INTERACTION OF AMINO ACID DEPENDENT AND INDEPENDENT STRAINS OF LACTIC ACID BACTERIA

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Lactobacillus arabinosus strain 17-5 fails to grow in the absence of phenylalanine or tyrosine during an incubation period in which maximum growth is attained in the presence of these amino acids. However, good growth is observed in media deficient in these amino acids when the incubation time is extended (Dunn et al., 1947; Prescott et al., 1949; James, 1950; Atkinson and Fox, 1951; Borek and Waelsch, 1951; Holden, 1956). This delayed demonstration of independence is also observed with Streptococcus faecalis strain R in a tyrosine-deficient medium. The data of James (1950) and of Atkinson and Fox (1951) indicate that the cells of L. arabinosus which grow in the absence of these amino acids are mutants, stably independent of the amino acid requirement found in the cells of the parent culture. This report describes experiments showing that when adaptation occurs in the presence of low levels of these amino acids cultures of L. arabinosus and S. faecalis grow less than they do in the complete absence of the amino acids. MacLeod (1951) has independently observed this anomalous growth response using S. faecalis and a tyrosine-supplemented medium. Similar experiments have been reported previously with a tryptophan-independent culture of L. arabinosus (Wright and Skeggs, 1945) and a histidine-requiring mutant of Escherichia coli (Ryan and Schneider, 1948). The experiments of Ryan and Schneider (1949 a, b, and c) demonstrated that a histidine-independent back mutant of E. coli, which overgrows the parent histidine-dependent culture in the absence of histidine, is inhibited in the presence of histidine by the dependent cells whose growth is fostered by additions of the amino acid. The studies with L. arabinosus and S. faecalis described here indicate that a similar mechanism is responsible for the depression of adaptive growth of these organisms.

MATERIALS AND METHODS

Adaptation experiments and measurements of growth response to amino acid supplements were carried out in tubes of liquid synthetic medium using the organisms and cultural conditions described previously (Holden et al., 1951). Growth in liquid medium was estimated turbidimetrically directly in matched culture tubes using an Evelyn colorimeter fitted with an adapter for these tubes, and is expressed as dry weight of cells per ml. L. arabinosus requires carbon dioxide for growth in phenylalanine- or tyrosine-deficient media (Lyman et al., 1947). Consequently, all incubations of this organism were carried out in a carbon dioxide enriched atmosphere.

The proportion of tyrosine-independent cells present in liquid cultures after adaptation was determined by a plating technique similar to that described by Ryan and Schneider (1948). Plates of tyrosine-deficient medium solidified with agar were used to estimate the number of tyrosine-independent cells. The agar had been washed as described by these authors (1949 d). Total viable cells were counted on plates containing medium originally supplemented with tyrosine. In addition, where no growth of independent cells had occurred in the deficient medium, the total population was estimated by layering such plates with 5 ml of tyrosine-supplemented agar medium and reincubating. Liquid cultures to be examined by this procedure were centrifuged twice, resuspending each time in sterile saline, and then diluted serially in sterile saline. Duplicate tubes of tyrosine-deficient and tyrosine-supplemented liquefied agar medium were inoculated and plated. The plates were incubated at 30 C for 40 hr. Several dilutions of each culture were examined.

