JOURNAL OF BACTERIOLOGY, Nov. 1973, p. 751-756
Vol. 116, No. 2

Escherichia coli K-12 Mutants Altered in the Transport Systems for Oligo- and Dipeptides

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Received for publication 18 June 1973

Two mutants of *Escherichia coli* K-12, defective in the oligopeptide and dipeptide transport system, are described. A mutant defective in the oligopeptide transport system (*opp-1*) was isolated as resistant to the inhibitory action of triornithine; this mutant is also resistant to glycylglycylvaline and does not concentrate ^{14}C-glycylglycylglycine, although it is still as sensitive as the parental strain to glycylvaline and valine. Starting from the *opp-1* strain, a mutant defective also in the dipeptide transport system (*dpp-1*) was isolated; this mutant is resistant to the inhibitory action of glycylvaline, valylleucine, and leucylvaline and does not concentrate ^{14}C-glycylglycine, although it is still as sensitive as the parental strain to valine. The apparent kinetic constants for oligopeptide and dipeptide transport were measured. The *opp* marker is co-transducible with *trp* at 27 min on the *E. coli* genetic map. The *dpp* locus is separated from *opp* and is located between *proC* (10 min) and *opp*.

We recently described (4) the isolation and some properties of *Escherichia coli* K-12 mutants that are resistant to the inhibition that valine exerts on the growth of this bacterium. The Val^r (valine-resistant) phenotype was expressed only if leucine were also present, and the observation that leucine could not be substituted by dipeptides containing it suggested that its action was exerted at the transport level. In fact, it is known that leucine and valine share a common transport system(s) (9) and that dipeptides are taken up by *E. coli* through a transport system(s) different from those used by the amino acids (7). For example, Levine and Simmonds (6) found that a glycine auxotroph altered in the transport system for glycine grew better in a medium containing glycylglycine than in one containing an equimolar concentration of glycine. We have exploited this as a criterion to isolate transport mutants. A mutant altered in valine transport should be Val^r but sensitive to glycylvaline inhibition (Glyval^s). A description of the transport systems for isoleucine, leucine, and valine is reported elsewhere (J. Guardiola, M. De Felice, T. Klopotowski, and M. Iaccarino, manuscripts submitted for publication; reference 4). Studies on the uptake and utilization of peptides in *E. coli* W have been described by Payne and Gilvarg (8) and Sussman and Gilvarg (10), who report that the uptake occurs through several systems for dipeptides and one system for oligopeptides. These authors also report the isolation of an *E. coli* W mutant (3, 8) that is altered in the oligopeptide transport system; unpublished evidence for an *E. coli* K-12 mutant altered in the same transport system is quoted in reference 8. A report on a *Salmonella* mutant altered in the oligopeptide transport system has also recently appeared (1).

In this paper we report experiments on the isolation and characterization of *E. coli* K-12 mutants altered in the oligopeptide and dipeptide transport systems. We also show that differential sensitivity to valine, glycylvaline, or glycylglycylvaline of these mutants is a valid criterion for defining them as transport mutants.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains of *E. coli* K-12 used.

Reagents and media. [Glycyl-*1*-^{14}*C*]glycine hydrochloride (12 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, England. *1,4,7*-^{14}*C*-glycylglycylglycine (181 mCi/mmol) was the generous gift of H. R. Kaback. The amino acids used were all L-forms of the highest purity available. Dipeptides and tripeptides were obtained from Miles Laboratories Inc., Elkhart, Ind., or from Nutritional Biochemicals Corp., Cleveland, Ohio. Dansyl-chloride was purchased from Sigma Chemical Co., St. Louis, Mo.,

TABLE 1. *Bacterial strains*[a]

Strain	Genotype and origin
X478	*thi, leuB, metE, proC, purE, trp, lysA, ara, xyl, lacZ, azi, str, tonA, tsx*, F^-; from P. Berg.
MI229	*thi, leuB, metE, proC, purE, trp, lysA, ara, xyl, lacZ, azi, str, tonA, tsx, opp-1*, F^-; spontaneous from X478.
MI232	*thi, leuB, metE, proC, purE, trp, lysA, ara, xyl, lacZ, azi, str, tonA, tsx, opp-1, dpp-1*, F^-; spontaneous from MI229.
Ca85	*thi, his, lac*^{am}, HfrH; from J. Beckwith.

