Isolation of Amino Acid Transport-negative Mutants of *Pseudomonas aeruginosa* and Cells with Repressed Transport Activity

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Methods are described for the isolation of amino acid transport-negative mutants of *Pseudomonas aeruginosa* and for the preparation of cells with repressed, specific amino acid permeases. *P. aeruginosa* was resistant to high concentrations of the majority of the 53 amino acid analogues examined and was unaffected by low concentrations of any of them. Cells which had been grown in the presence of sublethal concentrations of the few analogues which were inhibitory were subsequently more resistant to the analogues. These cells were also defective in the transport of the corresponding amino acid, as the analogue caused repression of the synthesis of the specific amino acid permease. The cells with repressed transport activity rapidly regained their normal level of constitutive permease when grown in the absence of the analogue. Higher levels of the permeases were induced when these cells were grown in the presence of the appropriate amino acid. The possible mechanisms for the mode of regulation of amino acid permeases are discussed.

The inhibitory effect of amino acid analogues on the growth of microorganisms is well known and has been attributed to the ability of the analogues to compete with the natural amino acids in various essential metabolic reactions (2, 13, 15, 16).

The development of resistance to these analogues has recently been described (4, 13, 16), and generally has been attributed to derepression of controlled biosynthetic systems (4) or to the loss of regulatory mechanisms, such as feedback inhibition. However, several investigators have isolated mutants which were resistant to analogues and which failed to accumulate either the analogue or the corresponding amino acid (3, 6, 7, 10, 18–20). The mechanism of transport, in these mutants, both for the natural amino acid and for its analogue was impaired. These mutants have been termed "transport-negative," Tr− (11).

The selection technique for transport-negative strains requires that the microorganisms be sensitive to very low concentrations of the appropriate analogue. Amino acid transport systems, in general, have very high affinities for their respective amino acids, and, as a result, selective procedures for the isolation of Tr− mutants have been most successful at low concentrations of amino acid analogues. At higher concentrations, the analogues enter the cells by diffusion, and, therefore, a mutation which confers resistance under these conditions presumably involves some cellular function other than the active transport system. *Pseudomonas aeruginosa* was generally insensitive to the inhibitory effects of amino acid analogues. The few analogues which effectively inhibited growth did so only when present at relatively high concentrations. Our attention was directed to the nature of the resistance of the microorganism to these analogues and to the selection and behavior of amino acid transport-negative mutants.

**MATERIALS AND METHODS**

*Microorganisms.* The organisms used were *P. aeruginosa* ATCC 9027, wild type; *P. aeruginosa* P22, a mutant unable to degrade proline as a carbon or nitrogen source; and *P. aeruginosa* P5, a proline transport-negative mutant. Growth conditions and media have been described previously (8).

The procedure for measuring 14C-amino acid incorporation into whole cells and cell fractions has also been described (8).

*Resistance to amino acid analogues.* Amino acid analogues were screened for their ability to inhibit growth of *P. aeruginosa* by placing a crystal of the...
analogue on the surface of a minimal medium (8) agar plate which previously had been seeded with approximately 10^8 organisms. Plates were incubated at 37 C for 14 to 20 hr, and inhibition was detected by a zone of inhibition around the crystal. When an analogue did inhibit growth, additional experiments were undertaken to establish the minimal concentration which was inhibitory. For these determinations, minimal medium, containing various concentrations of the analogues, was inoculated and incubated at 37 C for 14 hr.

Selection of transport-negative mutants. The procedure for mutation with N-methyl-N-nitro-N'-nitrosoguanidine has been described (9).

Preliminary attempts to isolate Tr^- mutants by the procedure of Lubin and Kessel (11), with the use of amino acid auxotrophs and streptomycin (400 μg/ml) instead of penicillin for enrichment, were repeatedly unsuccessful. Mutants of P. aeruginosa were isolated that were resistant to high levels of thiopeptide (1 mg/ml), dehydroproline (200 μg/ml), or canavanine (200 μg/ml), but these mutants were neither derepressed for proline or arginine biosynthesis nor were they Tr^-.

