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Genetics of NAD Metabolism in *Salmonella typhimurium* and Cloning of the *nadA* and *pnuC* Loci

SIMIN TIRGARI, MICHAEL P. SPECTOR, AND JOHN W. FOSTER*

Department of Microbiology, Marshall University School of Medicine, Huntington, West Virginia 25704

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The *nadA* and *pnuC* loci of *S. typhimurium* were cloned and found to reside within a 2.2-kilobase region. Two-dimensional O'Farrell gel electrophoresis of the proteins produced after chloramphenicol amplificaton and subsequent release from chloramphenicol inhibition revealed NadA and PnuC to be 43,000- and 25,000-molecular-weight proteins, respectively. The data indicated that *nadA* and *pnuC* represent two distinct genes.

The initial reactions in the biosynthesis of NAD in *Salmonella typhimurium* are catalyzed by the products of the *nadA* and *nadB* loci. These proteins form a loosely associated complex referred to as quinolinic acid synthetase. Both *nadA* (17 min) and *nadB* (55 min) are regulated coordinately at the transcriptional level by the product of the *nadR* locus (99 min), as determined through the construction of operon fusions (3). The *pnuC* locus is situated adjacent to *nadA* and is involved in the utilization of exogenous nicotinamide mononucleotide as a pyridine source (9). Operon fusion studies with this locus also indicate its regulation by the *nadR* product. Both *nadA* and *pnuC* are induced under anaerobic conditions, and at least *nadA* appears to be controlled in part by cyclic AMP. A study of these genes at the molecular level will provide important information about their genetic structure and also will identify regulatory sequences responsible for controlling their expression.

Strains used in this study are listed in Table 1. The initial cloning of the *nadA* and *pnuC* loci was accomplished by partially digesting wild-type strain LT2 chromosomal DNA with the restriction enzyme *Sau*3A. Digestion was carried out to the point at which the restriction fragment sizes were between 5 and 15 kilobases (kb). The chromosomal digest was ligated to bacterial alkaline phosphatase-treated *Bam*HI-cleaved pBR322 by using T4 ligase (5). The ligation mixture was then used to transform strain JF908 (Δ*nadA* Δ*pncA galE hutR recA1* Δ*srl*) by using the procedure of Lederberg and Cohen (4). The use of *galE* cells is important because of the tremendous increase in transformation ability afforded by this mutation. Transformed cells were plated directly on minimal E medium containing 10^{-5} M nicotinamide (NAm) and 30 μg of ampicillin per ml (9). Strain JF908 will not grow on NAm as a pyridine source because of the *nadA pncA* genotype. Recombinant colonies which grew on this media could be either *nadA*$^+$ or *pncA*$^+$ and were screened accordingly on minimal medium with and without NAm. Three *nadA*$^+$ clones were identified based upon their ability to grow on minimal medium, and one *pncA*$^+$ clone (pTF14) was identified as requiring NAm. The *pncA*$^+$ plasmid will be analyzed in detail elsewhere. The plasmids were extracted from these cells by using a miniextraction technique (1), and then they were retransformed into JF908. Subsequently, the transducing phage P22 HT105/1-int was propagated on the *nadA*$^+$ clones and was used to transduce the recombinant plasmids into strain JF1015 (*nadB pncA pnuC recA*), selecting for Ap^r or growth on NAm mononucleotide. One plasmid (pTF15) was capable of complementing the *pnuC* mutation as evidenced by growth on 10^{-4} M NAm mononucleotide, and another (pTF16) did not complement *pnuC* (Table 2). The recombinant plasmid pTF15 underwent restriction mapping (Fig. 1). The 1.9-kb *Hpa*I fragment was removed from pTF15, producing pTF15-10. This plasmid failed to complement either *nadA* or *pnuC* mutants, indicating that at least part of *nadA* and *pnuC* lay within this 1.9-kb region (Table 2). A spontaneous *Ava*I-*Hpa*I deletion that occurred during the initial cloning, resulting in pTF15-2, did not affect the ability to complement either locus. (This deletion was only observed in the initial clone as a second plasmid. Purified pTF15 has never spontaneously deleted this fragment.) This indicates that both *nadA* and *pnuC* are situated somewhere within the 2.4-kb *Hpa*I-*Bam*HI/*Sau*3A region. (*Bam*HI/*Sau*3A refers to a hybrid site.) To confirm this, the 4-kb *Ava*I-*Ava*I fragment from pTF15 was subcloned to pBR322 (pTF20) and tested for the ability to complement *nadA* and *pnuC*. As expected, both genes were complemented (Table 2). There were no restriction sites for *Eco*RI, *Hin*dIII, *Acc*I, *Sal*I, *Pvu*I, *Xho*I, *Sst*I, *Kpn*I, or *Bam*HI within the *Ava*I fragment. The 1.9-kb *Hpa*I-*Hpa*I fragment from pTF15 was subcloned into

