STUDIES ON TRANSFORMATION IN STREPTOCOCCI

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STUDIES ON TRANSFORMATION IN STREPTOCOCCI

INTRODUCTION

Among the various industries that use mixtures of different strains of bacteria to carry out a particular fermentation, the dairy industry may be cited as perhaps the outstanding example. Use of mixtures of microorganisms in controlled milk fermentations provides a great variety of dairy products to consumers the world over. These products, however, are not always uniform in flavor and texture despite apparent identical manufacturing and microbiological conditions in daily operations. This variation in the finished product has become a problem of increasing concern to dairy manufacturers in recent years as the consuming public and state and federal regulatory agencies have become more demanding where uniformity is concerned.

The recent demonstration of exchange of genetic material by phage-mediated (transduction), naked DNA-mediated (transformation) and sexual (conjugation) mechanisms has suggested the possibility that variability in mixed-strain starter cultures and products manufactured therefrom might arise by genetic exchange. It was the object of the present study to carry out preliminary

experiments designed to demonstrate transformation among lactic streptococcus organisms. The ultimate practical goal of this research, which will be continued by other workers, will be to determine the precise role that transformation plays in contributing to variability in lactic acid bacteria and dairy products.

Since transformation had not been demonstrated previously in the lactic acid streptococci, it was necessary to conduct a series of experiments designed to establish suitable transforming conditions. This involved nutritional and growth studies, isolation of mutants, lysis of cells and extraction of deoxyribonucleic acid. Subsequently, attention was given to the detection of strains that were suitable for use as donors and recipients of genetic material.

The major portion of this investigation was devoted to transformation of characters among strains of the same species. The characters used mainly were streptomycin resistance, tryptophan independence, arginine hydrolysis and citrate and mannitol fermentation.

HISTORICAL

In 1928, Griffith (26, p. 113-159) made a startling report of a specific genetic change which he induced in pneumococcus organisms. Interested in the conditions that would favor the conversion of avirulent pneumococci into virulent types, he carried out the subcutaneous injection of mice with virulent strains that previously had been killed by heat. It was already known that virulence in pneumococci depended upon the secretion around their outer walls of a capsule which was composed of polysaccharide. The chemical and antigenic specificity of the capsular polysaccharde was a genetic character of each strain or type of pneumococcus, and there were known, on this basis, many distinct types of virulent pneumococci. Moreover, many unencapsulated, virulent organisms had been found to arise, probably by mutation, from virulent strains both in vitro and in vivo. Griffith found that the animals he injected succumbed to infection and that virulent, encapsulated pneumococci could be recovered from each of them. It was significant that the type of virulent strain recovered did not correspond to the type of virulent strain from which the living, unencapsulated strain had been derived; rather, it corresponded to the type of virulent strain from which the heat-killed organisms or vaccine

had been prepared. This suggested that some substance had emanated from the heat-killed cells and converted the living, avirulent organisms into virulent types having the capacity to synthesize the capsular polysaccharide characteristic of the strain from which the heat-killed bacteria were prepared.

Since specific hereditary changes induced by nonliving agents represented a truly important discovery, it was imperative to exclude alternative explanations. The existence of a few viable organisms in the vaccine was ruled out by the fact that, not only was the vaccine alone incapable of causing any pneumococcal infection in the treated animals (26, p. 113-159), but the type transformation could occur in vitro (18, p. 681-699) and could be induced by cell-free extracts of the donor organisms (4, p. 91-99).

At this point, investigations of pneumococcal transformations concerned the determination of the chemical nature of the substance in the donor extract that possessed genetic properties. As a result of an extensive study, lasting over ten years, Avery and his co-workers at the Rockefeller Institute found that deoxyribonucleic acid (DNA) was transmitting the genetic information (8, p. 137-158). This was a remarkable conclusion because until that time, no direct evidence had been obtained for the function of the nucleic acids although their universal distribution

in living cells, especially the almost exclusive location of DNA in the chromosomes, had been established. Thus an important role was suggested for this substance (44, p. 1-45). Among the significant properties of the transforming agent shown by Avery and his collaborators (8, p. 137-158; 49, p. 89-96; 48, p. 63-71) was its insensitivity to proteolytic enzymes, protein denaturants, ribonuclease (RNAse), specific antibodies and other substances capable of binding pneumococcal protein and polysaccharides. However, it was rapidly inactivated by deoxyribonuclease (DNAse), the enzyme specifically depolymerizing DNA.

While these chemical and genetic studies were taking place, several other notable discoveries were being made which had to do with other modes of transfer of genetic information between bacteria. In 1946, Lederberg and Tatum reported a process of genetic transfer in Escherichia coli which resembled sexual conjugation in higher organisms. This process has been studied in detail since that time by Lederberg and his co-workers (39, p. 412-443; 41, p. 75-107), Jacob and Wollman (35, p. 75-92) and Hayes (28, p. 75-93). This mode of genetic transfer is characterized by an unidirectional transmission of DNA requiring contact between the donor and recipient strains and involving a portion of the donor genome. Generally, several

genes are transferred, although not necessarily integrated into the recipient organism. The requirement for physical contact distinguishes this process from transformation and it is to be noted that the ex-conjugant produces, among its progeny, bacteria possessing some genetic characters of the conor strain.

In 1952 a third type of genetic transfer was discovered in the Salmonella genus by Zinder and Lederberg (76, p. 679-699). In this type, referred to as transduction, a small portion of the genetic material of the donor bacterium was transferred to the recipient bacterium by means of a virus that had reproduced previously in the donor and had subsequently infected the recipient. Since the bacterial virus or bacteriophage (phage) serving as the vector need not be virulent and cause death of the recipient, the consequences of infection by genetic material of the donor may often be observed. These consequences are similar to those occurring in conjugation and transformation in that the progeny of the infected bacterium possess genetic characters of the donor strain. While being carried by the viral vector, the genetic material of the donor bacterium is insensitive to DNAse. There is considerable evidence to indicate that both in conjugation and transduction, DNA is the material transferred from donor to recipient. While this fact correlates

the mechanisms underlying transformation, transduction and conjugation, there are certain well-defined criteria for distinguishing between them. These are (a) conjugation: transfer requires donor-recipient contact; insensitive to DNAse; (b) transduction: transfer mediated by virus passing from former host (donor) to recipient; insensitive to DNAse; (c) transformation: transfer is sensitive to DNAse.

It has been found that transformation is not limited to the pneumococci. In recent years, conditions were found in which transformations would occur regularly in several species of the genera Hemophilus and Neisseria (2, p. 17-31; 13, p. 570-590). A few workers also have been able to carry out transformations in several species of Streptococcus, some of which are apparently closely related to the pneumococci (11, p. 247-259; 54, p. 319-323). Other transformable genera include Bacillus (70, p. 1072-1078), Agrobacterium (37, p. 308-313), Xanthomonas (14, p. 137-140; 15, p. 141-145; 16, p. 146-150), Rhizobium (9, p. 77-84), Escherichia (10, p. 7-17; 36, p. 392-415), and Staphylococcus (34, p. 187-190). Several other genera are reported to have undergone transformation but the results neither were reproducible or supported by adequate controls (7, p. 31-50; 60, p. 61-163). It is to be noted that among the genera listed above, several different

transformable species have been reported (60, p. 61-163).

Although the first character genetically altered by a DNA transforming agent was that of specific polysaccharide synthesis, the process of transformation does not appear to be limited in any way to a certain class or category of hereditary characters. Whatever be the species of bacterium in which transformation is studied, it generally has been found that any character that can be conveniently investigated is susceptible to hereditary transformation by the appropriate DNA agent. What guides the investigator as to the character used in transformation reactions is simply the availability of techniques for identifying the transformed bacteria from among the untransformed recipient population. Particularly when the frquency of transformation is low, the utilization of a selective screening technique becomes important. It is for this reason that investigators interested in transformation have studied changes that are readily seen on selective media. Included among the characters that have been transformed are filamentous type of growth, specific protein antigens, drug and antibiotic resistance, antibiotic dependence, synthesis of specific enzymes, sporulation, ability to infect plants, intermediate encapsulated types, mixed or binary

encapsulation, and abnormal capsular types (60, p. 61-163). This indicates that any portion of the genetic repertoire of an organism can be transformed.

Studies on transformation usually are begun with the isolation of certain mutants which may arise either spontaneously or by induction. Mutations in a population may be induced by treatment with ultraviolet light (UV) or X-rays (30), heat (75, p. 373-397), or any of several chemical mutagens such as nitrogen mustard, formaldehyde, peroxides, nitrous acid, ferrous and manganous ions, purine and pyrimidine analogues, and acridines. Such mutagens have been reviewed by Westergaard (74, p. 224).

Once mutations occur and are capable of expressing themselves in a population, the mutant organisms may be selected by one or a combination of methods. An extremely useful technique developed independently by Davis (17, p. 4267) and by Lederberg and Zinder (38, p. 4267) is based on the fact that penicillin kills only growing cells. Bacteria, usually treated with some mutagen to induce mutations, are allowed to grow in an appropriate complete medium to allow for phenotypic expression and then subjected to the action of penicillin in a so-called minimal medium. The minimal medium will lack a particular nutrient so that mutants requiring the missing compound, in contrast to non-mutant wild-type cells, will not grow. The wild-

type, or prototrophic, cells then are killed by the penicillin and the mutants, or auxotrophs, can be isolated. Important modifications of this technique by Gorini (25 p. 604-605) have made it possible to direct the selection of various amino acid and purine auxotrophs. Another simple mutant selection method was developed by the Lederbergs (40, p. 399-406). Colonies of bacteria are allowed to develop on a suitable complete medium and then are transferred in a single operation to plates containing different types of media by use of a velvet stamp. The stamp is pressed to the surface of the original plate to provide a master recording of the number and location of the colonies each of which may be replicated on different minimal media by pressing the velvet to a fresh agar surface. Mutants are recognized by comparison of the replicate plates with the original, the mutant strains not growing on certain of the minimal media.

Once mutant or wild-type organisms have been obtained for possible use in transformation, it is necessary to prepare cell-free extracts from which DNA carrying the desired genetic markers can be obtained. This can be accomplished if the cells are first disrupted under conditions which render DNA in a native, highly polymerized state. Methods commonly used for cell lysis have included mechanical disruption (27, p. 51-62) and lysis by lysosyme (70,

p. 1072-1078), extracts from Streptomyces albus (54, p. 319-323), and sodium desoxycholate (11, p. 247-259). It also has been found by Repaske that certain organisms ordinarily resistant to lysis by lysozyme were made susceptible when ethylenediaminetetracetic acid (EDTA) was incorporated in the lysing mixture (62, p. 225-232). Whatever method of cell disruption is used, it is followed by removal of protein and cell debris by denaturation and centrifugation, removal of RNA by RNAse and selective precipitation of the DNA with isopropanol (46, p. 208-218). DNAse and divalent metal ion contamination may be prevented by the addition of chelating agents and by the action of sodium lauryl sulfate. It is also necessary to protect the DNA from such adverse conditions as extremes in temperature and pH, sodium nitrite at low pH, formaldehyde, ferrous ions, dehydration, and low ionic strength (75, p. 373-397).

Among the species of bacteria in which genetic transformations have been found to occur, it is clear that certain internal and external conditions must prevail in order for the bacteria to become transformed when exposed to transforming DNA. If a strain secretes an active exocellular DNAse which depolymerizes homolgous as well as heterologous DNA, it may be impossible to transform such a strain unless the exocellular enzyme can be inactivated

without inactivating the transforming DNA (7, p. 31-50). Another hereditary character that definitely has an influence on transformation is the nature of the cell coat. The elaboration of a thick, gummy, or mucoid capsule around the cell wall or membrane may inhibit the penetration of transforming DNA into the cell. In the case of pneumococcal strains differing quantitatively in the amount of polysaccharide capsule they secrete, it has been shown that transformability is related inversely to the amount of capsule secreted (59, p. 335-348).

McCarthy et al. (50, p. 177-183) began the many investigations on biochemical conditions contributing to the transformability of pneumococci. Certain chemical factors in the medium, other than transforming DNA itself, appeared to be essential for transformation. One of these was inorganic pyrophosphate. Although its role was not established, it has since been suggested that the requirement for this compound probably depends on the nature of the medium; for it may be omitted from most media used today for transformation (1, p. 345-359; 58, p. 58-82).

Another environmental factor regarded important by early workers was the presence of antibodies capable of agglutinating the recipient bacteria (8, p. 137-158).

Antibodies *per se* probably were not necessary, since other physical means of holding the recipients *in situ*, such as increasing the viscosity of the medium through the addition of agar, could replace antibodies (50, p. 177-183; 58, p. 58-62).

Still another environmental factor that favored pneumococcal transformations was a substance identified as albumin (31, p. 300). The role of albumin in promoting transformations is still not clear. Thomas (72, p. 467-481; 73, p. 50-61) has shown that albumin promotes the development of, and possibly the maintenance of the physiological state of competence, i.e. it makes pneumococci more capable of making an effective contact with DNA, although it has little, if any effect on the genetic consequences of the contact. However, while the presence of serum is stimulatory, it is not essential, for transformation of pneumococci (53, p. 471-472) and of *Hemophilus* (2, p. 17-31) may occur in its absence.

The work of Hotchkiss (32, p. 49-55) conforms with the idea that competence is a physiological property that arises during a fraction of the generation period of the organism. Moreover, his evidence indicates that the period of competence corresponds to a particular period of bacterial division process. He found environmental

factors that induced synchronous division increased the waves of competence arising in a population. Fox and Hotchkiss (23, p. 1322-1325) used frozen cultures of competent pneumococcal cells to determine some of the factors necessary for the loss and reacquisition of competence. Upon prolonged storage at -20°C, or following melting and subsequent incubation at 37°C, bacteria lost their competence. The reacquisition of this state occurred within 20 minutes, and depended not only upon the presence of serum albumin, but also of calcium ions at a concentration of 10^{-3} to 10^{-4}M. The reacquisition of competence also appeared to require protein synthesis, for it was beneficial to include a mixture of amino acids and glucose as an energy source; chloramphenicol, a specific inhibitor of protein synthesis, prevented the renewal of transforming activity.

The transformation process usually is completed by assaying for transformants using techniques similar to those described above in the isolation and selection of mutants.

Bacterial transformation provides a mechanism by which closely linked genetic loci may be arranged in a sequential order or map (51, p. 52-55). Determination of the position of these loci with respect to each other has largely resulted from the earlier work of Hotchkiss and Marmur (33, p. 55-60). Since a unit of DNA transferred

from a donor bacterium to the recipient cell represents only a small fragment of the entire genome (51, p. 52-55), it is not possible to arrange many randomly distributed loci in any single transfer experiment. By the same token, when a pair of genes show an appreciable degree of joint transformation, it points directly to their genetic congruity or close association on the DNA strands.

