JOURNAL OF BACTERIOLOGY, June 1979, p. 783-787
0021-9193/79/06-0783/05$02.00/0

Vol. 38, No. 3

Genetic Mapping of a Mutation Affecting Pyridine Nucleotide Transhydrogenase in *Escherichia coli*

RONALD L. HANSON* AND CAROLINE ROSE

Department of Biochemistry, Columbia University, New York, New York 10032

Received for publication 6 February 1979

A mutation, *pnt-1*, causing loss of pyridine nucleotide transhydrogenase activity in *Escherichia coli*, was mapped by assaying for the enzyme in extracts of recombinant strains produced by conjugation, F-duction, and P1 transduction. The site of this mutation was near min 35, counterclockwise from *man*, and it cotransduced 59% with *man*. The mutation was associated with loss from the cell membrane fraction of energy-independent and adenosine 5'-triphosphate-dependent transhydrogenase activities, but reduced nicotinamide adenine dinucleotide dehydrogenase activity was not affected. Strains were constructed which lack phosphoglucoisomerase (*pgi-2*) and which carry either *pnt*$^+$ or *pnt-1*. Although such strains, when grown on glucose, are expected to produce a large excess of reduced nicotinamide adenine dinucleotide phosphate, the growth rate was not affected by the *pnt-1* allele.

Pyridine nucleotide transhydrogenase in *Escherichia coli* is a member of the class of membrane proteins which respond to the energized state of the cell membrane, produced either by ATP hydrolysis via the membrane ATPase or by respiration. To facilitate the analysis of the energy transduction mechanism and to assess the physiological roles of the enzyme, we previously isolated a mutant lacking transhydrogenase activity (19). The mutant was isolated by assaying for activity in survivors of nitrosoguanidine mutagenesis; it had no growth defects, but membrane preparations lacked both energy-independent and ATP-driven transhydrogenase. Strains containing *unc* mutations also lack ATP-driven transhydrogenase (9) due to various defects in the ATPase complex, but our mutant had ATPase activity and grew on succinate, indicating that the energy coupling systems were not impaired (19).

Because the absence of a phenotype could be due to leakiness of the mutant in vivo, we decided to map the mutation in order to eventually obtain deletion or insertion mutations. Mapping is also necessary to allow transfer of the mutation to other genetic backgrounds in which the mutation may have a physiological effect and may allow isolation of energy-uncoupled mutations in this gene by techniques of localized mutagenesis.

This report describes the mapping of the mutation (designated *pnt-1*), enzymatic properties of membrane fractions from two strains carrying the mutation, and the construction and properties of a strain lacking both phosphoglucoisomerase and transhydrogenase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used are listed in Table 1. Conjugations and transductions were carried out with strains grown on LB medium (14). Selective plates contained M63 (14) plus 1.5% agar with 1% carbon source, thiamine (2 μg/ml), and growth requirements at the recommended concentrations (14). For *aroD* strains, shikimic acid (0.7 mg/ml), phenylalanine (40 μg/ml), and tyrosine (8 μg/ml) were added (15). For enzyme assays, cells were grown at 37°C in a rotary shaker on M63 plus 1% glucose and growth requirements. Anaerobic growth was carried out in a BBL GasPak under H_2 and CO_2.

Genetic crosses. Mating mixtures for conjugation and F′ transfer were prepared by the method of Miller (14). After a 1-h conjugation period, a portion of the mixture was streaked on selective plates containing 100 μg of streptomycin per ml, but lacking histidine or tryptophan. After a 1-h F′ transfer, a portion of the mixture was streaked on plates lacking histidine. Donor strains required other amino acids which were not present.

Transductions were carried out with P1 *cml clr100* by established procedures (14).

After all crosses, recombinant colonies were grown out on M63 plus glucose and other requirements and sonicated for 30 s in the growth medium, and 0.1 ml of the whole sonicate was assayed for energy-independent transhydrogenase.

