JOURNAL OF BACTERIOLOGY, Dec. 1974, p. 1078-1084

Vol. 120, No. 3
Printed in U.S.A.

Genetic Basis of Nutritional Requirements in *Lactobacillus casei*

T. MORISHITA, T. FUKADA, M. SHIROTA, AND T. YURA

Yakult Institute for Microbiological Research, Kyoto, and Institute for Virus Research, Kyoto University, Kyoto, Japan

Received for publication 24 September 1974

In a study of the genetic basis of multiple nutritional requirements in *Lactobacillus casei*, systematic attempts were made to isolate mutants that can grow in the absence of a specific nutrient required by the parental organism. Such mutants have successfully been isolated with respect to seven of twelve amino acids (aspartic acid, leucine, isoleucine, lysine, methionine, serine, and threonine) and three of four vitamins (pantothenic acid, nicotinic acid, and pyridoxal) tested, after extensive screenings employing various mutagens. Mutants that can grow without tryptophan were not isolated, but those that can grow on anthranilate or indole as well as on tryptophan were obtained at a frequency expected for single-step mutations. Activity of tryptophan synthetase was demonstrated in extracts of these anthranilate-utilizing mutants, but not in the parental strain. These results suggest that the multiple nutritional requirements of *L. casei* are often, if not always, due to one or a few small lesions such as base substitution mutations rather than large deletions affecting the genes involved in each biosynthetic pathway. The data would also imply that many of the biosynthetic pathways that are not fully functional in *L. casei* were active at one time and became nonfunctional during evolution of the present species.

One of the striking characteristics of the genus *Lactobacillus* is the well-known multiple nutritional requirements that these bacteria require for their normal growth. They require a number of amino acids, vitamins, purines, and pyrimidines when grown in synthetic media. It has generally been thought that this characteristic may be the result of adaptation of these organisms to the natural environments to which they have been exposed. Thus, genetic and biochemical analysis of the mechanisms underlying the growth requirements in lactobacilli should contribute not only to the general problem of nutritional requirements among microorganisms found in nature but to ecological or evolutionary problems as well.

Although a number of mechanisms can be contemplated to explain growth requirements for a given nutrient, a specific question was asked as to whether these requirements are brought about by the lack of gene(s) responsible for its biosynthesis. Thus we made a systematic attempt to isolate mutants of *Lactobacillus casei* that had lost the growth requirement for a specific nutrient. As will be shown below, such mutants could indeed be isolated for a majority of nutrients required by the parental organism. Moreover, at least in one case studied, certain mutations were found to result in the appearance of a specific enzyme activity responsible for the biosynthesis of a specific amino acid. These results indicate that most, if not all, of the genes essential for biosynthesis of these nutrients are present in *L. casei*, but they do not produce active proteins due to one or a few deleterious mutations that occurred in the genes for each biosynthetic pathway.

MATERIALS AND METHODS

Bacterial strains. The wild-type *L. casei* used as the parental organism in this study was strain S1 from The Yakult Institute for Microbiological Research, Kyoto, Japan.

Media. Natural medium consisted of (per liter): 10 g of polypeptone (Wako Drug Co.), 10 g of yeast extract (Difco), 10 g of sodium acetate, and 20 g of D-glucose. Basal (synthetic) medium was a glucose-salts medium supplemented with all the nutrients required by the wild-type *L. casei*, strain S1, for its maximal growth (Table 1). Solid media contained 1.5% agar (Hakko Agar Co.). The pH of all media was adjusted to 7.2.

Isolation of mutants. Mutants that had lost a requirement for a given nutrient were isolated after mutagenic treatment of cells by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG), 2-aminopurine (AP), or ultraviolet light (UV).

