JOURNAL OF BACTERIOLOGY, Apr. 1989, p. 2173–2180
0021-9193/89/042173-08$02.00/0
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Vol. 171, No. 4

DNA Ligase and the Pyridine Nucleotide Cycle in *Salmonella typhimurium*

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Received 24 August 1988/Accepted 4 January 1989

Bacterial DNA ligases use NAD as an energy source. In this study we addressed two questions about these enzymes. First, what is the physiological consequence of completely removing the NAD-dependent enzyme and replacing it with an ATP-dependent DNA ligase? We constructed *Salmonella typhimurium* strains in which the endogenous NAD-dependent DNA ligase activity was inactivated by an insertion mutation and the ATP-dependent enzyme from bacteriophage T4 was provided by a cloned phage gene. Such strains were physiologically indistinguishable from the wild type, even under conditions of UV irradiation or treatment with alkylating agents. These results suggest that specific functional interactions between DNA ligase and other replication and repair enzymes may be unimportant under the conditions tested. Second, the importance of DNA ligation as the initiating event of the bacterial pyridine nucleotide cycle was critically assessed in these mutant strains. Surprisingly, our results indicate that DNA ligation makes a minimal contribution to the pyridine nucleotide cycle; the *Salmonella* strains with only an ATP-dependent ligase had the same NAD turnover rates as the wild-type strain with an NAD-dependent ligase. However, we found that NAD turnover was significantly decreased under anaerobic conditions. We suggest that most intracellular pyridine nucleotide breakdown occurs in a process that protects the cell against oxygen damage but involves a biochemical mechanism other than DNA ligation.

DNA ligases were first characterized in bacteria (14, 15, 21) and bacteriophages (19) and were found to use two different cofactors as an energy source for the synthesis of the phosphodiester bond in DNA. In all bacterial DNA ligases characterized, the energy source is NAD (12). In all phage enzymes (and in eucaryotic DNA ligases characterized subsequently), the cofactor used is ATP.

The discovery that NAD could serve as an energy source for DNA ligase demonstrated that in addition to its classical role as an oxidation-reduction cofactor, NAD could also serve as a substrate for an enzyme with a clearly defined metabolic function. In contrast to oxidation-reduction reactions, in which there is no net consumption of NAD, one DNA ligation event causes the breakdown of one NAD molecule to nicotinamide mononucleotide (NMN) and AMP (Fig. 1). To maintain steady-state levels of pyridine nucleotides, the NMN produced is recycled back into NAD by two pathways. Such recycling pathways are called pyridine nucleotide cycles (6), and the breakdown of NAD by DNA ligase is one potential event initiating recycling. The main pyridine nucleotide cycle that would be initiated by DNA ligase in an enteric bacterium is shown in Fig. 1.

In this study, we addressed two questions about bacterial DNA ligases. First, does DNA ligase play a physiological role in bacterial nucleic acid metabolism in addition to catalyzing DNA joining per se? For example, does DNA ligase interact with DNA polymerase I or other repair enzymes? A second related question is whether ATP-dependent ligases from other organisms can fully replace the NAD-dependent enzyme of *Salmonella typhimurium*.

In order to address these questions, we attempted to completely abolish the function of the resident DNA ligase and to substitute the ATP-dependent DNA ligase of bacteriophage T4. This required isolation of null mutants for the *Salmonella* DNA ligase gene (*lig*). Because DNA ligase is an essential enzyme, we isolated these mutants by the following strategy. The *lig* region of the chromosome was duplicated by means of a tandem chromosomal duplication; an insertion mutation was introduced into one of the *lig* gene copies; and the *lig* insertion was transduced into a *Salmonella* strain without the duplication but containing a plasmid carrying the gene for T4 DNA ligase, leaving a host chromosome with the *lig* insertion mutation.

In addition, we assessed the contribution of DNA ligation to NAD turnover. It was previously hypothesized that in *Escherichia coli*, DNA ligase may be the major enzyme cleaving intracellular NAD to NMN and AMP, thereby initiating the pyridine nucleotide cycle. Although this is an obvious hypothesis, it has never been rigorously tested and, as will be demonstrated below, turns out to be incorrect. The substitution of an ATP-dependent DNA ligase for the resident NAD-dependent DNA ligase in *Salmonella typhimurium* allowed critical evaluation of the role of DNA ligase in NAD turnover in this bacterium.

MATERIALS AND METHODS

Materials. [^{32}P]NAD was the product of New England Nuclear Co.; ^{14}C-labeled nicotinic acid and [^{3}H]adenine were purchased from Amersham-Searle. DEAE paper (Whatman DE91) was purchased from H. Reeve Angel Co. The dT_{20} and poly(dA) used in the ligation assay were purchased from Sigma Chemical Company. Bacterial alkaline phosphatase was the product of Worthington Biochemical Company. Polynucleotide kinase was a gift from Pat Higgins, University of Alabama.

