ADAPTATIONS IN GROWTH FACTOR REQUIREMENTS FOR LACTIC ACID BACTERIA^{1, 2}

VERNON H. CHELDELIN AND AGNAR P. NYGAARD³

Department of Chemistry, Oregon State College, Corvallis, Oregon

Received for publication January 24, 1951

Adaptive processes in bacteria, whereby growth of organisms in deficient or drug-enriched media can be initiated through the production of new or latent enzyme systems, have been widely described. Reviews on this subject by Karström (1938), Dubos (1940), and Monod (1947) have provided examples of adaptation in bacterial carbohydrases and proteases, in enzymes of fermentation and glycolysis, and in detoxication systems. Although Sevag (1946) and others have questioned whether these processes involve true adaptation or the selective growth of variants already present in the culture, it is evident that adjustments to deficient media, either by individual cells or through quantitative changes in large populations, are commonplace occurrences.

Adaptation with respect to vitamins and other accessory growth factors has been less frequently observed, particularly among the lactic acid bacteria. Notable exceptions are the loss of requirement for *p*-aminobenzoic acid by *Lactobacillus arabinosus* 17-5 (Lewis, through Pennington, 1946), for thiamine by *Lactobacillus fermenti* 9338 (Cheldelin, Bennett, and Kornberg, 1946), and for vitamin B₁₂ and the tomato juice (TJ) factor (vitamin B₆ phosphates) for *Lactobacillus lactis* Dorner (Shorb and Briggs, 1948).

In the present study, adaptations have been observed in the requirements for several accessory growth factors by *Lactobacillus gayoni* and *Leuconostoc mesenteroides*.

EXPERIMENTAL METHODS

Organisms. The organisms employed in this study were *L. gayoni* 8289 and *L. mesenteroides* 10100. Both were regularly maintained as stab cultures containing 0.5 per cent glucose, 1 per cent Difco yeast extract, 0.5 per cent Difco peptonized milk, and 1.5 per cent agar, although in earlier experiments (before 1949) the peptonized milk was omitted. Transfers were made monthly except when otherwise indicated. Cultures for inoculum were grown in 10 ml of basal medium containing 2.5 mg each of Wilson's liver fraction L and Difco yeast extract, after transfer from stabs and incubation at 37 C for 16 to 24 hours.

Basal media. The basal media employed are listed in table 1.

Assay procedure. Samples are arranged in test tubes containing 5 ml of medium

¹ Published with the approval of the Monographs Publications Committee, Oregon State College, as Research Paper No. 171, School of Science, Department of Chemistry.

² This work was supported by grants from Swift and Company, Inc., and the Williams-Waterman Fund of Research Corporation, Inc.

³ Norwegian National Research Council Fellow.

diluted to 10 ml with distilled water. Tubes are cotton-plugged and sterilized by autoclaving for 15 minutes at 15 pounds pressure. They are inoculated aseptically with one drop (0.05 ml) of an inoculum (optical density approximately 0.25)⁴ prepared by resuspending the centrifuged cells in sterile 0.9 per cent NaCl solution. The tubes are incubated at 37 C in a water bath. Growth is measured by turbidimetric readings in a suitable turbidimeter, or acid production is alternatively measured by titration with alkali.

TABLE 1
Composition of basal media

CONSTITUENTS	AMOUNT	
	<i>L. gayoni</i> 8289	<i>L. mesenteroides</i> 10100
Casein, vitamin-free, acid-hydrolyzed.....	10 g	10 g
Sodium acetate, anhydrous.....	12 g	12 g
Glucose.....	20 g	20 g
L-Cystine.....	200 mg	200 mg
DL-Tryptophan.....	200 mg	200 mg
Adenine sulfate, guanine hydrochloride, uracil.....	(each) 20 mg	20 mg
Salts A and B (Snell and Strong, 1939).....	(each) 10 ml	10 ml
Sorbitan monooleate ("tween 80").....	1 g	
Thiamine hydrochloride.....	200 µg	40 µg
Nicotinic acid.....	200 µg	200 µg
Calcium pantothenate.....	200 µg	100 µg
Pyridoxine hydrochloride.....	200 µg	100 µg
p-Aminobenzoic acid.....	200 µg	
Riboflavin.....	400 µg	4 µg
Biotin.....	0.4 µg	0.4 µg
Folic acid.....	20 µg	
Distilled water to 1,000 ml; pH 6.8.....		

