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Genetic Structure of the *nadA* and *nadB* Antivirulence Loci in *Shigella* spp.[▽]

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Comparison of *nadA* and *nadB* in 14 *Shigella* strains and enteroinvasive *Escherichia coli* versus *E. coli* showed that at least one locus is altered in all strains. These observations explain the characteristic nicotinic acid auxotrophy of *Shigella* organisms and are consistent with the previously identified antivirulence nature of these genes for these pathogens.

The evolution of bacterial pathogens from their commensal ancestors, allowing them to colonize a new ecological niche, is believed to occur by the acquisition of genes via horizontal transfer (6). The presence of virulence genes in pathogenicity islands reflects this form of gene acquisition (12). This first step of evolution is followed by progressive adaptation of the bacteria to their new niche (the host) by mutation and selection for improved fitness (23). An important element of this process of pathoadaptive evolution is the selection of "black holes" in pathogen genomes, that is, the inactivation or loss of genes that are incompatible with, and even antagonistic to, the new pathogenic lifestyle (14). These incompatible genes, which we define as antivirulence loci (AVL), are present in the genome of a nonpathogenic ancestor but absent or inactive in the pathogen because expression of AVL is detrimental to the expression of some virulence phenotypes.

Bacteria of the genus *Shigella* are the causative agents of bacillary dysentery. We previously demonstrated the contribution of black holes in the evolution of *Shigella* from the nonpathogenic gut commensal *Escherichia coli* (15). The *cadA* gene encoding lysine decarboxylase in *E. coli* K-12 was shown to be part of a large region deleted or altered in all *Shigella* spp., and its expression proved to be incompatible with *Shigella* virulence (3, 16).

The genetic similarities between *Shigella* and *E. coli* are strong enough to justify grouping them in the same genus. *Shigella* spp. and enteroinvasive *E. coli* (EIEC) are even more closely related, with the latter causing a disease very similar to dysentery. A recent study focusing both on housekeeping and virulence genes classified both species as a single pathovar of *E. coli* (9). However, there are several well-known auxotrophic requirements of *Shigella* that are not found among most isolates of *E. coli*. Most *Shigella* strains require nicotinic acid supplementation for growth on minimal medium (1). The nicotinic acid requirement of *Shigella flexneri* 2a strain 2457T is due to mutations in two unlinked loci, *nadA* and *nadB* (5), that encode enzymes in the L-aspartate-dihydroxyacetone pathway leading to de novo synthesis of NAD. In *S. flexneri* 5a strain M90T, Mantis et al. showed that *nadB* inactivation is responsible for this phenotype since nicotinic acid prototrophy can be restored by transformation with the cloned *nadB* gene from *Salmonella enterica* serovar Typhimurium (13). The *nadA* gene encodes the quinolinate synthetase A, while *nadB* encodes the L-aspartate oxydase (or quinolinate synthetase B). Both function together as a multienzyme complex catalyzing the oxidation and condensation of L-aspartate to quinolinate (QUIN). QUIN is subsequently ribosylated to nicotinic acid mononucleotide and converted to NAD by other enzymes in the pathway. In the absence of functional *nadA* and/or *nadB*, exogenous nicotinic acid can be used instead of QUIN to produce nicotinic acid mononucleotide, thus bypassing the need for *nadA* and *nadB* functionality and synthesis of QUIN.

We recently reported that QUIN is a strong and specific

TABLE 1. Primers used in this study

Gene	Primer name (direction)	Primer sequence (5′ to 3′)	Position[a]
nadA	nadAPF (sense)[b]	CAAGCAACTCTATGTCGGTGG	−184
	nadAPR (antisense)	CTTTGCACCGAAGCGCGCCAT	+231
	nadA2R (antisense)	GTCTTAAATTCATCATGCAC	+623
	nadA2 (sense)	CCACCAGTCAACTGATCGCT	+725
	nadAP (sense)	AAAGAACGTAATGCGGTG ATGG	+112
	nadAM (antisense)[b]	TGGCAAGGCCAATACACAGC	+1202
nadB	nadBPromF (sense)[b]	CAAAGGGTTAGAGTGTCTCG	−332
	nadBPF (sense)	CTGCCTGAAGAGTAACCCAAC	−191
	nadBPR (antisense)	ATCAAACACGGCGGCAATACC	+174
	nadB2 (sense)	GAAACCTGCCACGCAAAAGC	+577
	nadB3R (antisense)	CACGCCATAGCAATGCCAT	+686
	nadB2R (antisense)	CGCCCATGATCATCAACCA	+1091
	nadB3F (sense)	AGCCGCGTTGAGAACCCTGAC	+1273
	nadBP (sense)	CAAAGAAAATGAATACTCTCC CTGA	−8
	nadBM (antisense)[b]	CGTGGGCCAGACCAGAACTA TTCC	+1676

[a] Base numbering relative to ATG.
[b] Primer used for cloning.

