NUTRITIONAL REQUIREMENTS OF BACTERIOPHAGE-SENSITIVE AND BACTERIOPHAGE-RESISTANT STRAINS OF LACTIC STREPTOCOCCI

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INTRODUCTION

This investigation represents one phase of studies on the use of bacteriophage-resistant lactic acid starter bacteria for cheeses and cultured milks. Bacteriophages, or more simply phages, are known to be capable of lysing starter bacteria with a resulting lack of acid production. This form of infestation of the dairy plant may result in extensive economic loss due to production of inferior final products. Lack of acid production due to phage lysis of lactic acid bacteria also may lead in some instances to development of pathogenic bacteria in some products. Other studies conducted in the Department of Bacteriology at Oregon State College have indicated the practicability of using suitable bacteriophage-resistant culture developed from secondary growth following phage lysis of sensitive strains.

The study reported here was undertaken for the purpose of obtaining fundamental data on factors that effect continued virus-resistance and rate of acid production of lactic streptococci used in starter culture. Specifically, it is a study of: 1. Nutritional requirements of virus-sensitive strains of Streptococcus cremoris as compared with the requirements of virus-resistant mutants of

these organisms. 2. Effect of addition and deletion of a number of vitamins and other substances on the proliferation of bacteriophages homologous for the above strains.

HISTORICAL

General differences between phage sensitive and resistant mutants. It is well known that a culture of bacteria, subjected to lysis by bacteriophage, can give rise to secondary growth that is no longer susceptible to action by that specific virus. These resistant organisms may or may not exhibit morphological changes, but in many cases the only easily determined difference is their ability to grow in the presence of the homologous bacteriophage.

It was the opinion of d'Herelle (15, p. 188) that the resistant forms developed from cells which had recovered from infection by the virus and in the process had developed a hereditary immunity. He further concluded, "It is indeed probable that all fixed mutations occurring in bacterial species are produced through the action of the bacteriophage." (15, p. 222). Other investigators (7, pp. 31-37) (32, pp. 495-499) have offered rather conclusive evidence that resistant mutants are produced independent of action by the virus. Burnet (7, pp. 31-37) has actually isolated bacteriophage-resistant mutants of a normally sensitive culture which had not been subjected to lysis by any bacteriophage. At the present time it is generally accepted that the development of a bacteriophage resistant culture is one of selection rather than

"stimulated mutation". In recent years, evidence has been offered that these mutants may differ from their parent strain in their amino nitrogen requirements (2, pp. 398-399)(3, pp. 121-125), nucleic acid nutrition (11, pp. 187-196)(12, pp. 4-19)(19, p. 128), rate of growth (24, pp. 529-530), and in their type of biological output (34, pp. 3-5).

Nutritional requirements of lactic acid bacteria. The nutritional requirements of the lactic acid bacteria have recently received considerable study. This is due, in no small part, to the need by these organisms for a number of growth factors required by higher animals, and to the relative ease with which the metabolism of such substances may be studied in microorganisms. The extreme fastidiousness of some of the lactic acid bacteria is well illustrated by Leuconostoc mesenteroides P-60 which requires 17 different amino acids to achieve even the slightest amount of growth. This is in comparison to the higher animals and man which require only about 10 amino acids for growth.

The first accurate characterization of the complete nutritional requirements of Streptococcus lactis (a better known species closely related to S. cremoris) was made by Niven (38, pp. 343-349). Of 21 strains examined by him, all were found to require biotin, nicotinic acid, and pantothenate. Eighteen of 21 strains were shown to

require thiamin and 10 of 31 strains required riboflavin. Approximately half-maximum growth was obtained when pyridoxin was omitted from the medium. None of the strains tested were found to require folic acid. When single amino acids were omitted, valine, isoleucine, methionine, and aspartic acid were found essential for growth. About half-maximum growth was obtained when histidine or phenylalanine was omitted. Although the complete nutritional requirements were not determined, strains of this species were observed to grow in the complete medium. This same investigator also noted that glutamine was an essential requirement for the multiplication of S. lactis. The latter observation was in substantiation of an earlier report that glutamine plus asparagine were up to 25 times as effective as glutamic acid plus aspartic acid in the nutrition of S. lactis (43, p. 659). The findings of Niven (38, p. 347) that all strains of S. lactis do not require riboflavin are not in agreement with those of Orla-Jensen et al (40) who stated that all true lactic acid bacteria do require this vitamin.

In a survey of the hemolytic streptococci, Wooley (59, p. 160) found them to require riboflavin, pantothenate, pyridoxin, and a reducing agent in addition to purified protein derivatives. In 1943 Niven and Smiley

(39, p. 7) established the requirement of S. lactis for thiamine. This organism has also been shown to require nicotinic acid (53, p. 2825).

Stokes and Gunness (56, pp. 43-44) noted that the addition of pyridoxamine to a defined medium affected the requirement of Lactobacillus casei and Lactobacillus arabinosus for the amino acids: lysine, alanine, and threonine. No such effect was evident with S. lactis.

In recent years the purine-pyrimidine requirements of the lactic acid bacteria have received much interest. In 1941 Snell and Mitchell found that thymine, adenine, and guanine were essential for some strains of S. lactis. These requirements are not always specific. For example, Stoakstad (54, p. 476) reported that various combinations of hypoxanthine and xanthine would replace guanine as a requirement of S. lactis, but there was no substitute for thymine. Mitchell, Snell, and Williams (36, p. 2284) further substantiated the interchangability of the purine bases, but noted that adenine, guanine, and xanthine were necessary for optimum acid production by S. lactis. The only pyrimidine possessing any amount of activity was thymine. At least one pyrimidine (cytidine) has been shown to be inhibitory.

