fusB is an allele of nadD, encoding nicotinate mononucleotide adenyltransferase in Escherichia coli

Martin Stancek, Leif A. Isaksson and Monica Rydén-Aulin

Department of Microbiology, Stockholm University, S-106 91 Stockholm, Sweden

Isolation of the temperature-sensitive Escherichia coli mutant 72c has been described previously. The mutant allele was named fusB and causes a pleiotropic phenotype, the most striking features of which, besides temperature sensitivity, are the inability to grow on synthetic medium and supersensitivity to trimethoprim, an antibiotic that inhibits the C1 metabolism. This work shows that the fusB mutation is a frameshift mutation in the nadD gene that encodes nicotinate mononucleotide adenyltransferase. The frameshift leads to a change of the last 10 amino acids and an addition of 17 amino acids. This lesion, renamed nadD72, leads to very little NAD+ and NADPH synthesis at the permissive temperature and essentially no synthesis at the non-permissive temperature. As a comparison, a new mutation in the nadD gene, with an amino acid change in the ATP-binding site, has been isolated. Its NAD+ synthesis is decreased at 30 °C but the level is still sufficient to support normal growth. At 42 °C, NAD+ synthesis is reduced further, which leads to temperature sensitivity on minimal medium. This mutation was designated nadD74. Thus, a small decrease in NAD+ levels affects ability to grow on minimal medium at 42 °C, while a large decrease leads to a more pleiotropic phenotype.

Most of the metabolites produced during synthesis of NAD+ can either be taken up from the medium or be obtained by degrading NAD+. The only exceptions to this are NAMN and NAAD (Fig. 1) (Foster & Moat, 1980). Thus, deletion of any of the genes encoding enzymes leading up to NAMN causes auxotrophy but can be bypassed by processing of exogenous precursors. However, there are no known alternatives for the two last enzymic steps from NAMN to NAD+ in bacteria, with the possible exception of Haemophilus influenzae (Kurnasov et al., 2002). Accordingly, no auxotrophs can arise since the required metabolites are not taken up by the cell. However, the essentiality of the nadD gene has been disputed. On the one hand, transposon insertions have been isolated in nadD; on the other hand, the same authors claim that the entire gene cannot be deleted (Gerdes et al., 2002). It is possible that the transposon insertions affect a non-essential part of the gene.

The isolation of the temperature-sensitive (Ts) mutant 72c has been described previously (Isaksson et al., 1977). The mutant strain has a pleiotropic phenotype, the most striking

INTRODUCTION

The molecules NAD+ and NADP+ are essential for all living systems. On the one hand, they function as cofactors in oxidation–reduction reactions; on the other hand, NAD+ serves as a substrate in some enzymic reactions. The total concentration of NAD+, NADH, NADP+ and NADPH during exponential growth of Salmonella enterica serovar Typhimurium (S. typhimurium) is 1 mM, of which 0.8 mM is NAD+ (Bochner & Ames, 1982). In Escherichia coli and S. typhimurium there is a de novo pathway for synthesis as well as salvage pathways (Fig. 1).

De novo synthesis in bacteria starts with synthesis of quinolinate from aspartate, which is catalysed by a Nada–nadB complex (Suzuki et al., 1973). Thereafter, quinolinate is decarboxylated and converted to nicotinic acid mononucleotide (NAMN) by quinolinate phosphoribosyltransferase encoded by the nadC gene (Magni et al., 1999). Subsequently, NAMN condenses with ATP to form nicotinic acid adenine dinucleotide (NAAD), which in turn is amidated to form NAD+ (Fig. 1). NADN adenyllyltransferase (NAMNAT) (EC 2.7.7.18) and NAD+ synthetase catalyse the latter two reactions, respectively. The enzymes are encoded by the nadD and nadE genes. The nadD gene was first identified in S. typhimurium through isolation of mutants resistant to 6-aminonicotinamide (Hughes et al., 1983b). Recently, the nadD gene was identified in E. coli (Mehl et al., 2000). In addition to de novo synthesis from quinolinate, NAMN can be formed from two different salvage pathways (Fig. 1).

Abbreviations: 2D, two-dimensional; NAMN, nicotinic acid mononucleotide; S. typhimurium, Salmonella enterica serovar Typhimurium; Ts, temperature-sensitive.
features of which are its sensitivity to temperature, its inability to grow on minimal medium, its resistance to fusidic acid (the characteristic that gave the mutation its original name) and its supersensitivity to trimethoprim, an antibiotic that inhibits C1 metabolism. The mutation was mapped and named \textit{fusB} (Isaksson & Takata, 1978). In this report, we show that the \textit{fusB} mutation is a \textit{21} frameshift in the \textit{nadD} gene (\textit{nadD72}). That leads to essentially no NAD$^+$ synthesis. To extend the study of the enzyme, we have isolated another \textit{nadD} mutant with an intermediate production of NAD$^+$ (\textit{nadD74}). Here, we characterize and compare the two mutants and discuss their implications for NAD$^+$ synthesis.