ADAPTATION OF *L. GAYONI*

Nucleotides. It was reported by Hutchings, Sloane, and Boggiano (1947) that nucleotides were stimulatory for *L. gayoni*. This has been confirmed in this laboratory (Nygaard and Cheldelin, 1951). However, after more than a year of working with this organism, both cultures being used developed the ability to grow luxuriantly on the basal medium. A new stab obtained from the American Type Culture Collection also behaved in this manner, although the original ones were obtained from the same source. As a matter of interest, the original organisms were retested several months later, during which period they had not been used except for transfer at monthly intervals. They were then found to have assumed their previous requirement for nucleotides.

To test the hypothesis that these organisms had developed the ability to dispense with nucleotides after continued frequent use, they were subjected to daily transfer and tested for their dependence upon nucleotides (adenylic acid

⁴ Optical density = 2-log per cent transmission.

was used throughout). The results are summarized in table 2, where the cultures are labeled strain 45 and strain 47. These numbers refer to the year in which they were first studied in this laboratory, and serve to distinguish them from the nucleotide-independent strain that arose from them. The latter will be called strain 49. It will be seen that the transferred culture (strain 49) grew luxuriantly in the basal medium. However, the complete change to the nucleotide-independent type occurred at a much slower rate than has been observed for adaptations with respect to carbohydrases; for example, 20 transfers of strain 45 were required on the agar medium described above, and 15 transfers of strain 47 were needed in the broth used for the inoculum cultures. The slow rate of adaptation is not surprising in view of the fact that both the agar and broth media supplied

TABLE 2

Loss of requirement for nucleotides in L. gayoni after successive daily transfers in media containing liver

CULTURE	TRANSFER TREATMENT	ADENYLIC ACID ADDED TO TEST MEDIUM μg PER 10 ML	OPTICAL DENSITY			
			Time, hours			
			15	22	27	48
"45"	Transferred once to broth	0		0.01		0.01
		500		0.30		1.1
	Transferred daily on agar 20 times, then once to broth	0		0.94		1.1
		500		1.1		1.1
"47"	Transferred once to broth	0	0.00		0.00	
		500	0.06		1.1	
	Transferred daily in broth 15 times	0	0.31		0.87	

at least a part of the requirement for nucleotides, and that the extent of extra synthesis necessary to meet the total requirement could not therefore have been great.

Folic acid. Although the requirements for other growth factors have not been studied with each of the serial transfers from strains 45 and 47, strain 49 has been found able to dispense not only with nucleotides but also with folic acid, which is practically essential for strain 45 (Nygaard and Cheldelin, 1951). Whether these adaptations occur simultaneously with the change in the nucleotide requirement is not known. Evidence that the reverse processes (loss of ability of strain 49 to synthesize these factors) do *not* occur simultaneously is offered by the formation, through aging and infrequent transfer of strain 49, of a strain *requiring* folic acid that nevertheless grows abundantly in the absence of nucleotides.

An accessory liver factor. The existence of an unidentified growth factor for

L. gayoni 8289 has been previously demonstrated (Cheldelin and Riggs, 1946). This substance, obtained from liver, elicited luxuriant growth and heavy acid production by the organism. It was called for convenience the Gayoni factor. Subsequent experiments have revealed that the Gayoni factor probably represents the combined activity of nucleotides, high levels of folic acid, and an as yet unidentified stimulatory factor from liver. The relationship that exists among these metabolites is being investigated further. Of greater importance to the present study, however, is the observation that the loss of requirement for nucleotides described above was accompanied by a loss of requirement for liver preparations.

