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Cold-Sensitive Mutant of *Salmonella typhimurium* Defective in Nucleosidediphosphokinase

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A cold-sensitive mutant of *Salmonella typhimurium* defective in nucleosidediphosphokinase (ATP:nucleosidediphosphate phosphotransferase, EC 2.7.4.6) has been isolated and characterized. The mutant contains only 2% of the enzyme activity found in the parent, and the heat lability of this activity is 10 times that from the parent at 33 C. Mutant extracts lack the ability to convert any of 11 nucleoside diphosphates tested to the corresponding nucleoside triphosphates, but the nucleosidemonophosphatase activities are normal. Although the nucleoside triphosphate pools of the mutant are depressed significantly at the restrictive temperature (20 C), they are slightly elevated at the permissive temperature (37 C). The levels of guanosine pentaphosphate and guanosine tetraphosphate are dramatically increased. Two representative enzymes of pyrimidine de novo synthesis, aspartic transcarbamylase and dihydroorotate dehydrogenase, are fully repressed at both 37 and 20 C. Intracellular pools of uridine diphosphate are depressed at both permissive and restrictive temperature.

Nucleosidediphosphokinase (NDK) from a number of sources has been purified and studied (4, 18, 19, and review 17), and these investigations have established a remarkable similarity among the various enzymes. In spite of our rather detailed information about the properties and mechanism (5, 6, 14, 16, 17, 21) of NDK, important questions remain concerning its physiological role both in pathways of biosynthesis and in setting concentrations of effectors controlling various metabolic processes. These questions seemed most readily answerable by the study of mutants lacking NDK activity. In this paper we report the first isolation of such a mutant and describe the metabolic and regulatory consequences of a blockade in NDK.

MATERIALS AND METHODS

Strains. All strains used were derived from JL396, a strain of *Salmonella typhimurium* carrying the *galE1211* mutation.

Media and growth conditions. Basal salts medium 007 (3) was used for all experiments except for those to measure nucleotide pools when tris(hydroxymethyl)aminomethane (Tris) minimal medium containing 1 mM sodium phosphate (13) was used. All media contained (as indicated) either 0.02 or 0.2% glucose as carbon source. Supplements, when added, were at the following concentrations: purine and pyrimidine bases, 20 μg/ml; amino acids, 50 μg/ml; and vitamins, 1 μg/ml. Casamino Acids-supplemented media contained 0.15% of Norite-treated, vitamin-free casein hydrolysate (Nutritional Biochemicals Corp.). L-broth contained, per liter at pH 7.0: tryptone (Difco), 10 g; yeast extract (Difco), 5 g; NaCl, 10 g. Solid media were prepared by adding agar (Difco) to 1.5%.

Preparation of extracts for enzyme assays. Exponentially growing cells at A_{420} = 0.8 (4 × 10^8 cells/ml) were cooled rapidly, harvested by centrifugation (15,000 × *g* for 20 min), and washed once in distilled water. The pellets, if not used immediately, were frozen at −20 C. Cells were resuspended in 4 to 10 ml of TEA buffer (100 mM triethanolamine-hydrochloride, pH 7.0) for NDK assays, or Tris-Mg buffer (100 mM Tris-hydrochloride, 10 mM $MgCl_2$, 2 mM mercaptoethanol, pH 7.8) for nucleoside monophosphokinase assays, or phosphate buffer (40 mM potassium phosphate, pH 7.0) for aspartate transcarbamylase and dihydroorotate dehydrogenase assays.

Assays. NDK was assayed by either of two previously described methods (C. L. Ginther and J. L. Ingraham, J. Biol. Chem., in press). In most experiments the method employed measured the formation of [2-^{14}C]uridine triphosphate (UTP) from [2-^{14}C]uridine diphosphate (UDP) and adenosine triphosphate (ATP). In the survey of the activity of NDK against various nucleoside diphosphates, the method measuring the formation of [γ-^{32}P]nucleoside triphosphate from nucleoside diphosphate and [γ-^{32}P]ATP was used.

Nucleoside monophosphokinases were assayed using the method of Ingraham and Neuhard (8). Uridine monophosphate (UMP), cytidine monophos-

phate (CMP), thymidine monophosphate (TMP), or guanosine monophosphate (GMP) were present in the assay mixture at 1 mM, and a total of 20 to 50 nCi of [2-^{14}C]UMP, [2-^{14}C]AMP, [2-^{14}C]CMP, [2-^{14}C]TMP, or [8-^{14}C]GMP was added.