A number of investigations (26, p. 137)(31, p. 164) (55, pp. 204-207) have established thymine as the critical component in the purine-pyrimidine nutrition of lactic

acid bacteria. Stokes (55, p. 206) noted that the requirement of folic acid by S. lactis R (Streptococcus fecalis) could be partially satisfied by thymine or its nucleoside, thymidine. It was an unusual feature of these experiments that no matter what concentrations of pyrimidines were used, only half-maximum growth was obtainable if folic acid was not present. Thymidine has also been shown to prevent the toxicity of a competitive antagonist of folic acid (47, p. 2299). Furthermore, the requirement of Lactobacillus lactis Dorner for vitamin B_{12} may be satisfied by as little as 0.1 to 0.3 gamma thymidine per ml. Thymine was not active in concentrations as high as 1 to 10 gamma per ml. At about the same time, Wright et al (60, p. 176) reported that vitamin B_{12} was not necessary for growth of Lactobacillus lactis, provided that thymidine was present. Thymine was not effective. It was postulated by these authors that vitamin B_{12} functions in the biosynthesis of thymidine. Later, another group (28, pp. 993-994) found thymine as effective as thymidine in the nutrition of 11 out of 13 lactobacilli. The preceding data would seem to indicate that some lactobacilli possess the necessary mechanism of interconversion of these substances, while others clearly do not.

As early as 1929 Dubos (16, p. 566) noted that oxidation reduction potential was highly important in initiation of growth of facultative anaerobes. A later

investigator (5, pp. 514-516) found that ascorbic acid or thymine would considerably shorten the lag phase of the Diplococcus pneumoniae. McNutt and Snell (35, pp. 565-566), using media not containing vitamin B_{12}, have been able to obtain abundant growth of some organisms which normally require this substance by simply maintaining the medium in a reduced state through the addition of ascorbic acid or glutathione. Lactobacillus delbrueckii was shown to require thymidine or vitamin B_{12} specifically. It was the opinion of these investigators that many B_{12} requiring strains were able to synthesize vitamin B_{12} or agents equally effective if the oxidation-reduction potential was sufficiently low.

Elliker and Frazier (17, pp. 822-824) found that the oxidation-reduction potential of reconstituted sterile skim milk became significantly elevated upon aging. Addition of a reducing substance, thioglycollic acid, to the aged milk stimulated acid production by certain lactobacilli, but failed to significantly stimulate growth of S. lactis or Streptococcus thermophilus. The effect of addition of reducing substances to milk innoculated with lactic streptococci was also studied by a group of Canadian investigators (27, pp. 27-28). It was their observation that when glutathione was added to milk the activity of starter bacteria was increased. The difference in starter activity in reduced milk as compared with

milk in which no reducing agent had been added was considerably greater if the milks had been previously agitated. Cysteine showed starter stimulation only in agitated milks.

Other than a number of observations on the requirement of the tubercle bacillus for lipid-like materials, there has been little in the literature concerning these substances as growth requirements of other microflora. Considering the extreme fastidiousness of the lactic acid bacteria, it would not seem unusual to expect these organisms to require these substances or their definite precursers. This has proven to be the case. In 1946 Guirard et al. (21, pp. 371-375) found that certain lactobacilli required the acetate radical for growth. This requirement could be replaced wholly or in part by substances such as sterols, lipids, and related compounds. Williams and co-workers (58, pp. 625-627) have also noted that oleic acid was stimulatory to L. arabinosus, L. delbrueckii, and S. fecalis. The roles of acetate and oleate as related to the nutrition of S. cremoris were studied by Collins, Nelson, and Parmelee (13, pp. 70-71). In confirming the findings of Stoors and Anderson (57, p. 607) and other investigators, that the medium as proposed by Niven (38, pp. 343-349) is insufficient for growth of many of the lactic streptococci isolated from starter cultures, Collins and co-workers (13, pp. 70-71)

found that good growth of S. cremoris could be obtained by adding sodium acetate and sorbitan monooleate to the above medium. The requirement for acetate and oleate has very recently been associated with the pyruvate oxidation factor (POF) of O'Kane and Gunsalus (41, pp. 499-506) and may be replaced by it (33, p. 241). S. cremoris was shown to be one of very few organisms incapable of synthesizing POF upon prolonged incubation.

Metabolic differences associated with bacteriophage resistant organisms. To Anderson (2, pp. 398-399) must go the credit for the earliest report of qualitative differences in metabolism of virus-sensitive bacteria and their virus-resistant mutants. It was his observation that virus-resistant mutants of Escherichia coli failed to develop in a synthetic basal medium, but the parent strain did. Both were capable of developing in the basal medium plus yeast extract. None of the 23 amino acids or 8 vitamins, tested both singly and in various combinations, was able to replace yeast extract as a growth factor for the mutant strain. The amino acids and vitamins used and their concentrations were not stated. In a later study, this same investigator (3, pp. 121-125) noted that by increasing the concentration to 100 micrograms per ml. a number of amino acids would partially supplant yeast extract for many of the mutant strains. However, some strains resistant to bacteriophage T4 were found to

specifically require tryptophane for growth. This observation deserves special consideration in the light of later information (4, pp. 639-642) that tryptophane is also required by bacteriophage T4 as an adsorption cofactor. An adsorption cofactor is considered as a substance necessary for adsorption of the virus to the bacterium, which is a step necessarily preceding lysis. To the author's knowledge there has been no further study of correlation of virus adsorption cofactors and factors required by organisms resistant to a particular virus.

Henry and Henry (24, pp. 529-530) have reported that the virus-resistant strains of E. coli are slower growing and have a slower rate of aerobic oxidation of sugars than virus-sensitive strains, but have a higher rate of anaerobic glycolysis.

A rather interesting investigation by Fitzgerald and Lee (19, p. 128) showed that when strains of E. coli became resistant to the toxic effects of 2 amino 9 (p-amino phenyl) acridinium chloride they also became resistant to bacteriophage attack. This resistance was greatly decreased by the addition of ribonucleic acid (RNA). The conclusion drawn by these investigators was that acridine resistant mutants were insensitive to lysis because the cellular functions had been taken over by an alternate system which was able to bypass the drug and phage sensitive system and supplant it in the cell.

