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Characterization of Pyridoxine Auxotrophs of *Escherichia coli*: Chromosomal Position of Linkage Group I

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The chromosomal location of Group I pyridoxine mutations in *Escherichia coli* is shown to be adjacent to *dsdA*, *aroC*, and *purF* (old *purC*) in *E. coli* B × K-12 hybrids. All mutants previously classified into Group I by nutrition tests and transduction frequency tests are shown to be linked to *dsdA*.

Pyridoxine mutants of *Escherichia coli* B have been divided into five unlinked groups by transduction with Plbt phage (7). The chromosomal position of one of these groups, Group II, has been shown to lie near *pyrA* (7), a position similar to and perhaps identical with that described by Taylor and Trotter for *pdxA1* in strain K-12 (17). The present work reports the location of a second pyridoxine group. This group, Group I, is linked by transduction to *dsdA*, *aroC*, and *purF* (old *purC*) and may in fact lie between the latter two genes. This report also shows that all independent mutants previously assigned to Group I by nutritional tests and transduction frequencies are also linked to *dsdA*. Groups III, IV, and V remain unmapped.

Initial attempts to map the position of the *pdx* Group I locus by time of entry by using direct conjugation between B strains containing *pdx* loci and several Hfr K-12 strains gave meaningless data. Presumably this was due to the restriction between the two strains (3). To overcome this problem, B × K-12 hybrids were constructed by combining the method of Maas and Maas (13) with that of Jacob et al. (9). Conjugations between these hybrids were then used successfully to map the position of the *pdx* locus.

MATERIALS AND METHODS

Strains. The donors of the principal *E. coli* strains used here are listed in Table 1 together with the genotypes of the strains. All B strains used here but not listed in Table 1 are derived from WG1, an *E. coli* B strain provided several years ago by A. L. Koch (5). Many of these strains have recently been described elsewhere (7). Mutants with WG strain numbers higher than 999 and less than 3000 were all derived by the isolation technique presented here.

Phage. Phage P1*bt*, a gift of L. Bertani, was grown on a B strain *E. coli* exclusively. Phage P1*kc*, a gift of W. A. Newton, was grown on B × K-12 hybrids or on K-12 strain *E. coli*. Second cycle stocks of both of these phage strains were prepared as described earlier (7). P1*vir*O was derived from a stock of P1*vir* provided by H. Ikeda and J. Tomizawa by sequential selection of large (2 mm) clear plaques formed on WG1. This selection raised the efficiency of plating on B strain *E. coli* at least 1,000-fold. All three P1 strains were routinely titered on ATCC no. 11126 (*Shigella* sp). An initial stock of T6 phage was provided by J. McCorquodale. High titer stocks of T6 were prepared on WG1 by the method of Swanstrom and Adams (16).

Media. L broth has been described previously (7). VFCG medium contained 0.01% DL-diaminopimelic acid, 0.01% DL-tryptophan, 0.02% DL-alanine, 0.01% D-glutamic acid, and 0.8% charcoal-treated vitamin-free Casamino Acids (Nutritional Biochemical Co.) in glucose minimal medium. Charcoal treatment was accomplished by stirring 100 g of vitamin-free Casamino Acids in 1 liter of H_2O with 30 g of Norit A for 30 min. Charcoal was removed by filtration and the amino acid mixture was stored at 5 C under a layer of toluene. Glucose minimal medium contained 0.2% glucose, potassium phosphate *p*H 7.0 (63 mM), $MgSO_4$ (0.04 mM), $FeSO_4$ (0.01 mM), and $(NH_4)_2SO_4$ (15 mM). LSB agar was glucose minimal agar which contained 0.2% lactose, 0.024% streptomycin, 0.00001% pyridoxol, and 1.7% agar instead of glucose. GBA-agar was glucose minimal medium with 1.7% agar, 0.00001% pyridoxol, 0.005% L-tryptophan, 0.005% L-phenylalanine, 0.005% L-tyrosine, and 0.002% *p*-aminobenzoic acid.

Isolation of E. coli B mutants. Immediately after being treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Adelberg et al., (1), 10^9 saline-washed cells of WG1 were put in 10 ml of L broth at 45 C and incubated overnight at 45 C with shaking (15). The cells were centrifuged, washed once with 0.9% NaCl, and suspended in 10 ml of 0.9% NaCl.