RESULTS

In an otherwise adequate medium, the culture of L. arabinosus used achieves maximum growth.
within 24 hr of incubation when phenylalanine and tyrosine are present in the medium. When these amino acids are singly omitted, no visible growth is detected at this time, but maximum growth regularly occurs when incubation is continued to 45 hr, provided vitamin $B_6$ is present and incubation is carried out in an atmosphere of carbon dioxide. Omission of either of these amino acids and vitamin $B_6$ prevents visible growth for as long as 90 hr (L. arabinosus synthesizes vitamin $B_6$ (Holden et al., 1949), so that the vitamin $B_6$-dependency of growth in the absence of these amino acids is not absolute). S. faecalis behaves similarly in a tyrosine-deficient medium, except that the addition of carbon dioxide is not necessary (incubation temperature 30°C), and growth in the absence of vitamin $B_6$ and the amino acid rarely occurs even after incubation for 120 hr. Maximum growth of this organism regularly occurs in the absence of phenylalanine within 16 to 18 hr of incubation, and appears not to involve an adaptation. In the three instances of adaptation cited above, the dose-response curve obtained with graded amounts of the amino acid after incubation for 40 to 45 hr contains a distinctive depressed segment, as illustrated with S. faecalis in the upper curve of figure 1. The lower curve shows the amounts of growth obtained with tyrosine at this time in the absence of vitamin $B_6$ (i.e., when adaptation is prevented), and serves to demonstrate that the response to tyrosine, of the culture which has been permitted to adapt, is the same after the "dip" as is the response of the unadapted culture. After incubation for 20 hr, growth in the presence and absence of vitamin $B_6$ corresponds closely to that represented in the lower curve of figure 1. Substitution of $p$-hydroxyphenylpyruvic acid for tyrosine also gives a dipped dose-response curve in such an experiment. Table 1 summarizes data obtained with L. arabinosus in phenylalanine and tyrosine-deficient media, showing the depression of adaptive growth of this organism in the presence of small amounts of the respective amino and keto acids.

Cells of both organisms isolated from adapted cultures subsequently achieved maximum growth within 24 hr when incubated in media deficient in the respective amino acids. The ability to grow as rapidly in the absence of the amino acid as the parent culture does only in the presence of the amino acid is a stable characteristic, not being lost after many transfers in complete medium. These results are very similar to those obtained by Atkinson and Fox (1951), although it is apparent that the cultures of L. arabinosus used are not identical. Whereas the

![Figure 1. The response of Streptococcus faecalis to tyrosine at 40 hr in the presence and absence of pyridoxamine.](image)

**Table 1**

<table>
<thead>
<tr>
<th>Amount of Supplement</th>
<th>Growth in Basal Medium*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrosine-deficient</td>
<td>Phenylnalanine-deficient</td>
</tr>
<tr>
<td>Supplemented with l-tyrosine</td>
<td>Supplemented with $p$-hydroxyphenylpyruvic acid</td>
</tr>
<tr>
<td>mg dry cells/ml</td>
<td>mg dry cells/ml</td>
</tr>
<tr>
<td>0</td>
<td>1.13</td>
</tr>
<tr>
<td>10</td>
<td>0.59</td>
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<tr>
<td>20</td>
<td>0.74</td>
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<tr>
<td>40</td>
<td>1.04</td>
</tr>
<tr>
<td>100</td>
<td>1.71</td>
</tr>
<tr>
<td>200</td>
<td>1.84</td>
</tr>
</tbody>
</table>

* The basal medium contained 10 μg per 6 ml of pyridoxamine. Incubation was for 50 hr at 30°C in an atmosphere of CO$_2$. 

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2 Streptococcus faecalis failed to attain tyrosine independence at 40 hr in an experiment in which incubation was carried out at 37°C; suggesting that this organism also has a CO$_2$ requirement (Borek and Waelsch, 1951).
Iowa culture attained tyrosine and then phenylalanine independence in a stepwise fashion, the culture used here was found to acquire independence of both amino acids when adaptation occurred in a medium deficient in either amino acid. Thus, although both parent cultures fail to complete the synthesis of these amino acids, the interrupted reactions are not identical (Atkinson and Fox, 1951).

Examination of the population after adaptation in a series of tubes containing graded amounts of tyrosine originally inoculated with the parent, tyrosine-requiring culture of *S. faecalis* was undertaken to determine if the negative slope in the dose-response curve describes a progressive decrease in the number of tyrosine-independent cells. In the study of the histidine-independent back mutant of *E. coli*, Ryan and Schneider (1948) found that the dip in the dose-response curve after adaptation occurred for this reason. The constitution of the population in each tube was determined using the plating procedure described above. Results of one such experiment are summarized graphically in figure 2, which shows the variation in the size of the total population (upper curve) and of the tyrosine-independent population (lower curve) as the tyrosine concentration is increased. The tyrosine-independent cells were found to comprise a progressively smaller proportion of the population until in a tube containing approximately 20 µg of tyrosine they were no longer present in sufficient numbers to contribute significantly to the visible turbidity. It is clear then that when adaptation occurs in the presence of increasing amounts of tyrosine (where there is a progressive increase in the number of tyrosine-dependent cells) there is a concomitant decrease in the tyrosine-independent population.