[a] Symbols for genetic markers are those used by Taylor (11). The *opp* and *dpp* symbols are the loci defining genes for the transport of oligo- and dipeptides described in this report.

whereas micropolyamide plastic sheets were obtained from Ernst Schütt jun., Göttingen, Germany. Chloramphenicol was obtained from Boehringer, Mannheim, Germany; tri-L-ornithine·4HCl was from Miles-Yeda Ltd., Kankakee, Ill. Glycylglycylvaline was purchased from Nutritional Biochemicals Corp.; it contained roughly 10% impurity of valine and 10% of glycylvaline as judged by the intensity of the spot on a chromatogram (see below). "Minimal citrate" medium (12) was used in most experiments. Usual supplements, when required, were glucose (0.4%), L-tryptophan (25 μg/ml), L-valine (100 μg/ml), other L-amino acids (50 μg/ml), nucleosides (50 μg/ml), and thiamine (10 μg/ml).

Isolation of independent mutants resistant to glycylvaline. A suspension of bacteria was diluted to a concentration of about 100 cells/ml in a minimal medium containing all the supplements needed by the strain for growth. The suspension was divided into 1-ml portions which were incubated overnight. From each tube 0.1-ml samples were spread on plates supplemented with glycylvaline and other supplements. From each plate only one colony was purified and retained for further characterization.

Inhibition tests. A 0.1-ml amount of cell suspension grown overnight in minimal citrate medium was diluted in 3 ml of minimal citrate containing 0.7% agar and layered on supplemented minimal citrate plates; 10 μliters of a 5 mg/ml solution of the substance to be tested was pipetted onto a small disk (6 mm in diameter) of Whatman no. 3 MM paper applied on the agar surface. Inhibition was observed after overnight incubation at 37 C.

Transport assays. Bacteria were grown in minimal citrate medium, harvested by centrifugation in middle logarithmic phase, and washed twice with unsupplemented medium. Determination of rate of uptake was performed with a modification of the method already described (J. Guardiola et al., manuscript submitted for publication; references 4, 9). The washed cells were resuspended to give a final concentration of 10 mg/ml (dry weight) in minimal medium containing 300 μg of chloramphenicol per ml and 0.4% glucose. The bacterial suspension was kept at room temperature, and uptake was measured after 10 min by pipetting 0.1 ml of the bacterial suspension into a test tube containing 0.85 ml of a given concentration of the ^{14}C-substrate at 37 C. After 0.5 min the sample was filtered onto the center of a membrane filter (Millipore, type HA, 0.45 μm, 25 mm). The cells were immediately washed on the filter with 5 ml of unsupplemented medium at room temperature. Radioactivity of the dried filters was measured as described in reference 4. K_m and V_{max} values were evaluated by plotting $1/_c$ versus $1/_v$, where c is the concentration of the substrate expressed in micromoles per liter and v is the micromoles of ^{14}C-substrate incorporated per 0.5 min per gram of cells (dry weight).

Determination of valine and glycylvaline impurities in glycylglycylvaline. Equal volumes of 2×10^{-3} M valine, or glycylvaline, or glycylglycylvaline and dansyl-chloride (5 mg/ml in acetone) were mixed and incubated at 37 C for 2 h. Two dimensional chromatograms were developed by the method of Hartley (5) in (i) benzene:acetic acid (45:5), and (ii) water:formic acid (97:3). The dansylated derivatives were detected by using an ultraviolet lamp.