However, a method was devised which did allow the isolation of Tr^- mutants. Cells were first induced to use the particular amino acid as a sole carbon source and then mutated as previously described (9). The treated cultures were allowed to grow for 4 to 6 hr in minimal medium and then plated to give approximately 100 colonies per plate on minimal medium agar with the particular amino acid as the sole carbon source (0.1%). After 48 hr of incubation at 37 C, minute colonies were picked, washed in drops of saline, and patched onto minimal amino acid-agar plates. Slow-growing colonies (presumably Tr^-) were easily differentiated from the faster-growing wild-type and reverting colonies. Colonies isolated in this manner were transferred and grown in liquid minimal medium and were screened for their ability to transport low concentrations of the appropriate ^14C-amino acid.

Growth studies. Growth studies were carried out in 50 ml of minimal medium in 250-ml Erlenmeyer flasks equipped with test tube side arms and incubated in a temperature-controlled, Metabolite shaking water bath at 37 C (New Brunswick Scientific Co., New Brunswick, N.J.). Growth was followed with a Klett-Summerson photoelectric colorimeter equipped with a no. 54 filter. Appropriate corrections were carried out to establish linearity between Klett readings and turbidity. A relative absorbance value of 100 is equivalent to 0.375 mg (dry weight) of cells per ml.

Manometric studies. Manometric studies were carried out to determine whether growth of P. aeruginosa in the presence of amino acid analogues influenced the ability of the organism to oxidize the natural amino acids. Glucose minimal medium (8) containing the appropriate analogue was used for growth of the 20-hr inocula and the experimental cells. The experimental cultures were grown for 14 hr at 30 C in Roux flasks. Analogue concentrations in the growth medium were as follows: canavanine, 250 μg/ml; 3,4-dehydroproline, 100 μg/ml; thioproline, 1 mg/ml; p-fluorophenylalanine, 200 μg/ml; and 5-fluorotryptophan, 500 μg/ml. Cells were harvested from the late exponential phase of growth by centrifugation at 5,000 X g at room temperature. The cells were washed three times in 0.033 M phosphate buffer (pH 7.4) containing 0.05% MgCl₂ and 0.5 ppm of iron as FeSO₄·7H₂O and were resuspended in 0.05 M tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 7.4) at 5 mg (dry weight) of cells per ml. Oxygen-uptake studies were carried out at 37 C with a conventional Warburg respirometer.

Chemicals. Amino acid analogues were obtained from the following sources: 4-nitropyridine-N-oxide, 2,4-pyridinedicarboxylic acid (luttidin acid), 2,5-pyridinedicarboxylic acid (isocinchomeronic acid), 2,6-pyridinedicarboxylic acid (dipicolinic acid), o-hydroxyhippuric acid, 2,2'-benzoic acid, 3,3'-benzoic acid, and hippuric acid from J. T. Baker Chemical Co., Phillipsburg, N.J.; L-azetidine-2-carboxylic acid, L-aspartic acid, L-proline, L-serine methyl ester, L-canaeanine, D-tyrosine, DL-β-2-thienylalanine, thiazolyl-DL-alanine, DL-p-fluorophenylalanine, and 5-methyltryptophan from Calbiochem, Los Angeles, Calif.; o-hydroxyphenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, and phenylpyruvic acid from K & K Laboratories Inc., Plainview, N.Y.; pyridine hydrochloride from Matheson, Coleman and Bell, East Rutherford, N.J.; L-hydroxyproline, m-fluorophenylalanine, 5-fluorotryptophan, 6-fluorotryptophan, dibromotyrosine, diiodotyrosine, and ethionine from Nutritional Biochemicals Corp., Cleveland, Ohio; N-acetyl-DL-proline, DL-acetylglycine, L-serine, L-canaeanine, L-threonine, DL-α,methylene glutamic acid, DL-α-hydroxyethyl glutamic acid, DL-α-methylglutamic acid, DL-α-tyrosine, DL-β-methionine, L-mimosine, and 3,4-dihydroxyphenylalanine from Sigma Chemical Co., St. Louis, Mo.; 3,4-dehydro-DL-proline was kindly supplied by B. Witkop, National Institutes of Health, Bethesda, Md.; S-(β-amino-ethyl)-l-cysteine (l-thiosine), by J. Mauron, Laboratoire de Recherches des Produits Nestle, Vevey, Switzerland; and DL-α-methyltyrosine (2-methylphenylalanine), m-fluorotryptophan, 3-amino-l-tyrosine, and 4-amino-DL-phenylalanine, by G. Grant, University of California, La Jolla, Calif.

