution), antiflagellin (1:10,000 dilution), and secondary antibody (1:30,000) were carried out for 1 h at room temperature. The blot was developed with the ECL enhanced chemiluminescence Western blotting analysis system (Amersham Biosciences).

RNA extraction. Cultures of strains 86-24 and RCV02 were grown aerobically in LB medium at 37°C overnight and then were diluted 1:100 in DMEM and grown in a shaking incubator at 37°C to an optical density at 600 nm of 0.5. RNA from each strain was extracted using the RiboPure bacterial RNA isolation kit (Ambion).

Microarrays and data analysis. The Affymetrix GeneChip *E. coli* Genome 2.0 array was used to compare gene expression in strain 86-24 (wild type) to that in strain RCV02 (*cadA* mutant). The GeneChip *E. coli* Genome 2.0 array includes approximately 10,000 probe sets for all 20,366 genes present in four strains of *E. coli*: K-12 (lab strain MG1655), CFT073 (uropathogenic strain), O157:H7-EDL933 (enterohemorrhagic strain), and O157:H7-Sakai (enterohemorrhagic strain) (http://www.affymetrix.com/products/arrays/specific/ecoli2.affx). RNA processing, labeling, hybridization, and slide scanning procedures were performed as described in the *Affymetrix Gene Expression Technical Manual* (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). The output from the scanning of Affymetrix GeneChip *E. coli* Genome 2.0 was obtained using GCOS v 1.4 according to manufacturer's instructions. Comparisons of single replicates were performed using the analysis tools within GCOS v 1.4, by selecting 86-24 as the baseline for comparison. Data were normalized using robust multiarray analysis (RMA) via the RMAExpress website (http://rmaexpress.bmbolstad.com/), as previously described by Kendall et al. (13) The resulting data were compared to obtain features that were increased or decreased in response to the inactivation of the *cadA* gene. Custom analysis scripts were written in Perl to complete array analyses.

Quantitative real-time RT-PCR analysis. Results from microarray analyses were confirmed by performing real-time quantitative reverse transcription-PCR (RT-PCR) on seven representative genes found to be differentially expressed in the RCV02 mutant compared to the wild-type strain. Reactions were performed on the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, IN) using Sybr green I dye (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reaction mixtures were prepared containing 200 ng of RNA, 1 μg of each primer (Table 1), 10 μl of Sybr green PCR master mix, and 0.2 μl of reverse transcriptase in a total reaction volume of 20 μl. Thermal cycling conditions comprised an initial incubation at 50°C for 2 min; a *Taq* DNA polymerase activation at 95°C for 10 min; and 40 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Detection of the fluorescent product was performed at the end of the extension period at 80°C for 10 s. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Negative controls containing water instead of RNA were concomitantly run to confirm that the samples were not cross-contaminated. Targets were normalized to reactions performed using the housekeeping gene glyceraldehyde dehydrogenase (*gapA*) amplimers, and change (fold) was determined using the comparative threshold method (15). All of the experiments were performed at least in triplicate.

Electron microscopy. Strains were grown overnight at 37°C in DMEM under static conditions, centrifuged 8,000 × *g*, resuspended in PBS (pH 7.4), and allowed to adhere to Formvar carbon-coated copper grids (200 mesh; Electron Microscopy Sciences) as previously described (33). The samples were negatively stained with 2% potassium–phosphotungstic acid (pH 6.8). Specimens were examined in a Phillips 201 electron microscope.

Infant rabbit intestinal colonization assay. Infant rabbits (litters of 3-day-old New Zealand White infant rabbits, which were housed with their mothers) were infected following protocols that have been described previously (25, 26). In the competition assay, the animals were orogastrically inoculated (~5×10^8 CFU per 90 g of rabbit) with approximately 1:1 mixtures of EHEC wild-type strain 86-24 and an 86-24 *cadA*::*cat* mutant, RCV02, using size 5 French catheters with flexible tips. For these experiments, bacteria were grown overnight in L broth at 37°C in the presence of antibiotics, harvested by centrifugation, and then resuspended in sterile PBS (pH 7.2) and adjusted to a final cell density of ~10^9 CFU/ml. The rabbits were necropsied 5 days postinoculation, and intestinal samples were collected for microbiologic analyses. Any feces present in tissue samples at euthanasia were removed prior to the determination of bacterial CFU. The numbers of EHEC CFU in tissue samples were determined by plating tissue homogenates on sorbitol-MacConkey's agar plates to detect total EHEC and then replica plating onto chloramphenicol-containing sorbitol-MacConkey's agar plates to specifically detect the *cadA* mutant. Competition assays were performed in two independent litters, and competitive index (CI) values in each intestinal segment were compared to a theoretical value of 1 using the one-sample *t* test. Samples containing wild-type or *cadA* mutant cells only were excluded from the statistical analysis since calculation of CI values in these samples was not possible.

Microarray data accession number. Expression data can be accessed at the NCBI GEO database under accession no. GSE11927.