RESULTS

A mutant altered in the transport of valine is expected to be resistant to valine inhibition but sensitive to the inhibition caused by the valine contained in dipeptides or oligopeptides. Conversely, a mutant altered in the dipeptide or oligopeptide transport systems should be resistant to the inhibition caused by valine-peptides but sensitive to valine. To confirm this assumption, we isolated mutants altered in the oligopeptide and dipeptide transport system. A search was made for mutants resistant to glycylvaline and sensitive to valine but without success, probably because dipeptides use not only the dipeptide but also the oligopeptide transport system (8). We therefore looked for mutants resistant to glycylglycylvaline and sensitive to valine, again with no success. Since an *E. coli* W mutant altered in the transport of oligopeptides has been isolated on the basis of its resistance to triornithine (8), we decided to look for this mutation also in *E. coli* K-12. Among four different K-12 strains of our collection, two were already resistant to triornithine and two were sensitive but showed a great number of resistant mutants in their population. In Fig. 1A, an inhibition test of strain X478 shows that triornithine inhibits the growth of this strain and that in the inhibition zone many mutants appear. One of these mutants (MI229) was picked, purified, and tested for inhibition by triornithine. Data illustrated in Fig. 1B show that no inhibition occurs in strain MI229. Fig.

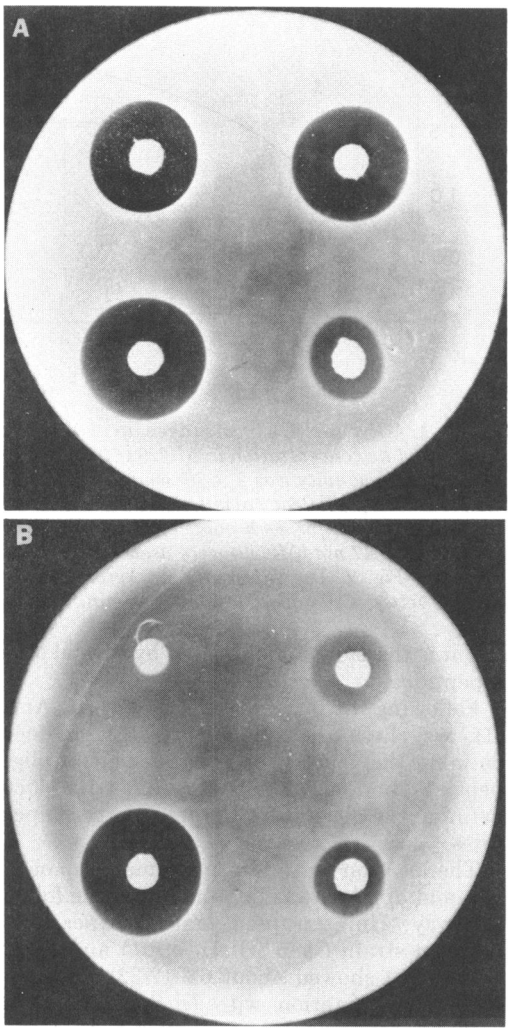

FIG. 1. *Inhibition of strain X478 (A) and MI229 (B) by different compounds. Top left, 75 nmol of triornithine; top right, 100 nmol of glycylglycylvaline; bottom left, 100 nmol of glycylvaline; bottom right, 250 nmol of valine.*

1A and B also show that glycylglycylvaline inhibits strain X478 but inhibits strain MI229 much less; this residual inhibition is probably due to glycylvaline and valine impurities which we detected (see Materials and Methods) in the sample of glycylglycylvaline we used. (These impurities explain the failure to isolate a mutant resistant to glycylglycylvaline.) An amount of glycylvaline equimolar to glycylglycylvaline gives an inhibition zone equal to that of the tripeptide in strain X478. Glycylvaline and valine give the same inhibition zone in strain X478 as in strain MI229. Since strain X478 is a leucine auxotroph, the plate contains leucine; therefore, since valine and leucine share a common transport system(s) (4, 9), valine inhibition cannot be compared on a molar basis with the inhibition caused by dipeptides containing valine.