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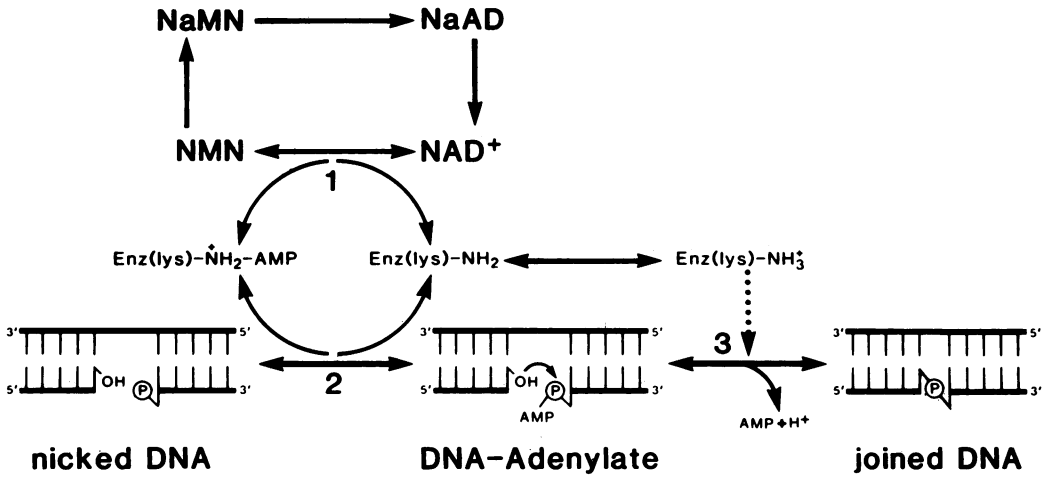

FIG. 1. DNA ligation and NAD turnover. The mechanism of the NAD-dependent DNA ligase is shown, with its relationship to the bacterial pyridine nucleotide cycle. DNA ligation in bacteria results in cleavage of NAD to NMN and AMP. The NMN is efficiently recycled back to NAD as shown, with nicotinic acid mononucleotide (NaMN) and nicotinic acid adenine dinucleotide (NaAD) as intermediates. DNA ligase = Enz(lys), indicating covalent state of Lys at active site. Reaction 3 is also catalyzed by ligase (dotted arrow).

Bacterial strains. All strains used in this study (Table 1) are *Salmonella typhimurium* strains derived from strain LT2. The construction of strain TT13616 will be described elsewhere (D. Hillyard and J. Roth, manuscript in preparation). The Mu d1 phage is the original construction of Casadaban and Cohen (1); Mu dJ is the transposition-defective mini-Mu d, Mu dI1734 (*lac* Km^r) of Castilho et al. (2).

Media. The E medium of Vogel and Bonner (18), supplemented with 0.2% glucose, was used as the minimal medium. Alternative carbon sources such as sorbitol were supplemented to 0.2% in E medium lacking citrate (4). Nutrient broth (Difco; 8 g/liter), with NaCl (5 g/liter), was used as the rich medium. Agar (Difco) was added at a final concentration of 1.5% for solid medium. Tetracycline and kanamycin sulfate were added to rich medium at final concentrations of 20 and 50 μg/ml, respectively, and to minimal medium at 10 and 125 μg/ml, respectively.

TABLE 1. List of strains

Strain	Genotype[a]	Source
TR1683	*ptsI*	Lab collection
TT7422	*dinB*::Mu d1(*lac* Ap); formerly GW1030	C. Kenyon (10)
TT10288	*hisD9953*::Mu dJ *hisA9944*::Mu d1	K. Hughes (9)
TT10543	LT2(pBR313/598/8/1b)	Lab collection
TT13253	Dup[(*pts hisH*) *Tn*10**(nadB pts*)]	This work
TT13254	Dup[(*pts hisH*) *Tn*10**(nadB lig-9*::Mu dJ *pts**)]	This work
TT13255	Dup[(*pts hisH*) *Tn*10**(nadB lig-7*::Mu dJ *pts*$^+$)]	This work
TT13256	Dup[(*pts hisH*) *Tn*10**(nadB lig-2*::Mu dJ *pts*$^+$)]	This work
TT13257	*lig-9*::Mu dJ(pBR313/598/8/1b)	This work
TT13258	*lig-7*::Mu dJ(pBR313/598/8/1b)	This work
TT13259	*lig-2*::Mu dJ(pBR313/598/8/1b)	This work
TT13260	Dup[(*pts hisH*) *Tn*10**(nadB zie-3639*::Mu dJ)]	This work
TT13261	Dup[(*pts hisH*) *Tn*10**(nadB zie-3640*::Mu dJ)]	This work
TT13262	Dup[(*pts hisH*) *Tn*10**(nadB zie-3641*::Mu dJ)]	This work
TT13616	Dup[(*hisH*) *Tn*10**(nadB)*]	D. Hillyard (8)
TT13895	Dup[*pts hisH*) *Tn*10**(nadB zie-3670*::Mu dJ)]	This work
TT13896	Dup[*pts hisH*) *Tn*10**(nadB zie-3671*::Mu dJ)]	This work

[a] Plasmid pBR313/598/8/1b carries the T4 ligase gene and an Amp^r determinant; asterisks designate Tn*10* at duplication joint point.

Transduction methods. For all transductional crosses, the high-frequency generalized transducing mutant of bacteriophage P22 (HT 105/1 *int*-201) was used (17). Cells (2×10^8) and phage (10^8 to 10^9) were spread on a nonselective plate and replica-printed to selective medium after 6 to 8 h of growth when selecting for resistance to kanamycin. Alternatively, cells and phage were spread directly on selective plates. Transductants were purified, and phage-free clones were isolated by streaking nonselectively onto green indicator plates (3). The phage-free clones were then checked for phage sensitivity by cross-streaking with P22 H5 (a clear-plaque mutant) phage.

