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Identification of Two Widely Separated Loci Conferring Nicotinic Acid Dependence on Wild-Type *Shigella flexneri* 2a

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Two widely separated loci causing the nicotinic acid dependence of wild-type *Shigella flexneri* 2a were identified by intergeneric mating procedures and found to be closely linked to the *gal* and *fuc* chromosomal determinants.

In recent years, our studies on *Shigella flexneri* have been concerned with the genetic control of virulence of this organism (2, 7), because such information may prove useful both in developing protective living vaccines against bacillary dysentery (4, 5) and in elucidating the pathogenesis of this disease (2, 3, 7). One genetic approach that we have employed has involved the construction, by intergeneric mating techniques, of *S. flexneri* strains hybridized with *Escherichia coli* K-12 chromosomal segments (2, 3, 7). *S. flexneri* 2a is particularly amenable to such intergeneric hybridization studies because of its close genetic homology with *E. coli* (1, 8, 9) and because it is naturally unable to utilize or ferment a number of carbohydrates (Table 1). The strain employed here is a typical *S. flexneri* of serotype 2a, being naturally unable to utilize lactose, fucose, xylose, and rhamnose. Hence, different hybrid classes can be recovered after mating *E. coli* K-12 Hfr donor strains with suitable *S. flexneri* recipients and selecting for the inheritance of the carbohydrate utilization alleles of the donor. Generally, the results of linkage analyses on hybrid classes have indicated that the order of genetic markers on the *S. flexneri* chromosome is similar to that established for the *E. coli* K-12 genome (6, 7, 9).

In addition to these carbohydrate markers, *S. flexneri* strains usually require nicotinic acid for growth in minimal salts medium. Although this Nic$^-$ phenotype is an excellent auxotrophic character, its use as a selective or nonselective marker in genetic analyses has been limited, due primarily to the low frequency with which Nic$^+$ hybrids are recovered from mating mixtures. This difficulty with the use of Nic as a genetic marker is now resolved by the finding, as reported in this communication, that there are two widely separated loci on the chromosome of *S. flexneri*, both in a negative allelic state, which affect the biosynthesis of nicotinic acid.

The pertinent characteristics of strains employed are summarized in Table 1. *S. flexneri* 2a 191b served as the recipient line in all mating experiments. In addition to the native fermentation markers of *S. flexneri* 2a previously mentioned, this strain was found on isolation to be maltose negative and was made by mutagenesis unable to ferment galactose. The aspartic acid marker, listed here as negative, is not an absolute requirement for growth in minimal medium. The addition of this amino acid appears to stimulate growth of this, as well as other *S. flexneri* 2a strains. The polarities of chromosome transfer of *E. coli* K-12 Hfr donor strains Hfr H and AB313 are presented in Fig. 1. The minimal selective medium and procedures employed for mating experiments and analyses of marker inheritance were similar to those previously reported (1, 6). Streptomycin, employed as a counter-selective agent against donor cells in matings, was incorporated into selective media at a concentration of 500 μg per ml.

Our initial suspicion that the Nic$^-$ phenotype of wild-type *S. flexneri* 2a was under the control of more than a single chromosomal locus was based on three experimental observations. First, it became apparent that the frequency of isolation of Nic$^+$ *S. flexneri* hybrids from matings was very low (about 10^{-8} to 10^{-9} per donor cell), regardless of the *E. coli* K-12 Hfr employed. In contrast, the frequency of recombination for other selected markers (Lac, Ara, Rha, Xyl, Fuc), was in a range of 10^{-2} to 10^{-6} per input male cell, depending on whether the marker was injected "early" or "late" by the Hfr donor strain used in the cross. Secondly, when heavy cell suspensions of *S. flexneri* 2a were plated on minimal medium

TABLE 1. *Characteristics of bacterial strains*[a]

Strain	Species	Mating polarity	*thr*	*leu*	*asp*	*lac*	*gal*	*fuc*	*mal*	*nic*	*xyl*	*rha*	*str*
Hfr H	*Escherichia coli* K-12	Hfr	+	+	+	+	+	+	+	+	+	+	S
AB313	*E. coli* K-12	Hfr	−	−	+	+	+	+	+	+	+	+	S
191B	*Shigella flexneri* 2a	F-	+	+	−	−	−	−	−	−	−	−	R

[a] Abbreviations: *thr*, threonine; *leu*, leucine; *asp*, aspartic acid; *lac*, lactose; *gal*, galactose; *fuc*, fucose; *mal*, maltose; *xyl*, xylose; *rha*, rhamnose; *nic*, nicotinic acid; *str*, streptomycin; +, synthesis or utilization; S, sensitive; R, resistant; F-, female recipient; Hfr, high frequency of recombination donor.