TABLE 1. *List of bacterial strains*

Number	Strain	Genotype		Source
AB444	K-12	*str*-8,-9 or 14 (R), *aroC4, purF1, ton-2, lac-1 or -4, tsx-23, or -25, thi-1,-2 or 12, arg-1, mtl-2, xyl-4, mal-1*	F^-	E. A. Adelberg
AT700	K-12	*his-1, strA1, malA2, aroE24, argG6, tsx-1, thi-1*? $(\lambda)^-$	F^-	A. L. Taylor
AT722	K-12	*strA1, malA1, argG6, pyrE41, rbs-1, thi-1,* (λ)	F^-	A. L. Taylor
EM1101	K-12	*dsdA1, dsdC1, lac, str*	F^-	E. McFall
E′ 36	K-12	lac^{del}, $F lac^{Ts}$	F^+	A. W. Newton
KL16	K-12	*thi*, λ^-	Hfr	B. Low
SB16	K-12	*met, nic, thi*	Hfr	R. Ghoulson
WG1010	Hybrid	*pdxB3, pyrE41, strA1, tsx*		This report
WG1012	Hybrid	*pdxB3, his-1, str, tsx-1*		This report
WG1013	Hybrid	*pdxB3, his-1, aroE24, str-1, tsx-1*		This report
WG1095	B	*dsdA*		This report
WG535	B	*pdxB3, lac, str*		
WG3	B	*pdxB3*		

Of this suspension, 0.5 ml was used to inoculate 50 ml of VFCG medium, and the resulting culture was shaken 90 min at 37 C. The cells were removed by centrifugation, washed with 0.9% NaCl, suspended in glucose minimal medium, and shaken 2 to 4 hr. Penicillin was then added to 1,000 units/ml, and shaking was continued 3 to 4 hr. Cells were then centrifuged, washed, and finally suspended in 1 ml of 0.9% NaCl. One-tenth milliliter each of $1:10^2$, $1:10^3$, and $1:10^4$ dilutions were spread on appropriate agar plates and mutants were detected by the replica plate technique. For the isolation of pyridoxineless mutants, the VFCG medium was 6×10^{-7} M in pyridoxol or pyridoxal. For the isolation of $DsdA^-$ (lacking D-serine deaminase) mutants the VFCG medium was 17 mM in D-serine (14).

Table 2 shows that this method of mutant isolation allows recovery of pyridoxineless mutants with the same frequency as amino acid auxotrophs. For these data, penicillin-treated cells from 11 separate experiments were plated on glucose minimal agar supplemented with threonine, methionine, and tryptophan each at 100 μg/ml and adenine and guanine each at 50 μg/ml in addition to pyridoxal. Requirement for each of these supplements was tested and the number of auxotrophic colonies of each type from each experiment was recorded.

Construction of strain WG1012. *E. coli* B strain WG535 was first converted to an Hfr strain by heat curing a thermosensitive F *lac* episome introduced from *E. coli* E′ 36 (9). To do this, 10^9 cells of the Lac^+ E′ 36 culture (grown overnight in L broth at 25 C with shaking) were mixed, in a total volume of 5 ml, with 10^9 cells of WG535 from a culture growing exponentially at 37 C in L broth. The mixture was held 1 hr at room temperature and then 0.1-ml samples were spread on LSB agar plates. Several large colonies developed on each plate after two days at 42 C. Sixty-four of these were purified by three consecutive single colony isolations at 42 C. As described by Jacob et al. (9), the resulting Hfr strains, segregated into Lac^+ and Lac^- colonies when grown at room temperature. Three separately isolated and randomly selected Lac^+ colonies were used as donors of the *pdxB3* locus to *E. coli* K-12 strain AT700.

TABLE 2. *Frequency of occurrence of mutant colonies*[a]

Phenotype	Number of colonies	Minimum number of separate genetic events	Frequency
Threonineless.....	15	6	0.13%
Methionineless....	39	10	0.34%
Tryptophanless....	6	6	0.05%
Threonine-methionineless.....	11	7	0.09%
Purineless.....	4	3	0.03%
Pyridoxineless....	15	6	0.13%

[a] Number of colonies examined, 11,443; separate experiments, 11.

