Regulation of NAD Biosynthesis in *Salmonella typhimurium*: Expression of *nad-lac* Gene Fusions and Identification of a *nad* Regulatory Locus

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Regulation of NAD biosynthesis was examined through the construction of *nad-lac* fusions in *Salmonella typhimurium*. The *nadA* (17 unit map position) and *nadB* (55 units) genetic loci involved with quinolinic acid biosynthesis were both found to be regulated by the product of a *nadR* locus (99 units) in a repression/derepression manner while *nadC* (3 units) expression appeared constitutive at the transcriptional level. Increases in *nadAB* transcription directly correlated with decreases in intracellular NAD(P) levels, and kinetic studies indicated that the NAD analogue 6-aminoNAD was ineffective in repressing either *nadA* or *nadB*. The presence of cAMP + cAMP receptor protein was essential for the complete derepression of *nadA* while no effect was evident upon *nadB*. Transfer of cultures from aerobic to anaerobic conditions, however, resulted in the partial derepression of both *nadA* and *nadB*. Thus, there appears to be a very complex set of controls regulating NAD biosynthesis.

INTRODUCTION

The biosynthesis of NAD and NADP in *Salmonella typhimurium* includes a *de novo* pathway and a series of recycling and salvage pathways termed pyridine nucleotide cycles (Foster & Moat, 1980). Four genes associated with *de novo* biosynthesis have been described for this enteric organism. They include *nadA* at 17 units (Foster & Moat, 1978; Langley & Guest, 1974), *nadB* at 55 units (Foster & Moat, 1978; Sanderson & Roth, 1983), *nadC* at 3 units (Foster & Moat, 1978; Langley & Guest, 1974) and *nadD* at 14 units (Hughes *et al.*, 1983a) on the *S. typhimurium* linkage map. The NadA and NadB gene products comprise the quinolinic acid (QA) synthetase system, the NadC protein is quinolinic acid phosphoribosyltransferase (EC 2.4.2.19) and the NadD product is nicotinic acid mononucleotide adenyltransferase (EC 2.7.7.18; Foster & Moat, 1980; Hughes *et al.*, 1983a, b). Fig. 1 illustrates the relationship between these genetic loci and NAD biosynthesis.

A powerful technique for examining gene expression involves the use of the mutagenic phage Mu cts d1(Ap^r *lac*) constructed by Casadaban & Cohen (1979). This phage contains an ampicillin resistance locus, a *lac* operon lacking a promoter and a temperature-sensitive phage repressor. When this phage is inserted in the proper orientation within a bacterial gene, expression of *lacZ* (β -galactosidase) is controlled by the promoter of the inactivated gene. Thus, transcriptional control of the locus in question can be examined easily by monitoring β -galactosidase activity.

Preliminary results from our laboratory using a *nadB-lac* and a *nadC-lac* gene fusion constructed using Mud1 suggested that transcription of *nadB* comes under repression control while *nadC* is transcribed constitutively (Holley & Foster, 1982). In this report we describe the isolation of additional *nadB-lac* and *nadC-lac* fusions as well as *nadA-lac* gene fusions using

Abbreviations: NA, nicotinic acid; 6ANA, 6-aminonicotinic acid; QA, quinolinic acid; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactoside.

Fig. 1. NAD metabolism in *Salmonella typhimurium* and relationship of genetic loci to the pathway. DHAP, dihydroxyacetone phosphate. Enzymes: (1) Quinolinic acid (QA) synthetase; (2) QA phosphoribosyltransferase; (3) nicotinic acid mononucleotide (NAMN) adenyltransferase; (4) NAD synthetase; (5) DNA ligase/NAD pyrophosphatase; (6) nicotinamide mononucleotide (NMN) glycohydrolase; (7) nicotinamide (NA) deamidase; (8) nicotinic acid (NA) phosphoribosyltransferase; (9) NMN deamidase.

both the Mud1 and the recently isolated Mud1-8 phage which requires an amber suppressor for transposition (Hughes & Roth, 1984). Expression of these loci is characterized in terms of derepression/repression kinetics, pyridine nucleotide levels during derepression, the effect of various *nad* mutations on the expression of *nad-lac* fusions, and the roles of oxygen and cAMP in *nadAB* regulon expression. In addition, the isolation of strains containing mutations (*nadR*) which alter *nadAB* expression is described.

METHODS

Bacteria and phage. The bacterial strains used are derivatives of *Salmonella typhimurium* LT-2 (Table 1). The phage used were the high-transducing derivative of P22, HT105/1-int (Schmieger, 1971), Mu cts d1(Ap^r *lac*) (Casadaban & Cohen, 1979), also referred to as Mud1, and a derivative of Mud1 designated Mu cts d8(Ap^r *lac*) or Mud1-8, constructed by Hughes & Roth (1984) and kindly provided by them for our use.

Chemicals and reagents. All reagents were of analytical quality. 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), nicotinic acid (NA) and 6-aminonicotinic acid (6ANA) were purchased from Sigma.