Transformation also is a valuable tool in studies involving the taxonomic relationship between various microorganisms. To date, a number of cases have been reported of transformations produced between populations regarded as distinct species by the bacterial taxonomist. Schaeffer first reported inter-specific transformation between Hemophilus influenzae and Hemophillus parainfluenzae (66, p. 1491-1493). Leidy et al. (42, p. 305-320) extended this finding for other species of the genus Hemophilus. Bracco et al. (11, p. 247-258) reported transformation of two Streptococcus organisms of the viridans group using DNA either from these same strains, from Streptococcus salivarus, or from pneumococci. Pakula and his associates (54, p. 319-323) extended the investigations on streptococci, and reported that of 45 viridans strains tested, only 13 were transformable. Each of these 13 strains was transformed by DNA from any one or more of five different viridans strains, three

S. salivarius strains, one Streptococcus SBE strain, four hemolytic Streptococcus strains and one enterococcus strain. None of 16 S. salivarius strains tested could be transformed, but since they were recently isolated from human saliva and presumably encapsulated, the failure could be due to poor penetrability of DNA into these bacteria. Of a number of hemolytic streptococci, only two strains belonging to group H were transformed; these strains were transformed by DNA from viridans streptococci, S. salivarius, S. SBE, and heterologous hemolytic streptococci. Streptococcus SBE was also transformed by these DNA preparations. Further studies on streptococci have been made by Perry and Slade (57, p. 443-449). They obtained transformation with serological groups F, H, O, and a serologically unclassifiable Streptococcus. The transformable streptococci incorporated DNA from both homologous and heterologous groups and strains of streptococci. Certain strains within a group served as DNA donors but not as recipients, and certain strains served as DNA recipients for some strains but not for others. Catlin (12, p. 608-610) also has reported interspecific transformation among Neisseria. It is noteworthy that DNA liberated into the medium by autolyzing Neisseria cells is as effective in inducing interspecific transformations as DNA extracted from the cells

by chemical means. Furthermore, Leidy *et al.* (42, p. 305-320) have applied an analysis of ratios of interspecific to intraspecific transformation to the taxonomy of the genus *Hemophilus* on the premise that such ratios reflect the degree of relationship of donor and recipient cells.

METHODS AND MATERIALS

Cultures Used

All bacteria used in this study were obtained from the culture collection of the Department of Microbiology, Oregon State University, except *Streptococcus* strains challis and SBE which were generously donated by Dr. R. Pakula, Department of Microbiology and Hygiene, School of Medicine, Warsaw, Poland. A list of the lactic acid bacteria employed is shown in Table 1. The original source along with considerable cultural data on most of these bacteria were presented by Sandine (64, p. 1-123)...Cultures were maintained by monthly transfer in stabs of the lactic agar of Elliker, *et al.* (21, p. 1611-1612) or the *Leuconostoc* medium of Seitz (65, p. 1-113). The composition of each of these media is shown in Tables 2 and 3 respectively..

Growth Studies in Synthetic Media

Stock vitamin solutions for use in preparing the synthetic medium were made up as shown in Table 4. Salts A and B were prepared as described by Snell and Wright (69, p. 675-685) and the recipes for each are shown in Table 5. An amino acid stock solution was prepared by dissolving 18 amino acids (2.0 g. of the L-forms

TABLE 1

Strains of bacteria of the *Streptococcus*, *Lactobacillus* and *Pediococcus* genera used in the present investigation.

Organism	Organism	Organism
Strain of *S. lactis*	Strain of *S. cremoris*	Strain of *S.* SBE
a	11D-1	582
E	C 13	589
27	31-9	
C2	11E	
C6	3D-19	Strain of *S. cremoris* var *aromaticans*
C10	31-1	
7962	W	
7963	CC 2	
11454	18-1	
11955a	C 3	Da-2
	27-1	Da-13
	36-4	
Strain of *S. diacetilactis*	114-F	
	9625	*S. faecalis* R
	9596	
	R 6	*S. durans* OSU
DRC 1	KH	
DRC 2	E 8	*S. liquefaciens* OSU
DRC 3	HP	
RM 1	R 1	*S. thermophilus* MC
Da-20	11602a	
CC-1	11603a	*L. arabinosus* Farr
4R-1	Da-1	
4R-5	Da-5	*L. arabinosus* 17-5
6B-1	18-10	
6B-3	31-6	*L. bulgaricus* GA
3D-1	4R-7	
11D-3	26-1	*L. lactis* 39-A
31-2	CC 9	
31-8	2R-1	*P. cerevisiae* P-60
18-16		
26-2		
QEL	Strains of *S. challis*	
CEL		
11007	588	
	622	

TABLE 2

Composition of lactic broth medium

Ingredient	Grams per liter
Tryptone	20.0
Yeast extract	5.0
Glucose	5.0
Lactose	5.0
Sucrose	5.0
Gelatin	2.5
Sodium chloride	4.0
Sodium acetate	1.5
Ascorbic acid	0.5
Agar*	15.0
pH was 6.8 to 7.0	

*Added when lactic agar was desired

TABLE 3

Composition of Leuconostoc medium

Ingredient	Grams per liter
Yeast extract	10.0
Tryptone	10.0
Whey-lac[a]	2.0
Sodium acetate	1.5
Sodium citrate	5.0
Glucose	10.0
Ascorbic acid	0.7
Tap water	33.0 ml
Sodium chloride	2.0
K_2HPO_4	2.0
KH_2PO_4	2.0
$MgSO_4$	2.0
Agar[b]	15.0
pH was 6.8 to 7.0	

[a]Deionized spray-dried whey (Consolidated Dairy Products, Seattle, Washington).

[b]Added when Leuconostoc agar was desired.

TABLE 4

Composition of stock vitamin solutions used to prepare the complete synthetic medium

Ingredient	Amount Weighed	Final Volume*	Concentration
Niacin	10.0	100 ml	100μg per ml
Calcium pantothenate	10.9	100 ml	100μg per ml
Pyridoxamine	10.0	100 ml	100μg per ml
Biotin	5.0	500 ml	10μg per ml

*Each ingredient was dissolved in distilled water

TABLE 5

Composition of salt solutions A and B used to prepare the complete synthetic medium

Ingredients	Grams used per 250 ml
Salts A	
K_2HPO_4	25.0
KH_2PO_4	25.0
Salts B	
$MgSO_4$	10.0
NaCl	0.5
$FeSO_4 \cdot 7H_2O$	0.5
$MnSO_4 . 4H_2O$	0.5
Concentrated HCl	5.0

TABLE 6

Composition of complete synthetic (CS) medium

Ingredient	Amount per liter
Amino acid stock solution	100 ml
Niacin	5 ml stock
Calcium pantothenate	5 ml stock
Pyridoxamine	5 ml stock
Biotin	5 ml stock
Salts A	2.5 ml stock
Salts B	2.5 ml stock
Sodium acetate	2.0 g
Glucose[a]	10.0 g
Agar[b]	15.0 g
pH was 6.8 to 7.0	

[a]Glucose was sterilized separately and added after cooling.
[b]Added when solid medium was desired.

and 4.0 g of the DL-forms) in 100 ml of water. The complete synthetic (CS) medium then was prepared in 1-liter amounts as shown in Table 6. The amino acids included in the stock solution were alanine, arginine, histidine, isoleucine, leucine, valine, glutamine, tyrosine, serine, glycine, cystine, aspartic acid, lysine, methionine, phenylalanine, proline, threonine, and tryptophan. The medium, without carbohydrate, was prepared double strength and dispensed in 5.0 ml amounts in 18 x 150 mm test tubes. Tubes of medium were sterilized by autoclaving for 5 minutes at 121°C, cooled and diluted to single strength with previously autoclaved (121°C for 15 minutes) 2.0 per cent glucose. Autoclaving for larger periods of time or in the presence of glucose was found to inhibit growth of the organisms.

Cultures were grown for 24 hours in lactic broth at 30°C. They were then centrifuged and washed twice with sterile physiological (0.85 per cent) saline. Five-hundredths of a ml of the resuspended cells was used to inoculate tubes containing 10 ml of CS medium. Results were recorded on basis of degree of growth in comparison to growth in lactic broth after incubation for 24 and 48 hours at 30°C. Several successive transfers were made in order to insure that each strain would grow in the CS medium on repeated sub-culturing.

Determination of amino acid requirements

A series of synthetic media were prepared in a manner similar to that described for the CS medium except that amino acid stock solutions lacking one each of the 18 amino acids listed above were used. Thus, 18 different media were compounded, each lacking only one amino acid. The amino acids which were found to be required or stimulatory for growth were used in preparing a minimal synthetic (MS) medium for one of the strains tested.

Incubation conditions for agar plates

When grown under aerobic conditions in a regular air incubator, colonies of lactic acid bacteria developing on agar plates were found to be very small (less than 0.5 mm in diameter). Since large colonies were desirable for subsequent genetic studies, it was felt that some method of inducing better surface growth by these bacteria would be desirable. Incubation under an atmosphere of carbon dioxide in a large dessicator containing moistened oats and a burning candle was employed. One-tenth of a ml of a 10^{-6} dilution of an 18-hour culture was spread on agar plates of MS medium and incubated in the oats jar at 30° C for 48 hours. Duplicate plates treated in a similar manner were incubated under an air atmosphere. At the end of the incubation period a comparison of colony sizes was made between the plates incubated under the two

different conditions.

Isolation of Tryptophan-Requiring Mutants

Determination of ultraviolet exposure time for 99.99 per cent kill

Ultraviolet irradiation was accomplished using a G. E. 30 watt germicidal lamp. The energy output was determined using a G. E. light meter and the following relationship:

$$Y = C \times I$$

Y indicates germicidal energy in ergs per cm^2 per second

C represents the conversion factor which equals 3.4 ergs per cm^2 per sec.

I represents the change in light intensity between readings made with and without light meter filter attachment.

Cells were grown for 5 hours on CS medium. Five ml were placed in a petri dish and irradiated at 7.215 ergs per cm^2 per sec. at a distance of 6 inches below the light source for 10, 15, 20 and 25 seconds. The irradiated cells were appropriately diluted and plated out on lactic agar. Aftern an incubation period of 48 hours at 30°C, the per cent kill was determined as follows:

$$\text{Number of cells per ml at zero time} - \frac{\text{Number of cells per ml after irradiation}}{\text{Number of cells per ml killed}}$$

$$\left(\frac{\text{Number of cells per ml killed}}{\text{Number of cells per ml at zero time}}\right) \times 100 \text{ per cent kill}$$

Ultraviolet irradiation and penicillin selection

Selection of tryptophan-requiring mutants was accomplished by a method similar to that described by Gorini (25, p. 604-605). A culture of S. diacetilactis 18-16 was grown in 10 ml of CS medium for 5 hours at 30°C. Five ml of this culture were placed in a petri dish and irradiated for 30 seconds at 7.215 ergs per cm^2 at a distance of 6 inches below the light source. The irradiated cells were transferred to lactic broth and held at 30°C for 2 hours in order to allow mutant stabilization. The cells then were centrifuged, and resuspended in CS medium and incubated for 14 hours. Following this incubation period, the cells were centrifuged, washed and resuspended in 1.5 ml of sterile physiological saline. Five-hundredths of a ml of this suspension was added to MS medium lacking tryptophan but containing 20 per cent sucrose and 0.01M magnesium sulfate. After incubation for four hours, 9,000 units per ml of penicillin were added and incubation was continued for an additional 12 hours. The culture was diluted from 10^{-4} to 10^{-5} and 0.1 ml samples were spread on the surface of lactic agar plates which previously had been dried for at least 24 hours. After incubation in an oats jar for 72 hours, the colonies were replicated onto plates of MS medium, MS medium containing tryptophan (MST) and lactic agar. Observations were made

for colonies growing on MST medium but not on the MS medium. In some instances this irradiation-selection procedure was repeated for mutants which seemed to be leaky or suppressed.

Verification of mutants

Mutants were verified in the following manner. After propagation in lactic broth for 8 hours, one loopful of culture was transferred to tubes of MS and MST media. Strains which grew in MST medium but which failed to grow in MS medium after an incubation period of 48 hours were regarded as tryptophan-requiring mutants. Each auxotroph was purified by repeated (3 times) single colony isolation with verification of its mutant nature being made after each plating.

Characterization of tryptophan-requiring mutants

These experiments were carried out to locate the metabolic blocks in tryptophan synthesis in each of the auxotrophs. Five-tenths of a ml of an 8-hour culture was added to cooled tubes of melted MS agar and the mixture was poured into a sterile petri plate. Sterile assay discs containing 0.2 mg per ml of shikimic acid, anthranilic acid, indole and tryptophan were added to various sections of the plate. Control plates containing innoculated MS agar also were prepared. All plates were

incubated for 72 hours at 30°C. Observations were made for growth around particular intermediates.

Isolation of Streptomycin-Resistant Mutants

Preparation of streptomycin solutions

Stock solutions of streptomycin were prepared by dissolving the desired amount of powdered Streptomycin sulfate (California Biochemical Corporation) in 0.1 M phosphate buffer at pH 6.0 to 8.0. Solutions were stored in the refrigerator at 4°C.

Step-wise mutants

One hundred micrograms (μg) per ml of streptomycin were added to a 12-hour lactic broth culture and incubation was continued at 30°C for an additional 12 hours. Five-tenths of a ml of this culture was added to a fresh tube of the same streptomycin-containing medium and the incubation continued. If good growth did not occur within 12 hours, incubation was continued for another 12-hour period. Five-tenths of a ml of this culture then was transferred to 10 ml of lactic broth containing 500 μg per ml of streptomycin and the cultures were incubated until an appreciable cell density had been reached. Finally, 0.5 ml of this culture was added to tubes of lactic broth containing 1000 μg per ml of streptomycin.

Colonies growing on these plates after incubation for 48 hours at 30°C were transferred to streptomycin-free lactic broth.

Single-step mutants

Cultures lacking previous exposure to streptomycin were grown in Leuconostoc broth for 24 hours. Two ml of each culture were used to inoculate bottles containing 100 ml of cooled (48°C) Leuconostoc agar containing 1000 μg per ml of streptomycin. The mixture was poured into petri dishes and incubated for at least 24 hours at 30°C. Visible colonies were picked and transferred to tubes of Leuconostoc broth containing 1000 μg per ml of streptomycin. After incubation at 30°C for 24 hours viable cultures were replated on Leuconostoc agar containing the same amount of antibiotic. Colonies were picked from these plates and transferred to streptomycin-free Leuconostoc broth.