Enzyme assays. Membrane fractions were prepared and optical assays were performed as described previously (19). Absorbance changes were followed at 380 nm for energy-independent transhydrogenase and at 340 nm for the other assays. The assay mixture for

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

Strain	Relevant characteristics	Source
AE62	F⁻ *argG6 his-1 trp-31 thyA str-104*	Anderson et al. (1)
KZ56	*pnt-1* derivative (lacking pyridine nucleotide transhydrogenase) of AE62	Hanson et al. (19)
KZ56-1	*pnt-1 trp*⁺ recombinant from conjugation of KL208 and KZ56	This work
KL16	Hfr (see Fig. 1 and ref. 13)	CGSC[a]
KL983	Hfr (see Fig. 1 and ref. 13)	CGSC
KL96	Hfr (see Fig. 1 and ref. 13)	CGSC
PK191	Hfr (see Fig. 1 and ref. 13)	CGSC
KL208	Hfr (see Fig. 1 and ref. 13)	CGSC
KL99	Hfr (see Fig. 1 and ref. 13)	CGSC
KLF48/KL159	Episome F148 (see Fig. 1 and ref. 12); chromosomal markers: *proA2 aroD5 his-4*	CGSC
F500/GMS724	Episome F500 (see Fig. 1 and ref. 16); chromosomal markers: *aroD6 metB1 man-4*	CGSC
DFF1/JC1553	Episome F150 (see ref. 7); chromosomal markers: *leuB6 hisG1 argG6 metB1*	CGSC
FP4102	Episome F410 (see ref. 18); chromosomal markers: *trpA9761 thyA25* Δ(*his-gnd*)	CGSC
GMS343	*aroD6 argE3 man-4*	CGSC
AT3196	*pdxH15 thi-1*	CGSC
DF40	*pgi-2*	CGSC
GMS 343-1	*argE3 man-4 aroD*⁺ *pdxH15* transductant of GMS-343 from AT3196	This work
RH1	*aroD6 argE3 man*⁺ *pnt-1* transductant of GMS343 from KZ56	This work
RH2	*aroD6 argE3 man*⁺ *pnt*⁺ transductant of GMS343 from KZ56	This work
RH3	*thi-1 pdxH*⁺ *pnt-1* transductant of AT3196 from KZ56	This work
RH4	*thi-1 pdxH*⁺ *pnt*⁺ transductant of AT3196 from KZ56	This work
DF11	*metA28 pgi-2 his-84*	CGSC
RH1-1	*aroD6 pnt-1 metA28* transductant of RH1 from DF11	This work
RH2-1	*aroD6 pnt*⁺ *metA28* transductant of RH2 from DF11	This work
RH1-2	*aroD6 pnt-1 pgi-2* transductant of RH1-1 from DF40	This work
RH2-2	*aroD6 pnt*⁺ *pgi-2* transductant of RH2-1 from DF40	This work

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

energy-independent transhydrogenase contained, in 1 ml: 0.1 M potassium phosphate (pH 7), 0.1 mM NADPH, 0.1 mM acetylpyridine adenine dinucleotide, and 10 mM mercaptoethanol. The assay mixture for ATP-driven transhydrogenase contained, in 1 ml: 0.1 M Tris-chloride (pH 7.8), 5 mM ATP, 5 mM $MgCl_2$, 0.1 mM NADP, 0.1 mM NADH, 10 mM mercaptoethanol, 50 μM 2-heptyl-4-hydroxyquinoline-*N*-oxide, 3 μg of yeast alcohol dehydrogenase, and 0.27 M ethanol. The assay mixture for NADH oxidation contained, in 1 ml: 0.1 M Tris-chloride (pH 7.8), 0.2 mM NADH, and 10 mM mercaptoethanol. The assay mixture for NADPH oxidation contained 0.1 M potassium phosphate (pH 7), 0.1 mM NADPH, ± 0.1 mM NAD, and 10 mM mercaptoethanol. Phosphoglucoisomerase was assayed by the method of Fraenkel and Levisohn (8).

Protein. A dye-binding assay (3) was used with bovine gamma globulin as the standard.