Effect of bacteriophage on host cell. The organized components of a host cell comprise the minimum substrate necessary for the proliferation of bacteriophage particles. It would then seem obvious that some consideration of the unusual metabolism of the infected host cell is necessary in any study of bacteriophage requirements. As the bacteriophage is apparently incapable of organizing its components to produce a like particle, the metabolism of the host must assume, at least in part, responsibility for this synthesis. Cohen (12, pp. 4-19) found that when a sensitive E. coli cell was infected with its homologous bacteriophage, the protein nitrogen content of the cell began to increase at once. The exact nature of this protein was not determined, but it was established that it was not phosphorylated. Approximately 7 minutes later the synthesis of desoxyribosenucleic acid (DNA) underwent a drastic increase. There was little if any increase in other protein-bound carbohydrate. While in a normal cell, the production of ribosenucleic acid (RNA) was about three times as great as that of DNA; the reverse was true in infected cells. This protein and DNA synthesis continued at a constant rate during the period of virus synthesis and ended with complete lysis. The concentrations of DNA and protein produced by bacteriophage infection were proportional to the length of the period required for synthesis or the so called latent period (11, pp. 187-190).

After final lysis, the level of phosphorus and nitrogen utilization of the residual resistant forms was approximately that of normal cells. It is of interest that all of the protein-bound phosphorus assimilated after infection is DNA, and all of the newly assimilated DNA goes into the virus particle (10, pp. 41-43).

This nucleoprotein synthesis is apparently dependent on some factors not influencing the growth of the host cell. Fitzgerald and co-workers (18, pp. 121-126)(19, p. 128) observed that the addition of 2 amino 9 (p-amino phenyl) acridinium chloride to a synthetic medium containing sensitive cells of E. coli and their homologous bacteriophage would greatly inhibit bacteriophage synthesis. This was not due to inhibition of cell activity as in this case the drug concentration was held to non-toxic limits. The antiviral activity of the drug was quantitatively reversible by the addition of RNA. Foster (20, pp. 805-807) found that another acridinium compound, proflavin, would produce a similar effect, but noted that DNA was more effective than RNA in the reversal of antiviral effects. The drug apparently blocked some essential process near the completion of synthesis of the active bacteriophage particle. This was demonstrated by the fact that the bacteriophage was still capable of lysing the host cell, but was incapable of proliferation by the infectious

process. Cohen (11, pp. 187-190) has noted a somewhat similar effect produced by an analog of tryptophane, but in this case the inhibition apparently occurred sooner in the lytic process as DNA synthesis was not stimulated by the infection and the cells were not lysed.

Further evidence of the importance of purine-pyrmidine-nucleic acid metabolism of the host cell in bacteriophage synthesis has been supplied by Roberts and coworkers (44, p. 710)(45, p. 711). They noted that the addition of vitamin B_{12} considerably increased the incorporation of phosphorus and DNA in normal cells of Lactobacillus leichmanii (44, p. 710). This discovery led them to a study of the effect of vitamin B_{12} on bacteriophage multiplication (45, p. 711). They found that there was an increase in the burst size and a shortening of the burst period. This was not due to incorporation of the vitamin in the bacteriophage particle, but rather it was presumed to aid in the synthesis of thymine-containing constituents in the virus.

Requirements of lactic streptococcus bacteriophage. As observed from preceding remarks, there is considerable literature on fundamental considerations of the host-virus relationship of E. coli and its homologous bacteriophages. However, there are at this writing only a few published reports on the biochemical considerations of lactic streptococcus bacteriophage and the effect of the virus on the

host cell. Nonetheless, some inference may be drawn from studies on other bacteriophages as their fundamental effect on host metabolism is believed to be the same.

Hunter (25, pp. 139-141) found that some races of bacteriophage effecting S. cremoris lysed the host cell at 30°C., but were incapable of doing so at 37°C. This could not be attributed to inactivation of the bacteriophage as this virus is considerably more thermo-resistant than the organisms which it effects (37, pp. 343-349). Any effect on cellular metabolism would be most difficult to explain as the organism is quite capable of rapid growth at this temperature.

The effect of electrolytes in a non-synthetic medium on production of bacteriophage particles, was determined by Cherry and Watson (9, pp. 113-117). Manganate and citrate were found to be inhibitory in all concentrations. However, a number of other salts were observed to be stimulatory within rather narrow limits of concentration. In particular, sodium-mono-hydrogen phosphate was stimulatory between concentrations of 0.01 and 0.005 molar and calcium chloride was similarly effective between 0.05 and 0.005 molar. Collins and co-workers (14, pp. 535-539) have recently reported on a rather complete study of the effect of individual constituents of a defined medium on bacteriophage proliferation. Using two strains of

S. lactis and their homologous bacteriophage, these investigators found that the omission of single components of a synthetic medium affected virus and host in a similar manner with the exception of calcium.

Eight out of 10 races of bacteriophage effecting S. lactis and S. cremoris were observed to require free calcium in the medium. Phosphate was required by the organism for growth, but concentrations above 0.1% rendered calcium unavailable to the virus and prevented its proliferation. It was noted that it was necessary to sterilize the medium by filtration or add the calcium after autoclaving as the effect of heat produced an unusable calcium-phosphate complex. Previous to this in 1949 Shew (46, pp. 492-493) had reported calcium as a requirement for eight strains of streptococcus bacteriophage, but failed to show that it was required in free form. Perlman (42, pp. 140-141) found that other agents capable of sequestering calcium such as oxalate and citrate were extremely inhibitory to an actinophage affecting Streptomyces griseus. These substances did not materially affect the metabolism of the host. In none of the above examples (14, pp. 535-539)(42, pp. 140-141)(46, pp. 492-493) was calcium required by the organism.

MATERIALS AND METHODS

Selection of bacterium and bacteriophage. The lactic streptococci used in these studies all were identified as Streptococcus cremoris by means of hydrolysis of arginine and growth at 40°C. The strains of S. cremoris used were obtained from the stock culture collection of the Oregon State College Department of Bacteriology and included the following: R6, W, 144, and 11E. Most of the homologous bacteriophages were isolated from Oregon dairy plants experiencing starter inactivity.

