GLYCYL-L-LEUCINE-RESISTANCE MUTATION AFFECTING TRANSPORT OF BRANCHED-CHAIN AMINO ACIDS IN *SALMONELLA TYPHIMURIUM*

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Branched-chain amino acids are incorporated in cells of *Salmonella typhimurium* through multiple, stereospecific transport systems, including at least three components (Kiritani and Ohnishi 1978). The transport activity in a wild-type strain is repressed when cells are grown in minimal medium containing leucine or several glycyl-dipeptides, especially glycyl-L-leucine (Kiritani and Ohnishi 1977).

Existence of multiple transport systems (two general systems, high- (LIV-I) and low-affinity (LIV-II), and a leucine-specific system) has also been reported in *Escherichia coli* (Furlong and Weiner 1970; Guardiola *et al.* 1974a, b; Rahmanian *et al.* 1973; Wood 1975). Two of these systems, LIV-I and the leucine-specific, are repressible by leucine (Guardiola *et al.* 1974a; Penrose *et al.* 1968; Quay *et al.* 1975b; Rahmanian *et al.* 1973; Templeton and Savageau 1974), apparently through participation of leucyl-transfer ribonucleic acid (Quay *et al.* 1975a; Quay and Oxender 1976). Two derepressed mutant strains in the transport of branched-chain amino acids were isolated (Rahmanian *et al.* 1973), and the mutant loci, *livR* and *lstR*, were located at 20 min on the genetic map (Anderson *et al.* 1976). The transport activity in a strain containing an altered transcription termination factor rho is also derepressed (Quay and Oxender 1977).

In *S. typhimurium*, we have recently found that growth of an isoleucine-valine requiring strain KA931 (*ilvC8*) can be strongly inhibited in minimal medium containing isoleucine and valine by excess glycylleucine, presumably due to repression of the transport systems for branched-chain amino acids (Kiritani and Ohnishi 1977). On the basis of this observation, we have attempted to obtain derepressed transport mutants for branched-chain amino acids from among those selected for glycyl-L-leucine-resistance. In this paper, we report on the selection procedure and characterization of glycyl-L-leucine-resistant strains having derepressed transport activity for branched-chain amino acids.

MATERIALS AND METHODS

Bacterial strain. All strains used in this study were derivatives of *S. typhimurium* LT2. Strains KA222, KA223, and KA224 were isolated as glycyl-L-leucine-resistant mutants (Gle^r) from strain KA931 (*ilvC8*).

Media. The composition of minimal medium was described previously (Kiritani 1974). Unless otherwise mentioned, supplements, when required, were L-isoleucine (10 μg/ml), L-valine (20 μg/ml), L-leucine (10 μg/ml), various amounts of glycyl-L-leucine as indicated in the text, and Ca-pantothenate (1 μg/ml). Penassay broth (antibiotic medium 3, Difco) was used as a nutrient broth. For an agar medium, 1.5% agar was added.

Bacterial growth. From an overnight culture in minimal medium supplemented with isoleucine, valine, leucine and pantothenate, cells were harvested by centrifugation, washed twice with minimal medium, and suspended in the original volume of minimal medium. The suspension was diluted 20-fold with minimal medium containing various supplements, and grown for 4 h on a shaker at 37°C. These cultures were again diluted 20-fold with the same media, and optical density of the cultures at 660 nm was measured at intervals with a Shimadzu-Bausch and Lomb Spectronic 20 colorimeter.

Mutagen treatment. Ethyl methane sulfonate was used as a mutagen. Cells of KA931 in minimal medium supplemented with isoleucine, valine, leucine and pantothenate were grown on a shaker at 37°C to the exponential phase (*ca.* 2×10^8 cells/ml). Mutagen treatment was carried out by the method described previously (Kiritani and Ohnishi 1978).