L-Thiazolidine-4-carboxylic acid (thioproline) was synthesized according to the procedures of Ratner and Clarke (14) and Schubert (17), as modified by Mackenzie and Harris (12), and was recrystallized twice.

The source and use of ^14C-labeled amino acids were described previously (8).

RESULTS

Resistance to amino acid analogues. Fifty-three amino acid analogues were examined for their ability to inhibit growth of P. aeruginosa. Only 3,4-dehydroproline, 4-nitropyridine - N-oxide, m-fluorophenylalanine, and m-fluorotryptophan markedly inhibited growth on minimal agar; canavanine, p-fluorophenylalanine, thioproline, and 5-fluorotryptophan were very weakly in-
hibitory. When these analogues were examined at defined concentrations in liquid minimal medium, it was generally found that very high concentrations (Table 1) were required for complete inhibition of growth and that at lower concentrations growth occurred slowly. p-Fluorophenylalanine was a more effective inhibitor in liquid medium than on agar, presumably owing to the slight solubility of this analogue.

This resistance of *P. aeruginosa* wild type to amino acid analogues made isolation of transport-negative mutants a rather difficult project. Both the apparent uniqueness and the extent of resistance suggested that further investigation into the nature of this resistance was warranted.

**Influence of analogues on growth.** When two proline analogues, thiprolone and 3,4-dehydroproline, were added to growing cultures of wild-type and P22 strains of *P. aeruginosa*, growth of both was altered from a logarithmic to a linear rate within 4 hr after the addition of 3,4-dehydroproline (100 μg/ml). As expected, the analogue had very little influence on the growth of the pro-Tr^- mutant P5, demonstrating that inhibition of growth by this analogue can be overcome at the transport level. Thiprolone (500 μg/ml) had only a minor inhibitory effect on the wild-type and P22 strains and had no discernible effect on P5.

When the analogues were added to the cultures at zero time, the effect of 3,4-dehydroproline on both the wild-type and the P22 strain was considerably more pronounced, but only minor inhibition occurred with P5 (Fig. 1). At a concentration of 100 μg/ml, dehydroproline did not completely inhibit growth but reduced the growth rate to approximately 6% of normal with the wild type and to 10% of normal with P22. With P5, presumably 3,4-dehydroproline entered the cell at a very slow rate by means of passive diffusion and competed with endogenously produced proline. Again, thiprolone (500 μg/ml) had only a minor influence on the growth of the three strains of *P. aeruginosa*.

The inhibitory effects of these analogues were overcome by the presence of proline, suggesting that competition occurred either for some intracellular reaction or at the transport level. Both thiprolone, and dehydroproline are transported by the specific proline permease in this organism (Kay and Gronlund, unpublished data).

**Growth in the presence of low concentrations of amino acid analogues.** From studies with inhibitors, it was observed that, at high concentrations of analogues, growth of the organism occurred but at a greatly reduced rate. In the presence of p-fluorophenylalanine, growth of the wild-type occurred after an 8-hr lag period, and

<p>| Table 1. Concentration of amino acid analogues required to inhibit growth of <em>P. aeruginosa</em> |
|-------------------------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Analogue</th>
<th>Inhibitory conc^a_</th>
<th>mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>Canavanine</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>dl-p-Fluorophenylalanine</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>3,4-Dehydroproline</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thioprolone</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-Nitropyridine-N-oxide</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Tryptophan</td>
<td>5-Fluorotryptophan</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>m-Fluorotyrosine</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

^a Complete inhibition of growth in minimal medium after 14 hr of incubation at 37 C.