Preparation of DNA

Lysis of cells

During preliminary phases of this study, it was discovered that lactic acid streptococci were resistant to lysis by lysozyme, sodium lauryl sulfate, and sodium desoxycholate when suspended in physiological saline or 0.1M

pH 7.0 phosphate buffer. Consequently it was necessary to develop a method of lysing these organisms in order to extract their DNA. The experiments carried out in the development of a suitable lysis technique are described in the results section. The final procedure adopted is described as follows:

Stock solutions of the appropriate reagents were prepared in a manner such that 0.1 ml of the stock solution would give the desired amount when added to a final volume of 3.0 ml. The EDTA, though insoluble in water at pH 7.0, was readily dissolved at pH 12. Then the solution was adjusted to pH 8.0.

Cells for lysis experiments were inoculated from lactic agar stabs into 10-ml broth tubes of the same medium. Following incubation for 12 hours at the optimum growth temperature, cells were centrifuged and washed twice. They were resuspended in 3.0 ml of the various lysing media; the complete system contained 100 micromoles of Tris(hydroxymethyl)aminomethane (Tris) buffer, 100 μg of lysozyme and 400 μg of EDTA. Lysis was measured by following the decrease in optical density from an initial value of about 0.7 using a Beckman model B spectrophotometer at 660 millimicrons (mμ).

When lysis of cells for the isolation of DNA was desired, the following procedure was employed. Cultures

were grown in flasks containing 2 liters of lactic or Leuconostoc broth for 8 to 12 hours at 30°C. The cultures then were centrifuged in a Serval SS-3 centrifuge at 12,000 rpm for 15 minutes. When larger volumes of culture were used, centrifugation was accomplished by a Sharples steam-driven, water cooled, centrifuge. Cells were washed once with a cold solution of 0.15M sodium chloride containing 0.015M sodium citrate: this was referred to as the saline-citrate solution. Following this treatment, the cells were resuspended in 18 ml of a solution containing 0.15M EDTA, 0.15M sodium chloride and 0.1M pH 8.0 Tris buffer (saline-EDTA). Ten mg of lysozyme (California Biochemical Corporation) were added and the mixture was incubated with magnetic stirring at room temperature for 2 to 6 hours, depending on the resistance of the cells.

Extraction of DNA

The procedure for the extraction of DNA was essentially that of Marmur (46, p. 208-218) which was carried out as follows: Following lysis, 2.0 ml of a 25 per cent solution of sodium lauryl sulfate was added and the mixture was heated in a 60°C water bath for 10 minutes. The salt concentration was increased to 1.0M by the addition of 5.0M sodium perchlorate. After cooling to

room temperature, an equal amount of chloroform-octanol (24:1) was added and the mixture was shaken on a reciprocal shaker for 30 minutes at room temperature. This was followed by centrifugation at 5 to 10,000 rpm in a Servall SS-1 centrifuge. The upper layer was carefully decanted and added to an 18 x 150 mm test tube. This was added to a sterile graduated cylinder containing 2.5 volumes of 95 per cent ethanol which had been previously cooled to 0°C. The long threads which precipitated were collected with the aid of a glass stirring rod. The threads were pressed free of ethanol and transferred to a tube containing 70 per cent ethanol. After standing 8 to 12 hours in this sterilizing solvent, the threads were pressed free of excess alcohol and dissolved in saline-citrate. The crude DNA solutions were stored at 4°C.

Purification of DNA

In most transformation experiments, crude DNA was used; however, when purified preparations were desired, it was necessary to remove contaminating protein. This was accomplished by shaking the crude DNA with an equal volume of chloroform-octanol, centrifuging and removing the aqueous layer and continuing this process until little or no protein could be seen at the interface. This usually required 7 chloroform-octanol treatments. The DNA then was precipitated with 95 per cent ethanol,

freed of excess ethanol and stored in saline-citrate at 4°C.

Qualitative and quantitative analyses of DNA

A 1:10 aqueous solution of purified DNA was prepared and an absorption spectrum plotted from 220 to 300 mμ using a Cary continuous-recording spectrophotometer.

When quantitative estimations of DNA were desired, the procedure of Dische (20, p. 285-305) was used. Diphenylamine was recrystallized twice from 70 per cent ethanol and 1.0 g of this purified material was added to 100 ml of reagent grade glacial acetic acid. Concentrated sulfuric acid (2.75 ml) was added to 100 ml of the diphenylamine solution. A standard DNA solution was prepared by dissolving 500 μg per ml of salmon sperm DNA (Nutritional Biochemicals Corporation) in saline-citrate. Various dilutions of this solution were added to the diphenylamine reagent and heated at 100°C for 10 minutes. The blue color which developed was measured at 595 mμ using a Beckman Model B spectrophotometer. A typical standard response curve from which the DNA content of unknown samples was determined is shown in Figure 1.

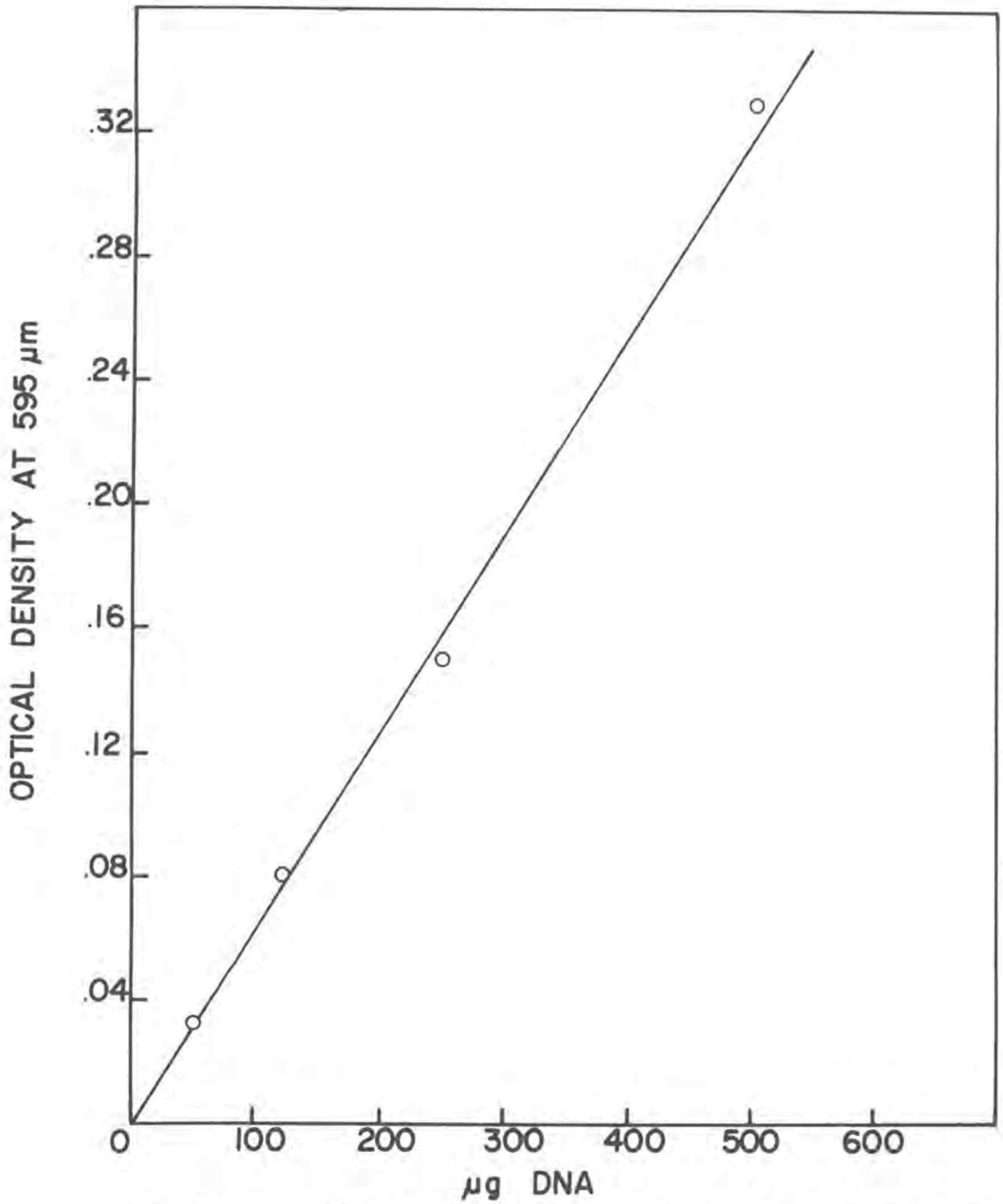

Figure 1. Standard reference curve used for determining the amount of DNA present in unknown samples.

Transformation of Transformable Streptococcus Organism

Of the many variables encountered in transformation experiments, two of the more important are conditions for lysis of donor cells and the selection of a medium that will yield competent cells of the recipient population. During preliminary phases of this investigation, transformation experiments were conducted on lactic acid streptococci without the use of transformable organisms as a control. It was felt that if strains of transformable streptococci related to the lactic streptococcus organisms were obtained, the method of cell lysis described above could be tested. Furthermore, this would allow an evaluation of the medium known to produce competent cells of transformable bacteria in transformation experiments with lactic acid streptococci. Cultures of transformable streptococci obtained were strain 588, resistant to 2,000 μg per ml of streptomycin and strain 622, streptomycin sensitive.

Preparation of charcoal adsorbed broth

Composition of charcoal adsorbed broth used for the growth of recipient cells is shown in Table 7. The broth was charcoal adsorbed according to the method of MacLeod et al. (45, p. 277-287) which involved the following steps: the broth, before the addition of glucose, was

TABLE 7

Composition of charcoal adsorbed broth

Ingredient	Grams per liter
Neopeptone	10.0
Yeast extract	5.0
Beef extract	5.0
Glucose	2.5

pH was 6.8

adjusted to pH 5.5 with concentrated hydrochloric acid (HCl). Two grams per 100 ml of activated charcoal were added and the mixture was heated to boiling over an open flame. This was followed by immediate filtration through a Buchner funnel. Glucose was added to the clear medium and the pH was readjusted to 6.8. Sterilization was accomplished by Seitz-filtration. The broth then was dispersed into 18 x 150 mm tubes and stored at 4°C until ready for use. When needed, bovine serum albumin (California Biochemical Corporation) was added to a concentration of 5.0 per cent by volume.

Preparation of competent cells

Recipient cells were grown in charcoal adsorbed broth containing 5.0 per cent bovine serum albumin (BSACAB) for two consecutive transfers. Aliquots (0.05 ml) of a 12-hour culture of the second transfer was added to 5.0 ml of fresh broth at 30°C. Growth was interrupted at 4 to 5 hours and the culture was supplemented with sterile 10 per cent glycerol. The mixture was divided into 1.5 ml portions and frozen immediately in ethanol-dry ice and stored at -20°C until ready for use.

Transformation procedure

The procedure used for transformation was the one

developed by Pakula (56, p. 79-85). Cells were removed from the deep freeze, thawed in tap water and diluted 1:10. Samples (1.8 ml) were added to tubes containing 0.2 ml of DNA prepared as described under preparation of DNA. Controls consisted of tubes to which no DNA was added, tubes to which DNAse had been added 5 minutes before the addition of DNA and tubes containing DNA, which had been heated at 100°C for 10 minutes. All tubes were held at 30°C for 30 minutes. This was followed by incubation at 37°C for 2 hours to allow for delayed expression.

Assay of transformants

The number of cells transformed to streptomycin resistance was determined by plating 0.1 ml samples of the DNA-treated and control cultures in lactic agar containing various concentrations of streptomycin. The total count in each culture was determined by plating out samples in the same medium containing no streptomycin. All plates were incubated at 37°C for 48 hours.

Factors affecting transformation in Streptococcus strain challis

The effect of DNA concentration was determined in an experiment using diluted and undiluted samples of DNA.

The effect of cultural conditions was determined by attempting transformation in a variety of media. In all

cases, the transformation and assay procedures were the same except that only one streptomycin concentration (100 μg per ml) was used in the assay of transformants. The media used for these experiments are listed in Table 8.

Growth of Lactic Acid Streptococcci in Charcoal Adsorbed Broth

Since a medium which would allow lactic acid streptococci to obtain a competent state was not known, the medium developed for transformation of the transformable strains was used. It was necessary, therefore, to determine the number of strains that would grow in this medium. Strains which gave rapid growth without additions were tested first for transformation. Strains which grew slowly or not at all were investigated further; a number were tested to see if an increased sugar concentration would facilitate growth.

Transformation Studies on Lactic Acid Streptococci

In most cases, the same procedures for lysis and transformation were employed as with the transformable strains. Where possible, shorter lysing periods were used.

TABLE 8

Media used to study the cultural conditions for transformation in Streptococcus strain challis

Number	Description
1	Leuconostoc medium
2	Leuconostoc medium minus ascorbic acid
3	Leuconostoc medium containing 5.0 per cent bovine serum albumin
4	Leuconostoc medium minus ascorbic acid plus 5.0 per cent bovine serum albumin
5	Charcoal adsorbed broth containing 0.5 per cent bovine serum albumin
6	Charcoal adsorbed broth containing 5.0 per cent bovine serum albumin
7	Charcoal adsorbed broth containing 10^{-4}M EDTA

Tryptophan independence

Most of this work was carried out before the transformable strains were obtained; therefore, many attempts were made to devise a suitable medium for competence. These experiments are described in the results section. The method finally adopted was the same as used with strain challis but the assay procedure was different. Following the 2-hour incubation period to allow for genetic expression of the new trait, the cells were centrifuged, washed and plated out on MS and MST media. After 48 hours of incubation the plates were compared.

Mannitol fermentation

Since it was known that significant differences existed among S. diacetilactis strains with regard to their ability to ferment mannitol (64, p. 1-123), the possibility that this could be used as a genetic marker was suggested. First, all S. diacetilactis strains were tested for their ability to ferment mannitol. Lactic broth containing 0.02 per cent brom cresol purple was used as the basal medium and when a sugar medium was needed, 1.0 ml of 5.0 per cent filter sterilized sugar (Difco) was added to 9.0 ml of the basal non-sugar (NS) medium. The appropriate media were inoculated with a loopful of each culture and these tubes were incubated for 48 hours at

30° C. This process was repeated and results were recorded after the second incubation period.

Those strains fermenting mannitol were used as DNA donors while those that did not ferment mannitol were used as recipients. Following exposure to DNA, the cells were incubated for 12 hours. Loopfuls of culture were transferred to mannitol broth and incubated for 48 hours; all recipient strains were exposed to DNA from all possible donors.