Culture medium and conditions. The minimal E medium of Vogel & Bonner (1956), supplemented with 0.4% glucose, was used throughout. Amino acids were supplemented at a concentration of 40 μg ml⁻¹, uracil at 30 μg ml⁻¹ and NA as described throughout the text. When used, ampicillin was added to 30 μg ml⁻¹. Tetracycline was used at 20 μg ml⁻¹ in complex medium and 10 μg ml⁻¹ in minimal media. Cultures were incubated at 30 °C with shaking (175 r.p.m.) in a gyratory incubator. Anaerobic conditions were achieved in a CO₂/H₂ environment using the anaerobic Gas-Pak system (BBL). The anaerobic container was placed inside the gyratory shaker and shaken at 175 r.p.m. Anaerobic conditions were attained within 2 h, as determined by a methylene blue indicator strip. Assays were performed 2–4 h later. Minimal lactose base medium was used when different carbon sources were examined (Foster *et al.*, 1979).

Kinetic studies involved growing the strain to be tested overnight in supplemented minimal medium. Immediately prior to the study the culture was washed twice in minimal medium lacking glucose (E buffer) to remove any remaining NA. The washed culture was resuspended and used to inoculate the media described in the text.

Table 1. *Strains of S. typhimurium*

Strain	Genotype	Source*
JF11	<i>purC7 proA46 ilv-405 rha-461 rpsL fla-56 pil nadB18</i>	
JF20	<i>purC7 proA46 ilv-405 rha-461 rpsL fla-56 pil nadB28</i>	
JF22	<i>purC7 proA46 ilv-405 rha-461 rpsL fla-56 pil nadB31</i>	
JF28	<i>purC7 proA46 ilv-405 rha-461 rpsL fla-56 pil nadA39</i>	
JF29	<i>purC7 proA46 ilv-405 rha-461 rpsL fla-56 pil nadC41</i>	

Table 1 (continued)

Strain	Genotype	Source*
JF153	<i>gal nadB1001::Mud1</i> (Lac ⁻)	
JF295	<i>pyrD95 nadB1002::Mud1</i> (Lac ⁺)	
JF296	<i>pyrD95 arg::Mud1</i> (Lac ⁺)	
JF297	<i>pyrD95 nadC1003::Mud1</i> (Lac ⁺)	
JF308	<i>pyrD95 nadB1019::Mud1</i> (Lac ⁻)	
JF466	<i>pyrD95 nadB1003::Mud1</i> (Lac ⁺)	
JF467	<i>pyrD95 nadC1004::Mud1</i> (Lac ⁺)	
JF468	<i>pyrD95 nadB1005::Mud1</i> (Lac ⁺)	
JF474	<i>pyrD95 nadC1007::Mud1</i> (Lac ⁺)	
JF475	<i>pyrD95 nadC1008::Mud1</i> (Lac ⁺)	
JF476	<i>pyrD95 nadB1009::Mud1</i> (Lac ⁺)	
JF477	<i>pyrD95 nadC1010::Mud1</i> (Lac ⁺)	
JF478	<i>pyrD95 nadA1011::Mud1</i> (Lac ⁺)	
JF479	<i>pyrD95 nadA1011::Mud1 nadB541::Tn10</i>	
JF484	<i>pyrD95 nadB1002::Mud1 nadA540::Tn10</i>	
JF489	<i>pyrD95 nadB1013::Mud1</i> (Lac ⁻)	
JF490	<i>pyrD95 nadB1014::Mud1</i> (Lac ⁻)	
JF491	<i>pyrD95 nadB1015::Mud1</i> (Lac ⁻)	
JF508	<i>purC7 proA46 ilv-405 rha-361 rpsL fla-56 pil zff-30::Tn10</i> (Tn10 near <i>nadB</i> ⁺)	
JF536	<i>trpA49 nadA56 pncA15 Δ(pnuA-nadR)1</i>	
JF596	<i>leu(am)515 su-19 nadA::Tn10 nadB1017::Mud1-8</i> (Lac ⁺)	
JF627	<i>pyrD95 nadB1002::Mud1 recA1 srl-202::Tn10</i>	
JF630	<i>pyrD95 nadA1011::Mud1 tyrA5</i>	
JF632	<i>ΔnadB120 nadA1011::Mud1 pyrD95 zff-29::Tn10</i>	
JF633	<i>ΔnadB122 nadA1011::Mud1 pyrD95 zff-29::Tn10</i>	
JF634	<i>ΔnadB127 nadA1011::Mud1 pyrD95 zff-29::Tn10</i>	
JF635	<i>ΔnadB130 nadA1011::Mud1 pyrD95 zff-29::Tn10</i>	
JF636	<i>pyrD95 nadA1011::Mud1 tyrA5 recA1 srl-202::Tn10</i>	
JF640	<i>pyrD95 nadB1002::Mud1 recA1 srl-202::Tn10/F'142 nadB</i> ⁺	
JF641	<i>pyrD95 nadA1011::Mud1 tyrA5 recA1 srl-202::Tn10/F'142 nadB</i> ⁺	
JF698	<i>ΔnadC nadB1017::Mud1-8 (Ap^r lac)</i>	
JF706	<i>supD10</i>	
JF714	<i>tyrA nadB1002::Mud1 ΔnadA ΔrecA srl-202/F'142 nadB</i> ⁺	
JF735	<i>purC7 proA46 ilv-405 rha-461 rpsL zff-1909::Tn10</i> (Tn10 near <i>nadB</i> ⁺)	
JF740	<i>zff-1000::Mud1-8 purC7 proA46 ilv-405 rha-461 rpsL M10 fla-56 pil</i>	
JF776	<i>zff-1909::Tn10 nadB1002::Mud1 pyrD95</i>	
JF797	<i>Φ(nadB-lac)1 pyrD95</i>	
JF801	<i>pyrD95 nadB1002::Mud1 cya::Tn10</i>	
JF802	<i>pyrD95 nadA1011::Mud1 cya::Tn10</i>	
JF803	<i>pyrD95 nadB1002::Mud1 cya::Tn10 crp</i> [*]	
JF804	<i>pyrD95 nadA1011::Mud1 cya::Tn10 crp</i> [*]	
JF836	<i>pyrD95 nadA1011::Mud1 tyrA5 recA1 srl-202::Tn10/F'152 nadA</i> ⁺	
JF864	<i>Φ(nadB-lac)1 nadR1 pyrD95</i>	
JF867	<i>zff-28::Tn10 nadR1 pyrD95</i> (Tn10 near <i>nadB</i> ⁺)	
JF868	<i>nadB1017::Mud1-8 zff-28::Tn10 nadR1 pyrD95</i>	
JF903	<i>nadA1011::Mud1 pyrD95 nadR2</i>	
JF904	<i>nadA1011::Mud1 pyrD95 nadR3</i>	
JF905	<i>nadA540::Tn10 nadR2 pyrD95</i>	
JF906	<i>nadA540::Tn10 nadR2 nadB1017::Mud1-8</i>	
JF985	<i>trpA49 nadA56 pncA15 Δ(pnuA nadR)1 nadB1017::Mud1-8</i>	
JF992	<i>nadA56 pncA15 pnuA7 trpA49 nadB1017::Mud1-8</i>	
JF993	<i>nadA56 pncA15 pnuA11 trpA49 nadB1017::Mud1-8</i>	
SF71	<i>nadA56 zff-1004::Tn10 pncA15 trpA49</i> (Tn10 near <i>pnuA</i> ⁺)	J. Roth
SF142	<i>hisD9953::Mud1-8</i>	J. Roth
SF145	<i>cya::Tn10 trpB223</i>	P. Postma
EK2 (KL709)	<i>pyrD34 trp-45 his-68 tyrA2 recA1 thi-1 galK35 xyl-7 mtl-2 rpsL118 λ⁻λ[*]/F'142</i> (<i>E. coli</i>)	B. Bachmann
EK62	<i>pyrD34 trp-45 his-68 tyrA2 recA1 thi-1 galK35 malA1 xyl-7 mtl-2 rpsL118</i> <i>λ⁻λ[*]/F'152 nadA</i> ⁺ (<i>E. coli</i>)	B. Bachmann

