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STUDIES ON LACTOBACILLI

by

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CHAPTER I

ORIGIN, AIMS AND HISTORICAL BACKGROUND TO THESE STUDIES

INTRODUCTION

The incidence of dental caries in South Africa is high (Ockerse, 1947) and in 1955 the Minister of Health and Nutrition instructed his departments to appoint a committee to investigate methods by which dental health in this country could be improved. In 1957 a report submitted by this committee emphasized, among other recommendations, the need for fundamental research into dental health. The report was accepted by the Minister and implementation of its proposals was begun in the same year by the establishment of the Nutrition and Dental Health Research Group of the South African Council for Scientific and Industrial Research under the honorary directorship of Professor C. L. de Jager, Head of the Department of Oral and Dental Pathology, and Dean of the Faculty of Dentistry of the University of Pretoria. The Group undertook the investigation of dental caries in two areas of South Africa in which the caries incidence rates differ markedly. The incidence in the Langkloof district is 99% and in the Nuwerus area only about 14% (de Jager, 1963). Details of the investigations that were to be carried out in the two areas have been published (de Jager, 1963). They included clinical and radiological dental surveys, a dietary survey, water and soil analyses and bacteriological studies of saliva and plaque material. Responsibility for the latter investigations fell on Professor J. N. Coetzee, Head of the Department of Microbiology of the Faculty of Medicine of the University of Pretoria. Professor Coetzee visited several laboratories overseas towards the end of 1957 to make a study of lactobacilli and other oral bacteria. These included the National Institute for Research in Dairying, Reading, England; the Department of Bacteriology, University of Birmingham; the Nederlands Instituut voor Zuivelonderzoek, at Ede; the Laboratory of Hygiene, University of Utrecht, Holland, and the State Dental School, Malmö, Sweden. Some months after his return I joined Professor Coetzee's department and he offered me the task of the isolation

and characterization of the lactobacilli from saliva and plaque material from Langkloof and Nuwerus. After some months of preparation and preliminary experimentation with media and the homogeneous dispersal of organisms in samples of plaque and saliva by sonic oscillation, work began in earnest early in 1959 and continued until the beginning of 1961. The great distances between the sources of the samples and the laboratory made the use of air transport essential, and the arrival of specimens in the evenings meant that the tedious initial treatment and plating of the samples took place throughout the night. This study is described in Chapter 3.

Compared with the classical thesis a single goal had not been set at the beginning, but the initial study had provided an extensive collection of lactobacilli and there were many aspects of these organisms which could be investigated. At this stage we had already observed morphological variants in some of the strains. Professor Coetzee was interested in bacteriophages and bacteriocins of the genus Proteus and I decided to investigate these elements among lactobacilli. It was in this way that these studies evolved.

HISTORICAL BACKGROUND

Examination of the fermented milk kefir, which contains lactic acid bacteria, yeasts and spore-forming organisms (Pederson, 1952), led to the discovery of the lactobacilli. In 1882 Kern observed a granular bacillus in this product which was probably a lactobacillus. The organism which he isolated and called Dispora caucasica was, however, a spore-bearer. Beijerinck (1889) later observed and isolated a non-spore-forming homofermentative organism from Kefir. He named it Bacillus caucasicus and this organism was designated as the type species of the genus Lactobacillus (Winslow et al. 1920). These observations were followed by reports of similar organisms in other milk products (see Briggs & Briggs, 1954). In 1892 Döderlein demonstrated Gram-positive bacilli in the vaginal secretions of pregnant women and suggested that the lactic acid produced by these organisms limited the growth of other bacteria in the vagina. Boas and Oppler (see Briggs & Briggs, 1954) observed similar Gram-positive organisms in stomach carcinomata. The advent of improved media, especially milk media with low pH values and added sugars facilitated the culture of lactic acid bacteria from various sources. Tissier

in 1900 described Bacillus bifidus from the faeces of breast-fed infants, and in the same year Moro (1900) isolated Bacillus acidophilus from the same source. The latter organism was found to occur widely in the faeces of many animal and bird species (Mereshkowsky, 1905). In their extensive review of the lactobacilli Briggs & Briggs (1954) have summarized the records of the isolation and early investigation of many Lactobacillus spp. They point out the absence of any logical sequence in these studies in that they consisted of many separate investigations more pre-occupied with names and sources of species than with stable and characteristic group properties on which their taxonomy could be based. These authors quote numerous examples where the identity of variously named species was established by later investigations. Early attempts to divide lactic acid bacteria into homogeneous groups on the basis of colonial and microscopic morphology, type of lactic acid formed, fermentation reactions and serological tests, were largely unsuccessful. There were many reports of the variability of lactobacilli particularly in fermentation reactions, (Harrison, 1939; Coolidge, Williams, Ebisch & Hodges, 1949). These tests were found to vary not only with different workers but also with the same individual at different times.

Similar difficulties were encountered with the identification and classification of oral lactobacilli and Rosebury (1944) summarized the contradictory evidence in the early literature on this group. The lack of proper classification led to the use of the term Lactobacillus acidophilus by many workers for any Gram-positive, non-motile, non-spore-forming anaerobic rod which occurred in the mouth. This attitude has been attributed (Rogosa et al. 1953) largely to the work of Hadley, Bunting & Delves (1930) and Hadley & Bunting (1932). These authors described 4 colonial types of lactobacilli and because they could easily dissociate smooth types from rough ones they maintained that the oral lactobacilli were simply colonial variants of L. acidophilus. It was also long held that the oral lactobacilli were a distinct group uniquely indigenous to the oral environment. Overshadowing the many early attempts to characterize individual Lactobacillus spp. and to differentiate between small groups of these organisms, the classic works of Orla-Jensen (1919, 1943) were devoted to the taxonomy of the entire group of lactic acid bacteria. He divided catalase negative rods of this group, which showed little surface growth and did not reduce nitrates into 3 groups, namely Thermobacterium

(Orla-Jensen, 1919) the members of which are homofermentative with relatively high optimum growth temperatures; Streptobacterium (Orla-Jensen, 1919) whose members are also homofermentative but with generally lower maximum growth temperatures; and Betabacterium (Orla-Jensen, 1919) with heterofermentative species. Despite the disadvantage that many intermediate and unclassifiable organisms resulted from the use of this classification it remains an outstanding contribution to the taxonomy of the genus Lactobacillus. In the 6th edition of Bergey's Manual (1948) the key to the species of the genus Lactobacillus used the terminology of Orla-Jensen (1919) and divided the genus in the same way. This nomenclature was questioned by Pederson (1952) on the grounds that it was illegitimate and Orla-Jensen's terminology was abandoned in the 7th edition of Bergey's Manual (1957). The subdivisions set forth by Orla-Jensen were however maintained.

The work of Tittsler, Gieb & Rogosa (1947) who evolved a simple key to strains of the genus Lactobacillus based on the correlation of various characteristics of 250 strains which they examined was applied to the classification of lactobacilli from the mouth by Rogosa et al. (1953). The latter authors described 2 new species among 500 strains examined and showed that oral Lactobacillus spp. are identical to organisms previously found in a wide variety of fermenting materials in Nature. They stressed the reproducibility of their tests and found gross variation of individual strains to be rare even when these were repeatedly tested. They discounted the earlier reports of variability on the grounds of poor technique and the use of nutritionally inadequate media. Rogosa, Tittsler & Gieb (1947) found a high correlation between vitamin requirements and cultural and biochemical characteristics of species of the genus Lactobacillus. These findings were confirmed by Rogosa et al. (1953) and extended in the work of Rogosa, Franklik & Perry (1961) who developed an improved medium for nutritional tests. Substantial contributions to the characterization and taxonomy of the lactobacilli have been made by the physiological studies of Briggs (1953), Davis (1955), Wheater (1955 a,b), de Man (1956, 1960) and Sharpe & Wheater (1957) and the serological classification of these organisms by Sharpe (1955). Rogosa & Sharpe (1959) presented an approach to the classification of the lactobacilli in which the characteristics of all the known species of this genus were defined in terms of the most recent knowledge and in which the terminology of Orla-

Jensen (1919) was accepted.

This brief outline of the history of the lactobacilli has been confined principally to taxonomic aspects of these organisms as it was against this background that the characterization of lactobacillus strains was undertaken in the first study (see Chapter 3). Such characterization was essential to the subsequent investigations. References to the literature concerning other aspects of these organisms are made in the appropriate chapters.

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CHAPTER 2

LACTOBACILLUS CULTURE MEDIA

SUMMARY

The stringent growth requirements of the lactobacilli necessitated the use of complex culture media for their study. The primary needs were for an adequately selective medium for the isolation of lactobacilli from a habitat which abounds with other bacteria and a general culture medium which would support good growth of all the Lactobacillus spp. isolated. In addition special media were required for the physiological tests used to identify the strains and a suitable storage medium was needed for their preservation. In this chapter the history of the development of selective and general culture media for lactobacilli is reviewed and the composition and methods of preparation of the media used in these studies is described. The possible functions of some of the constitutuents of these media are discussed.

INTRODUCTION

Different micro-organisms vary greatly in their nutritional demands and thus in the complexity of the culture media which fulfil their requirements for good growth. In this respect the lactobacilli have proved remarkably fastidious (Snell, 1952). In addition to utilizable sources of energy, nitrogen and carbon and various inorganic ions, lactobacilli require a variety of growth factors which include various vitamins, amino acids, purines, pyrimidines and even peptides (Snell, 1952; Rogosa, Franklin & Perry, 1961). All these requirements must be provided in an artificial culture medium which must also satisfy conditions of concentration and physical environment such as temperature, pH value and carbon dioxide tension, to ensure satisfactory growth of these organisms. An external supply of carbon dioxide stimulates, or in media with restricted amino acids is essential for, growth of several Lactobacillus spp. (see Snell, 1952). Since their discovery attempts have been made to grow lactobacilli on a wide variety of media and the lack of a

suitable general culture medium was until recently a serious handicap in the study of these organisms. The history of the development of culture media for lactobacilli and the media used in these studies are described in this chapter.

DEVELOPMENT OF LACTOBACILLUS CULTURE MEDIA

Non-selective media

The heterotrophic nature of lactobacilli was recognised by Orla-Jensen (1919, 1943). In his classic works on the lactic acid bacteria he stated that they required complex nitrogenous food and he used a medium which contained peptonized casein, yeast extract and phosphates and sulphates of potassium and magnesium. On this medium and also in milk he observed that different strains varied in their ability to grow. Other early workers also used milk or media derived from milk such as whey broth and agar. Among the strains studied on these media were Lactobacillus bulgaricus (Bertrand & Duchacek, 1909; Heineman & Hefferan, 1909), the Boas-Oppler bacillus (Galt & Iles, 1914-1915; Heinemann & Ecker, 1916) and Döderlein's bacillus (Thomas, 1928). Various media were tested by Bertrand & Duchacek (1909). They included a decoction of barley with peptone and calcium carbonate; malt extract plus the latter 2 ingredients and media prepared from onions, lentils, beans and urine. None of these supported growth of L. bulgaricus as well as milk. Whey broth and agar were time consuming and difficult to prepare and Rettger & Kulp (1922) sought a substitute for these media in a galactose agar which they reported to be satisfactory. The most important contribution towards a good general medium for lactobacilli was the introduction of tomato juice agar for the culture of L. acidophilus and L. bulgaricus (Kulp, 1927). The medium consisted of 20 - 40% (v/v) of filtered canned tomatoes 1% (w/v) Bacto-peptone (Difco) and agar 1.1% (w/v). The juice contained sufficient sugar (2-4%, w/v) to satisfy the requirements of these organisms. The success of this medium was ascribed to the possible presence of an accessory growth factor or factors in the tomato. The medium was improved (Kulp & White, 1932) by the addition of 1% (w/v) peptonized milk (Difco) and reduction

of its pH value from 7.2 to 6.0 and later (Valley & Herter, 1935) by the substitution of Neo-peptone (Difco) for the Bacto-peptone (Difco). These modifications resulted in increased colony size and higher plate counts of L. acidophilus. The evolution of this medium was continued by Hadley (1933) who reduced its pH value to 5.0 and raised the agar concentration to 2% (w/v) and by Weiss & Rettger (1934) who added 0.5% (w/v) yeast extract (Difco). These variations of Kulp's original formula and a glucose meat infusion broth (Jay & Voorhees, 1927) became the most commonly used media for the isolation of oral lactobacilli (Rosebury, 1944). Various other types of media for lactobacilli are to be found in the literature. They include casein digest media (Hunter, 1924; Kulp & Rettger, 1924; Orla-Jensen, 1943), some of which (Roberts & Snell, 1946; Henderson & Snell, 1948) were described by workers primarily concerned with the biological assay of vitamins and amino acids. To these may be added liver tryptone broth (Nymon & Gortner, 1946) and the rennet digest medium of Hunter (1950). Most of these media and the citrated milk of Browne & Howe (1922) were found inadequate for certain strains of lactobacilli, notably L. bulgaricus and L. acidophilus (Briggs, 1953a). The work of Briggs (1953a) and Cox & Briggs (1954) provided a medium which permitted consistently good growth of all known strains of lactobacilli. To accomplish this they measured the growth of 19 recalcitrant lactobacillus strains in a basal medium of tomato glucose broth, similar to that of Valley & Hurter (1935), to which varying concentrations and combinations of Tween 80 (polyoxyethylene sorbitan monooleate), sodium-oleate, soluble starch, albumin, and Yeastrel had been added. The results were subjected to statistical analysis and the best growth was achieved by the addition of Tween 80, 0.1% (v/v), Yeastrel, 0.6% (w/v) and starch, 0.05% (w/v). The addition of agar provided a good solid medium. These workers were prompted to test the effects of surface-tension depressants and fatty acids on the growth of lactobacilli by their earlier observation that low concentrations of bile salt improved the growth of some strains of L. acidophilus and the findings of other workers (see Briggs, 1953a) of a stimulatory effect on lactobacilli of sodium oleate, unsaturated fatty acids, non-ionic detergents and various Tween compounds. Although all the substances added to the basal medium caused increased growth, Briggs (1953a) found Tween 80 to be particularly important. It had

a greater stimulatory effect than Tween 20, 85 or sodium oleate. Its mode of action was uncertain but since sodium oleate had a similar effect she suggested that oleate was metabolized by these organisms (Hunter, 1924; Dubos, 1947). The wetting action of Tween 80 may affect the cell surface and facilitate the absorption of certain nutrients (Rogosa & Mitchell, 1950a). Another variation of tomato juice broth was successfully used by Davis, Bisset & Hale (1955). In 1960 de Man, Rogosa & Sharpe used a number of lactobacillus strains which grew poorly in Brigg's Medium to devise a more generally applicable non-selective medium for lactobacilli. They sought also to eliminate tomato juice because it was variable and inconvenient to prepare. The final composition of the medium (MRS) which answered these requirements was Oxoid peptone, 1% (w/v); Lab-Lemco (Oxoid), 1% (w/v); yeast extract (Difco or Oxoid), 0.5% (w/v); glucose, 2% (w/v); Tween 80, 0.1% (v/v); K_2HPO_4, 0.2% (w/v); $CH_3COONa.3H_2O$, 0.5% (w/v); triammonium citrate, 0.2% (w/v); $MgSO_4.7H_2O$, 0.02% (w/v); $MnSO_4.4H_2O$, 0.005% (w/v). The medium was sterilized for 15 min. at 120^{o} and the pH value was between 6.0 and 6.5. This work supported earlier findings that the addition of Tween 80 (Briggs, 1953a), citrate (Evans & Niven, 1951), acetate (Snell, Tatum & Peterson, 1937; Guirard, Snell & Williams, 1946) and Manganese (MacLeod & Snell, 1947; Evans & Niven, 1951) to lactobacillus media resulted in improved growth. The stimulatory effect of sodium acetate has been attributed (Guirard et al. 1946) to its buffer action against lactic acid production and to an unknown function concerned with the stimulation of early luxuriant growth of lactobacilli. It was suggested by these authors that acetate probably has a role in the synthesis of lipoid substances by lactic acid bacteria.

Selective media

The normal habitats of lactobacilli, especially dairy products and the intestinal tracts of man and various animals, abound with other bacteria and a selective medium for the primary isolation and for quantitative studies of lactobacilli is paramount. This need was felt particularly by workers engaged in the study of these organisms in relation to dental caries. Hadley (1933) attempted to overcome the problem by the modification of tomato juice agar (Kulp, 1927) already described. Although she increased its selectivity for

lactobacilli, yeasts, streptococci, staphylococci and Micrococcus tetragenus still grew on this medium. They could however be differentiated by their colonial morphology. The relative selectivity of low pH media for lactobacilli depended entirely on the aciduric qualities of these organisms until sodium azide was added by Diamond (1950). Concentrations of 0.01% (w/v) inhibited the growth of most strains of micrococci, staphylococci, aerobacter and proteus tested. This medium was used in dental caries studies by Grubb & Krasse (1954) and Krasse (1954), but it was criticized by Rogosa, Mitchell & Wiseman (1951b) on the grounds that they and other workers (Lichstein & Soule, 1944; Rogosa & Mitchell, 1950b) had shown an inhibitory effect of sodium azide on many lactobacilli. Krasse (1954) cited Rogosa in a personal communication that it was chiefly heterofermentative lactobacilli that were inhibited by this agent, but found that 2 heterofermentative strains of lactobacilli grew well on this medium. Krasse & Moller (1951) found no decrease in lactobacillus counts on tomato azide agar. In 1955 Sharpe studied the effect of thallous acetate 0.1% (w/v) added to tomato glucose agar (Briggs, 1953a). Although many of the species other than lactobacilli tested were almost totally inhibited the growth of streptococci was unsuppressed and staphylococci, a micrococcus, Bacillus licheniformis and Proteus miribilis were only partially inhibited. The isolation of lactobacilli from natural habitats with a mixed flora became possible on a number of highly selective media which allowed quantitative studies of lactobacilli from such sources as grass and silage (Keddie, 1951), oral and faecal material (Rogosa et al. 1951a, b) and dairy products (Mabbit & Zielinska, 1956). These media were designed by control of the concentrations of phosphate, citrate, salts, Tween 80 and hydrogen ions and by the incorporation of a suitable amount of sodium acetate into a nutritionally adequate basal medium. In a series of cultures of 122 human salivas and 156 samples from hamsters on their SL agar Rogosa et al. (1951b) found yeasts as the only extraneous organisms and these occurred on 3.3% or less of the plates. The selection of lactobacilli from Cheddar cheese (Mabbit & Zielinska, 1956) occasionally also yielded catalase positive micrococci. All other cheese organisms including the streptococcus starter were inhibited by their medium.