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TABLE 2. NadA/B sequence changes in various *Shigella* and EIEC strains compared to *E. coli* K-12

Strain	Serotype	Cluster[a]	Changes in NadA[b]	Accession no.[c]	Changes in NadB[b]	Accession no.[c]	Reference or source[d]
Shigella strains							
S. boydii BS511	14	1	**Q271***	EF473660	(I108V, E141Q, T142S, L149Q), **A226D**, **R234Q**, (Y412D, D415G, I416V), T468N	EF473669	CDC
S. boydii BS512	18	1	**A148V**	NZ_AAKA00000000	IS*600* between aa 178 and 179	NZ_AAKA00000000	CDC
S. boydii 227	4	1	G304D	NC_007613	IS*600* between aa 178 and 179	NC_007613	25
S. dysenteriae 1012	4	1	R35H, V45A, K77R, R134H, 1-bp insertion frameshift at position 442	NZ_AAMJ00000000	R138K, (V167I), W185*, (Y412D, D415G, I416V), T468N	NZ_AAMJ00000000	24
S. boydii BS686	17	2	R35H, V45A, R134H, 1-bp insertion frameshift at position 442	EF473663	**A19T**, **V67E**, **V102G**, E114Q, **D134Y**, R138K, (V167I, Y412D, D415G, I416V), T457K, T468N	EF473672	CDC
S. dysenteriae BS507	2	2	R35H, V45A, K77R, R134H, 1-bp insertion frameshift at position 442	EF473658	A75V, R138K, (V167I), W185*, (Y412D, D415G, I416V), T468N	EF473667	CDC
S. flexneri 2457T	2a	3	**A111V**, **C128Y**, **T252A**, **Q271R**, G304D	AF403415	R80H, **Q95P**, (I108V, E141Q, T142S, L149Q), **C354***, (D415G, I416V)	AF403416	4
S. flexneri BS510	3a	3	None	EF473659	**A73S**, (I108V, E141Q, T142S, L149Q), **C354***, (D415G, I416V)	EF473668	CDC
S. flexneri M90T	5a	3	**A111V**, **T252A**	EF473666	R80H, **Q95P**, (I108V E141Q, T142S, L149Q), **C354***, (D415G, I416V)	EF473657	21
S. dysenteriae 197	1	Outlier	R134H, (G191A), **P219L**, R257W	NC_007606	(V167I), **D218N**, (D415G, I416V)	NC_007606	25
S. dysenteriae BS681	8	Outlier	none	EF473662	**G44V**, (I108V, E141Q, L149Q), V180I, (Y412D, D415G, I416V)	EF473671	CDC
S. sonnei BS513	NA[e]	Outlier	IS21 between aa 292 and 293	EF473661	IS*600* in codon 233	EF473670	CDC
E. coli (EIEC) strains							
EDL1284	O124	NA	**G198S**, **V260G**	EF473665	Portion of IS*600* after aa 52; deletion aa 53 to 192	EF473674	7
E. coli (EIEC) strain 1	O136	NA	**G198S**	EF473664	IS*600* between aa 52 and 53	EF473673	L. Trabulsi

[a] Classification according to Pupo et al. (20).
[b] Changes are based on the comparison of each sequence to the translated *E. coli* K-12 sequences for *nadA* (gene identifier 945351) and *nadB* (gene identifier 947049). Nonsense mutations in DNA leading to the replacement of an amino acid by a stop codon are represented by an asterisk. Changes in boldface correspond to amino acids conserved among *E. coli* K-12 and four other bacterial species (Fig. 1). Parentheses indicate polymorphic changes also found in other *E. coli* strains. Total sequence length is 347 amino acids for NadA and 540 amino acids for NadB. aa, amino acids.
[c] The National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) was used to collect the available sequence data. NZ and NC accession numbers correspond to the whole genome or whole genome project. The remaining accession numbers correspond to the specific gene sequences determined for this study. The analysis was performed in October 2006.
[d] Centers for Disease Control and Prevention, Nancy Strockbine.
[e] NA, not applicable.

inhibitor of several virulence phenotypes of *Shigella* (19). We also found that complementation of strain M90T with functional copies of *nadA* and *nadB* from *E. coli* K-12, while conferring nicotinic acid prototrophy to the strain, strongly impaired its virulence. These results define *nadA* and *nadB* as AVL in *Shigella*. The model of pathoadaptive evolution predicts that strong selective pressure against expression of AVL will lead to their inactivation by mutation. In this study, we wanted to test this prediction for the *Shigella* AVL *nadA* and *nadB* by analyzing these loci in other strains of *Shigella* and EIEC.

