Properties of a Dicarboxylic Amino Acid Transport-deficient Mutant of *Streptococcus faecalis*

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SUMMARY

A mutant of *Streptococcus faecalis* has been isolated which has a 20-fold higher growth requirement for L-glutamic acid than the parent strain. The ability to utilize L-glutamine for growth is not affected. The mutant also has a greater dependence than the parent strain on an external supply of vitamin B₆.

The rate of glutamic acid transport at low extracellular concentrations (0.05 mM) was greatly reduced in the mutant but was only moderately affected at higher concentrations (5 mM). In contrast to the parent, in which kinetic studies revealed the operation of a high affinity and a low affinity system for glutamic acid transport, the mutant possessed a single catalytic component with kinetic constants equal to those of the low affinity system. These findings and the greatly elevated growth requirement for glutamic acid indicate that the high affinity system is inactive in the mutant. The transport of aspartic acid and D-glutamic acid by the mutant were similarly adversely affected, whereas essentially identical kinetic constants for glutamine, α-aminoisobutyric acid, and cycloleucine transport were obtained for the mutant and parent strains.

The absence of a functional high affinity dicarboxylic amino acid transport system in the mutant allowed a study of the specific properties of the low affinity system. In contrast to the parent strain, the initial rate of uptake of isotopically labeled glutamate in the mutant was not stimulated by glucose at any concentration. However, the accumulation of large amounts of this amino acid on extended incubation was dependent on an energy source. Glutamine competitively inhibited glutamic acid uptake in the mutant. There was no evidence that the mutant produced an inhibitor of the high affinity transport component.

An investigation of glutamic acid transport in *Streptococcus faecalis* has established the operation of two kinetically distinct catalytic systems for the uptake of this amino acid (1). Competition studies indicate that both systems also function in promoting aspartic acid transport. This report describes the properties of a mutant strain of *S. faecalis* with an unusually high glutamic acid growth requirement which was found to lack one of these transport components.

Multiple catalytic systems for several groups of amino acids have now been encountered in a variety of mammalian and microbial cells (2–10). The functional relationship of these systems is not understood. In particular, for many cases it is not known whether uptake of a given amino acid involves the operation of several independent catalysts or of a single allosterically modifiable catalyst. It is anticipated that transport mutants of the type described here will be helpful in investigating these questions. A preliminary summary of these findings has been reported (11).

EXPERIMENTAL METHODS

The procedures used for maintaining and growing cultures of the parent strain, *S. faecalis* R (ATCC 8043), henceforth designated ATCC, preparing cells for uptake experiments, and carrying out and evaluating the results of transport measurements are described in Reference 1.

Mutant strains were obtained by ultraviolet irradiation of a heavily inoculated exponential phase culture grown at 30° for 90 min in the regular synthetic medium. Irradiation was continued until 90.0% of the population was nonviable. Of the treated culture, 1.8 ml was used to inoculate 50 ml of a high glutamate medium containing 2.5 mg per ml of L-glutamic acid, 3.5 mg per ml of L-glutamic acid, and 50 μg per ml of L-aspartic acid. All other components were at previously described levels. After overnight growth, the cells were washed in growth medium lacking glutamic acid, aspartic acid, and glutamine and were used to inoculate (0.05 mg per ml) two flasks of a low glutamate medium containing 20 μg per ml of L-glutamic acid and 1 μg per ml of L-aspartic acid. After incubation for 45 min, 1000 units per ml of penicillin G were added to one flask and incubation at 30° was continued. Growth over a 2-hour period was monitored turbidimetrically and samples for further treatment were taken periodically from the penicillin culture. After washing twice with growth medium lacking glutamate and aspartate, the samples from the penicillin culture were used to inoculate overnight cultures in high glutamate medium. The penicillin treatment was repeated three times and the cells obtained after overnight growth on high glutamate medium were diluted and plated on high glutamate medium solidified with 1.5% washed agar. After 3 days of incubation these plates were replicated to agar medium.
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**L-GLUTAMIC ACID (~@mi)**

**L-GLUTAMINE (&g/ml)**

**FIG. 1.** Growth responses to (A) L-glutamic acid and (B) L-glutamine of the parent (ATCC) (○) and mutant (R-4) (○) strains of *S. faecalis*. Tube cultures were incubated at 30° for 17 hours in a medium containing 1 μg per ml of L-aspartic acid. In B, the same strain shows the identical response of both strains to glutamine in the absence of glutamic acid; ---, the responses in the presence of 500 μg per ml of L-glutamic acid.