Construction of Mu dJ pool. A pool of 300,000 random Mu dJ insertion mutants was made by using strain TT13616, which carries a tandem duplication including the *lig* gene and has a transposon Tn*10* (Tet^r) element between the two copies of the duplicated region. This pool was made by the transitory *cis* complementation method of Hughes and Roth (9). Phage P22 (10 ml) grown on strain TT10288 (carrying a Mu dJ and a Mu d1 insertion in the *his* operon) was mixed with 10 ml of an overnight culture of strain DH243, and 0.2 ml of the mixture was spread on nutrient broth plates containing tetracycline (20 μg/ml). Tetracycline was included to select for maintenance of the duplication in strain TT13616. The plates were incubated for 6 to 8 h at 37°C to allow expression of the Kan^r determinant of Mu dJ before being replica-printed to nutrient agar plates containing kanamycin sulfate (50 μg/ml). The final plates included EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (5 mM) to prevent further cycles of P22 growth. Liquid nutrient broth (2 ml) containing 5 mM EGTA was applied to each plate and used to suspend all colonies; in total, 30,000 colonies from 10 plates were pooled. Ten such independent pools were made from 100 plates. A P22 lysate was grown on each pool.

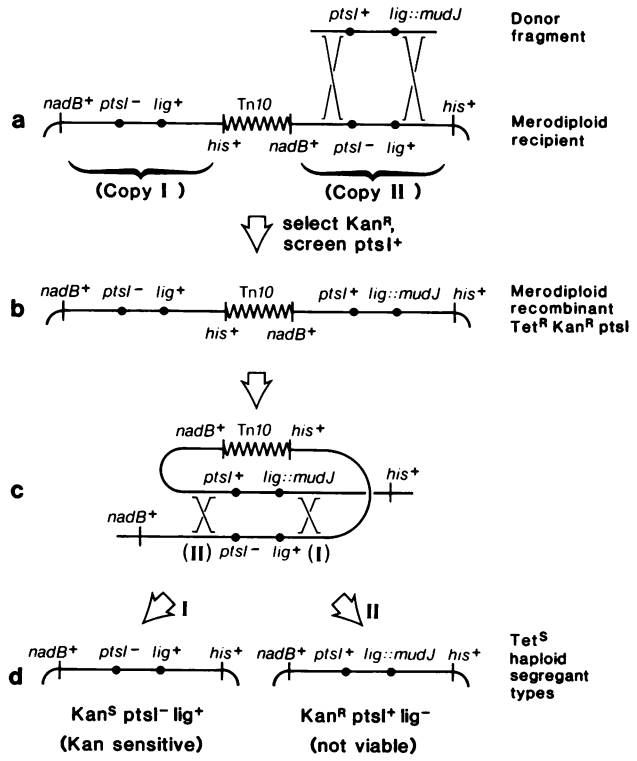

FIG. 2. Schematic representation of the strategy for making the DNA ligase insertion mutant, and the rationale for its segregation behavior. Recombination of a *lig*::Mu dJ transducing fragment obtained by screening for *pts*$^+$ is shown in the top diagram. The bottom diagrams illustrate segregation of the duplication to yield a tetracycline-sensitive haploid segregant type, which is a *lig* mutant and either kanamycin sensitive or kanamycin resistant but is not viable on kanamycin plates because of the *lig* mutation.

Isolation of Mu dJ insertions near *pts*. Phage grown on pools of random Mu dJ insertions were used to transduce a duplication strain with a mutation in both copies of the *ptsI* gene (TT13253); the *ptsI* mutation is linked to the *lig* gene (7). Selection was made for Kan^r (inheritance of a donor Mu dJ insertion), and recombinants were scored for the ability to grow on sorbitol (coinheritance of *pts*$^+$). Transductants which coinherited Kan^r and *pts*$^+$ are presumed to carry a Mu dJ insertion linked to the *pts* gene. This assumption was verified by subsequent tests.

Identification of insertions in essential genes within the duplication of strain TT13616. If insertions occur in one of the two copies of an essential gene in the duplicated region, such a strain would have a predictable phenotype. In the presence of kanamycin, which demands maintenance of the Mu dJ insertion, Tet^s Kan^r segregants would not be found (Fig. 2, and Results section), since they would lose the functional copy of the essential gene. A pool of random Mu dJ insertions (9) was made in a *lig*$^+$ duplication strain (TT13616). Insertions in the *lig* region were enriched by using linkage to the *ptsI* gene, which is closely linked to *lig* (90% cotransducible by P1 phage in *E. coli*) (7). The pools of Mu dJ insertions were used as donors to transduce a duplication strain (TT13253) in which both copies of the *lig* region have a *pts* mutant allele; Kan^r transductants were selected and *pts*$^+$ colonies were identified on the indicator media described above. Transductants which coinherit *pts*$^+$ and Mu dJ (Kan^r) have acquired a Mu dJ insertion within 31 kilobases (kb) of the *pts* gene, the length of a P22 transduced fragment. *lig* insertion mutants should be included in this set of *pts*$^+$ Kan^r transductants (Fig. 2b).

