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Pathoadaptive Mutations That Enhance Virulence: Genetic Organization of the *cadA* Regions of *Shigella* spp.

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Pathoadaptive mutations improve the fitness of pathogenic species by modification of traits that interfere with factors (virulence and ancestral) required for survival in host tissues. A demonstrated pathoadaptive mutation is the loss of lysine decarboxylase (LDC) expression in *Shigella* species that have evolved from LDC-expressing *Escherichia coli*. Previous studies demonstrated that the product of LDC activity, cadaverine, blocks the action of *Shigella* enterotoxins and that the gene encoding LDC, *cadA*, was abolished by large chromosomal deletions in each *Shigella* species. To better understand the nature and evolution of these pathoadaptive mutations, remnants of the *cad* region were sequenced from the four *Shigella* species. These analyses reveal novel gene arrangements in this region of the pathogens' chromosomes. Insertion sequences, a phage genome, and/or loci from different positions on the ancestral *E. coli* chromosome displaced the *cadA* locus to form distinct genetic linkages that are unique to each *Shigella* species. Hybridization studies, using an *E. coli* K-12 microarray, indicated that the genes displaced to form the novel linkages still remain in the *Shigella* genomes. None of these novel gene arrangements were observed in representatives of all *E. coli* phylogenies. Collectively, these observations indicate that inactivation of the *cadA* antivirulence gene occurred independently in each *Shigella* species. The convergent evolution of these pathoadaptive mutations demonstrates that, following evolution from commensal *E. coli*, strong pressures in host tissues selected *Shigella* clones with increased fitness and virulence through the loss of an ancestral trait (LDC). These observations strongly support the role of pathoadaptive mutation as an important pathway in the evolution of pathogenic organisms.

Classical models of evolution held that generation of new bacterial species was accomplished by accumulation of small changes in individual genes. Recently, however, two opposite mechanisms that shape the development and expression of novel traits in prokaryotes have gained recognition. Horizontal gene transfer is a widely acknowledged gain-of-function pathway used to confer new traits that enhance fitness in a current niche, or enable access to and survival in new environments (13, 25). A complementary loss-of-function system, termed antagonistic pleiotropy, fine tunes the fitness of organisms through mutation of ancestral genes encoding factors that interfere with the expression or function of traits, ancestral and newly acquired, necessary for success in an environment (6, 26). These complementary mechanisms are evident in the evolution of pathogenic bacteria.

Highly adapted, virulent bacteria evolve from commensal organisms through a series of genome modifications. Traits that permit commensal organisms access to new host environments, which often results in host disease, are incorporated into the genome via horizontal gene transfer of multiple linked genes contained on pathogenicity islands, bacteriophage, or plasmids. Selective pressures that confront the recently evolved pathogen in the new environment may be significantly different from those encountered in the ancestral niche. It has been suggested that the new pathogen is not optimally fit for the novel environment as it expresses both virulence factors (required for success in host tissues) and the full complement of ancestral traits (evolved for life in the ancestral niche) (40). Recent studies have demonstrated that, while a subset of ancestral traits may be neutral in the new environment, other traits, encoded by antivirulence genes, may negatively affect fitness by interfering with expression or function of ancestral or virulence factors required for survival within host tissues (2, 6, 16). The loss of ancestral traits encoded by antivirulence genes through negative selection has been termed pathoadaptive mutation (40). These alterations in the newly evolved pathogen genome may be generated through point mutations, which abolish protein function (non-sense mutations) or alter protein function (missense mutation), as well as deletion of antivirulence genes.

This expanded model of pathogen evolution can be traced in the generation of the shigellae. The four *Shigella* species, *S. boydii, S. dysenteriae, S. flexneri*, and *S. sonnei*, are closely related to the nonpathogenic enteric commensal organism *Escherichia coli*. Numerous studies have demonstrated that the two genera can be classified as a single species since their chromosomes are more than 90% homologous and largely colinear (3, 36). This observation is striking when one considers the very different habitats of the two closely related organisms. While nonpathogenic *E. coli* generally inhabit the lumen of the mammalian colon and do not cause disease, the shigellae are agents of bacillary dysentery, an acute inflammatory disease of the human colonic mucosa that results from invasion and intercellular spread of the pathogenic bacteria (29). It has been proposed that the salient factor required to transform the *E. coli* commensal to the *Shigella* phenotype is horizontal transfer of a large virulence plasmid that encodes an enterotoxin and fac-

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tors required for invasion of, and intercellular spread between, host cells (23, 32).

Antagonistic pleiotropy predicts that, since *E. coli* and the shigellae occupy very different environments, when *Shigella* evolved from its *E. coli* ancestor, loss of a subset of ancestral traits was required for optimal fitness and success of the pathogen in host tissues. Consistent with this idea, a number of traits commonly expressed in *E. coli* are not expressed by any *Shigella* species. Among these traits are nicotinic acid prototrophy and the ability to metabolize a number of carbon sources including melibiose, allose, and lysine. For example, greater than ninety percent of all *E. coli* isolates metabolize lysine via decarboxylation. However, none of the shigellae catabolize lysine due to a lack of lysine decarboxylase (LDC) activity (38). Restoration of LDC activity in *S. flexneri*, by supplying the gene encoding lysine decarboxylase (*cadA*) in *trans*, and examination of the strain's virulence phenotypes revealed that the product of LDC activity, cadaverine, blocks the action of *Shigella* enterotoxins (16). The inhibition of this virulence factor's function defines *cadA* as an antivirulence gene. Therefore, we proposed that mutation of the *cadA* locus in *Shigella* species represents a pathoadaptive mutation since elimination of LDC activity enhances enterotoxin activity and, presumably, fitness of the pathogens in host tissues. Interestingly, we showed that absence of LDC activity in each *Shigella* species was due to deletion of a large region (up to 90 kb) of each species' chromosome surrounding *cadA*. Furthermore, the end points of the deletions in each *Shigella* species appeared to vary, and at least one locus, *proP*, that mapped to the middle of the deleted region in the corresponding K-12 chromosome was retained somewhere in the shigellae genomes.

Several studies support the hypothesis that the four *Shigella* species are derived from different *E. coli* ancestors and do not form a subgroup of *E. coli* (27, 31, 32, 34). Our observation that the boundaries of the *cadA* deletion are different in each species of *Shigella* suggested that excision of the antivirulence gene occurred independently in each species after the split from an ancestral *E. coli*. Thus, lack of LDC activity in each *Shigella* species may reflect convergent evolution since the fitness of each newly evolved *Shigella* clone would be optimized through deletion of the *cadA* antivirulence locus. We therefore hypothesized that if the *cadA* pathoadaptive mutation occurred independently in each pathogen, the end points and nature of the *cadA* deletion would be distinct in each *Shigella* species. This report describes the distinct nature of the *cadA* deletions in the four *Shigella* species as determined through sequence analysis of remnants of the *cadA* region in each *Shigella* chromosome. Our findings indicate convergent evolution of the *Shigella cadA* pathoadaptive mutations and suggest that, in order to optimize virulence and fitness in host tissues, each newly evolved *Shigella* clone abolished an ancestral trait (LDC) in response to selective pressures encountered in the virulence niche. These results provide further evidence of the important role of pathoadaptive mutations in the evolution of pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains and growth media. Strains and species of *Shigella* and *E. coli* used in this study are described in Table 1. The four *Shigella* species employed, and the respective derivatives, are representatives of four separate, previously identified, *Shigella* lineages (32). *Shigella* species and *E. coli* strains were routinely cultured in Luria-Bertani broth at 30°C in a shaking water bath or on Luria-Bertani agar plates supplemented with the appropriate antibiotics in a 37°C incubator. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; erythromycin, 100 μg/ml; naladixic acid, 40 μg/ml; spectinomycin, 100 μg/ml; tetracycline, 5 μg/ml.