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containing low levels of glutamic acid (15 μg per ml) and 1 μg per ml of aspartic acid. Colonies on high glutamate medium which failed to replicate were selected and tested in tubes of liquid medium containing various levels of L-glutamic acid to determine the magnitude of their glutamic acid requirements. Strains with greatly elevated glutamic acid growth requirements were selected for further study. Growth was measured turbidimetrically at 640 nm and was expressed in dry weight equivalents. Most of the uptake experiments were carried out with a mutant strain designated R-4.

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**RESULTS**

**Growth Stimulation by Glutamic Acid and Glutamine**—The relative growth responses to glutamic acid and glutamine of the parent (ATCC) and mutant (R-4) strains are illustrated in Fig. 1. The parent strain achieved half-maximal growth in a medium containing approximately 40 μg per ml (0.27 mM) of L-glutamic acid. Half-maximal growth of the mutant in contrast was not attained unless the medium contained approximately 750 μg per ml (5.1 mM) of L-glutamic acid (Fig. 1A). As will be demonstrated below, these growth responses correlate well with the glutamic acid transport capabilities of the two strains.

In contrast to its defective ability to utilize glutamic acid for growth, the mutant utilized glutamine in place of glutamic acid as efficiently as the parent strain (Fig. 1B). Both strains attained half-maximal growth when the medium contained approximately 43 μg per ml of glutamine (0.3 mM). Figure 1B also illustrates a marked stimulatory effect of glutamine on the growth response to glutamic acid by the mutant when the medium contained 500 μg per ml of glutamic acid. As was shown in Fig. 1A, with this amount of glutamic acid, ATCC growth was essentially maximal, whereas R-4 grew only slightly. The addition of 1 μg per ml of glutamine permitted R-4 to attain maximum growth at this level of glutamic acid. The same type of stimulation can be seen with ATCC at an earlier incubation time and using lower levels of glutamic acid, demonstrating that this is not a unique property of the mutant. On the other hand, the mechanism of this stimulation is not understood, and it is apparently not damaged by the mutation which affects glutamate uptake in R-4. An apparently contradictory inhibitory effect of glutamine on glutamic acid uptake in the mutant strain will be described in a later section.

**Effect of Vitamin B₆ on Growth**—In a medium lacking vitamin B₆ containing 1 mg per ml of glutamic acid and 1 μg per ml of aspartic acid, the parent strain could be transferred successfully for many passes. Growth after the first pass generally reached only half-maximal levels and there was a tendency of the cells to lyse, but there was no difficulty in obtaining successive viable subcultures. In contrast, the mutant R-4 grew very poorly after the first pass in vitamin B₆-deficient medium and its growth with or without a previous subculture in vitamin B₆-deficient medium was markedly stimulated by the vitamin. The behavior on subsequent passes has been sporadic. In some cases, cells in the second pass in vitamin B₆-deficient medium were so fragile that further subcultures failed to grow. At other times, subsequent subcultures were viable, although growth always was inferior to that obtained by the parent strain in the absence of the vitamin. Raising the aspartic acid concentration to 100 μg per ml improved the stability of both strains, and the mutant could be transferred routinely beyond the second pass in vitamin B₆-deficient medium. However, the greater vitamin B₆ dependency of the mutant was not eliminated by increasing the aspartic acid content of the medium (Fig. 2). This observation argues against the possibility that the higher vitamin B₆ requirement originated in a greater dependency on a vitamin B₆-stimulated aspartate biosynthetic pathway resulting from a reduced...
The rate of aspartic acid uptake from the growth medium. We have not yet determined by back mutation studies whether the higher vitamin B₆ requirement of the mutant invariably accompanies the high glutamate requirement and the transport defect characteristic of this strain. The parent and mutant strains responded identically to growth-limiting levels of all the other vitamins normally provided in the medium.