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Construction of gene fusions. Mud-directed gene fusions were constructed in a manner similar to that described earlier (Holley & Foster, 1982). HT phage propagated either on a Mud1 lysogen (JF296) or on a Mud1-8 lysogen (SF142) were used to introduce Mud into *nad*⁺ recipients. Two methods of screening for Nad⁻ auxotrophs were used. The first method involved the direct plating of phage and cells on LB medium containing ampicillin. Resultant Ap^r colonies were replicated to minimal medium containing ampicillin either with or without 10⁻⁵ M-NA. NA-requiring mutants (*nad*:Mud) were purified and tested for the *lac*⁺ characteristic on minimal medium containing X-Gal. Alternatively, *nad*:Mud fusions were enriched using cycloserine selection. Cells and phage (0.25 ml each) were mixed and incubated stationary for 1 h at 30 °C. After the addition of fresh minimal medium (5.0 ml), ampicillin was added to 30 µg ml⁻¹ and the culture incubated overnight at 30 °C with shaking. The overnight culture was washed twice and resuspended to 10⁷ cells ml⁻¹ in 100 ml minimal medium. Cycloserine was added to 1 mM when the culture attained a density of 2 × 10⁸ cells ml⁻¹. After clearing (approximately 1–1.5 h), the culture was washed twice and resuspended in 10 ml minimal medium containing ampicillin and NA. This was allowed to grow overnight. The overnight culture was diluted and plated onto minimal medium supplemented with NA. Resultant colonies were then screened for their NA requirement by replicating onto medium with and without NA. Potential *nad*:Mud strains were analysed as described above. β-Galactosidase activity was assayed on all fusion strains as described by Miller (1972), and is expressed as Miller units. Values given represent means of two or more independent experiments.

