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Effects of an Insertion Mutation in a Locus Affecting Pyridine Nucleotide Transhydrogenase (*pnt*::Tn*5*) on the Growth of *Escherichia coli*

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The effects of a *pnt*::Tn*5* insertion mutation on the growth of strains lacking phosphoglucoisomerase or glucose 6-phosphate dehydrogenase were examined. The results support the idea that the energy-linked transhydrogenase is an important source of reduced nicotinamide adenine dinucleotide phosphate for *Escherichia coli* under some conditions.

The physiological functions of the energy-linked pyridine nucleotide transhydrogenase in *Escherichia coli* and animal mitochondria have not been established. In the absence of an energy source, membrane preparations catalyze the reduction of NAD by NADPH more rapidly than the reverse reaction, but ATP or substrates of respiration increase the rate of the reverse reaction several-fold. The enyzme could conceivably be used to channel electrons from NADPH into the electron transport chain or to generate NADPH. A mutant of *E. coli* lacking the activity was isolated after nitrosoguanidine mutagenesis (8, 12), but since no phenotype was associated with the *pnt-1* mutation, no conclusions could be drawn about the function of the enzyme.

Insertion mutations containing drug resistance transposons are well suited to investigating the physiological roles of an enzyme because the insertion assures the absence of the enzyme in vivo, and the drug-resistance phenotype allows positive selection when transducing the mutation to strain backgrounds where the lack of the enzyme may be critical (9). We report here on the isolation of a kanamycin-resistant mutant carrying *pnt*::Tn*5* and the growth properties of some strains carrying this mutation.

E. coli strains used are listed in Table 1.

A stock of λ_{467} *b*221 *rex*::Tn*5* *c*I857 *o*am29 *P*80 having a titer of 10^{11} per ml was prepared on plates containing strain AB1157, which has an amber suppressor. The stock contained 20 mM Tris chloride (pH 7.5), 0.1 M NaCl, and 10 mM $MgSO_4$. Suppressor-free strain KL96 was grown in lambda ym medium (10 g of tryptone, 2.5 g of NaCl, 2 g of maltose, and 0.1 g of yeast extract per liter) to Klett 100 (filter no. 66). A 10-ml sample of the culture was centrifuged, and the pellet was suspended in 1 ml of the λ_{467} lysate. After a 15-min adsorption period at room temperature, this culture was added to 50 ml of LB (10), containing 0.4% glucose and 10 mM sodium citrate (pH 7), and incubated for 1 h at 30°C on a rotary shaker. Kanamycin was then added to a concentration of 25 μg/ml, and the culture was grown overnight to saturation at 30°C. A 10-ml sample of the overnight culture was added to 50 ml of LB containing glucose, citrate, and kanamycin at the indicated concentrations, and the culture was grown to saturation at 42°C. This step was then repeated. *c*I857 encodes a heat-sensitive repressor, and *b*221 removes the lambda attachment site. Since lysogens cannot be formed at 42°C, surviving cells are those with translocations of Tn*5* to the bacterial chromosome.

A P1 *cml clr*100 lysate of the kanamycin-resistant culture was prepared on R plates according to Miller (10), but at 41°C to avoid lysogens. Strain GMS343-2 was tranduced with this lysate (10) and plated on M63 (10) plus 0.2% mannose plus 25 μg of kanamycin per ml at 41°C to select Tn*5* insertions in the vicinity of *man* (*man* is 59% cotransducible with *pnt* [8]). Of 62 transductant colonies grown on this selective medium and assayed for energy-independent transhydrogenase, two lacked the activity. One of these, RH5, was used for further work. A similar procedure carried out to generate Tn*10* insertions did not produce any *pnt* colonies among the 273 Man^+ Tet^r colonies examined. This may reflect the more random insertion of Tn*5* than Tn*10* into the chromosome (9).

Strains RH5 (*pnt-2*::Tn*5*) and GMS343-2 (*pnt*$^+$) had similar growth rates, compared to each other, with glucose, fructose, glycerol, or succinate as the carbon source (Table 2), and the growth yields at limiting glucose concentrations were also similar (data not shown). As with the *pnt-1* mutation, no distinctive phenotype was associated with *pnt-2*::Tn*5*. The insertion mutation allows the conclusion that transhydrogenase is not essential for the production of NADPH or ATP in *E. coli*.