MEDIA USED IN THESE STUDIES

The selective medium of Rogosa et al. (1951a, b) as modified by Mabbit & Zielinska (1956) was used. It was called acetate agar (AA) and was prepared as follows.

Nutrient solution. Six ml. of a salt solution containing $MgSO_4.7H_2O$, 11.5g.; $MnSO_4.4H_2O$, 2.81g.; $FeSO_4.7H_2O$, 0.68g. and distilled water, 100 ml. was added to yeast extract (Difco), 6.0g.; di-ammonium hydrogen citrate, 2.4g.; potassium dihydrogen phosphate, 7.2g.; d-glucose, 24.0g.; Tween 80, 1.2g. dissolved in distilled water, 100 ml. The ingredients were heated gently until dissolved. Then 60 ml. of a 4M-sodium acetate acetic acid buffer, pH 5.37, were added and the volume made up to 200 ml. with distilled water, the final pH being 5.0. This solution could be stored under refrigeration for long periods without prior sterilization because of its high acetate concentration and low pH value. The nutrient solution was added, as required, to the tryptic digest of milk which was prepared as follows.

Milk digest. One litre of separated raw milk (adjusted to pH 8.5); trypsin (British Drug Houses), 5g. and chloroform, 10 ml. were mixed and incubated at 37^{o} for 24 hr, steamed for 20 min, filtered hot and the pH adjusted to 6.65 with glacial acetic acid.

Complete medium. To 700 ml. of the tryptic digest 19g. of agar was added and dissolved by autoclaving at 121^{o} for 20 min. While hot this was mixed with 185 ml. of the nutrient solution previously warmed to 50^{o}. The volume was then made up to 1 litre with hot digest and the medium was kept at room temperature in sterile 8 oz. screw-cap bottles. When preparing plates the medium was melted without excessive heat to prevent the formation of a precipitate and darkening of the medium. The final pH was 5.3.

General culture medium

At the outset of these investigations extensive use was made of a tomato glucose medium in broth and agar forms (TGB, TGA) based on the media of Briggs (1953a, b) and Rogosa et al. (1951a, b). It consisted of Bacto peptone (Difco), 1.5% (w/v); Yeastrel (Brewers' Food Supply Company Ltd, Edinburgh), 0.3% (w/v); Tween 80, (Atlas Powder Company, Wilmington,

Delaware), 0.1% (v/v); salt solution (as for AA), 0.5% (v/v); sodium acetate, 0.5% (w/v); potassium di-hydrogen orthophosphate, 0.5% (w/v); di-ammonium hydrogen citrate, 0.2% (w/v); glucose, 2% (w/v) and tomato juice 10% (v/v). The medium was steamed for 30 min. adjusted to pH 6.1 and sterilized at 121° for 20 min. Concentrated tomato juice (Briggs, 1953b) was prepared by adding a litre of distilled water to 12 lb. of minced tomato which was then steamed for 1 hr. The juice was filtered, adjusted to pH 7.0 and autoclaved at 120° for 15 min. The solid medium was prepared by the addition of 2% (w/v) agar (Difco).

All the strains of lactobacilli isolated on AA medium in these studies (see Chapter 3) grew well in tomato broth and on agar. In the later studies the MRS medium of de Man et al. (1960), which has been described, was used exclusively for general culture. Fluid and solid (agar, 2% w/v) MRS media supported good growth of all the lactobacilli isolated here.

Media for physiological tests

Production of gas from glucose

The production of gas from glucose by heterofermentative lactobacilli cannot adequately be detected by means of the conventional Durham tube in a liquid medium (Gibson & Abd-el-Malek, 1945; Hayward, 1957). The solubility of the carbon dioxide formed and its escape into the atmosphere may lead to erroneous conclusions. Strålfors, (1950) reported that there were no gas-producing lactobacilli in the mouth using this method. Many quantitative and qualitative methods to determine gas production by lactic acid bacteria have been described (see Gibson & Abd-el-Malek, 1945). Those which require no special apparatus were considered unreliable by these workers. The technique devised by Gibson & Abd-el-Malek (1945) and the modification of the Durham tube method (Hayward, 1957) were both found adequate for testing gas production in most heterofermentative lactobacilli and a medium and technique based on those of Gibson & Ad-el-Malek (1945) was used successfully in this work. The medium was made as follows:

Litmus Milk. To 800 ml. of separated milk was added 10 ml. of a 1% (w/v)

litmus solution (Difco).

Nutrient agar. This was made up of Agar (Difco), 5.0 g.; peptone (Difco), 2.5 g.; Lab-Lemco, 2.5 g.; NaCl, 1.25 g.; distilled water to 250 ml. The medium was adjusted to pH 7.0 and sterilized by autoclaving at 121° for 20 min.

Complete medium. Eight hundred ml. of fresh litmus milk plus glucose, 55 g. were warmed. To this was added 200 ml. of melted nutrient agar; tomato juice (Briggs, 1953b), 100 ml.; Yeastrel, 2.8 g. and salt solution (as for AA), 5 ml. The pH was again adjusted to 7.0 and the medium tubed in 10 ml. quantities and sterilized by steaming for 30 min. on three consecutive days. Inoculated tubes were sealed with a layer of melted plain agar, 2% (w/v).

Production of ammonia from arginine

Briggs (1953b) successfully modified the method of Niven, Smiley & Sherman (1942) for the detection of ammonia production from arginine by streptococci, enabling its application to lactobacilli. Her method, with some modifications to the basal medium, was used in this work. The medium was prepared in two stages.

Basal medium. This consisted of tomato juice (Briggs, 1953b), 100 ml.; Bactopeptone (Difco), 15 g.; NaCl, 5 g.; Yeastrel, 3 g.; Tween, 1 ml. and distilled water, 900 ml. After it had been steamed for 30 min. the medium was adjusted to pH 6.6 and glucose, 20 g. was added. The medium was then sterilized in an autoclave at 121° for 20 min.

Complete medium. To this basal medium L-arginine hydrochloride (British Drug Houses) was added in a final concentration of 0.3% (w/v). Five ml. quantities were placed in MacCartney bottles and sterilized by steaming for 30 min. on three consecutive days. The same medium without the arginine served as a control.

Hydrolysis of aesculin

The basal medium described in the test above, but without the glucose, was used. Aesculin (Gurr), 0.2% (w/v) and ferric ammonium citrate 0.1%

(w/v) were added and the medium was dispensed in tubes in 10 ml. quantities and sterilized by steaming for 30 min. on three consecutive days.

Fermentation of carbohydrates

The fermentation of various carbohydrates by lactobacilli was tested by using the medium of Wheater (1955). The basal medium contained Neopeptone, 1.5% (w/v); Tween 80, 0.1% (v/v); Yeastrel 0.6% (w/v) and agar 0.15% (w/v) and was adjusted to pH 6.6. Chlorophenol red dissolved in 0.5 ml. ethanol was added as an indicator in a final concentration of 0.15% (w/v). The medium was tubed in 5 ml. quantities and sterilized in an autoclave at 121^{o} for 20 min. Two percent (w/v) Seitz-filtered solutions, of various carbohydrates (see Chapter 3) were added to the basic medium to give a final concentration of 2.0% (w/v) and sterility was checked by incubation for 48 hours at 37^{o} prior to inoculation.

Storage medium

Lactobacillus strains were stored at 4^{o} in yeast glucose litmus milk plus chalk, (YGLMC) (Davis, 1935). This medium had the following composition: separated raw milk 1000 ml.; Yeastrel, 3 g.; glucose, 10 g.; $CaCo_3$ (precipitated), 50 g. and litmus solution (1% w/v), 25 ml. The medium was dispensed in tubes in 15 ml. amounts and sterilized by steaming for 30 min. on 3 consecutive days. The tubes were inoculated with 1.0 ml. of overnight broth cultures of the strains and incubated for 24 hr at 37^{o} prior to refrigeration. These stock cultures were renewed at three-monthly intervals.

Other media

Certain of the investigations undertaken necessitated the use of media other than those described here or of modifications of these media. Details of these are given in the relative chapters.

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CHAPTER 3

LACTOBACILLI AND DENTAL CARIES IN TWO AREAS OF SOUTH AFRICA

SUMMARY

The quantitative and qualitative incidence of oral lactobacilli in children in 2 areas in South Africa was investigated. These areas, Langkloof and Nuwerus, have high and low dental caries incidence rates respectively. Lactobacilli in untreated saliva and oscillated saliva and plaque material were counted and different colonial morphological types of lactobacilli which occurred in the samples were identified. Specimens were examined over a period of 2 years. The results were subjected to statistical analysis to discover whether the incidence of these organisms in the 2 population groups could be correlated with the differences in their caries incidence. No significant differences in the lactobacillus counts of the 2 groups were observed. The same species of lactobacilli occurred in both areas and they were similar to those reported to occur in the human mouth by other workers. Lactobacillus acidophilus was found more frequently at Langkloof in the older children and this did not correlate with the higher caries incidence of the whole group.

INTRODUCTION

The factors responsible for dental caries may be classified under a factorial triad comprising the host, the diet and the oral microflora (Keyes, 1962). These factors are interdependant and all three contribute to the development of caries. The work of Miller between 1882 and 1890 (see Rosebury, 1944; Stephan, 1953) first clearly formulated the possible role of bacteria in dental caries. He attributed this disease to the dissolution of tooth enamel by the acids of bacterial fermentation with the subsequent destruction of the underlying dentine by the same process followed by proteolysis of the matrix of this tissue. Miller had little success in the cultivation of bacteria from carious teeth and because he observed different bacterial forms in stained sections of lesions he believed caries to be bacteriologically non-specific. The description by Goadby

in 1903 (see Rosebury, 1944) of organisms, which he called Bacillus necrodentalis, in carious dentine was probably the first association of lactobacilli with dental caries. He attached no significance to these organisms, but stressed the role of streptococci and staphylococci, also present in the lesions, as causative agents of caries. Kligler (1915) first proposed lactobacilli as the most important of the bacteria involved in the initiation of dental decay. He demonstrated a relative increase in lactic acid bacteria in carious mouths and suggested that the filamentous organisms also present served to attach the bacterial mass to the tooth surface in a network. This indictment of lactobacilli gained impetus through the studies of Howe & Hatch (1917) who found that only lactic acid bacteria which resembled the Moro-Tissier group of organisms were present in carious lesions which had been sealed beneath inert fillings for periods of up to 3 months. They postulated an aetiological significance for organisms most adapted to and therefore most able to survive in a carious environment. In 1922 McIntosh, James & Lazarus-Barlow again stressed the role of acid. They examined carious dentine for bacteria sufficiently acidogenic to induce caries yet aciduric enough to survive acid circumstances. They isolated organisms similar to B. acidophilus Moro which they called B. acidophilus-odontolyticus. Two morphological types were described. In the same year Rodriguez (1922) isolated 3 morphological types of lactobacillus from the clinically non-carious dentine beneath deep cavities and these he named B. odontolyticus. Similar evidence of the association of lactobacilli with dental caries was provided by Sierakowski & Zajdel (1924).

At about this time the emphasis shifted from the demonstration of lactobacilli in carious material and was centred on attempts to correlate caries with lactobacilli in saliva and material scraped from tooth surfaces. In a series of more than 1000 cases from which such material was cultured lactobacilli were found in 70% to 99% of various groups with caries and were absent in 60% to 100% of groups without caries (Bunting & Palmerlee, 1925; Bunting, Nickerson & Hard, 1926; Bunting, Nickerson, Hard & Crowley, 1928). This qualitative correlation was confirmed by Jay & Voorhees (1927), Morishita (1929), Thompson (1931) and Enright, Friesell & Trescher (1932). The prognostic significance of the presence of lactobacilli was stressed by Jay & Voorhees (1927) and Bunting et al. (1928). Tucker (1932) however reported that the presence of lactobacilli varied in individuals not only from day to day but also throughout the day. He

could not correlate the incidence of caries with one type of organism and frequently found lactobacilli in caries-free mouths. Similar findings were reported by Boyd, Zentmire & Drain (1933).

The above studies were all qualitative and a closer correlation between lactobacilli and caries was sought by the development of techniques for the quantitative estimation of lactobacilli in saliva. The first method (Rodriquez, 1930) was cumbersome but this author reported (Rodriguez, 1931) that the numbers of lactobacilli isolated from saliva were a presumptive index of caries activity. This work was followed by the introduction by Hadley (1933) of a selective medium on which dilutions of paraffin stimulated saliva were plated for the enumeration of lactobacilli. In this and subsequent work (Jay, Hadley, Bunting & Koehne, 1936) consistently high counts of lactobacilli were obtained in persons with dental caries and negative cultures or low counts were found in those free from the disease. Since the work of Hadley many studies of the quantitative aspects of the relationship between lactobacilli and caries have been published. Most of these have employed modifications of her methods and have involved various age-groups, various conditions of diet or the fluoridation of water. Many authors (Rosebury & Waugh, 1939; Snyder, 1942; Jay & Arnold, 1946; Dewar, 1950; Hewat, 1950; Sullivan & Storvick, 1950; Rizzio & Tilden, 1952; James & Parfitt, 1954; Krasse, 1954 a, b; Krasse & Odeen, 1955; Green & Dodd, 1956; Kesel, 1958; Rovelstad & Geller, 1958; Sims & Snyder, 1958; Davies, King & Collins, 1959; Geller & Rovelstad, 1959) have confirmed the direct relationship between the number of lactobacilli in saliva and the degree of caries activity. Other workers (Anderson & Rettger, 1937; Boyd, Cheyne & Wessels, 1949; Marshall-Day et al. 1949; Boyd & Wessels, 1951; Iyer, 1952; Slack & Martin, 1956; Green & Weisenstein, 1959) have shown no correlation between these two factors and have found salivary lactobacillus counts fallible as an index of caries activity or susceptibility. A clinical significance of lactobacillus counts established on a statistical basis has however been shown to exist for groups of subjects and to be unreliable at the individual level (Jay, 1958; Goldsworthy & Spies, 1958). Most of the studies mentioned so far have concerned lactobacilli in late lesions (carious dentine), or in saliva, mouth washings or swabbings and food particles removed from gingival crevices and tooth interstices and may not have reflected the conditions on the tooth surface at the sites of caries

initiation (Hemmens, Blayney & Harrison, 1941). Many workers (see Stephan, 1953) have studied the composition and activity of dental plaques because of the close physical association of these structures with early caries. Dental plaques are thin, tenacious, felt-like masses of mixed micro-organisms adherent to tooth surfaces (Hemmens et al. 1941). They were first implicated in the initiation of caries by Williams (1897, 1898, 1899) and Black (1898, 1899) who found that enamel caries developed beneath these deposits and they were considered to be a prerequisite for the development of caries. Early studies of dental plaques produced much controversy (see Stephan, 1953) but they confirmed their physical relationship to caries initiation, demonstrated low potential pH levels in carious plaques and showed that plaques contain large numbers of a variety of micro-organisms. Hemmens et al. (1941) compared the micro-organisms of cariogenic and non-cariogenic plaques. None of the 4 groups of aciduric organisms that they isolated was restricted to either type of plaque but lactobacilli were found more often in carious plaques. Subsequently these workers (Blayney, Brodel, Harrison & Hemmens, 1942; Hemmens, Blayney, Brodel & Harrison, 1946) demonstrated a marked increase in the occurrence of lactobacilli in pre-carious plaques prior to the appearance of clinically detectable caries. This was not so for the numerically superior aciduric streptococci. Quantitative studies of plaque material (Krasse, 1954b; Green, Dodd & Inverso, 1957) revealed low lactobacillus counts in plaque from caries immune subjects associated with low salivary counts and Green & Weisenstein (1959) correlated high counts in plaque with higher caries incidence rates.

Certain well-defined semi-isolated areas occur in South Africa in which the incidence of dental caries differs considerably. Two such areas situated in the Cape Province, Langkloof and Nuwerus, have caries incidence rates of 99% and 14% respectively (Ockerse, 1947). Expressed as the DMF surface percentage caries index (de Jager, 1963a) the incidence at Langkloof was found to be 58.6 and that at Nuwerus 20.2 (de Jager, 1963b). No investigations of the microbiology of dental caries had as yet been undertaken in South Africa, and these two areas were selected by the Nutrition and Dental Health Research Group of the South African Council for Scientific and Industrial Research for an investigation of the differences in their caries incidence rates. The scope of the research programme and the results of the dietary survey (de Jager, 1963b) and microbiological investigations (de Jager & de Klerk, 1964) have

been reported. In the latter study the sex and age group incidence of various micro-organisms in saliva and plaque material from children in the 2 areas was analysed. The work reported in this study is an assessment of the possible association between lactobacilli and caries based on the statistical analysis of the numbers and species of lactobacilli which occur in the mouths of children in the Langkloof and Nuwerus areas, and is an extension of the analysis of the data on lactobacilli previously reported (de Jager & de Klerk, 1964).

MATERIALS AND METHODS

Description of the two areas

Langkloof. This area extends from George in the west to the border of the Humansdorp district in the east (Fig. 1) and comprises the upper, lower and middle Langkloof. The latter, which was chosen for this survey, lies between Avontuur in the west and Heights in the east (Fig. 2). It consists of a fertile valley 50 miles long and 3 to 6 miles wide, bounded on the south by the Tsitsikama mountains and on the north by the Kouga mountain range. The area is about 2,000 feet above sea-level and the average annual rainfall is 18 in. Agriculture, particularly fruit farming, is the chief occupation of the district and the population is stable.

Nuwerus. The Nuwerus district (Fig. 1) is an arid and isolated area of about 1,000 sq. miles in the north-western Cape Province, 200 miles north of Cape Town. The climate is hot and dry with a rainfall which seldom exceeds 3 in. a year. Farming is limited to the keeping of sheep and goats, and practically no crops are grown. Poor roads make travel difficult in the area and the inhabitants seldom leave the district.