Arginine hydrolysis

Mutants of S. lactis 27 were donated by Mrs. N. Turner. They were obtained by ultraviolet irradiation of wild-type cells that were able to hydrolyze arginine. The wild type cells appeared pink when plated on Nivens' arginine medium (52, p. 651-660) containing 0.005 per cent 2,3,5-triphenyltetrazolium chloride (TTC) as an indicator. Mutants on the other hand, as a result of irradiation, had lost the ability to hydrolyze arginine and thus appeared white. The pink cells served as DNA donors while the white cells served as recipients.

Following exposure to DNA, the recipient cells were incubated for 12 hours. One-tenth ml samples were plated out on Nivens' arginine-TTC agar and these plates were incubated in an oats jar for 48 hours at 30° C. At the

end of this incubation period, plates were examined for a mixture of pink and white colonies.

Streptomycin resistance

It has been shown that the DNA of highly resistant mutants obtained by a stepwise procedure transform recipient cells also in discrete steps, whereas DNA from single step mutants transform recipient cells to high resistence in a single step (29, p. 1-9). For screening a large number of strains, it was felt that a single-step transformation procedure would be more desirable. For this reason, only the single-step mutants were used in this investigation.

The method described above for transformation of streptomycin resistance in transformable strains of streptococci was used.

Citrate fermentation

One of the characters used to separate species of lactic acid streptococci is the ability to ferment citrate. Strains of S. diacetilactis can ferment this compound while strains of S. lactis and S. cremoris cannot. Citrate-fermenting strains can be detected readily when grown in a medium containing 2.0 per cent sodium citrate; large volumes of carbon dioxide are

produced and may be detected by visual observation. To determine whether or not this character could be transferred to strains of S. lactis and S. cremoris, the following experiment was conducted:

A modified lactic broth with ascorbic acid omitted, but containing 5.0 per cent bovine serum albumin, was prepared and charcoal adsorbed as described previously. The medium was sterilized by Seitz-filtration and dispensed in 2.0 ml amounts in sterile 18 x 150 mm test tubes. This medium was used for strains of S. cremoris. Strains of S. lactis were cultivated in BSACAB containing 0.01M sodium citrate. One-tenth of a ml of an 18-hour culture of the strains to be tested was added to 2.0 ml of the same medium. Following incubation for 4 to 5 hours at 30°C, 0.1 ml of each culture was added to 1.9 ml of fresh broth containing 0.1 ml of DNA from S. diacetilactis DRC3. These cultures were incubated for 8 hours. At the end of this incubation period, 0.3 ml samples were added to tubes of citrate broth. The composition of the citrate broth is shown in Table 9. If the cultures showed no gas evolution in 24 hours, they were transferred to fresh tubes of citrate broth and observed for gas production. Controls, treated in the same manner, consisted of cells to which no DNA had been added.

TABLE 9

Composition of citrate broth

Ingredient	Grams per liter
Tryptone	10.0
Glucose	10.0
Sodium citrate.$2H_2O$	20.0
Yeast extract	5.0
K_2HPO_4	1.0
Magnesium sulfate	1.0
pH was adjusted to 7.0 with HCL	

Determination of Cellular DNA Content

Cultures of S. diacetilactis 18-16 and S. cremoris 144-F were grown in 2 liters of Leuconostoc medium for 12 hours. Appropriately diluted samples were spread on Leuconostoc agar in order to determine the total count. The cultures were centrifuged, washed once with saline-citrate and resuspended in 18 ml of saline-EDTA. Lysis was accomplished by the addition of 10 mg of lysozyme and incubation for 12 hours at room temperature. The DNA was prepared and extracted once with chloroform-octanol as described under preparation of DNA. The DNA concentration was determined by the diphenylamine reaction. This value was used to calculate the DNA content of individual cells of lactic acid streptococci. Comparisons were made with values that have been reported for other organisms.

RESULTS

Growth Studies in Synthetic Media

A study was made to determine the strains of lactic acid streptococci that were capable of growing in the complete synthetic medium which was described in Table 6. Those strains which were found to give rapid growth from small inocula on repeated subculturing in this medium are shown in Table 10. All strains of S. lactis and S. diacetilactis tested grew well, however, only four of the 32 S. cremoris strains examined were able to multiply in the synthetic medium. Those strains unable to grow are shown in Table 11.

Another finding of interest that was made during these studies concerned the possible existence of an unidentified growth factor in vitamin-free casein hydrolyzate. It was found that many strains of S. cremoris would not grow in a synthetic medium containing all the amino acids found in casein which were added individually as synthetic compounds. However, when vitamin-free, acid-hydrolyzed casein (Difco) was used as the amino acid source, good growth resulted. All S. diacetilactis and S. lactis strains grew well in both media.

Amino acid requirements

Two strains of S. diacetilactis were further

TABLE 10

Strains of lactic acid streptococci capable of growing in the complete synthetic medium

Strain of *S. lactis*	Strain of *S. cremoris*	Strain of *S. diacetilactis*
E	R6	DRC1
27	1	DRC2
C2	Da-5	DRC3
7962	11D-1	RM1
11454		Da-20
		CC-1
		4R-1
		4R-5
		6B-1
		6B-3
		3D-1
		11D-3
		31-2
		31-8
		18-16
		26-2

TABLE 11

Strains of S. cremoris incapable of growing in the complete synthetic medium

Strain designation	
144-F	27-1
C13	26-4
31-9	9625
11E	9596
3D-19	KH
31-1	E8
CC2	HP
18-1	R1
C3	11602a
4R-7	11603a
26-1	Da-1
CC9	Da-5
2R-1	18-10
	31-6

TABLE 12

The amino acid requirements of two strains of *S. diacetilactis*

Amino acid	Strain 18-16	Strain 26-2
Alanine	R	R
Histidine	R	R
Arginine	R	R
Isoleucine	R	R
Leucine	R	R
Valine	R	R
Glutamic Acid	R	R
Tyrosine	R	S
Cystine	S	S
Serine	-	-
Aspartic acid	S	R
Lysine	S	S
Methionine	R	R
Phenylalanine	S	S
Proline	-	S
Tryptophan	-	S
Threonine	A	A
Glycine	-	S

R indicates required for growth
S indicates stimulatory
- indicates not required or stimulatory

investigated in order to determine their amino acid requirements by single elimination experiments. From Table 12 it may be seen that each of the strains required 9 of the amino acids commonly found in casein, and that several other amino acids were stimulatory. Since fewer total amino acids were required or stimulatory for strain 18-16, it was felt that amino acid-requiring mutants would be more easily prepared from this strain than from strain 26-2. Consequently, strain 18-16 was selected for preliminary genetic studies.

When a medium consisting of the so-called required amino acids, glucose and the vitamin and mineral supplement was used as a minimal synthetic medium for S. diacetilactis 18-16, no growth of this strain occurred. Rapid growth could be obtained only when several stimulatory amino acids also were added. Thus, a minimal synthetic medium was developed for this strain; the composition of this medium is shown in Table 13.

Incubation conditions for agar plates

In most cases, comparison between colony sizes of cultures incubated under an atmosphere of carbon dioxide and those incubated under aerobic conditions were striking; colonies developing under the former condition were at least 0.5 of a mm in diameter. In addition,

TABLE 13

Composition of minimal synthetic medium for *S. diacetilactis* 18-16

Ingredient	Grams per liter
L-Alanine	2.0
DL-Histidine	4.0
L-Arginine	2.0
L-Isoleucine	2.0
L-Leucine	2.0
L-Valine	2.0
L-Glutamic acid	2.0
L-Tyrosine	2.0
DL-Cystine	4.0
DL-Aspartic acid	4.0
L-Lysine	2.0
L-Methionine	2.0
DL-Phenylalanine	4.0
L-Threonine	2.0
Sodium acetate	2.0
Glucose	10.0
	Ml of stock solution per liter
Niacin	5.0
Calcium pantothenate	5.0
Pyridoxamine	5.0
Biotin	5.0
Salts A	2.5
Salts B	2.5

pH was adjusted to 6.8

colonies developed more rapidly under a carbon dioxide atmosphere than under an atmosphere of air.

Isolation of Tryptophan-Requiring Mutants

Figure 2 shows the effect of ultraviolet light irradiation on a 5-hour culture of S. diacetilactis 18-16 when suspended in CS medium. From this curve, the irradiation time(s) corresponding to 99.99 per cent kill was determined. Although a 99.99 per cent kill of wild-type cells was obtained at all irradiation times used in this experiment, the highest exposure time was used in an effort to induce a maximum number of mutations. It was expected that if a large portion of the wild-type population were killed, the surviving population would include a number of mutants. Also, it was expected that the remaining wild-type cells would be killed off during the penicillin selection experiment; however, the concentration of penicillin used in these experiments failed to give the desired results.

Numerous attempts to obtain tryptophan-requiring mutants in this manner were unsuccessful until finally a colony growing slowly on MS medium was observed. A culture of this leaky, cross-fed, or suppressed mutant (no attempts were made to determine its actual nature) was plated out on MS and MST media before and after a

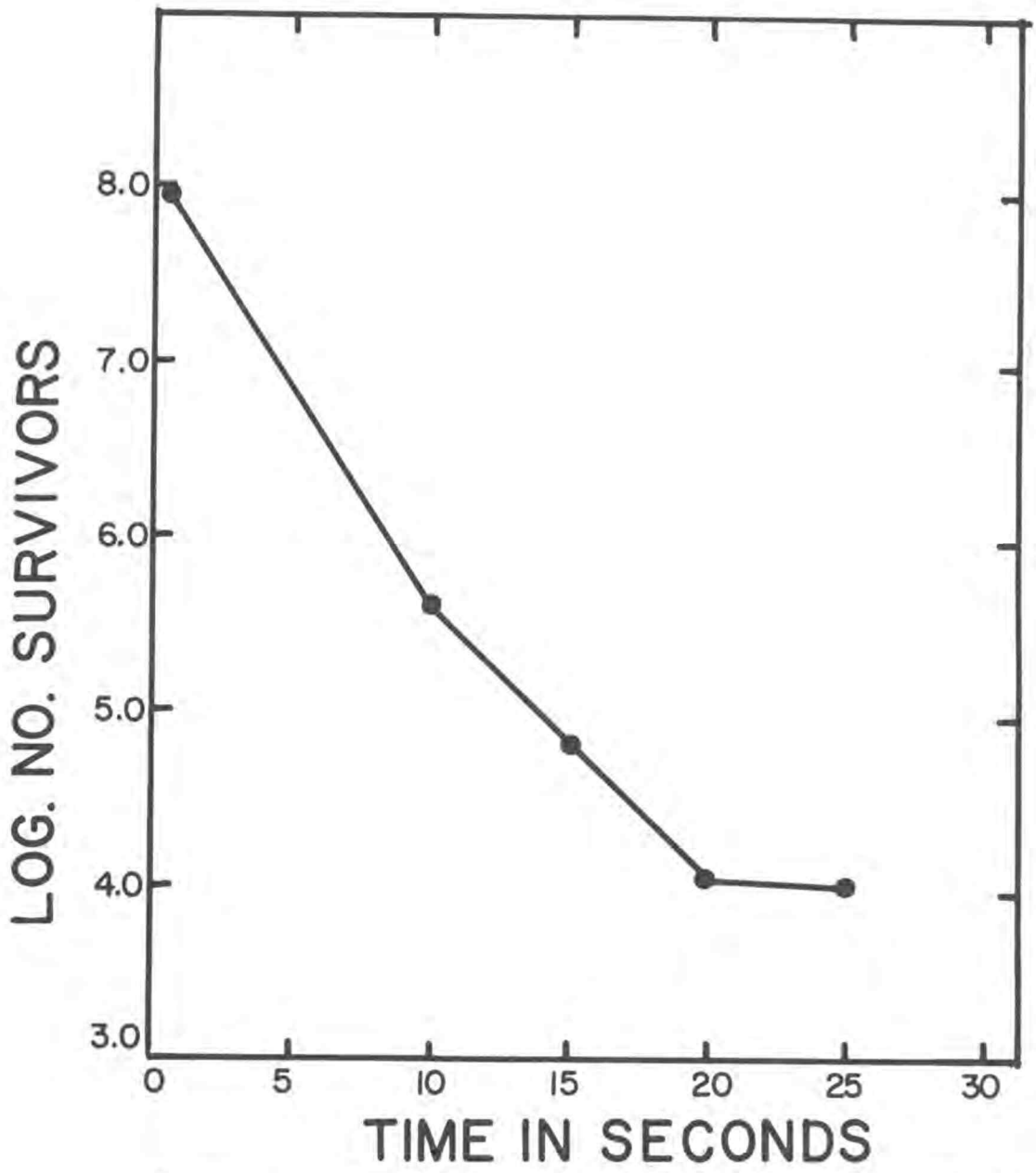

Figure 2. Effect of ultraviolet light irradiation of S. diacetilactis 18-16 for different lengths of time on the numbers of survivors.

second irradiation and the number of stable mutants determined. The results of this experiment are shown in Table 14. It may be seen that a significant increase in the number of stable auxotrophs resulted from the second irradiation treatment. When 17 of these mutants were examined for the location of their genetic blocks, it was found that all the mutants were blocked between indole and tryptophan (Figure 3).