Materials. Nucleotides were from P. L. Biochemicals; 2 heptyl-4-hydroxyquinoline-*N*-oxide was from Sigma Chemical Co.; the protein assay kit was from Bio-Rad Laboratories.

RESULTS

No growth requirement or altered phenotype has been detected for strain KZ56 which lacks transhydrogenase activity. Therefore, mapping was carried out by selecting recombinants for other markers and assaying extracts of these for transhydrogenase.

Conjugation with Hfr strains. The points of origin and direction of transfer of the Hfr strains used to obtain the approximate map position of *pnt-1* are shown in Fig. 1. Hfr strains KL16, KL983, KL96, and PK191 were conjugated with strain KZ56 in 1-h uninterrupted matings. *his*⁺ recombinants were assayed for transhydrogenase. Some *pnt*⁺ colonies were found after crosses with the first three Hfr strains, but none were found when PK191 was the donor (Table 2). Strains KL99 and KL208 were similarly crossed with KZ56 in 1-h uninterrupted matings, and *trp*⁺ recombinants were selected. Some of these colonies were *pnt*⁺ when KL99 was the donor, but no *pnt*⁺ colonies were found when KL208 was the donor. The results of these conjugations show that *pnt-1* is located between the points of origin of KL208 and PK191 in the 31- to 43-min region.

Episome transfers. A series of strains with

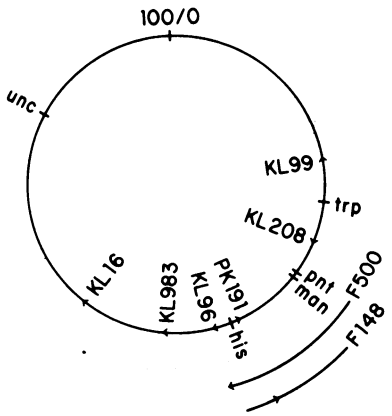

FIG. 1. *Map of E. coli chromosome showing relevant genes, points of origin of Hfr strains, and extent of F′ episomes used in this study.*

TABLE 2. *Conjugation of KZ56 with Hfr and F′ strains*

Donor	No. with selected marker assayed	No. *pnt*$^+$
Hfr strains:		
KL16	10 *his*$^+$	5
KL983	10 *his*$^+$	2
KL96	5 *his*$^+$	1
PK191	15 *his*$^+$	0
KL99	8 *trp*$^+$	2
KL208[a]	19 *trp*$^+$	0
F′ strains:		
KLF48/KL159	11 *his*$^+$	0
F500/GMS724	8 *his*$^+$	2
DFF1/JC1553	6 *his*$^+$	0
FP4102	4 *his*$^+$	0

[a] KZ56-1, a *trp*$^+$ recombinant from this conjugation, was used as recipient in episome transfers.

F′ factors covering different parts of this region were used as donors to further map *pnt-1*. Strain KZ56-1, a *his-1 trp*$^+$ derivative of KZ56, was used as recipient to allow counterselection of all F′ strains by amino acid requirements. Each of the F′ factors carried *his*$^+$, and *his*$^+$ recombinants were selected after a 1-h transfer period. Two of eight *his*$^+$ colonies produced by F500 transfer were *pnt*$^+$, but transfer of the other plasmids did not produce any *pnt*$^+$ colonies (Table 2). F148 extends from *his* through *aroD*, and F500 extends from *his* through *man* (Fig. 1). Episome F148 contains deletions which are covered in F150, and F410 covers a deletion in F150. The results from episome transfers imply that the *pnt-1* site is in the vicinity of *man* at 35 to 37 min.

Mapping by transduction. This location was confirmed by transduction using a P1 lysate of strain KZ56 and recipient strains with markers in this region (Table 3). With strain GMS343 as recipient, 14 of 21 *man*$^+$ transductants were *pnt-1*, but 0 of 23 *aroD*$^+$ transductants were *pnt-1*. *pdxH15* is located between *aroD* and *man*. Two of 20 *pdxH*$^+$ transductants of strain AT3196 were *pnt-1*.