**METHODS**

**Bacterial strains and media.** All strains used in this work are listed in Table 1. \textit{\lambda}-Phage clones were propagated as described by Chuang et al. (1993). NZC Broth for propagation of \textit{\lambda}-phage was purchased from Life Technologies. LB medium and M9 minimal medium were prepared according to Miller (1972). The concentrations of antibiotics were as follows: 2 \textmu g chloramphenicol ml$^{-1}$ in minimal plates and 4 \textmu g ml$^{-1}$ in LB plates, 10 \textmu g trimethoprim ml$^{-1}$, 400 \textmu g fusidic acid ml$^{-1}$, 25 \textmu g tetracycline ml$^{-1}$ and 200 \textmu g ampicillin ml$^{-1}$.

**DNA manipulations and DNA sequencing.** Standard recombination techniques were used (Sambrook et al., 1989). \textit{E. coli} strain XL-1 Blue was used as recipient for cloned DNA. Restriction and modification enzymes were purchased from New England Biolabs, Amersham Pharmacia Biotech or Life Technologies. Oligonucleotides were made by Cybergene AB. Plasmid DNA was purified with the Wizard Plus SV DNA miniprep kit (Promega). DNA fragments were separated by agarose gel electrophoresis, excised and purified using the Qiaex II Gel Extraction Kit (Qiagen). For the cloning of PCR fragments, the pMOSBlue blunt-ended cloning kit (Amersham Pharmacia Biotech) was used. For DNA sequencing, a BigDye sequencing kit from Applied Biosystems was used. DNA sequencing gels were run and analysed by Cybergene AB or Kiseq at the Karolinska Institute.

**Two-dimensional TLC (2D-TLC).** For the growth of bacteria, a modified protocol was used (Bochner & Ames, 1982). Because of the
inability of the nadD72 mutant cells to grow on synthetic media, they were grown on LB plates overnight. The resulting cells were resuspended in 0.9% NaCl (OD_{550} value similar to that of an overnight culture) and diluted 100-fold into medium containing 0.1% times LB and 10% MOPS medium (Bochner & Ames, 1982) supplemented with 0.4% glucose and proline. \(^{[2]}\)PPhosphoric acid (100 \(\mu\)Ci ml\(^{-1}\), 3-7 MBq ml\(^{-1}\); Amersham Pharmacia Biotech) was added concomitantly with the cells when grown at 30°C only, or after shift to non-permissive temperature. In all experiments, an identical parallel culture without radioactive phosphoric acid was inoculated and the growth of this culture was monitored. In the medium used, strain R18 enters stationary phase at an OD_{550} value of 0.5, so cultures were harvested when they reached an OD_{550} value of 0.25. When applicable, the cultures were shifted to 42°C when they had reached an OD_{550} value of 0.25 and incubated for another 30 min.

The preparation of samples for TLC was as described by Bochner & Ames (1982). PEI (polyethyleneimine)--cellulose TLC sheets (Macherey-Nagel) were purchased from Tamro. NAD\(^+\), NADPH and NADP\(^+\) were purchased from Sigma Aldrich.

### RESULTS AND DISCUSSION

#### Identification and characterization of the fusB mutation in strain 72c

The fusB mutation has previously been mapped to approximately 14 min on the E. coli linkage map between the genes lip and glnV (supE). We wanted to determine the exact position of the mutation.

We started with a \(\lambda\)-phage library made on E. coli strain MG1655 (Chuang et al., 1993). Based on the mapping data, two phage clones were chosen, DD139, which contains the region between the rna and ybeF genes, and DD142, which contains the region between the lipA and ybeL genes. The two clones overlap by approximately 2 kb. We used lysates of these two clones in a marker rescue experiment. Only clone DD142 gave a significant number of Ts\(^+\) colonies. We concluded that the mutation must lie within the area covered by clone DD142, minus the overlap with the DD139 clone (not shown).

To identify which specific gene is mutated, the region covered by the DD142 fragment was divided into four parts that were amplified by PCR (for primers see Table 2). Each of the four fragments was cloned into pMOSblue and transformed into strains 72c and P90A5c. A complementation test was performed on the assumption that the wild-type gene would be dominant. Only the fragment covering the region from rlpB to ybeA promoted weak growth for strain 72c at 42°C. The fragment was subcloned and re-transformed into 72c. We found that a wild-type copy of nadD could complement the Ts phenotype weakly. The nadD gene is located at 14.3 min on the E. coli map, and is most likely part of an operon.