Isolation of Streptomycin-Resistant Mutants

When strains of lactic acid streptococci were treated with increasing concentrations of streptomycin in successive experiments, mutants resistant to high levels of streptomycin (1000 μg per ml) could be obtained easily for all the strains tested. When initially exposed to such high levels of streptomycin, on the other hand, many strains failed to produce resistant colonies in a single step. In some instances it was necessary to use a 10-ml inoculum instead of 2 ml for bottles of agar containing 1000 μg per ml of streptomycin. Even when this procedure was employed, one-step resistant mutants were not obtained from all strains tested. A list of the single step mutants obtained by either procedure is shown in Table 15. Since all available strains were tested, a comparison between Tables 1 and 15 will show that single-step mutants were more easily obtained from strains of

TABLE 14

Effect of ultraviolet light irradiation on the percentage of mutant cells induced in a population of a "leaky" tryptophan-requiring mutant of S. diacetilactis 18-16

Non-irradiated mutant			Irradiated mutant		
Standard plate count x 10^7	Number of mutants x 10^7	Per cent mutants*	Standard plate count x 10^7	Number of mutants x 10^7	Per cent mutants
68	7	10.3	39	27	69.2
71	7	9.6	32	23	71.9
88	11	12.5	44	30	68.2

$$\left(\frac{\text{number of mutants per plate x } 10^7}{\text{standard plate count x } 10^7}\right) \times 100 = \text{per cent mutants}$$

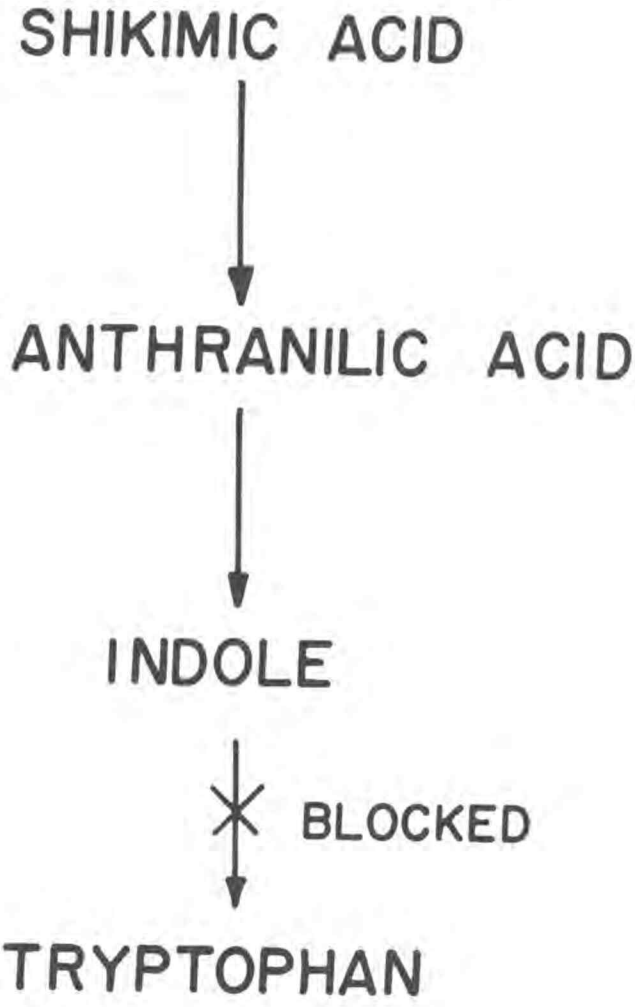

Figure 3. Location of genetic block in the tryptophan biosynthetic pathway for 17 tryptophan-requiring mutants of S. diacetilactis 18-16

TABLE 15

Single-step mutant strains of lactic acid streptococci resistant to 1000 μg per ml of streptomycin

S. lactis	S. diacetilactis	S. cremoris
E	31-2	31-1
11955a	DRC1	3D-19
C2	4R-5	11D-1
C6	18-16	
C10	11D-3	
27	CC-1	
7962	DRC3	
11454	6B-1	
	4R-1	
	6B-3	
	3D-1	
	11007	
	RM1	
	26-2	

S. diacetilactis and S. lactis than from strains of S. cremoris.

Preparation of DNA

Lysis of cells

During the early phases of this investigation, a suitable technique for lysing lactic acid streptococci was not known. The Streptomyces albus enzyme (47, p. 255-256; 67, p. 103-112) was an effective lytic agent for these organisms, but this material could not be obtained easily. During attempts to find a suitable lysing technique, it was found that when cells of S. diacetilactis were grown in a medium containing 2.0 per cent sodium citrate, nucleic acids were released without the addition of any lytic agent. The sensitization of bacterial cells by chelating agents was suggested by this finding. Subsequently, several experiments were designed to extend this observation. A system containing 0.005M EDTA, 0.005M sodium chloride and 10 mg per ml of lysozyme at pH 7.0 was tested against S. diacetilactis 18-16. The results of this experiment are shown in Figure 4. Here the lysis-accelerating effect of the EDTA, especially during the first 30 minutes, may be seen.

Before extending this system to other organisms,

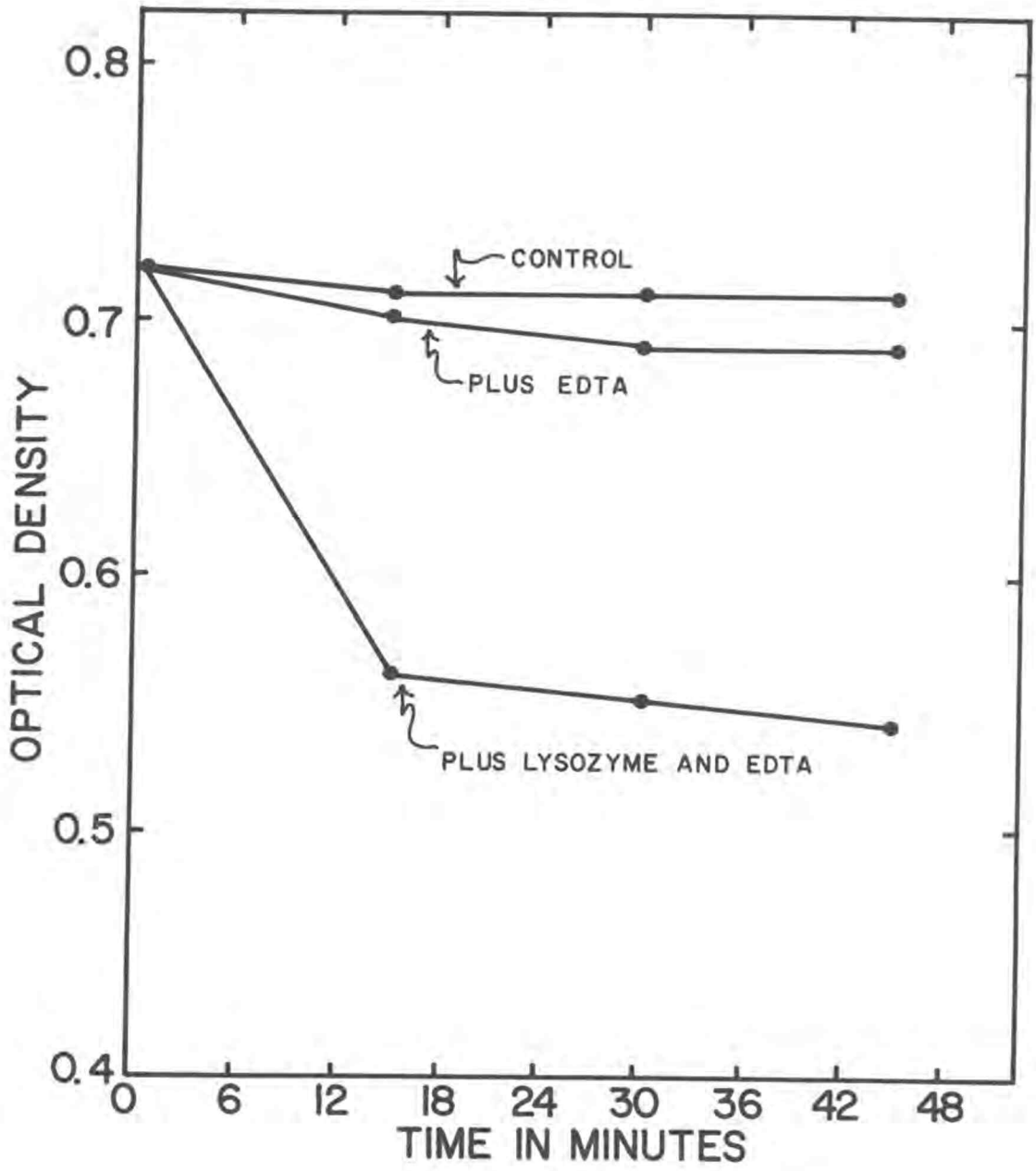

Figure 4. Lytic effect of EDTA and lysozyme plus EDTA on S. diacetilactis 18-16 cells suspended in 0.005M sodium chloride.

a series of experiments was conducted to determine the optimal conditions for lysis. Using a fixed quantity of lysozyme (1.0 mg per ml) and varying the concentrations of EDTA and sodium citrate, the optimal concentrations of chelating agents were determined. Similarly the optimal pH was determined using the optimal chelator concentration and 1.0 mg per ml of lysozyme. According to the results of these experiments, 0.005 M EDTA at pH 5.0 or 0.1 M sodium citrate at pH 8.0 provided conditions which were suitable for maximum lysis with 1 mg per ml of lysozyme. Experiments using sodium citrate were variable; therefore, further experiments using this compound were not attempted. Results obtained with EDTA, on the other hand, were reproducible and could be used in the determination of the optimal lysozyme concentration. The results of this determination are shown in Figure 5. Inspection of this figure reveals that a pronounced increase in lysis was obtained with low concentrations of lysozyme; high concentrations were inhibitory.

Initially, experiments were carried out at room temperature; however, once all other conditions were defined, an experiment was designed to determine the effect of temperature on the reaction. Thermostatically-controlled water baths at 30, 40, 50 and 60°C were used as incubators for the lysing mixtures which were held at these temperatures for 2 hours. The results of this experiment

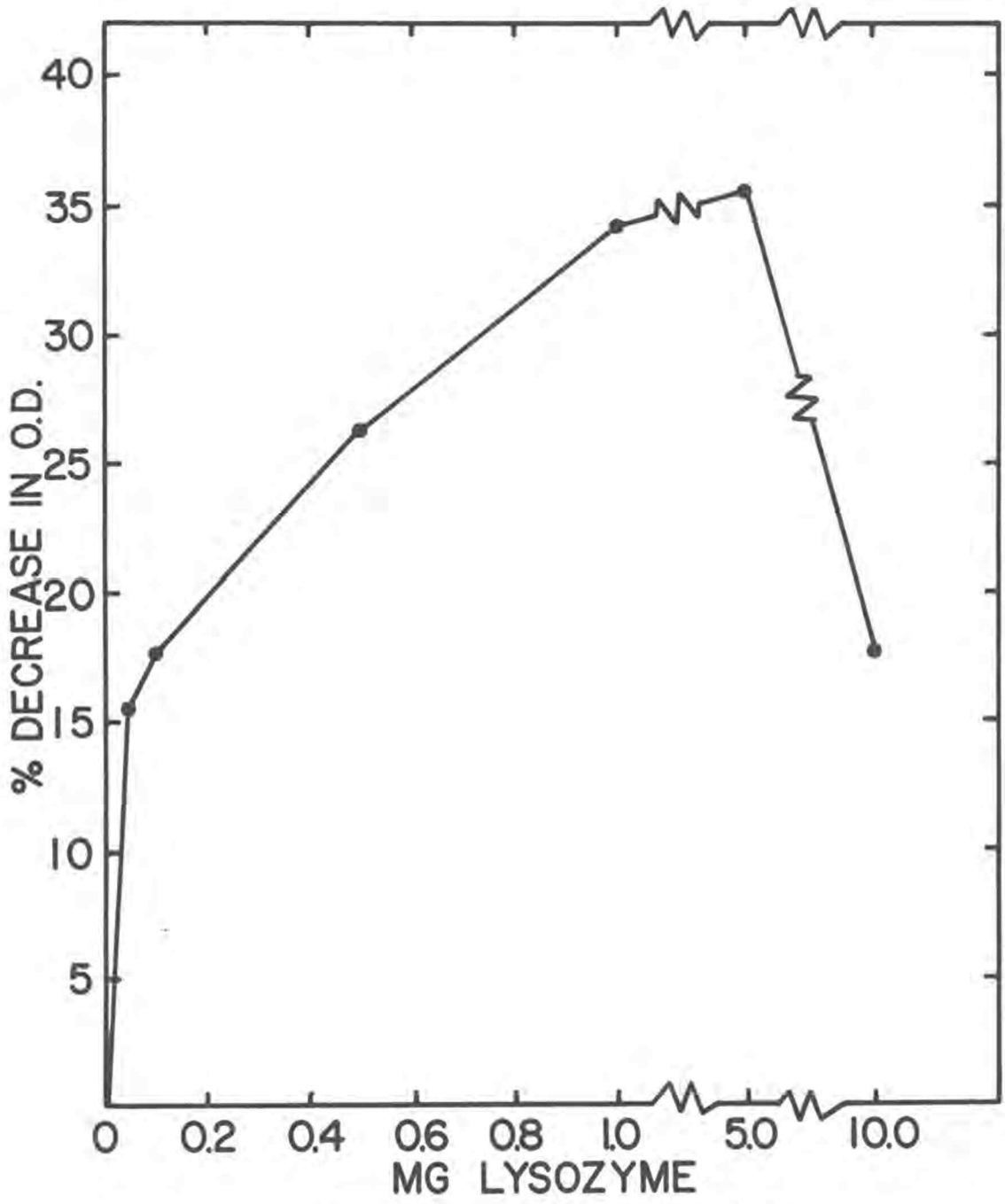

Figure 5. Effect of concentration of lysozyme in mg per ml on the lysis of S. diacetilactis 18-16 cells suspended in sodium chloride-EDTA, both at a concentration of 0.005M.

are shown in Figure 6. The apparent increase in lysis noted between 30 and 40°C may be insignificant; therefore, subsequent experiments were always carried out at room temperature. The most dramatic effect was the decrease in lysis obtained with temperatures above 40°C.

Thus, by varying conditions, a suitable lysis system was developed for S. diacetilactis 18-16 which consisted of 0.005M EDTA, 0.005M sodium chloride, and 1.0 mg per ml of lysozyme at pH 5.0. The system also was found to be effective against another lactic streptococus organism as shown in Figure 7, as well as a variety of other lactic acid bacteria (Table 16). In Table 16 it may be seen that similar degrees of lysis were obtained with S. diacetilactis, S. cremoris, and S. lactis. With other lactic acid bacteria, differences in lysis sensitivity were found. S. thermophilus and S. faecalis were highly sensitive to lysis under these conditions while L. arabinosus was highly resistant.

Following the development of this technique, it was discovered that Rapaske had reported a similar system for the lysis of gram-negative bacteria (62, p. 225-232). Attempts were made, therefore, to determine if the system developed by this investigator could be applied to the lactic acid bacteria. The procedure used in these experiments was described in the Methods section.