Transport assays. Transport was measured by the method described previously (Kiritani 1974). For preparing cell suspensions for transport assays, an overnight bacterial culture in minimal medium supplemented with isoleucine, valine, leucine and pantothenate was diluted 33-fold with minimal medium supplemented with isoleucine, valine, pantothenate and various amounts of glycylleucine as indicated in the text, or the medium without glycylleucine, and cells were grown for about 4 generations (*ca.* 4×10^8 cells/ml) on a shaker at 37°C. The cultures were harvested by centrifugation, washed twice with minimal medium, and suspended in the medium containing 100 μg of chloramphenicol per ml, adjusting the optical density to 0.15 at 660 nm.

Apparent K_m and V_{max} values were calculated by double-reciprocal plotting, 1/V and 1/S, where S is concentration of the substrate expressed in micromoles per liter and V is micromoles of ^{14}C-labeled amino acid incorporated per 15 sec per gram of cells (dry weight).

Chemicals. Glycyl-L-leucine was obtained from Tokyo Kasei Industries, Ltd., L-amino acids and Ca-pantothenate from Wako Pure Chemical Ind., and ethyl methane sulfonate from Sigma Chemical Co.. Uniformly ^{14}C-labeled L-amino acids were obtained from the Radiochemical Centre, Amersham, England.

RESULTS

Isolation of Gle^r mutants. Mutagenized cells of KA931 grown in nutrient broth were harvested by centrifugation, washed once with saline, and suspended in the same volume of saline. One tenth ml of the suspension was spread on minimal agar medium supplemented with isoleucine, valine and glycylleucine (200 μg/ml), then the plate was

incubated at 37°C for 48 h. Gle^r mutants formed colonies on the plate, the surface of which was covered with a thin, confluent growth of the parental cells. These colonies were purified by successive single colony isolations. Transport activity of the Gle^r mutants thus isolated was assayed with ^{14}C-isoleucine. Of eight strains, four showed increased incorporation of isoleucine into their cellular pool. Three of these strains, KA222, KA223 and KA224, were selected for further study.

Table 1. Growth of KA931 and Gle^r mutant strains in various media

No.	Supplement* (μg/ml)					Generation time (min)			
	Ile	Val	Leu	gL**	Pan	KA931	KA222	KA223	KA224
1	10	20				180	55	50	50
2	10	20		10		55			
3	10	20		50		55			
4	10	20		100		170			
5	10	20		200		360	70	70	60
6	10	20	10			60	55	50	55
7	10	20			1	55	55	55	60
8	10	20		200	1	160	50	55	60
9	50	20			1	90	60	55	55
10	100	20			1	180	100	70	65
11	200	20			1	200	120	90	90
12	10	20	200		1	160	50	55	60
13	10	20	500		1	220	95	100	90

Average values of two experiments are listed.
* Ile, L-isoleucine; Val, L-valine; Leu, L-leucine; gL, glycyl-L-leucine; Pan, Ca-pantothenate.
** Molecular weight of glycyl-L-leucine is 188.23.

Table 2. Uptake of L-(^{14}C)-isoleucine in KA931 and Gle^r mutant strains grown in the presence or absence of glycyl-L-leucine

Strain	gL* in growth medium	L-Isoleucine uptake per g of cells	
		μMoles	%
KA931	−	3.2	100
	+	1.1	34
KA222	−	6.5	203
	+	3.6	113
KA223	−	5.9	184
	+	3.6	113
KA224	−	6.7	209
	+	3.7	116

Uptake of 32 μM of L-(^{14}C)-isoleucine (specific activity: 8.5×10^6 cpm/μ mole) by bacteria was measured at 30 sec. Average values of two experiments are listed.
* gL, glycyl-L-leucine (1.0 mM).