To sequence the mutation, the nadD gene from strains 72c and P90A5c was amplified by using primers pYbeN1 and pYbeN2 (Table 2) and the amplified DNA was used as a template for sequencing with the same primers. A deletion of base T-613 in the nadD gene from strain 72c was found. This causes a \(-1\) frameshift, which leads to bypass of the nadD stop codon and gives rise to a longer protein. The wild-type nadD gene product is 213 aa long and the protein in the 72c mutant is 230 aa long. Hence, we conclude that the fusB mutation in strain 72c affects the nadD gene. The mutant allele was re-designated nadD72.

The nadD72 mutation was transferred to a new genetic background (CSH142) to test whether the pleiotropic phenotype is caused by this mutation only. Transposon zbe280::Tn10 was transduced from strain CAG12077 to strain 72c. A Ts clone was named MW3. A lysate made on MW3 was transduced to CSH142, selecting for tetracycline resistance and screening for the Ts phenotype. The presence of the nadD72 mutation was verified by sequencing and the isolate was named RI8; wild-type clone RI10 was also kept.

The phenotypic behaviour of strains RI10 and RI8 was tested by viable count under conditions used for strains 72c and P90A5c (Isaksson & Takata, 1978). Strain RI8 behaves just like 72c with one exception – it is not stimulated by sublethal amounts of chloramphenicol (Table 3). From this we conclude that all tested phenotypes observed for 72c, with the exception of chloramphenicol stimulation, are directly caused by the nadD72 mutation.

The phenotype of the Ts phenotype in strain 72c by wild-type NadD was only partial. To determine whether this observation can be explained by impaired expression of the downstream gene cobC, we extended the complementation analysis. Three DNA fragments were amplified and

### Table 2. Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer*</th>
<th>DNA sequence (5’–3’)</th>
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<tbody>
<tr>
<td>pMS72.1M</td>
<td>CCGGCCGTCGAGCAAGGCTTCGC</td>
</tr>
<tr>
<td>pMS72.2M</td>
<td>GAGCGCTGCACTTCACATTTCAG</td>
</tr>
<tr>
<td>pMS72.3M</td>
<td>TGGAAATAAAAAAGTGCCCTACTA</td>
</tr>
<tr>
<td>pMS72.4</td>
<td>CTACCGCGAACGCGGCTTATTT</td>
</tr>
<tr>
<td>pMS72.5</td>
<td>GGAGACGACCCACCAACCATCCTT</td>
</tr>
<tr>
<td>pMS72.6M</td>
<td>TCATTCTATCTGGTAGATAGTC</td>
</tr>
<tr>
<td>pMS72.7</td>
<td>TTGTGTATCTCTGGCGGTTTAA</td>
</tr>
<tr>
<td>pMS72.8M</td>
<td>TCCTAATGCAGCAATTGCCCAGGTTATAT</td>
</tr>
<tr>
<td>pYbeN1</td>
<td>ATGCGAGATTTTGCGCTGTCAATACCGCG</td>
</tr>
<tr>
<td>pYbeN2</td>
<td>AGACGCGATTTGCAAGCGCGCGACCCAG</td>
</tr>
<tr>
<td>pCob1u</td>
<td>GGACAAATGGTCGCCGAGATCGGTT</td>
</tr>
<tr>
<td>pCob1d</td>
<td>ATTTATCTGGCGTAAACGCGGCCT</td>
</tr>
<tr>
<td>pGR1u</td>
<td>GCTTTGCGGATCTTCTGATG</td>
</tr>
<tr>
<td>pGR2d</td>
<td>TACGGCAGCTGAGGGTTTATCGCT</td>
</tr>
</tbody>
</table>

*The pMS series of primers was used for initial complementation tests.
Table 3. Growth of strains RI10, RI8 and RI12 on different media and antibiotics

Each value is the mean of 5–6 independent measurements ± SEM.