Since DNA ligase is an essential gene, *lig*::Mu dJ insertion mutants will be viable only if the *lig*$^+$ copy is present. Therefore, the *lig*$^+$/*lig*::Mu dJ duplication should give rise only to haploid segregants which are *lig*$^+$ (Kan^s); one would not expect *lig*::Mu dJ (Kan^r) haploid segregants (Fig. 2d). Therefore, duplication strains carrying a Mu dJ insertion near *pts* were screened for the nature of their haploid segregants. Strains were sought which only gave rise to Kan^s, never to Kan^r segregants. Segregants can be identified because the duplication used carries Tn*10* (Tet^r) at the joint point; segregants become Tet^s. To test for essential insertions, 150 of the Kan^r *pts*$^+$ transductants (described above) were grown in 2 ml of nutrient broth containing kanamycin (50 μg/ml); 150 colonies were tested. Diluted overnight cultures (0.1 ml, diluted 10^5- to 10^7-fold) were spread on kanamycin plates. Segregants (Tet^s) that had lost the duplication were identified by replica-printing to plates containing tetracycline (20 μg/ml) and kanamycin (50 μg/ml). Mutants that failed to show Tet^s segregants in the presence of kanamycin were saved. Eight of 150 strains yielded no Kan^r Tet^s segregants; all Tet^s segregants from these strains were Kan^s. These eight strains were candidates for carrying insertions in an essential gene. This strategy has been developed as a general screen for mutants with insertions in essential genes (Hillyard and Roth, manuscript in preparation).

DNA ligation assay. The assay method of Olivera and Lehman (15) was used. The phosphate moiety at the 5′ end of homopolymer dT_{20} was labeled with ^{32}P by using [α-^{32}P]ATP and polynucleotide kinase.

The ligase reaction mixture contained 2.5 μM [5′-^{32}P]dT_{20} (specific activity, 4×10^5 cpm/nmol), 3.5 μM poly(dA), 30 mM Tris (pH 8), 4 mM $MgCl_2$, 0.5 mM EDTA, 10 mM $(NH_4)_2SO_4$, and either 0.03 mM NAD or 0.05 mM ATP. When ATP was used as a cofactor, 5 mM dithiothreitol was added. The ligase reaction was started by adding 0.02 to 0.2 μg of crude cell extract and incubating at 37°C for 15 to 30 min. Reaction mixtures were placed in an 80°C water bath for 10 min to stop the reaction. Bacterial alkaline phosphatase (30 μl) was added and incubated at 65°C for 15 min to remove any unligated ^{32}P-labeled 5′ end. Portions (0.045 ml) were spotted on a GF/C filter and acid precipitated in 10% trichloroacetic acid solution. The precipitated radioactivity was counted.

β-Galactosidase assay. β-galactosidase activity was measured as described by Miller (13). To test the effect of UV light on expression of β-galactosidase in *lig*::Mu dJ insertions, 2.5 ml of exponentially growing cells were placed in an empty petri dish and UV irradiated (λ, 254 nm) for 15 to 40 s at a dose of 1.8 W/m^2. UV-treated cells were removed from the plate and allowed to grow for 90 min; the β-galactosidase activity of the culture was then measured.

The effect of mitomycin on *lig*::*lac* fusion strains was tested as follows. Mitomycin (0.8 μg/ml, final concentration) was added to an exponentially growing culture, and growth was continued for 150 min. A fraction of the culture was used to assay β-galactosidase activity. Additional mitomycin (8 μg/ml, final concentration) was added to the culture, and growth was continued for 55 min before β-galactosidase activity was measured again.

Covalent bond formation with [^{32}P]NAD. Crude cell extract (25 to 60 μg of protein) was added to a reaction mixture (0.1 ml) which contained 1 μM [^{32}P]NAD with 1.2 μCi of ^{32}P, 4

TABLE 2. Kan^r transductants with and without T4 ligase

Donor/recipient with Mu dJ insertion in essential gene	No. of Kan^r transductants		
	LT2[a]	TT13616 (*his–NadB* duplicated)	TT10543 (LT2 containing T4 ligase plasmid)
TT13254	1	150	100
TT13255	3	650	380
TT13256	1	250	420
TT13260	0	100	1
TT13261	0	150	1
TT13262	2	250	2
TT13895	2	500	1
TT13896[b]	300	250	310

[a] LT2 sometimes generates spontaneous duplications. Mu d insertion in *lig* gene could be transduced if spontaneous duplication included *lig* region.

[b] This strain seems to have Mu dJ near the joint point and not in an essential gene.

mM $MgCl_2$, and 40 mM Tris buffer (pH 8.0). After incubation for 12 min at 37°C, addition of the sodium dodecyl sulfate (SDS) sample buffer of Laemmli (11) followed by boiling for 2 min stopped the reaction.

Electrophoresis, staining, and destaining of polyacrylamide gels were done as described by Laemmli (11). Vacuum-dried gel was exposed to Kodak X-OMat AR film at −70°C.

Assay of NAD turnover. NAD turnover measurements were made as described by Hillyard et al. (8). Cells were grown in 20 ml of nutrient broth containing 0.13 μmol of [^{14}C]nicotinic acid (total radioactivity, 7.5 μCi). When the absorbance at 595 nm was between 0.4 and 0.8, ^{3}H-labeled adenine (25 nmol, 1 mCi) was added. The culture was allowed to grow for 0.5 to 1.0 h more in the presence of adenine. Cells were harvested and diluted in identical medium without any radioactivity. The sample culture which was drawn at various times was analyzed as described previously. The ^{3}H/^{14}C ratio in the NAD peak was calculated and normalized to the ratio at time zero.