Considerable care was taken to insure homogeneity in individual strains of both bacteria and bacteriophage. Cultures were purified by plating and picking single colonies for three successive generations. For the remainder of this report the purified, sensitive culture has been referred to as the parent strain. Bacteriophages were purified by allowing them to produce lysis on a solid mat of organisms, and picking isolated plaques for two succeeding generations. The second generation plaque was picked into 100 ml. of skim milk seeded with sensitive culture. After 24 hours incubation at 30°C., the milk was coagulated with 10 percent sterile lactic acid and the whey filtered through a Selas candle. The filtrate was neutralized with calcium carbonate and refrigerated.

This filtrate was used in all portions of this study as the bacteriophage concentrate. The titer was approximately 10^9 in all cases.

Inocula. The bacteria were maintained in agar stabs. Each week the culture was transferred and at the same time another transfer was made into broth. The broth culture was used as a source of the organism for the ensuing week. Cell suspension for inoculation of the synthetic medium was grown in broth for 18 hours, centrifuged, washed in sterile tap water and then resuspended. This was repeated 3 times. Between the first and second washing the cells were starved for two hours at room temperature. One ml. of the final suspension was added to 10 ml. of distilled water and one drop used as an inoculum for each tube.

Mutant-strain. Bacteriophage resistant mutants of the parent strain were selected by transferring the virus sensitive bacteria in the presence of high concentrations of bacteriophage. As quickly as secondary growth occurred, the culture was transferred and recultured in the presence of more bacteriophage. This procedure was repeated 10 times and the parent strain (unexposed to bacteriophage) was transferred an equal number of times. In all procedures, an attempt was made to give the parent strain and its bacteriophage resistant mutant identical treatment.

Nomenclature. Except for the resistant mutants of culture 144, the nomenclature of the resistant strain was that of the parent strain plus the initials IM. Culture 144 had two mutants selected by two different bacteriophages. One mutant was referred to as 144co and the other as 144IM. Nomenclature of the bacteriophages was that of the sensitive organism followed by the letter "P". Bacteriophages active against culture 144 were identified as 144cP and 144IP. It is of interest that both 144co and 144IM were completely resistant to the bacteriophage used to select the mutant, but remained sensitive to an alternate bacteriophage effecting the parent strain.

From time to time during the progress of the study, cultures of the parent strain were mixed with the resistant strain and a filtrate prepared of the mixture. This was carried out to determine if there was any residual bacteriophage present. In none of these attempts was there any positive indication of presence of the virus.

Determination of Bacterial Requirements

As the possible mutations in nutritional requirements are almost endless in organisms as fastidious as those used in this study, it was considered impossible to predict changes (if any) in metabolic needs. For this reason it was necessary to use a synthetic medium which could be easily modified and yet satisfy a rather wide range in nutritional demand. The medium of Henderson and Snell

(23, pp. 15-29) appeared admirably suited for this purpose, but would not support growth of any of the organisms used in the study. Anderson and Elliker (1) have been able to obtain abundant growth of starter culture bacteria in the above medium by adding; lactose, sucrose, ascorbic acid, thymine, Tween 80, glutamine, and asparagine in the concentrations listed in table I. This was the basal medium in all studies on the requirements of bacteriophage-sensitive and bacteriophage-resistant strains of the organism. After all constituents had been added, the pH of the medium was adjusted to 6.8 with 2 normal potassium hydroxide and distilled water added to make up the desired volume.

Materials used in the medium were of the highest purity obtainable. Salts were C. P. or equivalent grade. Amino acids and vitamins were Nutritional Biochemicals "Purity" grade. Extreme care was used to prevent accidental contamination from the glassware. Before use it was washed in a sulfuric acid-potassium dichromate cleaning solution and then rinsed in running water. After each piece was completely clean, it was dried in a 55°C. oven and placed in a clean drawer. After cleaning, the culture tubes were placed in a stainless steel rack holding 60 tubes.

The medium was added by an automatic dispensing device known as the "Cannon Automatic Dispenser-Titrator".

This type of device is extremely accurate so only two ml. of medium were added to each tube. After the medium had been added, the tubes were closed with a loose fitting metal cap. This not only added greatly to the speed of operation, but also prevented possible nutrient contamination from the customary cotton plug. After the medium had been added to the tubes and capped the tubes were then autoclaved at 115°C. for 2 minutes, after which the pressure was immediately removed. This temperature appeared to destroy any aerobic acid forming bacteria without inactivating the thermolabile glutamine and asparagine. These substances could have been sterilized by filtration and added aseptically after the rest of the constituents were autoclaved. However, it was felt that both accuracy and convenience were more adequately served by dispensing the medium containing all desired constituents.

After some experimentation, it was decided that an incubation time of 48 hours at a temperature of 30°C. gave the most uniform and consistent results. At the end of the incubation period, growth of the organism was estimated by titration of the acid produced. This was performed semi-automatically with the same apparatus that was used to dispense the medium. This device is so constructed that it may easily be used to dispense a given volume of standard alkali over a given period of time. The amount of alkali delivered was determined by a

synchronous motor which operated a recording Veeder Root counter. The number of counts recorded served as an index to the volume of alkali delivered. In this study 3.8 ml. of 0.0489 normal sodium hydroxide was added per 100 counts.

The end point of titration was determined by a calomel and quinhydrone-platinum electrode system with a potassium chloride bridge reading across a 1000 ohm galvanometer. The galvanometer was zeroed at pH 7.0 using a standard Coleman buffer. The electrode system is more completely described by Henderson and Bickerson (22, pp. 15-29). Triplicate tubes were made of each factor varied. Considering the relatively high accuracy of the titration apparatus and the close general agreement between tubes, it was felt that three replications were sufficient as a basis for statistical comparison.

A strain of bacteria known to require each substance omitted was used in all trials as a control. This was usually Luconostoc mesenteroides P-60. Two trials were found to be contaminated and the results were discarded. The growth of the control strain is not reported in the data as it serves no useful purpose.