To do this, strain AT700 and the three presumed Hfr derivatives of WG535 were each grown to 4×10^8 cells/ml in L broth at 37 C with shaking. For each mating, the bacteria were brought into contact by filtering alternately three one-drop portions of both sexual types through the same spot on a 0.47-μm membrane filter (Millipore Corp., Bedford, Mass.) (13). The filter was put in a small flask, covered with 1 ml of L broth and left undisturbed for 2 hr at 42 C. The flask was then shaken 5 min at 37 C, 3×10^{10} T6 phage were added in 1 ml of L broth, and the mixture was shaken another 30 min (8). Three drops of each mating mixture were then plated on GBA agar plates containing either L-arginine (0.01%) or L-histidine (0.01%), and the plates were incubated at 37 C. His^+ and Arg^+ colonies from each mating were picked and streaked on plates identical to the original ones. Replica plating showed all of the 64 His^+ clones had WG535 phenotype, whereas, of

107 Arg$^+$ clones, 84 grew without pyridoxol, 19 had WG535 phenotype, two grew poorly on all media, and the remaining two, WG1012 and WG1013, had the characteristics listed in Table 1. A third hybrid strain, WG1010, was isolated in a similar manner from matings between the WG535 Hfr strains and a spontaneous T6-resistant mutant of AT722.

Interrupted mating between KL16 and WG1012. Exponentially growing aerobic cultures of both organisms were mixed in the ratio 5 × 10^9 WG1012 to 2.5 × 10^8 KL16 in 5 ml of L broth at 37 C. Five minutes after mixing, the mixture was diluted 1:1,000 in warm L broth and allowed to shake gently at 37 C. Samples of 0.2 ml were withdrawn at 1-min intervals, disrupted as described by Low and Wood (11), and plated alternately on glucose minimal medium containing 0.024% streptomycin and either 0.01% L-histidine or 0.00001% pyridoxol.

Other conjugations were performed with WG1010 and Hfr strains: Cavalli, AB312, and AB313. For these, 10^9 cells of exponentially growing WG1010 were mixed in a total volume of 15 ml of L broth at 37 C with 10^9 cells of an exponentially growing Hfr strain. These conjugations were stopped by adding T6 phage to samples withdrawn at various times (8). The mixtures were then plated on glucose minimal medium containing either 0.005% uracil or 0.00001% pyridoxol.

Miscellaneous. Transductions with K-12 strains were as performed by Luria et al., (12); B strains were transduced as reported earlier (7).

RESULTS

E. coli strains frequently restrict deoxyribonucleic acid (DNA) injected from other *E. coli* strains perhaps by using a nuclease which degrades foreign DNA (3). This property frequently makes interrupted conjugation experiments between strains useless as a means of genetic mapping by time of entry. Since the restriction phenomenon may extend to all foreign DNA regardless of how it entered the cell (2), and, since B × K-12 hybrids such as those isolated here may retain the B strain type of restriction, the restriction of each of the hybrids was tested by measuring their ability to plate three different strains of P1 phage, namely P1*bt*, P1*kc*, and P1-*vir*O grown on either K-12 or B strains. Table 3 shows that both WG1010 and WG1012 appeared to have K-12 type of restriction, and thus were probably suitable females for mating with K-12 Hfr strains.

Conjugations performed between WG1010 and AB312, AB313, and Hfr Cavalli indicated that *pdxB3* was at 40, 46, and 36 min, respectively, on the chromosomal map of *E. coli* B. The final determination of the chromosomal position of *pdxB3* was made from data obtained from WG1012 × KL16 crosses (Fig. 1). In three separate experiments the time of entry for *pdxB3* was 6.7, 6.8, and 6.9 min before *his*, placing *pdxB3* at minute 45 on the most recent chromosomal map of *E. coli* (17).

TABLE 3. *Efficiencies of plating of P1 phage on E. coli B strains as a measure of interstrain restriction*[a]

Donors	P1*bt*		P1*kc*		P1*virO*
	WG1095	WG3	WG1012	EM1101	SB16
WG3	0.4	0.2	0.005	0.002	0.01
AT700	0.01	0.003	0.3	0.06	0.3
AT722	0.02	0.002	0.2	0.2	0.4
B×K12 hybrids					
WG1010	0.01	ND[b]	ND	0.3	0.6
WG1012	0.1	0.002	0.5	0.3	0.5

[a] Numbers of plaques formed on ATCC number 11126 (*Shigella* sp.) was assigned an efficiency of 1 in each case.