TABLE 1. Bacterial strains

Strain	Genotype
JF908	Δ*nadA101* Δ*pncA1 galE hutR49 recA1* Δ*srl*
JF1015	*nadB51 pncA15 trpA49 pnuB1 pnuC1 recA1 srl-202*::Tn*10*
JF1028	JF908(pTF15) *nadA*$^+$ *pnuC*$^+$
JF1029	JF908(pTF16) *nadA*$^+$
JF1067	JF908(pTF15-10); *Hpa*I deletion from pTF15
JF1122	JF908(pTF15-2); *Hpa*I-*Ava*I deletion from pTF15
JF1123	JF908(pTF20); *Ava*I-*Ava*I fragment from pTF15 subcloned in pBR322
JF1124	JF908(pTF22); *Ava*I-*Ava*I fragment from pTF16 subcloned in pBR322
JF1131	JF908(pTF23); *Hpa*I fragment from pTF20 subcloned in pACYC177
JF1206	JF908(pTF26); *Bgl*I fragment from pTF20 subcloned in pACYC177

* Corresponding author.

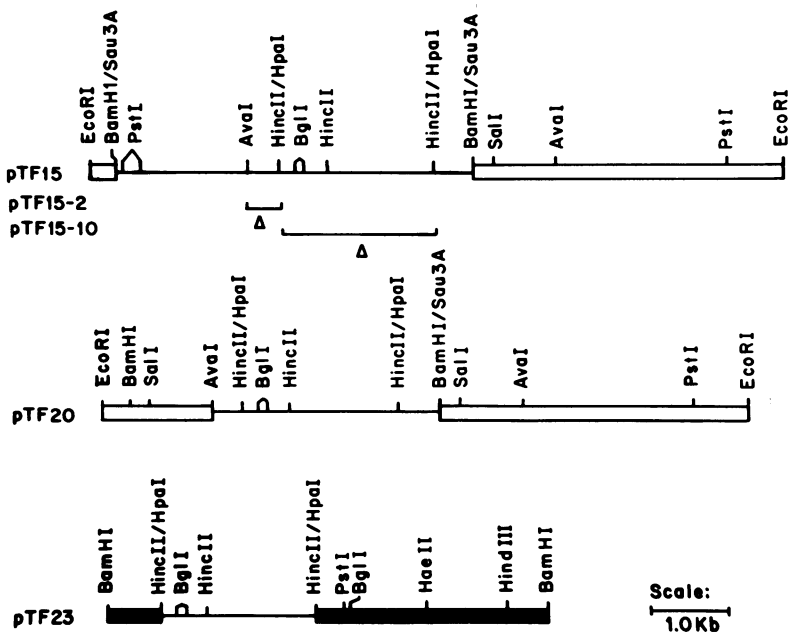
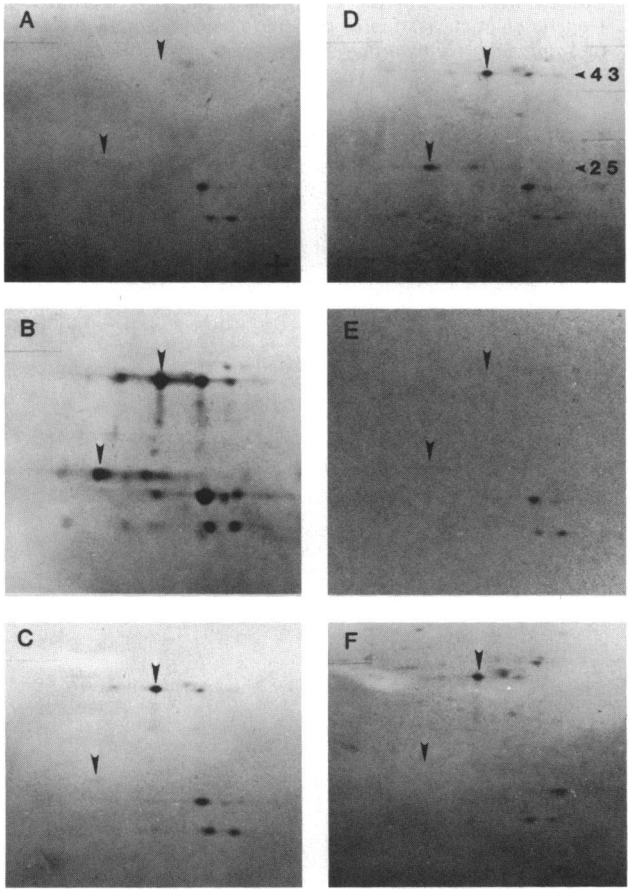