Molecular methods. Plasmids generated in the course of these studies are described in Table 1. Analyses of DNA, plasmid constructions, genomic extractions, and electroporations of *E. coli* were performed using manufacturers' suggested conditions or standard protocols described elsewhere (35). All restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.). Amplification of products less than 4 kb long employed Accu-POL DNA polymerase (PGC Scientifics, Frederick, Md.); amplification of products greater than 4 kb employed Long Taq Plus DNA polymerase (Stratagene, La Jolla, Calif.). Each thermostable DNA polymerase was used according to the manufacturer's protocols. Templates for DNA sequencing were prepared using the ABI Prism Dye Terminator cycle Sequencing Kit. Products were analyzed on an ABI Prism 377 DNA sequencer in the Uniformed Services University of the Health Sciences (USUHS) Biomedical Instrumentation Center. Oligonucleotide synthesis was performed using Applied Biosystems automated solid-phase synthesis with standard chemistry at the USUHS Biomedical Instrumentation Center.

Cloning of the *S. flexneri* 2a *cadA* deletion region. To clone regions counterclockwise (CCW) to *yjdC* in the *S. flexneri* 2a chromosome the *dsbD* locus was tagged with a cassette carrying an antibiotic resistance marker and multiple restriction sites. PCR amplification of the *yjdC-dsbD* region from the *S. flexneri* 2a chromosome was accomplished using primers corresponding to the 3′ end of *yjdC* and the 5′ end of *dsbD*. Employing restriction sites engineered on the 5′ ends of each primer the product was cloned into *Xba*I/*Hind*III-digested pUC19 to generate pEBD173. The *ery* gene from transposon Tn*10*CamErmRCP2, which contains a multiple restriction site cassette immediately upstream of the erythromycin resistance locus (14), was amplified from *E. coli* strain χMZ115 and cloned into the unique *Bgl*II site in *dsbD* on pEBD173. The resulting plasmid was designated pEBD176. Replacement of the *S. flexneri* 2a wild type *dsbD* allele with the *dsbD*::*ery* allele on pEBD176 was accomplished by linear DNA transformation. Briefly, the pEBD176 insert was PCR amplified using primers derived from vector sequences outside the multiple cloning site, and the products were digested with *Dpn*I to degrade the plasmid template, concentrated via ethanol precipitation, and electroporated into BS600, a *S. flexneri* strain competent for linear DNA substrates. BS660, a double crossover recombinant that harbors the *dsbD*::*ery* allele, was identified by its resistance to erythromycin and scored for ampicillin sensitivity. The recombinant allele structure of BS660 was confirmed by subsequent PCR analysis. BS660 genomic DNA was then completely digested with *Xma*I, which cuts upstream of *ery* and CCW in the *cadA* deletion region (not in *dsbD* or *yjdC*). The products were ligated to pUC19, and electroporated into *E. coli* DH5α (Fig. 1A). Selection of ampicillin and erythromycin resistant transformants permitted recovery of pEBD194 which contained DNA CCW to *yjdC* in the *S. flexneri* 2a chromosome. The insert in pEBD194 was sequenced by primer walking.

Cloning of the *S. boydii* 18, *S. dysenteriae* 1, and *S. sonnei cadA* deletion regions. To clone regions CCW to *yjdC* in *S. boydii* 18, *S. dysenteriae* 1, and *S. sonnei*, a modification of the above strategy was employed. The *spc* gene from transposon Tn*10*SpcRCP2, which contains a multiple restriction site cassette immediately downstream of the spectinomycin resistance locus (14), was amplified from *E. coli* strain χMZ2125 and cloned into the unique *Eco*RV site of pEBD173 (see above), located 32 bp from the 3′ end of *yjdC*. The insert was oriented such that the restriction sites were situated between the *yjdC*′ gene fragment and the *spc* resistance marker to generate pEBD218. To construct a suicide plasmid that would integrate into the *Shigella* chromosome at *yjdC* and enable retrieval of regions CCW to *yjdC*, the *yjdC*′-*spc* allele from pEBD218 was cloned as a *Pvu*II fragment into the *Fsp*I-*Sma*I sites of the R6K-based suicide vector pGP704 (19), generating pEBD221 (Fig. 1B). pEBD221 was introduced into *E. coli* SM10λ*pir* and delivered to *S. boydii* 18, S. *dysenteriae* 1, and *S. sonnei* by conjugation. Selection for single crossover recombinants harboring pEBD221 integrated at the *yjdC* locus was made by plating for resistance to spectinomycin. Counterselection against the *E. coli* donor utilized antibiotics appropriate for each recipient *Shigella* strain. The recombinant allele structure of each strain (i.e., integration of pEBD221 at *yjdC*) was confirmed using PCR. Genomic DNA extracted from the recombinants BS650, BS661, and BS663 was completely digested with *Bgl*II or *Xma*I, which cut downstream of *spc* and CCW in the *cadA* deletion region (not in the integrated suicide vector or *yjdC*). The products were self-ligated, and electroporated into *E. coli* DH5αλ*pir*, a K-12 strain that encodes the π protein and permits replication of plasmids carrying the R6K *ori*. This