Strain GMS343-1 *pdxH15 man-4 pnt*$^+$ was constructed by transducing GMS343 to *aroD*$^+$, using strain AT3196 as donor and selecting a *pdxH15 man-4* colony from the transductants. Results from a three-factor cross of this strain with the donor KZ56 *pdxH*$^+$ *man*$^+$ *pnt-1*, with *pdx*$^+$ as the selected marker, were consistent with the gene order *pnt man pdxH* (Table 3). If *man* is the middle marker, then formation of the rarest class, *pnt-1 man-4 pdxH*$^+$, would require four cross-overs. *pnt-1* is apparently located next to the genetically silent gap counterclockwise from *man* at about 35.4 min (2). When *man*$^+$ was the selected marker, there was no class of infrequent recombinants, which is consistent with this gene order.

Enzymatic properties of membranes from strains carrying *pnt-1*. Both energy-independent and ATP-driven transhydrogenase activities were missing from the original mutant strain KZ56. It appeared that a single enzyme catalyzing hydride transfer was missing or defective in this strain and was used for both transhydrogenase reactions, but this conclusion was tentative considering the mutagenized background of the strain (19). Of 25 *man*$^+$ transductants of GMS343-1 from KZ56, 13 lacked both activities and 12 had both activities, which confirms the previous conclusion. The loss of both transhydrogenase activities in two strains carrying *pnt-1* is shown in Table 4. NADH dehydro-

TABLE 3. *Mapping of pnt-1 by transduction from KZ56*

Recipient	Selected marker (no. scored)	Unselected marker(s) (no. found)
GMS343	*man*$^+$ (21)	*pnt-1* (14)
GMS343	*aroD*$^+$ (23)	*pnt-1* (0)
AT3196	*pdxH*$^+$ (20)	*pnt-1* (2)
GMS343-1[a]	*pdxH*$^+$ (72)	*man-4 pnt*$^+$ (37)
		man-4 pnt-1 (1)
		man$^+$ *pnt-1* (19)
		man$^+$ *pnt*$^+$ (15)
GMS343-1[b]	*man*$^+$ (25)	*pdxH15 pnt*$^+$ (7)
		pdxH15 pnt-1 (4)
		pdxH$^+$ *pnt*$^+$ (5)
		pdxH$^+$ *pnt-1* (9)

[a] Transduction and reversion frequencies for *pdxH*$^+$ were 3×10^{-5} and 1×10^{-6}, respectively.

[b] Transduction and reversion frequencies for *man*$^+$ were 3×10^{-6} and $<10^{-8}$, respectively.

TABLE 4. *Membrane enzyme specific activities*[a] *in parental and mutant strains*

Strain	Energy-independent transhydrogenase	Energy-dependent transhydrogenase		NADH dehydrogenase	
		+ATP	−ATP	−HHQNO	+HHQNO[b]
RH1 *pnt-1*	0	0	0	230	19
RH2 *pnt*$^+$	65	24	6	270	26
RH3 *pnt-1*	0	0	0	89	11
RH4 *pnt*$^+$	97	12	4	71	3

[a] Nanomoles per minute per milligram of protein.
[b] 50 μM 2-heptyl-4-hydroxyquinoline-*N*-oxide.

genase activity is not decreased by *pnt-1* and responds similarly to the electron-transport inhibitor, 2-heptyl-4-hydroxyquinoline-*N*-oxide (4), in all four strains. Therefore, NADH dehydrogenase appears to be the result of enzyme(s) distinct from transhydrogenase.

Construction of a strain lacking phosphoglucoisomerase and transhydrogenase. The mapping of *pnt-1* facilitates its combination with other mutations, which may allow a better assessment of its physiological role. Strains carrying *pgi-2* lack phosphoglucoisomerase and grow slowly on glucose but at normal rates on fructose, compared to *pgi*$^+$ strains (8). From growth on glucose exclusively via the hexose monophosphate shunt, the *pgi-2* mutants are calculated to produce about 46 nmol of NADPH per mg of cells, whereas the calculated requirement is for only 17 nmol per mg (5). The disposal of excess NADPH could be limiting growth in this situation and could require transhydrogenase.