We conclude from this experiment that strain MI229 is altered in the oligopeptide transport system because triornithine does not inhibit the growth of the mutant and glycylglycylvaline shows less inhibition, whereas the inhibition caused by glycylvaline and valine is not decreased.

Figure 2 shows the initial rate of uptake of ^{14}C-glycylglycylglycine in strains X478 and MI229 as a function of its concentration. A striking difference is evident in the rate of uptake between these two strains; the apparent kinetic constants of glycylglycylglycine uptake are: K_m, 1.1 μM, and V_{max}, 2.5 μmol per 0.5 min per g of cells. These constants could not be measured in the case of strain MI229 because uptake was too low.

Strain MI229 was used to look for a dipeptide transport mutant: if the failure to find such a mutant was due to the passage of the dipeptide through the oligopeptide transport system, it should now be found starting from strain MI229. In fact, among 30 spontaneous mutants independently isolated as resistant to glycylvaline, 13 were sensitive to valine. One of these (MI232), after purification, was chosen for fur-

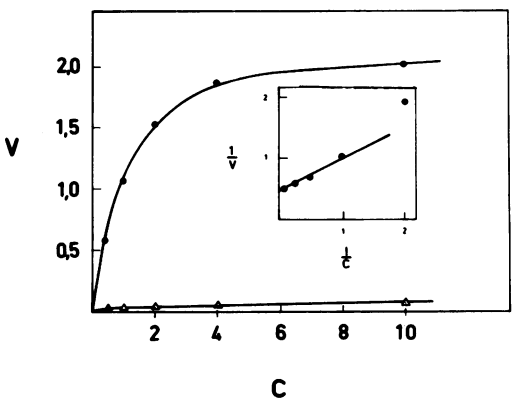

FIG. 2. *1,4,7^{14}C-glycylglycylglycine taken up in 0.5 min as a function of its concentration in X478 (●) and MI229 (▲). Specific activity 10^7 counts per min per μmol (counting efficiency about 85%). Each point was in duplicate, and for each point 0.72 mg of bacteria (X478) or 0.64 mg (MI229) were used. v, Micromoles of ^{14}C-substrate taken up per 0.5 min per gram of cells. c, Micromolar concentration of substrate. A background value was determined at each concentration used and is subtracted in the data of the figure. It ranged between 90 and 350 counts per min.*

ther study. This strain (Fig. 3) is still sensitive to valine, but it is resistant to glycylvaline and also to valylleucine or leucylvaline. The initial rates of uptake of ^{14}C-glycylglycine in strains MI229 and MI232 as a function of concentration are reported in Fig. 4. The figure shows a striking difference in the rate of uptake between these two strains; the apparent kinetic constants of glycylglycine uptake are: K_m, 1×10^{-5} M, and V_{max}, 5 μmol per 0.5 min per g of cells in the case of MI229; they are not measurable in the case of MI232. We conclude that the transport system for glycylvaline is also used by valylleucine, leucylvaline, and glycylglycine,