TABLE 1. *Strains of E. coli K-12*

Strain	Relevant characteristics	Source
GMS343-1	F⁻ *argE3 man-4 pdxH15*	Hanson and Rose (8)
GMS343-2	F⁻ *argE3 man-4*	Transductant of GMS343-1
KL96	Supressor-free strain	CGSC[a]
AB1157	*supE44*	L. Csonka
RH5	*argE3 man*⁺ *pnt-2*::Tn*5*	Transductant of GMS343-2 (see text)
DF40	*pgi-2*	CGSC
DF40-K	*pgi-2 pnt-2*::Tn*5*	Transductant of DF40 from RH5 (see text)
DF2001	*zwfA2*	CGSC
DF2001-K	*zwfA2 pnt-2*::Tn*5*	Transductant of DF2001 from RH5 (see text)
KL16-99	Hfr *recA1*	CGSC
GMS343-2T	F⁻ *thyA argE3 man-4*	Selected from GMS343-2 by trimethoprim resistance (10)
GMS343-2R	*thy*⁺ *argE3 man-4 recA1*	Recombinant from conjugation of GMS343-2T and KL16-99, identified by UV light sensitivity
F500/GMS724	Episome F500 (see ref. 8); chromosomal markers: *aroD6 metB1 man-4 recA1*	CGSC
F500/GMS343-2R	Episome F500; chromosomal markers: *argE3 man-4 recA1*	Episome transfer from F500/GMS724

[a] *E. coli* Genetic Stock Center.

TABLE 2. *Effects of transhydrogenase mutation on aerobic growth rates*[a]

Strain	Doubling time (min)						
	Glucose[b]	Fructose[b]	Glycerol[b]	Succinate[b]	Glucose,[b] Leu	Glucose,[b] Casamino Acids	LB
GMS343-2 (*pnt*⁺)	61	82	124	88			
RH5 (*pnt-2*::Tn*5*)	64	84	116	78			
DF2001 (*zwfA2*)	52 ± 4	74 ± 10	86 ± 8	74 ± 8	85 ± 6	36 ± 3	32 ± 0
DF2001-K (*zwfA2 pnt-2*::Tn*5*)	108 ± 19	119 ± 10	125 ± 5	80 ± 5	128 ± 19	41 ± 7	32 ± 4
DF40 (*pgi-2*)	150 ± 2	64 ± 4	242 ± 50	390 ± 98	140 ± 5	62 ± 11	36 ± 3
DF40-K (*pgi-2 pnt-2*::Tn*5*)	144 ± 12	114 ± 5	265 ± 57	313 ± 11	146 ± 10	57 ± 6	31 ± 0

[a] Values are the mean of two or three cultures (with standard deviation). Phosphoglucoisomerase (4), glucose 6-phosphate dehydrogenase (4), and transhydrogenase (8) were assayed at the end of the growth period in each medium to verify the enzyme deficiencies.

[b] M63 medium containing 0.2% carbon source and 2 mM leucine or 0.1% Casamino Acids when indicated.

Strains lacking transhydrogenase and either glucose 6-phosphate dehydrogenase (*zwfA2* [3]) or phosphoglucoisomerase (*pgi-2* [4]) were constructed to assess the importance of transhydrogenase in producing or oxidizing NADPH. During growth in glucose minimal medium, the shunt has been estimated isotopically to supply 20 to 50% of the NADPH for *E. coli* (2). When all glucose is metabolized via the shunt in a *pgi* mutant, a threefold excess of NADPH is calculated to be produced (2). A P1 *cml clr*100 lysate of RH5 was used to transduce strain DF40 to kanamycin resistance (25 μg/ml) on M63 plus fructose at 41°C. Of 10 colonies examined, all lacked transhydrogenase, and one of these, DF40-K (*pgi-2 pnt-2*::Tn*5*), was used for further work. Strain DF2001 was similarly transduced to kanamycin resistance on M63 plus glucose or LB plates. When four colonies were examined, two of two colonies from LB plates lacked transhydrogenase, but both colonies from M63 plus glucose had the enzyme. One of the colonies from an LB-kanamycin plate, DF2001-K (*zwfA2 pnt-2*::Tn*5*), was saved for further work.