RESULTS

CadA regulates *E. coli* O157:H7 adherence. We previously performed a transposon mutagenesis analysis to identify bacterial factors involved in the control of *E. coli* O157:H7 adherence to HeLa cells (34). Using this approach, we identified a *cadA*::Tn*p* insertion mutant that exhibited increased adherence to HeLa cells. Here we constructed a defined *cadA*::*cat* mutation in EHEC 86-24 to confirm and extend these previous observations. The 86-24 *cadA*::*cat* mutant strain, designated RCV02, was unable to decarboxylate lysine (data not shown). In contrast, the wild type and complemented strain RCV02(pCadABC) were LDC positive. Like the transpososn *cadA*::Tn*p* mutant strain P9C12D4, RCV02 was hyperadherent to HeLa cells (Fig. 1). The enhanced adherence of the *cadA* mutant was evident in micrographs of Giemsa-stained HeLa cells and in quantitative analyses of adherent bacteria recovered from HeLa cells (Fig. 1A, B, and D). Introduction of a pCadABC into RCV02 reduced its adherence back to the levels observed in the wild-type strain (Fig. 1C). Quantitative analysis of the number of adherent bacterial cells confirmed these observations (Fig. 1D). Interestingly, adherence to plastic wells without cells was minimal, and the levels were similar in the wild type and the isogenic mutants (~2.1×10^3 to 3.8×10^3 bacteria per well). Furthermore, autoaggregation assays were performed to determine whether this was the reason for the hyperadherence phenotype but the results indicated that the wild type and the mutants aggregate at the same rate (data not show). Therefore, our results provide

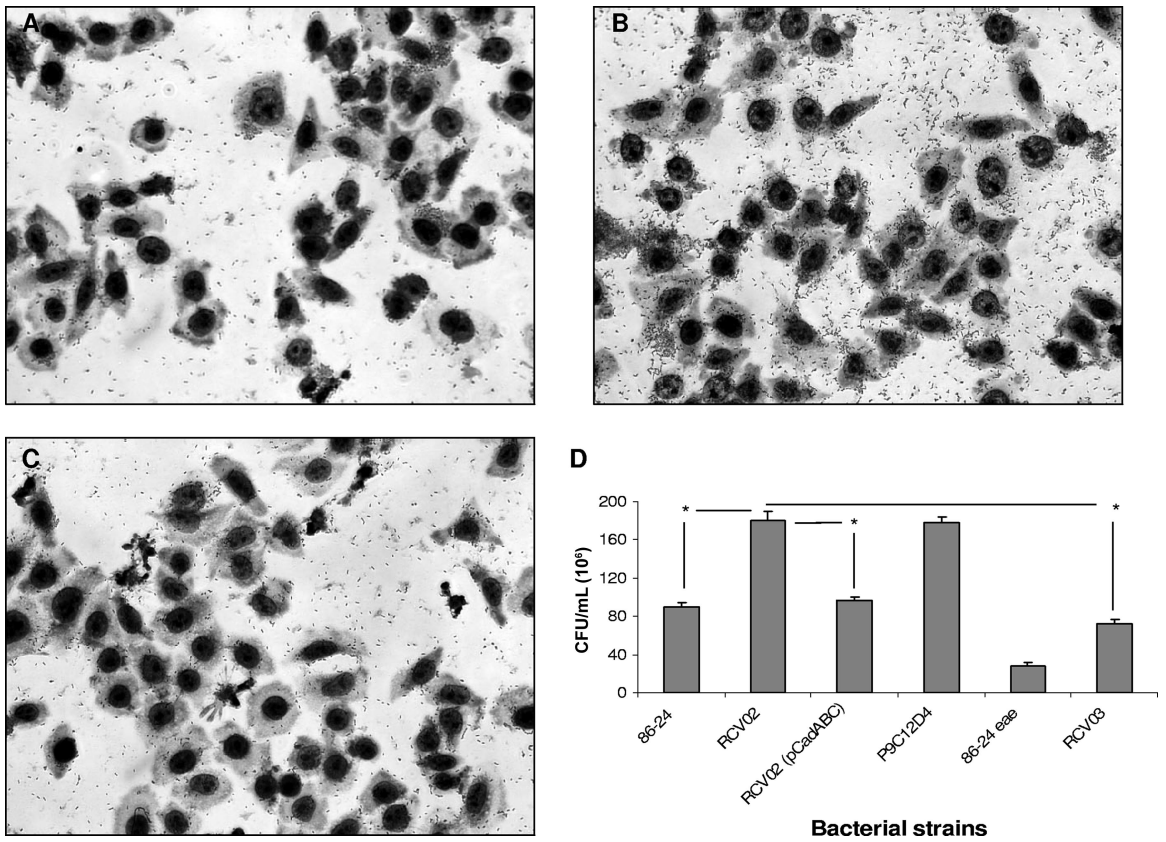

FIG. 1. Adhesion to HeLa cells after 3 h of incubation of EHEC O157:H7 strain 86-24 (A), RCV02 (*cadA*::*cat*) (B), and RCV02(pCadABC) (*cadA*$^+$) (C). (D) CFU/ml of EHEC O157:H7 isolate 86-24 and the mutant strains RCV02, RCV02(pCadABC), P9C12D4 (*cadA*::Tn*p*), 86-24 *eae*, and RCV03 (*eae cadA*::*cat*) adherent to HeLa cells. The error bars indicate the standard deviation. *, $P < 0.01$.

strong support for the idea that CadA negatively regulates EHEC adherence to tissue-cultured cells.