Bacterial growth in various media. Growth of KA931 and Gle^r mutants in minimal medium containing various supplements is shown in Table 1. KA931 grew slowly in medium containing isoleucine and valine (No. 1), and the growth was stimulated by addition of leucine, pantothenate, or 10 to 50 μg of glycylleucine per ml (No. 2, 3, 6 and 7). Gle^r mutants grew well on isoleucine and valine in the absence of leucine or pantothenate (No. 1, 6 and 7). Since leucine and pantothenate are derived from α-ketoisovalerate, these results indicate that addition of these compounds spares the requirement of KA931 for valine. However, when the amounts of glycylleucine added in the medium were increased over 100 μg per ml, growth of KA931 was inhibited (No. 4 and 5). Pantothenate relieved partially the inhibitory effect of glycylleucine on growth (No. 8). Growth of Gle^r mutants was little or not affected by glycylleucine (No. 5 and 8). Since the uptake of valine is competitively inhibited by addition of large amounts of isoleucine, growth rates of both KA931 and Gle^r mutants were reduced in the presence
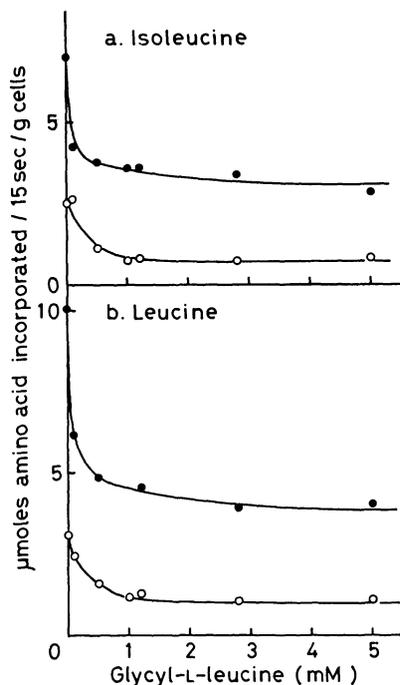
Fig. 1. Effect of glycyl-L-leucine concentration on the uptake of L-(^{14}C)-isoleucine and L-(^{14}C)-leucine. Bacteria were grown in minimal medium supplemented with L-isoleucine, L-valine, Ca-pantothenate, and various amounts of glycyl-L-leucine. The concentrations of L-(^{14}C)-isoleucine and L-(^{14}C)-leucine were 75 μM, and the specific radioactivities were 6.7×10⁶ and 6.8×10⁶ cpm/μ mole, respectively. Open and closed circles indicate strains KA931 and KA224, respectively.