Aspartic transcarbamylase was assayed by the method of Gerhart and Pardee (7).

Dihydroorotate dehydrogenase was assayed by the method of O'Donovan and Gerhart (15).

Protein was determined by the method of Lowry et al. (10) using recrystallized bovine serum albumin as the standard.

Specific activities were calculated from the linear portions of plots relating enzyme activity to protein.

Purification of NDK. The method used to purify the enzyme from the mutant was similar to that previously described for the purification of the enzyme from the parent (Ginther and Ingraham, in press).

Kinetic studies. The K_m values for ATP and UDP were determined using the purified NDK from strains JL2097 and JL396 at 20 C. The K_m for ATP was calculated from assays containing 1 mM UDP and various ATP concentrations in the standard ^{14}C assay mixture which were incubated at 20 C for 10 min. The K_m for UDP was similarly calculated from assays containing 6.25 mM ATP with various concentrations of UDP.

Nucleoside triphosphate pools. Nucleoside triphosphate pools were determined using methods and chemicals that were previously described (13).

UDP pools. The nucleotide extractions used to determine triphosphate pools were also used to determine the UDP pools. Chromatography was done on PEI-Avicel plates as previously described (13) except that the solvents were changed. First-dimension solvents were (i) absolute methanol to the origin, (ii) 2 M sodium formate (pH 3.4) to 2 cm above the origin, and (iii) 4 M sodium formate (pH 3.4) to 12 cm above the origin. The chromatogram was dried with warm air, washed for 5 min with 0.01 M Tris in methanol, dried and washed again for 15 min in absolute methanol, and dried. The second-dimension solvents were: (i) 0.3 M LiCl in 0.9 M acetic acid to 3 cm above the origin and (ii) 0.8 M LiCl in 1.0 M acetic acid to 15 cm above the origin. The location of UDP was determined by the position of unlabeled UDP, and the position of the labeled spots was determined by autoradiography.

RESULTS

Isolation of an NDK mutant. The following rationale was used for the isolation of a mutant lacking NDK. We presumed that NDK is essential for the biosynthesis of nucleoside triphosphates from nucleoside diphosphates, and since nucleotides cannot be exogenously fed to *S. typhimurium* a temperature-conditional mutant was sought. However, some additional selection procedure was clearly required to enrich for mutants blocked at NDK. We chose to use resistance to 8-azaguanine which is toxic to *S. typhimurium* because it is converted in vivo to 8-azaguanosine triphosphate and then into ribonucleic acid (RNA), rendering it nonfunctional (9, 11). Mutations in any of the several enzymes required for the incorporation of 8-azaguanine into RNA should confer resistance. Among these would be mutations in the structural gene for NDK that would modify the enzyme such that natural nucleoside diphosphates but not 8-azaguanosine diphosphate could serve as a substrate. Such modification should have a reasonable possibility of also conferring a temperature-conditional phenotype. We chose a cold-sensitive phenotype since (for reasons not fully understood) cold sensitivity is a frequent consequence of nucleotide kinase defects (8; unpublished results). Thus strain JL396 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (2), grown overnight in Casamino Acids-supplemented glucose basal salts medium at 37 C, and counterselected with 20,000 IU of penicillin per ml at 20 C for 16 h while growing in L-broth. The surviving cells were plated on Casamino Acids-supplemented glucose basal salts plates containing 150 μg of 8-azaguanine per ml, and incubated at 37 C for 48 h. Clones that grew on these plates, on L-medium plates, and on glucose basal salts plates at 37 C, but which grew on none of these plates at 20 C were considered candidates for producing a mutant NDK. These clones were assayed for NDK activity and one, strain JL2097, was found to contain only 2% of the activity produced by the parent (JL396). The levels of the various nucleoside monophosphokinases in strain JL2097 were normal (Table 1).

Phenotype of strain JL2097. A culture of strain JL2097 growing exponentially at 37 C stops growing immediately when shifted to 20 C, and resumes growth immediately when returned to 37 C (Fig. 1). However, even at 37 C the mutant grows more slowly than its parent (Table 2), and there is evidence of appreciable cell lysis in liquid cultures growing at 37 C. Due to the blockade at NDK, one might expect cultures of strain JL2097 to excrete purines and pyrimidines which cannot be converted to the triphosphate level, but, although some excretion can be detected on feeder plates, the amount of excretion is very small as compared to that excreted by other strains carrying mutations affecting pruine (GMP kinase mutants [unpublished data]) and pyrimidine nucleotide (UMP kinase [8]) mutants.