CadA regulates expression of the *E. coli* O157:H7 intimin protein. Because we have previously demonstrated that the lack of LDC activity has an effect on the expression of the adhesin intimin in EHEC strains of serotypes O111 and O26 (36), here we investigated, at the transcriptional and protein expression levels, whether CadA affects expression of EHEC O157:H7 intimin and whether this regulatory mechanism results in a change of bacterial binding to tissue-cultured cells. We used transcriptional fusions of the promoter regions of *eae* (intimin gene) and *tir* with a *lacZ* reporter gene and examined its expression during exponential growth in minimal essential media (DMEM [Fig. 2A]). These conditions are known to induce optimal transcription of LEE-encoded genes and other putative adhesin-encoded genes (29, 32, 35). (Note that *tir* and *eae* are located in the *LEE5* operon, and the main promoter controlling expression of the genes in this operon is the one located upstream of *tir* [27].) The EHEC wild-type strain 86-24 and its isogenic *cadA* mutant strain RCV02 were transformed with the parent plasmid (pRS551) or the plasmids containing the *eae* (pVS50Z) and *tir* (pVS24Z) promoter regions. Measurements of β-galactosidase activity revealed that the transcription of the LEE5 operon was increased in the mutant strain compared with the wild type [1.97-fold in RCV02(pVS50Z) and 1.48-fold in RCV02(pVS24Z) (Fig. 2A)]. Overall β-galactosidase activity was higher in strain RCV02 containing the promoter P_{tir} than P_{eae}, and in both cases, the activity in the mutant was significantly greater ($P < 0.01$) than that of the wild-type strains. In support of our transcriptional data, we analyzed the expression of intimin in the EHEC wild-type strain 86-24, RCV02 (*cadA*), and P9C12D4 (*cadA*::Tn*p*) strains, and in the complemented strain RCV02(pCadABC). As indicated in Fig. 2B, Western blotting using anti-intimin sera showed that expression of intimin is slightly enhanced in the *cadA* and *cadA*::Tn*p* mutants, and such increases in expression were reduced when the *cad* mutant is complemented in *trans*.

To confirm that the hyperadherence phenotype in the *cadA* mutant was due to increased expression of intimin, we examined whether a mutation in the intimin gene combined with the *cadA* mutation eliminated adhesion of *E. coli* O157:H7 strains to HeLa cells. The *cadA eae* double mutant (strain RCV03) was compared to the wild-type and single *eae* mutant strains, and as shown in Fig. 1D, the double mutant displayed a reduction in adherence (0.8-fold reduction compared to the wild type and 2.5-fold compared to the *cadA* mutant). To our surprise, the *cadA eae* double mutant adhered better than the *eae* single mutant, suggesting that an additional CadA-regulated factor(s) is expressed and participating in adherence of this strain. We have also tested adherence of these strains to Caco-2 cells, and the results were similar to those obtained