RESULTS

Isolation of Mu dJ insertions in the ligase structural gene. Since DNA ligase is an essential enzyme, an insertion mutation, which abolishes enzymatic function, would ordinarily be lethal. However, the insertion has been isolated on an *S. typhimurium* strain carrying a tandem duplication of the DNA ligase gene. One copy of the *lig* gene is disrupted with a Mu dJ insertion, and the wild-type copy supplies DNA ligase function. Since a fully functional *lig* gene is present, there is no restriction on the nature of the *lig* insertion mutation. In fact, it is identified by virtue of its lethality when haploid. The basic strategy for constructing the *lig* insertion is shown in Fig. 2 and described in Materials and Methods; eight candidate insertion mutants were obtained.

The eight insertions were further characterized by transductional tests. An attempt was made to transduce each Mu dJ insertion both into haploid recipients (LT2) and into strain TT13616, which is diploid for the *lig* region of the chromosome (Table 2). Seven of the eight putative essential insertions were transduced at high efficiency into duplication strain TT13616, whereas only rare transductants were found when haploid strain LT2 was the recipient (these few are presumed to be due to spontaneous duplications in the recipient population). In contrast, Mu dJ insertions from one strain could be transduced at similar frequencies into both LT2 and the duplication strain (data not shown). These results suggest that 7 of the 150 *pts*-linked Mu dJ insertion mutations can only persist when complemented by wild-type functions. Each of these insertions is therefore presumed to disrupt an essential gene within the duplicated chromosomal segment and is a candidate for carrying an insertion in DNA ligase.

Complementation with T4 DNA ligase plasmids. In principle, the scheme above should yield Mu dJ insertions in the DNA ligase gene at quite a high frequency, since *lig* and *pts* are closely linked. However, any other essential gene within the duplication that can be cotransduced with the *pts* marker would behave similarly in segregation tests.

The availability of a plasmid carrying the gene for T4 DNA ligase (20) provides a possible complementation assay for determining which (if any) of the *pts*-linked Mu dJ insertions disrupt the *Salmonella* ligase gene. To determine whether the lethal phenotype of any of the seven Mu dJ insertions could be complemented by T4 ligase activity, we attempted to transduce each Mu dJ insertion into a haploid *Salmonella* strain (LT2) carrying the T4 *lig* plasmid. If the T4 enzyme can substitute for host NAD-dependent ligase activity, then transduction of this strain by *lig*::Mu dJ insertions should yield viable colonies at high frequency. The data for the different mutants are shown in Table 2. It is clear that the mutants fell into two classes; three could transduce LT2 containing the T4 plasmid, and four could not. Those results therefore identify a class of three potential *lig*::Mu dJ insertions (in duplication strains TT13254, T13255, and TT13256); after transduction into the T4 ligase plasmid-bearing strains, corresponding haploid strains presumably bearing only the *lig*::Mu dJ insertion copy of the *lig* gene in the chromosome were saved (strains TT13257 to TT13259).

The duplication strains carrying potential *lig* insertions were also tested for their ability to segregate Kan^r Tet^s segregants when the T4 plasmid was present. The plasmid, which carries an ampicillin resistance marker, was transferred to duplication strains carrying the seven lethal Mu dJ insertions in the *lig* region, selecting for Ap^r and Kn^r. For strains TT13254 to TT13256, the loss of Tet^r (by duplication segregation) was very rapid, and tetracycline-sensitive segregants were the majority population even during the preincubation period. In contrast, for strains TT13260 to TT13262, presumably containing an essential insertion not in the DNA ligase gene, very few tetracycline-sensitive transductants were found. These results support the conclusion that three strains with putative insertions carry *lig*::Mu dJ insertions and can be complemented by the plasmid carrying T4 ligase; four strains (TT13260 to TT13262) presumably carry insertions in another essential gene(s) linked to the *ptsI* locus.

Biochemical assays. Haploid strains carrying a *lig*::Mu d insertion in the chromosome and a T4 *lig* plasmid should show no host ligase activity (NAD dependent); extracts of these strains should show only the ATP-dependent T4 ligase activity. This prediction was tested by biochemical assays.

Since the host *Salmonella* DNA ligase requires NAD while the complementing T4 DNA ligase requires ATP, it is possible to assay the two enzymes independently in crude extracts. Such assays are shown in Table 3. The results clearly demonstrate that the original duplication strain (TT13256) had the NAD-requiring ligase and no ATP-requiring ligase, while LT2 with the plasmid (TT10543) had both types of activity. However, haploid *lig*::Mu dJ strains carrying the T4 *lig* plasmid (TT13259) showed only the

TABLE 3. DNA ligation assay with different cofactors[a]

Strain (description)	Cofactor	End ligated (pmol/min per mg of protein)
TT13259 (*lig*::Mu dJ, T4 ligase plasmid)	NAD	<1.0
	ATP	560
	NAD + ATP	540
TT10543 (LT2 with T4 ligase plasmid)	NAD	240
	ATP	340
	NAD + ATP	430
TT13256 (*lig*$^+$/*lig*::Mu dJ duplication strain)	NAD	80
	ATP	<1.0

[a] DNA ligation assays were carried out with the assay of Olivera and Lehman (15) as described in the text.

ATP-dependent DNA ligase activity; no NAD-dependent DNA ligase was detected.