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant information	Source or reference
Shigella strains		
2457T	Wild type *S. flexneri* 2a (cluster III *Shigella* lineage)	10
60R	Wild type *S. dysenteriae* 1 Tet^r (clonal *S. dysenteriae* 1 lineage)	22
BS109	2457T *galU*::Tn*10* Tet^r	Laboratory stock
BS600	BS109 (Δ*recB ptr recC recD*)::P_{lac} *bet exo cat galU*::Tn*10* (P1L4 transductant from KM32)	This study
BS601	Wild-type *S. sonnei* Nal^r (clonal *S. sonnei* lineage)	Laboratory stock
BS649	*S. boydii* 18 *galU*::Tn*10* Tet^r (cluster I *Shigella* lineage)	Laboratory stock
BS650	BS649::pEBD221 Spc^r Tet^r	This study
BS660	BS600 *dsbD*::*ery* Erm^r	This study
BS661	60R::pEBD221 Spc^r Tet^r	This study
BS663	BS601::pEBD221 Spc^r Nal^r	This study
E. coli strains		
MG1655	K-12 reference strain	11
χMZ115	MG1655 *zii-215*::Tn*10* CamErmRCP2	1
χMZ2125	MG1655 *zjh-225*::Tn*10* SpcRCP2	1
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* (Δ*lacIZYA-argF*)*U169 deoR* (φ80 *dlac*Δ(*lacZ*) *M15*)	12
DH5α λ*pir*	λ*pir* lysogen of DH5α	Laboratory stock
KM32	K-12 (Δ*recB ptr recC recD*)::P_{lac} *bet exo cat*	20
SM10λ*pir*	*thi-1 thr-1 leuB6 tonA21 lacY1 supE44 recA*::*RP4-2-Tc*::*Mu* Kan^r λ*pir*	39
ATM558	pEBD221 transformant of SM10λ*pir*	This study
EC07	ECOR strain, group A phylogeny	18
EC12	ECOR strain, group A phylogeny	18
EC15	ECOR strain, group A phylogeny	18
EC30	ECOR strain, group B1 phylogeny	18
EC40	ECOR strain, group D phylogeny	18
EC42	ECOR strain, group E phylogeny	18
EC47	ECOR strain, group D phylogeny	18
EC54	ECOR strain, group B2 phylogeny	18
EC59	ECOR strain, group B2 phylogeny	18
EC72	ECOR strain, group B1 phylogeny	18
Plasmids		
pUC19	Cloning vector, ColE1 *ori*, Ap^r	NEB
pGP704	Suicide vector, R6K *ori*, RP4 *mob*, Ap^r	19
pEBD173	*S. flexneri* 2a *yjdC-dsbD* region cloned into *Xba*I/*Hind*III sites of pUC19, Ap^r	This study
pEBD176	Derivative of pEBD173, *ery* marker and restriction sites of Tn*10*CamErmRCP2 cloned into unique *Bgl*II site in *dsbD*, Ap^r Erm^r	This study
pEBD194	8.5-kb *Xma*I fragment of BS660 chromosome (*S. flexneri* 2a) cloned into *Xma*I site of pUC19, Erm^r Ap^r	This study
pEBD218	Derivative of pEBD173, *spc* gene and restriction sites of Tn*10*SpcRC cloned into unique *Eco*RV site located 32 bp from the *yjdC* stop codon, Ap^r Spc^r	This study
pEBD221	Derivative of pGP704, *yjdC'-spc* allele of pEBD218 cloned into *Fsp*I/*Sma*I sites of the suicide vector, Spc^r	This study
pEBD249	14-kb self-ligated *Bgl*II fragment of BS661 (*S. dysenteriae* 1) chromosome, Spc^r	This study
pEBD273	32-kb self-ligated *Bgl*II fragment of BS650 (*S. boydii* 18) chromosome, Spc^r	This study
pEBD303	26-kb self-ligated *Xma*I fragment of BS663 (*S. sonnei*) chromosome, Spc^r	This study

strategy permitted recovery of plasmids pEBD249, pEBD273, and pEBD303 that contain chromosomal DNA CCW of *yjdC* in *S. dysenteriae* 1, *S. boydii* 18, and *S. sonnei*, respectively (Table 1). Sequencing of all inserts was accomplished by primer walking.

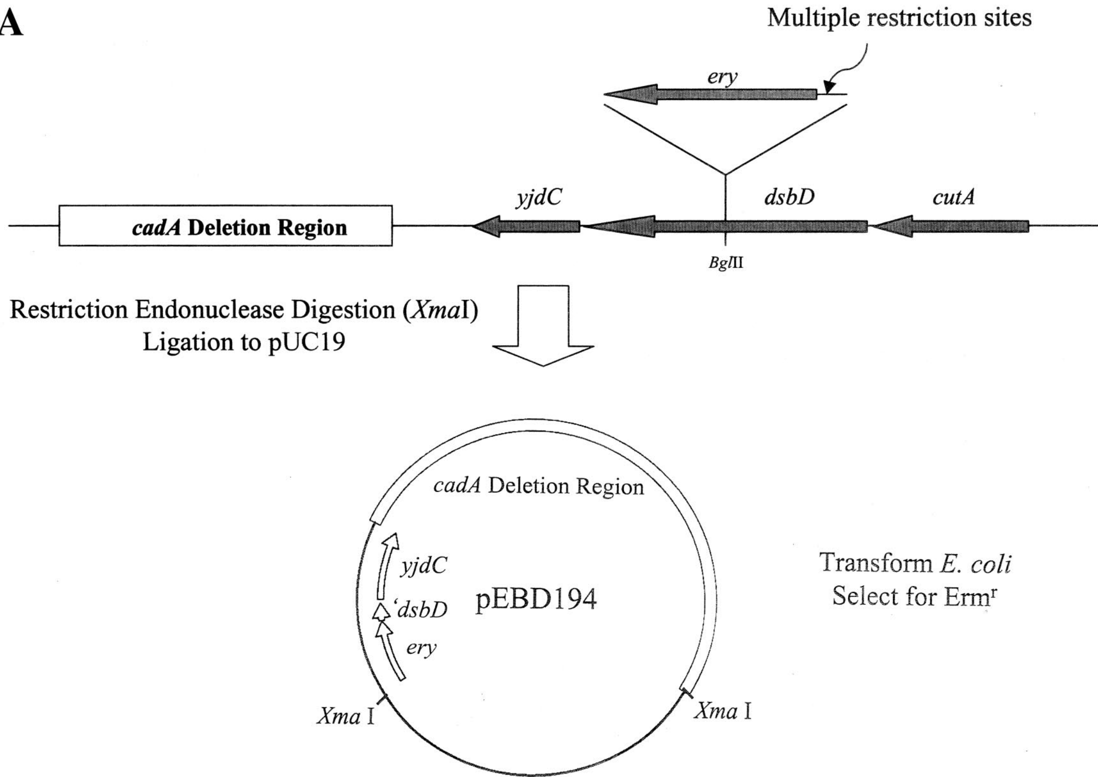
Microarray hybridization and analysis. Microarray nylon membranes containing all 4290 open reading frames (ORFs) from *E. coli* K-12 were purchased from Sigma-Genosys Biotechnologies (Woodland, Tex.). Hybridization, signal detection, and stripping of microarray filters were performed as directed by the manufacturer. Radiolabeled DNA probes derived from the *Shigella* genomes were generated using random hexamer primers (Roche Molecular Biochemicals, Indianapolis, Ind.) and Klenow DNA polymerase (New England Biolabs) to incorporate [^{33}P]dCTP into newly synthesized products. Unincorporated nucleotides were removed using G-50 Sephadex spin columns (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.). Microarray filters were prehybridized with 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 2% sodium dodecyl sulfate (SDS), 1× Denhardt's, and sheared salmon sperm DNA (100 mg/ml) for 2 h at 65°C. Hybridization with 3 ml of probe in fresh hybridization solution was performed overnight (18 h) at 65°C. Unbound and nonspecifically bound probe was removed by washing the filters three times in 0.5× SSPE–0.2% SDS for 10 min at 25°C followed by three washes in the same buffer for 20 min at 65°C. Washed filters were air dried and exposed overnight to a phosphorimager screen which was then scanned on a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) at a resolution of 50 μm. The filters were stripped to allow rehybridization by incubation in 50% formamide–0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 30 min at 65°C and rinsing in 0.1× SSC–0.1% SDS for 30 min at 65°C. Removal of bound probe was assessed by scanning the stripped filters overnight with the phosphorimager.