Source of Material

Saliva and plaque material from children between the ages of 6 and 12 years were examined. The groups consisted of 41 children at Nuwerus and 121 children at Langkloof. Their code numbers, names, and ages at the outset of

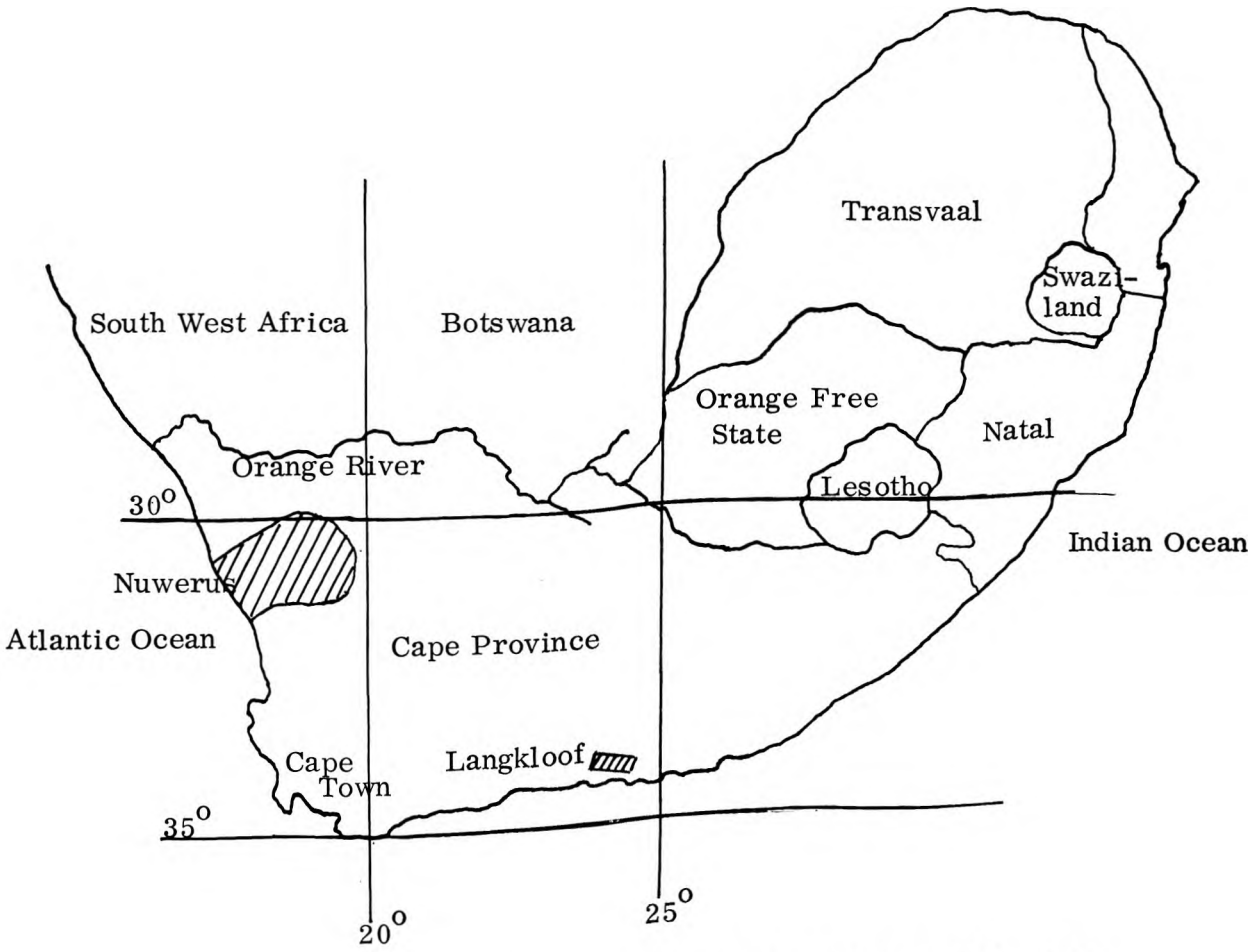


FIG. I. Map of the Republic of South Africa showing Langkloof and Nuwerus Areas.

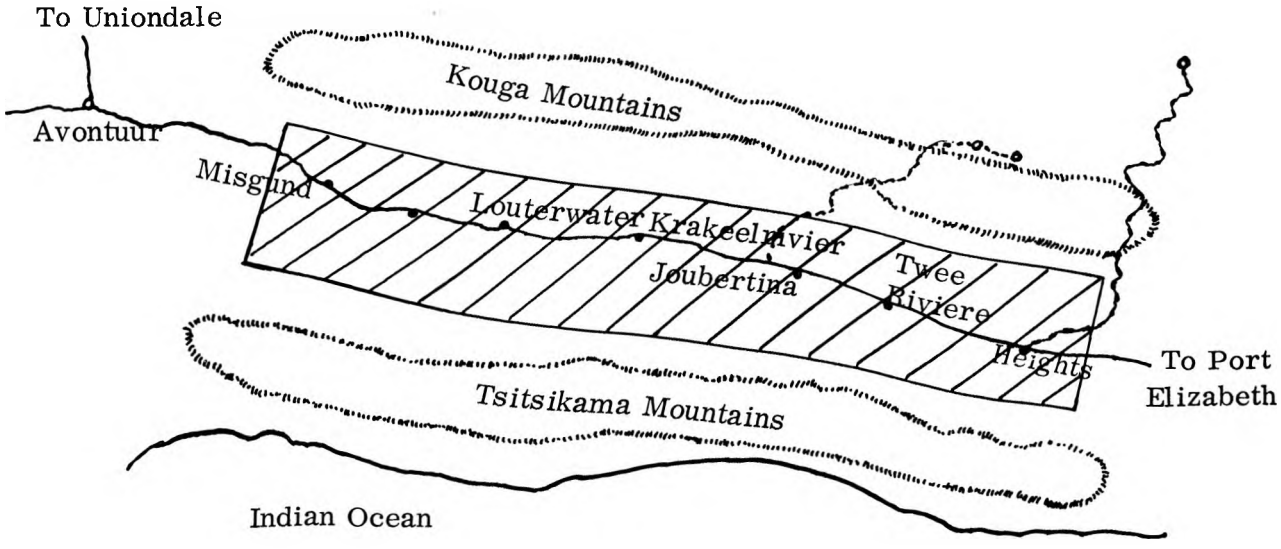


FIG. II. Map of the Middle Langkloof. Shaded portion denotes survey area.

the investigation are listed in appendices V, VI. Samples of saliva and plaque material were taken at half-yearly intervals during 1959 and 1960 and from 1 to 5 specimens were obtained from each child during this period.

Collection of saliva and plaque material. Paraffin-stimulated saliva was collected in sterile wide-mouthed glass specimen bottles. Plaque material scraped from the buccal and gingival surfaces of incisor, premolar and molar teeth was placed in sterile glass tubes (2 in. x 3/8th in.) which had previously been weighed and which contained 1.0 ml. of nutrient broth (Difco). The cork stoppers used to seal the tubes were soaked in molten paraffin wax. Prior to the collection of specimens, each tube was numbered and the level of the meniscus of the fluid marked on the side. Tubes showing a fall in the fluid level on arrival at the laboratory were discarded. Re-weighing of the tubes enabled the calculation of the weight of each sample of plaque material. All samples were immediately placed on ice in vaccuum flasks and maintained at 4^{o} in transit. They reached the laboratory within 12 hr. of collection.

Treatment of samples. Each saliva specimen was diluted 1/3 in nutrient broth (Difco) and 5 ml. of this dilution served as a control. A further 10 ml. of diluted saliva was treated in a sonic oscillator (Raythenon, model S-102A) at a frequency of 9 kilocycles / sec at 22^{o} for 6 min. The plaque material was washed from the tubes into the oscillator with 9 ml. of broth and treated for 12 min. at 22^{o}. These oscillation times were found to give maximum dispersal of lactobacilli in preliminary tests of 10 samples each of saliva and plaque material. Inocula of 0.1 ml. of suitable dilutions in sterile 0.85% (w/v) saline of the control and treated saliva and the oscillated suspensions of plaque material were pipetted onto plates and rubbed dry with a sterile glass rod. The plates were incubated for 3 days at 37^{o} in an atmosphere of carbon dioxide and the colonies counted. The counts were subjected to statistical analysis.

Media. The selective medium for the enumeration and primary isolation of lactobacilli was acetate agar. The general culture media were tomato glucose agar and broth (TGA, TGB). These and the media used for the identification and storage of the isolates have been described in Chapter 2.

Identification of lactobacillus strains

General methods for the study of lactobacilli have been described by Rogosa et al. (1953), Briggs (1953), Davis (1955), Sharpe (1955), and Wheater (1955a, b) and collated by Rogosa & Sharpe (1959). From these the following methods were selected to identify the strains isolated here. Plates were incubated in an atmosphere of CO_2 for 48 hr. Unless otherwise stated the incubation temperature for all tests was 37° and inocula in fluid media were 1-2 drops of overnight broth cultures.

Preliminary tests. From each acetate agar culture 3 to 6 colonies, which represented as many different colony morphologies, were Gram-stained and picked onto TGA. Single colony isolations were repeated at least twice. Catalase production was tested by pipetting a drop of 3% (v/v) hydrogen peroxide over 48 hr surface growth of strains on TGA and examining for effervescence. A culture of Staphylococus aureus on TGA served as a positive control. Gram-positive, catalase negative rods were transferred to TGB and subcultured 48 hourly. Cultures were stored at 4° in YGLMC (see Chapter 2) and subcultured at 3 monthly intervals.

Physiological tests

Growth at 15° and 45° was tested in broth. Cultures were incubated in accurately controlled water-baths for 21 and 7 days respectively. Tubes were inspected daily and levels in the water-baths were kept well above the surface of the broth.

Gas production from glucose. The medium (see Chapter 2) was inoculated with 0.5 ml. of an overnight broth culture and sealed with a layer of melted agar, 2% (w/v). Cultures were incubated for 7 days and inspected 48 hourly.

Ammonia production from Arginine. The medium used has been described (Chapter 2). Cultures were tested on the third and seventh days of incubation by the addition of a drop of Nessler's solution to drops of the cultures placed in the cups of a porcelain agglutination tray. The presence of ammonia was indicated by the development of a bright orange colour (Briggs, 1953).

Hydrolysis of aesculin. Cultures in aesculin medium (see Chapter 2) were incubated for 7 days and hydrolysis was assessed by the loss of fluorescence or blackening of the medium (Davis, 1955; Naylor & Sharpe, 1958).

Fermentation of Carbohydrates. The following carbohydrates were incorporated into the basal medium described in Chapter 2: D-Maltose, D-Lactose, D-Mannitol, salicin, D-cellobiose, D-melibiose, L-rhamnose, D-trehalose and D-mannose. The tests were incubated for 7 days and inspected at 48 hr intervals.

RESULTS

Quantitative analysis

A total of 774 samples were examined. They comprised 293 salivas and 228 samples of plaque material from Langkloof and 162 salivas and 91 plaque specimens from Nuwerus. The lactobacillus counts of the specimens are presented in appendices I to IV.

Saliva. In the statistical analysis of these counts the effects of 4 factors were investigated namely areas (Langkloof and Nuwerus), sexes, two methods of treatment of samples (control and oscillation) and age (6,7,8,9,10,11 and 12 years old). Because of unequal number of observations per cell and heterogeneity of observations within cells, a "smoothing" of data was affected by taking averages in cells and treating them as true observations. Since the observations are counts of bacteria the underlying distribution is certainly non-normal (probably Poisson). Accordingly, in order to use an analysis of variance to discriminate between the mean values of the levels of the factors the data were subjected to the transformation $y = \sqrt{x + 3/8}$ to normalise the distribution and stabilize the variance. The results of the analysis showed a significant difference in lactobacillus counts at the 5% level of significance only between the sexes but not between the other factors considered.

Plaque material. Counts were done only on oscillated samples of plaque material and thus only 3 factors were considered in their statistical analysis. No significant differences could be shown for areas, sexes or age groups.

Qualitative analysis

From the 2 areas a total of 1,248 strains of lactobacillus were isolated. On the basis of the tests used 1,202 (96.3%) of the strains were classified into 8 species. The differentiation and the number of strains of each species are shown in Table 3. All 8 species occurred in both areas. Only 46 strains (3.7%) could not be classified with the limited number of taxa used and they were not studied further. The incidence of the various species of lactobacillus was analysed as follows. Children from the 2 areas in each age group were scored for the presence or not of each species of lactobacillus. The hypothesis that P1 (the incidence at Langkloof) = P2 (the incidence at Nuwerus) was tested for each of the species observed. No significant difference in the incidence of the various lactobacillus species in the two areas could be shown except for Lactobacillus acidophilus in the 9 and 10 year age groups. The incidence of this species is significantly lower among 9 and 10 year-olds in the Nuwerus area.

DISCUSSION

Recent studies have continued to show a relationship between oral lactobacilli and dental caries activity. Canby & Burnett (1960) isolated only lactobacilli from deep portions of extracted teeth. Adams & Cramer (1961) demonstrated inhibitory and stimulatory effects on lactobacilli of salivas from caries immune and susceptible subjects respectively. The occurrence of lactobacilli in the mouth has been found to correspond to the positions of carious lesions and plaques (Bahn & Quillman, 1963) by the culture of mouth impressions. A quantitative correlation between salivary lactobacilli and caries activity in children was reported by Hafer (1960) and this finding was supported by Blayney & Hill (1965) in a survey of 2,991 children for a period of 11 years. The latter investigation encompassed a transition to the use of fluoridated water and the salivary lactobacillus counts and untreated carious tooth surfaces declined proportionately over 8 years. The correlation was present in different age groups and the authors stressed the validity of lactobacillus counts as an index of caries attack rates in large populations. A

Table 3. Differentiating characteristics and incidence of oral Lactobacilli isolated at Langkloof and Nuwerus

Species of Lactobacillus	Growth at 15°	Growth at 45°	Gas from glucose	NH_3 from arginine	aesculin cleavage	Lactose	Mannitol	Salicin	Cellobiose	Melibiose	Rhamnose	Mannose	Trehalose	No. of strains isolated	Incidence (%)
acidophilus	-	+	-	-	+	+	-	+	+	-	-	+	+	365	29.2
salivarius var. salivarius	-	+	-	-	-	+	+	-	-	+	+	+	+	91	7.3
salivarius var. salicinus	-	+	-	-	+	+	+	+	-	+	-	+	+	11	0.9
casei var. casei	+	-	-	-	+	+	+	+	+	-	-	+	+	76	6.1
casei var. rhamnosus	+	±	-	-	+	+	+	+	+	-	+	+	+	208	16.7
plantarum	+	-	-	-	+	+	+	+	+	+	±	+	+	20	1.6
fermenti	-	+	+	+	-	±	-	-	-	±	-	±	±	386	30.9
brevis	+	-	+	+	±	±	-	-	-	+	-	-	-	45	3.6

All species fermented maltose. ± = variable reaction, majority of the strains positive.

positive relationship between dental caries and lactobacilli has also been found in experimental animals (Rogosa, Johansen, Disraely & Beaman, 1957; Herrmann, Rozeik & Lammers, 1960). The findings quoted above are offset by the results of other workers who have recently found no association between these 2 factors, (Dvir, Gedalia & Sulitzeanu, 1962; Snyder, Porter, Claycomb & Sims, 1962; Askew & Lamb, 1963) and with whose findings the present results are in broad agreement.

In this study no attempt was made to correlate the incidence of lactobacilli with the caries status of the individuals in the 2 groups, but there was no significant difference in the epidemiology of these organisms in the 2 areas despite the contrast in caries incidence. The difference in the incidence of lactobacilli in the sexes cannot be related to difference in caries activity and is unexplained. The higher rate of dental decay at Langkloof has been attributed partly to the greater intake of refined carbohydrates in this region, (de Jager, 1963b). Although increased dietary carbohydrate has been correlated with increased caries activity and the number of oral lactobacilli (Koehne, Bunting & Morel, 1934) and the converse has been shown by Hadley (1933), Jay (1940) and Becks, Jensen & Millarr (1944), a concomitant higher incidence of lactobacilli was not found at Langkloof. Krasse (1954a) also failed to correlate diet with lactobacillus counts. It has been shown (see de Jager, 1963b) that caries will not develop in the absence of carbohydrates in the diet but that their effect in caries is due partly to a systemic action.

In the course of identification of the organisms isolated from Langkloof and Nuwerus all colonies picked from acetate agar plates were Gram-positive, catalase negative rods. The preliminary acceptance of these strains as lactobacilli was endorsed by their subsequent characterization. No other bacteria were found on the selective agar although it has been reported to support the growth of Leuconostoc spp. and some strains of micrococci from cheese (Mabbit & Zielinska, 1956). No catalase positive lactobacilli were encountered in this study although catalase activity on TGA has been described in 3 strains of Lactobacillus plantarum (Dacre & Sharpe, 1956). Other workers (Whittenbury, 1960; Scheibner, 1962; Johnston & Delwiche, 1962) have reported catalase positive strains of L. casei, L. plantarum, L. brevis, L. fermenti, L. viridescens and L. leichmanii by the use of special media. The species of lactobacilli isolated from saliva and plaque material from both areas correspond

to those previously described as occurring in the mouth (Rogosa et al. 1953; Davis, 1955; Hayward & Davis, 1956; Bisset, 1958). The frequency distribution of the species among the combined populations of these areas (Table 3) is similar to that reported by Rogosa, Mitchell & Fitzgerald (1950) and Rogosa et al. (1953). These workers however found a higher incidence of L. casei (39%) and a much lower incidence (11%) of L. acidophilus. Grubb & Krasse (1953) reported the predominance of L. casei (69%) in the mouths of 67 subjects. This may have resulted from the inhibitory effect of sodium azide in their medium on certain strains of L. fermenti (Rogosa, Mitchell & Wiseman, 1951) and their use of aerobic incubation. An attempt to relate lactobacillus species and caries was made by Clapper & Heatherman (1949, 1955) who found caries associated with a high incidence of a "Lactobacillus type I" in saliva and dental plaques. From their limited description this organism may have been L. casei var. rhamnosus. In 45 children with high lactobacillus counts Tilden & Svec (1952) found more L. casei than other species of lactobacillus and the majority of these fermented rhamnose. L. acidophilus was present only in small numbers. In the present work the only correlation with caries incidence was that of L. acidophilus in the 9 and 10 year age groups. This species which occurs more frequently only among the older children examined at Langkloof cannot be held responsible for the higher caries incidence of the whole group studied in this area.

The evidence in support of an association between dental decay and lactobacilli implies a role for these organisms in the caries process which is based by most of the authors quoted on the acidogenic and aciduric properties of these organisms. Krasse (1954a) argued that this relationship does not necessarily mean that lactobacilli are the cause of caries. He proposed (Krasse, 1954c) that they may act as part of a chain of processes which lead to the development of these lesions or that they may indicate conditions which favour caries. Bibby (1961) summarised the evidence against a specific role for lactobacilli in this disease. He pointed out that demonstration of an association of these organisms with tooth decay does not prove the association to be causal. Furthermore, the association is not constant and lactobacilli form only a small proportion of the plaque population. The numerically superior aciduric streptococci in the mouth produce far more acid and he believes the presence of lactobacilli is an effect and not a cause of dental caries. Similar views have

been expressed by Winkler & Backer Dirks (1959).