**Fig. 1. Inhibitory effects of proline analogues on the growth of wild-type and mutant strains of *P. aeruginosa*. Cultures were grown in minimal medium (8). Symbols: ○, control; △, thiprolone (500 μg/ml); ●, dehydroproline (100 μg/ml). The inhibitors were added at zero time. A relative absorbance value of 100 is equivalent to 0.375 mg (dry weight) of cells per ml.
the ensuing growth rate was a function of the inhibitor concentration (Fig. 2). With this analogue at concentrations of 25 μg/ml or less, growth proceeded at the normal rate after the initial lag period. When wild-type cells which were growing in the presence of 100 μg of p-fluorophenylalanine per ml were transferred into fresh medium containing various concentrations of p-fluorophenylalanine, growth commenced almost immediately (Fig. 3), indicating that the cells had acquired some resistance to the inhibitory effects of the analogue. The growth rate at high inhibitor concentrations was slow, but the long lag period previously observed (Fig. 2) was no longer evident. It is unlikely that the cells grown in the presence of p-fluorophenylalanine subsequently grew slowly in the presence of high concentrations of p-fluorophenylalanine because of an alteration of some essential protein as a result of analogue incorporation. When these cells were resuspended in minimal medium without p-fluorophenylalanine, they grew at a rate which was only slightly slower than that of non-treated cells.

From these growth studies, it was apparent that some physiological alteration of a cellular function or functions occurred after exposure of P. aeruginosa to the analogues, and this alteration conferred resistance to the inhibitory effects of the amino acid analogues.

Mechanism of resistance to analogues. P. aeruginosa, and the pseudomonads in general, catabolize a wide array of substrates, including amino acids (21), and it was possible that the analogues had caused the induction of high levels of enzymes capable of degrading amino acids and their analogues. This could have significantly reduced the concentration of the analogues and thus their effectiveness.

Cells, which had been grown in the presence of amino acid analogues, were tested for their ability to oxidize the corresponding amino acid. The oxidative patterns of cells grown in the presence and absence of arginine and proline analogues are shown in Fig. 4. With the exception of canavanine-grown cells, faster rates of amino acid oxidation were not evident. Although the initial rate of arginine oxidation by canavanine-grown cells was greater than with the control cells, the final degree of oxidation was lower, suggesting the accumulation of an intermediate. Cells grown in the presence of aromatic amino acid analogues similarly were not induced to oxidize the corresponding amino acids at a rate which was more rapid than the constitutive rate (Fig. 5). On the contrary, most of the analogue-grown cells either oxidized the amino acid more slowly or to a significantly lesser extent. The implications of this latter observation will be discussed.

When the cells which had been grown in the presence of analogues were tested for their ability to transport the corresponding amino acids, present at low external concentrations, it was found that the rate of transport was reduced to...
proline grown in wild cells.

Table 3,4-dehydroproline (5 umoles): O, by control cells; A, by cells grown in the presence of 3,4-dehydroproline (100 μg/ml); □, by cells grown in the presence of thioproline (1 mg/ml).

Fig. 4: Oxidation of arginine and proline by P. aeruginosa wild type, control cells, and analogue-grown cells. Resting-cell suspensions were prepared as described in Materials and Methods. Oxidation of arginine (5 μmoles): O, by control cells; ●, by cells grown in the presence of canavanine (250 μg/ml). Oxidation of proline (5 μmoles): △, by control cells; ▲, by cells grown in the presence of 3,4-dehydroproline (100 μg/ml); □, by cells grown in the presence of thioproline (1 mg/ml).

Fig. 5: Oxidation of aromatic amino acids by normal cells and analogue-grown cells. Resting-cell suspensions were prepared as described in Materials and Methods. Oxidation of tyrosine (5 μmoles): O, by control cells; ●, by cells grown in the presence of p-fluorophenylalanine (200 μg/ml). Oxidation of phenylalanine (5 μmoles): △, by control cells; A, by cells grown in the presence of p-fluorophenylalanine (200 μg/ml). Oxidation of tryptophan: □, by control cells; cells grown in the presence of 5-fluorotryptophan (300 μg/ml) showed no oxygen uptake.