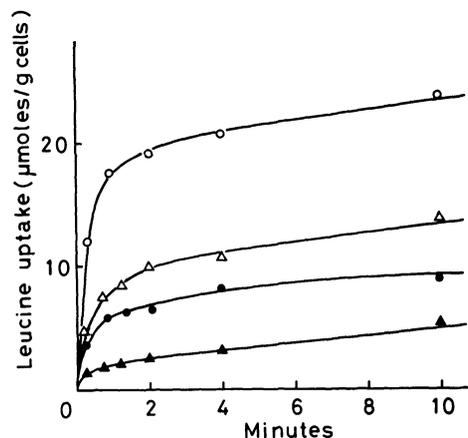
Fig. 2. Time course of L-(^{14}C)-leucine uptake. Bacteria were grown in minimal medium supplemented with L-isoleucine, L-valine, Ca-pantothenate, and 5 mM glycyl-L-leucine (△, KA224; ▲, KA931), or the medium without glycyl-L-leucine (○, KA224; ●, KA931). Concentration of L-(^{14}C)-leucine was 75 μM and the specific activity was 6.8×10⁶ cpm/μ mole.
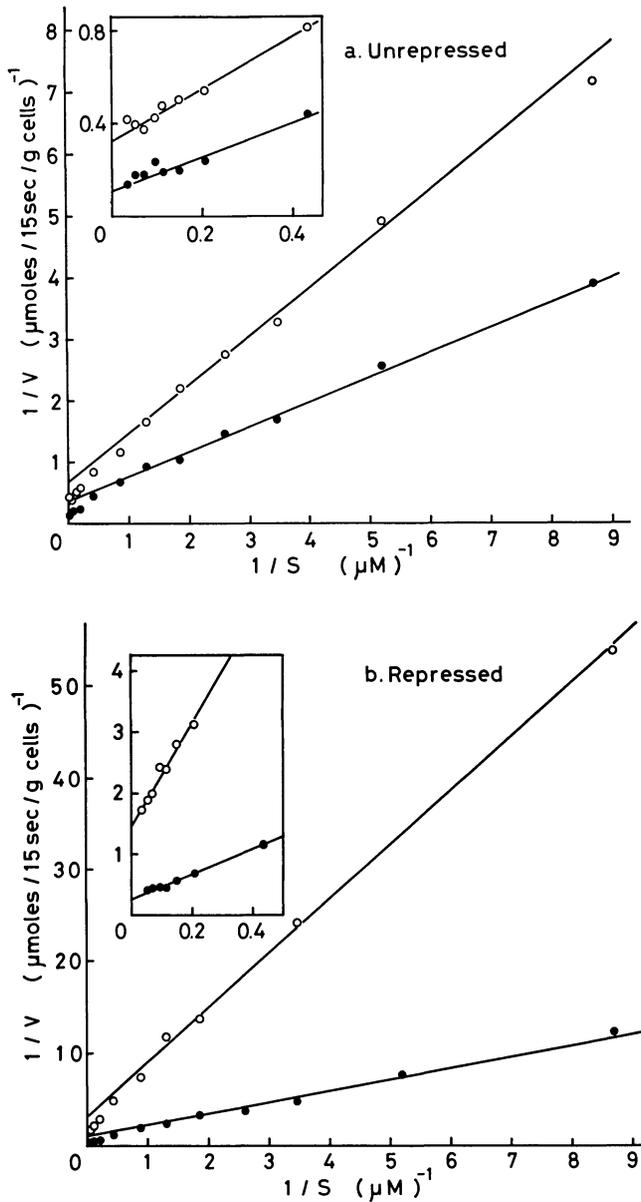
Fig. 3. Double-reciprocal plots of initial rate of uptake of L-(^{14}C)-isoleucine in KA931 (open circle) and KA224 (closed circle). Bacteria were grown in minimal medium supplemented with L-isoleucine, L-valine, Ca-pantothenate, and 5 mM glycyl-L-leucine (b), or in the medium without glycyl-L-leucine (a). Specific activities of L-(^{14}C)-isoleucine were 2.5×10^8 cpm/μmole in the concentration range of 0.12 to 2.3 μM, and 6.6×10^6 cpm/μmole in the range of 2.4 to 29 μM. K_m and V_{max} were as follows; KA931 ($K_m 1 = 0.7$, $K_m 2 = 4.0$, $V_{max} 1 = 0.5$, $V_{max} 2 = 2.6$) and KA224 ($K_m 1 = 0.7$, $K_m 2 = 7.5$, $V_{max} 1 = 1.0$, $V_{max} 2 = 8.5$) under the unrepressing condition: KA931 ($K_m 1 = 1.0$, $K_m 2 = 6.3$, $V_{max} 1 = 0.1$, $V_{max} 2 = 0.6$) and KA224 ($K_m 1 = 1.0$, $K_m 2 = 8.4$, $V_{max} 1 = 0.3$, $V_{max} 2 = 3.6$) under the repressing condition.