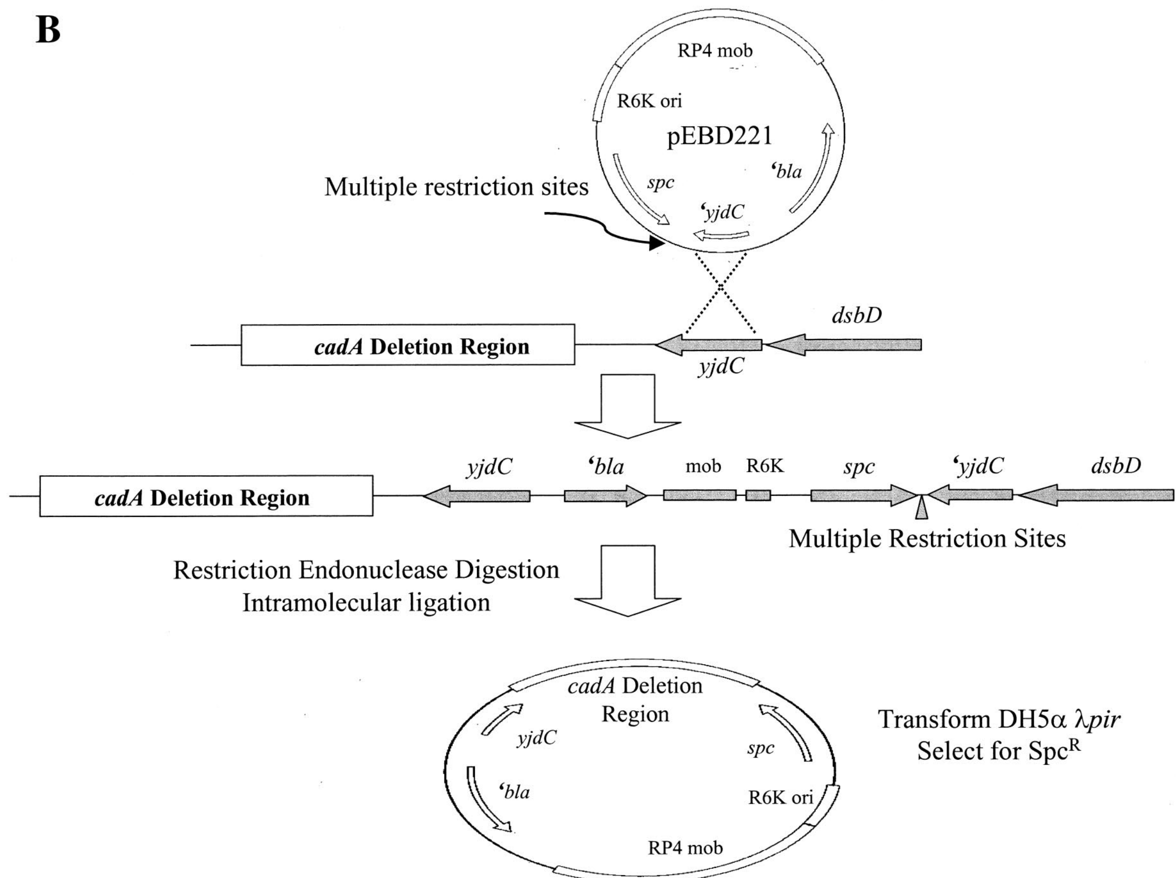
Analysis of the scanned microarray autoradiographs was performed as described by Ochman and Jones (24).

Nucleotide sequence accession numbers. The sequences of the *S. flexneri* 2a, *S. boydii* 18, *S. dysenteriae* 1, and *S. sonnei cadA* deletion regions (pathoadaptive mutations) have been deposited in GenBank under accession numbers AF417476, AF417477, AF417478, and AF417480, respectively.

RESULTS

***yjdC* is a conserved anchor locus linked to the *Shigella cadA* deletion region.** Our previous studies suggested that, relative to


A
Multiple restriction sites
ery
yjdC
dsbD
cutA
cadA Deletion Region
BglII
Restriction Endonuclease Digestion (XmaI)
Ligation to pUC19
cadA Deletion Region
yjdC
'dsbD
pEBD194
ery
Xma I
Xma I
Transform E. coli
Select for Erm^r


B
RP4 mob
R6K ori
pEBD221
Multiple restriction sites
spc
'bla
'yjdC
dsbD
cadA Deletion Region
yjdC
yjdC
'bla
mob
R6K
spc
'yjdC
dsbD
cadA Deletion Region
Multiple Restriction Sites
Restriction Endonuclease Digestion
Intramolecular ligation
cadA Deletion
Region
yjdC
spc
'bla
R6K ori
RP4 mob
Transform DH5α λpir
Select for Spc^R

E. coli K-12, the *Shigella* chromosomes contain large (up to 90 kb) deletions surrounding the *cadA* antivirulence locus (16). As a first step to characterize this region of the shigellae chromosomes, we determined the end points of the deletion for each species chromosome. *dsbD*, a gene whose role in *Shigella* virulence was previously studied (42), is located 4,689 bp clockwise (CW) from *cadA* in *E. coli* K-12 (http://genolist.pasteur.fr/Colibri/genome.cgi). Since this earlier report demonstrated the presence of *dsbD* in the *S. flexneri* 2a genome, a PCR-based analysis was performed to determine whether the linear arrangement of genes neighboring this locus is similar to that found in *E. coli* K-12. The generation of identically sized PCR products from both *S. flexneri* 2a and *E. coli* K-12 chromosomes, using overlapping primer sets, demonstrated that linkage of *dsbD* to CW loci as far away as *psd* (located roughly 30 kb in the CW direction) is identical in *S. flexneri* 2a and *E. coli* K-12 and indicated colinearity of the two chromosomes in this region (Fig. 2 and Table 2). Moreover, since *dsbD* is located just CW of *cadA* in *E. coli* K-12, we reasoned that *dsbD* may be located close to the edge of the *S. flexneri* 2a *cadA* deletion. Therefore, PCR-based linkage analysis was performed to determine the distance between *dsbD* and the *S. flexneri* 2a *cadA* deletion border. Consistent with our previous findings, colinearity was lost CCW of *yjdC*, the locus immediately adjacent to *dsbD* (Table 2). No products were amplified from the *S. flexneri* 2a chromosome using a *yjdC* primer with primers derived from *cadC, cadB, cadA*, or *yjdL* (Table 2 and data not shown). PCR analysis further demonstrated that linkage of loci CW of the *cadA* deletion region (from *yjdC* to *dsbD*) is identical in all other species of *Shigella* examined, suggesting that *yjdC* is a conserved anchor point that maps to the edge of the *cadA* deletion region in the shigellae (Table 2).

Composition of the *S. flexneri* 2a *cadA* deletion region indicates rearrangement. To gain a better understanding of the nature of the *cadA* deletion in *S. flexneri* 2a, an 8.5-kb region of the *S. flexneri* 2a chromosome, CCW to *yjdC* and corresponding to the *cadA* deletion, was cloned (as pEBD194) and sequenced (Fig. 1A; Materials and Methods). Analysis of the region revealed that all remnants of the *cad* operon, except for a fragment of the *cadB* gene, were absent and were replaced with insertion sequences linked to novel ORFs not found in the *E. coli* K-12 genome (Fig. 2). Homology between the *S. flexneri* 2a and *E. coli* K-12 chromosomes is reestablished two-thirds of the way through the *ytfA* gene, which is present as a gene fragment in the K-12 genomic sequence (http://genolist.pasteur.fr/Colibri/genome.cgi). BLASTp searches using the *S. flexneri* 2a *ytfA* ORF revealed significant global homology (25% identity and 47% similarity) to the transcription factor TetR (30). The relatedness of the predicted YtfA and TetR proteins is highest in the N-terminal domain that contains a helix-turn-helix motif required for DNA binding in the TetR/AcrR family of transcription factors (41). The observation that this conserved region is missing in the truncated *E. coli* K-12 *ytfA* ORF indicates that the K-12 chromosome is rearranged at this locus and suggests that *E. coli* K-12 (of *E. coli* group A phylogeny) is not a likely ancestor for *S. flexneri* 2a. Proteins encoded by *orf2* and *orf3* have most significant global homology to *Mesorhizobium loti* 3-oxoacyl-acyl carrier protein reductase (43% identity), which is involved in fatty acid elongation, and an *M. loti* tagatose 3-epimerase (31% identity), which is involved in carbohydrate catabolism, respectively.