That the reduction in the number of independent cells in the adapting culture is not produced directly by an inhibitory effect of the amino acid on these cells was established by comparing the growth response of isolated, independent cultures of *L. arabinosus* in the presence and absence of tyrosine or phenylalanine, and of the independent culture of *S. faecalis* in the presence and absence of tyrosine. In all cases, the isolated, independent cultures grew maximally in 24 hr in the presence of low and high concentrations of the amino acids and in their complete absence. The absence of any effect of tyrosine on the size of the tyrosine-independent population of *S. faecalis* was confirmed by plate counts. Since an obvious difference between the isolated, independent culture and the parent culture adapting in the presence of small amounts of tyrosine is the previous growth and presence of dependent cells in the latter, the possibility was considered that these, in some way, are responsible for the inhibition of growth of the independent population.

Only a limited investigation of the mechanism of this effect will be reported here. Ryan and Schneider (1949a, b, and c), in a thorough study of the interaction between histidine-dependent and histidine-independent cells reported several mechanisms of inhibition by the dependent population, these being depletion of carbohydrate, secretion of inhibitors, and increase in acidity of the medium. Examination of culture filtrates of the parent, tyrosine-requiring strain of *S. faecalis*, taken before adaptation, showed them to be markedly inhibitory. A series of tubes

![Figure 2](http://jb.asm.org/)

*Figure 2.* Variation in the total number (upper curve) and the number of tyrosine-independent (lower curve) cells of *Streptococcus faecalis* after adaptation in the presence of increasing concentrations of tyrosine. The numbers in parentheses indicate the per cent of the total number of cells in a tube found to be tyrosine-independent.
Figure 3. Inhibition of an isolated tyrosine-independent strain of *Streptococcus faecalis* by culture filtrates of the parent dependent strain grown with varying concentrations of tyrosine. Column height represents the amount of growth attained in the presence of the indicated volume of filtrate. Positions along the abscissa indicate the amount of tyrosine originally present in the tube from which the filtrate was prepared. The horizontal line at the top of the figure indicates the amount of growth attained in the absence of filtrate.

containing basal medium and graded amounts of tyrosine was inoculated with the dependent strain and incubated for 26 hr. Growth was comparable to that shown in the lower curve of figure 1. The cells were removed by centrifugation, the supernatant medium neutralized with potassium hydroxide and filter-sterilized. Aliquots of the sterile filtrates were added to previously autoclaved tubes containing 3 ml of double-strength tyrosine-deficient medium and sufficient water to make 6 ml after addition of the filtrates. The tubes were inoculated with an isolated tyrosine-independent culture of *S. faecalis* and incubated for 19 hr. The amounts of growth observed are illustrated in figure 3. All the filtrates inhibited growth to some extent, the most active being those from tubes which contained 4 to 10 µg of tyrosine. Such a result was unexpected, in the light of the experiments of Ryan and Schneider, which showed that the inhibitory activity of culture filtrates could be correlated with the size of the population in that tube. Since filtrates from tubes which had been supplemented with large amounts of tyrosine were least inhibitory, the inability of tyrosine-independent cells to grow in these tubes (figure 2) indicates the possible action of other growth-limiting factors such as low pH or nutrient depletion. In a parallel experiment, filtrates were examined from tubes in which the isolated independent strain had grown in the presence of graded amounts of tyrosine. Growth in all these tubes was maximal, and in all cases, these filtrates were found to be only slightly inhibitory. The maximum inhibition obtained was less than the least active filtrate from the dependent culture. Examination of uninoculated tubes showed them to be uninhibitory in these experiments.