The divergence of opinion on the existence of a quantitative correlation between lactobacilli and tooth decay and its possible significance may be rooted in several factors. These include differences in the methods used in various studies, possible true daily or even hourly fluctuations of lactobacilli in the individual (Grubb & Krasse, 1954), unequal sampling (Hadley, 1933) and inadequate homogenization and experimental error in the dilution and plating of samples (Permar, Kitchin & Robinson, 1946). In addition the variety of methods used to evaluate caries incidence or activity seriously hamper comparison of the findings of different workers (de Jager, 1963a). Many of the indices used do not give reproducible results (Backer Dirks, van Amerongen & Winkler, 1951). Conflicting quantitative findings have recently extended to caries research with gnotobiotic techniques. Some workers (see Keyes, 1962) have induced caries in germ-free rats and hamsters by mono-infection with strains of enterococci and streptococci but not with L. fermenti or L. acidophilus. Blayney & Hill (1965) cited F.J. Orland in a personal communication that germ-free rats showed definite evidence of caries after mono-infection with a strain of lactobacillus. The aetiology of dental caries is obscure and it is likely to remain so until more is known about the fundamental characteristics of the various micro-organisms which may be involved in its pathogenisis and the factors which control their ecology in the mouth. It is improbable that dental decay is caused solely by lactobacilli or any other specific micro-organism.

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CHAPTER 4

MORPHOLOGICAL VARIANTS OF LACTOBACILLI

SUMMARY

Colonies of 4 strains of Lactobacillus casei var. casei and 3 strains of L. casei var. rhamnosus investigated developed rough outgrowths while growing on a medium containing no fermentable carbohydrates. In addition to rough outgrowths they also formed smooth secondary colonies situated on the primary colonies. These secondary colonies arose after about 6 days of incubation and were of 2 types: when situated centrally they formed papillae; when near the margin of the parent colony they often split over and formed fan-shaped outgrowths adjacent to the parent colony. Cultures of several different species of Lactobacillus were examined and smooth secondary colony formation was confined almost exclusively to strains of L. casei. None of the rough or smooth outgrowths appeared on this medium when it contained more than 0.5% (w/v) glucose. Rough elements were present in the latter colonies, but because of lack of a selective advantage did not show phenotypically. The rough outgrowths possessed the characteristics of R variants. The mutational origin of the R variants from wild S types was demonstrated by a method involving the direct observation of rough clones. The mutation rate involved is presented for 6 of the strains. In continuous culture in broth both the S and the R types were stable. On agar the R variant was also stable. The fan-shaped outgrowths were pure cultures of organisms possessing a shorter generation time than those comprising the parent colony. An inverse relationship existed on crowded and sparsely inoculated plates between the numbers of papillae and fan-shaped outgrowths/colony observed. The distribution of colonies bearing different numbers of secondary colonies did not follow a Poisson distribution and the random origin of fast-growing variants could not be established. On sub-culture the fast-growing variants produced colonies with a slightly different morphology from that of the wild-types. This difference was also stable and persisted through numerous subcultures. Both rough and fast-growing variants had biochemical reactions identical to those of the parent strains.

INTRODUCTION

Many workers (see Barber & Frazier, 1945; Davis, 1956) have encountered colonial variants of lactobacilli (see Hadley, Bunting & Delves, 1930, for illustrations). Most of the variants described have belonged to the subgenus Thermobacterium (Rogosa & Sharpe, 1959). If strains of the Bacillus acidophilus group I of Hadley et al. (1930) are accepted as Lactobacillus casei, then these authors must be regarded as the first to have succeeded in systematic attempts to isolate morphological variants of this species. Barber & Frazier (1945) and McDonald & Frazier (1951) also attempted the isolation of colonial variants of L. casei. The former failed with their type strain 28 but succeeded with 8 other unnamed strains, and the latter workers were unsuccessful. Weinstein, Anderson & Rettger (1933) attempted the isolation of rough variants from 37 unidentified oral strains of lactobacilli; they were successful with only one of the strains. No quantitative experiments have been reported to establish how variants derive from one another in a particular environment. In an attempt to isolate mutants of lactobacilli, strains of L. casei were plated on solid media which contained sugars not normally fermented by these strains. The colonies which arose were studied daily for the presence of papillae which might indicate the presence of clones capable of using the extra source of energy provided by the carbohydrate. Many smooth papillae were seen on the colonies but none were found on subculture to contain cells capable of fermenting the carbohydrate. More striking was the appearance on many of the old colonies of rough outgrowths projecting from their margins. Both the smooth papillae and the rough outgrowths also appeared when the strains were plated on a carbohydrate-free medium. A cursory examination of subcultures of the smooth papillae on the same medium revealed colonies similar in appearance to the primary colony. On crowded plates most of the smooth papillae which were situated peripherally continued to develop to form fan-shaped outgrowths which extended beyond the margins of the parent colony. The appearance of these 2 types of outgrowth on colonies of L. casei was remarkable and it was decided to investigate their nature and derivation from the parent strains.

METHODS

Organisms. Strains C2, C9 and H2 were kindly supplied by Dr. M. Elizabeth Sharpe (National Institute for Research in Dairying, Reading, Berkshire, England). The first 2 represent the serological groups B and C respectively of Lactobacillus casei var. casei (Sharpe & Wheater, 1957; Rogosa & Sharpe, 1959) and the last is a L. casei var. rhamnosus (Rogosa et al. 1953). In In addition 2 strains of L. casei var casei (no. 300 and 316) and 2 strains of L. casei var. rhamnosus (no. 430 and 542) isolated in this laboratory from human saliva were investigated for rough outgrowth formation. Strains C9, 300 and 316 were studied in detail for the production of smooth papillae and fan-shaped outgrowths and other local salivary strains comprising 15 of L. casei var. casei, 10 strains of L. casei var. rhamnosus, 5 of L. plantarum, 8 of L. acidophilus, 6 of L. salivarius, 10 of L. fermenti and 10 of L. brevis were examined for smooth outgrowth formation. The origin, identification and maintenance of the local strains have been described in Chapter 3. Bacteriophages 300 and 316 (Coetzee, de Klerk & Sacks, 1960) were isolated from sewage (see Chapter 5) and are lytic for L. casei strains 300 and 316 respectively. The phage techniques were those of Adams (1956).

Media. The media used were the tomato glucose agar and broth (TGA, TGB) described in Chapter 2 and the same media without tomato juice and glucose called Medium I and Medium I broth. The concentration of reducing sugars in the tomato juice was determined by the method of King, Haslewood, Delroy & Beale (1942) and the constituent sugars were identified by chromatography (Horrocks & Manning, 1949). As tomato juice and Yeastrel are antigenic (Sharpe, 1955a) the medium for growing organisms to give antigens for injection into rabbits had the following composition: peptone (Difco), 1.0% (w/v); Tween 80, 0.01% (v/v); glucose 2.0% (w/v); Malt extract (Difco), 0.1% (w/v). The medium was adjusted to pH 6.1 and sterilized by autoclaving at 121^{o} for 20 min. Cultures were incubated at 37^{o} in an atmosphere of 100% CO_2.

Serology. Antigens for rabbit inoculation and the corresponding antisera were prepared by the methods of Sharpe (1955b). Agglutination and agglutinin-absorption tests were done according to the methods of Mackie & McCartney (1953). Where spontaneous clumping occurred, the organisms were suspended

in distilled water and the serum dilutions made in 0.2% (w/v) NaCl solution.

Continuous culture. Continuous cultures of organisms in medium I broth were started by subculturing 0.05 ml. of a 10^{-7} dilution of an overnight growth in this broth into 1000 ml. prewarmed broth. This inoculum when plated on medium I yielded about 3 colonies for both smooth and rough growths. At the first indication of growth (usually after 36 hr at 37^{o}) cultures were well-shaken to homogenize them and 0.2 ml. subcultured into a further 1000 ml. of the warmed broth. By repeating this process at 24 hr intervals cultures never reached their maximum concentrations and could be maintained in exponential growth. Mixtures of smooth and rough organisms of strain C9 were also maintained in logarithmic growth by the same method.

Mutational origin and mutation rates of organisms in rough outgrowths. For reasons to be mentioned the enumeration of individual organisms was avoided as far as possible and the work on mutation and mutation rates was based on the modification by Ryan, Schwartz & Fried (1955) of the method of Luria & Delbrück (1943) for the determination of mutation rates of bacteria. This method is independant of the number of mutants and also of the growth rates of the parent and the mutant. It is based on the assumption that the clonal progeny of each mutation with a selective advantage, which occurs during the development of a colony, will become visible as a papilla. The mean number of mutations/colony may then be determined by direct observation. On the assumption that mutations are randomly distributed, the mean number of mutations/colony can also be calculated from the proportion of colonies without papillae. Rough outgrowths may be regarded as papillae of a different nature which possess certain selective advantages. This reasoning has been applied to other systems (Coetzee & Sacks, 1960b) and is used here. Cultures were accordingly seeded on medium I to yield about 50 colonies/plate. This was done by pipetting 0.1 ml. of suitable dilutions and rubbing to dryness with a sterile glass rod. The number of outgrowths/colony was recorded daily until no more appeared. Plates were incubated between counting sessions. At this stage the total number of colonies was counted and a number of entire colonies, with no visible rough outgrowths, were streaked individually on medium I plates and incubated to discover any hidden rough elements within smooth colonies. These would be expected to appear as entirely rough colonies. After scanning with a dissecting microscope the proportion of plates showing no

rough colonies was used in the Poisson distribution to obtain an estimate of the mean number of hidden rough outgrowths/colony.

The total population of smooth colonies with no outgrowths at the end of the period of observation was estimated with the use of a Petroff-Hauser counting chamber and a phase-contrast microscope according to the method of Ryan et al. (1955). Cytological observations were made on bacteria stained with acid Giemsa according to the method of Bisset (1950).

Secondary colony formation. The plating technique described above was used. The effects of crowding on the development of smooth papillae and fan-shaped outgrowths was studied by spreading 0.1 ml. of various dilutions of overnight cultures grown in TGB on to medium I agar. After 8 days of incubation the distribution of secondary colonies in the form of papillae and fan-shaped outgrowths on parent colonies was determined in areas of 3 cm^2. The effect of the depth of the medium on the incidence on the 2 types of smooth outgrowth was investigated by using different volumes of agar in similar Petri dishes. The effect of amino acids and vitamins on secondary colony formation was tested by incorporating vitamin-free pancreatic casein digest (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) at 10 g./1. into medium I. Ascorbic acid, 0.5 g./1.; nicotine acid, thiamine hydrochloride, calcium-D-pantothenate, and riboflavin, all 1.0 g./1.; p-aminobenzoic acid and DL biotin, each 10 µg./1.; folic acid and vitamin B_{12}, each 1 µg./1. (all obtained from Nutritional Biochemicals Corporation) were incorporated in medium I by the methods of Rogosa, Franklik & Perry (1961).

Effect of carbohydrates on outgrowths formation. Rough outgrowth formation by strain 300 was studied on medium I with different concentrations of glucose (0.1%, w/v to 4.0%, w/v) and on medium I with concentrations of tomato juice ranging from 10% (v/v) to 40% (v/v). The effect of fermentable and non-fermentable carbohydrates on the development of smooth secondary colonies was similarly tested using glucose, cellobiose, rhamnose and melibiose.

Colony diameters. Suspensions were made of samples of organisms from fan-shaped outgrowths and from areas of the parent colonies which were devoid of secondary colonies. Suitable dilutions to yield about 50 colonies/plate were seeded on to medium I agar. Colonies derived from these 2 sources will subsequently be referred to as F variants and wild-types, respectively. The

diameters of 50 well-separated colonies were measured after incubation for 48 hr. This was done with a micrometer eyepiece at X41.25 magnification.

Generation times. Overnight cultures of single F variant and wild-type colonies in 5.0 ml. medium I broth were transferred to 100 ml. quantities of fresh pre-warmed medium I broth. These cultures were maintained at 37° and at suitable intervals samples removed. The optical densities of these samples were read with a Zeiss spectrophotometer at 660 m μ. Total counts on the samples, suitably diluted in cold distilled water were done with a Petroff-Hauser counting chamber as described above. Dilutions of the samples were also plated for viable counts.

Changes in pH value of cultures in medium I. Cultures of F variants and wild-type strains were grown in 250 ml. medium I broth for 170 hr. These cultures were sampled at intervals and the pH values of the samples determined with a Beckman pH meter.

RESULTS

On TGA well separated 48 hr colonies of all the strains examined were circular, about 2 mm. in diameter, dome-shaped with an entire edge. The surface was very finely granular and glistening and the colonies white and opaque. On medium I the colonies were smaller, less raised and had a smooth glistening surface (Pl. 1, fig. 1). On further incubation colonies on TGA became larger but did not change in character. On medium I colonies of Lactobacillus casei strains C2, C9, H2, 300, 316, 430, 542, became slightly larger and flatter and developed on their surfaces various smooth excrescences. In addition to these excrescences, rough outgrowths which might appear anywhere from the surfaces or margins of the colonies became discernible in some cases after incubation for about 72 hr. When first seen they might consist of a few protruding whispy strands of growth which resemble the curled hair-lock appearance associated with colonies of Bacillus anthracis (Pl. 1, figs. 2, 3) or they might be much more impressive florid structures (Pl. 1, fig. 4). Whatever the begining, this growth was out of step with the remainder of the colony and continued to increase in size when the rest of the colony had ceased to grow. For this reason it was important, in quantitative experiments,

to detect outgrowths at an early stage of development before they coalesced with others on the same colony. The surfaces of these outgrowths were extremely rough and contrasted sharply with the adjoining surfaces of the colony. On a few occasions colonies with rough sectors were also seen. When rough areas of colonies were subcultured on to medium I agar they gave rise to typical medusa-head colonies (Pl. 1, fig. 5) (Bisset, 1938; Davis, Bisset & Hale, 1955). Apart from an increase in size, these medusa-head colonies underwent no other change in morphology with longer periods of incubation. They also bred true when streaked for single colonies on medium I agar and no smooth colony progeny were ever detected in numerous platings.

On TGA subcultures of rough outgrowths of strains C9 and 316 yield smoother colonies which closely resemble the original smooth parent colony but slightly duller in appearance. Rough outgrowths from strain 300 persisted as rough colonies on TGA. To prove that differences in appearance on the two kinds of media are not due to selection of variants, a medusa-head colony of strain C9 was repeatedly restreaked on medium I and a single colony finally picked off and suspended in sterile distilled water. From this suspension suitable dilutions were made and from the same dilution tube 0.1 ml. volumes were seeded on plates of TGA and medium I. After 48 hr the colonies were examined and counted. There were 326 colonies on medium I and 311 colonies on TGA. Those on medium I were all typical medusa-head colonies while the colonies on TGA were all smooth dome-shaped colonies only slightly duller in appearance than the original smooth colonies.

In broth smooth colonies produced a uniform turbidity with a slight finely granular deposit after overnight incubation. Subcultures from rough outgrowths produced a granular stringy growth at the bottom of the tube and a clear supernatant fluid. In 0.85% (w/v) NaCl solution rough growths were unstable and soon settled out. Microscopically the organisms from rough areas, whether on agar or in broth, consisted of a tangled mass of chains comprising thousands of bacilli. The appearance was that of a long ribbon folded upon itself. Chain formation of rough growths occurred despite the presence of smooth-colony inducing agents (Tween 80, acetate) in the media used (Rogosa & Mitchell, 1950; Rogosa et al. 1953; Davis, 1956; Rogosa & Sharpe, 1959). The addition of tryptophan (Green, Dodd & Radike, 1955) also had no effect on chain formation. Organisms from smooth areas were usually single or in

short chains of 3 or 4 bacilli. On no occasion in all the plating done from stock cultures during these experiments were primary medusa-head colonies detected. All colonies were initially smooth, and rough outgrowths were generally first detected after 4 days but they sometimes appeared as late as the 13th day of incubation.

Colonies of all strains of L. casei and one strain each of the L. fermenti and the L. brevis strains tested formed secondary colonies on medium I which appeared as papillae anywhere on their surfaces or margins. These outgrowths generally appeared between the fifth and seventh days of incubation. No secondary colony formation was ever seen on colonies of the other species examined. The number of secondary colonies formed by the single strains of L. fermenti and L. brevis mentioned was negligible in comparison with their numbers on colonies of the strains of L. casei. Secondary colony formation on this medium thus appears to be a feature of the latter species. The secondary colonies varied in size and were smooth and glistening (Pl. 2, figs. 6, 7). When situated centrally they soon stopped growing; but those at the periphery often split over the margin of the parent colony. Once in contact with the medium, they grew rapidly into large fan-shaped outgrowths which retained their smooth glistening surfaces and entire edges (Pl. 2, figs. 8, 10). Adjacent fan-shaped outgrowths did not coalesce (Pl. 2, fig. 9) like rough outgrowths. Forty-eight hr colonies of organisms from the parent colony (wild-type) and the fan-shaped outgrowths (F variants) on medium I, were smooth but differed in that the latter were larger and slightly more raised and opaque (Pl. 3, fig. 11). This morphological difference persisted through numerous subcultures. Subcultures from fan-shaped outgrowths were homogeneous and yielded only F variants. Subcultures from papillae situated centrally usually yielded mixtures of 2 types of colony, corresponding to the descriptions of F variant and wild-type given above. On other occasions only one or other of the colony types was recognised in subcultures from papillae. It was not possible to tell the composition of a papilla from its appearance. Microscopically, organisms from the parent colony and fan-shaped outgrowths or from subcultures of these on agar or in fluid medium possessed identical morphologies. The organisms were arranged singly and in chains of 2 to 6 bacilli. The difference in size of the two colony types on medium I agar was verified by direct measurement. The mean diameter of 48 hr colonies of the

wild-type strain 300 was 0.46 mm. and that of colonies of the F variant of this strain was 0.55 mm. Wild-type and F variant colonies of the other 2 strains showed similar differences. This difference in diameter was paralleled by results of total and viable counts which are shown in Table 1.

Table I. Total and viable counts of organisms in 48 hr-colonies of wild strains and F variants of Lactobacillus casei

Six whole colonies of each strain were cut out with the underlying agar and thoroughly emulsified in separate 0.25 ml. volumes of distilled water. The organisms present in samples were counted in a Petroff-Hauser chamber with a phase-contrast microscope. Suitable dilutions were plated on tomato glucose agar for viable counts. Colonies were enumerated after incubation for 48 hr at 37°.