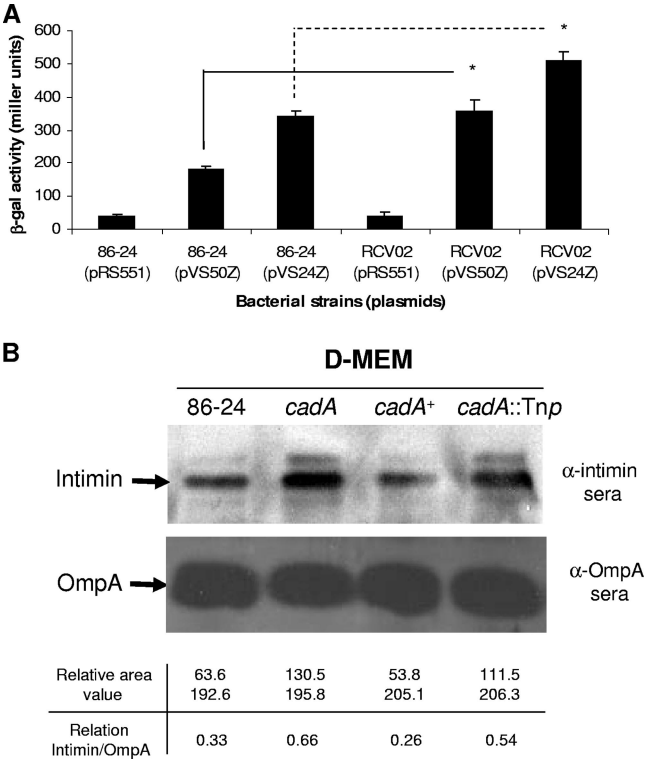

FIG. 2. CadA modulates expression of the *eae* gene, and the intimin protein participates in the hyperadherence phenotype. (A) β-Galactosidase (β-gal) assays of EHEC strains 86-24 and RCV02 carrying plasmids pRS551 (vector control), pVS50Z (*eaep*::*lacZ*), and pVS24Z (*tirp*::*lacZ*) in DMEM during the exponential growth phase. The error bars indicate standard deviation. *, $P < 0.01$. (B) Western blot with anti-intimin (α-intimin) and anti-OmpA (α-OmpA [control]) sera of EHEC 86-24, RCV02 (*cadA*::*cat*), RCV02(pCadABC) (*cadA*$^+$), and P9C12D4 (*cadA*::Tn*p*) grown in DMEM and fractionated by SDS–12% PAGE. The intimin and the OmpA proteins are identified with black arrows. The Western blot results were analyzed using the NIH ImageJ program. The areas of intensity were quantified and corresponded to each of the protein bands. The relationship between intimin and OmpA was calculated from the relative values obtained from each area quantified and represents the transcription level for *lpfA* and l6S in each strain analyzed.

with HeLa cells (data not shown). Thus, our data confirmed that under in vitro culture conditions CadA regulates transcription of *eae*, that enhanced expression of intimin is observed in the *cadA* and *cadA*::Tn*p* mutants during growth in DMEM, and that deletion of the intimin adhesin caused a reduction in the ability of strain RCV03 to bind tissue-cultured cells but suggest that additional adhesins might also be regulated by CadA.

CadA-regulated adherence is not dependent on pH or concentration of lysine or cadaverine. Because the Cad system is thought to sense signals induced by external pH and lysine, we determined whether changes in the pH of the medium or addition of lysine or cadaverine (by-product of lysine decarboxylation) had an effect on the hyperadherence phenotype of the *cadA* mutant. Adherence assays were performed with the wild-type and RCV02 (*cadA* mutant) strains in DMEM only, DMEM with pH buffered with MOPS (pH 7.4), or DMEM supplemented with lysine or cadaverine, and the pH of the media was monitored at the start and at the end of the experiment. As shown in Fig. 3A, RCV02 adhered better than the wild type in DMEM, and we found that bacteria acidified the medium (pH from 7.42 to 7.23). When MOPS was used to buffer the medium, no significant change in pH was observed after 3 h of infection, and although we observed an overall reduction in adherence, a similar trend was observed: RCV02 adhered better than the wild type. Furthermore, addition of cadaverine to the medium did not eliminate the hyperadherence phenotype, as previously observed (36). In contrast, addition of 50 mM lysine to the medium had a significant impact in the ability of the wild-type and the mutant strains to adhere to HeLa cells (Fig. 3A); however, the pH of the medium changed significantly with the addition of this amino acid (the start pH was 9.26, compared with 7.4 for DMEM only).

To determine whether the presence of external lysine or a change in the pH was responsible for the reduction in adherence, we performed the experiment with 10-fold dilutions of lysine and monitored the changes in pH in the assay. As observed in Fig. 3B, adherence to HeLa cells of the wild type and the *cadA* mutant was restored when the pH of the medium approached neutrality. Interestingly, the increased adherence displayed by the *cadA* mutant was observed in the presence of 5 or 0.5 mM lysine, and overall, these results suggest that this phenotype is independent of the concentration of lysine or cadaverine in the medium or the pH (because an increase in pH had an effect on the adherence of the wild type as well as the mutant strain, but the hyperadherence phenotype was maintained).

Global gene expression analysis of *E. coli* O157:H7 lacking CadA. To examine the genome-wide variations in gene expression in the *cadA* mutant, we utilized an Affymetrix GeneChip *E. coli* Genome 2.0 array. The EHEC O157:H7 strain 86-24 and the *cadA* mutant strain RCV02 were grown in DMEM, total RNA was extracted from cultures at exponential phase, and cDNA was synthesized and hybridized to the arrays. Gene expression was normalized to strain 86-24 and expressed as a ratio of the *cadA* mutant to the parent strain. Genes exhibiting a greater than twofold change in expression between the wild-type and mutant strains were examined in greater detail. In total, 1,464 genes (132 upregulated and 1,332 downregulated) were identified to be differentially regulated by more than twofold (with 99% confidence) in the *cadA* mutant versus the parent strain (see Tables S1 and S2 in the supplemental material). When we examine the expression profile based on the origin of the gene probes, a pattern of pathogen-specific regulation becomes evident (see Tables S1 and S2 in the supplemental material). The number of genes that have decreased expression in the *cadA* mutant from other EHEC strains is greater than twice that of the genes decreased in the laboratory-adapted K-12 strain (EDL933, 20.83%; Sakai, 20.11%; and K-12, 9.51%), suggesting that CadA regulates gene expression in an EHEC-specific manner. Similarly, the intergenic regions also display an altered level of expression, but the reason for this observation is unclear at this time. The difference in the number of genes upregulated versus downregulated suggests that CadA is a positive regulator of a number of critical processes in EHEC.