TABLE 1. *Composition of basal medium*[a]

Compound	Concn (mg/ml)	Compound	Concn (mg/ml)
D-Glucose	10	L-Lysine	0.1
Sodium acetate	30	L-Methionine	0.1
Ammonium chloride	3	L-Phenylalanine	0.1
KH_2PO_4	2	L-Serine	0.1
K_2HPO_4	2	L-Threonine	0.1
Tween 80	1	L-Tryptophan	0.1
Sodium chloride	0.02	L-Tyrosine	0.1
$MgSO_4 \cdot 7H_2O$	0.2	L-Valine	0.1
$MnSO_4 \cdot 7H_2O$	0.04	Riboflavine	0.001
$FeSO_4 \cdot 7H_2O$	0.02	Pantothenic acid	0.001
L-Arginine	0.1	Pyridoxal	0.001
L-Aspartic acid	0.4	Nicotinic acid	0.001
L-Cystine	0.1	Folic acid	0.0001
L-Glutamic acid	0.4	Adenine	0.02
L-Isoleucine	0.1	Uracil	0.02
L-Leucine	0.1	Xanthine	0.02

[a] This medium is based on the synthetic medium routinely used at the Yakult Institute (H. Endo, personal communication) and contains all the nutrients required by the wild-type *L. casei*, strain S1, for its maximal growth. It includes some compounds that are not absolutely required but do exhibit strong stimulatory effects (see Table 2).

(i) Mutagenesis with NG. Cells of the wild-type strain grown in natural medium overnight at 37 C were collected, washed in saline, and treated with NG (700 μg/ml) in 0.2 M acetate buffer (pH 5.0) at 37 C for 3 h. Cells were collected, washed twice in saline, and plated onto appropriate selective agar media. In some experiments, NG mutagenesis was performed by placing crystals of NG at the center of a petri dish containing selective agar media seeded with a lawn of washed wild-type cells.

(ii) Mutagenesis with AP. Cells were grown in natural medium containing AP (50 μg/ml) overnight at 37 C. The cells were harvested, washed twice in saline, and plated on selective agar medium directly, or, in some experiments, after incubation in liquid selective medium for 2 days.

(iii) UV irradiation. UV irradiation was carried out by exposing a saline suspension of cells to a Toshiba germicidal lamp (15 watt) for 1 min at a distance of 60 cm. Irradiated cells were incubated at 37 C overnight in natural medium collected, washed, and plated on selective agar media.

All agar plates were incubated at 37 C, and colonies that appeared after 4 to 5 days were purified by single colony isolation on appropriate selective agar plates. Presumptive mutants thus obtained were then tested for their ability to grow in liquid selective media lacking a given nutrient. All liquid cultures were incubated without aeration, and optical densities were determined with a Klett-Summerson colorimeter using a no. 54 filter.

Assay for tryptophan synthetase. Either cell suspension or crude extract was used for assay of tryptophan synthetase (EC 4.2.1.20). Cultures were grown with limiting amounts of L-tryptophan (1 to 2 μg/ml) or indole (2 μg/ml) in the otherwise standard basal medium. After standing at 37 C for 3 days, cells were harvested, washed, and suspended in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.8). For preparation of crude extracts, 5 mM 2-mercaptoethanol and 10% glycerol were added to the cell suspension, which was then disrupted in a Raytheon 10-kc sonic oscillator and centrifuged at 10,000 × *g* for 20 min. The resulting supernatant fluids were used as crude extracts.

Tryptophan synthetase activity was assayed by the standard procedure used for the enzyme of *Escherichia coli* (7). The reaction mixture (0.5 ml) contained the following (micromoles): Tris-hydrochloride (pH 7.8), 35; indole, 0.2; D,L-serine, 40; pyridoxal phosphate, 0.04; 0.015 ml of saturated NaCl solution; and 0.25 ml of cell suspension or crude extract. After 30 min of incubation at 37 C, the reaction was stopped by adding 0.1 ml of 1 N NaOH to each tube. Disappearance of indole during incubation was estimated by the colorimetric precedure (7).

Protein was determined by the method of Lowry et al. (3), and optical density was measured with a Klett-Summerson colorimeter with a no. 54 filter.

RESULTS

Mutants that lost a specific nutritional requirement. We first determined the nutritional requirements of the parental *L. casei* in liquid culture (Table 2). Several compounds not present in the basal medium were also included to observe their possible stimulatory effects on growth. It can be seen that this strain required 12 amino acids and 4 vitamins for normal growth. In addition, several other nutrients, including amino acid, vitamin, purine, and pyrimidine, were found to stimulate growth of the organism appreciably under the conditions employed. These results generally confirmed

TABLE 2. *Nutritional requirements of the wild-type L. casei*[a]