Genetic manipulations. Conjugations and transductions were done by mixing donor cells or phage with recipient cells directly on selective medium. Transductants were screened for nonlysogens on green medium as described by Chan *et al.* (1972). Tn10-generated deletions were selected on the medium described by Maloy & Nunn (1981) with some modification. Depending upon the strain, Maloy & Nunn medium was formulated using lower quantities of tryptone, usually one-half to one-quarter the normal amount. Mud-directed deletions were constructed by selecting for either Lac⁺ Ap^rTs⁺ or Lac⁻ Ap^rTs⁺ colonies as described by Maloy & Roth (1983). All deletions were checked by crosses with known point mutations. Point mutations were made using the hydroxylamine comutagenesis procedure described by Hong & Ames (1971).

Construction of a pool of Mud1-8 insertions. Since Mud1-8 will not transpose unless it is present in a *supD* background, it was possible to use Mud1-8 to construct a pool of insertions in manner similar to that commonly used for Tn10. P22 phage grown on a Mud1-8 lysogen (SF142) was used to infect a *supD* strain (JF706). The resulting Ap^r colonies (~2000), comprising a Mud1-8 transposition pool, were collected in approximately 2 ml saline containing 15% (v/v) glycerol. A 0.5 ml sample of these cells was grown to mid-exponential phase in 500 ml LB and infected with P22. After overnight incubation, 200 ml of the phage preparation was harvested and the supernatant lysate used for subsequent screening of Mud1-8 insertions near *nadB*.

Measurement of NAD and NADP concentrations. Pyridine nucleotides were measured using cold 40% (v/v) acetic acid extraction of [¹⁴C]NA-labelled nucleotides as described previously (Foster *et al.*, 1979). Cells were grown overnight in supplemented minimal medium, washed to remove unlabelled NA and resuspended in fresh minimal medium containing 10⁻⁵ M- or 10⁻⁷ M-[¹⁴C]NA (10 µCi µmol⁻¹; 370 kBq µmol⁻¹). Samples were filtered, washed with cold E buffer and the intracellular nucleotides extracted with 0.5 ml cold 40% (v/v) acetic acid. An equal volume of extract for each strain was chromatographed on an isobutyric acid/ammonium hydroxide/H₂O (66:1:33, by vol.) solvent system.

RESULTS AND DISCUSSION

Isolation, characterization and expression of nad-lac fusions

Mu d1(Ap^r *lac*)- and Mu d1-8(Ap^r *lac*)-directed *lacZ* fusion strains were isolated as described in Methods using either direct isolation or cycloserine enrichment. All independently isolated Ap^r NA-requiring strains were mapped to the *nadA*, the *nadB* or the *nadC* locus by transducing the fusion strain to *nad*⁺ using phage propagated on known *nadA* (JF28), *nadB* (JF22) or *nadC* (JF29) mutants. Failure of a particular phage preparation to repair the *nad*:Mud insertion indicated the *nad* locus into which the fusion had occurred. Scoring of *nad*⁺ transductants for the loss of Ap^r and the acquisition of temperature resistance was used to indicate the presence of a single Mud insertion in the *nad* locus. Table 2 lists the *nad*:Mud insertions isolated and presents the results of *in vitro* β-galactosidase activities obtained following growth in various concentrations of NA. The results indicate that the *nadA* fusion and five of the 10 *nadB* fusions are controlled at the transcriptional level by end-product repression. The *nadB*:Mud insertions which exhibit low unregulated β-galactosidase activities probably represent Mud insertions in the wrong orientation for transcription from the *nadB* promoter. Transcription of *nadC* is constitutive in nature (Table 2).

Table 2. Expression of various *nad* :: *Mud* fusions

Strain	Pertinent genotype	β -Galactosidase activity		
		10^{-4} M-NA	10^{-7} M-NA	Increase (fold)
JF478	<i>nadA1011</i> :: <i>Mud1</i>	54.75	819	15
JF153	<i>nadB1001</i> :: <i>Mud1</i>	0.0	0.0	—
JF295	<i>nadB1002</i> :: <i>Mud1</i>	18.8	395	21
JF308	<i>nadB1019</i> :: <i>Mud1</i>	0.0	0.0	—
JF466	<i>nadB1003</i> :: <i>Mud1</i>	1.2	6.1	5.1
JF468	<i>nadB1005</i> :: <i>Mud1</i>	27.6	703	25
JF476	<i>nadB1009</i> :: <i>Mud1</i>	1.0	1.24	—
JF489	<i>nadB1013</i> :: <i>Mud1</i>	3.38	2.72	—
JF490	<i>nadB1014</i> :: <i>Mud1</i>	41.8	701	17
JF491	<i>nadB1015</i> :: <i>Mud1</i>	1.56	1.2	—
JF596	<i>nadB1017</i> :: <i>Mud1</i> -8	11.5	361	33
JF297	<i>nadC1003</i> :: <i>Mud1</i>	37	21	—
JF467	<i>nadC1004</i> :: <i>Mud1</i>	2.3	2.7	—
JF474	<i>nadC1007</i> :: <i>Mud1</i>	2.19	2.32	—
JF475	<i>nadC1008</i> :: <i>Mud1</i>	6.7	5.5	—
JF477	<i>nadC1010</i> :: <i>Mud1</i>	3.65	3.71	—