We first analyzed the *nadA* and *nadB* loci in strain M90T. Table 1 shows the overlapping primers used for amplification and sequencing of both loci. PCR amplification for sequencing, cloning, and plasmid screening purposes utilized the *Taq* DNA polymerase (QIAGEN). For sequencing PCR-generated prod-

TABLE 3. Results of *nadA*/*B* complementation experiments

Strain[a]	Relevant characteristics[b]	Growth on MM
E. coli strains		
K-12 MC4100	$nadA_{EC}$ $nadB_{EC}$	+
K-12 CAG12147	*nadA*57::Tn*10*	−
K-12 CAG18480	*nadB*51::Tn*10*	−
ATM876	CAG12147 + pBluescript/$nadA_{EC}$	+
ATM839	CAG12147 + pGEM-T/$nadA_{SF}$	−
ATM877	CAG18480 + pGEM-T/$nadB_{EC}$	+
ATM878	CAG18480 + pGEM-T/$nadB_{SF}$	−
S. flexneri 5a strains		
M90T	$nadA_{SF}$ $nadB_{SF}$	−
BS830	M90T + pBluescript/$nadA_{EC}$	−
BS831	M90T + pGEM-T/$nadB_{EC}$	−
BS813	M90T + pBluescript/$nadA_{EC}$-$nadB_{EC}$	+

[a] *E. coli* K-12 MC4100 was the source of DNA for PCRs to amplify the functional *nadA* and *nadB* loci. Strains CAG12147 and CAG18480 were constructed by C. Gross (17, 22).

[b] Genes from *S. flexneri* 5a M90T carry the subscript SF, and genes from *E. coli* K-12 MC410 carry the subscript EC.

ucts, samples were prepared with an ABI Prism Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems) and analyzed using an ABI Prism 377 DNA sequencer (Applied Biosystems). Clone Manager (SciEd software) was used to perform DNA translation to protein and comparative alignments. We found that *nadA* and *nadB* sequences from M90T were identical to those from the published sequence for *S. flexneri* 5b strain 8401 (18). Both genes contained point mutations that result in 2- and 8-amino-acid substitutions compared to the functional NadA and NadB of *E. coli* K-12, respectively (Table 2). In addition, the *nadB* gene of M90T contained a single base change that created a stop codon at amino acid 354, resulting in truncation of the C-terminal third of the *nadB* product. Gemski et al. originally reported that *nadA* and *nadB* of *S. flexneri* 2a strain 2457T are both mutated (5). Mantis et al. reported defects limited to *nadB* in M90T, and since the nicotinic acid auxotrophy of M90T could be satisfied by introduction of the *nadB* gene from *S. enterica* serovar Typhimurium, they suggested that its *nadA* gene was functional (13). In order to confirm these latter results, the nadAPF/nadAM and nadBPromF/nadBM primers (Table 1) were used to amplify the full *nadA* and *nadB* loci and their promoters from M90T. The fragments obtained were cloned into pGEM-T (Promega) and tested for their ability to complement *nadA* or *nadB* mutants of *E. coli* K-12 on M9 glucose minimal medium (MM). Nicotinic acid (Sigma) at a concentration of 10 μg/ml was used as a supplement in control plates. Table 3 shows that neither clone from M90T was able to complement the respective mutant strains of *E. coli* K-12. In reciprocal experiments, the wild-type *nadA* and *nadB* genes of *E. coli* K-12 were cloned into pBluescript (Stratagene) and pGEM-T, respectively, and the constructs obtained were transformed into M90T, generating strains BS830 and BS831, respectively. Neither transformant showed nicotinic acid independence. However, when both genes cloned together on pBluescript were transformed into M90T, the resulting strain (BS813) grew on MM without nicotinic acid (Table 3). These data extend the initial work of Gemski et al. (5) to another *S. flexneri* serotype and suggest that, contrary to the observations of Mantis et al. (13), the nicotinic acid auxotrophy of M90T is due to loss of function of both *nadA* and *nadB*. Bergthorsson and Roth (2) identified an A111V substitution in NadA as responsible for the nicotinic acid auxotrophy of the cattle pathogen *S. enterica* serovar Dublin, indicating that this residue is essential for the functionality of the protein. The same change was found in M90T (Table 2), supporting our conclusion that NadA is not functional in this strain. Regarding NadB, while a nonsense mutation alone usually leads to a mutant phenotype, one or more of the other amino acid changes in this protein must also lead to a null phenotype since we were unable to select spontaneous $nadB^+$ revertants of BS830 on MM. The reciprocal experiment using BS831 failed to yield spontaneous $nadA^+$ revertants (Table 3). These results suggest that multiple point mutations altered essential amino acid residues in both loci, emphasizing a strong selective pressure for their loss of functionality.