Closer examination of the genes with functional annotation allowed the identification of overrepresented functional groups,

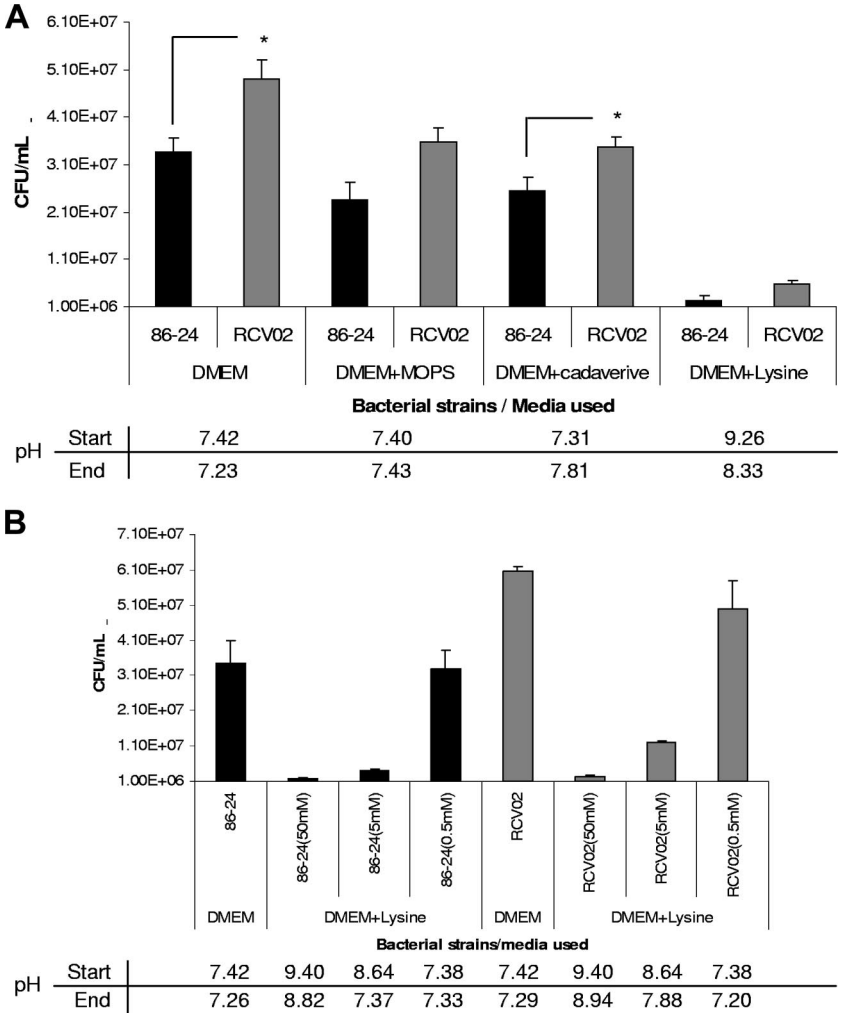

FIG. 3. (A) Quantification of EHEC 86-24 and RCV02 (*cadA*) strains adherent to cultured HeLa cells after 3 h of incubation in DMEM, MOPS-buffered DMEM (pH 7.4), or DMEM supplemented with cadaverine (500 μM) or L-lysine (50 mM). (B) Effects of different concentrations of L-lysine on adhesion of EHEC 86-24 and RCV02. The error bars indicate standard deviations. *, $P < 0.05$.

as determined by gene ontology (2) in the *cadA* mutant. These functional group overrepresentations provide insight into the possible role of CadA in EHEC regulation (data not shown). Some of the most upregulated genes in the *cadA* mutant are those involved in flagella and motility, and although we have previously proposed that the flagella of EHEC O157:H7 do not mediate adherence to epithelial cells (8), recent evidence indicate that the H7 flagella possess adhesive properties that may contribute to colonization of mucosal surfaces (6). Our initial analysis of the genes with upregulated expression profiles in the *cadA* mutant also identified the Z2200 gene, which encodes a fimbrial protein of the F9 fimbriae (16). The F9 fimbria might act as the additional adhesin because it promotes an increase in the levels of *E. coli* K-12 binding to bovine epithelial cells (16).

Microarray results were independently confirmed by measuring gene expression changes of five upregulated genes (LDC 1 gene *cadA*; flagellar basal body and hook-encoding genes *flgB* and *flgE*, respectively; the F9 major fimbrial subunit gene Z2200; and the metabolic synthetase gene *purD*) and two downregulated genes (putative fimbrial chaperone and major fimbrial subunit-encoding genes Z4969 and Z3601, respectively) selected based on their ability to potentially contribute to adhesion or as random controls, and analyzed by real-time RT-PCR (Table 2). The real-time RT-PCR results confirmed the elevated expression of these five genes and decreased expression of the other two genes, in concordance with the microarray results.