of high concentrations of the amino acid, but the reduction in the Gle[r] mutants was less marked (No. 9, 10 and 11). Similarly, when incorporation of isoleucine and valine was limited by excess leucine, the growth inhibition was more remarkable in KA931 than in the Gle[r] mutants (No. 12 and 13). These results suggest that the transport system for branched-chain amino acids may be in the derepressed state in the Gle[r] mutants.

Transport activity for branched-chain amino acids. Table 2 shows the uptake of isoleucine by the Gle[r] mutants and the parental strain KA931 grown in the presence and absence of excess glycylleucine. The transport activity of the mutants was about two-fold higher than that of KA931 in the absence of glycylleucine. The activity in the mutants as well as KA931 was repressed by glycylleucine, but the extent of repression was 39 to 44% in the mutants as compared to 66% in KA931. Thus the activity in the repressed mutant cells was about equal to that in the unrepressed wild-type cells.

As shown in Fig. 1, maximal repression of the transport in KA931 and KA224 was achieved at about 1 mM of glycylleucine. The time course of leucine uptake by cells is illustrated in Fig. 2. The internal pool of bacterial cells for leucine grown with or without excess glycylleucine was almost saturated with ^{14}C-labeled leucine after 4 min of incubation. The saturation levels in the unrepressed and repressed cells of KA224 were about three times as high as those of KA931. The results obtained with KA222 and KA223 were analogous to those with KA224 (data not shown). Thus the internal pool size of the mutant cells for branched-chain amino acids was apparently increased by the Gle[r] mutations.

Apparent K_m and V_{max} values. Initial uptake of ^{14}C-labeled isoleucine by cells of KA931 and KA224 was measured in the concentration range of 0.12 to 29 μM, and double-reciprocal plots of the data are illustrated in Fig. 3. The plots of the two strains,

Table 3. Apparent K_m and V_{max} for the transport systems

Strain	gL* in growth medium	Isoleucine				Leucine			
		High-affinity system		Low-affinity system		High-affinity system		Low-affinity system	
		K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
KA931	−	0.7	0.5	4.1	2.6	0.5	0.3	5.0	3.4
	+	1.1	0.1	6.6	0.6	0.8	0.03	8.6	1.0
KA222	−	1.1	0.7	7.9	5.1	0.7	0.4	7.3	7.1
	+	0.7	0.2	7.5	2.9	0.5	0.1	8.4	4.1
KA223	−	0.8	0.7	5.5	5.9	0.6	0.3	6.3	8.1
	+	0.7	0.2	4.1	2.3	0.8	0.2	8.7	3.7
KA224	−	0.9	0.8	7.2	8.6	1.1	0.7	6.8	9.1
	+	0.9	0.4	7.8	3.6	0.7	0.1	6.9	4.4

Specific activities of L-(^{14}C)-isoleucine and L-(^{14}C)-leucine were 2.5×10^8 and 1.5×10^8 cpm/μ mole in the concentration range of 0.12 to 2.3 μM, respectively, and 6.6×10^6 and 7.3×10^6 cpm/μ mole in the range of 2.4 to 29 μM, respectively. K_m (micromolar) and V_{max} (micromoles/15 sec/gram of cells) are average of at least two experiments.
  * gL, glycyl-L-leucine (5 mM).

Table 4. Uptake of ^{14}C-labeled amino acids

[14]C-Amino acid	KA931	KA222	KA223	KA224
	(μMole/20 sec/g cells)			
Glycine	1.9	1.8	1.9	1.9
L-Isoleucine	3.3	6.2	6.5	9.5
L-Leucine	3.9	9.1	9.7	13.9
L-Methionine	2.1	2.6	2.7	2.5
L-Proline	2.3	2.6	1.7	2.0

The concentration of ^{14}C-glycine, L-(^{14}C)-isoleucine, L-(^{14}C)-leucine, and L-(^{14}C)-proline was 75 μM, and that of L-(^{14}C)-methionine was 77 μM. Specific activities of ^{14}C-labeled amino acids were 2.1×10^7 (glycine), 6.7×10^6 (isoleucine), 6.8×10^6 (leucine), 7.9×10^6 (methionine) and 2.2×10^7 (proline) cpm/μ mole.

grown in the presence (Fig. 3b) and absence (Fig. 3a) of 5 mM glycylleucine, show an apparent break in the lines at approximately 1 μM, indicating that at least two transport systems exist for uptake of isoleucine.

Apparent K_m and V_{max} values of isoleucine and leucine for the transport systems of KA931 and its Gle[r] mutants are presented in Table 3. These values were calculated from the double-reciprocal plots according to the formula of Neal (1972). The K_m values of isoleucine and leucine obtained with the Gle[r] mutants were not significantly different from those with KA931, and not altered by repression of the transport by glycylleucine, although these values were somewhat variable with different mutants. In the absence of glycylleucine, the V_{max} values of the high-affinity system in the mutants, except KA224, were equal to, or slightly higher than, those in KA931, whereas V_{max} for the low-affinity system was approximately two-fold higher in the mutants. The V_{max} values of the two systems in KA224 were about two- to three-fold higher than those in KA931. As judged from the V_{max} values, the low-affinity system in the mutants appears to be more resistant than that in KA931 for repression induced by glycyl-leucine; that is, the V_{max} values for isoleucine were reduced 43 to 61% and those for leucine 42 to 52% in the mutant cells grown on glycylleucine, whereas for isoleucine 77% and for leucine 70% in KA931. Glycylleucine repressed strongly the high-affinity system of both the Gle[r] mutants and KA931. However, a difference between the mutants and KA931 is not clear with respect to the sensitivity of the high-affinity system to repression. The transport of glycine, methionine and proline is not significantly altered in the mutants (Table 4).