The reductions in rates of transport of amino acids were specific in that cells grown in the presence of dehydroproline did not transport proline but rapidly transported 12 amino acids from a 14C-protein hydrolysate. In this respect, the cells behaved exactly as the pro Tr- strain P5 isolated after treatment of the cells with a mutagen. It was possible that the analogues bound irreversibly to the amino acid permeases and thereby inactivated them. However, when cells were grown in minimal medium, incubated with 5 × 10⁻⁴ M dehydroproline for 30 min, and washed with medium, they were still able to transport proline as effectively as cells that had not been exposed to the analogue. Also, the kinetics of inhibition of proline uptake in the presence of dehydroproline and thioproline, in

Fig. 6: Transport of 14C-proline by P. aeruginosa previously grown in the presence or absence of 3, 4-dehydroproline. Symbols: O, control cells; ●, cells grown in the presence of 3, 4-dehydroproline. Cells were washed three times with minimal medium prior to the initiation of the transport experiments. External proline concentration was 10⁻⁴ M, and the concentration of P. aeruginosa cells was 0.1 mg (dry weight) per ml.
Table 2. Inhibition of amino acid transport in cells grown in the presence of amino acid analogues

<table>
<thead>
<tr>
<th>Amino acid analogue</th>
<th>14C-amino acida</th>
<th>Inhibitionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canavanine</td>
<td>Arginine</td>
<td>65.6%</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>21.5%</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>—</td>
</tr>
<tr>
<td>Dehydroproline</td>
<td>Proline</td>
<td>94.4%</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>—</td>
</tr>
<tr>
<td>Thioproline</td>
<td>Proline</td>
<td>96.1%</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>—</td>
</tr>
<tr>
<td>p-Fluorophenylalanine</td>
<td>Phenylalanine</td>
<td>96.3%</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>92.6%</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>—</td>
</tr>
<tr>
<td>m-Fluorotyrosine</td>
<td>Tyrosine</td>
<td>93.8%</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>95.1%</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5-Fluorotryptophan</td>
<td>Tryptophan</td>
<td>93.1%</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>—</td>
</tr>
</tbody>
</table>

a Uptake studies were performed with amino acid concentrations of 2.5 × 10⁻² M and 0.1 mg (dry weight) of cells/ml.

b Per cent inhibition of normal transport rate. c Within experimental error of no inhibition.

Despite the inhibition of transport of amino acids by these analogues, this organism, as has been shown with Escherichia coli (24), has been shown to strictly transport competitive (Kay and Gronlund, "unpublished data").

Recovery of the transport capacity. When cells were grown in the presence of p-fluorophenylalanine and then resuspended in minimal medium, they quickly regained their original transport capacity (Fig. 7). The cells also rapidly induced higher levels of their transport system when tyrosine (1 mg/ml) was added to the medium. The rate of tyrosine transport increased faster than the rate of growth; that is, during the time interval that the cell mass increased by a factor of 2, the rate of tyrosine transport increased by a factor of 5.5 for those cells in minimal medium, and by a factor of 9 for those cells in the presence of tyrosine. This indicated that the permease was preferentially synthesized by the cells with repressed transport activity.

Transport-negative mutants. The nature of P. aeruginosa as a transport-negative mutant has not been thoroughly investigated as yet. These transport defects were specific for proline, and the kinetics of amino acid uptake were similar to those of cells with repressed proline transport activity, obtained by growth in the presence of partially inhibitory amino acid analogues.

**DISCUSSION**

The complete resistance of P. aeruginosa to inhibition of growth by many amino acid analogues imposes severe restrictions on the isolation of excretor mutants and especially of amino acid transport-negative mutants. The isolation of the latter class of mutants requires that the analogue be an effective inhibitor when present at low concentrations. In instances where analogues do not inhibit growth, the method described here can be employed to isolate transport-negative strains. Although somewhat laborious, this technique is free from most of the inherent errors.
attributed to selection by other methods (3). It also permits the successful isolation of mutants which have lost transport proteins not only for amino acids but also, theoretically, for any compound that will support growth of the organism. This technique should be particularly useful for organisms such as the pseudomonads, which degrade a wide range of substrates. We have successfully isolated transport-deficient mutants for arginine, proline, isoleucine, and tyrosine by this approach, and properties of these mutants are presently under investigation.

The amino acid analogues which caused some inhibition of growth were few, and strong inhibition was demonstrated only at relatively high concentrations. The mode of this inhibition is presumably through the formation of defective protein (5), or possibly by competition for an essential cellular reaction. The proline analogues, azetidine-2-carboxylic acid, dehydroproline, and thioproline, have been shown to be incorporated into the protein of E. coli (23). However, the inhibition of growth by thioproline was considered to act most strongly by preventing the attachment of proline to acceptor ribonucleic acid (24).