Studies on Bacteriophage Requirements

Medium. The medium used for the study of the requirements of the sensitive and resistant bacteria (table I) was not suitable for the study of bacteriophage

requirements, and the following modifications in the medium were made: Lactose concentration was adjusted to 0.1 gm./100 ml., sucrose to 0.1 gm./100 ml., glucose to 0.1 gm./100 ml., and potassium phosphate (monobasic) to 0.1 gm./100 ml. DNA and sodium citrate were omitted. After all constituents had been added the pH was adjusted to 6.4. In addition 0.2 gm./100 ml. of sterile calcium carbonate was added after sterilization.

Determination of the effect of the various constituents of the medium on bacteriophage proliferation followed the same general pattern of the study on requirements of the host organism. Single substances were omitted or added to a synthetic medium, sensitive bacteria were provided, and the virus population estimated after a given period.

Ten ml. of the medium were added to large (25 x 140 mm.) pyrex tubes and autoclaved for 2 minutes. The large tubes were necessary to avoid the boiling out of any of the constituents. A washed cell suspension was prepared in the same manner as for the study of bacterial requirements and one drop added to each tube. After inoculation, the suspension was incubated for 30 minutes at 30°C. At the end of this time 1 ml. of a 10^7 dilution of the original bacteriophage filtrate was added. The incubation allowed in this study was 5.5 hours after which the virus population was determined.

Determination of bacteriophage population. Bacteriophages are usually counted by one of two general methods. The first is the so called plaque technique. This allows a given dilution of the virus to develop on a solid mat of the sensitive organism. The small area of lysis caused by the multiplying virus is called the "plaque". Bacteriophage population can then be assessed by the number of plaques, in the same way as bacterial colonies are counted. The other general technique is the determination of the titer or maximum dilution possessing bacteriophage activity. The latter method was chosen for this experiment, as the plaques formed by the bacteriophages used are small and consequently quite difficult to count. Also their occurrence is characterized by some inconsistency.

As used in this study, the "titer method" was given statistical validity by making three such determinations and applying "Most Probable Number" tables (6, p. 12). Dilutions were made into tubes of skim milk containing resazurin and seeded with sensitive culture. These dilutions were incubated at 30°C. until a control tube containing only the culture was reduced and coagulated. At this time the tubes were examined to determine the titer. When the virus was diluted to extinction, normal growth of the culture occurred. The titer was considered the highest dilution showing virus activity.

RESULTS

Table II presents a comparison of growth in the basal medium of all cultures used in the study. It may be seen that the growth produced by the resistant mutant of 11E was considerably in excess of the parent strain. The only other significant difference between parent and mutant strains in the basal medium occurred with 144co which produced slightly more acid than either the parent strain or the other resistant mutant. Thus, two bacteriophage selected mutants of the same strain of organism show different growth characteristics in the basal medium. As demonstrated later, there is considerable difference in their nutritional requirements. Any significant difference in acid production between parent and mutant was in the nature of an increase.

For convenience in comparison, tables III and IV are expressed as a percentage of growth in the complete medium. The significant differences indicated in these tables were obtained by pairing the raw data of the mutant against that of the parent strain and applying the chi square test.

In table III there are 24 significant changes in nutritional requirements indicated; 23 of these are in the direction of greater fastidiousness by the resistant mutants. The one exception to the general trend of more

complex requirements by the virus-resistant strain was the complete requirement for histidine by 11E while the 11EIM was able to demonstrate slight growth. This strain did follow the general pattern, however, with distinct increase in requirements for tyrosine, tryptophane, and valine. The most outstanding difference between parent and mutant of this strain was in the requirement for tyrosine. Although the virus-sensitive strain was able to execute maximum growth in the absence of this substance, the resistant strain produced only slightly more than half-maximum growth.

The greatest difference in nutritional requirements between any of the parent and mutant strains tested occurred between 144 and 144IM. There was a decrease from a maximum of growth by 144 in the absence of proline to less than half-maximum growth by the 144IM. Similarly the parent strain was able to develop about two-thirds of maximum growth, while the resistant mutant produced only about 13 per cent if tryptophane was omitted. The greatest number of mutations of a nutritional nature occurring simultaneously with mutation to virus resistance occurred between 144 and the resistant forms of this organism. There were distinct increases in requirements for seven different amino acids by 144IM and six increases by 144co. There was considerable difference in the requirements of the two resistant forms. Strain 144co displayed increased

need for cystine, methionine, tryptophane, histidine, threonine, and the mixture of glutamine and asparagine. Strain 144 did not produce acid as rapidly as the parent strain when methionine, tyrosine, tryptophane, valine, glycine, and glutamine plus asparagine were omitted from the medium.

Strain WI executed the least number of changes in nutritional requirements coexistant with mutation to virus-resistance. Only the removal of tryptophane and serine had a significantly greater adverse effect upon the mutant than on the parent strain.

The mutant of R6 did not develop as well as the parent strain when cystine, tyrosine, phenylalanine, isolucine, and glutamine plus asparagine were omitted. This was the only resistant strain not showing an increased requirement for tryptophane.

Although most strains did not appear to require glutamic acid the requirement for this substance is at least partially satisfied by the glutamine present.

Vitamin needs of resistant strains (Table IV) were considerably less altered than their amino acid requirements. Only five significant differences in vitamin requirements could be detected, and two of these were

mutations to a lessening of requirement. The removal of p-aminobenzoic acid (PABA) adversely affected the growth of 11E and 144, but had no apparent effect on the mutant strains.

Requirements of two of the mutant forms for pyridoxal were significantly increased. The parent strain of 144IM was able to show 70 per cent growth, but the mutant was only able to produce 49 per cent of maximum. Also strain R6 produced 82 per cent of basal growth while R6IM displayed half development in the absence of pyridoxal.

The removal of PABA and folic acid was definitely detrimental to the growth of one mutant strain. With both of the above vitamins absent from the medium, 144co produced only 61 per cent of maximum growth, although when either vitamin was omitted singly, acid production was not materially affected.

All strains studied definitely required riboflavin, nicotinic acid, biotin, and the pantothenate radical even for minimal activity.