NAD-dependent DNA ligase can also be detected in crude extracts by an autoradiographic assay. DNA ligase is detected as a labeled band after incubation of crude extracts with [^{32}P]NAD, followed by SDS gel electrophoresis and autoradiography. The DNA ligase band is the major protein labeled by [^{32}P]NAD, with a molecular weight above 30,000 (Fig. 3). This labeled adduct is a consequence of ligase forming a covalent enzyme-adenylate intermediate (12). This labeled band was not seen when the extract was preincubated with a large excess of NMN (data not shown); the enzyme-adenylate intermediate of DNA ligase would be discharged into free enzyme and NAD in the presence of a large excess of NMN (Fig. 1).

As shown in Fig. 3, neither one of the putative *lig*::Mu dJ insertion mutants tested showed the bacterial DNA ligase band. The absence of labeling of DNA ligase protein by [^{32}P]NAD is additional evidence for the absence of NAD-dependent ligase. The autoradiographic assay results are consistent with the mutants being defective not only in DNA ligation, but also in the first catalytic step of the ligation reaction, the formation of enzyme adenylate.

Physiological studies of *S. typhimurium* mutants lacking NAD-dependent DNA ligase. The three *lig* mutant strains TT13257, TT13258, and TT13259 therefore appear to be devoid of NAD-dependent DNA ligase activity but are viable by virtue of the T4 ATP-dependent ligase. These strains exhibited no unusual growth phenotype. The generation time and general physiology appeared to be normal.

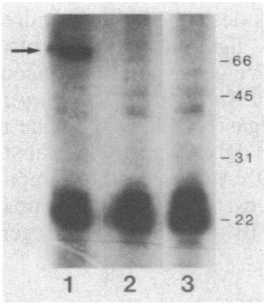

FIG. 3. Polyacrylamide gel electrophoresis of DNA ligase enzyme adenylate intermediate in wild-type and mutant strains. Two lanes show an analysis of the *lig*$^+$ and the T4 ligase-substituted strains. Crude extracts were incubated with [^{32}P]NAD as described in the text, and the mixture was then boiled in SDS and run on polyacrylamide gel electrophoresis. Lane 1, *lig*$^+$ strain TT10543; the arrow indicates the band corresponding to *Salmonella* DNA ligase. Lanes 2 and 3, Mutant strains TT13257 and TT13259, respectively; the bacterial DNA ligase band is missing in these strains. Size markers are in kilodaltons.

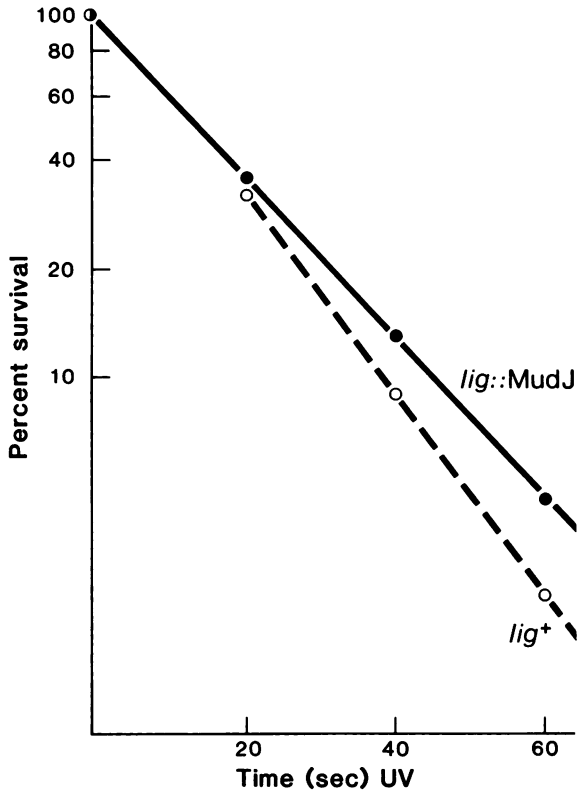

FIG. 4. Killing curve by UV light. Cells were irradiated at 1.8 W/m^2 as described in the text for the times indicated, and the cells were plated on nutrient broth and counted.

We also examined a number of physiological conditions under which a higher demand for DNA ligation would be expected, i.e., treatment with UV light and DNA-alkylating agents. A UV survival curve of the *lig* mutant strains was determined. These results are shown in Fig. 4. It can be seen that the absence of an NAD-dependent DNA ligase did not cause any significant difference in killing compared with the wild-type strain. The strains were not detectably mitomycin sensitive, nor were they differentially sensitive to UV irradiation after starvation (results not shown). The relatively normal physiology of the three mutant strains, which do not contain the normal host NAD-dependent DNA ligase, suggests that T4 DNA ligase can completely substitute for the *Salmonella typhimurium* NAD-dependent DNA ligase in both replication and DNA repair.

Regulation of the *lig* gene. All of the mutants carrying Mu dJ insertions into the ligase gene that were obtained formed blue colonies on medium containing the chromogenic β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Therefore, β-galactosidase expression is presumably under the control of the DNA ligase promoter in these strains (1). Direct evidence from DNA sequencing that the *lig* promoter controls β-galactosidase gene transcription has been obtained for the *lig-2*::Mu dJ allele. Two sequencing runs were carried out with each Mu end used as a primer. The sequencing results are consistent with the insertion occurring between amino acids 4 and 12 from the N-terminus of the *lig* structural gene; the insertion could not be localized

TABLE 4. β-Galactosidase assay of Mu dJ insertion in *lig*

Stimulus	Treatment	β-Galactosidase sp act[a] (nmol/min per ml)	
		TT7422[b] (*dinB*::Mu d1)	TT13259 (*lig*::Mu dJ)
UV irradiation	None	7.6	46.0
	27 J/m^2	14.0	40.0
	72 J/m^2	26.0	36.0
H_2O_2	None	8.9	62.0
	15 min, 6 mM	9.4	55.0
	120 min, 6 mM	14.0	47.0
Mitomycin	None	4.0	46.0
	15 min, 8 μg/ml	28.0	44.0
	120 min, 2 μg/ml	52.0	53.0

[a] Specific activity expressed as nanomoles per minute (using 1 ml of cells at OD_{650}).