Several studies have demonstrated that *Shigella* lineages or species have evolved from other *E. coli* phylogenies (groups B1, B2, D, or E). To determine whether the K-12 genomic sequence in the *cadA* region is representative of linkages in other *E. coli* strains that may be ancestors of *Shigella* species, gene order in the *cad* region was examined in representatives of the five *E. coli* phylogenies. Ten strains of *E. coli*, representing all *E. coli* phylogenies, were obtained from the ECOR collection (18), and the gene order in the *cad* region of these strains' chromosomes relative to the K-12 *cad* region was examined by PCR. Amplification of identically sized products from all *E. coli* strains examined, in both directions relative to *cad*, demonstrates the colinearity in this region of all 10 *E. coli* strains (Table 2). Moreover, colinearity of the *cadA* region in representatives of all five *E. coli* phylogenies suggests that disruptions of the *cadA* antivirulence gene are unique to the *Shigella* species and have not occurred in other *E. coli* strains.

To determine whether the novel gene arrangements in the *S. flexneri* 2a *cadA* deletion region were present in the *cadA* pathoadaptive mutations of other *Shigella* species, PCR analysis was conducted using primers derived from a number of loci present in the region (Fig. 2). These analyses demonstrate the novelty of the *S. flexneri* 2a *cadA* deletion region since similar linkage (specifically, *ytfA* to *yjdC*) was not observed in other *Shigella* species (Table 2). However, linkage of the complete *ytfA* allele to *orf3* was observed in *S. sonnei* suggesting the likelihood that this linkage existed in *E. coli* phylogenies that are ancestral to *S. flexneri* 2a and *S. sonnei*. Collectively, these observations indicate that the *S. flexneri* 2a *cadA* deletion region is unique to this species and demonstrate rearrangement in this region of the chromosome.

Distinct composition of the *cadA* regions in each *Shigella* species. In order to compare the *cadA* deletion of each *Shigella* species, a strategy was devised that simplified cloning of regions CCW to the *yjdC* anchor (Fig. 1B; Materials and Methods). This strategy permitted recovery of regions CCW to *yjdC*

FIG. 1. Strategy used to clone regions CCW of *yjdC*. (A) To clone the regions CCW of *yjdC* from the *S. flexneri* 2a chromosome the *ery* gene from Tn*10*dCamErmRCP2 was introduced at the *Bgl*II site of *dsbD* oriented so that, following replacement of the wild-type *dsbD* with the marked allele (see Materials and Methods), restriction with endonucleases that cut within the multiple restriction site upstream of *ery* retains linkage of the resistance marker with regions CCW to *yjdC*. The *ery*-tagged fragment was cloned into pUC19, transformed into *E. coli*, and selected by plating for Erm^r. (B) To clone the regions CCW to *yjdC* from the *S. boydii* 18, *S. dysenteriae* 1, and *S. sonnei* chromosomes, the circular suicide plasmid pEBD221 was introduced into each *Shigella* species by conjugation. pEBD221 integrates into the *Shigella* chromosome at *yjdC* by single-crossover recombination to generate the spectinomycin resistance allele depicted in the second chromosome. Restriction with endonucleases that cut within the multiple restriction site downstream of the *spc* resistance marker and at a CCW site in the *cadA* deletion region generates a self-replicating fragment containing the regions CCW to *yjdC*, the R6K *ori*, and the *spc* selectable marker. Self-ligated plasmids are recovered in a host carrying the *pir* gene (see Materials and Methods).

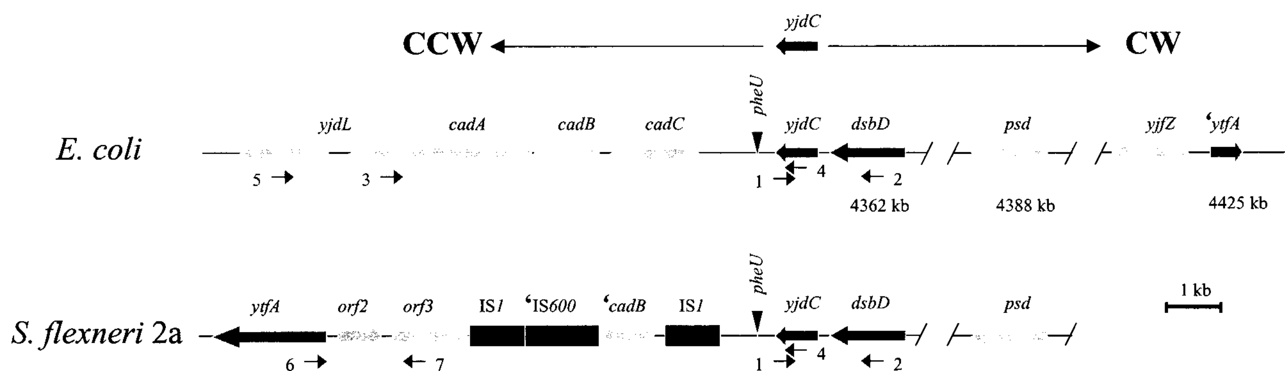


FIG. 2. Alignment of chromosomal regions adjacent to *yjdC* in *E. coli* and *S. flexneri* 2a based on sequence comparison of pEBD194 with *E. coli* K-12 and PCR-based linkage analyses. Orientation of the depicted region relative to *yjdC* of *E. coli* K-12 is indicated above the figure: CW, clockwise; CCW, counterclockwise. Gene loci are depicted as arrows, insertion sequences are depicted as black rectangles, the *pheU* tRNA locus is depicted as an inverted triangle; truncated ORFs and insertion sequences are indicated by a single quotation mark. The two chromosomal maps are aligned at the *yjdC* locus to facilitate comparison. Locations of primers used in the linkage analyses (Table 2) are depicted below the appropriate ORF. The physical map locations (in kilobase pairs) of *dsbD, psd*, and *'ytfA* on the *E. coli* K-12 chromosome are indicated below each ORF.

in *S. boydii* 18, *S. dysenteriae* 1, and *S. sonnei* via linkage to a suicide plasmid (pEBD221) integrated at *yjdC*. The strains selected to represent each *Shigella* species also represent three different *Shigella* lineages as described by Pupo et al. (see Table 1) (32). As was the case for *S. flexneri* 2a, comparison of *Shigella* DNA sequences CCW of *yjdC* to the *E. coli cad* region revealed the nature of the pathoadaptive mutations (Fig. 3). In *S. boydii* 18, all remnants of the *cad* operon are replaced by genes homologous to those encoding a P4-like prophage that has integrated immediately CCW of the *pheU* (phenylalanine tRNA) region. While the presence of the prophage did not exclude the possibility that the *cad* operon existed CCW to the phage genome, hybridization analysis using an *E. coli* K-12 microarray demonstrated that the *cad* genes were not present in the *S. boydii* 18 genome (Fig. 4; see below). Preliminary downstream sequence data indicate that this region of the *S. boydii* 18 genome is allelic with, and identical to, the recently described SHI-3 pathogenicity island which is comprised of not only P4-like prophage loci but also encodes an iron-uptake system (33). In *S. dysenteriae* 1 the *cad* operon is replaced by an IS*1* element. Homology between the *S. dysenteriae* 1 and *E. coli* K-12 chromosomes is re-established upstream of *kgtP*, a locus found at kb 2724 on the *E. coli* K-12 physical map. The novel linkage CCW of *yjdC* in the *S. dysenteriae* 1 chromosome indicates extensive rearrangement in this region. In *S. sonnei*, the entire *cad* operon linked to *yjdL* (CCW) and *yjdC* (CW) was recovered indicating that no rearrangement had occurred in this region of the chromosome relative to *E. coli*. The *cadA* pathoadaptive mutation in *S. sonnei* resulted from an iso-IS*3* element inserted in *cadA* 259 bp from the stop codon. Two other IS elements, IS*4* and iso-IS*3*, were present in this region and disrupt the *cadB* ORF.