The reverse adaptation process, i.e., development of a requirement for the foregoing metabolites is evidently much slower. Although the adapted strain appeared to revert spontaneously to a nucleotide-requiring type, studies to date with strain 49, which have attempted to set a minimum time for this reversal, have proceeded beyond 8 months' duration with no definite adaptation toward a more fastidious character. As Monod has pointed out (1947), this is perhaps to be expected, since in any process $A \leftrightarrow C \leftrightarrow D$, if the regular sequence $A \leftrightarrow C$

can be by-passed by development of the reaction route $B \leftrightarrow C$, reversion to the $A \leftrightarrow C$ route will be slowed by the continued production of B through its equilibrium with C . The enzyme system that catalyzes the latter transformation will thus be maintained in an active state over a relatively long period of time. Braun (1947), in discussing the phenomenon of dissociation (change from smooth to rough colonies), explains the lack of reversibility (rough to smooth) on the basis that the rough variant is usually one of high viability. Smooth variants arising in a rough population will thus have little chance to establish themselves because of competition with the much greater number of more viable rough cells. Although no systematic attempt has been made in the present study to relate the biochemical changes observed to a dissociation process, examination of about a dozen colonies of each strain revealed no obvious differences in colony appearance.⁵ In any case, Braun's explanation for slow reversibility may also apply to *L. gayoni*, since the new variant (strain 49) is the more viable one.

The possibility, of course, exists that alterations in growth requirements for strain 45 might be due to preferential growth of variants already present in the culture, as pointed out by Sevag (1946). The data presented here are not intended to bear upon this controversy, although it was observed that in the approximately 12 isolates from each strain mentioned above, growth requirements were uniform and characteristic of their respective strains.

Maintenance of nutilite dependence in strain 45 has lately been successful for more than a year. This has been accomplished by including 0.05 per cent ammonia-hydrolyzed yeast nucleic acid in the agar stabs, then preparing several agar transfers simultaneously from each stock culture and aging all of them for

⁵ We wish to thank Dr. P. R. Elliker and Mr. A. W. Anderson for their kind assistance in conducting these studies.

at least one month before use. Serial transfers are never made oftener than at monthly intervals, in contrast to the usual practice of transferring lactobacilli weekly or oftener when these are used as assay organisms. Other techniques such as enriching the agar with additional liver, or lyophilization, are also being considered.

ADAPTATION OF *L. MESENEROIDES*

A growth factor for L. mesenteroides. An unknown factor from liver, necessary for optimum growth of *L. mesenteroides* 10100, has been studied by the authors for several years. The concentration and properties of this factor, which has been shown very recently to be probably identical to the vitamin B₆ phosphates, are being reported in a separate publication.

TABLE 3

Adaptation of L. mesenteroides 10100 to growth in the absence of a liver factor (B₆ phosphate)

LIVER FRACTION L	OPTICAL DENSITY		
	Time, hours		
	20	15	60
	Organism I	Organism II	Organism III
mg			
0	0.094	0.360	0.040
0.1	0.245	0.388	
0.2	0.300	0.400	
0.5	0.365	0.428	0.120
1.0	0.405	0.450	0.260
5.0			0.470

Organism I = stock culture, first transfer.

Organism II = stock culture, transferred twice weekly approximately 15 times.

Organism III = organism II, after being transferred monthly over three-year period.

As observed for *L. gayoni*, *L. mesenteroides* has also improved in its ability to grow on the basal medium (table 1) employed in its culture. Growth became nearly as heavy in the "blank" tubes as in those containing liver extract, as may be seen from table 3. With the first transfer from a stock culture, the turbidities ranged from 0.09 in the blank tube to 0.41 in the tube containing 1 mg of liver fraction L. In a culture that had been transferred frequently over a period of several weeks, the corresponding turbidities were 0.36 and 0.45, respectively. The similarity that this phenomenon seemed to bear to the process described above for *L. gayoni* was accentuated by the observation, made 3 years later, that *L. mesenteroides* cultures that had meanwhile been transferred at monthly intervals were again dependent upon liver extracts for growth even after incubation periods of 60 hours or longer. Although the columns in table 3 are not strictly comparable because of the fact that the response curves were obtained at different times with different liver standards, the low blank value of 0.04 in the aged organism indicates that synthesis of the factor was at a minimum.