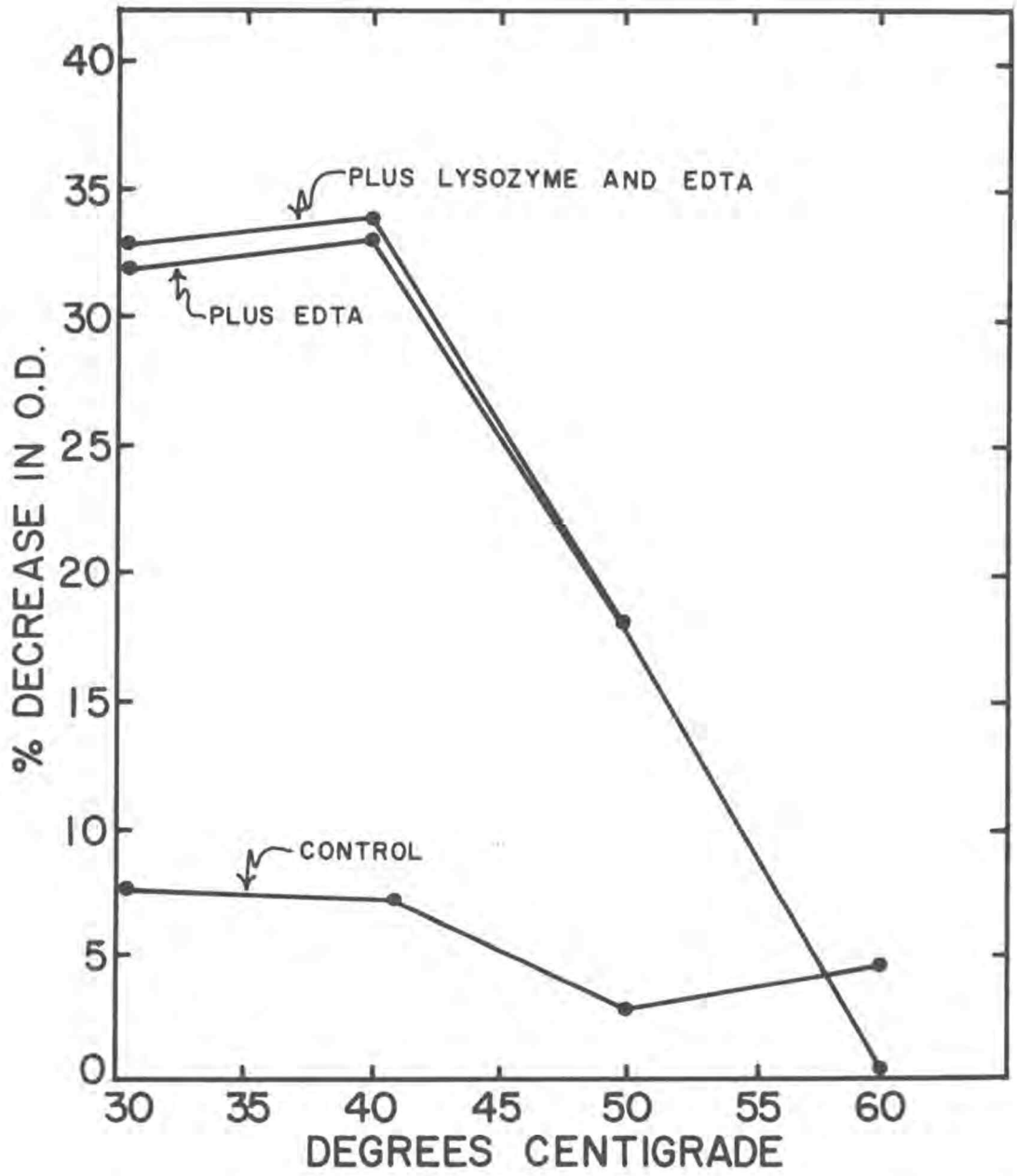

Figure 6. Effect of temperature on the degree of lysis observed when S. diacetilactis 18-16 cells were suspended in 0.005M sodium chloride (control) and two other lytic systems.

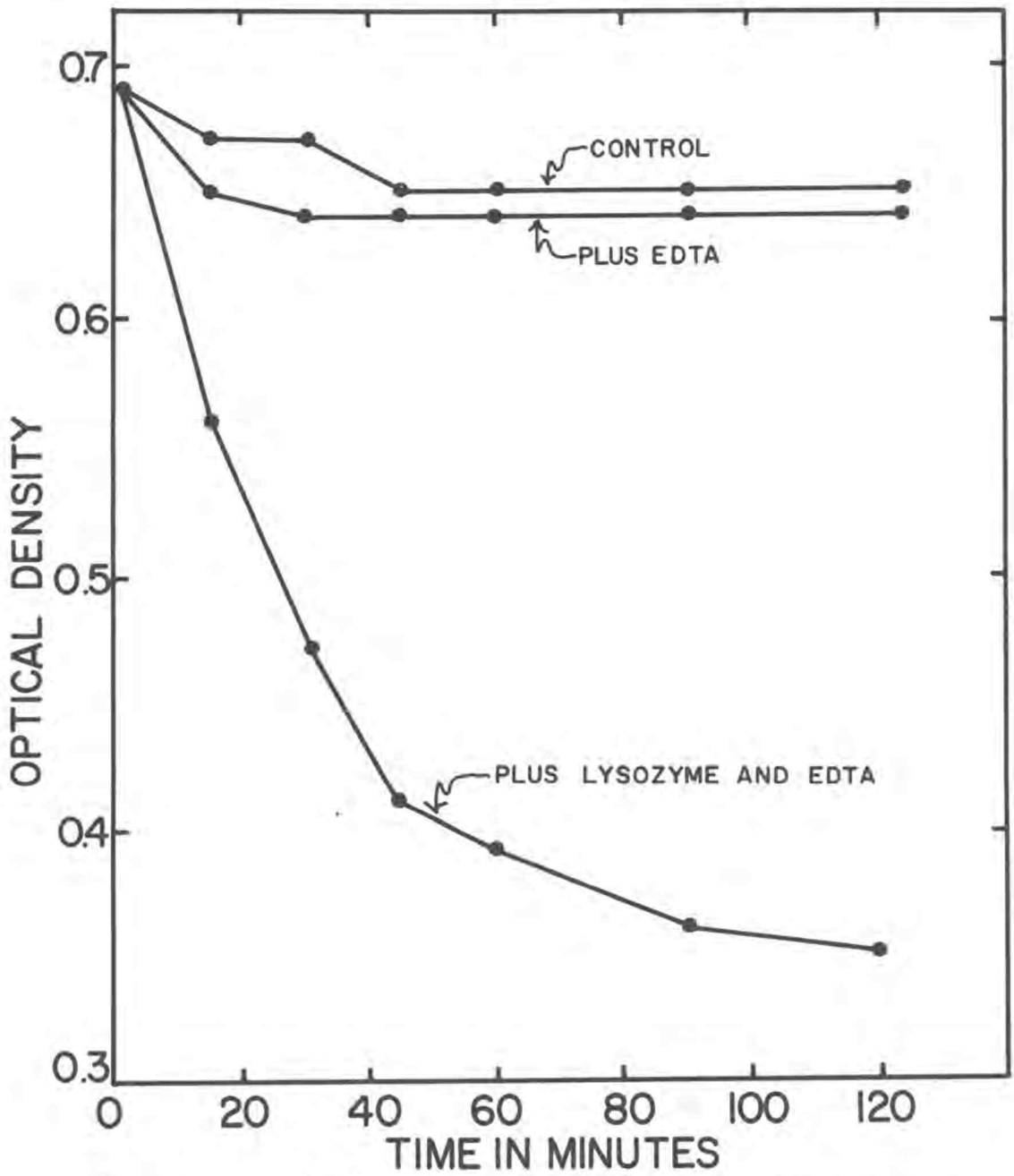

Figure 7. Lytic effect of EDTA and lysozyme plus EDTA on S. cremoris 144F cells suspended in 0.005M sodium chloride.

TABLE 16

The effect of lysozyme and EDTA on lactic acid bacteria

Organism	Per cent lysis in 2 hours[a]		
	Control[b]	Plus EDTA	Plus lysozyme and EDTA
S. diacetilactis 18-16	7.1	12.7	40.0
S. cremoris 144-F	4.5	4.2	38.0
S. lactis 27	0	0	43.5
L. arabinosus 17-5	6.0	6.1	9.4
S. faecalis	12.8	14.1	63.0
S. thermophilus MC	22.5	22.2	81.0

[a]Using the values from Figure 7, per cent lysis equals

$$((69-35)+69)\ 100 = 49 \text{ per cent}$$

[b]Control consisted of cells suspended in 0.005M sodium chloride

Results of a typical experiment using S. lactis 27 are shown in Figure 8. Application of this system to other lactic acid bacteria again revealed the wide differences in lysis sensitivity as shown in Table 17. A comparison of Tables 16 and 17 show that similar degrees of lysis of S. lactis and S. cremoris were obtained with both systems; However, an apparent lysis-inhibition by EDTA was shown for S. diacetilactis with the system of Repaske. On the other hand, the lysis of L. arabinosus, S. faecalis and S. thermophilus was greater when the system of Repaske was used. Also, the stimulatory effect of the Tris buffer is interesting to note. Where all organisms tested were resistant to lysozyme in saline and phosphate buffer they showed some degree of lysozyme sensitivity in Tris buffer. Although DNA could be obtained from the organisms tested under both systems, the method of Repaske was adopted. Concentrations of the various components of the lysing mixture were maintained in the same relative proportions but adjusted as appropriate to account for differences in mass of cells collected from small and large volumes of media.

Qualitative and quantitative analyses of DNA

An ultraviolet absorption spectrum of purified S. diacetilactis 18-16 DNA is shown in Figure 9. It

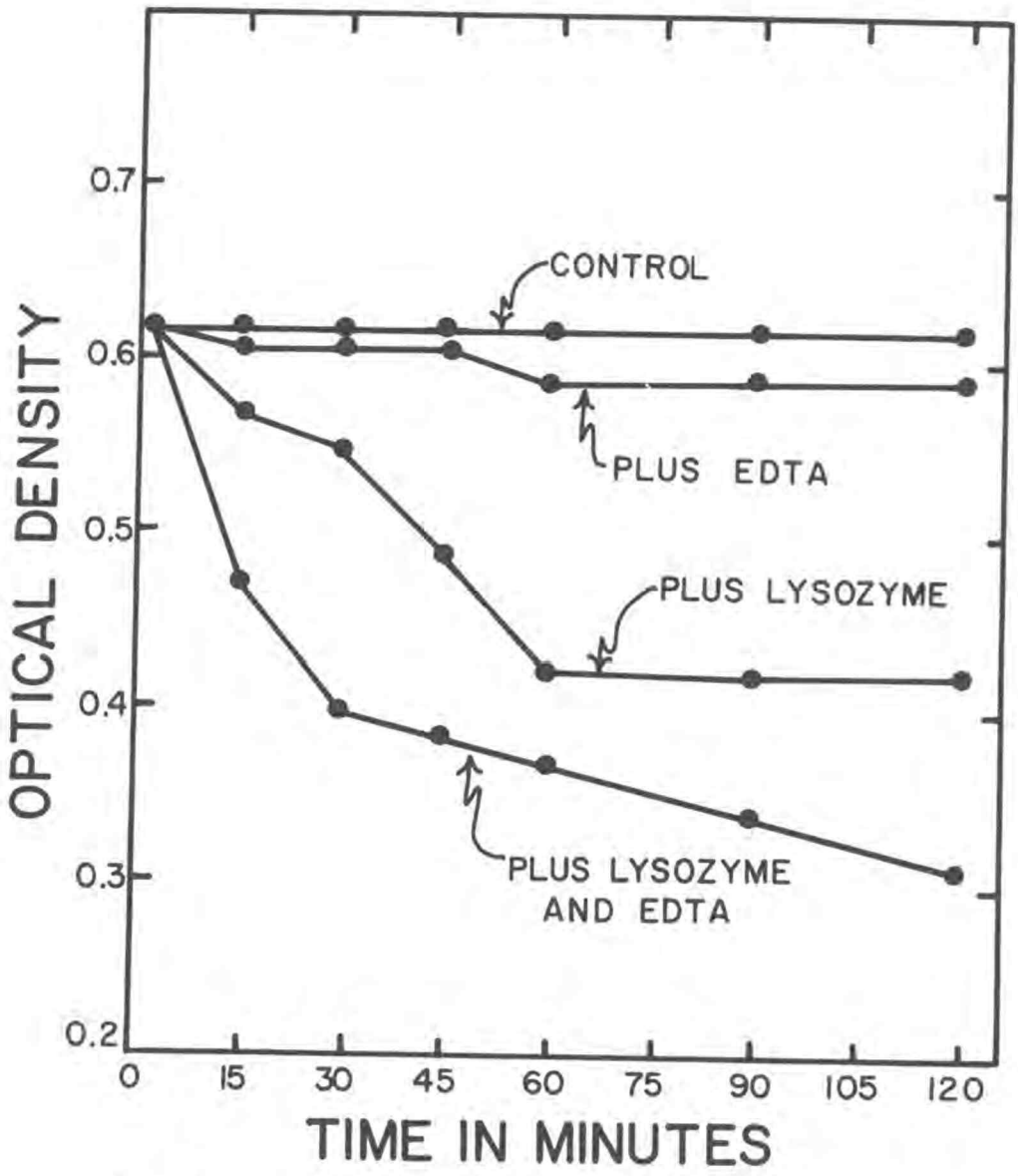

Figure 8. Comparison of rates of lysis of S. lactis 27 suspended in tris buffer (control), tris plus EDTA, tris plus lysozyme, and tris plus lysozyme and EDTA.