TABLE 2. PCR-based linkage analysis of loci adjacent to *yjdC* in *E. coli* and *S. flexneri* 2a[a]

Strain	Site of amplification product					
	dsbD-psd (primer walk)[b]	*yjdC-dsbD* (pr1-pr2)	*cadA-yjdC* (pr3-pr4)	*yjdL-yjdC* (pr5-pr4)	*ytfA-yjdC* (pr6-pr4)	*ytfA-orf3* (pr6-pr7)
S. flexneri 2a	26.7	1.6	NPA[c]	NPA	7.5	1.8
S. boydii 18	26.7	1.6	NPA	NPA	NPA	NPA
S. dysenteriae 1	26.7	1.6	NPA	NPA	NPA	NPA
S. sonnei	26.7	1.6	NPA	NPA	NPA	1.8
E. coli K-12	26.7	1.6	6.4	8.0	NPA	NPA
EC07	ND[d]	ND	6.4	8.0	ND	ND
EC12	ND	ND	6.4	8.0	ND	ND
EC15	ND	ND	6.4	8.0	ND	ND
EC30	ND	ND	6.4	8.0	ND	ND
EC40	ND	ND	6.4	8.0	ND	ND
EC42	ND	ND	6.4	8.0	ND	ND
EC47	ND	ND	6.4	8.0	ND	ND
EC54	ND	ND	6.4	8.0	ND	ND
EC59	ND	ND	6.4	8.0	ND	ND
EC70	ND	ND	6.4	8.0	ND	ND

[a] Loci examined for linkage are provided as gene pairs; loci located between the gene pairs are not indicated. Primers (pr1, pr2, etc.) used in PCR analyses, derived from the gene pairs examined, are indicated below each pair of genes and their positions are depicted in Fig. 2. Sizes of amplification products (in kilobase pairs) are indicated below each pair of genes analyzed.

[b] Overlapping primer sets were used in long PCRs to demonstrate linkage of these loci.The product reported (26.7 kb) is the sum of amplification products.

[c] NPA, no product amplified.

[b] ND, not done.

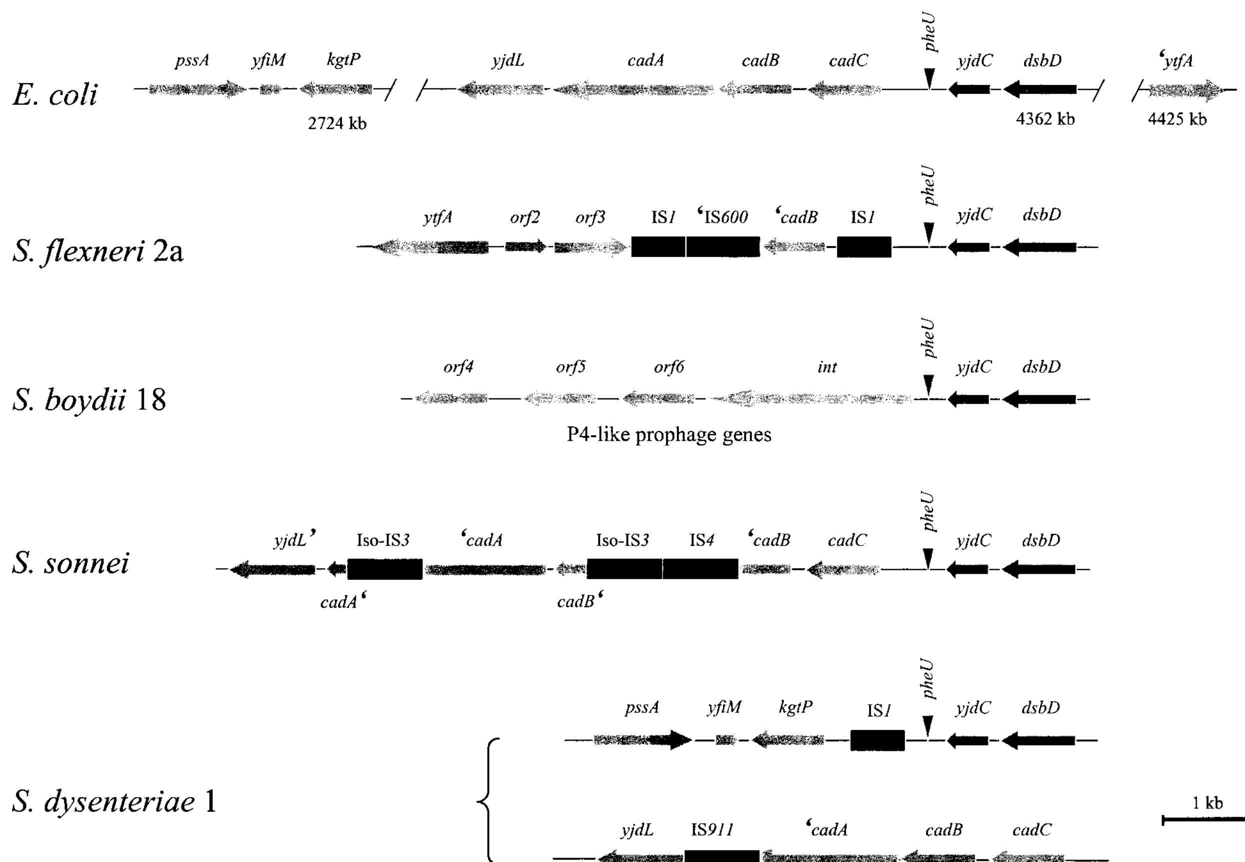


FIG. 3. Alignment of chromosomal regions CCW to *yjdC* in *E. coli* and the four *Shigella* species. All features are depicted as in Fig. 2. The chromosomal maps are aligned at the *yjdC* locus to facilitate comparison. The locations (in kilobase pairs) of *kgtP, dsbD*, and *'ytfA* on the *E. coli* K-12 chromosome are indicated below each ORF. The *S. dysenteriae* 1 *cad* operon, which is displaced and not linked to *yjdC*, is depicted below the region CCW to *yjdC*.