**DISCUSSION**

In discussing the results of their investigation into the nature of the growth depression of a histidine-independent back mutant of *E. coli* by the parent, dependent cells, Ryan and Schneider (1949c) emphasized that published data can be interpreted to illustrate such interactions, and on this basis they postulated a general occurrence of this phenomenon. The data presented here together with those of James (1950) and of Rose and György (1955) demonstrate the existence of four additional instances in which parent cells with a growth requirement in some way antagonize the subsequent growth of mutant cells independent of this requirement. The sizeable number of such interactions involving organisms expressing diverse growth requirements precludes a direct relation of the inhibition to the nutrient required. The phenomenon probably is nonspecific with regard to the growth requirement and is more a reflection of the response of the growth factor independent cells to the absence of a nutrient or the presence of an inhibitor in the medium which had previously supported growth of the parent growth factor requiring cells.

Inhibitor production is clearly involved in the growth inhibition of tyrosine-independent cells of *S. faecalis* described here. The degree of inhibition produced by the histidine-dependent strain of *E. coli* described by Ryan and Schneider (1949a) varied directly with the size of this population. With the organisms studied here, which are cultured in complex growth media,
 maximal inhibitor production occurred when the parent strain had achieved much less than maximum growth. Heavy growth of the parent cells resulted in relatively low inhibitor production. It is likely therefore that in this case the inhibitory compound(s) is derived from components of the medium. Thus, heavy growth either of the dependent or independent strains would lead to low inhibitor production due to depletion of the precursor in the course of cell synthesis. The identification of the inhibitory substance should be greatly facilitated if this is true.

It is of considerable interest that the pattern of adaptation in L. arabinosus observed in this study is different from that reported for this organism by Atkinson and Fox (1951). Presumably these cultures were originally derived from the same source. Nevertheless, since the culture used here attained phenylalanine and tyrosine independence simultaneously, while the Iowa culture attained independence in a stepwise fashion, it appears certain that the interruptions in the syntheses of these amino acids in the two cultures are not identical. As a point of departure, if one considers the scheme proposed by Atkinson and Fox (1951), the organism used here is apparently blocked prior to the appearance of the last common precursor, as they believe their culture to be, but, unlike their culture, not at any subsequent point. The extent to which these differences can be ascribed to differences in cultural conditions and to alterations which occurred while the cultures were maintained separately in the two laboratories cannot be estimated without a comparative study of the cultures under defined conditions. The variability in amino acid requirements of L. arabinosus has been discussed in the past (Dunn et al., 1947) and it would seem futile to make definitive statements concerning the characteristic amino acid requirements of this organism on the basis of studies with a single culture.

The finding that different cultures of the same species (indeed, supposedly the same strain) appear to be enzymatically (and possibly genetically) distinct suggests that examination of a large number of cultures might reveal a series of naturally occurring mutants, all requiring the same growth substance but blocked in its synthesis at different sites. A study of such organisms might fruitfully augment the valuable contributions made through the study of the artificially induced mutants. The experiments of Volcani and Snell (1948), in which the route of synthesis of arginine in the lactic acid bacteria was charted by determining the ability of various species to utilize for growth the postulated precursors of this amino acid, represents an initial step in this direction.

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SUMMARY

A culture of Lactobacillus arabinosus which regularly achieves independence of tyrosine and phenylalanine, and a culture of Streptococcus faecalis which achieves tyrosine independence, were studied to determine why adapting cultures grow less in the presence of small amounts of the respective amino acid than they do in the complete absence of the amino acid. Growth of isolated independent strains of these organisms is not inhibited by the amino acids. Counting experiments demonstrated that the number of tyrosine-independent cells of S. faecalis present after adaptation was progressively reduced as the concentration of tyrosine (and consequently the number of tyrosine-dependent cells) was increased. Filtrates of tyrosine-dependent cultures were found to inhibit growth of the independent cells.

The culture of L. arabinosus used here attains phenylalanine and tyrosine independence simultaneously when adaptation occurs in media deficient in either amino acid.

REFERENCES


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