Kinetics of nadA-lacZ and nadB-lacZ expression

Strains JF478 (*nadA* :: *Mud1*) and JF295 (*nadB* :: *Mud1*) were examined more precisely during NA starvation to determine at what point derepression of these genes occurs and how this derepression is related to internal pyridine nucleotide concentrations. A parallel culture was used for the labelling of internal pyridine nucleotides. The results are shown in Figs 2 and 3. Both *nadA* and *nadB* were derepressed at approximately the same time, 1–1.5 h after transfer to 10^{-7} M-NA. During this period the total pyridine nucleotide content declined to 0.6–0.7 mM (Fig. 3). NAD(H) decreased from its normal value of 0.9 mM to 0.5 mM while NADP(H) decreased from 0.3 mM to 0.15 mM. Similar values for intracellular pyridine nucleotides were reported by Bochner & Ames (1982). These data illustrate the direct relationship between internal pyridine nucleotide concentrations and the expression of the *nadAB* regulon. The NADP(H)/NAD(H) ratio, while remaining constant at 0.32–0.34 during balanced growth, rose dramatically during NA starvation. The increase probably reflects a need in the stressed cell to maintain NADP(H) levels for biosynthetic purposes. Alternatively, the increased ratio may represent a signal which affects the expression of the NAD biosynthetic genes. A similar effect was observed with *Escherichia coli* by Lundquist & Olivera (1973).

Repression kinetics and the effect of 6ANA on nadA-lacZ and nadB-lacZ expression

The addition of NA or nicotinamide to a growing *nad-lacZ* fusion strain results in the repression of *nadA* and *nadB*, presumably through conversion to NAD. To determine how quickly external pyridine sources can generate a repression signal (i.e. increased internal NAD levels) the experiments presented in Fig. 4 were performed. Both *nadA-lacZ* (JF478) and *nadB-lacZ* (JF295) fusion strains were grown overnight in 10^{-4} M-NA-supplemented minimal medium. After washing twice in E buffer, the cells were transferred to a starvation medium containing 10^{-6} M-NA to derepress *nadA* and *nadB*. Growth, and the increase in β -galactosidase activity, were monitored during derepression. At the time indicated, the culture was split into three subcultures; one received NA (10^{-4} M), another received the analogue 6ANA (4×10^{-4} M) and the third received no addition. Addition of NA had an immediate effect in stopping the derepression of both *nadA* and *nadB* (Fig. 4). The subculture containing the NA analogue, however, appeared to show an increased derepression of these genes for 75–90 min (at which point growth was affected). The delay observed with the analogue does not appear to be the result of a lower rate of uptake and utilization since we and others (Hughes *et al.*, 1983*b*) have

Fig. 2. Kinetics of *nad-lac* derepression. Cultures of strains JF478 (*nadA::Mud1*) and JF295 (*nadB::Mud1*) were transferred from 10^{-5} M-NA to 10^{-7} M-NA at time zero. ●, Growth; ○, β -galactosidase activity (Miller units).

Fig. 3. Pyridine nucleotide concentrations during NA starvation. Strain JF478 (*nadA::Mud1*), after overnight growth in 10^{-5} M-[14 C]NA, was washed and transferred to 10^{-7} M-[14 C]NA. Samples were taken and analysed for intracellular pyridine nucleotides as described in Methods. ○, Total pyridine nucleotide concentration; ●, NAD(H); □, NADP(H); ▲, NADP(H)/NAD(H) ratio.

found that the addition of analogue to unstarved cells results in an increase of intracellular 6-aminoNAD concentrations within 10–15 min after addition. Our results indicate that 6-aminoNAD does not represent a repression signal for *nadA* or *nadB*. The initial increase in β -galactosidase is most probably due to the accelerated depletion of NAD levels which has been shown to occur following the administration of these analogues (Hughes *et al.*, 1983b).

All the data presented above indicate that *nadA* and *nadB* are controlled at the transcriptional level by a repression–derepression mechanism. Saxton *et al.* (1968) and Chandler & Ghoson (1972) have presented data suggesting repression control of this system in *E. coli*. The present study, however, is the first to demonstrate unequivocally in either *E. coli* or *S. typhimurium* that this regulation occurs at the transcriptional level.

Fig. 4. Repression kinetics of *nad-lac* fusion strains JF478 (*nadA::lacZ*) and JF295 (*nadB::lacZ*) grown with NA or 6ANA. Derepression was initially accomplished in 10^{-6} M-NA. Vertical arrows indicate the time of addition. ○, Growth with no addition; □, growth with 4×10^{-4} M-6ANA; △, growth with 10^{-4} M-NA; ●, β -galactosidase (Miller units) with no addition; ■, β -galactosidase with 4×10^{-4} M-6ANA; ▲, β -galactosidase with 10^{-4} M-NA.