Ultrastructural studies were performed to explore whether surface-associated structures such as fimbriae and flagella were detected in RCV02 and EHEC 86-24. Analysis of multiple electron microscopic fields revealed that the RCV02 mutant strain possesses more flagellar filaments than the wild type grown under the same conditions (Fig. 4A and B), although the motilities of both strains were similar on motility agar plates (data not shown). One striking feature of the electron microscopy analysis of RCV02 was that this mutant strain expressed fimbrial structures that protrude and form aggregates around the bacteria (Fig. 4C and D). In contrast, we were not able to detect fimbrial production by the wild-type strain under the

TABLE 2. Real-time RT-PCR confirmation of representative genes up- and downregulated in the *cadA* mutant compared to the EHEC O157:H7 wild-type strain

Gene	Product	Fold change by:	
		Microarray	Real-time RT-PCR
flgB	Flagellar basal body rod protein	+2.4	+4.1
cadA	LDC 1	+2.2	+2.5
flgE	Flagellar hook protein	+2.0	+3.0
Z2200	Putative major fimbrial subunit, F9 fimbriae	+2.2	+5.8
purD	Phosphoribosylglycinamide synthetase	+2.0	+2.8
Z4969	Putative fimbrial chaperone	−2.1	−3.6
Z3601	Putative major fimbrial subunit	−2.0	−3.0

conditions tested (Fig. 4A and B). To further characterize the presence of these surface structures on RCV02, we heat extracted proteins from wild-type and *cadA* mutant cells, separated the resulting protein preparations by SDS-PAGE, and stained them with Coomassie blue (Fig. 4E). This analysis revealed that one protein was clearly upregulated in the RCV02 strain compared to the heat-extracted proteins of the wild-type strain. The protein was excised and sent for matrix-assisted laser desorption ionization–time of flight analysis, which revealed that the protein was the flagellin FliC protein. Western blot analysis further confirmed that the protein cross-reacted with anti-FliC serum (Fig. 4E). Our inability to detect F9 fimbriae subunits in our heat-extracted preparations could be due, perhaps, to the gentle procedure used, because it has been reported that purification of F9 fimbriae from uropathogenic *E. coli* requires blending to detach fimbriae and acid