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Growth of strain (c.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RI10 (wild-type)</td>
</tr>
<tr>
<td>LB 30 °C †</td>
<td>1</td>
</tr>
<tr>
<td>LB 30 °C, Fus</td>
<td>&lt; 10⁻³</td>
</tr>
<tr>
<td>LB 30 °C, Tri</td>
<td>0·92 ± 0·05</td>
</tr>
<tr>
<td>LB 30 °C, Cm</td>
<td>0·84 ± 0·1</td>
</tr>
<tr>
<td>LB 42 °C</td>
<td>1·1 ± 0·1</td>
</tr>
<tr>
<td>LB 42 °C, Cm</td>
<td>0·91 ± 0·07</td>
</tr>
<tr>
<td>Min. 30 °C</td>
<td>0·96 ± 0·06</td>
</tr>
<tr>
<td>Min. 30 °C, Cm</td>
<td>0·90 ± 0·07</td>
</tr>
<tr>
<td>Min. 42 °C</td>
<td>0·76 ± 0·04</td>
</tr>
<tr>
<td>Min. 42 °C, Cm</td>
<td>0·54 ± 0·04</td>
</tr>
</tbody>
</table>

*Fus, 400 μg fusidic acid ml⁻¹; Tri, 10 μg trimethoprim ml⁻¹; Cm, 2 μg chloramphenicol ml⁻¹ in M9 minimal (Min.) plates and 4 μg ml⁻¹ in LB plates.
† Growth on LB plates at 30 °C was normalized to one.

cloned: the first fragment with only the cobC gene, the second with both cobC and nadD, and the third with only the nadD gene. The two clones with the nadD gene complemented both the Ts phenotype and the inability to grow on minimal medium to the same extent (data not shown). Thus, less expression from cobC is not the cause for the partial complementation.

Analysis of intracellular nucleotide pools by 2D-TLC

Since NAD⁺ is a molecule essential to the living cell, we wanted to analyse the effect of the nadD72 mutation on NAD⁺ synthesis. To do this, 2D-TLC was used to analyse intracellular nucleotide pools in RI10 (wild-type) and RI8 (nadD72) (Bochner & Ames, 1982). Quantifications of these chromatograms are difficult since the background varies, not only from sheet to sheet, but also within one sheet. Our statements about changes are only qualitative and are based on an overall inspection of the intensity of the spots. When chromatograms on nucleotides from RI10 (wild-type) and RI8 (nadD72) grown at 30 °C are compared, it is clear that two spots are greatly reduced in the nadD72 mutant sample (Fig. 2c) as compared to the parental strain RI10 (Fig. 2a). The chromatographic pattern is similar to what has been published previously (Bochner & Ames, 1982) and, according to their identifications, the missing spots should be NAD⁺ and NADPH. Both spots reappear in an extract from the mutant strain containing a plasmid with the nadD wild-type gene (Fig. 3). Interestingly, the amount of NADP⁺ is not greatly affected. This result is in accordance with the observation that when an E. coli strain, auxotrophic for nicotinamide, is starved for nicotinamide, the NADP⁺:NAD⁺ ratio increases from 0·3 to 2 (Lundquist & Olvera, 1973). The reason for this is not known. When the two strains are grown at 42 °C, RI8 does make some nucleotides before it dies but no NAD⁺, NADPH or NADP⁺ can be observed.

The identity of the NAD⁺ spot was confirmed by adding 10 μl of 10 mM NAD⁺ together with a radioactive sample from the wild-type strain. The cold NAD⁺ spot was visible in UV 260 nm light and migrated with the large spot that is present for RI10 but reduced for RI8. The NADP⁺ and NADPH positions were confirmed similarly.

Selection and characterization of a new nadD mutant

Mutations in nadD have been isolated before in S. typhimurium (Hughes et al., 1983b). These mutants are seemingly less severe than the nadD72 mutation, i.e. they are Ts only on minimal medium and can grow on LB plates at 42 °C. We wanted to isolate a mutant of the same type and compare it to nadD72 to see if something could be learnt about the enzyme.

Three separate cultures of strain RI10 were grown and plated on 6-aminonicotinamide (6-Nm) minimal plates. The resistant colonies from each culture were collected, resulting in three independent pools that were transduced to CSH142 with selection for tetracycline resistance and 6-Nm³. Only a few colonies were obtained from one of the pools; each was tested for Ts on minimal medium. One Ts mutant was sequenced and a new mutation in the nadD gene was found. A change of A-38 to T changes Asp-13 to Val in the ATP-binding site (Zhang et al., 2002). The mutant allele was named nadD74 and the strain RI12.

The nadD74 mutant was tested with respect to phenotype to compare it with strains RI10 and RI8 (Table 3). It is clear that RI12 differs from the wild-type only in that it is Ts on minimal medium and more resistant to fusidic acid, although not to the same extent as RI8. This makes this mutation very similar to previously isolated mutations (Hughes et al., 1983a) and distinct from nadD72.