The growth response of P. aeruginosa to the effects of 3,4-dehydroproline, when added to an exponentially growing culture, resembled the response caused by amino acid analogues known to be incorporated into protein (15); that is, inhibition of growth proceeds slowly after the addition of the analogue. This effect has been attributed to competition between the analogue and the proline synthesized by the organism (5, 15).

The specific mechanism of inhibition of the growth of P. aeruginosa by analogues was not the prime concern of this study. The determination of the nature of the acquired resistance was considered pertinent to our needs. The resistance was not due to mutation, since, with the exception of p-fluorophenylalanine, a slow rate of growth generally ensued immediately in the presence of the analogue, a phenomenon which would require the presence of a large number of spontaneous, resistant mutants. Further, the transport function, which was lost during growth of the organism in the presence of the analogue, was quickly regained by growth of the organism in the absence of the analogue.

Cells grown in the presence of analogues did not oxidize the respective amino acids rapidly, indicating that the capacity to degrade amino acids had not been induced above the constitutive level. The important factor in determining resistance was the ability of this microorganism to repress the synthesis of particular amino acid permeases. From studies on the uptake of amino acids, it was learned that the analogue-treated cells had essentially lost their ability to transport the specific natural amino acid.

The mechanism whereby the cells accomplish this is unclear; however, three possibilities are most likely at present. Firstly, amino acid transport in P. aeruginosa can be induced to higher than constitutive levels, by growth in the presence of the particular amino acid (Kay and Gronlund, unpublished data). Amino acid transport and degradation are co-induced in this organism (Kay and Gronlund, unpublished data); therefore, it is possible that a degradation product, not the original amino acid, acts as the inducer of transport. If the analogue prevented formation of the inducer, then even the constitutive level of transport protein would be lowered. From the oxidation data presented, this explanation perhaps would seem to be a possibility since the oxidation of the corresponding amino acid was incomplete with cells that had been grown in the presence of analogues. In Bacillus subtilis, it has been suggested that the inducer for arginine transport is linked to arginine degradation, since mutants which were constitutive for arginine degradation were also constitutive for arginine transport. In P. aeruginosa, the constitutive level of the enzymes which degrade arginine is high and the transport rate for arginine is also high (8) relative to other amino acids. Incomplete oxidation of arginine by canavanine-grown cells occurred; however, the
rate of transport of arginine was affected to a lesser extent, relative to cells treated with other analogues. This suggests that this hypothesis may be incorrect unless the intermediate which accumulated during arginine oxidation was, in fact, the inducer. Arginine can also be transported into \textit{P. aeruginosa} via a lysine permease (Kay and Gronlund, unpublished data) and, more likely, this explains the less effective reduction of arginine transport in cells grown in the presence of canavanine.

Secondly, it is possible that the transport proteins specifically incorporated high concentrations of the analogues, resulting in defective permeases. This is somewhat unlikely, as most cellular functions were essentially unaffected by growth in the presence of the analogue and these cells grew quite normally when resuspended in minimal medium.

Thirdly, it is possible that the first enzyme in the pathway of amino acid biosynthesis was partially inhibited by the analogue by feedback inhibition. This would effectively reduce the level of pool amino acid and result in the synthesis of a lower basal level of transport protein if, in fact, the amino acid and not a degradation product is the inducer. End-product inhibition by proline has been observed by Strecker (22). Baich and Pierson studied the regulation of the proline pathway in \textit{E. coli} (1), and also found that the amino acid analogues thio proline, dehydroproline and azetidine-2-carboxylic acid acted as false feedback inhibitors of the pathway (24). The result of such an effect would be repressed transport activity in \textit{P. aeruginosa}. It is likely that this would also be true for \textit{E. coli}, since a high rate of proline transport is also induced by growth in the presence of the amino acid (R. J. Britten, personal communication).

The preparation of transport-deficient cells by the analogue technique described in this report makes possible the study of the specificities of the transport systems for compounds such as amino acids, and obviates the necessity of isolating Tr mutants.

ACKNOWLEDGMENT

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LITERATURE CITED