Nutrient omitted	Growth	Nutrient omitted	Growth
Amino acids		Vitamins	
Alanine	+	Thiamine	+
Arginine	−	Riboflavine	−
Aspartic acid	−	Pyridoxal	−
Cystine	±	Biotin	+
Glutamic acid	−	Pantothenic acid	−
Glycine	+	Nicotinic acid	−
Histidine	+	Folic acid	±
Isoleucine	−	*p*-Aminobenzoic acid	+
Leucine	−		
Lysine	−	Purines and pyrimidines	
Methionine	−	Adenine	±
Phenylalanine	±	Cytosine	+
Proline	+	Guanine	+
Serine	−	Thymine	+
Threonine	−	Uracil	±
Tryptophan	−	Xanthine	±
Tyrosine	−		
Valine	−		

[a] Cells of the wild-type strain grown in natural medium overnight at 37 C were collected, washed twice in saline, and inoculated into a series of liquid synthetic media containing all but one of the nutrients listed. These cultures were incubated at 37 C for 3 days, during which optical density was measured at intervals of 24 h. At least duplicate tubes were employed for each test. Symbols: +, normal growth; ±, partial growth; −, no growth.

those obtained previously by H. Endo (personal communication).

Systematic attempts were then made to isolate mutants that no longer required a specific nutrient for growth. Thus, cells of the wild-type *L. casei* were treated with various mutagens and plated on appropriate media to select for possible mutants. Such mutants were successfully isolated with respect to seven of twelve amino acids, and three of four vitamins required by the parental strain (Table 3). These mutants were obtained only after treatment by mutagens; no mutants have so far been obtained spontaneously. The frequencies of mutants shown in Table 3, however, should be considered only as approximations, in view of the procedures employed for selecting these mutants.

A number of mutants obtained from each selection were purified by single colony isolation, and their nutritional properties were confirmed by streaking tests on agar media with appropriate supplements. The rate of growth of these mutants on selective agar varied widely among different mutants, and some of the faster-growing mutants were further examined in liquid media. Typical growth curves obtained for some of the mutants in the corresponding selective medium are shown in Fig. 1. It can be seen that each of the mutants tested grew in the medium lacking one of the amino acids required by the parental organism. Other nutritional characters of these mutants remained unchanged, however, indicating that the effect of such mutations is strictly limited to a specific biosynthetic pathway. It was also found that the maximal growth of these mutants attained in natural medium was generally somewhat reduced as compared to that of the wild-type strain.

TABLE 3. *Frequencies of mutations leading to the loss of a specific growth requirement*

Nutrient omitted[a]	Spontaneous mutation	Induced mutation[b]	
		Mutagen	Frequency
Aspartic acid	$<6 \times 10^{-10}$	NG	10^{-5}
Leucine	$<5 \times 10^{-10}$	NG	10^{-6}
Isoleucine	$<5 \times 10^{-10}$	AP	10^{-8}
		UV	10^{-8}
Lysine	$<6 \times 10^{-10}$	NG	10^{-7}
Methionine	$<6 \times 10^{-10}$	AP	10^{-7}
Serine	$<6 \times 10^{-10}$	NG	10^{-5}
Tyrosine	$<5 \times 10^{-10}$	NG	10^{-7}
Pantothenic acid	$<7 \times 10^{-10}$	NG	10^{-7}
Nicotinic acid	$<7 \times 10^{-10}$	AP	10^{-7}
Pyridoxal	$<7 \times 10^{-10}$	NG	10^{-8}

[a] Each of these nutrients was omitted from the basal medium used for selection of the mutants.

[b] Mutagenesis was carried out as described in Materials and Methods. Frequency of mutants shown gives an approximate order of magnitude estimated from several experiments.