Strain	Mean populations of six 48 hr colonies.	
	Viable count (millions)	Total count (millions)
C9 wild	1.2	4.3
C9 F variant	2.9	8.0
300 wild	1.0	3.6
300 F variant	2.5	6.0
316 wild	3.3	5.0
316 F variant	4.4	7.5

The tendency of these organisms to grow in chains of different lengths is one of the factors responsible for the viable counts being lower than the total counts. On further incubation of F variant and wild-type colonies on medium I, rough outgrowths often appeared on both types of colony but secondary colony formation in the form of smooth papillae and fan-shaped outgrowths was detected only on wild-type colonies. Rough outgrowths were occasionally seen on fan-shaped outgrowths (Pl. 3, fig. 12) and frequently a wild-type colony bore both rough and fan-shaped outgrowths (Pl. 3, fig. 13).

Effect of carbohydrates, amino acids and vitamins. Rough outgrowth formation of strain 300 (the strain with the highest incidence of outgrowths/colony) was studied on medium I with different concentrations of glucose or tomato juice added. The colonies were observed for 14 days and the results are presented in Table 2.

Table 2. Rough outgrowth formation of Lactobacillus casei strain 300 on various media

Plates were inoculated with a dilution of L. casei strain 300 yielding about 50 colonies/plate. About 500 colonies on each medium were studied for 14 days. Numbers in brackets denote the concentration (w/v) of sugars present in media.

Media.	No. outgrowths/colony.
Medium I. (0)	0.40
Medium I plus 10% (v/v) tomato juice (0.24)	0.41
Medium I plus 20% (v/v) tomato juice (0.48)	0.39
Medium I plus 40% (v/v) tomato juice (0.96)	0.
Medium I plus 0.1% (w/v) glucose	0.39
Medium I plus 0.25% (w/v) glucose	0.40
Medium I plus 0.5% (w/v) glucose	0.38
Medium I plus 1.0% (w/v) glucose	0.
Medium I plus 2.0% (w/v) glucose	0.
Medium I plus 4.0% (w/v) glucose	0.
Tomato glucose agar (2.24)	0.

The total concentration of reducing sugars present in the tomato juice used was 2.4 g./100 ml. As the method of preparation of this juice involved the hydrolysis of any sucrose, this figure reflects the total concentration of sugar present. Paper chromatography proved the bulk of the latter to be glucose. Up to a concentration of 0.5% (w/v) sugar the mean number of rough outgrowths/colony on medium I + glucose and on medium I + tomato juice was similar to that on medium I. Above this concentration the incidence was zero.

The presence of 2.24% (w/v) sugar in TGA thus appeared to account for the absence of outgrowths on this medium. As with the rough outgrowths of L. casei neither form of secondary colony appeared on medium I supplemented with glucose or cellobiose in excess of 0.5% (w/v). The addition of rhamnose or melibiose (not normally fermented by L. casei) did not prevent secondary colony formation. The morphological differences between wild-type and F variant colonies noted on medium I disappeared when these were subcultured on TGA. Forty-eight hr colonies of both types on the latter medium were circular, white, opaque, about 2 mm. in diameter, dome-shaped with an entire edge and a finely granular and glistening surface. No change in the incidence of smooth papillae and fan-shaped outgrowths on the strains of L. casei tested was detected on medium I supplemented with amino acids and/or vitamins.

Continuous culture experiments. These were done with purified rough and smooth growths of L. casei strains 300, C9 and 316 maintained in exponential growth for 400 hr and plated for single colonies on 10 plates each of medium I at 24 hr intervals. Both smooth and rough elements were perfectly stable under these conditions and no primarily smooth or medusa-head colonies appeared on the plates from rough or smooth continuous cultures respectively. A similar atavistic deficiency was noted by Tracy (1938) for R variants of L. plantarum. Kopeloff (1934) however, could readily select S variants from R cultures of L. acidophilus, but not vice versa. In continuous cultures started with mixtures of smooth and rough growths of L. casei strain C9 the smooth-colony growth overgrew and entirely replaced the medusa-head colony forming growth within 144 hr. Because of chain formation of the rough growths these experiments were subject to gross errors of sampling and enumeration. Yet the above results were obtained when platings of initial inocula indicated a starting ratio of 10 medusa-head colonies to 1 smooth colony. Under these conditions cultures were almost certainly initiated with a great excess of rough organisms.

Psysiological characters. The biochemical reactions and growth temperatures of purified rough growths were identical to those of the parent smooth strains as were those of the wild-types and their corresponding F variants.

Serological tests. The results of agglutination and agglutinin absorption tests

of sera prepared against smooth and rough variants of strains C9, 300 and 316 are presented in Table 3. These results indicate antigenic differences between

Table 3. Results of agglutination and agglutinin absorption tests on antisera prepared against smooth and rough growths of Lactobacillus casei strains C9, 300 and 316

Antigens were suspended in distilled water and serum dilutions made in 0.2% (w/v) NaCl solution. Equal volumes of antigen and antiserum were mixed and tests were kept in a water bath at 50^{o} for 6 hr and then at 4^{o} overnight. Agglutinin absorbtions were done by adding an excess of tightly packed heated antigen to a dilution of serum corresponding to sixty-four times the concentration of its titre. The contents of these tubes were well mixed and kept at 37^{o} for 4 hr. The controls with 0.2% (w/v) NaCl solution without serum were satisfactory in all cases.

Antiserum	Serum absorbed with	Antigen	Titre
C9 smooth	-	C9 smooth	1/8192
C9 smooth	-	C9 rough	1/4096
C9 smooth	C9 rough	C9 rough	< 1/128
C9 smooth	C9 rough	C9 smooth	1/512
C9 rough	-	C9 rough	1/2048
C9 rough	-	C9 smooth	1/1024
C9 rough	C9 smooth	C9 smooth	< 1/32
C9 rough	C9 smooth	C9 rough	< 1/32
300 smooth	-	300 smooth	1/8192
300 smooth	-	300 rough	1/4096
300 smooth	300 rough	300 rough	< 1/128
300 smooth	300 rough	300 smooth	1/512
300 rough	-	300 rough	1/2048
300 rough	-	300 smooth	1/512
300 rough	300 smooth	300 smooth	1/32
300 rough	300 smooth	300 rough	< 1/16
316 smooth	-	316 smooth	1/4096
316 smooth	-	316 rough	1/4096
316 smooth	316 rough	316 rough	< 1/128
316 smooth	316 rough	316 smooth	1/512
316 rough	-	316 rough	1/2048
316 rough	-	316 smooth	1/128
316 rough	316 smooth	316 smooth	< 1/4
316 rough	316 smooth	316 rough	< 1/4

smooth and rough growths of all 3 strains. The difference in each case appears to be an antigenic deficiency of the rough growths. This is the usual change associated with S-R variation (Topley & Wilson's Principles, 1955). The results of agglutination and agglutinin absorption tests with sera prepared against wild-types and F variants of strains C9 and 316 are shown in Table 4.

Table 4. Results of agglutination and agglutinin absorption tests on antisera prepared against the wild-types and the F variants of Lactobacillus casei strains C9 and 316

Antigens were suspended in distilled water and serum dilutions made in 0.85% NaCl solution. Equal volumes of antigen and antiserum were mixed and tests were kept in a waterbath at 50^{o} for 6 hr and then at 4^{o} overnight. Agglutinin absorptions were done by adding an excess of tightly packed heated antigen to a dilution of serum corresponding to thirty-two times the concentration of its titre. The contents of these tubes were well mixed and kept at 37^{o} for 4 hr. Controls with 0.85% (w/v) NaCl solution were satisfactory.

Antiserum	Serum absorbed with	Antigen	Titre
C9 (wild-type)	-	C9 (wild-type)	1/1024
C9 (wild-type)	-	C9 (F variant)	1/1024
C9 (wild-type)	C9 (F variant)	C9 (F variant)	< 1/8
C9 (wild-type)	C9 (F variant)	C9 (wild-type)	< 1/8
C9 (F variant)	-	C9 (F variant)	1/1024
C9 (F variant)	-	C9 (wild-type)	1/512
C9 (F variant)	C9 (wild-type)	C9 (wild-type)	< 1/16
C9 (F variant)	C9 (wild-type)	C9 (F variant)	< 1/16
316 (wild-type)	-	316 (wild type)	1/2048
316 (wild-type)	-	316 (F variant)	1/2048
316 (wild-type)	316 (F variant)	316 (F variant)	< 1/16
316 (wild-type)	316 (F variant)	316 (wild-type)	< 1/16
316 (F variant)	-	316 (F variant)	1/1024
316 (F variant)	-	316 (wild-type)	1/1024
316 (F variant)	316 (wild-type)	316 (wild-type)	< 1/8
316 (F variant)	316 (wild-type)	316 (F variant)	< 1/8

Unlike the rough variants of these strains F variants of strains C9, 316 and 300 were found to be antigenically identical to the corresponding wild-types.

Phage susceptibility. Cultures from smooth colonies of Lactobacillus casei strains 300 and 316 are susceptible to lytic phages 300 and 316 respectively. Rough outgrowths of strain 316 did not absorb phage 316, but the efficiency of plating of phage 300 on rough cultures of strain 300 was identical to that of the smooth variant. The efficiencies of plating of these 2 phages on the F variants of their hosts were identical to those on the corresponding wild strains. No phage active on strain C9 was available.

Mutational origin of rough outgrowths. Counts of the total bacterial population of 14 day smooth colonies on medium I with no outgrowths are presented in Table 5. These counts may be regarded as reliable because smooth colonies formed homogeneous suspensions mainly composed of single organisms. The chains present were short and constituent organisms could be counted. Results of the enumeration of rough outgrowths are presented in Tables 6 and 7. Because of the great crowding on plates used to determine the number of hidden rough outgrowths/colony, these estimates must be regarded as minimal.

Table 5. Total counts of organisms present in 14-day smooth colonies of various strains of Lactobacillus casei with no visible outgrowths

Six entire colonies of each strain were cut out with the underlying agar and thoroughly emulsified in separate 1 ml. volumes of distilled water. Organisms present in samples were then counted in a Petroff-Hauser chamber by means of a phase-contrast microscope.

Strain	Mean population of 6 colonies (N)
C2	7.0×10^7
C9	1.8×10^8
300	7.4×10^7
316	1.3×10^8
H2	4.0×10^7
430	5.5×10^7
542	7.0×10^7

Table 6. Results of two sets of experiments with Lactobacillus casei strains C9, 300 and 316 to determine the mutational origin and mutation rates of rough from smooth growths

	Experiment 1			Experiment 2		
	Variant colonies (no.)			Variant colonies (no.)		
	Strain C9	Strain 300	Strain 316	Strain C9	Strain 300	Strain 316
No outgrowths	233	250	549	1208	576	1786
1 outgrowth	21	100	55	129	226	138
2 outgrowths	2	24	5	10	47	9
3 outgrowths	0	1	0	0	6	0
4 outgrowths	0	0	0	0	1	0
5 outgrowths	0	0	0	0	1	0
Mean no. outgrowths/colony (m)	0.1	0.4	0.11	0.11	0.4	0.08
Probability of drawing a similar sample of outgrowths from a Poisson distribution with m as mean (P)	0.40	0.62	0.25	0.29	0.89	0.15
No. of 14-day old colonies with no outgrowths streaked out	30	30	30	30	30	30
No. of these plates revealing rough colonies	0	2	1	0	2	1
Mean no. concealed rough growths/colony (mc)	0	0.07	0.03	0	0.07	0.03
Mutation rate/bacterium/generation (m+mc) 1n2/N	3.9×10^{-10}	3.8×10^{-8}	7.5×10^{-10}	4.3×10^{-10}	3.8×10^{-8}	5.9×10^{-10}

N = mean total count of six 14-day colonies showing no outgrowths.

Colonies on medium I agar were examined daily for rough growths. No new rough elements appeared after 14 days incubation.

Table 7. Results of experiments with Lactobacillus casei strains C2, H2, 430 and 542 to determine the mutational origin and mutation rates of rough from smooth growths

	Variant colonies (no.)			
	Strain C2	Strain H2	Strain 430	Strain 542
No outgrowths	1360	5015	2918	1299
1 outgrowth	139	2	235	241
2 outgrowths	4	0	7	22
3 outgrowths	0	0	1	4
Mean no. outgrowths/colony (m)	0.1	-	0.08	0.19
Probability of drawing a similar sample of outgrowths from a Poisson distribution with m as mean (P)	0.25	-	0.61	0.69
No. of 14-day old colonies with no outgrowths streaked out	20	45	20	20
No. of these plates revealing rough colonies	1	0	0	1
Mean no. concealed rough growths/colony (mc)	0.05	0	0	0.05
Mutation rate/bacterium/generation (m+mc) 1n2/N	1.5×10^{-9}	-	1.1×10^{-9}	2.4×10^{-9}

N = mean total count of six 14-day colonies showing no outgrowths.

Colonies on medium I agar were examined daily for rough growths. No new rough elements appeared after 14 days incubation.

The P values given in Tables 6 and 7 represent the chances of drawing similar samples from Poisson distributions with means equal to the mean number of growths/colony. The values obtained are taken to indicate that the rough outgrowths were randomly distributed and support the theory of the mutational origin of the events which started the rough clones (Ryan et al. 1955). Mutation rates/bacterium/generation (Luria & Delbrück, 1943) are also presented in Tables 6 and 7; these are low and may be overestimated. The reason is that acid Giemsa staining of organisms in smooth growths reveal from 1 to 4 chromatinic bodies/organism and the mutation rate is expressed as such, and not per nucleus. There is a possibility that rough variants did arise during colony formation on TGA but failed to register phenotypically because of lack of selective advantage. This was investigated by streaking 50 entire 5-day colonies of strain 300 from TGA on to 4 plates each of medium I. After incubation for 48 hr plates were examined with a dissecting microscope for pure medusa-head colonies.. Plates representing 10 colonies had these rough clones present. Application of the zero Poisson distribution gives 0.22 for the mean number of unobserved rough clones present in 5-day smooth colonies on TGA. Very similar results were obtained when 5-day colonies of strain 300 on medium I containing 2% (w/v) glucose were likewise plated on medium I agar.

The effect of inoculum-size on the formation of papillae and fan-shaped outgrowths by strain C9 is shown in Table 8. The incidence of fan-shaped outgrowths/colony bore a direct relationship to the number of colonies/unit area. The number of papillae on the other hand had an inverse relationship. Experiments with strains 300 and 316 yielded similar results. Distribution of colonies bearing different numbers of fan-shaped outgrowths or papillae, or the sum of the latter 2 did not approach Poisson distributions at any of the degrees of crowding used in the above experiments. Differences in the thickness of the layer of medium had no effect on secondary colony formation.

Growth rates of parent and F variant strains. Measurement of the optical density of cultures growing in medium I broth are shown in Fig. 1. The slopes of these lines show that the F variants of L. casei grew more rapidly than the wild strains under the conditions of the experiment. Generation times of wild-types and F variants calculated from viable counts during the exponential period of growth always showed those of the F variant to be the shorter. The actual times obtained with any pair of strains were, however, so non-reproducible

that this method of obtaining absolute generation times was soon abandoned.

Table 8. The effect of crowding on the development of papillae and fan-shaped outgrowths by Lactobacillus casei strain C9

Samples (0.1 ml.) of 4 different dilutions of overnight growth of cultures in tomato glucose broth were spread on medium I in similar Petri dishes. These were incubated at 37° in an atmosphere of carbon dioxide for 8 days. Counts were done on 8 areas of 3 $cm.^2$ for each dilution.

Dilution	No. of colonies/ 72 $cm.^2$	Mean no. of secondary colonies: Fan-shaped outgrowths/ colony	Papillae/ colony
1×10^{-5}	1304	0.44	1.95
5×10^{-6}	488	0.28	3.24
1×10^{-6}	160	0.04	3.49
5×10^{-7}	56	0	5.72

This irregular behaviour was attributed to the fact that L. casei grows in chains of different lengths. Generation times of the wild-type and F variant of strain 300, calculated from total counts in 6 different experiments, varied from 173 to 185 min. for the wild-type and from 147 to 154 min. for the F variant. These results are considered reliable since individual organisms in the chains could be counted and deaths during the period of the experiment (usually about 10 hr) would be minimal. Experiments with wild-types and F variants of strains C9 and 316 yielded similar results. These results confirm the indications above that F variants have shorter generation times than wild-types.

The changes in pH value in medium I broth cultures of wild-types and F variants were identical and minimal. During incubation for 17 hr there was a gradual change from pH 6.35 to 6.7.