The pyrimidine, thymine in concentration of 10 gamma per ml., was beneficial to 11E, but apparently had little effect on 11EIM. On the basis of total acid produced, however, strain 11EIM retained superiority. Thymine was not particularly stimulatory to any of the other cultures.

Although it slightly increased the development of 11E and 11EIM, the addition of vitamin B_{12} in concentration

of 0.005 gamma per ml. did not appear stimulative to the other seven strains. In fact, it did appear to inhibit WIIM, and this effect was not understood. It can be stated that this was not due to toxic properties as a concentration 100 times as great allowed significantly more acid to be produced. The higher concentration was inhibitory to several of the other strains.

Tables V, VI, VII, and VIII show that although slightly less demanding, the vitamin requirements of the virus appeared to approximate those of the host organism. The one notable exception was the abundant increase in virus population even though calcium pantothenate was omitted. Increases in virus population as great as one million-fold were possible when this unit was omitted. The host cell was unable to produce more than trace growth under these conditions.

Slight increases in virus titer were allowed when pyridoxine, riboflavin, nicotinic acid, and biotin were omitted although these substances were definitely required by the host. It could not be determined if this was due to lack of requirement by the virus or residual vitamin in the cell in amount sufficient for organism activity. The addition of vitamin B_{12}, thymine, or DNA did produce a slight increase in virus titer, but with the exception of thymine did not significantly affect acid production by the organism.

Ribonucleic acid in concentration of 500 gamma per ml., which was five times as great as the concentration of desoxyribose nucleic acid almost completely inhibited bacteriophage multiplication. Although this level of RNA definitely had a limiting effect on acid production by the organism, the inhibition was only one-half to two-thirds complete.

TABLE I

DEFINED BASAL MEDIUM

Concentrations given per 100 ml. medium

Glucose	1.00 g.	Alanine	0.4 g.
Sucrose	1.00 g.	Aspartic acid	0.4 g.
Lactose	1.00 g.	*L Glutamic acid	0.4 g.
Sodium citrate	1.00 g.	L Arganine	80 mg.
Sodium acetrate	0.10 g.	L Lysine	80 mg.
Ammonium chloride	0.30 g.	Histidine	80 mg.
		Isolucine	80 mg.
Dipotassium phosphate	0.50 g.	Leucine	80 mg.
		Methionine	80 mg.
Salt C	2.00 ml.	Phenylalanine	80 mg.
		Proline	80 mg.
Adenine sulfate	1 mg.	Threonine	80 mg.
Guanine hydrochloride	1 mg.	Tyrosine	80 mg.
		Valine	80 mg.
Uracil	1 mg.	Tryptophane	80 mg.
		Cystine	80 mg.
DNA	100 gamma	Serine	80 mg.
Xanthine	100 gamma	Glycine	80 mg.
Thiamine	100 gamma		
Riboflavin	100 gamma	Glutamine	0.4 mg.
Pyridoxal	20 gamma	Asparagine	0.4 mg.
Ca-panto-thenate	100 gamma	Tween 80	
		Ascorbic acid	0.05 g.
Niacin	100 gamma		
P.A.B.A.	20 gamma		
Folic acid	1 gamma		
Biotin	1 gamma		

* All amino acids assumed to be racemic or DL form unless otherwise specified.

TABLE II

Growth in the Defined Basal Medium by Bacteriophage-Sensitive and Bacteriophage-Resistant Strains of S. cremoris.

ORGANISM STRAIN	GROWTH[1] Average of 15 trials
11E	36
11EIM	54
144	52
144co	49
144IM	57
W1	48
W1IM	47
R5	49
R5IM	47

[1] Value represents number of counts on automatic titration apparatus required to neutralize two ml. of medium. One hundred counts equals 3.8 ml. of 0.0489 N sodium hydroxide.

TABLE III

Effect on Omission of Individual Amino Acids from a Defined Basal Medium on Growth[1&2] of Bacteriophage-Sensitive and Bacteriophage-Resistant Strains of S. cremoris[3].

NUTRIENT OMITTED	ORGANISM STRAIN								
	11E	11EIM	144	144co	144IM	W1	W1IM	R6	R6IM
Glutamine & Asparagine	2	1	27	16*	10*	8	2	27	10*
Methionine	18	16	19	0*	1*	8	0	0	0
Cystine	98	98	99	64*	100	101	100	92	58*
Aspartic acid	99	99	100	101	96	91	92	100	98
Tyrosine	100	61*	100	99	77*	82	81	88	54*
Glutamic acid	96	92	89	84	80	100	101	92	92
Trypto-phane	21	10*	67	32*	13*	56	33*	12	19
Phenyl-alanine	17	14	9	4	11	27	33	30	10*
Histidine	0	15*	49	38*	41	27	30	8	2
Serine	14	19	15	14	23	17	7*	23	20
Isolucine	14	16	15	11	17	12	11	13	4*
Valine	20	3*	17	4*	6*	17	15	6	7
Threonine	97	92	95	93	95	94	90	94	98
Arganine	17	18	20	15	22	18	22	22	23
Proline	102	98	102	87	44*	88	86	90	88
Leucine	14	8	8	3	4	6	6	4	1
Glycine	98	93	99	90	77*	95	90	107	98
Lysine	0	0	2	0	3	3	2	1	0

[1] Value represents comparison with basal medium; growth in basal medium considered as 100 per cent as determined by titratable acidity.

[2] Values are any average of three trials.

[3] Asterisk indicates significant difference in nutritional requirement of mutant strain.

TABLE IV

Effect of Modification of the Defined Basal Medium on Growth[1&2] of Bacteriophage-Sensitive and Bacteriophage-Resistant Strains of S. cremoris[3].