Mutants similar to those described here have

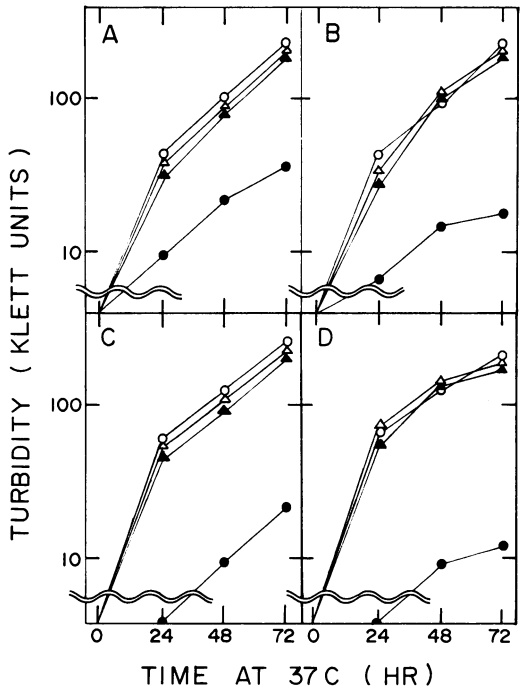

FIG. 1. *Typical growth curves of the mutants that have lost a specific nutritional requirement. (A) Aspartate-independent mutant, (B) serine-independent mutant, (C) lysine-independent mutant, and (D) leucine-independent mutant. Washed cell suspension of each mutant was used to inoculate the basal medium (△) and a selective medium lacking a specific amino acid (▲) at time zero. Cultures were incubated at 37 C without aeration, and optical density was measured at the times indicated. The parental strain was grown in the basal medium (○) as well as in the selective medium (●) as a control for each mutant tested.*

also been isolated from another species, *L. acidophilus*, at least with respect to several amino acids, including aspartic acid, tyrosine, and isoleucine. This suggests the general occurrence of such mutants among various lactobacilli.

Mutants capable of utilizing precursors of tryptophan. The above results strongly suggest that all the genes determining the structure of enzymes involved in most biosynthetic pathways are present in the wild-type *L. casei* and that one or a few mutations can lead to the normal functioning of each pathway. With respect to the nutrients for which no such mutants could be isolated by one-step selection, however, it is not clear whether the defect is due to an "irreparable" damage such as large deletion or to a number of reparable damages. In the latter case, we might expect to find mutants that can grow on certain precursors of a nutrient that cannot be utilized by the parental organism. The tryptophan biosynthetic pathway was chosen to investigate this possibility.

It was first shown that the tryptophan requirement of the wild-type *L. casei* was not satisfied by anthranilate or indole, possible intermediates on the biosynthetic pathway. Experiments were then carried out to find possible mutants that can grow on indole or anthranilate as well as on tryptophan in otherwise standard basal medium. Wild-type cells grown in the presence of 2-aminopurine were harvested and washed, and samples were plated on basal agar medium containing anthranilate or indole instead of tryptophan. After incubation at 37 C for 5 days, mutant colonies appeared on the agar plates at a frequency of about 10^{-6}. These mutants grew on anthranilate or indole, as well as on tryptophan, in contrast to the parental strain, whose requirement for tryptophan was not satisfied by these compounds (Table 4). This was true whether the selection was made on the medium containing indole or anthranilate.

These results suggest that anthranilate and indole are probably involved in tryptophan biosynthesis in *L. casei* as in *E. coli*, and that the wild-type strain carries genetic blocks at the stages of both anthranilate synthesis and the

TABLE 4. *Growth of mutants that can utilize precursors of tryptophan*

Strain	Growth (optical density)[a]			
	Basal medium	−Tryptophan	−Tryptophan +Anthranilate	−Tryptophan +Indole
Wild type	207	7	9	11
Mutant 2[b]	162	5	151	143
Mutant 4[b]	167	2	149	150
Mutant 8[b]	174	2	156	160
Mutant 9[b]	158	0	159	147
Mutant 10[b]	189	3	178	175
Mutant 11[c]	155	0	141	132
Mutant 12[c]	181	1	143	155
Mutant 15[c]	176	2	159	162
Mutant 17[c]	163	6	156	147
Mutant 18[c]	158	0	155	144

[a] Washed cell suspensions were used to inoculate the media as indicated and were incubated at 37 C for 48 h. Values represent optical density as expressed in Klett units.

[b] Mutants selected for their ability to grow on indole.

[c] Mutants selected for their ability to grow on anthranilate.

conversion of indole (or indole glycerol phosphate) to tryptophan (Fig. 2).

Tryptophan synthetase activity in anthranilate-utilizing mutants. To find out whether the utilization of anthranilate or indole by these mutants was due to an increased activity of tryptophan synthetase or to other factors such as increased permeability to these compounds, tryptophan synthetase activity was assayed in the mutant and in the wild-type strains. Cultures were grown in basal medium with limiting concentrations of L-tryptophan or indole, and washed cell suspensions were used for enzyme assay. Tryptophan synthetase activities in terms of serine-dependent disappearance of indole were demonstrated in all the mutants tested, whereas little or no activity was detected in the wild-type strain (Table 5).