Table 3. Effect of *nad* mutations on the expression of *nad-lac* fusions in haploids and merodiploids

Strain	Pertinent genotype	β -Galactosidase activity		
		10^{-4} M-NA	10^{-7} M-NA	Increase (fold)
JF478	<i>nadA1011::Mud1</i>	54.7	819	15.0
JF479	<i>nadA1011::Mud1 nadB541::Tn10</i>	55.1	726	13.2
JF295	<i>nadB1002::Mud1</i>	60.0	765	12.8
JF484	<i>nadB1002::Mud1 nadA540::Tn10</i>	75.0	990	13.2
JF698	<i>nadB1017::Mud1-8 ΔnadC</i>	12.6	347	27.5
JF627	<i>nadB1002::Mud1</i>	60.1	712	11.9
JF640	<i>nadB1002::Mud1/F'142 nadB⁺</i>	80.9	239	2.9
JF714	<i>nadB1002::Mud1 ΔnadA/F'142 nadB⁺</i>	67.3	842	12.5
JF636	<i>nadA1011::Mud1</i>	54.8	927	16.9
JF641	<i>nadA1011::Mud1/F'142 nadB⁺</i>	80.8	1309	16.2

Regulation and merodiploid studies

Reports suggest the presence of a regulatory locus in *E. coli*, designated *nadR*, adjacent to *nadB*; mutations in this locus appeared to decrease the amount of both NadA and NadB protein (Tritz & Chandler, 1973). It was unclear, however, whether *nadR* actually represented a class of *nadB* mutant affecting some autoregulatory feature of the *nadB* gene product (Foster & Moat, 1980). To answer this question in *S. typhimurium* we constructed a series of strains containing either a *nadA-lac* fusion and a *nadB::Tn10* insertion (JF479) or a *nadB-lac* fusion and a *nadA::Tn10* insertion (JF484). Presumably if the *nadB* gene product regulated its own expression as well as that of *nadA* then the insertion of *Tn10* into *nadB* should eliminate control over *nadA-lacZ* expression. The data presented in Table 3 reveal that this is not the case. Thus, the *nadR* locus, if it exists, is not allelic with *nadB*.

To determine if *nadR* was situated adjacent to *nadB* we isolated a series of *Tn10* and *Mud1-8* insertions near *nadB* for the purpose of generating deletions through *nadB*. Certainly if *nadR* occupied a position next to *nadB*, some *Tn10*- or *Mud*-generated deletions should remove *nadR*

and render *nadA-lacZ* expression constitutive. Numerous *nadB* deletions were transferred to JF478 or constructed in JF479 (*nadA::Mud nadB::Tn10*) as described in Methods. Screening for the *nadR* phenotype involved plating the potential deletions on minimal medium containing X-Gal supplemented with 10^{-5} M or 5×10^{-7} M-NA. None of the deletions appeared altered with respect to regulation (data not shown). These results suggest that *nadR*, at least in *S. typhimurium*, is probably not situated near *nadB*.

Merodiploid studies were done to determine if the *E. coli nadA* and *nadB* loci could complement the analogous loci of *S. typhimurium*. F'142 (*nadB*⁺) and F'152 (*nadA*⁺) were transferred to several of the fusion strains constructed during this study. The results (Table 3) reveal that F'142 does complement a chromosomal *nadB* mutation (JF640) but does not appear to have an effect upon *nadB-lacZ* (JF714) or *nadA-lacZ* (JF641) expression as long as a requirement for NA is maintained. Strain JF640, which no longer requires NA for growth, does not show as great a level of *nadB-lacZ* derepression. This can be explained by the fact that the merodiploid can maintain intracellular NAD concentrations through *de novo* synthesis. Similar results were obtained with *nadA* merodiploids. Studies with *E. coli* indicate that the NadB protein complexes with the NadA protein to form an active QA synthetase (Nasu *et al.*, 1982). The fact that the F' strains used in this study were derived from *E. coli* strongly suggests that the NadB and NadA proteins of *S. typhimurium* also form a complexed QA synthetase in which an *E. coli* NadB or NadA protein can substitute for the missing *Salmonella* component.

Isolation of nadR mutants altered in nadAB regulon expression

A stabilized *nadB-lac* fusion strain (JF797) would not grow on minimal medium containing lactose as the sole carbon source if a repressive concentration of NA (10^{-4} M) was included. This strain was heavily streaked on this same medium containing X-Gal as an indicator in the hope that spontaneous *nadR* mutations would result in the constitutive expression of the *nadB-lac* fusion. The subsequent overproduction of β -galactosidase would allow growth on minimal lactose medium containing a repressive concentration of NA.

Several strains were isolated by this method. In order to determine if the mutation occurred in a locus separate from *nadB* (i.e. *nadR*) each strain was transduced to *nadB*⁺ prototrophy and checked for loss of the Lac⁺ characteristic. Next a *nadB::Mud1-8* insertion was reintroduced and expression analysed. Three of the regulatory mutants initially isolated were apparently altered in the *nadB* region since reintroduction of *nadB::Mud1-8* resulted in normal repression-depression of *nadB*. One strain, however, upon reintroduction of *nadB::Mud1-8* still produced β -galactosidase constitutively (Table 4). Similar *nadR* mutations were isolated in a *nadA-lac* fusion strain. These, too, caused deregulation of *nadB-lac* fusions (Table 4). The results indicate that the *nadA* and *nadB* loci form a regulon controlled by *nadR*.