NUTRIENT OMITTED	ORGANISM STRAIN								
	11E	11EIM	144	144co	144IM	W1	W1IM	R6	R6IM
Thiamine	93	93	67	70	58	93	93	82	76
Pyridoxal	85	73	70	70	49*	93	84	82	56*
Riboflavin	0	0	0	7	0	0	0	2	4
Nicotinic acid	0	3	0	3	1	1	5	0	1
Biotin	3	2	0	0	2	6	4	2	6
PABA	80	103	85	106	100	100	93	100	100
Folic acid	96	98	101	96	94	99	101	93	99
PABA and Folic acid	83	96	86	61*	108	108	108	70	69
Ca-panto-thenate	18	12	8	5	0	0	0	0	0
NUTRIENT ADDED									
Thymine[4]	122*	100	100		97	101	100		102
Vitamin B_{12} [5]	117	111	99		101	100	73*		103
Excess vitamin B_{12}[6]	89	80	95		100	85	83		93

[1] Value represents comparison with basal medium; growth in basal medium considered as 100 per cent as determined by titratable acidity.

[2] Values are an average of three trials.

[3] Asterisk indicates significant difference in nutritional requirement of mutant strain.

[4] Concentration of 10 gamma per ml.

[5] Concentration of 0.005 gamma per ml.

[6] Concentration of 0.5 gamma per ml.

TABLE V

Effect of Modification of the Defined Basal Medium on Multiplication of S. cremoris 11E and Bacteriophage 11EP.

NUTRIENT OMITTED	ORGANISM GROWTH[1] Per Cent	BACTERIOPHAGE TITER[2] (5.5 HR)
None (basal medium)	100	2.5×10^8
Thiamine	93	2.5×10^5
Pyridoxal	85	9.5×10^6
Riboflavin	0	3.0×10^2
Nicotinic acid	0	4.5×10^1
Biotin	3	3.0×10^2
PABA	80	1.5×10^7
Ca-pantothenate	18	1.5×10^7
NUTRIENT ADDED		
Vitamin B_{12}[3]	117	4.5×10^8
Thymine[4]	122	1.5×10^8
DNA[5]	103	1.5×10^8
DNA plus RNA[6]	40	1.5×10^1

[1] Value represents comparison with basal growth; growth in basal medium considered as 100 per cent. Values given are an average of three trials.

[2] Initial titer 2.5×10^1.

[3] Concentration of 0.005 gamma per ml.

[4] Concentration of 10 gamma per ml.

[5] Concentration of 100 gamma per ml.

[6] Concentration of 500 gamma per ml.

TABLE VI

Effect of Modification of the Defined Basal Medium on Multiplication of S. cremoris 144 and Bacteriophages 144cP and 144IP.

NUTRIENT OMITTED	ORGANISM[1] GROWTH Per Cent	BACTERIOPHAGE[2] 144cP TITER (5.5 HR)	BACTERIOPHAGE[3] 144IP TITER (5.5 HR)
None	100	9.5×10^7	1.5×10^8
Thiamine	67	9.5×10^3	4.5×10^2
Pyridoxal	70	7.5×10^7	1.5×10^7
Riboflavin	0	4.5×10^3	2.5×10^4
Nicotinic acid	0	2.5×10^2	2.5×10^2
Biotin	0	1.5×10^2	1.5×10^2
PABA	85	4.5×10^7	9.5×10^7
Ca-pantothenate	8	9.5×10^5	1.5×10^3
NUTRIENT ADDED[4]			
Vitamin B_{12}	99	4.5×10^8	2.5×10^8
Thymine	100	7.5×10^8	7.5×10^8
DNA	100	1.5×10^8	9.5×10^8
DNA plus RNA	41	4.5×10^1	1.5×10^2

[1] Value represents comparison with basal growth; growth in basal medium considered as 100 per cent. Values given are an average of three trials.

[2] Initial titer 2.0×10^1.

[3] Initial titer 1.5×10^2.

[4] Concentrations listed in Table V.

TABLE VII

Effect of Modification of the Defined Basal Medium on Multiplication of S. cremoris W and Bacteriophage WP.

NUTRIENT OMITTED	ORGANISM GROWTH[1] Per Cent	BACTERIOPHAGE TITER[2] (5.5 HR)
None (basal medium)	100	1.5×10^8
Thiamine	93	2.5×10^3
Pyridoxal	93	4.5×10^7
Riboflavin	0	1.5×10^3
Nicotinic acid	1	1.5×10^3
Biotin	6	9.5×10^2
PABA	100	1.5×10^8
Ca-pantothenate	0	7.5×10^5
NUTRIENT ADDED[3]		
Vitamin B_{12}	100	1.5×10^9
Thymine	100	2.5×10^9
DNA	104	7.5×10^8
DNA plus RNA	33	2.5×10^1

[1] Value represents comparison with basal growth; growth in basal medium considered as 100 per cent. Values given are an average of three trials.

[2] Initial titer 1.5×10^1.

[3] Concentrations listed in Table V.

TABLE VIII

Effect of Modification of the Defined Basal Medium on Multiplication of S. cremoris R6 and Bacteriophage R6P.

NUTRIENT OMITTED	ORGANISM GROWTH[1] Per Cent	BACTERIOPHAGE TITER[2] (5.5 HR)
None (basal medium)	100	9.5×10^7
Thiamine	82	1.5×10^5
Pyridoxal	82	1.5×10^7
Riboflavin	2	7.5×10^3
Nicotinic acid	0	1.5×10^2
Biotin	2	9.5×10^3
PABA	100	4.5×10^8
Ca-pantothenate	0	4.5×10^4
NUTRIENT ADDED[3]		
Vitamin B_{12}	99	4.5×10^8
Thymine	103	9.5×10^8
DNA	105	1.5×10^8
DNA plus RNA	35	9.5×10^1

[1] Value represents comparison with basal growth; growth in basal medium considered as 100 per cent. Values given are an average of three trials.

[2] Initial titer 2.0×10^2.

[3] Concentrations listed in Table V.

DISCUSSION

The data presented suggest that mutation from bacteriophage-sensitivity to bacteriophage-resistance results in a more fastidious nature. It is interesting that none of the changes in amino acid requirements were from complete dispensability to complete indispensability. Considering the dynamic relationships possible between the large number of amino acids that are present, this is not particularly surprising. It is possible that by omitting two amino acids (neither of which are required) some of the mutations would be shown to vary from complete dispensability to complete indispensability. The closest approach to this change was shown in culture 144IM which dropped to 13 per cent in the absence of tryptophane. The small amount of growth obtained with either the mutant or parent strain of most cultures when tryptophane was omitted is further evidence of the more complex demands of S. cremoris. Niven (38, p. 346) has reported that S. lactis was able to grow as well in the absence of this substance as in its presence.