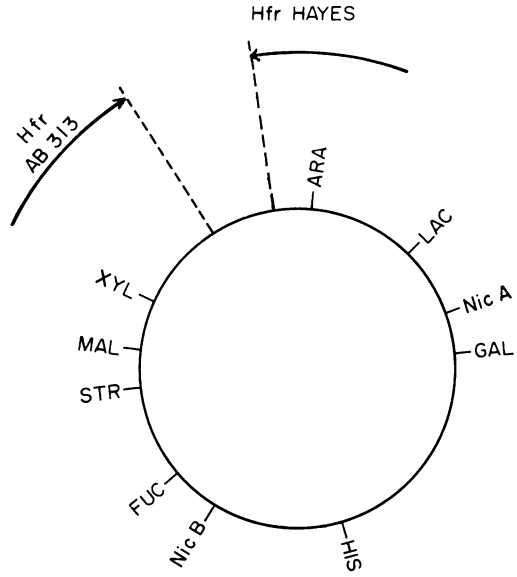

FIG. 1. *Chromosomal map of Escherichia coli.*

lacking nicotinic acid, no Nic$^+$ revertants were ever recovered. Although this finding could be the result of a deletion within a single Nic locus, it is consistent with the notion that more than one locus is involved with the Nic$^-$ phenotype. Thirdly, when unselected marker analyses were performed on hybrids derived from intergeneric matings, an anomolous pattern of Nic$^+$ inheritance was observed. The results of such an analysis of hybrids obtained from a cross of *E. coli* K-12 Hfr H with *S. flexneri* 2a 191b are summarized in Table 2. When selections were made for Nic$^+$ recombinants, a significant proportion of the hybrids also inherited the donor unselected markers Lac$^+$ (52%), Gal$^+$ (95%), or Fuc$^+$ (31%). Such high genetic linkage between these three loci and Nic was not detected, however, when either Lac$^+$, Gal$^+$, or Fuc$^+$ served as the selective marker and Nic$^+$ was used as an unselected marker. As can be seen, the linkage was very low between Nic$^+$ and these three selective markers. This type of linkage behavior for the Nic marker suggested that the Nic$^-$ phenotype of *S. flexneri* 2a was affected by at least two distinct chromosomal loci, one near the *lac—gal* chromosomal segment and another distally located near the *fuc* region (*see* Fig. 1). Selections for Nic$^+$ prototrophy would require the inheritance of both loci from *E. coli* K-12, thus accounting for the observed linkage of Nic$^+$ to such widely separated markers as Gal and Fuc. On the other hand, although selection for either Gal$^+$ or Fuc$^+$ hybrids might result in the inheritance of the closely linked Nic locus, it is unlikely that inheritance of the distal Nic locus would result. As is shown in Table 2, unselected marker analysis of Lac$^+$ and Gal$^+$ hybrids revealed a low frequency of inheritance of known distal markers (Fuc, Mal, Xyl, and Rha).

Further evidence for the existence of these two

TABLE 2. *Analysis of hybrids obtained from a cross of Escherichia coli K-12 Hfr H with Shigella flexneri 2a 191b*

Donor-selected character	Lac+	Gal+	Fuc+	Mal+	Xyl+	Rha+	Nic+
Nic+	52[a]	95	31	1	1	1	100
Lac+	100	77	1	1	1	1	1.9
Gal+	62	100	1	1	1	1	1
Fuc+	14	19	100	14	1	1	1

[a] Percentages based on analysis of an average of 100 clones.