Map position of nadR

Each of the *nadR* mutants was crossed with phage propagated on strains containing Tn10 insertions near various NAD-related loci. In each instance, the *nadR* mutation was found to map near *pnuA* (pyridine nucleotide uptake) at 99 units (Table 5). This clearly indicates that *nadR* is not located at *nadB* as suggested for *E. coli* and that the *nadR* gene product probably acts in *trans* upon *nadA* and *nadB*. Spector *et al.* (1985) have shown that *nadR* mutations also affect the regulation of *pnuC*. It would appear that *nadR* is distinct from *pnuA*. Several *pnuA* point mutations had no effect upon *nadB-lacZ* repression (Table 6). However, a *pnuA* deletion clearly resulted in the constitutive expression of *nadB*, suggesting that *nadR* is situated very close to *pnuA* (Table 6).

Effect of nadR on intracellular NAD concentrations

To determine if the *nadR* regulatory mutation also altered pyridine nucleotide cycle metabolism the *nadB-lac nadR1* strain JF864 and its *nadR*⁺ parent JF797 were used for *in vivo* labelling experiments with [¹⁴C]NA. Both strains were grown overnight in supplemented minimal medium, washed twice to remove unlabelled NA and then used to inoculate minimal medium supplemented with [¹⁴C]NA at 10^{-5} M. Periodic samples were taken and intracellular

Table 4. Effect of *nadR* on *nadA* and *nadB* expression

Strain	Pertinent genotype	β -Galactosidase activity	
		10^{-4} M-NA	10^{-6} M-NA
JF797	$\Phi(nadB-lacZ)$	18.4	320
JF864	$\Phi(nadB-lacZ) nadR1$	667	680
JF867	<i>nadB</i> ⁺ <i>nadR1</i>	0.0	0.0
JF868	<i>nadB</i> ::Mud1-8 <i>nadR1</i>	868	921
JF478	<i>nadA</i> ::Mud1	48.3	1194
JF903	<i>nadA</i> ::Mud1 <i>nadR2</i>	1296	1336
JF905	<i>nadA</i> ⁺ <i>nadR2</i>	0.0	0.0
JF906	<i>nadB</i> ::Mud1-8 <i>nadR2</i>	849	930

Table 5. Cotransduction of *nadR* to *zjj-1004*::Tn10 (Tn10 near *pnuA*⁺)

Donor phage was grown on SF71. Initial selection was for Tet^R.

Recipient	Pertinent genotype	Number scored	Linkage to <i>zjj-1004</i> ::Tn10 (%)
JF864	$\Phi(nadB-lac) nadR1$	95	81
JF903	<i>nadA</i> ::Mud1 <i>nadR2</i>	106	46
JF904	<i>nadA</i> ::Mud1 <i>nadR3</i>	99	30

Table 6. Effect of *pnuA* mutations on *nadB-lac* expression

Strain	Pertinent genotype	β -Galactosidase activity		
		10^{-4} M-NA	10^{-6} M-NA	Increase (fold)
JF596	<i>nadB</i> ::Mud1-8	18.5	441	24
JF985	$\Delta pnuA nadB$::Mud1-8	801	661	—
JF992	<i>pnuA7 nadB</i> ::Mud1-8	22.2	312	14
JF993	<i>pnuA11 nadB</i> ::Mud1-8	18.4	473	26

nucleotides extracted with cold 40% (v/v) acetic acid. Chromatography was performed in several solvent systems. The results (not shown) suggested no significant difference between intracellular [¹⁴C]NAD levels of the *nadR* mutant as compared with the *nadR*⁺ control. Samples were taken at comparable cell densities. Previous studies have indicated that the intracellular NADP/NAD ratio remains relatively constant at 0.3–0.34 during exponential growth (Foster & Moat, 1980; Lundquist & Olivera, 1973; Foster *et al.*, 1984). Our results confirmed this, with a ratio of 0.33.

Effect of carbon source and anaerobiosis on *nad-lac* expression

The isolation of *nad-lac* fusions and the construction of a *nadB-lac/F' nadB*⁺ strain (JF640) allowed the evaluation of carbon source and oxygen supply on the regulation of NAD biosynthesis at the transcriptional level. Preliminary experiments revealed that the *nadB-lac/F' nadB*⁺ strain, with no NA requirement, expressed β -galactosidase at a constant level during exponential growth. Thus, analyses of conditions resulting in different growth rates could be compared. Table 7 presents the β -galactosidase activities obtained after growth on glucose, glycerol and acetate under aerobic conditions and on glucose under anaerobic conditions. The data indicate that regardless of the carbon source and subsequent growth rate *nadB* is expressed at a relatively constant level. However, transfer of the culture to anaerobic conditions resulted in at least a twofold derepression of β -galactosidase, suggesting an increased requirement for NAD under anaerobiosis. Similarly, transfer of a *nadA-lac/F' nadA*⁺ strain to anaerobic conditions resulted in a threefold increase in β -galactosidase. These results were very reproducible.

