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Natural Isolates of *Salmonella enterica* Serovar Dublin Carry a Single *nadA* Missense Mutation

Ulfar Bergthorsson† and John R. Roth*

Department of Biology, University of Utah, Salt Lake City, Utah

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Nicotinic acid is required by most isolates of *Salmonella enterica* (serovar Dublin), a pathogen of cattle. A single *nadA* missense mutation causes the nutritional requirement of all serovar Dublin isolates tested. Models for persistence of this allele are tested and discussed.

While most natural isolates of *Salmonella enterica* and *Escherichia coli* grow on minimal medium, a small percentage are auxotrophs, many with a defect in pyridine (NAD) synthesis (1, 29, 36, 42). Pyridine auxotrophy (3, 11) is characteristic of particular bacterial subgroups including the cattle pathogen *S. enterica* (serovar Dublin) (7, 10) and *Shigella* spp. (3, 43). Persistent auxotrophy may reflect important aspects of bacterial life history and population structure. The following explanations are tested here.

(i) Relaxed selection for prototrophy. Natural conditions might provide nutrients and allow particular auxotrophic mutations to escape purifying selection and drift to high frequency. This seems particularly likely for nutrients required at low levels.

(ii) Conditional auxotrophy. The auxotrophy seen in the laboratory might be corrected under frequently encountered natural conditions. Many laboratory mutants are corrected by low temperature or high salt (19), as are some naturally occurring pyridine auxotrophs of *E. coli* (1, 21, 46).

(iii) Selection for auxotrophy. An auxotrophic mutant allele could be selectively maintained if it provided a fitness advantage under some conditions. Resistance to some antibiotics can result from reduced proton motive force (PMF), which is required for their import. Starvation of a *nad* auxotroph might reduce PMF by limiting NAD levels and compromising electron transport. A precedent is streptomycin resistance (Str^r) seen under anaerobic conditions (38) or when respiration is compromised by impaired heme synthesis (37). Mutations (*strB* = *isc*) cause Str^r by reducing PMF by their failure to repair oxidized iron-sulfur clusters (40). A leaky *nadD* mutation causes resistance to fusidic acid and trimethoprim (41).

(iv) Mutation pressure. Mutator alleles are found in 1 to 2% of natural isolates of *E. coli* and *S. enterica* and might generate auxotrophic mutations faster than selection can remove them (23). Furthermore, some genes contain sequence motifs that are local mutational hot spots at which the same mutation is generated repeatedly (2, 8, 14, 27).

These possibilities were tested for pyridine-requiring strains of the cattle pathogen, *S. enterica* serovar Dublin. Analysis was facilitated because serovar Dublin strains can be transductionally crossed with characterized pyridine (*nad*) auxotrophs of serovar Typhimurium (33).

Auxotrophy of serovar Dublin isolates. The auxotrophy of serovar Dublin is satisfied by extremely low levels (<1μM) of nicotinic acid or nicotinamide and by the intermediate quinolinic acid, suggesting a deficiency in one of the first two steps in the pathway, *nadA* or *nadB*. Serovar Dublin strains tested were TR6325, obtained from J. Farmer at Centers for Disease Control and Prevention (Atlanta, Ga.), and strains TR7209 to -12 (SL1 to -4) from Steven Libby, University of Washington (24). Strains from the *Salmonella* reference collection B (SARB) collection (6) were SARB12, the most common serovar Dublin type based on multilocus enzyme electrophoresis (39), SARB13 (requiring thiamine in addition to a pyridine), and SARB14 (the only serovar Dublin isolate tested that did not require a pyridine compound).

Reversion tests. Auxotrophic serovar Dublin strains revert to growth on minimal glucose. By a fluctuation test (25), serovar Dublin isolate TR7209 was found to revert at a rate of 10^{-10}/cell/division, a rate typical for alterations of a particular base pair (15). Mutation rate was estimated as $-\ln(P_0)/n$, where n is the number of cells in a culture and P_0 is the fraction of 26 (1-ml) cultures with zero mutations (scored on day 3).

Initial evidence for a *nadA* mutation. Serovar Dublin strains TR7209, TR7211, and SARB13 were transduced to prototrophy using phage P22 grown on *nadA*, *-B*, or *-C* insertion mutants (Tn*10*) of serovar Typhimurium. Only the *nadA* mutant donor showed reduced ability to repair the Na requirement. This suggested that serovar Dublin's defect is in the *nadA* gene and repair efficiency is reduced because it requires an exchange between the donor and recipient *nad* mutations.