Subcultures were also made from centrally situated papillae of colonies of

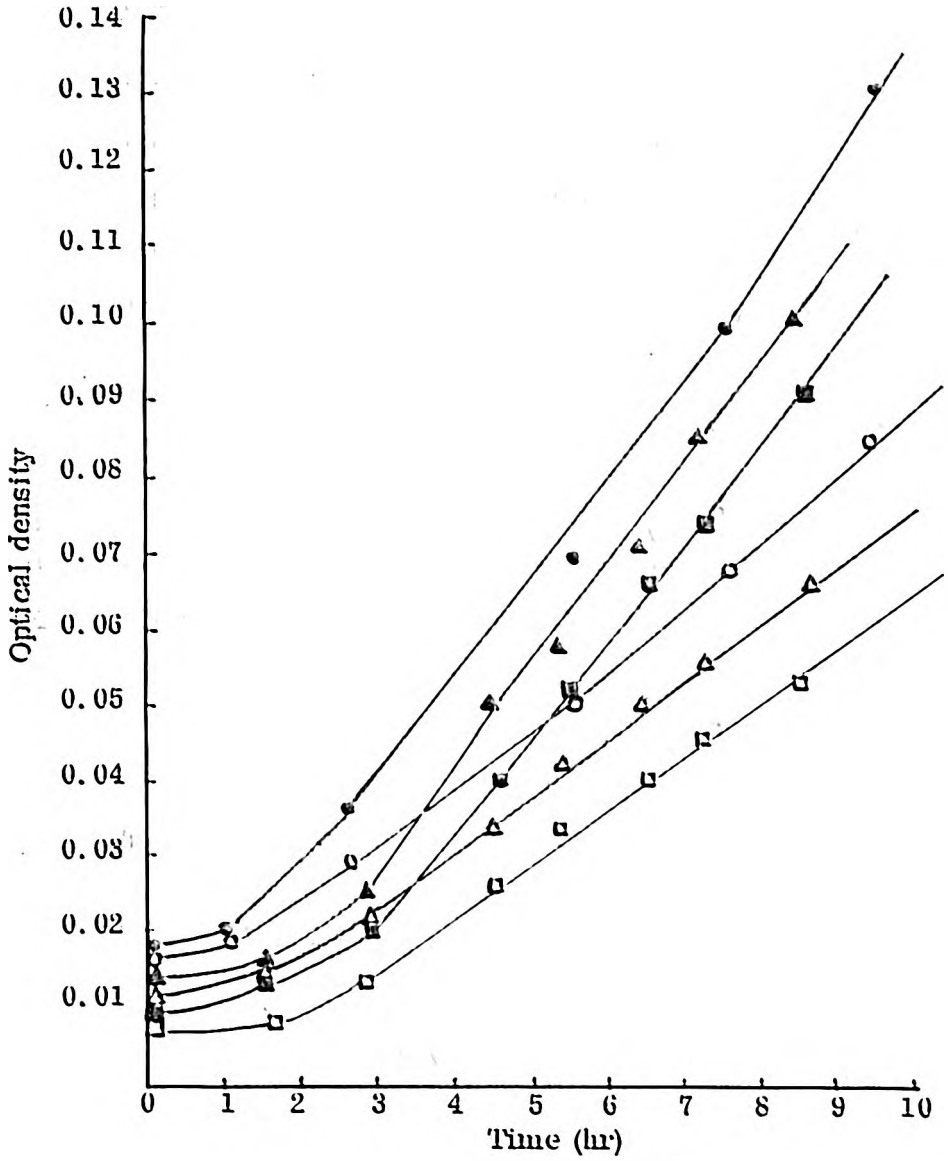


Fig. 1. Photometric determinations of growth of wild-types and F variants of strains C9, 300 and 316. Overnight cultures of wild-type and F variant colonies in 6 ml. of medium 1 broth were transferred into 100 ml. volumes of fresh pre-warmed broth and maintained at 37° for 10 hr. At intervals samples were removed and optical densities read on a Zeiss spectrophometer at 660 mμ. Strain 300 wild-type, ○; strain 300 F variant, ●. Strain C9 wild-type, △; strain C9 F variant, ▲. Strain 316 wild-type, □; strain 316 F variant, ■.

the 3 strains examined. A limited number of F variant-like colonies and wild-type-like colonies so obtained were examined by all the above methods. Organisms comprising the 2 types of colony were found to possess properties identical with those in F variant and wild-type colonies, respectively.

DISCUSSION

The rough outgrowths present on colonies of Lactobacillus casei form unstable suspensions in 0.85% (w/v) NaCl solution and broth, and differ antigenically from the parent colony. They thus conform to R variants of bacteria described by Arkwright (1920, 1921). Arkwright (1924), Hadley (1926) and Burnet (1927) also demonstrated that S and R variants of a strain might have different bacteriophage specificities. Strain 316 conforms to this rule in that only the S variant adsorbed phage 316. Both the S and the R forms of strain 300 are susceptible to phage 300. This indicates that the change in surface structures associated with the S-R variation is not necessarily of the same nature or extent in all strains. Arkwright (1920) noted this in his first communication on the S-R variation and Bisset (1938) could not demonstrate serological differences between S and R variants of a strain of Shigella dysenteriae (Flexner). No biochemical differences were detected as between the S and R variants of the strains examined. Rogers (1934) also observed that the fermentation reactions and growth temperatures of S and R variants of a strain of L. acidophilus were identical. Hadley et al. (1930) noted biochemical differences between S and R variants of their group I (? L. casei) strains. Tracy (1938) encountered a strain of L. plantarum in which the S-R variation was associated with a loss of saccharolytic properties and Pederson (1947) claimed that L. acidophilus and L. casei were simply rough and smooth variants of the same organism. Pederson's claim has never been confirmed and was criticized by Davis (1956) on the grounds of faulty technique.

Entire populations of lactobacilli may change their colonical form abruptly when plated on different media. This has been shown by Rogosa & Mitchell (1950), McDonald & Frazier (1951) and by Green et al. (1955). Similar changes have been produced with the colonial morphology of Lactobacillus casei strains C9R and 316R. This altered morphology of a population is not the result of genetic changes but is merely a difference in

phenotypic expression in different environments. The rough growths encountered in all the strains of L. casei studied here started as randomly distributed events during the development of the colony and may be regarded as mutations which were selected by the particular ecological conditions prevailing in the colony and its environment. The results obtained indicate that the absence of fermentable carbohydrates in medium I provided some selective advantage for the R mutants. Hadley et al. (1930), however, observed smooth colonies of their group I (? L. casei) to undergo 'marginal dissociation' to R forms on a glucose infusion agar. Barber & Frazier (1945) selected R variants of L. casei by repeated subculture in carrot liver broth at 48°, with platings on the agar. Weinstein et al. (1933) used a tomato juice broth and agar without any additional carbohydrate. A selective advantage for mutants is the essence of the papillary method of determining mutation rates. This was shown in the present work by finding genetically rough clones concealed in colonies of L. casei strain 300 on TGA and on medium I + 2% (w/v) glucose. Apart from L. casei var. casei strain 300 which has a high S-R mutation rate and L. casei var. rhamnosus strain H2 which forms very few rough outgrowths, the remaining strains of both species had fairly uniform S-R mutation rates and the latter feature did not appear to possess any taxonomic significance. The mutation rates from S to R are low for morphological variants of bacteria (Coetzee, 1959a) but there is no reliable independent method of confirming these results. The reason is that all other methods of determining these mutation rates involve enumeration of R organisms. Because of chain formation viable counts are misleading. Total counts are also unsatisfactory because, despite thorough shaking of cultures of rough growths, numerous chains of organisms which extend across the microscopic field are invariably present and constituent organisms cannot be counted.

No back mutation of the R to the S form was demonstrated on agar or in continuous culture. The S form is perfectly stable in continuous culture, but if R variants should derive from the S form in nature the latter must possess some selective advantage to maintain itself as the wild or modal type (Rogosa & Sharpe, 1959). With due regard to the limitations of the experiments, the fact that in mixed continuous cultures the S form invariably entirely replaced the R form may be taken to indicate that the former possesses a shorter generation time (Armitage, 1952). Barber & Frazier (1945) main-

tained that S forms of Lactobacillus bulgaricus divided more rapidly than the corresponding R variants. With the R cultures studied here the growth rates could not be determined because of the difficulties of enumeration previously mentioned. Metabolically the 2 forms appear similar, but it may be that in Nature the individualistic S form possesses some other subtle advantage over the gregarious long-chain R mode of existence.

Organisms that are able to grow out above their relations in a colony must possess some selective advantage. This has been proved many times, (see Ryan et al. 1955). These workers described the development of papillae arising from colonies of a histidineless strain of Escherichia coli, growing in the presence of subminimal quantities of histidine. The selective advantage enjoyed by the mutant organisms comprising these papillae was found to be due to the fact that they were histidine independent. Shah & Iyer (1960) showed that organisms in secondary colonies of Bacillus subtilis were favoured by their greater ability to neutralize the acids initially produced by fermentation in colonies on a medium containing glucose and peptone. The Lactobacillus casei F variants encountered in the present work differed from the wild-types in that colonies of the former were larger, more opaque and contained more bacilli than the latter when growing on medium I. These differences may be associated with the observation that the F variants had relatively shorter generation times than the wild-types under the conditions of study. Absolute generation times could only be determined by means of total counts. The times obtained were much longer than those of L. casei and other lactobacilli determined under optimal conditions by a variety of methods, Mason (1935). This was probably due to the poor growth supporting qualities of medium I. It is interesting to note that L. casei has the shortest generation time of all the species of lactobacilli listed by Mason (1935). It might have been anticipated that fast growing mutants of other species of lactobacilli which have longer generation times would possess a greater selective advantage and be encountered more frequently, yet secondary colony formation was practically confined to strains of L. casei var. casei and L. casei var. rhamnosus. Fast-growing variants of E. coli detected in continuous cultures have been reported by Novick & Szilard (1950) and by Moser (1955) In the present work the fan-shaped outgrowths were pure cultures of F variants but uncertainty still remains regarding the nature of many papillae. Most

papillae apparently consisted of mixtures of F variant and wild-type organisms. Some consisted of pure cultures of F variant-producing organisms, but subculture of many papillae produced only wild-type colonies. It may be argued that a papilla is a dynamic structure and that it might, at a particular stage of its development, still be contaminated with parent organisms. Despite the limited means of identification available this could hardly explain the many instances where no F variant-like colonies were detected in subcultures of well-developed papillae. Loss of viability of F variants in papillae with survival of contaminating wild-type organisms appears unlikely as an explanation of the latter finding. First, because there were no indications that F variant organisms tended to die off more rapidly than the wild-type and secondly because F variants were often present in other papillae on the same colony.

The fact that all secondary colonies failed to appear on media which contained some carbohydrate is no argument for the basic homogeneity of these colonies. The R mutants also lose their selective advantage under these circumstances. Another argument in favour of the basic heterogeneity of secondary outgrowths described here is the fact that the distribution of colonies bearing homogeneous outgrowths and papillae often follow a Poisson distribution (Ryan et al. 1955; Coetzee, 1959b; Coetzee & Sacks, 1960a, b). The fact that the distribution of colonies of L. casei bearing varying numbers of secondary colonies did not follow such a distribution is another factor against the inherent identity of all these structures. However, an inverse relationship existed between numbers of papillae and fan-shaped outgrowths/colony on crowded and on sparsely inoculated plates. This might be explained on the basis that on crowded plates the colonies were smaller and secondary colonies which arose in the form of papillae thus stood a greater chance of spilling over the margins of the colonies and developing into fan-shaped outgrowths. This suggests that many papillae and fan-shaped outgrowths were of the same composition. Because other undetected phenotypic differences (and possible selective advantages) might be involved it was decided not to couple the single manifestation of fast growth with secondary colony formation. For reasons mentioned above it was not possible to obtain proof of the random origin of the F variants and to determine the mutation rates involved. The fan-shaped outgrowths, however, may be regarded as fast-growing variants of L. casei which possess a selective advantage under the austere conditions

prevailing in growth on medium I. These variants maintained their characteristics through numerous platings and were stable in the environment tested. Loss of their selective advantage in the presence of small amounts of fermentable carbohydrates is, as in the case of the R variants, a possible explanation of why the wild-type maintains itself as the modal form.

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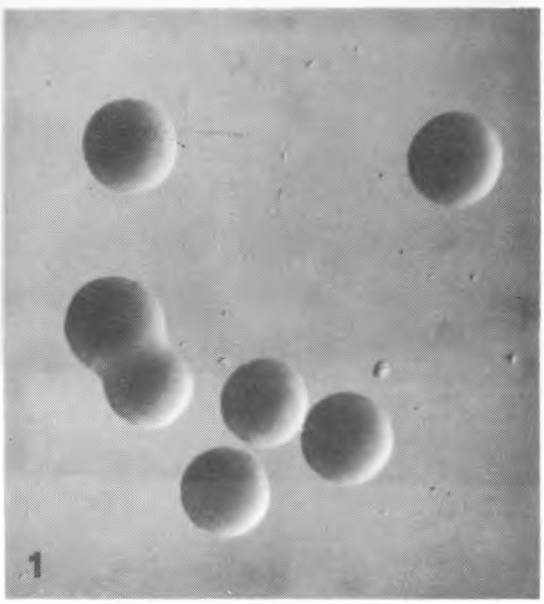
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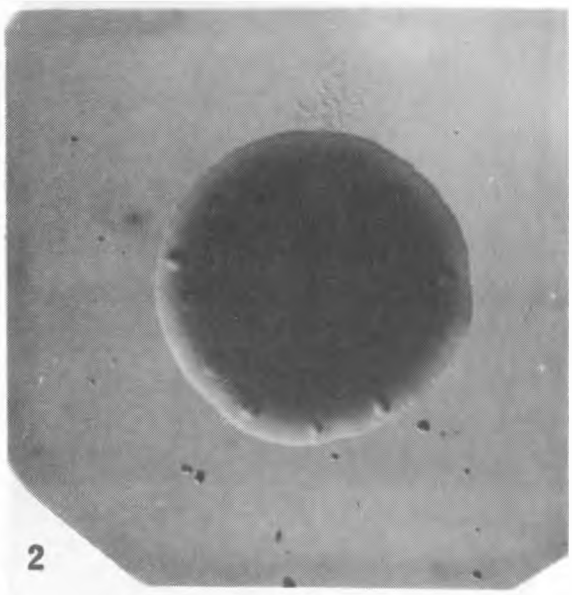
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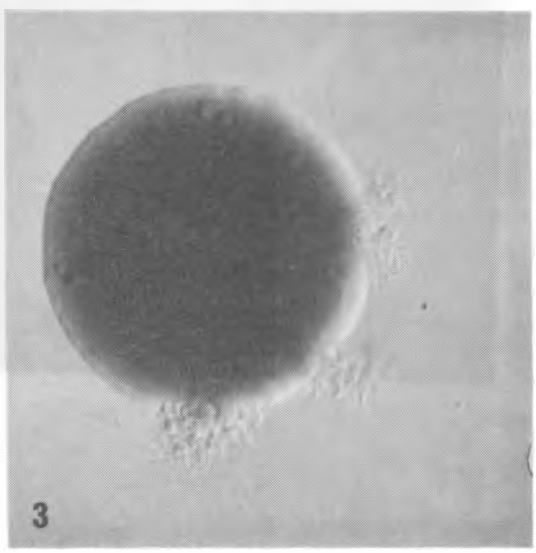
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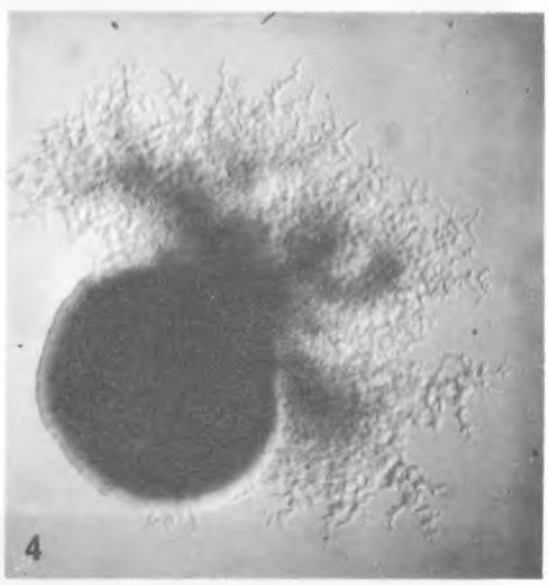
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Plate 1









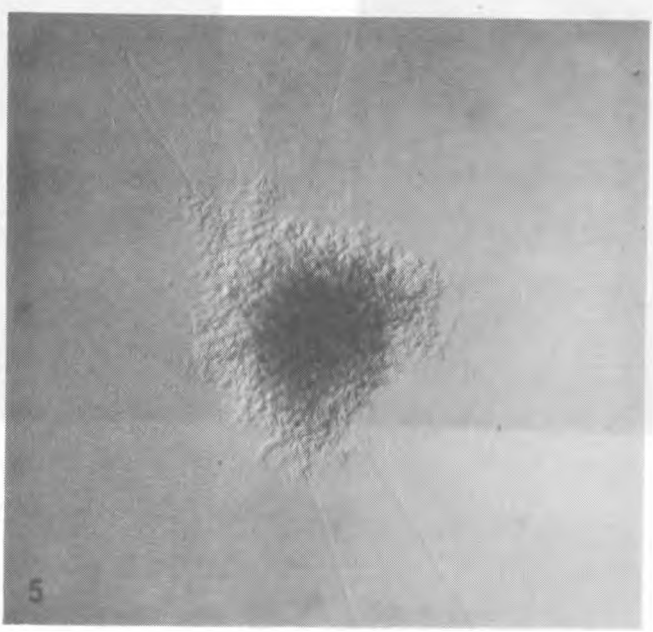
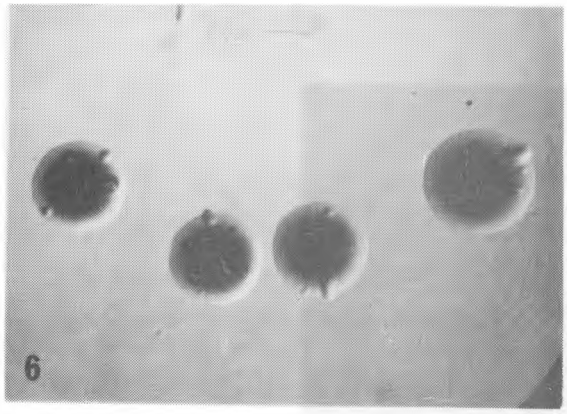
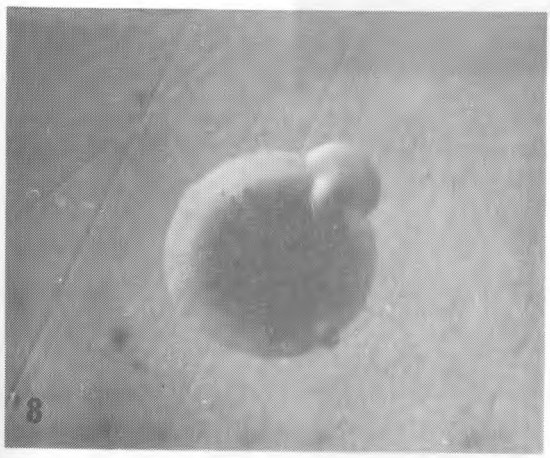
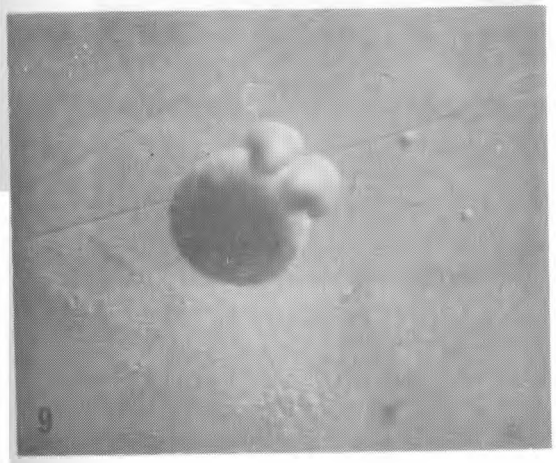