Table 7. *Effect of carbon source and oxygen on nad-lac expression*

Strain	Pertinent genotype	Carbon source	Atmosphere	Generation time	β -Galactosidase activity
				(min)	
JF640	<i>nadB-lac/F' nadB⁺</i>	Glucose (30 mM)	Aerobic	75	213
		Glycerol (50 mM)	Aerobic	170	208
		Acetate (50 mM)	Aerobic	460	216
		Glucose (30 mM)	Anaerobic	140	424
JF836	<i>nadA-lac/F' nadA⁺</i>	Glucose (30 mM)	Aerobic	70	307
		Glucose (30 mM)	Anaerobic	145	1107

Table 8. *Effect of adenyl cyclase and cAMP on nad-lac expression*

Cells were inoculated to an initial OD₆₀₀ of 0.06; cAMP was added at OD₆₀₀ 0.2 and β -galactosidase activity measured at OD₆₀₀ 0.6.

Strain	Pertinent genotype	NA concn (M)	cAMP concn (M)	β -Galactosidase	
				Activity	Increase (fold)
JF478	<i>nadA1011::Mud1</i>	10 ⁻⁴	—	58	
		10 ⁻⁴	5	62	
		10 ⁻⁶	—	1039	17.9
		10 ⁻⁶	5	1077	18.6
JF802	<i>nadA1011::Mud1 cya::Tn10</i>	10 ⁻⁴	—	62	
		10 ⁻⁴	5	59	
		10 ⁻⁶	—	331	5.4
		10 ⁻⁶	5	813	13.2
JF804	<i>nadA1011::Mud1 cya::Tn10 crp*</i>	10 ⁻⁴	—	37	
		10 ⁻⁶	—	726	19.6
JF295	<i>nadB1002::Mud1</i>	10 ⁻⁴	—	29	
		10 ⁻⁴	5	32	
		10 ⁻⁶	—	760	26
		10 ⁻⁶	5	703	24
JF801	<i>nadB1002::Mud1 cya::Tn10</i>	10 ⁻⁴	—	40	
		10 ⁻⁴	5	36	
		10 ⁻⁶	—	549	13.8
		10 ⁻⁶	5	553	13.8
JF803	<i>nadB1002::Mud1 cya::Tn10 crp*</i>	10 ⁻⁴	—	19	
		10 ⁻⁶	—	293	15.4

This phenomenon is not due to some anaerobic effect on β -galactosidase since several other fusions isolated in our laboratory were unaffected by the presence or absence of oxygen. Wimpenny & Firth (1972) have reported that when *E. coli* is switched to anaerobic conditions NAD concentrations drop precipitously and then rise slowly. This would explain the increase in *nadB* expression demonstrated here. The reported drop in NAD concentration could be related to the oxygen requirement reported by Nasu *et al.* (1982) for *nadB*.

The tight control exerted over QA synthetase and thus NAD biosynthesis was also evident in these studies. Merodiploids grown in medium containing various carbon sources resulting in a wide range of growth rates nevertheless expressed *nadB* at a constant level. Studies with *Pseudomonas aeruginosa* have revealed that NAD(H) concentrations remain relatively constant on a wide variety of carbon sources (Martin & Gottschal, 1976). The constant level of *nadB-lac* expression in *Salmonella* suggests a similar consistency in NAD(H) levels. Transfer to anaerobic conditions, however, resulted in a definite, reproducible two- to threefold increase in *nad-lac* expression.

Role of cAMP in the expression of *nadA*

Various nutrient limitations, including phosphate, ammonia and glucose, were tested for their effects on the expression of the *nadAB* regulon. Only glucose starvation resulted in an observable increase in β -galactosidase activity of *nadA* fusion strains. The effect was evident on solid media but could not be demonstrated in liquid media (data not shown).

One obvious possible signal molecule suggested by the glucose limitation study is cAMP. Thus, a *cya::Tn10* insertion was introduced into the *nadA-lac* and *nadB-lac* fusion strains to prevent cAMP synthesis and β -galactosidase production was measured during NA starvation conditions. The results presented in Table 8 show that *nadA* expression was dependent upon adenylate cyclase and cAMP while the effect was not evident with *nadB*. Derepression of *nadA* was greatly reduced in a *cya::Tn10* background but was increased upon the addition of 5 mM-cAMP. Additionally, a *nadA-lac cya::Tn10* strain containing a *crp** mutation, which codes for a cAMP receptor protein that does not require cAMP for activity, displayed normal derepression. This phenomenon has been demonstrated in a second, independent *nadA-lac* operon fusion strain (data not shown).

The results presented in this communication suggest dual regulation of *nadA*: by NAD levels through *nadR* repression and by cAMP and its receptor protein (which are apparently required for full derepression). The reason for this cAMP-dependence is not immediately apparent. However, it is interesting that *nadB* does not appear to display this dual regulation. Further studies of the *nadAB* regulon at the molecular level will be necessary to determine precisely the various levels of regulation and reveal other potential signal molecules involved with controlling NAD biosynthesis.

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