We next compiled the available *nadA* and *nadB* sequences of *Shigella* species from the databases and complemented these data by sequencing *nadA* and *nadB* in representative isolates of each species and in two EIEC strains that are also nicotinic acid auxotrophs. All the strains studied displayed changes in at least one of the loci compared to *E. coli* K-12 (Table 2). The EIEC strains, two of the *Shigella boydii* strains, and a *Shigella sonnei* strain showed a significant disruption of *nadB* by insertion of insertion sequence (IS) elements. EIEC O124 contained an insertion of a portion of IS*600* after codon 52, and the gene also showed a large deletion of the coding region for amino acids 53 to 192. Interestingly, EIEC O136 also had an IS*600* insertion at codon 52, but neither the IS element nor the surrounding open reading frame showed any rearrangements. Both strains also displayed the same amino acid change in NadA at codon 198 compared to *E. coli* K-12, with an additional amino acid change in EIEC O124. These results strongly suggest that both strains are related and that they share a common ancestor, even though they correspond to different serotypes. The *nadB* sequences of *S. boydii* serotypes 4 and 18 were both disrupted by insertion of IS*600* after codon 178. This result also suggests a common ancestor for these strains. Finally, both the *S. sonnei nadA* and *nadB* loci showed disruption by IS*21* and IS*600*, respectively. Their sequences are identical to the *nadA* and *nadB* loci of *S. sonnei* 46, the sequenced strain from the databases. These results are consistent with the well-known clonal nature of *S. sonnei* strains (8). In all of these strains, it is very likely that the IS-related disruptions of *nadA* and/or *nadB* loci are responsible for the nicotinic acid auxotrophy by preventing the production of functional proteins.

Numerous amino acid changes were found among all the *Shigella* and EIEC strains studied compared to *E. coli* K-12. In order to determine if some of these changes were due to polymorphisms of the *nadA* and *nadB* loci, we compared the deduced NadA and NadB protein sequences from the 17 DNA sequences of *E. coli* available in the databases. This analysis revealed 12 and 33 polymorphic positions in NadA and NadB, respectively. Several of these changes were also found in some of our strains and are indicated in parentheses in Table 2. However, all the *Shigella* strains studied were found to contain additional and specific changes in at least one locus compared to *E. coli*. Several strains had nonsense mutations or frameshifts, which likely result in production of nonfunctional pro-