We investigated further the possibility that the higher vitamin B₆ requirement reflected a need of the mutant to utilize a vitamin B₆-dependent pathway to biosynthesize an essential cell component as a consequence of its failure to take up glutamic acid at a normal rate. None of a large variety of substances synthesized or metabolized by catalysts for which vitamin B₆ is a cofactor consistently modified the heightened dependence of the mutant strain on the vitamin. The only exceptions were the amino sugars, glucosamine and muramic acid, which irregularly stimulated, but also occasionally inhibited growth of the mutant. The effects were highly variable and seemed to be related to the rate of appearance of revertants with a diminished glutamic acid growth requirement.

Comparative Uptake of Glutamic Acid in Parent and Mutant Strains—Fig. 3A shows that glutamate uptake by the mutant at low extracellular glutamate concentrations is markedly lower than in the parent strain. In contrast, Fig. 3B shows that this defect is much less pronounced at higher extracellular glutamate levels. This behavior led to the recognition that the parent strain possesses multiple catalytic systems for glutamic acid transport, only one of which is defective in the mutant. As shown in the accompanying report (1), using initial rate data for glutamate uptake, hyperbolic Lineweaver-Burk plots were obtained for the parent strain. The experimental data corresponded closely to a curve predicted by the sum of two Michaelis-Menten terms for catalytic systems with a relatively high affinity and another with a distinctly lower affinity. In comparable studies, glutamate uptake by the mutant R-4 yielded a linear Lineweaver-Burk plot describing the operation of a catalytic system with constants comparable with those of the parent LA₁ system (Fig. 4A). Therefore, it appears, on the basis of these observations and the results of the growth studies, that the R-4 mutant lacks

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**Fig. 2.** Growth of parent and mutant strains in media containing limiting amounts of vitamin B₆. Inoculum cells were prepared by transferring cells from a stab to a liquid culture of regular medium containing an excess of pyridoxamine. The cells were collected by centrifugation, washed once in water, resuspended in water, and used to inoculate tubes containing 5 ml of regular medium except for the vitamin B₆ content which was as indicated in the figure. Curve 1, ○—○, R-4, incubation 18 hours; Curve 2, ●—●, ATCC, incubation 18 hours; Curve 3, X—X, R-4, incubation 22 hours.

**Fig. 3.** Comparative uptake of L-glutamic acid by the parent (ATCC) and mutant (R-4) strains at (A) low and (B) high extracellular concentrations.
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GLUTAMIC ACID

Fig. 4. Reciprocal plot of initial rates of (A) L-glutamic acid and (B) L-aspartic acid uptake at various extracellular concentrations by the parent (ATCC) and mutant (R-4) strains. The data are represented by the points which were obtained using the filtration method and measuring radioactivity in cell extracts. A, the lines describe glutamate uptake assuming catalytic components with the following constants. R-4: $K_m = 0.5 \text{ mM}$, $V_{max} = 8.0 \mu\text{moles min}^{-1} \text{ 100 mg}^{-1}$; ATCC: $K_1 = 0.05 \text{ mM}$, $V_1 = 0.80 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$, $K_2 = 12.0 \text{ mM}$, $V_2 = 6.5 \mu\text{ moles min}^{-1} \text{ 100 mg}^{-1}$. B, the lines were drawn using values for aspartate $v_1$ obtained by substituting the following constants in Equations 2 and 3 described previously (1). R-4: $K_m = 0.30 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$, $K_b = 2.5 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$; ATCC: $K_1 = 0.05 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$, $K_2 = 0.65 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$, $K_b = 0.10 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$.