In vivo function of NDK. It has been established that NDK is capable of phosphorylating

all naturally occurring nucleoside diphosphates plus xanthine diphosphate and inosine diphosphate to triphosphates at the expense of the γ-phosphate of ATP (Ginther and Ingraham, in press). The availability of a mutant strain allowed us to determine whether NDK is the only enzyme capable of catalyzing such phosphorylations. Accordingly, crude extracts from both parent (JL396) and mutant (JL2097) were assayed for activity against the various nucleoside diphosphates (Table 3). The activity against all 11 substrates was at least 20-fold greater in the extract from the parent than in that from the mutant, allowing us to conclude that NDK is the only enzyme in *S. typhimurium* with significant activity for phosphorylating nucleoside diphosphates utilizing ATP as the phosphate donor.

TABLE 1. *NDK and nucleoside monophosphokinase levels in JL396 and JL2097 grown at 37 C*

Strain	Sp act[a]					
	NDK	GMP kinase	AMP kinase	UMP kinase	CMP kinase	TMP kinase
JL2097	96	20.2	278	35.7	15.1	3.02
JL396	3960	19.5	285	33.6	16.0	3.15

[a] Specific activities are all expressed in nanomoles of substrate utilized per milligram of protein per minute at 20 C.

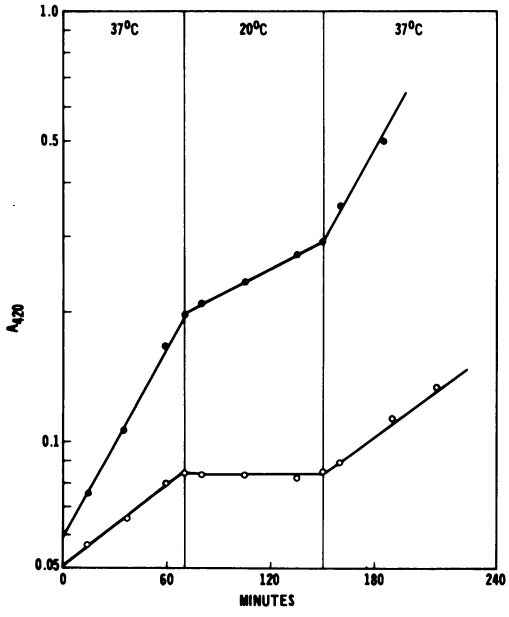

FIG. 1. *Growth of JL396 (●) and JL2097 (○) at 37 and 20 C. Cultures (50 ml) were grown in Casamino Acids-supplemented glucose basal salts medium, and growth was followed by measuring A_{420}. At times indicated by the vertical lines, temperature shifts were effected by transferring the culture from one shaking water bath to another.*

TABLE 2. *Growth rates of JL396 and JL2097 at 37 C*

Strain	Specific growth rate (per h)[a]	
	Glucose basal salts	Casamino Acids-glucose basal salts
JL396	0.80	1.15
JL2097	0.28	0.42

[a] Growth was followed by measuring A_{420}.

TABLE 3. *Activity of NDK in crude extracts of JL396 and JL2097 in a phosphotransferase reaction between ATP and various nucleoside diphosphates*

Nucleoside diphosphate	Sp act[a]	
	JL396[b]	JL2097[b]
ADP[c]	3.0	0.02
UDP	2.8	0.02
GDP	2.2	0.08
CDP	1.8	0.07
dADP[c]	2.0	0.05
dGDP	2.3	0.08
dCDP	1.3	0.04
XDP	2.0	0.03
IDP	1.3	0.03
dUDP	0.8	0.01
dTDP	0.9	0.01

[a] Specific activity is expressed in micromoles of substrate utilized per milligram of protein at 20 C.

[b] Cultures were grown at 37 C.

[c] [^{14}C]nucleoside diphosphate assay was used; in all others [^{32}P] assay was used. "d" indicates "deoxy-."