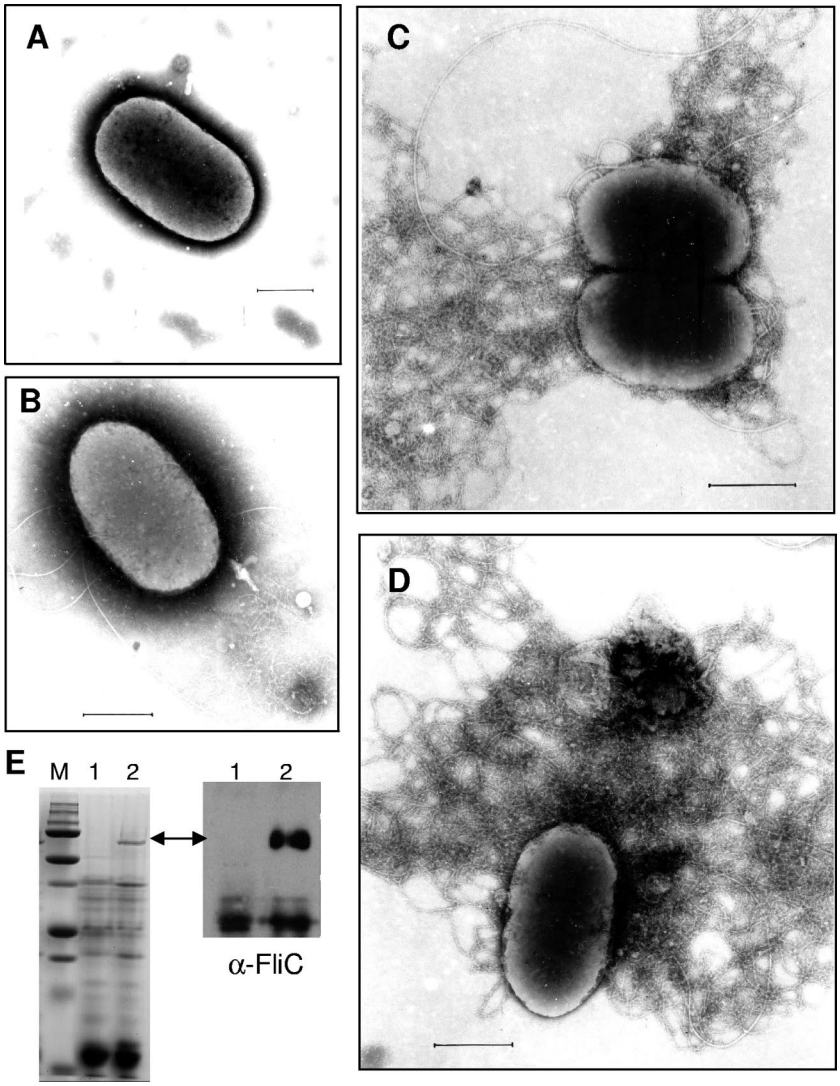

FIG. 4. Flagellum and fimbria expression on the surface of RCV02. Flagella and fimbriae were visualized by transmission electron microscopy. Bars, 0.5 μm. (A to D) EHEC strain 86-24 (A) and strain RCV02 (*cadA*::*cat*) (B to D) grown in DMEM. (E) Heat-extracted proteins from EHEC strains 86-24 (lanes 1) and RCV02 (lanes 2) were fractionated by SDS-PAGE and stained with Coomassie blue. The identity of the FliC protein (black arrow) was confirmed by matrix-assisted laser desorption ionization–time of flight analysis and Western blotting with anti-FliC (α-FliC) serum. M, molecular mass markers.

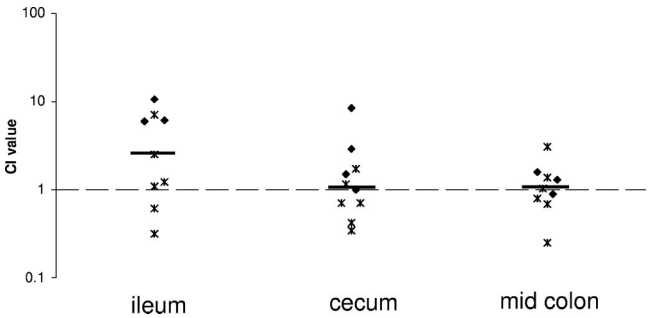

FIG. 5. Role of CadA in EHEC O157:H7 intestinal colonization in infant rabbits. Shown are CI values in ileal, cecal, and mid-colon samples taken from rabbits coinfected with the *cadA* mutant and wild type strain 86-24, at day 5 postinoculation. CI values were calculated as (no. of mutant cells/no. of wild-type cells in the output pool)/(no. of mutant cells/no. of wild-type cells in the input pool). Symbols represent individual rabbits from the two litters: litter A, diamonds; litter B, stars. Bars represent the overall median.

treatment to separate the subunits (38). Overall, our results showed that (i) CadA regulates the expression of multiple genes, (ii) the majority of those genes are downregulated, (iii) flagella and F9 fimbriae are upregulated, and (iv) expression of these two proteins on the surface of EHEC could contribute to the adherence phenotype.

The *cadA* mutant outcompetes the wild-type strain in colonization of the infant rabbit. Next, we tested whether the *cadA* mutation influenced *E. coli* O157:H7 intestinal colonization using the infant rabbit model (25, 26). For these studies, we carried out competition assays in which approximately equal numbers of RCV02 and 86-24 cells were oro-gastrically coinoculated into 3-day-old New Zealand White rabbits. Numbers of RCV02 and 86-24 cells were determined in ileal, cecal, and mid-colon samples at 5 days postinoculation (Fig. 5). The recovery of bacterial strains is expressed as a CI, a ratio of mutant to wild-type cells detected in tissue homogenates divided by the ratio of mutant to wild-type cells present in the inoculum. Overall, CI values obtained from two independent litters indicated that the *cadA* mutant outcompeted the wild type in the ileum ($P < 0.05$), but not in the cecum or mid-colon. As can be seen in Fig. 5, this phenotype was more pronounced in litter A than in litter B; indeed, in litter A, one rabbit appeared to contain only *cadA* mutant cells in most of the samples at the dilutions examined.

DISCUSSION

The gastrointestinal tract plays a crucial "balancing act" by confining the microflora in the lumen, protecting the host from potential disease, and maintaining compartmentalization of different environmental conditions (1). In order to cause disease, enteric pathogens like *E. coli*, *Salmonella*, and *Shigella* have to overcome several barriers, including the dramatic pH fluctuations found during their transit through the gastrointestinal lumen. To withstand the extremely low pH of the stomach, these pathogens have evolved complex, acid-inducible survival strategies. Despite the beneficial role that the CadBAC lysine-dependent acid resistance system might have in the survival of enteric organisms in the intestine, cumulative evidence indicates that the absence of these genes, due to deletions or insertional mutagenesis, in some members of the family *Enterobacteriaceae* results in an enhancement of their virulence phenotype (31). For example, it is well documented that attenuation of virulence phenotypes in *Shigella flexneri* 2a has been linked to expression of LDC and, specifically, to the production of cadaverine (7, 18, 19). These studies demonstrated for the first time that the *cadA* gene acts as an antivirulence gene for *Shigella*. Furthermore, our own studies also show that some clinical EHEC isolates, belonging to the serogroups O111 and O26, possessed disruptions of the *cad* system and that these mutations were associated with increase adherence to tissue-cultured cells (36). Finally, the absence of LDC activity due to inactivation of the *cad* system has been documented in other categories of pathogenic *E. coli* and *Salmonella* strains associated with outbreaks and human disease (11, 28).