GLUTAMINE

Fig. 5. Reciprocal plot of initial rates of L-glutamine uptake by the ATCC and R-4 strains. The points represent experimental data. The lines were drawn using values obtained by substituting the following constants in Equation 2 (1). ATCC: $K_m = 0.003 \text{ mM}$, $V_{max} = 1.0 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$, $K_b = 2.5 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$; R-4: $K_m = 0.0025 \text{ mM}$, $V_{max} = 1.0 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$, $K_b = 2.5 \mu\text{ mole min}^{-1} \text{ 100 mg}^{-1}$.

a functional HIA system. As indicated previously, a comparative study of glutamic acid metabolism in the parent and mutant revealed no significant differences between these strains in the distribution of isotope derived from glutamic acid among several intracellular glutamate metabolites. These results indicate that the mutation has not affected a glutamic acid-metabolizing system but involves damage to a transport catalyst with a relatively high affinity for this amino acid.

It is interesting to note that glutamate uptake by the mutant at low concentrations can be accounted for entirely by the activity of the low affinity system. For example, at 0.1 mM R-4 takes up glutamate at 17% of the ATCC rate. As shown in Fig. 3, Reference 1, at this external concentration the low affinity system accounts for 19% of the initial rate of transport by the parent strain. The growth response of the mutant also is consistent with the absence of a functional high affinity system, if allowance is made for a competitive effect of L-aspartic acid in the growth medium on glutamic acid uptake.

Aspartic Acid Transport in Parent and Mutant Strains—Competition studies in the parent strain indicated that aspartic and glutamic acids are both transported by the same low and high affinity systems. This is further supported by the results described in Fig. 4B which show that the mutant R-4 possesses a single saturable system for aspartic acid in contrast to the parent strain which has two saturable systems in addition to a diffusion component also detected in the mutant. The system found in the mutant has kinetic constants similar to those of the low affinity system in the parent, indicating again that only the high affinity system has been rendered nonfunctional in the mutant.

Transport of Other Amino Acids—The mutational inactivation
of the HA dicarboxylic amino acid transport system did not affect the uptake of other amino acids except those such as α-methylglutamic acid (12) and 2-amino-3-phosphonopropionic acid (13) which bear a close structural and charge distribution relationship to glutamic and aspartic acids. As shown in Fig. 5, the uptake of L-glutamine, which involves a single catalytic component and a nonsaturable, apparently diffusion-controlled process in the parent strain, is not affected by the R-4 mutation. Closely similar kinetic constants also were obtained for α-aminoisobutyric acid and cycloleucine transport in the parent and mutant strains (Table I). Only a single catalytic system for 2-amino-3-phosphonopropionic acid was detected in ATCC and no measurable uptake was observed in the mutant, indicating that this structural analogue of aspartic acid is transported only by the high affinity system.

The conclusion of these kinetic studies, i.e. that the R-4 mutation exclusively inactivated the HA dicarboxylic amino acid carrier and did not alter the activity of other systems, is further supported by comparisons of the equilibrium level accumulation of several amino acids in the parent and mutant strains (Table I). Only a single catalytic system for 2-amino-3-phosphonopropionic acid was detected in ATCC and no measurable uptake was observed in the mutant, indicating that this structural analogue of aspartic acid is transported only by the high affinity system.

The relative rates of L- and D-glutamic acid uptake in ATCC and R-4 are summarized in Table III. The marked reduction in D-glutamic acid uptake rate at 0.65 mM in the mutant clearly indicates that the HA system can transport the D isomer. The similarity of the relative rates of L- and D-glutamic acid uptake in both strains at 5.0 mM indicates that the LA system also transports the D isomer.