nic− loci on the *S. flexneri* 2a chromosome was provided by genetic crosses in which we mated *E. coli* K-12 donors with recipients which were recombinants from a previous cross in which *S. flexneri* strain 191b had obtained either the *gal* or the *fuc* chromosomal region of an *E. coli* Hfr donor. It occurred to us that Gal+ and Fuc+ hybrids may also have inherited their respective closely linked Nic+ marker but, due to a failure to inherit the distally located Nic+ determinant, were still expressing a Nic− phenotype. To test for this possibility, *S. flexneri* Fuc+ hybrids (derived from a mating with *E. coli* K-12 Hfr AB313) were remated with *E. coli* Hfr H, and selection was made for Nic+ and Gal+. Likewise, *S. flexneri* Gal+ hybrids (prepared from a mating with *E. coli* K-12 Hfr H) were remated with *E. coli* K-12 Hfr AB313, selections this time being made for Fuc+ and Nic+ recombinants. If our suspicion that many Gal+ and Fuc+ recombinants are hybridized also for their closely linked Nic determinant is correct, then one would expect from such backcross matings a significant increase in the frequency of recovery of Nic+-selected recombinants (since a single locus, rather than two widely separated loci, is being selected). In addition, genetic analysis of such hybrids should reveal a strong linkage of the Nic+ marker either to *gal* or to *fuc*, depending on which hybrid recipient is employed. These findings were indeed the case as is shown in Tables 3 and 4. The frequency of recovery of Nic+-selected recombinants was about 1,000-fold higher with Fuc+ and Gal+ hybrid recipients than with the unhybridized original parent, *S. flexneri* 2a 191b. Unselected marker analysis (Table 4) showed high linkage of Nic+ to the Gal or Fuc markers according to which hybrid was used, under conditions in which Nic+ was employed either as a selected or unselected marker.

On the basis of these studies, we conclude that two loci on the chromosome of *S. flexneri* 2a are responsible for the Nic− phenotype of this organism. One locus, termed here as *nic*A, is closely linked to the *gal* cluster, whereas another locus, referred to here as *nic*B, is located near the *fuc* gene(s). Both loci express a negative allelic state, since a Nic+ phenotype for *S. flexneri* is detected

TABLE 3. *Selection of Nic+ hybrids from matings of Escherichia coli K-12 Hfr strains with Shigella flexneri 2a 191b Gal+ and Fuc+ hybrid recipients*

Donor	Recipient	Selection	Frequency of recombination
Escherichia coli K-12 Hfr H	*Shigella flexneri* 2a Fuc+ hybrid	Nic+	10^{-5} [a]
E. coli K-12 Hfr H	*S. flexneri* 2a	Nic+	3×10^{-8}
E. coli K-12 AB 313	*S. flexneri* 2a Gal+ hybrid	Nic+	2.5×10^{-5} [a]
E. coli K-12 AB 313	*S. flexneri* 2a	Nic+	10^{-8}

[a] Average of three independent hybrid recipients.

TABLE 4. *Analysis of recombinants obtained from crosses of Escherichia coli K-12 Hfr strains with Shigella flexneri 2a Gal+ and Fuc+ hybrids*

Donor strain	Recipient strain	Donor-selected character	No. analyzed	Nic+	Gal+	Lac+	Fuc+
Escherichia coli K-12 Hfr H	*Shigella flexneri* Fuc+ hybrid	Nic+	80	80	80	56	—[a]
		Gal+	80	80	80	76	—
E. coli K-12 AB 313	*S. flexneri* Gal+ hybrid	Nic+	40	40	—	NT[b]	37
		Fuc+	23	23	—	NT	23

[a] Recipient strain is positive as result of previous hybridization.
[b] Not tested.

only after both loci have been genetically altered. Although *S. flexneri* 2a wild-type cells of strain 191b and other strains appear not to revert to a Nic$^+$ state, we have observed Nic$^+$ revertants in cell populations of *S. flexneri gal*$^+$ *nic*A$^+$ and *S. flexneri fuc*$^+$ *nic*B$^+$ hybrids. This finding suggests that the native *nic*A and *nic*B alleles of *S. flexneri* are not deletions but rather point genetic alterations resulting in an inability to synthesize nicotinic acid. Genetic studies on *E. coli* K-12 mutants unable to produce nicotinic acid have revealed two loci for nicotinic acid dependence; *nad*A (previously termed *nic*A) which has been positioned between the *lac* and *gal* genes (minute 16) and *nad*B (previously called *nic*B) which is located at minute 49 between the *fuc* and *his* loci (10). Because of previous studies showing a close genetic homology and similar chromosomal organization between *E. coli* and *S. flexneri* (1, 6–9), we suspect that the loci for *nic* in these two genera are similar in position and function.

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