Plate 2









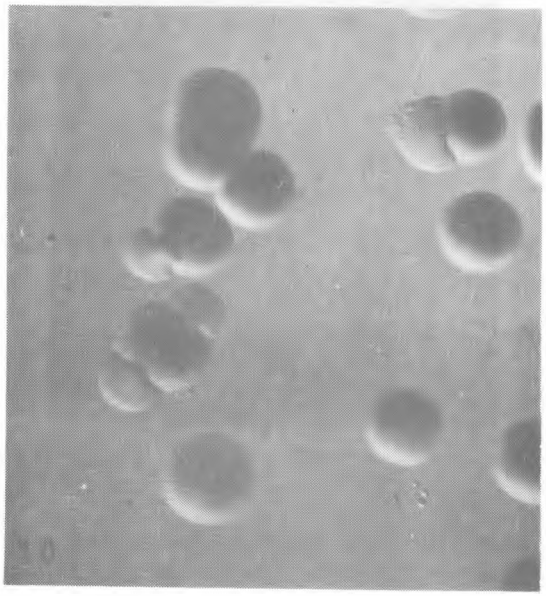
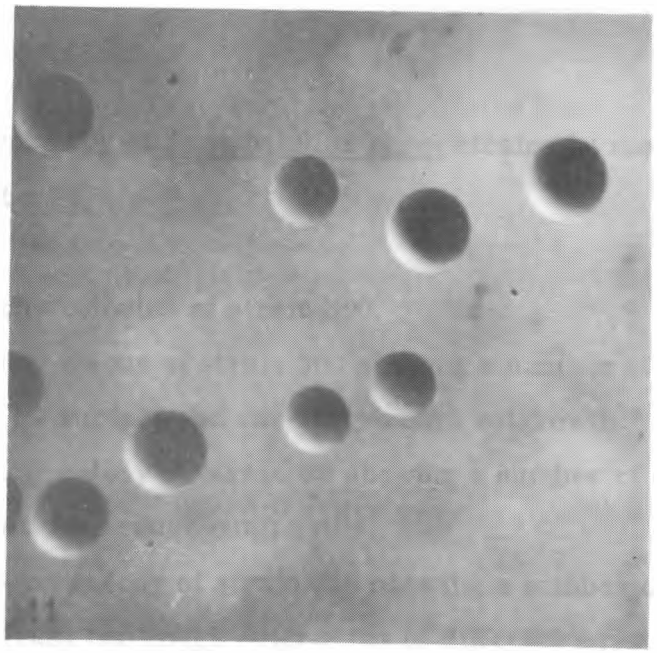
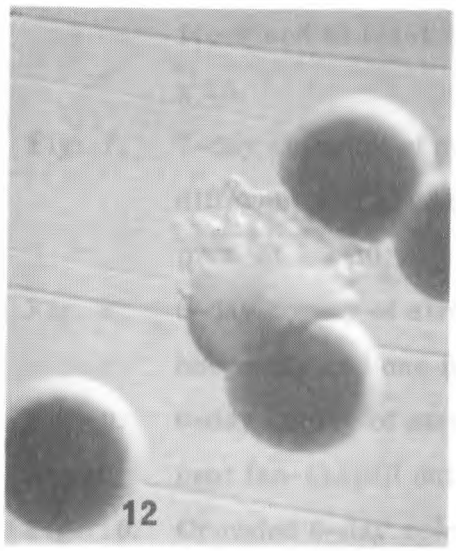
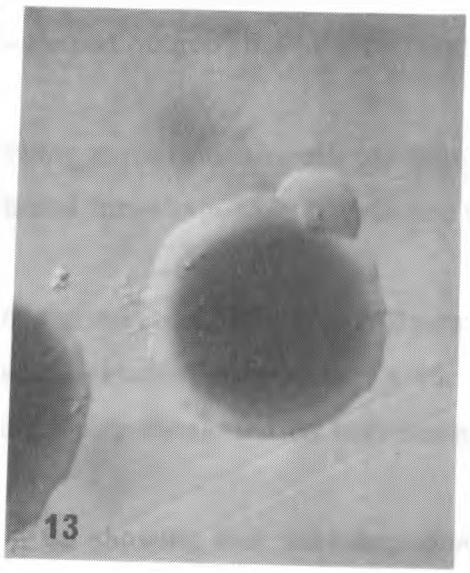


Plate 3







EXPLANATION OF PLATES

Plate 1

Colonial morphology of Lactobacillus casei strains on medium I agar; magnification about x 25.

Fig. 1. 2-day colonies of strain 300.

Fig. 2. 8-day colony of strain 300 showing a number of smooth excrescences on its surface and one early rough outgrowth.

Fig. 3. 8-day colony of strain C9 showing a number of excrescences and at least four rough outgrowths.

Fig. 4. 10-day colony of strain 316 showing a number of excrescences and at least two rough outgrowths of different ages.

Fig. 5. 2-day medusa-head colony of strain C9. This is a subculture of a rough outgrowth from a C9 colony.

Plate 2

Fig. 6. 4-day colonies of strain 300 showing smooth papillae on their surfaces and at least two early fan-shaped outgrowths at the margins. x 25.

Fig. 7. 7-day colonies of strain 300 showing numerous smooth papillae of different sizes. An R outgrowth and fan-shaped outgrowth are also present. x 40.

Fig. 8. 6-day colony of strain 316 showing numerous smooth papillae and one early and one fully developed fan-shaped outgrowth. x 40.

Fig. 9. 6-day colony of strain C9 showing sharp demarcation between adjacent fan-shaped outgrowths. x 25.

Fig. 10. Crowded 6-day colonies of strain C9 showing four fan-shaped outgrowths and numerous minute papillae. An R outgrowth is seen on one of the fan-shaped outgrowths. x 25.

Plate 3

Fig. 11. 2-day colonies of the wild-type and F variant of strain 316. F variant colonies are clearly distinguishable by their larger diameter and greater density. x 25.

Fig. 12. A fan-shaped outgrowth on a 6-day colony of strain C9 showing the development of an R outgrowth from its margin. x 40.

Fig. 13. 8-day colony of strain 300 with a fan-shaped and R outgrowth on its margin. Numerous papillae are present on the surface. x 40.

CHAPTER 5

ISOLATION AND CHARACTERIZATION OF LACTOBACILLUS BACTERIOPHAGES

SUMMARY

Four phages active on strains of Lactobacillus casei and 40 active on strains of L. fermenti have been isolated from sewage. The properties of the 4 L. casei phages and 11 of the L. fermenti phages are presented. Most of these phages are species specific. The 2 groups of phages are serologically distinct but the members of each group are related serologically. All the phages have slow adsorption rates. The L. casei phages are citrate insensitive, while some of the L. fermenti phages are citrate sensitive. The L. fermenti phages have shorter latent periods than the L. casei phages and this correlates directly with the generation times of their hosts. The phages are large and have a tadpole appearance. The susceptibility of 121 strains of L. fermenti to the 40 L. fermenti phages was tested by the use of an RTD of the phages. Ninety-three of the strains could be divided into 3 phage groups. comprising a number of different types. In all 76 different phage types were revealed.

INTRODUCTION

Bacteriophages were discovered independantly by Twort (1915) and d'Herelle (1917) and their discovery was followed by much controversy as to whether they were inanimate autocatalytic agents or self-reproducing organisms like viruses, (see Stent, 1963). The latter hypothesis which was adopted at an early stage by d'Herelle has prevailed and their properties of infectivity, absence of metabolic activity, possession of only one type of nucleic acid (see Hayes, 1965) have led to the proposal of phages as a model of host-virus relationship (Lwoff, 1959). Much of what is known of the nature of phage and the physiology of phage infection has accrued from research into the behaviour of the set of 7 virulent phages of Escherichia coli B known

as the T series (Tl - T7) which were collected by Demerec & Fano (1945). Many of the properties of these phages have been found to apply to phages active on other genera (see Discussion). The properties of phages and methods for their study have been the subject of several detailed reviews (Adams, 1959; Garen & Kozloff, 1959; Stent, 1959; Sinsheimer, 1960; Kellenberger, 1961; Mahler, & Fraser, 1961; Stent, 1963).

Phages active on a wide variety of Eubacteria have been described (see Stent, 1963). The first phage active on lactobacilli was reported by Kopeloff (1934). This phage was isolated from sewage. No further details were given apart from the statement that it attacked certain strains of Lactobacillus acidophilus and L. bulgaricus and did not attack R variants of these strains. Kiuru & Tybeck (1955) isolated 2 phages from starters used in the manufacture of Swiss cheese. These phages had very restricted host ranges. The one was active on a strain of L. lactis and the other on 2 strains of L. helveticus. Meyers, Walter & Green (1958) isolated from saliva a phage active on a strain of L. casei. This phage was characterized by Walter (1958). The purpose of this study was the isolation and characterization of further phages active on members of this genus and the possible development of a phage-typing scheme for these organisms. Fifteen of the phages investigated here were isolated by Coetzee, de Klerk & Sacks (1960) and de Klerk, Coetzee & Theron (1963).

METHODS

Media. The media used were tomato glucose broth and agar (TGB, TGA) described in Chapter 2. Top layer agar contained 1% (w/v) agar. In some adsorption experiments the broth was supplemented with vitamin-free pancreatic casein digest (Nutritional Biochemicals Co., Cleveland, Ohio) 10g./1. and/or 50% (v/v) of Hanks balanced salt solution (Weller, Enders, Robbins & Stoddard, 1952). In other experiments Tween 80 was omitted from all media. Cultures were incubated at 37^{o} in an atmosphere of 100% carbon dioxide.

Organisms. Three hundred and seventy-three strains of lactobacilli comprising 181 strains of Lactobacillus acidophilus, 57 of L. salivarius, 17 of L. casei, 38 of L. casei var rhamnosus, 8 of L. plantarium, 61 of L. fermenti, and 11 of L. brevis, were used as possible hosts for bacteriophages. In subsequent experiments 121 strains of L. fermenti were used in attempts to isolate further phages active against strains of this species. The isolation and characterization of these strains has been described in Chapter 3. In addition L. acidophilus strain A1, L. salivarius strain 8C, L. casei strains C2 and C9, L. casei var. rhamnosus strain H2, L. plantarum strain 3J, L. fermenti strain F1 and L. brevis strain X1 kindly supplied by Dr. M. Elizabeth Sharpe of the National Institute for Research in Dairying, Reading, Berkshire, England, were also used.

Isolation of phages. The enrichment technique of Adams (1959) was used. Overnight growths of different lactobacillus strains in TGB (1 ml.) were mixed in groups of 12 in different experiments. Five millilitres of the mixtures were transferred to 100 ml. of fresh warmed TGB. The mixed cultures were inoculated with 10 ml. of the supernatant of samples of untreated sewage which had been centrifuged for 15 min. at 6000 g. Similar mixed cultures were enriched with pooled saliva or vaginal washings. Cultures were incubated for 10 days and aliquots were centrifuged daily and tested for the presence of phage. This was done by spotting the supernatants on cultures of each of the 12 strains inoculated into a top layer by the agar layer method (Adams, 1959). After overnight incubation plates were examined for lytic activity. Phage was purified by repeated picking of single plaques into young broth cultures of the host organism.

Host range determination. In addition to their host strains the phages were tested against 246 strains of lactobacilli comprising 47 strains of Lactobacillus acidophilus, 19 of L. salivarius, 25 of L. casei, 22 of L. casei var. rhamnosus, 39 of L. plantarum, 56 of L. fermenti, 37 of L. brevis and 1 strain of L. helveticus. This was done by titration of 0.1 ml. of undiluted and 10^{-2} and 10^{-4} dilutions of lysates (10^{8} plaque-forming particles/ml.) by the agar layer method (Adams, 1959) and by spotting a large drop of the corresponding dilution in the middle of the plate after the

top layer agar had set. Subsequently the host ranges of the L. fermenti phages were tested against 121 strains of L. fermenti, and an attempt was made to classify the latter into phage types. Routine Test Dilutions (RTD) which gave confluent lysis of the propagating strains were determined for each phage and the susceptibility of each strain was tested by spotting standard drops of twice the concentration of this RTD on lawns of all the organisms. After overnight incubation areas of confluent lysis were recorded.

Phage lysates. These were prepared by inoculating 40 ml. of a young broth culture from a plaque. After overnight incubation the cultures were clear and had plaque forming titres of about 1×10^9/ml. Phage suspensions were stored above 0.1 vol. of chloroform at 4^o.

Assay of phage. Phages were assayed by the agar layer method (Adams, (1959). Ten-fold serial dilutions of lysates in TBD (0.1 ml.) were transferred to 2.5 ml. of melted toplayer held at 45^o and seeded with 2 drops of overnight broth culture of the host organism. The mixtures were poured on to agar plates. Plaques were counted after overnight incubation.

Antiphage sera. Phage antisera were prepared in rabbits (Adams, 1959). Lysates (10^9 plaque-forming particles /ml.) were injected subcutaneously (5 ml.) and intravenously (1 ml.) on alternate days for 3 weeks. Rabbits were bled 7 days after the last injection. Host-cell antibodies were removed by absorption with bacterial suspensions. Sera were stored frozen.

Assay of antiphage sera. Neutralization constants of sera were determined with homologous and heterologous phages by the method of Adams (1959). Dilutions of antisera were mixed with phages at 37^o. Phage was assayed at minute intervals and the proportion of surviving phage was plotted against time. Neutralization constants (K) were derived from the equation

$$K = 2.3\ D/t \times \log po/p$$

where po = phage assay at zero time, p = phage assay at time t min., D = final dilution of serum. The equation is valid where 90 - 99% of the phage in the reaction mixture is neutralized, (Adams, 1959).

Adsorption rates. Adsorption constants in broth at 37^o were determined by the titration of unadsorbed phage (Adams, 1959) in mixtures of phage and

host organisms at intervals of 1 minute. The adsorption constants (ml./min.) were derived from the formula

$$K = 2.3/(B)t \times \log po/p$$

in which po = phage assay at zero time, p = unadsorbed phage at time t min. and B = concentration of bacteria. The latter was determined by direct counts as described in Chapter 4.

Latent periods and burst sizes. These were determined in one-step growth experiments (Ellis & Delbrück, 1939). The method used was that of Adams (1959). Phages were adsorbed to their hosts in broth at 37^{o} at an input ratio of 0.1 for 10 min. Unadsorbed phage was neutralized with antiserum. Infected bacteria were suitably diluted to growth tubes, held at 37^{o} and assayed for phage at 1 minute intervals.

Phage resistant mutants. These were selected by the method of Adams (1959). Phage was adsorbed to host cells (1×10^{8}/ml.) in broth at 37^{o} at a multiplicity of input of 10 for 15 min. The mixtures were spread on TGA and incubated for 3 days. Single colonies were picked suspended in a little broth and restreaked on fresh TGA. Single colony isolations were repeated to free the cultures of contaminating phage.

Sodium chloride. The effect of sodium chloride (25%. w/v) on the stability of the phages was tested by the method of Kiuru & Tybeck (1955).

Citrate sensitivity. The citrate sensitivity of the phages was expressed as their relative efficiency of plating (e.o.p.) on media supplemented with 2% (w/v) sodium citrate.

Heat inactivation. Heat inactivation constants were determined by maintaining the phages at 55^{o} in broth and assaying samples at intervals. The samples were brought to 20^{o} before plating. The constants were derived from the formula

$$K = 2.3/t \times \log po/p$$

where po = phage assay at zero time and p = phage assay at time t min.

Generation times. The generation times of the lactobacilli were determined by total counts as described in Chapter 4.

Electron Microscopy. Phage lysates were purified and concentrated by differential centrifugtion (alternate cycles at 12,350g for 90 min. and 3090g for 10 min.) in a Servall centrifuge (Model SS-4). Purified suspensions (plaque-forming titre 1 x 10^{12}/ml.) were sprayed on to collodion covered grids, air-dried, shadowed with a gold+palladium alloy and examined with a Philips EM100 electron microscope.

RESULTS

Isolation and host range of Phages

From mixed cultures of various Lactobacillus spp. enriched with 10 batches of sewage 15 bacteriophages were isolated. They are identified according to the number of the original host organisms. These are 41, 69, 206, 222, 222a, 276, 315, 514, 517, 535, 547 isolated on Lactobacillus fermenti hosts and 300, 316, 356, 780 isolated on L. casei hosts. Lytic activity was never demonstrated before the second and usually only after the sixth day of incubation. No phage was isolated from mixed cultures supplemented with pooled human saliva or vaginal secretions. Subsequently a further 29 phages were isolated from mixed cultures of the 121 L. fermenti strains in various combinations enriched with 6 batches of sewage in different experiments. These phages were No. 308, 360, 544, 549, 757, 762, 766, 839, 885, 937, 968, 970, 991, 1010, 1025, 1056, 1058, 1072, 1074, 1126, 1128, 1132, 1154, 1158, 1182, 1216, 1287, 1301, F1.

Host range. The results of preliminary host range experiments on the 11 L. fermenti phages and 3 L. casei phages first isolated are shown in Table 1. Discrepancies were observed in these host ranges as determined by the 2 methods. In many instances an area of lysis was present where the drop of undiluted lysate was placed but the plaque-forming titre of the phage on the particular organism was zero. This effect which was specific and reproducible always extended the intra-species and often the inter-species host-range. Dilution of the lysate before application of the drop in these cases never resulted in the occurrence of individual plaques in the area of the drop but simply in the disappearance of the entire lytic area. This phenomenon

Table 1. Host range of Lactobacillus phages

Phages	Lactobacillus species						
	acido-philus (10)*	sali-varius (7)	casei (11)	casei var. rhamnosus (11)	plant-arum (10)	fer-menti (21)	brevis (10)
41	0	0	0	0	0	7	1
	2	0	1	0	0	9	3
69	0	0	0	0	0	5	1
	0	0	1	0	0	11	3
206	0	0	0	0	0	9	1
	0	0	1	0	0	6	1
222	0	0	0	0	0	12	1
	1	0	2	0	0	5	2
222a	0	0	0	0	0	12	3
	0	0	1	0	0	6	1
276	0	0	0	0	0	6	1
	1	0	1	0	0	10	1
300	0	0	2	1	1	1	0
	0	0	4	4	0	0	0
315	0	0	2	0	0	10	1
	0	0	0	0	0	0	1
316	0	0	4	0	0	0	0
	0	0	3	8	3	1	0
356	0	0	1	1	1	0	0
	0	0	2	0	1	0	0
514	0	0	0	0	0	3	0
	0	0	0	0	0	7	2
517	0	0	0	0	0	7	1
	0	0	1	0	0	10	1
535	0	0	0	0	0	11	1
	0	0	0	0	0	8	3
547	0	0	0	0	0	4	1
	0	0	1	0	0	11	3

* Figures in brackets indicate number of strains tested.

Upper figure, number of strains with phage efficiency of plating equal to host strain.