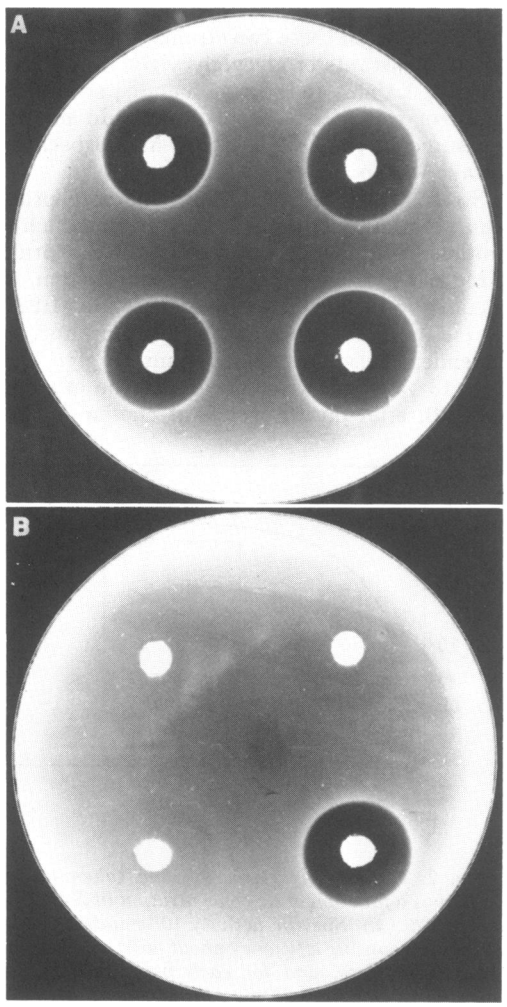

FIG. 3. *Inhibition of MI229 (A) and MI232 (B) by different substances. Top left, 100 nmol of glycylvaline; top right, 100 nmol of valylleucine; bottom left, 100 nmol of leucylvaline; bottom right, 200 nmol of valine.*

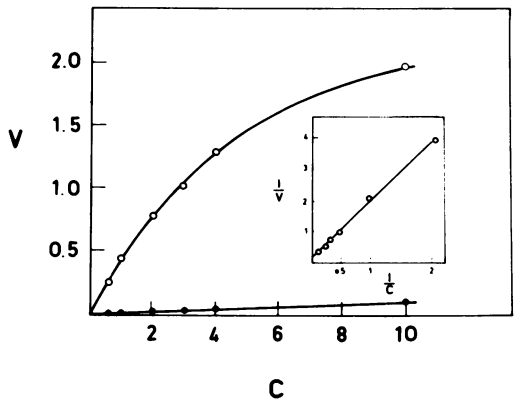

FIG. 4. *[Glycyl-1^{14}C]glycine taken up in 0.5 min as function of its concentration in MI229 (○) and MI232 (●). Specific activity was 2×10^7 counts per min per μmol (counting efficiency about 85%). Each point was in duplicate, and for each point 0.49 mg of bacteria (MI229) or 0.47 mg (MI232) were used. v and c units are as in Fig. 2. The subtracted background values ranged between 80 and 360 counts per min.*

and it is therefore possibly used by several other dipeptides.

Following the terminology proposed by Ames (1), we chose the symbol *opp* for the locus defining the gene(s) for the transport of oligopeptides and the symbol *dpp* for the locus defining the gene(s) for the transport of dipeptides.

The location on the chromosomal map of the *opp* and *dpp* loci was determined in the following way. Uninterrupted mating experiments between strain Ca85 (HfrH, *opp*$^+$) and MI229 (F$^-$, *opp*$^-$) showed about 6% (2/32) linkage of the *opp-1* mutation with *leuB*, 12.5% (4/32) with *proC*, 73% (22/32) with *trp*, 25% (8/32) with lysA, and 15% (5/32) with *metE*. This result indicates that the *opp-1* mutation is located very close to the *trp* mutation of strain MI229, and for this reason we performed the three-point test described in Table 2. Recombinants (160) from a new mating between strains Ca85 and MI229 were analyzed for the linkage of *trp* or *opp-1* with *proC*. The rarest class was *pro*$^+$, *trp*$^-$, *opp*$^+$, suggesting that the *opp* locus lies on the right side of *trp* (Table 2).

The *opp-1* mutation is co-transducible with *trp*. When strain MI229 was treated with P1 grown on a wild-type *E. coli* and *trp*$^+$ transductants were selected, 54% (48/88) of them were *opp*$^+$ (sensitive to triornithine). The three-point test of the previous experiment suggests that *opp* lies close to *cysB* and *pyrF*. An analysis of the deletion present among the selected *tonB* mutants should easily confirm this location.