TABLE 17

Lysis of lactic acid bacteria by lysozyme and EDTA

Organism	Per cent lysis in 2 hours			
	Control[a]	Plus EDTA	Plus lysozyme	Plus lysozyme and EDTA
S. lactis 27	0	13	25	48
S. cremoris 144-F	4	7	0[b]	36
S. diacetilactis 18-16	7	9	46	24
S. faecalis R	7	7	75	89
S. durans OSU	5	33	40	35
S. liquefaciens OSU	12	6	33	13
S. thermophilus MC	22	37	30	50
L. acidophilus Farr	4	10	23	29
L. arabinosus 17-5	2	12	10	19
L. bulgaricus GA	19	14	17	64
L. lactis 39A	7	37	30	17
Pediococcus cerevisiae P-60	7	11	56	74

[a]Cells suspended in 0.1M Tris buffer at pH 8.0

[b]An increase in optical density was observed

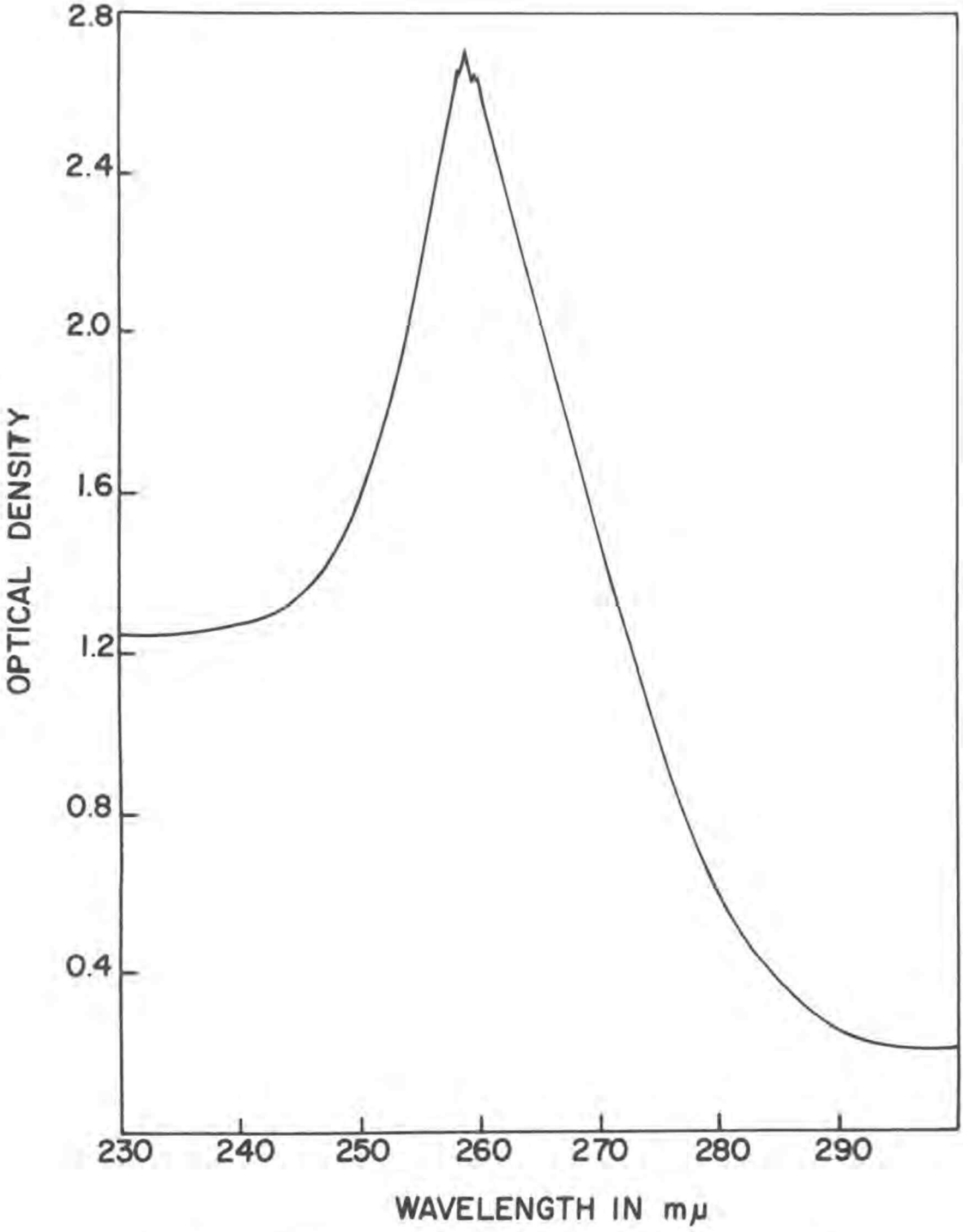

Figure 9. Absorption spectrum of purified DNA isolated from S. diacetilactis 18-16.

may be seen that this material had an absorption maximum at 259 mμ which is characteristic of nucleic acids.

Before the response curve shown in Figure 1 was prepared, the absorption spectrum of the salmon sperm DNA-diphenylamine solution was plotted from 400 to 700 mμ. Absorption maxima for this compound were found at 513 and 595 mμ as shown in Figure 10. It may be seen that the greatest absorption occurred at 513 mμ but 595 mμ was used routinely since this represented the wavelength commonly used by other investigators.

Transformation of Transformable Strains of Streptococci

The results of a typical transformation experiment obtained with the transformable streptococci are shown in Table 18. It may be seen that transformation occurred only in the presence of active DNA. Data obtained using diluted and undiluted samples of DNA were unexpected. Stock solutions usually contained from 2,000 to 2,500 μg of DNA per ml and even at a 1:10 dilution, where significant reduction in the number of transformants was observed, recipient cells were exposed to 50 μg of DNA. This greatly exceeded the amount used by Pakula et al. (56, p. 79-85) in transformation of these same organisms. It was noticed, also, that fewer transformants were obtained in these experiments than Pakula

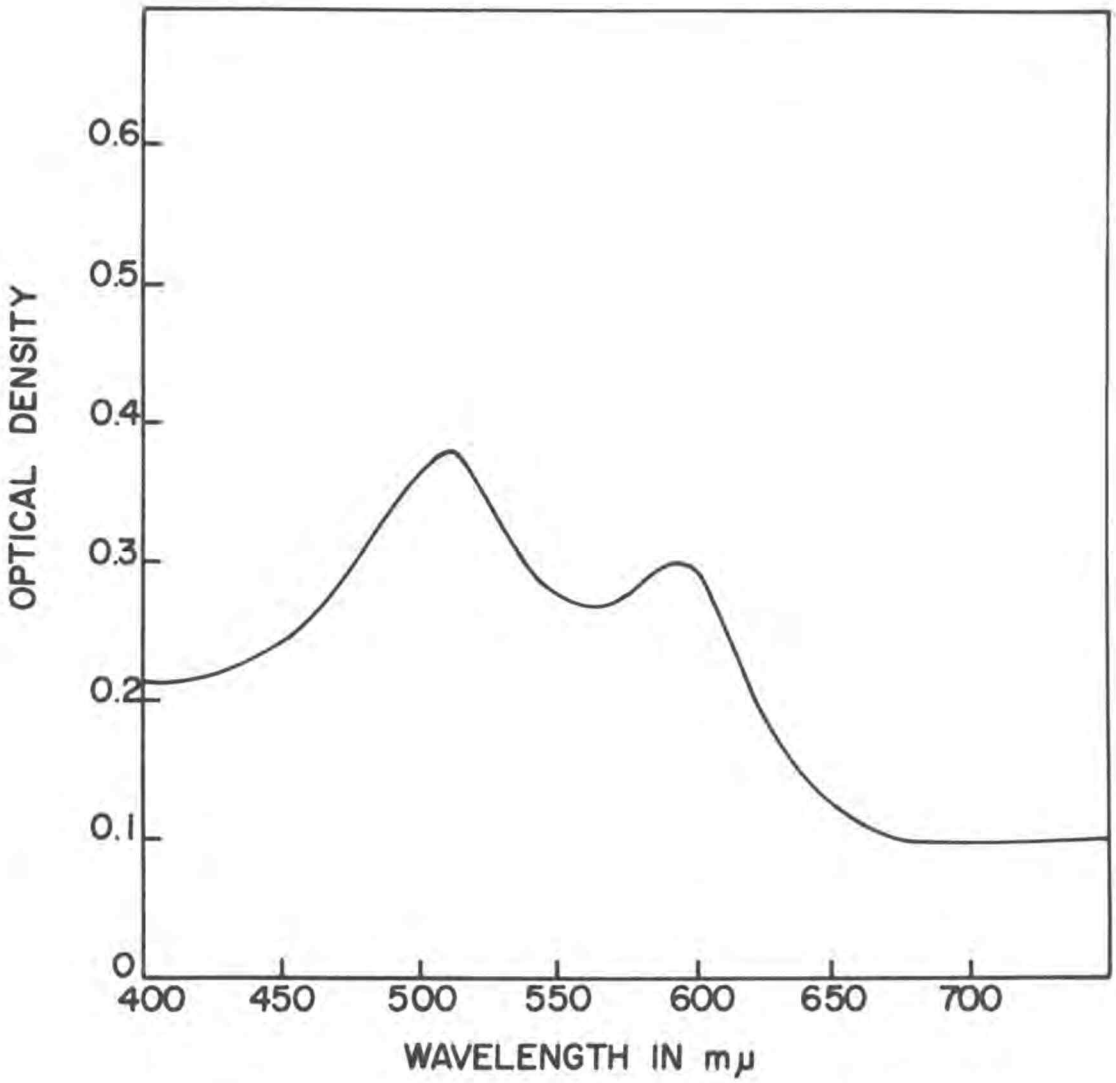

Figure 10. Adsorption spectrum of salmon sperm DNA-diphenylamine solution prepared by the method of Dische

TABLE 18

Number[a] of Streptococcus strain challis transformants resistant to 100 and 1000 μg per ml of streptomycin

Transformation carried out with	Control[b]		Plus DNA		Plus DNA Plus DNAse		Plus boiled DNA	
	100	1000	100	1000	100	1000	100	1000
Undiluted DNA	0	0	454	480	0	0	0	0
Diluted DNA[c]	0	0	48	0	0	0	0	0
Deproteinized DNA	0	0	454	480	0	0	0	0
Undeproteinized DNA	0	0	178	160	0	0	0	0

[a] The total number of cells was 3.0×10^7 per ml
[b] Control cultures contained cells to which no DNA was added
[c] Stock DNA solution diluted 1:10 to provide a concentration of 200 to 250 μg per ml

had observed.

As was expected, deproteinization of DNA caused an increase in the number of cells transformed to streptomycin resistance. Spizizen made a similar observation with B. subtilus DNA (70, p. 1072-1078).

Although good growth of the recipient strain occurred in all the media tested (Table 8), transformation of these organisms could be obtained only in BSACAB. The requirement for high concentrations of serum (5.0 per cent) was evident. No transformation occurred when serum was added at a level of only 0.5 per cent.

Growth of Lactic Acid Streptococci in Charcoal Adsorbed Broth

Most strains of S. lactis and S. diacetilactis grew rapidly in charcoal adsorbed broth. Lack of growth in this medium was found primarily among strains of S. cremoris. Since it was felt that the medium developed for Streptococcus strain challis would also facilitate the competence of lactic acid streptococci, the majority of the transformation experiments were carried out in this manner.

Transformation Studies on Lactic Acid Streptococci

Tryptophan independence

A series of media was prepared to test their effectiveness in supporting competence in lactic acid streptococci. The composition of each medium is shown in Table 19. The obvious difficulty encountered in this experiment was that known transformable strains were not available with which to test each medium. Failure to detect transformation of tryptophan independence in auxotrophs of S. diacetilactis 18-16, therefore, could not be attributed solely to the ineffectiveness of the media. When the same strain was tested with the charcoal absorbed broth used by Pakula (Table 7) still no transformants could be detected; hence, it was concluded that S. diacetilactis 18-16 was not transformable to tryptophan independence under these conditions.

Mannitol fermentation

It has been shown by Hotchkiss and Marmur (33, p. 55-60) that the ability to ferment mannitol is dependent on the presence of mannitol dehydrogenase; organisms deficient in this enzyme are unable to produce an acid reaction in mannitol broth containing brom cresol purple. Table 20 records results of an experiment to identify

TABLE 19

Composition of different media used to test lactic streptococcus organisms for transformation competence

Medium	Ingredients	Amount[a]
1	Lactic broth	100 ml
	Calcium chloride	1 mg
	Bovine serum albumin	750 mg
2	Lactic broth	100 ml
	Calcium chloride	1 mg
	EDTA	3 mg
	Bovine serum albumin	750 mg
3	Lactic broth	100 ml
	Bovine serum albumin	750 mg
4	Neopeptone	1 g
	Yeast extract	500 mg
	Glucose	100 mg
	Bovine serum albumin	1 g
5	Neopeptone	1 g
	Yeast extract	500 mg
	Glucose	100 mg
	Bovine serum albumin	1 g
	Calcium chloride	1 mg
	EDTA	3 mg
6	Neopeptone	1 g
	Yeast Extract	500 mg
	Glucose	100 mg
	Bovine serum albumin	1 g
	Calcium chloride	1 mg
7	Acid-hydrolyzed casein	30 mg
	Tryptophan	200 µg
	Glucose	200 µg
	Vitamins	500 µl
	Salts A	500 µl
	Salts B	500 µl
	EDTA	3 mg

Table 19 - continued

Medium	Ingredients	Amount[a]
8	Acid-hydrolyzed casein	20 mg
	Tryptophan	200 µg
	Glucose	500 mg
	Vitamins	500 µl
	Salts A	500 µl
	EDTA	3 mg
	Calcium chloride	1 mg

[a]The final volume of each medium was 100 ml

[b]Stock vitamin solutions described in Table 4

[c]Solutions of salts A and B described in Table 5

TABLE 20

Production of acid from mannitol and glucose in 48 hours at 30°C by strains of *S. diacetilactis*

Strain	No sugar	Glucose	Mannitol
11007	-	A	A
DRC3	-	A	A
18-16	-	A	A
4R-5	-	A	A
4R-1	-	A	A
Da-20	-	A	A
31-8	-	A	-
31-2	-	A	-
RM1	-	A	-
6B-1	-	A	-
DRC2	-	A	-
6B-3	-	A	-
26-2	-	A	-
DRC1	-	A	-
3D-1	-	A	A
CC1	-	A	A
11D-3	-	A	-

A indicates acid produced
- indicates no acid produced

mannitol-fermenting strains of S. diacetilactis. The ability to produce acid from glucose also was tested for control purposes. The enzyme was found in eight strains while an apparent absence was noted for nine. The nine strains deficient in mannitol dehydrogenase were used as recipients in transformation experiments while the eight strains containing the enzyme were used as donors. Even though all recipients were exposed to DNA from all donor strains, no transformants could be detected.

Arginine hydrolysis

A mutant strain of S. lactis 27 incapable of hydrolyzing arginine was exposed to DNA from the arginine-hydrolyzing wild type strain. Since mutants were white in contrast to pink wild type colonies when plated on Nivens' arginine agar containing TTC, it was expected that any tranformants would be detected readily among mutant colonies on this medium. Results obtained in this experiment were confusing. Although a mixture of pink and white colonies was seen on plates containing cells that had been exposed to DNA, the same results were obtained with cells that had not been exposed to DNA. Realizing that transformation could have occurred in this experiment, a large number of pink and white colonies

were picked from both plates and tested, using the procedure of Nivens et al. (52, p. 651-660), for their ability to hydrolyze arginine. The cells were subcultured twice in Nivens' arginine broth for 48 hours. The presence or absence of ammonia was detected in the cultures by the addition of one drop of culture to one drop of Nessler's reagent in a spot plate. Failure to produce a brown color with Nessler's reagent indicated a negative test. All cultures tested failed to give evidence of ammonia production, suggesting that transformation had not occurred.

Streptomycin resistance

In many instances some growth (20 to 30 colonies) of the wild-type strains which had been exposed to DNA obtained from a streptomycin resistant mutant was observed on plates containing 1000 μg per ml of streptomycin. However, in these instances there was no significant difference between the growth on experimental and control plates. In spite of the large number of strains used in these experiments, no transformation was found.

Citrate fermentation

Ten strains of S. lactis and 13 strains of S. cremoris were used as recipients of DNA from S. diacetilactis DRC3. In spite of repeated subculturing in citrate broth,

evolution of carbon dioxide by any of these strains was not detected. In addition, characteristic aroma compounds were not noticeable in these cultures.