**Effect of Glucose on Uptake**—The absence of a functional high affinity system in the mutant permitted a more refined study of the isolated low affinity system than was possible in the parent strain. For example, the initial rate of glutamic acid uptake by the R-4 strain over a wide concentration range either was unaffected by the presence or absence of glucose, or, as shown in Fig. 6, was depressed by glucose at glutamate concentrations greater than 0.1 mM. This is in contrast to the behavior of ATCC, in which the strict energy dependence of a functional HA system was reflected in a marked stimulation of the initial rate by glucose at low extracellular concentrations (1). At higher concentrations, at which the LA system predominates, glucose inhibition also can be observed in ATCC, although it is clearly evident only at much higher levels than was encountered in R-4. The initial entry of glutamate in the absence of glucose most likely represents exchange of labeled extracellular amino acid with a pre-existing intracellular glutamic acid pool.

When the time of observation was extended, as in ATCC, a glucose dependence for glutamate pool accumulation also was observed in R-4 (Fig. 7A) except at lower concentrations, at

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which it was difficult to demonstrate any stimulation (Fig. 7B), most likely because the pre-existing gradients already were quite high. At an extracellular concentration of 5 mM and assuming an intracellular water space of approximately 2 ml per g, uptake in the absence of glucose tended toward a plateau at an estimated intracellular concentration of approximately 4 to 5 mM, indicating an equilibration of the glutamate in the two phases (Fig. 7A).

In the absence of glucose, linear Lineweaver-Burk plots were obtained with R-4 and its parent ATCC (Fig. 8). The kinetic constants are comparable with those obtained previously for the low affinity system in the parent strain. There is no indication that the high affinity system is functional under these conditions in either strain. Since both strains contain significant pools of freely extractable glutamic acid, this observation suggests that the HA component may not participate in a glutamate-exchange reaction in the absence of an energy source.

Aspartic acid transport in the R-4 strain showed the same activity variations in the absence and presence of glucose as was observed with glutamic acid.

Inhibitory Effect of Glutamine—An examination of substrate specificity in the parent strain suggested that glutamic acid uptake by the low affinity system was more sensitive to inhibition by L-glutamine than was uptake via the high affinity system. The absence of a functional HA component in the R-4 strain allowed a more reliable study of the effect of glutamine specifically on the LA component. As shown in Fig. 9, glutamine clearly inhibited the initial rate of glutamic acid uptake in an apparently competitive manner. The relative ineffectivity of glutamine as a competitor against the HA component argues against the possibility that the amide is rapidly converted extracellularly or within the membrane to glutamic acid which then acts as the competitive agent, since greater sensitivity of the HA system to inhibition should have been observed if this was the case. The deamination of glutamine prior to the transport of this amino acid into the cell also is unlikely in view of its ability to support growth of the parent and mutant strains equally well (Fig. 1B). Glutamic acid transport in Escherichia coli also has been shown to be competitively inhibited by glutamine (14).

The inhibition of glutamic acid uptake by glutamine however, would appear to be in conflict with its marked stimulation of R-4

**Fig. 6.** Effect of glucose on the initial rate of glutamic acid uptake in the R-4 mutant strain. Initial rates were determined using the filtration, cell extraction procedures. Note that both scales are logarithmic.

**Fig. 7.** Time course of glutamic acid uptake by the R-4 mutant strain at an extracellular concentration of (A) 5.0 mM and (B) 0.05 mM in the absence (○) and presence (●) of glucose. Uptake was determined using the filtration and cell extraction procedures.
growth on suboptimal levels of glutamic acid (Fig. 1B). It might be pertinent to note, therefore, that in contrast to the inhibitory effect of glutamine on the initial rate, when the incubation period was extended, there was instead a distinct enhancement in the final amount of glutamic acid accumulated intracellularly at all levels of this amino acid, even with the lowest glutamine concentration tested (0.02 mM).

Since the transport of glutamine is not affected by the R-4 mutation, the high affinity glutamic acid component is not the major catalyst utilized for glutamine uptake. The competitive effect of glutamine in the mutant raises the possibility that the low affinity system might be utilized for transport of the amide. However, the data presented in Fig. 9 indicate that the $K_i$ value for glutamine as a competitive inhibitor of the LA system is almost two orders of magnitude higher than its apparent $K_m$ value for catalyzed transport, arguing strongly against identity of the LA dicarboxylic and the glutamine transport systems.