DISCUSSION

Since 1 mM glycylleucine added in growth medium of KA931 (*ilvC8*) represses the transport of branched-chain amino acids, and consequently inhibits the bacterial growth, we have attempted to select derepressed mutants of the transport system from glycylleucine-resistant mutants (Gle[r]). The Gle[r] mutants thus obtained appear to be

derepressed in the transport systems for branched-chain amino acids because of (i) the increased uptake of isoleucine and leucine under unrepressing and repressing conditions (Table 2 and Fig. 2), (ii) the altered kinetics of the high- and low-affinity systems (Table 3), (iii) the observed growth response to isoleucine and valine (Table 1). As judged from the V_{max} values obtained, the low-affinity system of the mutants appears to be derepressed. It is not clear whether the high-affinity system is affected by the Gle^r mutations, although the activity of KA224 seems to be increased (Table 3).

The two k_m values of the high- and low-affinity transport systems for isoleucine and leucine, calculated for KA931 and the Gle^r mutant strains, correspond roughly to those of the high-affinity and the low-affinity-1 systems for the wild-type strain (Kiritani and Ohnishi 1978), although the values for the low-affinity system are somewhat lower in these strains. The low-affinity-2 system, which is found in the multiple transport mutants (Kiritani and Ohnishi 1978), is apparently undetectable in KA931 and the Gle^r mutant strains.

Two derepressed mutants in the transport of branched-chain amino acids have been isolated in *E. coli* as D-leucine utilizers (Rahmanian *et al.* 1973). The mutant loci, *lstR* and *livR*, result in derepression of the leucine-specific transport system and both the leucine-specific and the general high-affinity (LIV-I; K_m for leucine uptake=0.2 μM) (Wood 1975) for branched-chain amino acids, respectively. These two loci have been mapped near *aroA* at 20 min on the genetic map (Anderson *et al.* 1976). The Gle^r mutation induced in *S. typhimurium* appears to be different from the *livR* mutation, because the low-affinity transport system (K_m for leucine uptake=*ca.* 8 μM) is mainly derepressed. Furthermore, the mutant locus responsible for the Gle^r mutation of strain KA224 is found to be closely linked to *ilvT* (defective in the low-affinity transport system; Kiritani 1974, Kiritani and Ohnishi 1978) (unpublished data). Thus the results suggest that the Gle^r mutation of strain KA224 may reside in the regulatory or the operator gene for the transport of branched-chain amino acids. Experiments to clarify this are in progress.

SUMMARY

Three mutants, KA222, KA223 and KA224, derepressed in the transport of branched-chain amino acids were isolated as glycyl-L-leucine-resistant (Gle^r) strains from an isoleucine-valine-requiring mutant, KA931, of *Salmonella typhimurium* LT2. These Gle^r strains grow normally in minimal medium supplemented with L-isoleucine (10 μg/ml), L-valine (20 μg/ml) and large amounts of glycyl-L-leucine (1 mM: 188 μg/ml), where growth of the parent strain, KA931, is markedly inhibited. When cells were grown in the absence of glycyl-L-leucine, the Gle^r mutants incorporated two- to three-fold higher amounts of L-isoleucine or L-leucine than did KA931. Although the transport activity of Gle^r strains was repressible by glycyl-L-leucine, the activity in the repressed state was equal to, or even higher than, the activity of KA931 in the unrepressed state. The increment of uptake in the Gle^r strains is mainly due to derepression of the low-affinity transport system. In the Gle^r strains, the transport of glycine, L-methionine,

and L-proline was normal.

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