[b] In this strain, β-galactosidase transcription is dependent on the *dinB* promoter, which is induced under SOS conditions.

more precisely because the sequence near the primers could not be determined unambiguously. However, since *lig* sequence was obtained from both ends of Mu, the insertion was clearly in the *lig* gene.

We therefore assayed β-galactosidase activity in strains with *lig*::Mu dJ fusions grown under different physiological conditions. Under conditions which clearly induced *dinB*, a gene of the SOS regulon (10), there was no change in β-galactosidase activity in the *lig*::Mu dJ strain (Table 4). In addition, treatment with hydrogen peroxide did not cause any change in transcription of the DNA ligase gene. The most straightforward interpretation of the results is that the level of transcription of the DNA ligase gene does not change under these different physiological conditions.

NAD turnover. The major pyridine nucleotide cycle in *E. coli* involves the cleavage of NAD to NMN and AMP (8). DNA ligase is an obvious intracellular activity which can cause such an NAD cleavage event, and it was suggested that most NAD turnover in enteric bacteria may be due to DNA ligation. This hypothesis predicts that *Salmonella* mutants which lack NAD-dependent DNA ligase should have severely decreased NAD turnover.

The results of NAD turnover experiments carried out on LT2 as well as two *Salmonella typhimurium* mutants lacking NAD-dependent DNA ligase are shown in Fig. 5 and Table 5. NAD turnover was measured as described by Hillyard et al. (8). It can be seen from the results in Fig. 5 that wild-type and mutant strains had indistinguishable rates of NAD turnover. These results indicate that NAD-dependent DNA ligation does not make a major contribution to NAD turnover in exponentially growing *Salmonella typhimurium*.

Another enzyme known to break down NAD in *Salmonella typhimurium* is NAD pyrophosphatase. Although this enzyme appears to be located on the membrane facing outside the cell (5, 16), this activity is sufficiently potent to make it conceivable that as the enzyme is transferred to the membrane, it cleaves significant levels of intracellular NAD. Recently, insertion and deletion mutations have been isolated at the newly described *pnuE* locus; these mutants lack detectable NAD pyrophosphatase activity (16). In order to determine whether NAD pyrophosphatase is involved in NAD turnover, a double mutant lacking both the NAD-dependent DNA ligase and the NAD pyrophosphatase was constructed, and the NAD turnover rate was determined. It

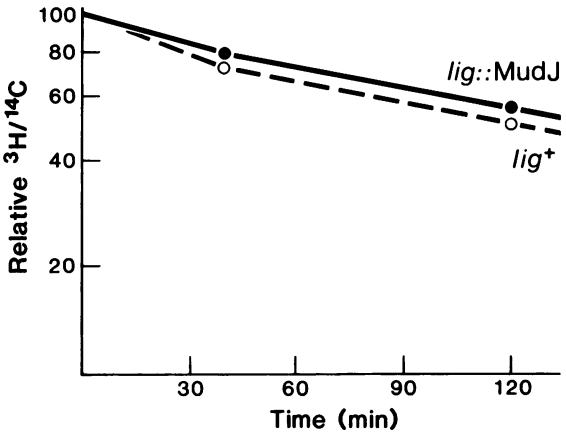

FIG. 5. NAD breakdown in a *lig*$^+$ strain versus *lig*::Mu dJ strain. The decay rate of NAD based on chromatographic analysis of radiolabel in the intracellular NAD peak at the time of labeling (100%) and after the chase times indicated is shown. In this experiment, [^{14}C]nicotinamide and [^{3}H]adenine were used as label, and the ratio of ^{14}C to ^{3}H is plotted as a function of time. It is clear that radioactivity in mutant and wild-type strains did not decay at significantly different rates.

was found that NAD turnover in the double mutant was not significantly different from that in a wild-type strain.

Among all the metabolic conditions tested, only one factor seemed to significantly change the rate of intracellular NAD turnover in *Salmonella typhimurium*: anaerobic growth. Strains grown aerobically had a much faster NAD turnover time than strains growing anaerobically. Since the generation time under anaerobic conditions was longer than the generation time under standard aerobic conditions, we also examined the effect of generation time. Surprisingly, generation time had relatively little effect as long as the culture was growing aerobically (Table 5). NAD turnover was approximately four times faster at all generation times than the turnover rate under anaerobic conditions. These results suggest that a significant fraction of NAD turnover is linked to some aspect of aerobic metabolism, independent of the growth rate.