Due to the importance of the *cad* locus during acid adaptation of enteric bacteria, a question that arises is whether the lysine decarboxylation system is fully required for survival or whether compensation with other decarboxylation systems might give rise to clinical isolates which are LDC negative and which possess virulence phenotypes enhanced due to mutations within this locus. We believe our present study helps to resolve some aspects of this question. First, we demonstrated that disruption of the *cadA* gene in EHEC O157:H7 enhanced the ability of this strain to adhere to tissue-cultured cells without affecting the growth of the bacteria in the different media tested (data not shown). Second, we found that several adhesins, and a large number of genes with other functions, were differentially regulated in the *cadA* mutant, indicating that in addition to its enzymatic activity, the CadA protein plays an important role in modulating the expression of specific adhesins and, possibly, other virulence factors. Third, we found that the *cadA* mutant was able to survive transit through the acidic milieu of the infant rabbit stomach and colonize the intestine. Furthermore, our data suggest that the *cadA* mutant exhibits an interesting tissue-specific phenotype as it was able to outcompete the wild type in the rabbit small intestine (~2.5-fold) but not the large intestine. So, in the context of EHEC O157:H7, which are LDC-positive strains, the Cad system might be playing a modulator role, allowing the bacteria to survive in low pH, but it might be also acting as a signaling mechanism to repress colonization mechanisms once the bacteria reach the ileum.

It is well known that *Salmonella* and *E. coli* are able to activate three major amino acid decarboxylase systems which operate on glutamate, arginine, and lysine and can 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 (3). In the case of 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, 40). The CadA protein plays a key role in the defense of *Salmonella* at lower pH (24) but was thought to be ineffective in *E. coli* since the protection of *E. coli* challenged at low pH (e.g., pH 2.5) by lysine is much weaker than the protection by the inducible GadABC glutamate-dependent acid resistance system. However, it has been proposed recently

that *E. coli* can survive in the gut because of the activity of both the GadABC glutamate- and CadBA lysine-dependent acid resistance systems (22). The data in our lab suggest that the downregulation of several genes observed in the *cadA* mutant can be reverted when the bacteria are grown in different media (unpublished observations), but the increased-adherence phenotype seems to be unaffected by changes of pH during the assay or the concentration of lysine and cadaverine. However, further studies are required to elucidate the true mechanism of the CadBA lysine-dependent acid resistance system in *E. coli* O157:H7, alone or in combination with the GadABC glutamate-dependent acid resistance system.

Our microarray and real-time PCR experiments were performed to characterize the CadA regulon and identified putative adhesins. In support of our initial phenotypic analysis, results indicated that upregulation of flagellar and F9 fimbrial genes occurs at the transcriptional level. The downregulation of so many genes in the *cadA* mutant is perhaps surprising, as the mutated gene encodes an LDC enzyme. A plausible explanation for these findings is that the Cad system might be playing a modulator role, allowing the bacteria to survive at different pHs, and the absence of this enzyme may act as a signaling mechanism to repress a large number of genes, allowing activation of proteins associated with colonization mechanisms required at a particular pH.

Another interesting observation of our study is that expression of specific adhesins is induced under in vitro culture conditions in the EHEC O157:H7 *cadA* mutant strain, and deletion of one of these adhesins (intimin) caused a decrease but did not abolish the adherence phenotype in this strain. The possible role of flagella and F9 fimbriae in the adhesion of an EHEC O157:H7 *cadA* mutant still needs to be elucidated; however, recent evidence explored the adhesive properties of H7 flagella and their binding properties to mucus and mucins, and the data suggested that these structures may have a biologically relevant role within the context of colonization of the host (6). It is well known that within the gut, epithelial cells and extracellular matrix proteins form barriers to prevent microorganisms from penetrating these tissues and has been speculated that A/E-producing pathogens, like EPEC or EHEC, might have evolved mechanisms for breaking tight junctions that lead to flagellum-mediated binding to extracellular matrix proteins in the basal lamina (6). We can also speculate that overexpression of flagella might be advantageous to the bacteria to colonize the intestine in response to certain environmental conditions. Our studies suggest that LCD-negative *E. coli* strains might have an advantage over LCD-positive *E. coli* strains because they might be using one or more of these factors to colonize the intestine, and furthermore, they also suggest that these factors might be important in colonizing a different region of the intestine.

Whether the accumulation of mutations and/or deletions of the *cad* locus in *Enterobacteriaceae* represents an evolutionary advantage to the pathogenic style of some members of this family remains to be fully elucidated. However, our data present interesting evidence indicating that the *cad* locus encodes more than just a mechanism to degrade lysine in *Enterobacteriaceae* and in EHEC O157:H7; disruption of the *cad* locus confers an advantage for adhesion and pathogenesis within the host.

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