FIG. 1. Restriction maps of recombinant plasmids. ▭, pBR322 sequences; ▬, pACYC sequences. pTF20 was constructed by inserting the *Ava*I-*Ava*I fragment of pTF15 into the *Ava*I site of pBR322. pTF23 was constructed by inserting the *Hpa*I-*Hpa*I fragment of pTF15 into the *Hin*cII site of pACYC. ⌂, Multiple sites; △, deletion.



pACYC177, producing pTF23 (2). This plasmid only complemented *nadA*, indicating that part of *pnuC* mapped within the 0.5-kb *Hpa*I-*Bam*HI/*Sau*3A fragment. Subsequently, the 2.4-kb *Bgl*I fragment was subcloned into pACYC177, yielding pTF26, which complemented both *nadA* and *pnuC*, indicating that both structural genes lay within a 2.2-kb region from *Bgl*I to the *Bam*HI/*Sau*3A joint.

Identification of the *nadA* and *pnuC* gene products was accomplished subsequent to the recovery of plasmid-containing cells from chloramphenicol inhibition as described by Neidhardt et al. (6). Plasmid-containing cells were grown exponentially and then treated with chloramphenicol (80 μg/ml) overnight to amplify the plasmid. The overnight culture was washed in MOPS (morpholinepropanesulfonic acid) medium lacking sulfate and then suspended in 75 μl of the same medium containing 25 μCi ^{35}S-labeled methionine. Cells were labeled for 30 min at 37°C, harvested by centrifugation, and processed for two-dimensional gel electrophoresis as described by O'Farrell and co-workers (7, 8). Extracts were prepared by boiling in a sodium dodecyl sulfate lysing solution.

The results of these chloramphenicol recovery experiments are displayed in Fig. 2. Plasmids pTF15 and pTF20, which complemented *nadA* and *pnuC*, produced proteins with molecular weights of approximately 43,000 (NadA) and 25,000 (PnuC) that did not correspond to pBR322 proteins. Plasmids pTF16 and pTF23, which complemented *nadA* but not *pnuC* mutants, only produced the 43-kilodalton protein,

FIG. 2. Two-dimensional analysis of plasmid-encoded proteins after chloramphenicol release. (A) pBR322; (B) pTF15; (C) pTF16; (D) pTF20; (E) pTF15-10; (F) pTF23. Each panel represents only a section of an entire gel. The overexpression of both proteins resulted in multiple isoelectric forms which can be observed to the left and right of NadA (upper arrow) and PnuC (lower arrow). + and −, Isoelectric focusing electrodes.

TABLE 2. Complementation studies[a]

Plasmid	Complementation[b] of plasmid with gene:	
	nadA	*pnuC*
pTF15	+	+
pTF16	+	−
pTF15-2	+	+
pTF15-10	−	−
pTF20	+	+
pTF23	+	−
pTF26	+	+

[a] Complementation for *nadA* was tested by transferring the plasmid to strain JF908 and screening for growth on minimal medium. Testing for *pnuC* followed plasmid transfer to JF1015, with subsequent screening for growth on minimal medium containing 10^{-4} M NAm mononucleotide.

[b] +, Complementation; −, lack of complementation.

whereas pTF15-10, which complemented neither gene, did not produce either protein. The molecular weights for these proteins were determined by using internal standards and by comparison with a standard two-dimensional peptide map of *Salmonella* proteins (M. P. Spector and J. W. Foster, unpublished observation). The estimated coding region for the *nadA* and *pnuC* genes calculated from the molecular weights of their gene products was 2 to 2.2 kb, which agreed nicely with the 2.2-kb region estimated from the cloning studies.

These studies indicated that *pnuC* is a locus distinct from *nadA* in that two proteins are produced from this region, one (43,000 molecular weight) which complemented *nadA* mutations and another (25,000 molecular weight) which corrected lesions in the *pnuC* locus. The data did not clearly indicate whether both genes are under the control of a single promoter. Although genetic studies suggest two promoters (3), additional analyses of these recombinant plasmids should clarify this point and eventually reveal the molecular interaction between the *nadR* repressor and the operator(s) for these genes.

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