DISCUSSION

The experiments described above address the physiology of bacterial DNA ligation and the intracellular pyridine nucleotide cycle. We successfully replaced the NAD-dependent DNA ligase of *Salmonella typhimurium* with the ATP-dependent DNA ligase of phage T4. The results lead to the conclusion that T4 DNA ligase can substitute for the host NAD-dependent DNA ligase without apparent adverse metabolic consequences. The T4 ligase-substituted strain appears to be normal by all criteria tested: generation time, UV

TABLE 5. NAD turnover rate[a]

Growth conditions	Doubling time (min)	Half-life of NAD (min)
Aerobic, glucose medium	40	90
Aerobic, glycerol medium	65	90
Anaerobic, glucose medium	65	330

[a] The half-life of NAD was determined by the method of Hillyard et al. (8); also see Fig. 4 and text. Wild-type *Salmonella typhimurium* LT2 was used in these experiments.

sensitivity, and mitomycin sensitivity. Since there is no major deficit in either DNA replication or repair, either direct interactions between the host DNA ligase and other host replication or repair enzymes are unimportant or the T4 enzyme has the same interactions. In addition, substitution of an ATP-dependent for an NAD-dependent enzyme does not cause detectable metabolic aberrations.

We have implicitly assumed above that each insertion mutant is null in DNA ligase. We believe that NAD-dependent DNA ligase activity is indeed missing from these strains. This conclusion is based on a variety of evidence. (i) Direct enzyme assays of DNA ligase activity: mutant strains showed no DNA ligase activity when NAD was provided as the cofactor, but clearly showed detectable ATP-dependent DNA ligase. (ii) Assays for the formation of the enzyme-adenylate intermediate: in mutant strains, no covalently linked [^{32}P]AMP-labeled ligase band was seen. (iii) Sequencing: sequencing data indicate that the *lig-2*::Mu dJ insertion occurred very near the N-terminus of the gene.

Despite the evidence above that the DNA ligase of *Salmonella typhimurium* has been completely replaced (and complemented) by the T4 ligase, we cannot totally eliminate the possibility that some (unknown) function of DNA ligase is still being carried out in these extracts. However, we should emphasize that three independently isolated insertion strains can be complemented by T4 DNA ligase. The screen used to identify *lig*::Mu dJ insertion-carrying strains did not require complementation by T4 ligase; rather, we identified duplication strains with a diploid complement of *lig* genes which failed to segregate. Since the initial insertion mutagenesis resulted in strains with a wild-type copy of the bacterial *lig* gene, it was presumably nonselective with respect to the type of *lig* mutants that might be recovered. Thus, we think it unlikely that a special class of *lig* mutants was preselected; the most straightforward interpretation is that these insertion mutants are totally devoid of DNA ligase activity.

Our results raise the question of why most if not all bacteria use NAD as a cofactor for DNA ligation. The selective factors that lead to this unusual cofactor requirement are clearly more subtle (or operate on a more long-term evolutionary time scale) than can be detected by the present investigation.

We previously hypothesized that DNA ligation might be the major metabolic phenomenon responsible for initiating intracellular pyridine nucleotide cycles in bacteria. In this report, we tested that prediction by assessing the rate of NAD turnover when an ATP-dependent ligase was substituted for the host NAD-dependent ligase. Our expectation was that the rate of NAD turnover would be severely decreased; instead, we found that NAD turnover was not measurably affected by the substitution of the ATP-dependent enzyme for the host ligase. This indicates that DNA ligation makes only a minor contribution to initiating the pyridine nucleotide cycle of *Salmonella typhimurium*. Experiments with a *lig pnuE* double mutant strain indicate that NAD pyrophosphatase also plays a minor role in the in vivo cycle (although the latter enzyme is responsible for most NAD breakdown observed in vitro in crude extracts of *Salmonella* strains).

While the true identity of the event that initiates the pyridine cycle remains unknown, there are several specific experimental leads to explore.

(i) We recently detected a second major covalent NAD-dependent protein modification unrelated to DNA ligase. The proteins modified and the function of this covalent modification are unknown. Under certain conditions (such as in Fig. 4), the extent of labeling of these small protein bands by NAD can exceed labeling of DNA ligase. These heretofore-undefined NAD-linked protein modification reactions could be central to the bacterial pyridine nucleotide cycle.

(ii) A second possible initiating event would be α-pyridine nucleotide degradation. The α-pyridine nucleotides should be spontaneously and continuously formed from the natural β-pyridine nucleotides in vivo. It is believed that the isomerization of β-NAD to α-NAD probably proceeds mainly when the nucleotides are in the reduced form under physiological conditions. The metabolic fate of any α-NAD or α-NADH which is formed remains unknown. If α-NAD derivatives were rapidly broken down, this might account for some fraction of NAD turnover. Such a hypothesis needs to be tested experimentally.

One experimental result obtained from this study suggests a third possibility for the metabolic origin of NAD turnover (the three possibilities are not mutually exclusive). This is the discovery that at two very different generation times, the rate of NAD breakdown was relatively constant under aerobic conditions but was drastically decreased under anaerobic conditions. Perhaps NAD or NADH plays an additional role, besides serving as a cofactor for DNA ligase, in protecting the cell from oxygen damage to membranes or DNA. In such a role, the NAD (or NADH) molecule would have to be broken down and resynthesized. We have *Salmonella* strains which lack the two major enzymes (NAD pyrophosphatase and DNA ligase) which break down NAD in vitro. We are presently examining extracts of this strain to identify new biochemical events which lead to NAD breakdown.

ACKNOWLEDGMENTS

We acknowledge the technical contributions of Maren Marsh and Pauline Cordray; the T4 ligase-containing plasmid strain was a gift of Noreen Murray.

This work was supported by Public Health Service grant GM-25654 from the National Institute of General Medical Sciences.

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