The serovar Dublin *nadA* allele is necessary and sufficient for auxotrophy. To verify the serovar Dublin *nadA* mutation, a nonauxotrophic *pnuC*::MudJ(Kn^r) insertion closely linked to *nadA* in serovar Typhimurium was transduced into several serovar Dublin isolates (SARB13, TR7209, and TR7212) selecting kanamycin resistance. Of Kn^r recombinants, 83% lost their pyridine requirement, showing linkage of the serovar Dublin mutation to the *nadA* region. A Kn^r serovar Dublin transductant that retained its pyridine requirement was used as donor with wild-type serovar Typhimurium. Among the serovar Typhimurium transductants inheriting *pnuC*::MudJ(Kn^r),

* Corresponding author. Present address: Microbiology Section of the Division of Biological Sciences, Center for Genetics and Development, University of California, Davis, Davis, CA 95616-8665. Phone: (530) 752-6679. Fax: (530) 752-7663. E-mail: jrroth@ucdavis.edu.

† Present address: Department of Biology, Indiana University, Bloomington, IN 47405.

90% inherited auxotrophy from serovar Dublin. An analogous set of crosses using a *nadA*-linked Tn*10*dTc insertion (*zbh-3652*::Tn*10*dTc) produced similar results. Thus, a mutation in or near the *nadA* gene is both necessary and sufficient for serovar Dublin's nutritional requirement.

Comparing *nadA* sequences of enteric bacteria. The *nadA* genes of several serovar Dublin strains and derived Nad^+ revertants were compared to those of serovars Typhimurium and Typhi (9, 13, 28, 32). As seen in Table 1, a valine residue at position 111 (codon GTG) correlates with pyridine auxotrophy (boxed columns). An alanine residue (codon GCG) is found at this position in prototrophic strains (serovar Typhimurium and the one prototrophic serovar Dublin isolate SARB14). Furthermore, prototrophic revertants of serovar Dublin strain TR7209 (strains TR7275 and TR7276) replace the valine of parental serovar Dublin with the alanine seen in prototrophic isolates. Thus, alanine 111 appears essential for NadA activity and valine at this position causes the auxotrophy of serovar Dublin isolates.

Like prototrophic *Salmonella* strains, *E. coli* has alanine at this site (5, 12, 34, 45). However, valine is found there in *Shigella flexneri* 2a (17, 44), consistent with the pyridine requirement of most *Shigella* isolates. This may not conflict with a report that *S. flexneri* is defective in *nadB*, not *nadA* (26), because *Shigella* lineages have evolved several times from *E. coli* (35) and thus may carry different mutations.

Differences between serovars Typhi and Typhimurium are distributed across the entire *nadA* gene. However, the 5′ half of the serovar Dublin sequence resembles that of serovar Typhi, while the 3′ half resembles that of serovar Typhimurium (Table 1). A portion of the 3′ half of serovar Typhi is more similar to *E. coli* and *Shigella* than to other salmonellae (data not shown). This suggests multiple recombination events within the *nadA* loci of enteric bacteria.

A search for conditions that suppress the auxotrophy of serovar Dublin. The auxotrophy of serovar Dublin was tested under the following conditions: (i) aerobic with various carbon sources (acetate, ethanolamine, glucose, glycerol, propanediol, propionate, or ribose); (ii) anaerobic with one of the above carbon sources and nitrate or tetrathionate as electron acceptor; and (iii) aerobic and anaerobic with glucose plus aspartate (a precursor of pyridines), plus thiamine (required with pyridines by some low-level streptomycin resistant [*strB*] mutants), plus NaCl (crystals and concentrations up to 1 mM), which corrects many missense mutations (19), plus iron (40 μM $FeSO_4$) since oxidative stress stimulates the pyridine nucleotide cycle (31), or plus Casamino Acids, shown to correct some leaky *nad* mutants (16, 18, 41).

Slight correction was provided anaerobically by Casamino Acids, but not by any of the other conditions tested. Casamino Acids did not correct *nadA*, *nadB*, or *nadC* insertion (null) mutants of either serovar Dublin or serovar Typhimurium, eliminating contamination by pyridines. We suggest that the serovar Dublin defect is slightly leaky and growth is improved by reducing the demand for NAD (NADP). Provided amino acids may spare the need for NADP in biosynthesis and glucose, by allowing fermentation, may spare the need for NAD in respiration.

Pyridine limitation does not provide antibiotic resistance in

TABLE 1. Polymorphic sites in *nadA* of serovar Typhimurium, Typhi, and Dublin isolates