Uninterrupted mating experiments between strains MI232 (F^-, *opp-1*, *dpp-1*) and Ca85 (HfrH, opp^+, dpp^+) were performed to locate the *dpp-1* mutation. pro^+ recombinants were analyzed for glycylvaline or triornithine resistance; as shown in Table 3, the three-point test indicates that (i) *opp* can be separated from *dpp*, and (ii) *dpp* lies between *proC* and *opp*, probably closer to the former. An analogous result was obtained when leu^+ recombinants were selected in a conjugation of the same strains.

DISCUSSION

The use of radioactive precursors shows that the *opp-1* and *dpp-1* mutations described in this paper cause an alteration of the respective transport systems and not of the peptidases by which oligo- and dipeptides are utilized. Furthermore, the Michaelis-Menten type of kinetics (Fig. 2, 4) shows that these systems can be saturated, as is usual for a transport system. The very low uptake of substrate found in strains MI229 and MI232 indicates that other transport systems for the same substrates but with different affinities are not present in these mutants; the experiments of Fig. 2 and 4 cannot exclude, however, a system with much lower V_{max} or a system with much lower affinity.

The results reported in this paper show that it is possible to isolate mutants in the oligopeptide and dipeptide transport system. Results of Tables 2 and 3 show that the altered genes are chromosomal and are separated from each other. The K-12 strain of *E. coli* is particularly suited for genetic experiments, and therefore combined genetic and biochemical studies of oligo- and dipeptide transport can be initiated. These transport systems might be useful for utilization by the cell of substances otherwise excluded (see discussion in references 1 and 3).

We also show that resistance to one of the following substances, valine, or glycylvaline, or glycylglycylvaline, accompanied by sensitivity to the other two substances is a good criterion for defining transport mutants. This criterion can also be used in the case of mutants resistant to an analogue of an amino acid, provided that a dipeptide or oligopeptide of the analogue is available and that it is taken up by the cell. If the mutant is resistant to the inhibitory action of all classes of the analogue, it should be a regulatory mutant; otherwise, it should be a transport mutant. Differential sensitivity to an amino acid and its dipeptides should also be useful in all cases in which it is possible to isolate a mutant sensitive to an amino acid because of abnormal feed-back inhibition of any biosynthetic enzyme (for example, see reference 2). These mutants could give pseudorevertants that might be extragenic suppressors; differential resistance or sensitivity to oligo- or dipeptides containing that amino acid would establish whether the additional mutations determine a regulatory or a transport alteration. We are regularly using the criterion of resistance to valine and glycylvaline to isolate regulatory mutants in which the biosynthesis of isoleucine and valine is affected.

TABLE 2. *Conjugation of strain MI229 (F^-, opp-1) with strain Ca85 (HfrH, opp^+) and selection of Pro^+ recombinants*

trp	*opp*	Frequency (%)
+	+	19 (31/160)
−	−	65 (105/160)
+	−	14 (23/160)
−	+	0.6 (1/160)

	Ca85	+	+	+
		proC	*trp*	*opp-1*
MI229		−	−	−

TABLE 3. *Conjugation of strain MI232 (F^-, opp-1, dpp-1) with strain Ca85 (HfrH, opp^+, dpp^+) and selection of Pro^+ recombinants*

dpp	*opp*	Frequency (%)
+	+	6 (6/96)
+	−	64 (63/96)
−	+	<1 (0/96)
−	−	28 (27/96)

	Ca85	+	+	+
		proC	*dpp-1*	*opp-1*
MI232		−	−	−

ACKNOWLEDGMENTS

We thank H. R. Kaback for a very generous gift of ^{14}C-glycylglycylglycine and Bruno Esposito for skillful technical assistance.

ADDENDUM IN PROOF

Whereas our data show 50% co-transduction of *opp* with *trp*, Z. Barak and C. Gilvarg (J. Biol. Chem. in press) find a higher (80 to 95%) co-transduction frequency of these markers. We think that the discrepancy might be due to one of the following reasons: (i) the genes involved are different; (ii) our co-transduction frequency is lowered by either the reported appearance of frequent *opp* strains in a culture of strain X478 (see Fig. 1) or by a deletion possibly present in this strain.

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