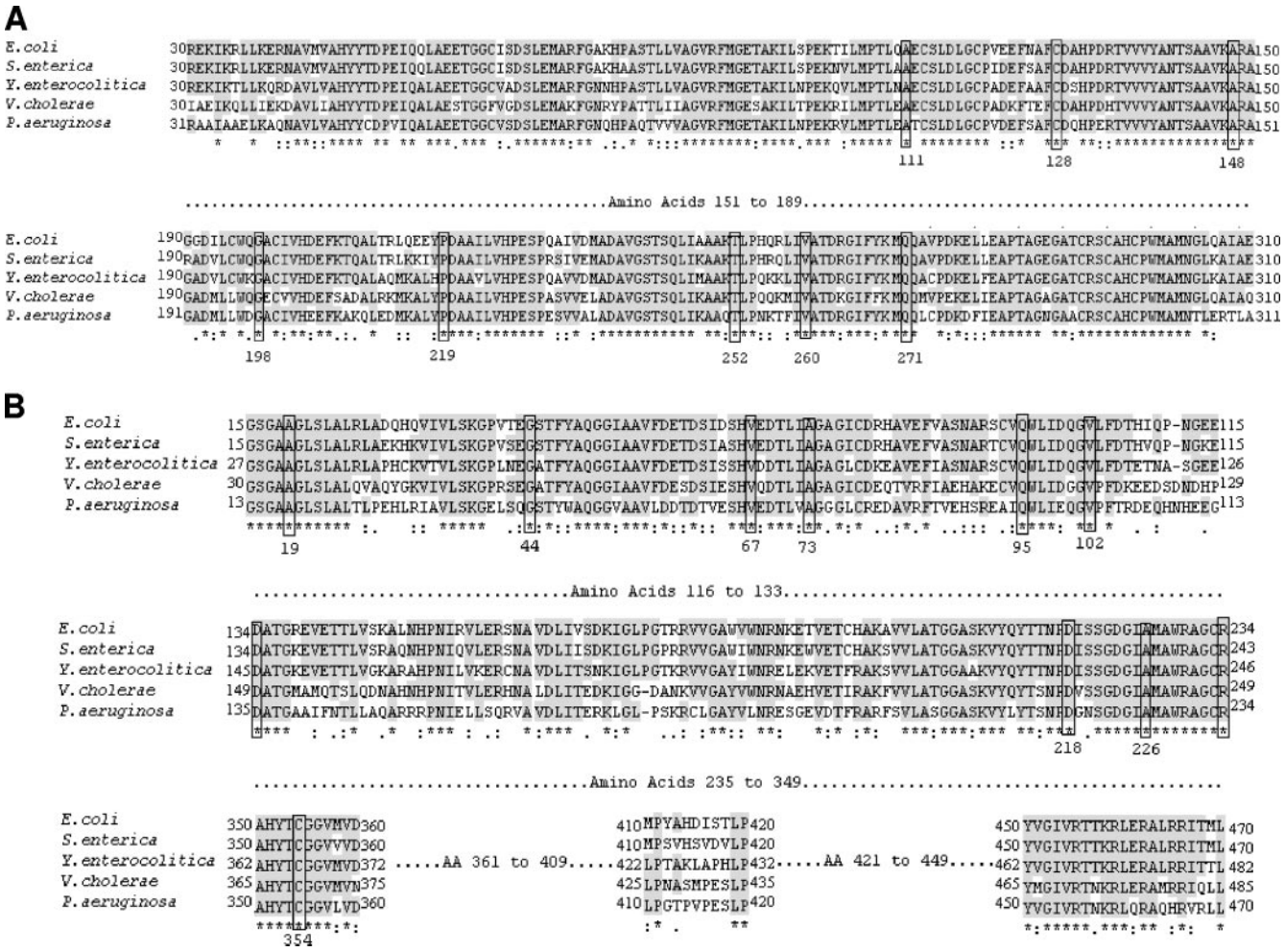

FIG. 1. Partial alignments of NadA and NadB sequences from various bacterial species. The National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) was used to collect the protein sequence data. The accession number is indicated for each sequence. (A) Partial alignment of NadA from *E. coli* K-12 (NP_415271), *S. enterica* CT18 (NP_455307), *Y. enterocolitica* 8081 (YP_001007117), *V. cholerae* N16961 (NP_231467), and *P. aeruginosa* PAO1 (NP_249695). (B) Partial alignment of NadB from *E. coli* K-12 (NP_417069), *S. enterica* CT18 (NP_457117), *Y. enterocolitica* 8081 (YP_001005342), *V. cholerae* N16961 (NP_232098), and *P. aeruginosa* PAO1 (NP_249452). Both alignments were obtained via ClustalW (http://www.ebi.ac.uk/clustalw/) and edited using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Conserved amino acids among three or more of the five sequences are shaded. An asterisk indicates a conserved amino acid among all five species, a colon indicates conservative amino acid substitutions, and a period indicates semiconservative substitutions. Boxes show conserved amino acids that were found to be mutated in one or more of the *Shigella* and EIEC strains studied, with *E. coli* numbering indicated underneath. The analysis was performed in February 2007.