Rearrangement in the *cadA* region is not accompanied by deletion of neighboring loci. Disruption of *yjdL-yjdC* linkage and the disparity in loci linked to the *yjdC* anchor in three of the *Shigella* species examined suggested that these chromosomes were extensively rearranged in this region relative to their respective ancestral *E. coli*. Our previous studies suggested that a number of loci CCW of *yjdC* were deleted from each *Shigella* genome, including *S. sonnei* (16). To determine whether the novel compositions observed CCW to *yjdC* in the shigellae were accompanied by deletion of neighboring, intervening, or displaced loci, a microarray containing all of the *E. coli* K-12 ORFs spotted in duplicate on nylon membranes was screened with labeled genomic DNA from each *Shigella* species. Hybridizations were also performed with genomic DNA from ECOR47, a group D *E. coli* (18). MG1655, the *E. coli* K-12 from which the microarray was generated, was used as a positive control. Figure 4 is a schematic representation of array signals obtained with each genome probe corresponding to loci present CCW of *yjdC* in *E. coli* K-12. The pattern of hybridization signals indicated the presence of all non-*cad* operon loci CCW to *yjdC* in each *Shigella* genome examined, suggesting that none of the rearrangements shown (Fig. 3) resulted in deletion of loci previously linked to *cadA*. Therefore, these displaced loci are present elsewhere in the *S. boydii* 18, *S. dysenteriae* 1, and *S. flexneri* 2a genomes, unlinked to *yjdC*. In contrast, hybridization signals for the *cad* operon varied between *Shigella* species. Consistent with sequence data CCW to *yjdC* in *S. flexneri* 2a, a hybridization signal was observed for *cadB* alone. Hybridization signals were observed for both *cadB* and *cadC* in *S. sonnei*. No signal was observed for the *cadA* ORF. These data are inconsistent with sequence analysis of the *S. sonnei cad* operon (Fig. 3). However, amplification of the interrupted *cadA* ORF from two different *S. sonnei* isolates (data not shown) substantiates our sequence data and indicates that the hybridization screenings are not as reliable (i.e., may yield false negatives) as sequence data. Sequence data of the region CCW to *yjdC* in *S. boydii* 18 did not suggest a fate for *cadA* in this species (Fig. 3). However, the absence of hybridization signals for any of the genes in the *cad* operon indicated that insertion of the phage genome at the *pheU* locus did not simply displace the *cad* genes. Rather, deletion of the *cad* operon may have preceded, coincided with, or occurred after integration of the prophage. Surprisingly, hybridization signals were observed for *cadC, cadB*, and *cadA* with the *S. dysenteriae* 1 probe, suggesting that the rearrangement resulting in the novel linkage CCW of *yjdC* was not accompanied by deletion of these genes. A positive *S. dysenteriae* 1 hybridization signal to *yjdL*, which is located immediately CCW of *cadA* in all *E. coli* phylogenies (Table 2), suggested that the apparently intact *cad* operon may retain CCW linkages present in the species

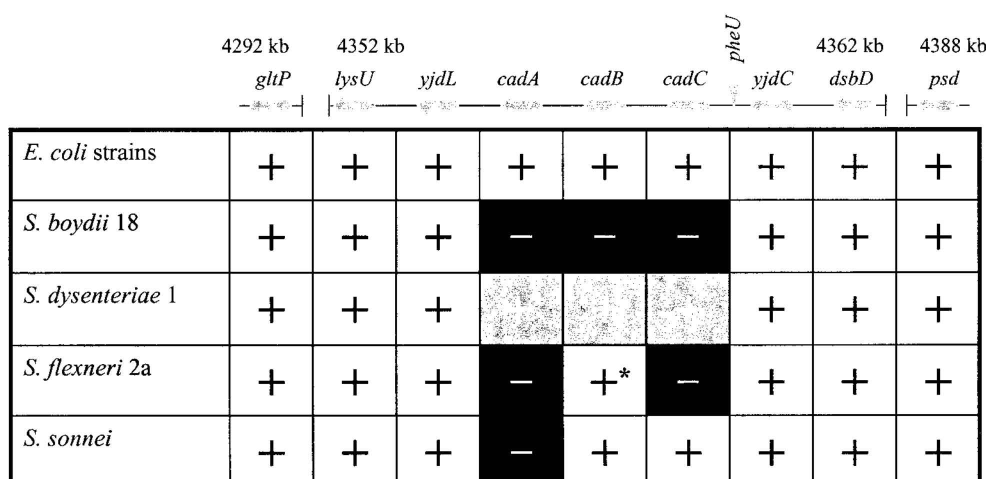


FIG. 4. Microarray hybridization signals of loci adjacent to or displaced from the *Shigella cadA* regions. Gene loci (not drawn to scale) adjacent to *yjdC*, as ordered in this region of the *E. coli* K-12 chromosome, are depicted as arrows; the *pheU* tRNA locus is depicted as an inverted triangle. Loci between *gltP* and *lysU* and between *dsbD* and *psd* (indicated by the gapped line) demonstrated positive hybridization signals with DNA probes from all six genomes. The locations in kilobases on the *E. coli* K-12 chromosome are indicated above boundary loci. The plus symbol indicates the presence of a hybridization signal; the minus symbol on a black background indicates the absence of a hybridization signal. The plus symbol on a gray background indicates rearrangement of these loci; *, nonfunctional ORF (see text and Fig. 3). Identical hybridization patterns for this chromosomal region were obtained with genomic probes derived from two different strains of *E. coli*, MG1655 (K-12, group A *E. coli* phylogeny) and EC47 (group D *E. coli* phylogeny).

ancestor. Using primers internal to *yjdL* and *cadB*, a PCR product of 3.0 kb was amplified from the *S. dysenteriae* 1 genome. Sequence analysis of the product indicated that the *cadA* ORF was interrupted by an IS*911* insertion sequence 146 bp from the stop codon (Fig. 3). Together with sequence data obtained from the *yjdC* region of the *S. dysenteriae* 1 chromosome, these results indicate that the intact *cad* operon was moved to another region of the chromosome and contains a mutation in *cadA*. Collectively, our results demonstrate that the *cadA* deletion regions are unique in each *Shigella* species. Moreover, these findings strongly suggest that mutation of the *cadA* anti-virulence gene was accomplished by different mechanisms in each species.

DISCUSSION

It has been proposed that *Shigella* species evolved from commensal *E. coli* that acquired the *Shigella* virulence plasmid via horizontal transfer and, with it, the ability to occupy a new host niche (inside colonocytes) (23, 32). Pathoadaptive mutations arose within the genome of the new pathogen in response to selective pressure for optimal fitness in host tissues. This mutation pathway eliminates or modifies traits that inhibit fitness (i.e., antivirulence genes). Recent insights into the evolution of the shigellae suggest that these organisms may serve as a model for the study of pathoadaptive mutations and the evolution of pathogenic bacteria. Several investigators have suggested that the evolution of new pathogen lineages expressing the *Shigella* phenotype has occurred multiple times (27, 31, 34). These observations predict that the four *Shigella* species evolved from separate *E. coli* ancestors and do not form an individual *E. coli* subgroup. Most recently, Pupo et al. identified seven separate *Shigella* lineages (32). Three lineages (cluster I [which includes *S. boydii* 18], II, and III [which includes *S. flexneri* 2a]) are comprised of several pathogenic strains identified by metabolic characteristics and/or serotype. The remaining four lineages are comprised of single clones, including *S. dysenteriae* 1, and *S. sonnei*. An important implication of these observations is that expression of traits unique to and shared by all *Shigella* species reflects convergent evolution, whether the traits were acquired through gain-of-function (via horizontal gene transfer) or loss-of-function (pathoadaptive and random mutation) mutations.