Purification of NDK from strain JL2097. To establish that the mutation carried by strain JL2097 is in the structural gene rather than in some regulatory gene, evidence for physical alteration of the residual activity in strain JL2097 was sought. Measurements of the heat stability of NDK in crude extracts from strains JL396 and JL2097 showed that the activity from the mutant was less stable than that from the parent. However, this comparison is significantly complicated by the grossly different levels of activity in the two extracts. Both protection by other proteins and interference by phosphatases tend to minimize the apparent differences in heat stability. To assure that the apparent lesser stability of the mutant enzyme was a change in the enzyme rather than an indirect consequence of the lower level of activity, NDK from parent and mutant were purified. The purification of NDK from JL396 has been described (Ginther and Ingraham, in

press), and that from strain JL2097 was purified similarly (Table 4). The mutant and parent enzyme behaved similarly during purification.

Heat stability of NDK. The heat stabilities of purified NDK from strains JL396 and JL2097 were compared at 33 C (Fig. 2); the mutant enzyme is at least 10-fold more labile than the parent enzyme. Clearly the primary structure of the enzyme is altered by the mutation. In spite of the dramatic heat instability of the enzyme, it is quite easy to purify and assay because the presence of substrates completely stabilizes it at 33 C.

Kinetic studies. The K_m values of the mutant and normal enzyme were determined from Lineweaver-Burk plots. There is no significant difference between the K_m of the enzyme from mutant and parent for either substrate (data not shown). The K_m for UDP was 0.5 mM, and that for ATP was 1.2 mM.

Nucleoside triphosphate pools. Clearly the mutation carried by strain JL2097 is in the structural gene encoding NDK because the residual activity of the mutant is physically altered (see above). Also, the inability of the mutant strain to grow at 20 C is a direct consequence of the mutation in the gene encoding NDK, because recombinants and revertants which gain the ability to grow at 20 C produce normal levels of NDK (data not shown). From these facts and the observation (see above) that NDK is required for the synthesis of triphosphates, it seems reasonable to conclude that the inability of strain JL2097 to grow at 20 C is a consequence of starvation for triphosphates. Accordingly, the nucleoside triphosphate pools of strains JL396 and JL2097 were measured at 37 and 20 C (Table 5). As expected, the triphosphate pools of the mutant drop significantly upon shift of a culture from 37 to 20 C, whereas those of the parent increase. In Fig. 3 the difference in the pool levels of strains JL396 and JL2097 at 20 C is clearly shown. Pools of all triphosphates in the mutant drop by approximately the same factor. The mutant pools are also unique in that very large amounts of guanosine tetraphosphate, guanosine pentaphosphate, and a third unknown spot are present at both 37 and 20 C (Fig. 3); the levels of these compounds are unaffected by temperature. We do not know what significance to attach to this observation. At 37 C, although

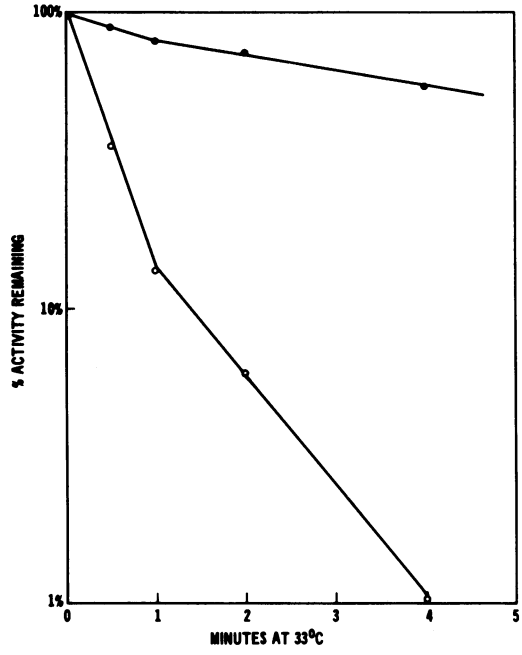

FIG. 2. *Temperature stability of purified NDK from JL396 and JL2097. Dilutions of purified preparations were added to buffer to 0 C containing: ovalbumin, 1 mg/ml; 10 mM triethanolamine-hydrochloride (pH 8.0); 10 mM $MgCl_2$; and 2 mM mercaptoethanol. The dilutions (1 ml) were placed in a water bath at 33 C and at 0, 0.5, 1, 2, and 4 min were removed and placed at 0 C. Samples were assayed at 20 C for 10 min for residual NDK activity using the [2-^{14}C]UDP method. One test sample contained 0.2 ng of purified NDK from JL396 (●), and the other contained 470 ng of purified NDK from JL2097 (○).*