The effects of the *pnt2*-::Tn*5* mutation on the growth of strains with altered use of the hexose monophosphate shunt are shown in Tables 2 and 3. During growth on glucose, fructose, or glycerol, strain DF2001-K, which lacks glucose 6-phosphate dehydrogenase and transhydrogenase, had increased doubling times compared to strain DF2001. When succinate was the carbon source, the absence of transhydrogenase had little effect on the growth rate of strain DF2001-K. Here the cells can use a NADP-specific malic enzyme in the pathway from succinate to pyru-

TABLE 3. *Effects of transhydrogenase mutation on anaerobic growth*[a]

Strain	Colony diam (mm)	
	Glucose	Fructose
GMS343-2 (*pnt*$^+$)	1.3	0.8
RH5 (*pnt-2*::Tn*5*)	1.3	0.9
DF40 (*pgi-2*)	0	1.0
DF40-K (*pgi-2 pnt-2*::Tn*5*)	0	0.8
DF2001 (*zwfA2*)	1.2	0.5
DF2001-K (*zwfA2 pnt-2*::Tn*5*)	0.3	0.2

[a] Plates containing M63, 0.2% sugar, and 1.5% agar were incubated for 48 h at 37°C under H_2 and CO_2. The diameters of three well-spaced colonies were measured after the plates were projected on a screen with an overhead projector, to give about a 10-fold magnification. Standard deviations were less than 10%.

vate (7) as a source of NADPH. In *E. coli* W, the NADP-specific malic enzyme has been shown to be strongly repressed during growth on glucose and partially repressed during growth on glycerol, compared to the enzyme level in cells grown on succinate medium (11).

Strains lacking phosphoglucoisomerase grow slowly on glucose and are calculated to produce a threefold excess of NADPH under this condition (2). Since strains DF40 and DF40-K had similar growth rates on glucose (Table 2), transhydrogenase is not essential for oxidation of excess NADPH, although most of the in vitro capacity for NADPH oxidation in the presence of NAD results from transhydrogenase (8). These *pgi* mutants have glucose 6-phosphate dehydrogenase but cannot synthesize glucose 6-phosphate from fructose. Strain DF40-K had a longer doubling time than DF40 on fructose, suggesting the importance of transhydrogenase for supplying NADPH in this situation. Both strains had similar slow growth rates on succinate or glycerol, and factors other than transhydrogenase apparently limit growth under these conditions.

The results in Table 2 are in agreement with studies on the regulation of transhydrogenase. The enzyme was increased twofold in strains lacking the shunt dehydrogenases grown on glucose (6) and apparently serves as a source of NADPH under these conditions. Wild-type strains grown on succinate had half the transhydrogenase specific activity of cells grown on glucose (1, 6). Our interpretation is that the NADP-specific malic enzyme supplies more NADPH during growth on succinate than on glucose. During growth on LB medium or glucose plus Casamino Acids, transhydrogenase is almost completely repressed (1, 6), and the presence or absence of *pnt-2*::Tn*5* had no effect on growth rates (Table 2). Leucine also represses transhydrogenase by 80%, and this regulation is affected by the *livR* and *lstR* mutations, which affect the regulation of leucine transport (6). The presence of leucine did not increase the slow growth rate of strain DF2001-K on glucose (Table 2). Hence, the repression by leucine is apparently not directly related to the NADPH requirement of the cell. The doubling time of strain DF2001 was increased by leucine (Table 2), but effects in addition to repression of transhydrogenase are probably involved.

Anaerobically, strain DF2001-K produced much smaller colonies than strain DF2001 when plated on M63 plus glucose or fructose (Table 3). Strain DF40-K produced only slightly smaller colonies than strain DF40 anaerobically on fructose, although the hexose monophosphate shunt is not operative in this case. The inability of strains lacking phosphoglucoisomerase to grow anaerobically on glucose has been noted previously (5, 8).

The F500 episome carries the *pnt* locus (8). A threefold gene dosage effect was obtained from this episome on the specific activity of energy-independent transhydrogenase in both the whole sonic extract and the membrane fraction (Table 4). This is evidence that, unless there are unusual regulatory effects, *pnt* is the structural gene for transhydrogenase. Therefore it may be possible to obtain an energy-uncoupled transhydrogenase by localized mutagenesis at this site. In addition to providing information about the physiological function of transhydrogenase, the phenotype of microcolonies during anaerobic growth on glucose of a *zwf pnt* mutant, which presumably reflects the lack of energy-linked NADPH formation, could allow the identification of transhydrogenase mutants that are defective in energy coupling.

TABLE 4. *Effect of F500 episome on specific activity of transhydrogenase*

Strain	Transhydrogenase sp act[a] (nmol/min per mg)	
	Sonic extract	Membranes
GMS343-2R	16.6 ± 3.9	47.3 ± 4.8
F500/GMS343-2R[b]	56.8 ± 4.7	133 ± 5

[a] Membranes were prepared (12) and energy-independent transhydrogenase was assayed (8) as described previously. Values are the mean of four cultures with standard deviation.

[b] Strain was constructed according to Table 1 because acridine orange treatment of F500/GMS724 did not cure it of the episome.

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