Another finding of interest was that a number of S. cremoris strains would grow well in lactic broth but not in charcoal adsorbed lactic broth. They were 11602a, 9596, 9625, R6, 31-1, Da-5, 2R-1, Da-1, 26-1, W, CC2, and Da-2.

Cellular DNA Content of Lactic Acid Streptococci

The cellular DNA content of S. diacetilactis 18-16 and S. cremoris 144F is shown in Table 21. It may be seen that S. cremoris cells have a higher DNA content than those of S. diacetilactis. This was surprising since cells of both these organisms have about the same average diameter. Fox (22, p. 53-55) reported a value of 1.5×10^{-15} g per cell for pneumococci. Goodgal and Herriott (24, p. 590) provided data which indicated a DNA content of 3.0×10^{-15} g per cell for Hemophilus influenzae.

TABLE 21

Cellular DNA content of two lactic streptococcus organisms

Organism	Total number of cells	Grams of DNA	Grams of DNA per cell
S. diacetilactis 18-16	2.1×10^{12}	4.68×10^{-3}	2.2×10^{-15}
S. cremoris 144-F	2.7×10^{11}	2.5×10^{-3}	9.3×10^{-15}

DISCUSSION

It was found that most of the S. cremoris strains used in this investigation grew well in a medium containing vitamin-free, acid-hydrolyzed casein as the amino acid source; no growth, however, occurred when the amino acids found in casein were added individually as synthetic compounds. S. lactis and S. diacetilactis grew well in media using either of these two sources for amino acids. This suggested the existence of a non-protein growth factor in acid-hydrolyzed casein which was required by S. cremoris; strains of S. lactis and S. diacetilactis were either able to synthesize this compound or did not require it. In this regard, Demain et al. (19, p. 839-843) reported the presence of fatty acids in acid-hydrolyzed casein which were stimulatory for the growth of Sarcina species in a synthetic medium. An observation made in the present study that also may be relevant was that some of the strains of S. cremoris listed in Table 11 grew well in lactic broth but poorly or not at all in charcoal adsorbed lactic broth. This finding indicated that the growth factor required by strains of S. cremoris was heat-labile, or charcoal adsorbed.

Anderson and Elliker (6, p. 161-167) found considerable

variation in amino acid requirements for strains of S. cremoris and S. lactis. The results in Table 12 suggest a similar variation among strains of S. diacetilactis. Both strains tested required isoleucine, valine, leucine, histidine, methionine, arginine and glutamine or glutamic acid but differences were found with regard to other requirements. It was surprising that strain 18-16 would not grow in a minimal synthetic medium consisting of the so-called required amino acids, glucose and a vitamin and mineral supplement. When rapid growth of this strain occurred following the addition of several stimulatory amino acids, it appeared likely that certain combinations of amino acids were necessary for maximum growth. Reiter and Oram (61, p. 63-67) recently reported the amino acid requirements for several strains of S. diacetilactis. They found that glutamic acid, valine, methionine, histidine, leucine and isoleucine were required by all strains tested. In addition, several strains showed a requirement for phenylalanine.

Incubation under an atmosphere of carbon dioxide increased the colony sizes of lactic acid streptococci developing on agar plates. It is known that at least one strain of S. diacetilactis is capable of carbon dioxide fixation (64, p. 1-123) and this might explain the stimulating role of carbon dioxide in this organism;

however, similar experiments with S. lactis and S. cremoris apparently have not been performed.

It seemed extraordinary in the early phases of this study that S. diacetilactis 18-16 wild-type cells were not killed by penicillin concentrations from 1000 to 9,000 units per ml. While concentrations of the antibiotic below 1000 units per ml were not used in this investigation, it is now known that 20 units per ml and less are lethal for this organism; Allen (3) found that S. diacetilactis 18-16 was killed by 5, 10, 15 and 20 units per ml of penicillin but not by 100 units. Perhaps the high levels of penicillin were not effective because the synthesis of penicillinase was induced. The effective use of high concentrations of the antibiotic (2000 units per ml) in the selection of nutritional auxotrophs of E. coli is probably due to the fact that gram-negative organisms are not as sensitive to penicillin as gram-positive organisms. The attempted application of the penicillin selection technique in this study led to the use of the ineffective high levels and undoubtedly explains why tryptophan-requiring mutants were not easily obtained. Since the wild-type population was not sufficiently decreased, it was necessary to screen for mutants on plates seeded with highly diluted (10^{-4} to 10^{-5}) culture. At these dilutions, small

numbers of mutants present in the population would likely not be detected.

Whether the "leaky" mutant finally isolated arose spontaneously or was induced by ultraviolet light irradiation is not known. However, it was surprising that the stable tryptophan-requiring mutants obtained by further irradiation of this mutant were all blocked at the same point (Figure 3). This might be explained by assuming that all the mutants tested were the result of a single nutational event, followed by division and growth of the auxotroph. The possibility that these organisms synthesize tryptophan by a pathway different from that shown in Figure 2 is unlikely. It is possible, however, that additional intermediates exist between indole and tryptophan. If these intermediates could be determined, different blocks might be found among the mutants.

As noted in Table 15, single-step mutants resistant to 1000 g per ml of streptomycin were not as easily obtained with S. cremoris. This also was true when much lower concentrations of the antibiotic were used. It appears, therefore, that strains of S. cremoris have a much lower mutation rate to streptomycin resistance than strains of either S. lactis or S. diacetilactis.

Highly polymerized extracellular DNA has been found

in a variety of bacteria; cultures of Micrococcus halodenitrificans (71, p. 687-694), Pseudomonas fluorescens and Neisseria meningitidis (13, p. 510-590) accumulated slime under conditions associated with loss of viability by some cells of the population. The accumulated capsular material contained DNA which was liberated by lysis of the cells and it was found by Catlin (13, p. 570-590) to be active in transformation. Since the liberation of DNA in broth cultures of S. diacetilactis 18-16 was observed only when 2.0 per cent sodium citrate was present, it was felt that this phenomenon was associated with some property of the citrate rather than with the ordinary release of DNA due to autolysis of the cells. It was assumed that sodium citrate altered the cell walls of these organisms and stimulated lysis. Since citrate is known for its ability to chelate metals, it was felt that other chelating agents, such as EDTA, may also be effective in this regard. Furthermore, it was necessary to abandon the use of citrate because it was rapidly metabolized by S. diacetilactis. Tables 16 and 17 clearly show that EDTA was a very effective lysis-accelerating agent when used with lysozyme. However, whether or not this compound would by itself cause the release of DNA from actively growing cells in a manner similar to that of citrate was not tested.

Experiments using sodium citrate to lyse cells and obtain DNA might have been successful when applied to S. lactis and S. cremoris, since these organisms cannot use this compound. However, since this phase of the investigation was devoted to the development of a suitable lysing technique as rapidly as possible, further studies of this phenomenon were not made. Therefore, EDTA, the chelating agent which gave the best results during preliminary experiments, was used. Although the method of Repaske and the one developed in this investigation gave similar degrees of lysis, the method of Repaske finally was adopted because it had been found suitable for gram-negative organisms as well. This latter system offered an advantage in the use of Tris buffer near physiological hydrogen ion concentrations to provide a protective environment for the DNA.

The cell walls of gram-positive bacteria are mucopolysaccharide in nature, containing peptide, amino sugar and carbohydrate moieties but little or no lipid. Cell walls of gram-negative bacteria are similarly constructed but in addition are believed to be surrounded by a lipoprotein complex. It is thought that reagents such as EDTA are capable of disaggregating this surrounding complex in gram-negative bacteria (63, p. 481-490). Gram-positive bacteria, such as those shown in Tables 16 and

17 which ordinarily are resistant to lysozyme but become susceptible in the presence of chelating agents might, therefore, contain small amounts of the lipoprotein complex. On the other hand, the lysis-stimulating effect of EDTA might be explained by assuming that the chelating agent combines with metals that interfere with the active site for lysozyme action (62, p. 225-232).

An inspection of Figure 10 will show that the maximum absorption of the DNA-diphenylamine solution occurred at 413 mμ. Since it has been a common practice among other investigators to use a wavelength of 595 to 600 mμ for quantitative estimation of DNA (70, 1072-1078; 57, p. 443_449), it was assumed that the 513 mμ-absorbing material represented non-DNA impurities in this sample. Dische reported that substances with absorption maxima at 510, 530 and 560 mμ may interfere with the diphenylamine reaction (20, p. 285-305). Consequently, all readings in the present study for the quantitative determination of DNA were made at 595 mμ.

It is evident from the transformation data presented above that DNA with some degree of activity can be prepared by the method developed in this investigation. However, it must be pointed out that the relative activity of DNA prepared in this manner is questionable. If the DNA used was highly polymerized, then it seems

unlikely that such high concentrations as were necessary in these studies (500 μg per ml) would be required in transformation experiments. Pakula (56, p. 79-85) using DNA liberated by the S. albus enzyme transformed the challis and SBE streptococci using as little as 10 μg per ml of DNA. This suggests that DNA prepared by the lysozyme-EDTA method was damaged. On the other hand, the low efficiency of transformation might be explained by assuming that maximum competence in these organisms is obtained only as a result of subculturing in blood broth, prior to exposure to DNA. This technique, used by other investigators in the transformation of these strains (54, p. 319-323; 57, p. 443-449) was omitted during this investigation since it was felt that studies on maximum transformation efficiency would be more meaningful when applied later to competent strains of lactic streptococci.

It seems likely that transformation of strain challis would have occurred in media other than BSACAB that were charcoal adsorbed. The apparent need for charcoal adsorption, however, was emphasized by the observation that serum-containing media that were not so treated, although similar in composition to CABBSA, would not support transformation. This is a reasonable assumption in view of the recent finding with the pneumococcus

transforming system that serum was stimulatory but not indispensable (53, p. 471-472).

The media shown in Table 19 were developed as a result of two considerations. One consideration was based on the finding by Fox and Hotchkiss (22, p. 83-85) that the development of competence in pneumococci required (a) a medium that supported good growth of the organism (b), a serum factor, and (c) calcium ions. On the other hand, Anagnostopolous and Spizizen (5, p. 741-746), demonstrated with B. subtilus that relatively large amounts of amino acids, such as are present in acid-hydrolyzed casein, reduced the sensitivity to transformation presumably by the synthesis of cell wall components which may prevent DNA uptake; a 0.02 per cent solution of casein-hydrolyzate was optimal. They also showed that chelating compounds were effective in the development of competence. On the basis of this finding, they suggested that the role of these compounds probably was analogous to the role played by serum in other systems. Media 7 and 8 were formulated with these factors in mind. Of course, it cannot be concluded that none of the media shown in Table 19 would facilitate competence in lactic acid streptococci since only one strain was tested. This conclusion could be made only after each medium

was tested with all the strains used in this investigation.

The possibility that an exocellular DNAse was affecting the transforming ability of these strains has been obviated, at least for strains of S. lactis, by some recent unsuccessful transformation experiments in a medium containing 0.01M sodium citrate. Using this technique, unsuccessful attempts also have been made by Pakula (54, p. 319-323) to induce transformation among groups of streptococci that are known to produce DNAse.

It is possible that the cell mass or cytoplasmic membrane was acting as a barrier to the entrance of DNA into nontransformable cells. In a recent study by Perry and Slade (51, p. 493-499), it was shown that substances which affected cell permeability (trypsin-Streptomyces albus enzyme, and cetyltrimethlammonium bromide) would not effectuate the transformation of nontransformable strains. However, some of these nontransformable strains would incorporate DNA in amounts comparable to the transformable strains. Lerman and Tolmach, in some earlier studies (43, p. 68-82) showed that nontransformable strains did not incorporate significant amounts of DNA. On this basis, they suggested that the term competence might appropriately be applied to a cell's capacity to incorporate significant amounts of DNA. If future

experiments are conducted with lactic streptococcus organisms to relate the amount of DNA incorporation to their competence or incompetence, the data on the normal cellular DNA content will be useful.

It must be kept in mind that tremendous variations may exist between organisms in their response to different substances, even among those that are related. Future experiments may indicate whether or not some of the compounds that affect cell wall permeability, used unsuccessfully with other groups of streptococci, would bring about transformation in the lactic streptococcus organisms.

SUMMARY

The ability of lactic acid streptococci to grow in a complete synthetic medium was investigated. All strains of S. lactis and S. diacetilactis gave satisfactory growth in the synthetic medium while strains of S. cremoris failed to develop. These latter organisms grew, however, in a medium containing vitamin-free acid-hydrolyzed casein as the amino acid source. Later findings suggested the importance of a heat-labile substance in the growth of S. cremoris and the probably existence of this compound in acid-hydrolyzed casein was indicated.

The amino acid requirements of two strains of S. diacetilactis were determined. The results indicated that variations existed among these organisms where their amino acid requirements were concerned. Determination of the amino acid requirements allowed the development of a minimal synthetic medium for S. diacetilactis 18-16.

Incubation under an atmosphere of carbon dioxide proved to be a useful technique for obtaining large colonies on the surface of agar plates. This facilitated the replica plating procedure subsequently used in this investigation.

During these studies, a number of mutants were isolated. Tryptophan-requiring mutants of S. diacetilactis 18-16 were obtained by ultraviolet light irradiation of wild-type cells. Seventeen of these mutants were identical with respect to their blocks in the tryptophan biosynthetic pathway. Streptomycin-resistant mutants of several lactic streptococcus organisms were obtained by selection on agar plates containing the appropriate concentrations of the antibiotic. The numbers of mutants obtained suggested that strains of S. cremoris generally were more susceptible to streptomycin than strains of S. lactis or S. diacetilactis.

A method employing EDTA, lysozyme, and a weak sodium chloride solution at pH 5.0 was developed for the lysis of a number of lysozyme-resistant gram-positive organisms. Another method previously reported for the lysis of gram-negative bacteria also was found to be effective when applied to the organisms used in this investigation. This method employed EDTA, lysozyme, and Tris buffer at pH 8.0. The decision to use the latter method for the routine preparation of DNA was based primarily on the assumption that Tris buffer at pH 8.0 would offer more protection for the DNA during the isolation procedure. The relative activity of the DNA prepared according to this procedure was tested using transformable strains

of streptococci. The results indicated that either the DNA prepared by this method was damaged or that a relatively small number of recipient cells were competent.

Experiments with the transformable strains showed also that a charcoal adsorbed medium containing high concentrations of bovine serum albumin was necessary for transformation. Moreover, these strains could be transformed with either deproteinized or undeproteinized DNA, but more transformants were obtained with deproteinized samples.

In spite of the wide variety of characters and transformation procedures employed, it was not possible to demonstrate transformation with the lactic acid streptococci. After it was shown that strains of S. lactis were not transformed even in the presence of 0.01 M sodium citrate, it appeared unlikely that the presence of an exocellular DNAse was inhibiting transformation.

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