TABLE 4. *Purification of NDK from JL2097*

Treatment[a]	Protein (mg/ml)	Total activity (nmol/min)	Vol (ml)	Sp act (nmol/min/mg of protein)	Fold purification
Crude extract	39.5	13,244	35	9.58	1
Calcium phosphate gel	0.9	2,755	110	27.0	2.8
Ammonium sulfate	2.0	1,500	10	75.0	7.8
DEAE-cellulose[b]	0.05	832	18	925.0	97.0
Ammonium sulfate	0.23	551	2	1200.0	126.0
Sephadex G200	0.00475	187	10	3940	411.0

[a] Methods for the various purification steps have been previously described (Ginther and Ingraham, in press).
[b] DEAE, Diethylaminoethyl.

TABLE 5. *Nucleoside triphosphate pools of JL396 and JL2097 grown at 20 and 37 C*

Pool component	Pool size[a]					
	JL396			JL2097		
	37 C	20 C	20 C/37 C	37 C	20 C	20 C/37 C
ATP	0.84	1.3	1.5	1.3	1.1	0.84
UTP	0.45	0.68	1.5	0.42	0.31	0.73
GTP	0.34	0.54	1.6	0.45	0.24	0.53
CTP	0.17	0.33	1.9	0.29	0.15	0.51
pppGpp	ND[b]	ND	ND	0.15	0.16	1.10
ppGpp	ND	ND	ND	0.11	0.10	0.91
UDP	0.13	0.10	0.77	0.07	0.02	0.28

[a] Cells were grown at 37 C in Casamino Acid-supplemented glucose Tris medium and [^{32}P] phosphoric acid (final concentration of 20 μCi/μmol phosphoric acid). Growth was followed by measuring A_{420}. After at least two doublings, 5-ml samples were removed for the extraction of triphosphates (13), and the culture was shifted to 20 C. After 30 min at 20 C, a 5-ml sample was removed for triphosphate extraction. The figures are absolute pool sizes in nanomoles per 8 $\times$ 10^8 cells.

[b] ND, Not detectable.

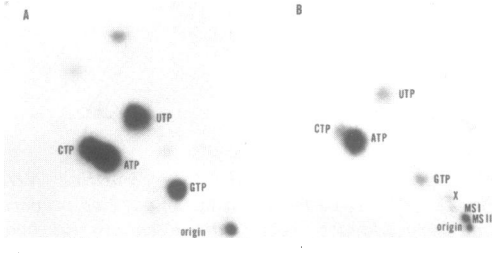

FIG. 3. *Autoradiograms of nucleoside triphosphate pool chromatograms for JL396 (A) and JL2097 (B). Cells were grown at 37 C for two doublings and then shifted to 20 C for 0.5 h. The extraction procedure and chromatography have been described (13). The known spots are designated with the compound name. The major unknown spot which is discussed in the text is marked with an X. Both cultures were harvested at A_{420} = 0.480.*

the mutant still grows slowly, the triphosphate pools of the mutant seem to be slightly elevated.

Levels of enzymes of de novo synthesis of pyrimidines. Aspartate transcarbamylase (ATCase), the first enzyme unique to the pathway leading to the synthesis of pyrimidines, is repressed by a uracil-containing nucleotide (12), and several other enzymes of the pathway, including dihydroorotate dehydrogenase (DHODase) are repressed by a cytosine-containing nucleotide (1, 20). In a mutant lacking UMP kinase (*pyrH*), ATCase is highly derepressed (8), and thus UMP cannot be the effector, rather it must be either UDP or UTP. To distinguish between the di- and triphosphates as repressors. the levels of ATCase and DHODase were measured in the NDK mutant growing at 37 C and after a shift to 20 C (Table 6). Both ATCase and DHODase are repressed in strain JL2097. When uracil is present in the culture medium, ATCase is severely repressed.

UDP pools. Since ATCase and DHODase are repressed, and UTP pools are low in this mutant, UTP evidently is not repressing the synthesis of these enzymes in this case. Since UDP is the alternative candidate for repressor, it became important to measure the UDP levels of the mutant and wild-type strains. Surprisingly, the levels of UDP were also low in strain JL2097 at 20 C (Table 5).