[b] ND = No data.

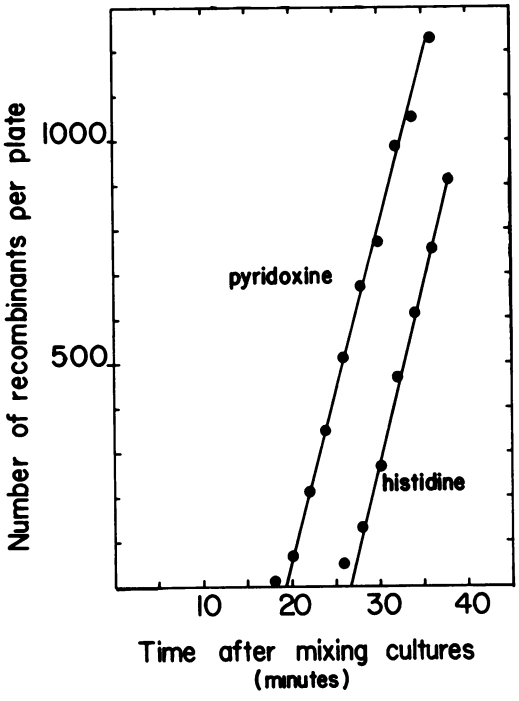

FIG. 1. *Pdx$^+$ and His$^+$ recombinants as a function of time after conjugation.*

A more exact location of the *pdxB3* gene was then made by transduction. To do this, crosses mediated by P1*kc* were performed between WG1012 and both EM1101 a strain carrying the *dsdA1* marker (14) and AB444 a strain carrying both *aroC* and *purF* (old *purC*). The position of these markers is shown in Fig. 2. The data in

Table 4 show that *pdxB3* appears to lie between *aroC* and *purF*.

This latter conclusion was drawn from consideration of the two possible configurations, namely *pdx* lies between the other two markers (I, Fig. 2) or *pdx* lies outside the other two markers (II, Fig. 2). The phenotype Aro$^+$ Pdx$^+$ Pur$^+$ is obtained in AB444 × WG1012 crosses by two crossovers in II regardless of which strain is recipient, but four crossovers are required in I when WG1012 is donor. In I, then, Aro$^+$ Pdx$^+$ Pur$^+$ should occur rarely when WG1012 is donor; instead Aro$^+$ Pdx$^-$ Pur$^+$ transductants should predominate whenever Aro$^+$ Pur$^+$ is selected. The data in Table 4 show that the phenotype Aro$^+$ Pdx$^+$ Pur$^+$ does occur only rarely in crosses with WG1012 as donor, and, therefore, the probable configuration is that in I. Further support is lent to this configuration by the data in Table 4 which show only 30–40% coinheritance of Pdx$^-$ when selection for either outside marker alone is made.

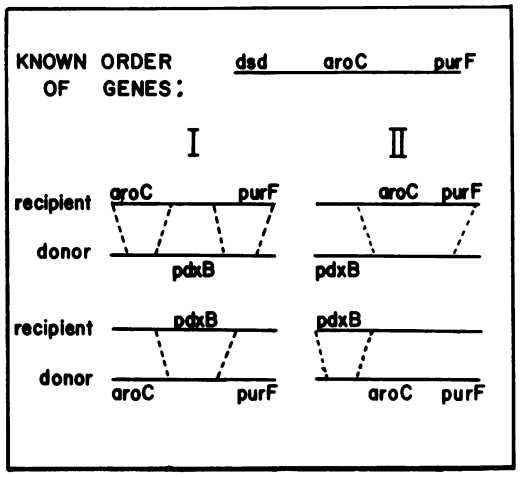

FIG. 2. *Schematic representation of possible orientation of genes near Group I pdx mutations in E. coli.*

With the above information, it was now reasonable to test whether all pyridoxineless mutants in *E. coli* strain B which had been assigned to Group I on the basis of both nutritional properties and transduction frequencies (7) were also linked to *dsdA*. To do this, a DsdA$^-$ mutant was isolated in *E. coli* by the general procedure described by McFall (14) and was then used to prepare a stock of P1*bt* phage. All the *pdx* mutants in Group I were transduced to Pdx$^+$ with this phage stock, and the percentage of Dsd$^-$ phenotypes among them was measured. One representative of each possible nutritional type in Group I was used as a phage donor for reciprocal crosses. The data in Table 5 show that all markers previously classified as Group I are linked to *dsdA*. For completeness, this table shows data from other experiments in which members of each of the other linkage groups were used as recipients.