To examine this possibility, *pgi-2* was introduced by two transductions into strain RH1 and its otherwise isogenic counterpart RH-2 to produce RH1-2 *pnt-1 pgi-2* and RH2-2 *pnt*$^+$ *pgi-2*. Both strains grew anaerobically on fructose but not glucose, which is consistent with the idea that disposal of excess NADPH requires electron transport to oxygen. Aerobically, however, both strains had doubling times similar to each other when grown on glucose, glycerol, or succinate. From these results it appears that transhydrogenase is not necessary for growth on glucose of a strain lacking phosphoglucoisomerase. However the question should be reexamined with a deletion or insertion mutation to assure that transhydrogenase is definitely absent in vivo.

Specific activities for NADPH oxidation by whole sonicates of strains RH1-2 and RH2-2, grown on glucose, are shown in Table 5. In the presence of NAD, oxidation of NADPH by the *pnt*$^+$ strain was at a seven times greater rate than in the *pnt-1* strain. In the absence of NAD, the specific activities were similar in both strains and appeared sufficient to remove, during one generation time, the excess NADPH produced by exclusive use of the hexose monophosphate shunt. In the presence of NAD, oxidation of NADPH by the *pnt*$^+$ strain was sensitive to an inhibitor (4) of NADH oxidation, but oxidation by the *pnt-1* strain was not inhibited. In the absence of NAD, NADPH oxidation was not inhibited by HHQNO in either strain, indicating that the residual rates were independent of NADH oxidation.

TABLE 5. *Oxidation*[a] *of NADPH by strains lacking phosphoglucoisomerase*

Substrates	NADPH, NAD		NADPH	
	−HHQNO	+HHQNO[b]	−HHQNO	+HHQNO
Strain				
RH1-2 *pnt-1*	4.7	8.9	8.2	13
RH2-2 *pnt*$^+$	35	12	7.8	11

[a] Nanomoles per minute per milligram of protein.
[b] 50 μM 2-heptyl-4-hydroxyquinoline-*N*-oxide.

DISCUSSION

The *pnt-1* mutation causes loss of both energy-independent and energy-dependent transhydrogenase activities. The energy coupling systems are not affected, as evidenced by the growth of the mutant on succinate, and the mutation appears to produce loss of the enzyme catalyzing hydride transfer for both activities. There is no loss of NADH dehydrogenase activity, indicating that transhydrogenase is distinct from this activity in *E. coli* and that the mutation does not cause the loss of any electron-transferring prosthetic group used for NADH oxidation. At least two NADH dehydrogenase activities have been detected in detergent extracts of *E. coli* membrane vesicles (6, 17), but Dancey et al. (6) have suggested that one form is derived from the other.

Kinetic studies of the transhydrogenase reaction catalyzed by *E. coli* membrane fractions and by the partially purified enzyme showed nonlinear double reciprocal plots (10) for both

substrates in the energy-linked direction (reduction of NADP by NADH). In impure preparations, nonlinearity could always be due to the presence of more than one enzyme, but the loss of all transhydrogenase activity in *pnt-1* strains is evidence that only a single enzyme is involved.

A major reason for isolating the transhydrogenase mutant was to determine the physiological function of the enzyme. Thus far, we have not found any growth defects in strains carrying *pnt-1*. Determination of the function will require obtaining a deletion or insertion mutation and possibly transferring it to other genetic backgrounds where the loss of the enzyme is critical. The mapping will facilitate both tasks. For example it may be possible, based on the 59% cotransducibility with *man*, to insert the tetracycline resistance element Tn*10* into the *pnt* locus. This procedure is expected to definitely eliminate the enzyme in vivo and allow positive selection when transducing the mutation to other strains (11).