DISCUSSION

By selection for both resistance to the guanine analogue, 8-azaguanine, and for cold sensitivity, a mutant strain has been isolated that produces very low levels of NDK activity even at permissive temperature. The residual enzyme activity produced by the mutant has dramatically decreased heat stability, and hence we conclude that it is physically altered by the mutation, which, as a consequence, must lie in the structural gene (*ndk*) encoding nucleoside diphosphokinase (NDK).

The availability of the mutant strain JL2097 (*galE1211, ndk-1*) allows certain conclusions to be drawn concerning the metabolic role of NDK. The enzyme is capable of catalyzing a very large number of reactions, probably as many as 121, since at least 11 diphosphates are substrates, and probably all the corresponding triphosphates can also serve as substrates

TABLE 6. *Levels of two enzymes of the pyrimidine biosynthetic pathway in JL396 and JL2097*

Strain	Uracil[a] present	Sp act[b]		
		ATCase		DHODase
		37 C	20 C	37 C
JL396	–	20.3	20.7	26.3
	+	10.0		22.2
JL2097	–	13.9	9.6	20.5
	+	1.5		18.3

[a] Uracil when added was at a final concentration of 20 μg/ml.

[b] Specific activities were expressed as nanomoles of product formed per minute per milligram of protein for ATCase, and as ($\Delta OD_{460} \times 10^3$/20 min)/(mg protein/0.05 ml extract) for DHODase.

(Ginther and Ingraham, in press). We presume, however, that the most significant reactions metabolically are those which result in the synthesis of the various essential nucleoside triphosphates at the expense of the γ-phosphate of ATP. We have previously reported that NDK from *S. typhimurium* is capable of catalyzing all of these reactions. Now, on the basis of the fact that crude extracts from strain JL2097 contain only about 2% of the wild-type activity for all these reactions, we can conclude that NDK is the only enzyme with significant activity for synthesizing nucleoside triphosphates at the expense of ATP.

Certain aspects of the phenotype of strain JL2097 were surprising. The growth defect at 37 C and the cold sensitivity of the strain are clearly direct consequences of the lesion in the *ndk* gene, because recombinants and revertants which gain normal growth properties simultaneously gain the property of producing normal levels of NDK activity. We expected the growth defects to be a direct consequence of a deficiency of the essential products of the NDK-catalyzed reactions, namely, nucleoside triphosphates. But other important factors, probably regulatory mechanisms, must intervene because, although the nucleoside triphosphate pools at the restrictive temperature are significantly depressed, those at the permissive temperature (where growth rate is slowed as a consequence of the mutation in *ndk*) are slightly elevated. Although there is no direct evidence for a "master switch" type of mechanism which conserves nucleoside triphosphate pools when a culture is deprived of energy, it seems inevitable that one must exist. It is well known that energy-starved cells maintain nucleoside triphosphate pools sufficient to allow continued synthesis of nucleic acids after such synthesis has completely stopped. Were there no master switch mechanism, energy starvation would certainly be a lethal event, because, owing to the activity of adenylate kinase, depletion of ATP would simultaneously deplete ADP and there are no known biochemical mechanisms for the regeneration of ATP in the absence of ADP and other nucleoside triphosphates. We suspect that the master switch mechanism is the factor that slows the growth of strain JL2097 at 37 C and stops it at 20 C.

Strain JL2097 would certainly be expected to have an altered ratio of di- to triphosphates, particularly at 20 C. It has been shown previously that depletion of UDP and UTP maximally derepresses the synthesis of ATCase. The availability of the NDK mutant offered the possibility of distinguishing between these two candidates for co-repressor. ATCase levels in strain JL2097 are maximally repressed at both 37 and 20 C, and in the presence of exogenous uracil they fall to levels lower than we have seen under any conditions. These results, of course, suggest that UDP is the co-repressor, but studies on diphosphate levels indicate the UDP levels are also low in strain JL2097. Similarly, DHODase, which is known to be regulated by a cytosine nucleotide, is also fully repressed in strain JL2097. However, the levels of all known nucleoside monophosphokinases are unaffected by the mutation in *ndk*. Thus we are presented with a dilemma in which all candidates for repressor are present at low levels, but the system is still severely repressed.

We are unable to explain why the mutant is cold sensitive because the purified residual activity of the mutant is not, but we suspect that the cold sensitivity is a consequence of a temperature effect of some associated control mechanism.

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