DISCUSSION

The location of two groups of pyridoxine mutations in widely separated places on the bacterial chromosome confirms the previous evidence that showed these two groups were unlinked and suggests as a corollary that control by repression, if it occurs in the biosynthesis of pyridoxine, must be of the regulon rather than the operon type. That is, it must be analogous to that system which controls arginine biosynthesis (10), instead of being like the one which controls histidine biosynthesis. It is pertinent now to point out that all these Pdx$^-$ organisms are of this phenotype because they all grow fully in minimal medium supplemented only with normal or physiological amounts of pyridoxine (6 ×

TABLE 4. *Results of Plkc transductions*

Donor strain	Recipient strain	Selected marker	No. of transductants	Unselected marker	No. of cotransductants	Cotransduction
						%
AB444	WG1012	Pdx$^+$	327	Pur$^-$ Aro$^+$	94	29
				Aro$^-$ Pur$^+$	65	20
				Aro$^-$ Pur$^-$	91	28
WG1012	AB444	Pur$^+$	47	Pdx$^-$ Aro$^-$	13	28
				Aro$^+$ Pdx$^+$	9	19
				Aro$^+$ Pdx$^-$	4	8
		Aro$^+$	10	Pdx$^-$ Pur$^-$	4	40
				Pur$^+$ Pdx$^+$	1	10
				Pur$^+$ Pdx$^-$	4	40
		Aro$^+$ Pur$^+$	78	Pdx$^-$	72	92
EM1101	WG1012	Pdx$^+$	224	Dsd$^-$	57	25

TABLE 5. *Linkage of E. coli B Group I mutants to dsd*

Donor	Recipient	Selected marker	No. of transductants	Unselected marker	No. of cotransductants	Linkage
						%
WG1095	WG3 (*pdxB3*)	Pdx+	213	Dsd−	63	29
	WG59 (*pdxB59*)		68		28	41
	WG142 (*pdxB142*)		42		10	24
	WG82 (*pdxB147*)		118		31	26
	WG150 (*pdxB150*)		90		27	30
	WG139 (*pdxC139*)		190		67	35
	WG140 (*pdxC140*)		204		73	36
	WG15 (*pdxD15*)		251		102	41
	WG60 (*pdxD60*)		121		44	36
	WG1009 (*pdxD137*)		124		52	42
	WG1080 (*pdxD158*)		67		27	40
	WG73 (*pdxE73*)		127		0	0
	WG5 (*pdxF5*)		128		1	0
	WG25 (*pdxG25*)		224		0	0
	WG2 (*pdxH2*)		137		0	0
	WG1027 (*pdxJ151*)		133		0	0
WG2	WG1095 (*dsd*)	Dsd+	155	Pdx−	0	0
WG3			175		47	27
WG5			38		0	0
WG15			72		26	36
WG25			145		0	0
WG139			160		37	23
WG140			153		32	21

10^{-7} M; 6). It has already been shown that this amount of pyridoxol is both similar to the amounts found in a fully grown culture and adequate, when added to a culture, to stop de novo biosynthesis of vitamin B_6 (5), but, in the absence of information about pyridoxine biosynthesis, enzymatic tests have not been made on the nature of the mutations in the mutants studied here.

The method for isolation of pyridoxine mutants was derived from the following considerations. (i) Most amino acids have a pyridoxal 5′-phosphate-requiring enzyme involved in their biosynthesis. (ii) Most amino acid biosynthesizing systems would be repressed in *E. coli* growing in media containing all amino acids. Starvation of a Pdx− mutant for amino acids at the same time starvation for pyridoxine was initiated would derepress the amino acid biosynthesis system which might then bring growth to a more rapid halt than is usually found in vitamin starvations (18), by forcing the amino acid pools to be used for making the amino acid biosynthetic enzymes. A Pdx− mutant would be unable to make the pyridoxal 5′-phosphate required as coenzyme at some step in most amino acid biosyntheses and therefore the depleted amino acid pool could not be replenished. The organisms would then stop growing quickly enough to survive penicillin treatment better than they did in simple pyridoxine starvations (6).

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