In order to determine the extent of synthesis of the *Leuconostoc* factor by growing cultures, the following experiment was performed: two 30-ml volumes of medium were inoculated with a culture that was capable of good growth on the basal medium after prolonged incubation (36 hours or longer). One flask contained no added liver, whereas the other contained about half of the amount needed for optimum growth in 14 to 18 hours (0.6 mg liver fraction L per 10 ml). The latter culture was removed to a refrigerator after growing to an optical density approximately 0.40, and the culture without added liver was allowed to remain at 37 C until its optical density also reached 0.40. Both crops of cells were then harvested, and, after autoclaving, the organisms and the media were tested for the presence of the *Leuconostoc* factor. The results in table 4 indicate that synthesis occurred in each culture, but that it was about three times as great in the culture that had grown without the added liver factor. It seems reasonable

TABLE 4

*Synthesis of Leuconostoc factor (B₆ phosphate) during growth of L. mesenteroides 10100**

FACTOR ADDED mg liver L activity	ACTIVITY FOUND	
	mg liver L activity	
0	Cells	0.6
	Medium	1.1
	Total	1.7
	Total synthesized	1.7
1.8	Cells	1.1
	Medium	1.3
	Total	2.4
	Total synthesized	0.6

Volume = 30 ml.

* Stock culture used.

to conclude that adaptation of *L. mesenteroides* to more rapid growth through transfer on the basal medium is caused by increased synthesis of the factor by the organism. The loss in requirement for B₆ phosphates by *L. lactis* Dorner (Shorb and Briggs, 1948), during dissociation of the culture, presumably also involves an adaptation toward synthesis of these factors. As with *L. gayoni*, *L. mesenteroides* is at present being maintained in a liver-factor-dependent state through aging. Intervals of at least a month are placed between successive serial transfers.

General experience with lactic acid bacteria has indicated that adaptive enzymes do not exist in these organisms for the production of most vitamins. However, it is now evident that adaptation occurs toward the synthesis of thiamine, *p*-aminobenzoic acid, vitamin B₁₂, folic acid, and B₆ phosphates. With the exception of *p*-aminobenzoic acid, these nutrilities possess compound structures consisting of two or more moieties. These may likely arise (as does thiamine in *L. fermenti*) through the coupling of constituent parts. It seems probable that

many other species that require vitamins and coenzymes for growth could be trained to synthesize them, not only from the moieties but possibly also from other precursors. Successful development of such variant strains would furnish much useful material for studying metabolic transformations that occur in these organisms, particularly at the enzyme level, through the use of cell-free systems.

REFERENCES

- BRAUN, W. 1947 Bacterial dissociation. *Bact. Revs.*, **11**, 75-104.
- CHELDELIN, V. H., BENNETT, M. J., AND KORNBERG, H. A. 1946 Modifications in the *Lactobacillus fermenti* 36 assay for thiamine. *J. Biol. Chem.*, **166**, 779-780.
- CHELDELIN, V. H., AND RIGGS, T. R. 1946 A growth factor for *Lactobacillus gayoni* 8289. *Arch. Biochem.*, **10**, 19-32.
- DUBOS, R. J. 1940 The adaptive production of enzymes by bacteria. *Bact. Revs.*, **4**, 1-16.
- GALE, E. F. 1943 Factors influencing the enzymic activities of bacteria. *Bact. Revs.*, **7**, 139-173.
- HUTCHINGS, B. L., SLOANE, N. H., AND BOGGIANO, E. 1947 The nutrition of *Lactobacillus gayonii*. *J. Biol. Chem.*, **162**, 737-738.
- KARSTRÖM, H. 1938 Enzymatische Adaption bei Mikroorganismen. *Ergeb. Enzymforsch.*, **7**, 350-376.
- LEWIS, J. C., through PENNINGTON, D. 1946 Assay of *p*-aminobenzoic acid. *Science*, **103**, 397.
- MONOD, J. 1947 The phenomenon of enzymatic adaptation. *Growth*, **11**, 223-289.
- NYGAARD, A. P., AND CHELDELIN, V. H. 1951 Nutrition studies of two variants of *Lactobacillus gayoni*. *J. Bact.*, **61**, 497-505.
- SEVAG, M. G. 1946 Enzyme problems in relation to chemotherapy, "adaptation," mutations, resistance, and immunity. *Advances in Enzymol.*, **6**, 33-121.
- SHORB, M. S., AND BRIGGS, G. M. 1948 The effect of dissociation in *Lactobacillus lactis* cultures on the requirement for vitamin B₁₂. *J. Biol. Chem.*, **176**, 1463-1464.
- SNELL, E. E., AND STRONG, F. M. 1939 A microbiological assay for riboflavin. *Ind. Eng. Chem., Anal. Ed.*, **11**, 346-350.