These results were also confirmed by enzyme assays performed with crude extracts (Table 6). The enzyme in the mutant extract required serine for maximal activity. Furthermore, mixing of equal amounts of wild-type and mutant extracts resulted in activity about equal to that of the mutant extract alone. It seems unlikely, therefore, that the lack of enzyme activity in the wild-type extract is due to the presence of an enzyme inhibitor. Nor does it seem likely that the appearance of enzyme activity in the mutant is due to the production of an enzyme activator. It may tentatively be concluded that these mutations affected one of the genes determining the structure or amount of tryptophan synthetase.

Attempts to isolate tryptophan-independent mutants starting from the anthranilate-utilizing mutants obtained above have so far failed, despite many efforts that were made, employing several different mutagens.

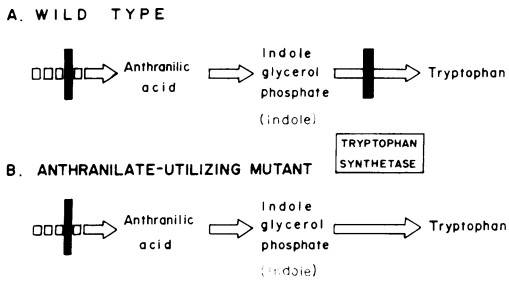

FIG. 2. *Location of genetic blocks on the postulated pathway of tryptophan biosynthesis in L. casei, strain S1.*

DISCUSSION

Mutants of lactobacilli that have lost the growth requirement for a specific nutrient have been reported previously in several different species. For example, a thiamine-independent mutant of *L. fermenti* (1), tryptophan-

TABLE 6. *Tryptophan synthetase activity in crude extracts of an anthranilate-utilizing mutant*

Extracts (mg of protein)	Reaction system	Disappearance of indole (μmol)	Specific activity[a]
Wild (0.58)	Complete	<0.01	<0.17
Wild (0.29)	Complete	<0.01	<0.37
Mutant 2 (0.61)	Complete	0.097	1.59
Mutant 2 (0.31)	Complete	0.063	2.03
Mutant 2 (0.61)	−Serine	0.015	0.25
Wild (0.29) + Mutant 2 (0.31)	Complete	0.053	0.88

[a] Expressed as units per milligram of protein. One unit is defined as that amount of enzyme which causes disappearance of 0.1 μmol of indole in 30 min under standard conditions.

TABLE 5. *Tryptophan synthetase activity in cells of anthranilate-utilizing mutants*[a]

Expt	Strain	Addition to the medium (μg/ml)	Cell suspensions used[b]	Disappearance of indole (μmol)	Specific activity[c]
I	Wild type	Tryptophan 1	1.00	<0.01	<0.01
	Wild type	Tryptophan 2	1.00	<0.01	<0.01
	Mutant 2	Tryptophan 1	0.72	0.06	0.083
	Mutant 2	Tryptophan 2	1.69	0.08	0.047
II	Wild type	Tryptophan 2	1.00	<0.01	<0.01
	Mutant 8	Indole 2	1.04	0.077	0.074
	Mutant 9	Indole 2	0.75	0.047	0.063

[a] Cells were grown in basal medium containing limiting concentrations of L-tryptophan or indole, as indicated, to stationary phase, and washed cell suspensions were used for assay of tryptophan synthetase activity.

[b] Expressed as optical density, which was measured and represented in Klett units divided by 100.

[c] Expressed as micromoles/optical density.

independent mutants of *L. arabinosus* and *L. casei* (6), and a mutant capable of growing in the absence of nucleotides and folate in *L. fermenti* (2, 5) have been described. Mutants with altered nutritional requirements were also found among those selected for resistance to certain antibiotics in various species of lactobacilli (4). However, in all these instances, the kinds of mutants obtained are limited, and mutants altered with respect to only a few nutrients were obtained from any specific parental organism employed.

In our study, extensive search for such mutants using several different mutagens led to the successful isolation of mutants for the majority (10 of 16) of nutrients required by the parental strain of *L. casei*. These mutants most probably represent single-step or at most two-step mutations. In the case of tryptophan, tryptophan-independent mutants could not be isolated by single-step mutations, but anthranilate-utilizing mutants were obtained as a result of mutation affecting the activity of tryptophan synthetase (Fig. 2). No attempts were made, however, to see if an inactive tryptophan synthetase is produced by the parental strain. Therefore, the question of whether the present mutations affected one of the structural genes for the enzyme rather than a regulatory gene that controls the amount of enzyme synthesized remains open for future investigation.