We hypothesized that deletion of the *cadA* antivirulence locus occurred independently in each species or lineage of *Shigella* and was accomplished by different mechanisms. To test this hypothesis, the studies we report here examine the nature of the *cadA* pathoadaptive mutation in four *Shigella* species representative of four separate lineages through sequence analysis of what was once the *cadA* region of these organisms' chromosomes. Implicit in this analysis is the axiom that the regions studied are, in fact, remnants of the disrupted *cadA* regions. Data from the linkage studies support this assumption. First, linkage analysis of the *cad* region to *yjdC* in ten ECOR strains demonstrated that gene order in this region is conserved in all *E. coli* phylogenies. Since the shigellae evolved from commensal *E. coli*, it is reasonable to assume that *yjdC* was linked to the *cad* operon in ancestors of the shigellae. Second, the shigellae and *E. coli* chromosomes are colinear in the region CW to *yjdC*. Since colinearity between *E. coli* and each *Shigella* chromosome is disrupted immediately CCW to *yjdC*, this gene is positioned at the edge of the *cadA* pathoadaptive mutation in each pathogen's chromosome. Consequently, our studies focused on characterization of the regions CCW to *yjdC* that are linked to remnants of the *cad* operon in each *Shigella* chromosome.

The discrete compositions of loci present in this region of each *Shigella* chromosome support our hypothesis. These dis-

similar linkages argue that deletion of the *cadA* antivirulence gene occurred independently, and by different mechanisms, in each species. The disparity in the remnants of the *Shigella cadA* regions also supports the idea that each *Shigella* species evolved independently and that lack of LDC activity in the shigellae results from convergent evolution. Collectively, these observations reinforce the concept of *cadA* as an antivirulence gene in the shigellae and that ablation of LDC activity in these pathogens occurred as a pathoadaptive mutation. Indeed, our findings suggest that each time an *E. coli* commensal acquired the *Shigella* enterotoxin gene *senA* (encoded on the virulence plasmid), it encountered strong selective pressure for deletion of the *cadA* antivirulence locus as LDC-deficient clones would permit optimal enterotoxin activity. The action of the toxin expressed in each new *Shigella* clone (21) likely enhanced dissemination of the pathogens into the environment where they may infect new hosts.

Our previous study reported that removal of the *cadA* locus from each *Shigella* chromosome was accomplished by deletion of distinct large (>90 kb) regions of the chromosomes (16). The novel linkages to *yjdC* in each *Shigella* chromosome raised the possibility that the previously reported large deletions resulted from rearrangements that generated the observed linkages. This possibility was particularly plausible for *S. dysenteriae* 1 and *S. flexneri* 2a, in which different sections of the *E. coli* chromosome are linked to *yjdC*. However, our data indicate that rearrangement at *cadA* was not accompanied by large deletions outside the *cad* operon. Hybridization screens of an *E. coli* K-12 microarray demonstrated that essentially all non-*cad* operon loci displaced to make way for the observed linkages are still present in the *Shigella* genomes. This observation indicates rearrangement of at least one large region of the *S. dysenteriae* 1 and *S. flexneri* 2a chromosomes. Similar observations have been reported by Shu et al. as pulsed field analysis revealed rearrangement of the *S. dysenteriae* 1 and *S. flexneri* 2a chromosomes that map close to the *cadA* deletion regions (37). While the extent and nature of rearrangements vary between *S. dysenteriae* 1 and *S. flexneri* 2a lineages, both rearrangements involve *cadA* and are associated with gene inactivation. No evidence of rearrangement was observed in any of the five *E. coli* phylogenies. In fact, gene order in this region of the commensal's chromosomes is highly conserved, suggesting that disruption of the shigella *cadA* regions, through insertion (IS) elements or rearrangement, did not first occur in an *E. coli* ancestor. Rather, we propose that disruption of *cadA* was selected in each *Shigella* clone following acquisition of the enterotoxin gene *senA* encoded on the virulence plasmid.

All four pathoadaptive mutations characterized in these studies involve insertion of heterologous elements in the form of IS elements or a prophage genome. The remnant *cadA* region in *S. dysenteriae* 1 contains two IS elements. In *S. flexneri* 2a and *S. sonnei* the remnant *cadA* region contains three IS elements. The ubiquitous presence of these mobile genetic elements suggests that each, particularly the IS elements, may have played an important role in generation of the *cadA* pathoadaptive mutation. Several studies have demonstrated the presence of many IS elements in the *Shigella* genomes and one report estimated that over 300 IS elements exist in the *S. sonnei* genome (5, 15, 28). While many of these elements are present in the *Shigella* virulence plasmid (4), most are located in the pathogens' chromosomes. Transposition of IS elements into new areas of the *Shigella* chromosome, such as *cadA*, may provide an effective means of niche adaptation, as interruption of an ORF with an IS element is more likely to abolish the trait associated with that ORF than random accumulation of mutations, which may occur at a lower rate than transposition and often form silent or conservative missense mutations. It is reasonable to speculate that the original *cadA* pathoadaptive mutation in *S. flexneri* 2a, *S. sonnei* and perhaps *S. dysenteriae* 1, was accomplished by an IS element. This DNA sequence may have provided a region of homology with other portions of the chromosome containing identical IS elements that could recombine to rearrange the chromosome and form the novel linkages we observed (7, 9, 17). This scenario requires two discrete steps, including an intermediate ancestral clone that harbors *cadA* interrupted by IS elements, to generate the novel linkages observed in these most virulent *Shigella* lineages. PCR analysis of the *yjdC* linkages in five other cluster III *Shigella* strains (that share a common ancestor with *S. flexneri* 2a) revealed identical gene arrangements consistent with the clonal nature of these strains (data not shown). The failure to identify an intermediate strain harboring a *cadA*::IS allele linked to *yjdC* suggests that either the *cadA* deletion that occurred in evolution of this lineage was accomplished by a one-step mechanism or rearrangement of the chromosome generated a strain that was more fit than the ancestor harboring only the virulence plasmid and an interrupted *cadA*.

The niche occupied by a pathogenic species is often very different than the environment of the ancestral organism. Not surprisingly, fitness in the ancestral niche does not guarantee fitness in the new environment. A subset of traits (encoded by antivirulence genes) antagonizes the function of virulence and ancestral factors required for growth and survival in host tissues (6, 40). Our previous studies defined *cadA* as an antivirulence gene for the shigellae. The results we report here indicate convergent evolution of *cadA* mutations in the shigellae and reinforce the important role of pathoadaptive mutations in the evolution of pathogenic bacteria. Thus, the mechanism of pathoadaptive mutation is one by which antivirulence genes are removed from the genomes of newly evolved pathogens through selection of clones for optimal fitness in the new host environment. These observations suggest criteria for the identification of antivirulence genes: the antivirulence gene must be present (and expressed) in closely related or ancestral species occupying the nonvirulent ancestral niche but absent from pathogenic clones living in host tissues. Most importantly, expression of the antivirulence gene by the pathogen in host tissues must attenuate virulence and inhibit fitness. These criteria, which essentially constitute the converse of molecular Koch's postulates (8), have been demonstrated for the *cadA* antivirulence locus in the shigellae. Application of converse Koch's postulates to additional loci in *Shigella* and other pathogenic organisms holds great promise for identification of antivirulence genes lost by pathoadaptive mutation. These new insights into the ecology of bacterial pathogens will identify factors, proteins, or products of biochemical pathways (such as the product of LDC activity, cadaverine) that block the activity of traits required for pathogen survival in host tissues. These natural agents, which are expressed by nonpathogenic ances-

tral organisms, may aid in the development of novel pathogen-specific therapies.

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