**Additional Comparative Properties**—Several other parameters relating to glutamate uptake were compared in the parent and mutant strains and were found not to differ significantly, including the effect of pH and reaction to a variety of structurally related analogues. The effects of cell extracts and supernatant growth media from both strains on uptake by either strain were comparable, indicating that the mutant does not produce a transport inhibitor or overproduce one of the amino acid substrates of the functionally defective system. The metabolism of L-glutamic acid by the mutant during uptake at relatively low and high extracellular concentrations has been summarized in the accompanying report (1) to facilitate comparison to the parent strain, which it closely resembles in this respect.

**DISCUSSION**

The growth and transport properties of the R-4 mutant strain of *S. faecalis* described above are consonant with the specific inactivation of a dicarboxylic amino acid transport catalyst with a relatively high affinity for its substrates. There appear to be no changes either in another dicarboxylic amino acid transport system of lower affinity, or in several other systems specific for structurally unrelated amino acids such as a-aminoisobutyric acid, cycloleucine, phenylalanine, and lysine. There was no indication that the strain overproduces or excretes competitive substrates for this transport component, or, as described elsewhere (1), that there is a change in the metabolism of glutamic acid.

The mutant has been useful in distinguishing the properties of the two dicarboxylic amino acid transport components as, for example, in allowing a study of the competitive interaction of glutamine specifically with the low affinity system (Fig. 9) or in demonstrating that the phosphonic acid analogue of aspartic acid, 3-amino-3-phosphonopropionic acid, is transported solely by the high affinity system (13). It has also been shown in a comparative study of the parent and mutant strains that a-methyl-L-glutamic acid is not transported by the LA system. The mutant should be useful later in a chemical dissection of these transport systems since presumably an essential component of the LA system has been structurally altered. So far, however, we have been unable to distinguish parent and mutant strains, for example, on the basis of membrane protein composition. Osmotic shock procedures which have been so useful recently in studying transport systems in gram-negative bacteria have not produced comparable inactivation of the amino acid transport systems in *S. faecalis* and, therefore, have not aided in the comparative study of the mutant.

There is no indication in our studies that the curvilinear Lineweaver-Burk plots of initial rate data for glutamic acid uptake...
can be explained in this organism by an allostery interconversion of two forms of the same transport catalyst differing in their substate affinities. However, if the normal system were capable of reacting in two affinity states, the apparent absence of one state in the mutant could be explained either by an alteration in the catalyst which prevents it from undergoing the affinity change, or by a failure of this strain to produce an endogenous effector substance. The latter possibility could be tested experimentally, although, admittedly, only to a limited extent. For example, it was found that transport kinetics in the mutant could not be modified using all available glutamate metabolites as well as extracts of normal cells which had accumulated small and large glutamate pools. Likewise, mutant extracts failed to alter the kinetics of uptake by the parent strain. Although far from conclusive, these experiments provide no indication that the mutant is incapable of producing an allosteric modifier substance, a conclusion which is supported also by the glutamate metabolism experiments (1), indicating identity of the parent and mutant strains in this regard.

As yet, there are no indications of the biological significance of the existence of multiple transport systems for a given amino acid. The possibility that the two systems might transport the amino acid into spatially or functionally distinct regions of the cell is not supported by our metabolic studies which showed that the glutamic acid molecules entering on both systems seemed to be metabolized in an identical manner, at least within the limits of the measurements applied. The very large differences in the apparent $K_m$ values for several of these multiple systems have suggested that they may have evolved successively to enable primitive organisms to obtain adequate amounts of nutrients from a progressively depleted extracellular source (15). In this context the low affinity dicarboxylic amino acid system might have evolved sooner than the high affinity system and the respective catalysts might be structurally related. Such questions, of course, cannot be experimentally evaluated until the respective catalysts have been isolated in a chemically pure state.

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