In view of the results obtained for the tryptophan pathway, it is possible that similar situations may be found with other biosynthetic pathways for which single-step mutants able to grow without the respective end product have not so far been isolated, i.e., arginine, glutamate, threonine, valine, and riboflavine. In any event, it may be concluded that growth requirements for specific nutrients in lactobacilli are usually, if not always, due to one or a few minor defects in the bacterial genome which can easily be eliminated or phenotypically suppressed by a single or a small number of mutations. The defects presumably involve point mutations rather than large deletions.

A wild-type strain of *L. casei* whose tryptophan requirement can be satisfied by anthranilate or indole has been described by Snell (8). Although the precise nature of difference(s) between the two strains of *L. casei* remains obscure at present, strain S1 used in our laboratory appears to carry an additional defect in the conversion of indole to tryptophan, and the latter defect can be overcome by single mutational events.

The maximal growth of most of the mutant strains obtained was significantly lower than that of the wild-type strain when cultured in natural medium. Furthermore, we have occasionally observed that these mutants can revert to the original phenotype with respect to the nutritional requirement after successive transfers in natural medium. It seems as though the wild-type strain of *L. casei* represents the most well-adapted form in the natural environments, and any deviations from this genetic constitution by single-step mutations are likely to be deleterious to the whole organism.

This notion seems to be further supported by the following experiments. Starting with one of the isoleucine-independent mutants listed in Table 3, tyrosine-independent mutants were selected after nitrosoguanidine mutagenesis. As expected, the double mutants obtained required neither isoleucine nor tyrosine for growth. When grown in natural medium, they appeared to grow even more slowly than their immediate parental strain. By repeating the same selection procedure, we finally obtained quintuple mutants that had lost the growth requirements for serine, aspartic acid, and leucine, in addition to isoleucine and tyrosine. The comparison of growth rates among these mutants in natural medium revealed the inverse relationship between growth rate and the number of mutational steps involved to obtain the mutant, with the quintuple mutants being the slowest (T. Morishita, unpublished data). It remains possible, however, that some unrelated deleterious mutations accumulated during the repeated treatments with nitrosoguanidine.

As to the mechanisms of nutritional requirements in wild-type *L. casei* and other related organisms, the present status of our knowledge is too limited to permit meaningful discussion at this time. It should be stated, however, that a genetic as well as a biochemical approach would prove most fruitful in solving this long-lasting problem. The importance of discovering genetic mechanisms such as those found in *Escherichia coli* cannot be overemphasized for future development in this field.

LITERATURE CITED

1. Cheldelin, V. H., M. J. Bennett, and H. A. Kornberg. 1946. Modifications in the *Lactobacillus fermenti* 36 assay for thiamine. J. Biol. Chem. **166**:779-780.
2. Cheldelin, V. H., and A. P. Nygaard. 1951. Adaptations in growth factor requirements for lactic acid bacteria. J. Bacteriol. **61**:489-495.
3. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
4. Miyazawa, S. 1962. Studies on the nutritional requirements of lactic acid bacteria in the presence of antibiotics. Part XI. The vitamin and amino acid requirements of antibiotics resistant strains in lactic acid bacteria.

Nippon Nogei Kagaku Kaishi **36**:514-520.
5. Nygaard, A. P., and V. H. Cheldelin. 1951. Nutrition studies of two variants of *Lactobacillus gayoni*. J. Bacteriol. **61**:497-505.
6. Sbarra, A. J., and M. M. Hardin. 1951. Attempts to develop strains of Lactobacillus arabinosus 17-5 and *Lactobacillus casei* ATCC 7649 independent of certain growth factors. J. Bacteriol. **61**:99-100.
7. Smith, O. H., and C. Yanofsky. 1962. Enzymes involved in the biosynthesis of tryptophan, p. 794-806. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
8. Snell, E. E. 1943. Growth promotion of tryptophan-deficient media by o-aminobenzoic acid and its attempted reversal with orthanilamide. Arch. Biochem. **2**:389-394.