The F'500 factor carries the *pnt* gene. We did not determine whether this plasmid produces a gene dosage effect, since our recipient strain was *rec*$^+$. If a gene dosage effect is produced in a *recA* strain, the addition of this or another plasmid carrying the *man-pnt* region could be of value for the purification of transhydrogenase.

Using the linkage to *man*, it should be possible to isolate additional transhydrogenase mutants by techniques of localized mutagenesis. If *pnt* proves to be the structural gene for transhydrogenase, mutants which carry out hydrid transfer but are not energy coupled would be of particular interest.

ACKNOWLEDGMENTS

We thank B. Bachmann for providing strains and L. N. Csonka for helpful discussions.

This work was supported by Public Health Service Grant GM-23092 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Anderson, J. J., S.C. Quay, and D. L. Oxender.** 1976. Mapping of two loci affecting the regulation of branched-chain amino acid transport in *Escherichia coli* K-12. J. Bacteriol. **126**:80–90.
2. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Cox, G. B., N. A. Newton, F. Gibson, A. M. Snoswell, and J. A. Hamilton.** 1970. The function of ubiquinone in *Escherichia coli*. Biochem. J. **117**:551–562.
5. **Csonka, L. N., and D. G. Fraenkel.** 1977. Pathways of NADPH formation in *Escherichia coli*. J. Biol. Chem. **252**:3382–3391.
6. **Dancey, G. F., A. E. Levine, and B. M. Shapiro.** 1976. The NADH dehydrogenase of the respiratory chain of *Escherichia coli*. I. Properties of the membrane-bound enzyme, its solubilization, and purification to near homogeneity. J. Biol. Chem. **251**:5911–5920.
7. **Fraenkel, D. G., and S. Banerjee.** 1971. A mutation increasing the amount of a constitutive enzyme in *Escherichia coli*, glucose 6-phosphate dehydrogenase. J. Mol. Biol. **56**:183–194.
8. **Fraenkel, D. G., and S. R. Levisohn.** 1967. Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucoisomerase. J. Bacteriol. **93**:1571–1578.
9. **Gibson, F., J. A. Downie, G. B. Cox, and J. Radik.** 1978. Mu-induced polarity in the *unc* operon of *Escherichia coli*. J. Bacteriol. **134**:728–736.
10. **Hanson, R. L.** 1979. The kinetic mechanism of pyridine nucleotide transhydrogenase from *Escherichia coli*. J. Biol. Chem. **254**:888–893.
11. **Kleckner, N., J. Roth, and D. Botstein.** 1977. Genetic engineering *in vivo* using translocatable drug-resistance elements. New methods in bacterial genetics. J. Mol. Biol. **116**:125–159.
12. **Low, K. B.** 1972. *Escherichia coli* K-12 F-prime factors, old and new. Bacteriol. Rev. **36**:587–607.
13. **Low, B.** 1973. Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. J. Bacteriol. **113**:798–812.
14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
15. **Novel, G., and M. Novel.** 1973. Mutants d'*Escherichia coli* K 12 affectés pour leur croissance sur méthyl-β-D-glucuronide: localisation du gène de structure de la β-D-glucuronidase (*uidA*). Mol. Gen. Genet. **120**:319–335.
16. **Novel, M., and G. Novel.** 1976. Regulation of β-glucuronidase synthesis in *Escherichia coli* K-12: constitutive mutants specifically derepressed for *uidA* expression. J. Bacteriol. **127**:406–417.
17. **Owen, P., and H. R. Kaback.** 1978. Molecular structure of membrane vesicles from *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **75**:3148–3152.
18. **Parkinson, J. S.** 1976. *cheA*, *cheB*, and *cheC* genes of *Escherichia coli* and their role in chemotaxis. J. Bacteriol. **126**:758–770.
19. **Zahl, K. J., C. Rose, and R. L. Hanson.** 1978. Isolation and partial characterization of a mutant of *Escherichia coli* lacking pyridine nucleotide transhydrogenase. Arch. Biochem. Biophys. **190**:598–602.