The increased requirement of two of the resistant strains for pyridoxal is in general agreement with the increased amino requirements. The conception of the function of this vitamin is that it serves in an amino acid-transamination system. Thus, some relationship of

increased pyridoxal requirement to increased amino acid requirement was suggested. The fact that this occurred with only two of the five resistant strains, however, would indicate that transamination activity, or the lack of it, is not an integral part of virus-resistance.

The difference in amino acid requirements of the mutants indicate a possible relationship to the findings of Knight (29, p. 127) that mutations in virus specificity are associated with changes in amino acid content. Also the relative inefficiency of the mutants in synthesizing certain amino acids from the constituents of the substrate suggests that the specificity of the virus for a particular organism is in some way related to the specific activity of the enzyme systems of the host. The observation by Cohen (12, pp. 4-19) that protein synthesis is increased immediately after adsorption of the bacteriophage would further indicate that the first effect by the virus is on or through the protein-synthesizing enzyme systems of the infected bacterium.

An attempt to classify as a systematic group those amino acids that figured in change in nutrition requirements due to mutation was not successful. All structural classifications were represented in the group required additionally by the resistant strain. The extreme

diversity of structural and functional types represented would seem to indicate that the type or function of the amino acid required by sensitive and resistant strains is not as important as mutation *per se*.

The failure of 11E to develop as well as the mutant in the basal medium demonstrates that the parent strain requires some substance not required by 11EIM. Thymine partially satisfied this requirement, but 11EIM remained significantly superior in activity.

It is generally accepted that the metabolism of the host cell is responsible for the synthesis of many of the essential bacteriophage constituents. Therefore, it would not seem unreasonable to expect that the minimum requirements of the virus be at least those of the host. The findings of this study do not agree with this supposition. Although the pantothenate radical is specifically required by the host, the virus is capable of tremendous multiplication in the absence of this substance. Also a slight increase in virus population was possible when a number of other essential substances were omitted. It is possible that these effects are due to the small amount of vitamin remaining in the host cell. The amount of vitamin and energy required for virus synthesis is undoubtedly quite small in comparison to the vitamin level necessary for production of a significant amount of acid.

As it had been previously reported that vitamin B_{12} stimulated the production of bacteriophage effective against E. coli (45, p. 711), it was anticipated that the addition of this substance would result in higher titers of the bacteriophages used in this study. Vitamin B_{12}, thymine, and other substances usually associated with nucleoprotein anabolism, were not effective in promoting more extensive virus synthesis. However, these results are perhaps not in actual conflict with the earlier findings. The titers obtained in this study were near the maximum usually obtainable even with enriched non-synthetic medium. Thus, although the addition of vitamin B_{12} and associated substances may accelerate the rate of virus synthesis, the absolute concentration would remain stable. A titer at a shorter time interval may have also demonstrated the synthesis of this virus to be more rapid.

The definitely depressing effect of RNA on bacteriophage synthesis gives further confirmation to the opinion voiced by Skegs et al. (49, p. 813) that RNA serves as a regulatory mechanism by maintaining a normal balance between the two types of nucleic acids. As the bacteriophages contain large amounts of DNA, the limiting effect of RNA is much more pronounced on bacteriophage synthesis than on organism activity.

SUMMARY AND CONCLUSIONS

Bacteriophage resistant strains of S. cremoris were obtained by culturing bacteriophage sensitive strains in the presence of the homologous virus and selecting the secondary growth. Resistant mutant cultures developed acid at a rate equal or superior to that of the parent strain.

The limitation of growth produced by omission of a number of amino acids in a synthetic medium was much more pronounced on the mutant strains. All resistant strain displayed significantly increased requirements for a number of different amino acids. One showed increased growth with 2 and another strain required as many as 7 additional amino acids to attain growth comparable to the parent strain. Only one example of less fastidiousness on the part of any of the mutant strains was apparent. This was the ability of 11EIM to produce slight growth in the absence of histidine, a condition under which the parent strain failed to develop.

Increased requirement by one or another of the mutants was shown for all structural types of amino acids, and there was no detectable relationship between the amino acids represented. The extreme diversity of structural

and functional types of these amino acids may indicate that mutation to virus resistance is associated with a non-specific alteration of the amino acid enzyme systems.

The removal of tryptophane had a significantly greater adverse effect on the mutant in four of the five resistant strains studied and this was the most common increased requirement. Omission of tryptophane from the basal medium strain resulted in 67 per cent of maximum development by strain 144 and only 13 per cent of maximum development by 144IM.

Vitamin B_{12} was stimulatory to strains 11E and 11EIM, but thymine stimulated 11E only. Extremely high concentrations of vitamin B_{12} were slightly inhibitory to some strains. Pyridoxal was required for maximum growth by two resistant strains, but not by their sensitive parent strains. Most vitamin requirements of the sensitive and resistant strains were found to be similar.

The fact that the resistant mutants usually are more active than the parent strain in milk culture may indicate that more fastidious cultures generally are more active in a highly nutritious medium. The observation that S. cremoris frequently is considered a superior lactic starter to the less fastidious S. lactis would lend some support to this consideration.

Studies also were made on the effect of modification of the defined basal medium on bacteriophage multiplication. Some bacteriophage proliferation was possible even though substances essential for organism growth were omitted. Increases in virus population as high as one million-fold were possible in the absence of the pantothenate radical in spite of the fact that only trace growth by the host organism was permitted under these circumstances. The removal of a number of other vitamins essential for growth by the host did not completely prohibit bacteriophage multiplication.

The addition of an excess of RNA in the basal medium completely prohibited the multiplication of bacteriophage. This substance also was inhibitory to the host organism, but did permit from one-third to slightly less than one-half of maximum growth.

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