Serovar or strain	Nad	Polymorphic codon[a]																
		7	24	35	68	76	83	102	103	104	108	111[b]	142	190	195	198	208	220
Typhimurium	+	CCA	GAT	CGG	TCA	ACG	CTG	AAG	ACC	ATT	ACC	GCG	ACC	GGG	TGC	GGA	ACT	GAT
SARB12	−	---	--C	--T	--G	G--	T-A	--A	-A-	---	--G	-T-	-T-	---	--T	--G	--C	T--
SARB13	−	---	--C	--T	--G	G--	T-A	--A	-A-	---	--G	-T-	---	---	--T	--G	--C	T--
SARB14	+	---	---	---	---	G--	---	---	---	---	--G	---	---	---	--T	--G	--C	---
TR7209	−	---	--C	--T	--G	G--	T-A	--A	-A-	---	--G	-T-	-T-	---	--T	--G	--C	T--
TR7211	−	---	--C	--T	--G	G--	T-A	--A	-A-	---	--G	-T-	-T-	---	--T	--G	--C	T--
TR7275 Rev	+	---	--C	--T	--G	G--	T-A	--A	-A-	---	--G	---	-T-	---	--T	--G	--C	T--
TR7276 Rev	+	---	--C	--T	--G	G--	T-A	--A	-A-	---	--G	---	-T-	---	--T	--G	--C	T--
Typhi	−	T--	--C	--T	--G	G--	T-A	--A	-A-	G--	--G	-T-	---	A--	--T	--G	--C	---

Serovar or strain	Polymorphic codon[a]																			
	231	260	274	275	276	278	279	282	283	285	287	289	291	292	296	303	330	333	334	347
Typhimurium	CAG	GTG	GTG	CCT	GAA	GAA	CTG	GCG	CCC	GCT	GAG	GCG	TGC	CGT	CAC	AAT	GGC	CTG	CCG	GCG
SARB12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SARB13	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
SARB14	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
TR7209	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
TR7211	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
TR7275 Rev	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
TR7276 Rev	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
Typhi	-C-	--C	--T	--G	--T	--G	T--	--A	--A	--G	--A	--A	--T	--C	--T	--C	-GC	--T	--C	-T-

[a] All sequences are compared to that of *S. enterica* (serovar Typhimurium, LT2). Dashes indicate identity; indicated bases show differences. Only polymorphic sites are listed.
[b] TR7275 and TR7276 are independent Nad^+ revertants of serovar Dublin auxotroph TR7209.

serovar Dublin. Streptomycin resistance was tested on minimal medium with growth-limiting levels of nicotinic acid (10^{-6} M, 3×10^{-7} M, 10^{-7} M, and 3×10^{-8} M). Pyridine limitation of Nad^- serovar Dublin strains did not increase Str^r above that seen for Nad^+ isolates (or recombinants) or for Nad^- strains with excess pyridine.

Frequency of Nad auxotrophs in natural isolates of *S. enterica* and *E. coli*. Auxotrophy is more common among clinical isolates than among isolates from healthy individuals or environmental sites (36). To test a wider range of isolates, *S. enterica* and *E. coli* strains from three collections were tested—SARB (6), SARA (4), and ECOR (30).The SARB collection includes three serovar Dublin isolates, two of which are NAD auxotrophs. SARB12 requires Na, SARB13 requires Na plus thiamine, but SARB14 is prototrophic. The only other Na auxotrophs in the SARB and SARA collections were SARB4 and -6, both of serovar Choleraesuis. Of the 72 ECOR strains of *E. coli*, only ECOR23 and ECOR29 required nicotinate.

Natural auxotrophy is not associated with mutators or hot spots. In addition to pyridine auxotrophs, the ECOR collection contained one strain (ECOR47) that required proline and another (ECOR49) that required cysteine. In the SARA collection, SARA6 and -28 required purine and SARA33 required cysteine. Surprisingly, 23 of 72 strains in the SARB collection (32%) required one or more growth factors; the most common was purine (nine isolates).

The high auxotroph frequency among SARB strains does not reflect mutators. None of the SARB auxotrophs exhibited a high rate of mutation to rifampin resistance (Rif^r). Only one SARB strain (nonauxotrophic strain 38) acquired Rif^r at a rate suggestive of a mutator allele. The *nadA* mutation found in serovar Dublin strains is not at a cytosine methylation site, which can cause a local mutation hot spot (8).

Summary. Evidence is provided that independent serovar Dublin isolates owe their auxotrophy to a single *nadA* missense mutation. None of the tests supported suppression, positive selection, or high mutation rate as explanations for persistence of this allele. The low level of pyridine required (<1 μM nicotinamide) is likely to be found in host organisms but may not be present in soil or water. Thus selection for pyridine synthesis may be weak in bacteria that transfer directly from one host to another without considerable time in soil or water. This might apply to serovar Dublin and pathogenic strains of *E. coli* and is consistent with higher auxotroph frequencies among clinical isolates (20, 36). The fact that serovar Dublin has not accumulated additional *nad* mutations suggests that mutation rates are low and/or the mutation observed is of recent origin. Consistent with weak selection, sequence data suggested repeated loss and recombinational repair of the *nadA* locus. The *nadA* gene may be mutationally lost during extended periods of relaxed selection and reacquired by horizontal transfer when selection is reimposed (22). Missense mutations like that described here cannot be detected by genome comparisons alone and may be common in natural isolates.

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