We also measured growth rate in LB medium at 30 °C. Strains RI10 and RI12 have the same doubling time (40 min), whereas the doubling time for RI8 is 57 min. Again, the nadD72 allele confers a more severe phenotype than does nadD74.

An analysis of intracellular nucleotide pools by 2D-TLC was also performed on RI12. As seen in Fig. 2(e, f), the nadD74 mutant strain has a decreased amount of NAD⁺ at 30 °C and an even lower amount of NAD⁺ at 42 °C.

The pleiotropic phenotype caused by nadD72

Mutant strain 72c is characterized by a pleiotropic phenotype (Isaksson & Takata, 1978); it is Ts for growth at 40 °C.
and cannot grow on minimal medium. The \textit{nadD72} mutant is also supersensitive to trimethoprim and resistant to fusidic acid. The Ts phenotype can be reverted by chloramphenicol, by some mutations in RNA polymerase and by mutations in ribosomal protein S12. Most of these phenotypes indicated that the mutation is in a gene important for translation, transcription or both. Unexpectedly, we have found that the mutation affects NAD\(^+\) metabolism. In light of this, how does one explain all the phenotypes observed?

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{2D-TLC of intracellular nucleotides. Solvents Ta and Sb were used for the first and second dimensions, respectively (Bochner & Ames, 1982). Solid-line arrows show the position of NAD\(^+\). The broken-line arrow shows the position of NADP\(^+\) and the dot-line arrow shows the position of NADPH. Cells were grown at 30\(\degree\)C (a, c, e) or at 42\(\degree\)C (b, d, f). Extracts from RI10 are shown in (a, b), extracts from RI8 (\textit{nadD72}) in (c, d), and extracts from RI12 (\textit{nadD74}) in (e, f). The experiment was repeated four times and these are representative figures.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{2D-TLC of intracellular nucleotides. Solvents Ta and Sb were used for the first and second dimensions, respectively (Bochner & Ames, 1982). Solid-line arrows show the position of NAD\(^+\). The broken-line arrow shows the position of NADPH. Wild-type cells P90A5c (a) and 72c cells containing a plasmid with the \textit{nadD} wild-type gene (b) were grown at 30\(\degree\)C.}
\end{figure}
The supersensitivity to trimethoprim can be explained as a direct effect of NAD$^+$ deficiency. This antibiotic inhibits the last step in tetrahydrofolate (THF) synthesis, a step where NAD$^+$ is needed as a cofactor. If, as in the mutant strain, the concentration of NAD$^+$ is very low, it is likely that this step will become extra sensitive to trimethoprim, which is what we observed.

Both mutant alleles impair growth on minimal medium; a strain with nadD72 cannot grow on minimal medium at all, whereas a strain carrying nadD74 is Ts at 42 °C. To investigate the cause of this growth deficiency, experiments testing different nutritional requirements were performed. First, we supplemented minimal plates with either nicotinic acid, nicotinamide or nicotinamide mononucleotide (NMN); none of the additions could support growth for either mutant (not shown). Another possibility is that the pools of aspartate and dihydroxyacetone phosphate are drained in an attempt to compensate for the NAD$^+$ deficiency. The hypothesis was tested with negative results (not shown). Another possibility is that, because the amount of available NADPH is low, the reducing power might be poor and this power is needed for the synthesis of cysteine. We found that addition of cysteine could partially compensate the growth deficiency on minimal plates; R112 (nadD74) is able to grow on minimal plates at 42 °C and R18 (nadD72) grows enough to form revertants at 30 °C (data not shown). Coincidentally, we found that vitamin B$_6$ also has a positive effect on growth. The vitamin is synthesized from pyrimidines, a process that is in turn unbalanced nucleotide pools.

We have shown that the effect of chloramphenicol is strain-dependent. The suppressors in rpoB and rpsL have not been tested. Their effect may be strain-dependent, like the chloramphenicol effect, or may be indirect through unbalanced nucleotide pools.

Finally, NAD$^+$ is a substrate in some enzymic reactions (e.g. DNA ligation) in E. coli. To investigate whether the UV sensitivity of the mutants was affected by NAD$^+$ deficiency, we tested the effect of UV on the mutants, but we did not see any difference as compared to the wild-type (data not shown).

**Conclusion**

We have characterized two mutations in the nadD gene. One mutant is changed in the ATP-binding site and not too severely affected as judged by growth characteristics. The other mutant, changed in the C terminus, is more interesting in that it has a very low level of NAD$^+$ and shows a pleiotropic phenotype. Both mutants can be the starting point for studies of NAD$^+$ metabolism and the function of NAMN adenylyltransferase (NAMNAT) in E. coli.

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