tein(s). Overall, among the 14 strains studied, only two (*Shigella dysenteriae* 1 and 8) contain no more than a few specific amino acid substitutions compared to *E. coli* K-12 and are still likely to produce full-sized NadA and NadB proteins. To determine whether these mutations might be in essential residues, we performed alignments of NadA and NadB sequences for *E. coli* K-12, *S. enterica* CT18, *Yersinia enterocolitica* 8081, *Vibrio cholerae* N16961, and *Pseudomonas aeruginosa* PAO1, all of which contain functional alleles of *nadA* and *nadB* (Fig. 1). The P219L substitution in NadA found in *S. dysenteriae* 1, as well as the D218N and G44V substitutions in NadB found in *S. dysenteriae* 1 and 8, respectively, represent alterations in residues that are conserved among the five different species. This observation strongly suggests that these residues are important for the function of the protein(s). Therefore, it is likely that some or all of these amino acid changes in the *S. dysenteriae* loci lead to loss of protein function. Additionally, among the 12 other *Shigella* and EIEC strains studied, eight contained one or more changes in conserved amino acids in NadA and/or NadB (Table 2; Fig. 1). This observation provides further evidence of a strong selective pressure toward the loss of function of both proteins in *Shigella* species. Finally, only two strains, *S. flexneri* 3a and *S. dysenteriae* 8, displayed no changes in NadA compared to *E. coli*. In both strains, the ability to grow on MM was restored by the reintroduction of a functional *E. coli* copy of *nadB*, confirming that mutations in *nadB* were responsible for their nicotinic acid auxotrophy (data not shown).

The phylogenetic relationships of *Shigella* strains to each other and to *E. coli* strains have been analyzed by sequencing two virulence plasmid genes and eight housekeeping genes in

four separate regions around the chromosome (9–11, 20). These data show that the majority of *Shigella* strains fall into three main clusters within *E. coli* and that EIEC and *S. sonnei* strains, as well as *S. dysenteriae* serotypes 1, 8, and 10, lie outside these three clusters. Our results show a good correlation of *nadA* and *nadB* mutations with these clusters (Table 2). Only a few of the amino acid substitutions were common to strains from several clusters and even outliers (i.e., R35H, V45A, K77R, R134H, and G304D in NadA and R138K and T468N in NadB) while the majority of the amino acid substitutions were either unique to strains from a single cluster (i.e., A111V and T252A in NadA and R80H, Q95P, and C354* in NadB for cluster 3) or even unique to one single strain. In summary, most of the substitutions we found were likely selected for because they affected essential residues of the proteins (Table 2), and very few may represent polymorphisms due to their position in a particular "hot spot" for mutation within the gene. Overall, these results support the idea that a strong selective pressure toward the loss of *nadA* and *nadB* function occurred in the evolution of *Shigella* species.

Inactivation of either *nadA* or *nadB* would satisfy this selective pressure to block the synthesis of QUIN, but mutations would tend to subsequently accumulate in both genes because of the absence of selective pressure to maintain gene function. It is difficult to definitely distinguish which gene is the preferred target for inactivation. However, several of our observations suggest that mutation of NadB was the first event in the evolution of *Shigella* species toward nicotinic acid auxotrophy. First, two strains, *S. dysenteriae* 8 and S. *flexneri* 3a, had no alterations of *nadA* and produced functional NadA protein. Their defect in NAD synthesis is solely due to alterations in NadB (missense mutations in conserved residues and/or a nonsense mutation that truncates one-third of the protein). Second, in the two EIEC strains, as well as in the two *S. boydii* serotypes 4 and 18, the *nadB* gene sustained a major disruption by IS elements while *nadA* showed only point mutations compared to the *E. coli* K-12 sequence. The latter could be the result of genetic drift of *nadA* after inactivation of *nadB* as a result of the loss of the selective pressure to maintain a functional *nadA* gene. In general, we also found that the majority of the strains studied (except the *S. sonnei* strain) show a higher number of modifications in NadB than in NadA. This phenomenon could be explained by the larger size of the *nadB* locus relative to *nadA*, which makes it a more frequent target for random mutation during the selection process for nicotinic acid auxotrophs.

In summary, we have found that among 14 strains of *Shigella* and EIEC, all contained alterations in at least one of the *nadA* or *nadB* loci that could explain their nicotinic acid auxotrophy. These observations are consistent with our previous report that the *nadA* and *nadB* genes are AVL in *Shigella* (19). Additionally, the distinct nature of mutations defining the *nadA* and *nadB* loci of the four different species of *Shigella* suggests their acquisition and accumulation after *Shigella* speciation. This conclusion is in accord with the model that clones of *Shigella* evolved multiple times from different lines of *E. coli* ancestor strains (20). Our findings confirm and extend the concept that bacterial pathogen evolution proceeds through convergent evolution toward removal or inactivation of AVL by whatever alteration (IS, deletion, or point mutation) leads to a more virulent phenotype or to improved fitness for survival in the host (14).

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