Lower figure, number of strains on which only a lytic drop area was demonstrated.

was investigated by mixing phage and the organism at 37°. After a period of 20 min. for adsorption an aliquot was centrifuged to clarity and the supernatant titrated on the host strain for unadsorbed phage. Simultaneously another aliquot was titrated for viable bacteria and unadsorbed phage plus infective centres on both the organism under investigation and the host strain. All titrations were completed within 30 min. of mixing. Results of a typical experiment are presented in Table 2. Abortive infection with killing of the

Table 2. Investigation of phage 222 which produces a drop area of lysis on Lactobacillus casei 316 without individual plaque formation. Adsorption 20 min. at 37°C

Initial concentration L. casei 316	7.0×10^8/ml.
Initial concentration of phage 222	4.5×10^8/ml.
Multiplicity of infection	0.64
Free phage after adsorption	5.8×10^6/ml.
Phage adsorbed (per cent)	98.7
Surviving bacteria	3.6×10^8/ml.
Bacteria killed by phage infection (per cent)	48.6
Unadsorbed phage + infective centres on L. casei 316	0
Unadsorbed phage + infective centres on host L. fermenti 222	1.0×10^7/ml.
Infective centres on L. fermenti 222	4.2×10^6/ml.
Killed L. casei 316 not yielding progeny phage (per cent)	98.8

organism provides an adequate explanation for the phenomenon. A similar phenomenon has been encountered with Salmonella and Staphylococcus phages (Anderson & Williams, 1956) and with Proteus phages (J.N. Coetzee, personal communication). Extension of the host range experiments with additional strains of lactobacilli and inclusion of phage 780 in the tests confirmed the above findings. Individual L. fermenti phages productively lysed 25%-40% of a further 35 L. fermenti strains tested and caused abortive infection with host killing in 14.3%-66% of these organisms. The L. casei phages productively

lysed 12%–40% of an additional 25 L. casei strains tested, and caused abortive infection with host death in 15% of these organisms. A few of the L. fermenti phages caused abortive infections with host killing of 3 L. acidophilus, 3 L. casei and 3 L. brevis organisms and productive infections in 2 L. casei and 3 L. brevis strains. The L. fermenti host no. 296 on which L. casei phage 300 caused lytic infection has been lost and extra-species activity of the L. casei phages is limited to the action of 3 of these on one or more of 3 L. plantarum organisms. The host ranges of the 11 L. fermenti phages differ from one another, while those of 2 of the L. casei phages (nos. 300, 780) are identical. These 15 phages were also tested against 20 strains each of Escherichia coli, Proteus, Salmonella and Shigella spp. and 27 strains of alpha haemolytic streptococci, 21 strains of beta haemolytic streptococci, 6 of Streptococcus pneumoniae, 6 of enterococci and 26 strains of Staphyloccus aureus. No activity was detected on any of these organisms.

The 40 L. fermenti phages tested against 121 strains of L. fermenti showed lytic activity on 14%-66% of these strains when undiluted lysates (1×10^8 p.f.u./ml.) were spotted on lawns of these organisms. Many of the strains were attacked by the same phages. When the RTD of each phage was spotted and only areas of confluent lysis recorded the host ranges of the phages were markedly curtailed and patterns of sensitivity became evident among the L. fermenti strains. Many areas which gave complete lysis with high titre lysates showed varying numbers of discrete plaques when the RTD was used, illustrating differences in e.o.p. of the phages on heterologous sensitive strains. Other drop areas showed a uniform turbidity with the latter method, suggestive of abortive infection. By this method 93 of the strains could be divided into 3 phage groups each comprising a number of different types. These results are summarized in Table 3. The groups were determined by the number of phages in the patterns of individual types. In all, 76 different phage types were revealed by this procedure. The remaining 28 strains gave only turbid spots or no reactions and were untypeable with the RTD used. When tested with higher concentrations of phage (1×10^9 p.f.u./ml.) 18 of the 28 strains could be differentiated into a further 17 types while 10 strains were not susceptible to any of the phages. This scheme has been found to be reproducible but test results are greatly influenced by the phage concentration and critical dilutions of the typing phages must be used.

Properties of Lactobacillus phages

The 11 Lactobacillus fermenti and 4 L. casei phages initially isolated were characterized in detail and have the following properties. All these phages were stable in chloroform thus differing from a series of clostridium perfringens phages (Smith, 1959) and Brucella phages (McDuff, Jones & Wilson, 1962).

Electron microscopy. Electron micrographs were taken of the 4 Lactobacillus casei phages and 4 of the L. fermenti phages. These revealed tad-pole morphologies. The L. casei phages had larger heads (average dimensions 105 x 87 mμ) than the L. fermenti phages (average dimensions 83 x 64 mμ). The tails of the former were slightly shorter and thicker than the L. fermenti phages (average dimensions 194 x 21 mμ and 210 x 18 mμ respectively). The average overall length of the phages was similar (299 and 293 mμ respectively). All the phages showed terminal knobs like Listeria monocytogenes phages (Sword & Pickett, 1961).

Table 3. Phage groups and types of Lactobacillus fermenti

Group	Phage types	No. of phages in type patterns	No. of strains
A	1 - 21 (21)	10 - 20	21
B	22 - 37 (16)	6 - 9	16
C	38 - 76 (39)	1 - 5	56
Untypeable	-	-	28

No. of phages used: 40. No. of strains tested: 121.
Figures in brackets denote number of phage types.

Adsorption rates. The adsorption of all these Lactobacillus phages followed first order reactions. The L. fermenti phages all adsorbed slowly (Table 4) and their adsorption rates could not be correlated with their other properties. The L. casei phages (Table 5) also had slow adsorption rates, with the exception

Table 4. Some properties of Lactobacillus fermenti phages

Phage no.	E.o.p. on citrate media	Growth constants at 37°		Adsorption constants (ml./min.) at 37°	Heat inactivation constants at 55°
		Latent period (min.)	Burst size		
41	1	83	82	2.3×10^{-10}	0.044
315	1	77	60	1.5×10^{-11}	0.156
514	1	85	88	1.5×10^{-11}	0.620
222	1	85	63	4.0×10^{-10}	0.643
222a	1	97	30	2.8×10^{-11}	0.098
69	0.0001	99	35	5.7×10^{-12}	0.088
206	0.0002	72	100	5.7×10^{-11}	0.066
517	0.0001	72	90	2.6×10^{-11}	0.087
535	0.0001	85	73	1.6×10^{-11}	0.093
547	0.0003	87	60	1.6×10^{-11}	0.112
276	0.0001	72	70	1.2×10^{-11}	0.095

Table 5. Some properties of Lactobacillus casei phages

Phage no.	E. o. p. on citrate media	Growth constants at 37°		Adsorption constants (ml./min.) at 37°	Heat inactivation constants at 55°
		Latent period (min.)	Burst size		
300	1	220	20	5.0×10^{-12}	0.190
780	1	220	20	2.1×10^{-11}	0.204
316	1	220	6	3.3×10^{-11}	0.442
356	1	180	33	3.2×10^{-9}	0.104

of phage 356, which differed in many respects from the other L. casei phages. The adsorption of all the phages was unaffected by the omission of Tween (White & Knight, 1958) or the addition of co-factors to the media. The optimal concentration of organisms was 1.0×10^9 colony forming units/ml.; smaller values than this slowed the adsorption rate.

Plaque morphology. The Lactobacillus casei phages formed minute clear plaques of average diameter 0.3 mm. and a shelving edge. The L. fermenti phages formed similar but larger plaques. Phages 41, 69, 276, 535 and 547 formed plaques of average diameter 0.5 mm. and phages 206, 222, 222a, 315, 514 and 517 formed plaques with a diameter of 1 mm. The plaques of individual phages varied somewhat in size and this variation may be explained (McDuff et al. 1962) as due to vagaries of adsorption. The phages are large and the smallness of the plaques may be ascribed to this factor, but the slow adsorption rates may be a contributary factor (McDuff et al. 1962).

Serology. The inactivation of the phages by antisera followed first order kinetics; Table 6 shows the neutralization constants of the phage antisera. These constants are low. The sera of a second series of rabbits immunized with the phages yielded lower K values. Table 6 shows that the Lactobacillus casei phages and L. fermenti phages are serologically distinct and are classified into Groups I and II respectively.

Citrate sensitivity. The Lactobacillus casei phages did not require Ca^{2+} for adsorption (Table 5). L. fermenti phages 41, 222, 222a, 315 and 514 had an e.o.p. value of 1 in the presence of sodium citrate, while phages 69, 206, 276, 517, 535 and 547 had a plating efficiency of 10^{-4} (Table 4).

Heat inactivation. Heat inactivation of the phages at 55° followed first order kinetics and the constants varied from 0.044 to 0.643 (Tables 4, 5). These values did not correlate with other properties of the phages.

Latent period. The latent periods of the Lactobacillus casei phages at 37° ranged from 180 min. (phage 356) to 220 min. (phages 300, 316 and 780). Their burst sizes were below 50 (Table 5). The latent periods of all the L. fermenti phages were less than 100 min. (Table 4), and their burst sizes were between 50 and 100 (Table 4). The latent periods correlated directly

Table 6. Neutralization constants of Lactobacillus phage antisera at 37°

Dilutions of homologous and heterologous phage antisera were mixed with the phages at 37°. Phage was assayed at intervals. The proportion of phage surviving was plotted against time. The survival was a first order reaction from this neutralization constants were calculated.

Phage antiserum no.	Phage no. 41	69	206	222	222a	276	315	514	517	535	547	300	780	316	356
	Minutes $^{-1}$														
41	4	3	2	4	4	4	4	4	4	4	4	0	0	0	0
69	8	11	3	10	10	6	9	8	10	10	10	0	0	0	0
206	8	5	9	6	6	7	2	3	4	3	2	0	0	0	0
222	12	12	5	11	11	7	12	11	13	11	12	0	0	0	0
222a	15	14	9	12	21	17	13	16	16	18	7	0	0	0	0
276	5	5	8	4	4	8	4	3	5	4	3	0	0	0	0
315	3	3	1	3	3	3	9	4	5	4	2	0	0	0	0
514	3	1	1	2	4	1	4	2	3	4	3	0	0	0	0
517	4	5	2	5	6	3	7	4	4	3	5	0	0	0	0
535	10	8	4	8	8	8	10	9	14	12	6	0	0	0	0
547	3	3	6	5	3	2	3	3	3	4	6	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0	0	76	60	3.6	1.2
780	0	0	0	0	0	0	0	0	0	0	0	29	30	3.0	1.0
316	0	0	0	0	0	0	0	0	0	0	0	17	12	72	29
356	0	0	0	0	0	0	0	0	0	0	0	0.80	2.0	10	16
	L. fermenti phages Serological group II											L. casei phages Serological group 1			

with the generation times (McDuff et al. 1962). The generation time of L. casei organisms at 37° was 62 min. as compared with 50 min. for the L. fermenti organisms. By premature lysis of the host organisms with chloroform (Séchaud & Kellenberger, 1956) L. casei phage could be detected at 85 min. and L. fermenti phages were demonstrated in this manner after 60 min.

Effect of sodium chloride. Like the phages of Kiuru & Tybeck (1955) the Lactobacillus casei phages showed no significant decrease in plaque forming units when suspended in 25% (w/v) NaCl solution for 2 days. The L. fermenti phages were unstable in this solution. The titre of all the phages of this latter group decreased by more than 90% under these conditions.

Cross-resistance tests. Cross-resistance tests afforded another method of distinguishing phages with similar properties. Phage-resistant mutants of Lactobacillus casei strains 300 and 780 and of L. fermenti strains 41, 69, 206, 514, 517, 535 and 547 were obtained on first attempt and are presumably one-step mutants. The phages adsorbed at the same rate to the resistant mutants but no progeny phage was liberated and the bacteria were unaffected (Garen & Puck, 1951). All the mutants were of this type. No phage non-adsorbing mutants of these strains were obtained. Resistant mutants of L. casei strain 316 and L. fermenti strains 222, 222a and 276 were not obtained despite repeated attempts at selection. L. casei phages 300 and 780 could not be differentiated by cross-resistance tests and, as the other properties examined are similar, they are presumably identical. Table 7 shows that L. fermenti 41/41 is also resistant to phage 315. L. fermenti 315/315 is also resistant to phage 514 but is lysed by phage 41. L. fermenti 514/514 is lysed by phages 41 and 315. Table 8 shows the results of cross-resistance tests with a group of citrate-sensitive L. fermenti phages. Some of the mutants again show multiple resistance (Demerec & Fano, 1945) but the lytic pattern enables phages 69, 517 and 514 to be differentiated from one another. Phages 206 and 535 have the same host range on these mutants but can be differentiated by plaque size.

Table 7. Cross-resistance grouping of citrate-insensitive Lactobacillus fermenti phages

Phages were added to organisms and after adsorption for 15 min. unadsorbed phage was assayed. The contents of the adsorption tube were then diluted 1/100 in fresh pre-warmed broth and maintained at 37°. Samples were assayed at intervals.

	Phage no.		
Bacteria no.	41	315	514
41 / 41	-	-	+
315 / 315	+	-	-
514 / 514	+	+	-

+ = Productive infection; - = adsorption, but no phage production.

Table 8. Cross-resistance grouping of citrate-sensitive Lactobacillus fermenti phages

Phages were added to organisms and after adsorption for 15 min. unadsorbed phage was assayed. The contents of the adsorption tube were then diluted 1/100 in fresh pre-warmed broth and maintained at 37°. Samples were assayed at intervals.

	Phage no.				
Bacteria no.	69	206	517	535	547
69 / 69	-	+	+	+	+
517 / 517	-	+	-	+	-
547 / 547	+	+	+	+	-
206 / 206	-	-	+	-	-
535 / 535	-	-	-	-	-

+ = Productive infection; - = adsorption, but no phage production.

DISCUSSION

Recent interest has centred on phages other than those which attack organisms of the family Enterobacteriaceae. Smith (1959) described phages active against strains of Clostridium perfringens and Sword & Pickett (1961) described Listeria monocytogenes phages. Brucella phages were described by McDuff et al. (1962) and Bacillus phages have been characterized by Meynell (1962). A revival of interest has also been shown in the mycophages, Streptococcus phages and Staphylococcus phages (Groman, 1961). All these phages have properties of a similar nature to the T phages. The Lactobacillus phages are no exception. The characteristics of the 4 phages isolated on homofermentative hosts correspond to descriptions of the Lactobacillus casei phage characterized by Walter (1958). Thus Walter's phage has the same overall length as these L. casei phages, although the tail is shorter and lacks a terminal knob. His phage also forms small plaques and has a host range limited to 1 strain of L. casei. The latent period (140 min.) is shorter than the latent periods of the L. casei phages described here but the burst size is similar. The long latent periods of the L. casei phages approach those of certain mycophages (Bowman & Redmond, 1959). Walter's phage had the same heat sensitivity as the L. casei phages described here. Apart from L. casei phage 356 the other phages investigated had slow adsorption rates which could not be increased. Walter (1958) encountered the same phenomenon with his phage. The media used contained Tween 80 and it was hoped to increase the adsorption rate by omitting it (White & Knight, 1958). This omission had no effect. Kiuru and Tybeck (1955) investigated their phages along different lines, but their 2 phages were relatively stable at 55^{o} and like these L. casei phages were also not inactivated by 25% (w/v) NaCl solution.

The 11 phages isolated on heterofermentative hosts which were characterized in detail differ in some respects from the Lactobacillus casei phages. The most striking properties of the former phages are the larger plaques, the very much shorter latent periods and their inactivation by 25% (w/v) NaCl solution. These 11 phages are serologically related but are not homogeneous as regards citrate sensitivity.

The results of preliminary phage-typing by titration and spotting (Table 1) serve to confirm some of the serological relationships among lactobacilli

encountered by Sharpe (1955) and Sharpe & Wheater (1957). Thus Lactobacillus casei phages 300, 316 and the L. casei var. rhamnosus phage 356 all act on different strains of L. casei, L. casei var. rhamnosus and L. plantarum. The former 2 phages also acted on an L. fermenti strain. The serological relationship which was detected between L. fermenti, L. casei and L. casei var. rhamnosus strains may be mirrored in the fact that many of the L. fermenti phages also act on L. casei strains. Davis (1955) includes L. lactis in his L. acidophilus group and Sharpe (1955) found L. brevis and L. lactis to share a major antigenic component. It is thus of some interest that 3 L. fermenti phages which act on L. brevis strains also produce abortive infections in 4 homofermentative L. acidophilus strains. While Sharpe (1955) has not indicated any serological relationship between the heterofermentative species L. fermenti and L. brevis many of the phages isolated on the former also lyse strains of the latter species.

Host range experiments with the 11 L. fermenti phages and the 4 L. casei phages indicated that they were not only genus specific but practically species specific. With the exception of some inter-species abortive and productive infections their host ranges support the serological grouping of these phages into 2 groups.

Phage typing schemes of taxononic and epidemiological value have been applied to a number of genera and species of bacteria, (see Adams, 1959). Phage typing of Salmonella typhosa was introduced by Craigie & Yen (1938) who recognized an unusual property of their Vi-phage II. This phage which attacked only S. typhosa strains containing the Vi antigen (Felix & Pitt, 1934) acquired a high specificity (high e.o.p.) for the last strain of this organism on which it was propagated. The change in host specificity of the phage was shown to be due in many instances to host-induced or phenotypic modification (Anderson & Felix, 1952, 1953a) imposed upon the phages by a small proportion of infected heterologous host cells in which the phage DNA did not undergo restriction (see Luria, 1953; Hayes, 1965). From the few plaques which arose on heterologous S. typhosa strains "adapted" Vi-phages with a high e.o.p. on the particular strain of S. typhosa could be isolated. This new host range was maintained as long as the modified phage was allowed to multiply in the new host. If the phage was plated on the original host the few particles which escaped restriction reverted in a single growth cycle to

the host range of the original phage. Altered host ranges of Vi-phage II may result from mutation or a combination of phenotypic and mutational change (Anderson & Fraser, 1955, 1956). In addition the susceptibility of many Vi-strains of S. typhosa to "adapted" Vi-phages and their ability to modify them is dependent on the lysogenicity of the strains for phages unrelated to Vi-phage II (Craigie, 1946; Felix & Anderson, 1951; Anderson, 1951; Anderson & Felix, 1953b), called type-determining phages. The typing scheme for S. typhosa in current international use contains 72 types based on their reactions with various typing adaptations of Vi-phage II (Bernstein & Wilson, 1963). Phage-typing schemes also exist for other Salmonella spp.; S. paratyphi B and S. typhimurium (Felix & Callow, 1943, 1951; Callow, 1959), S. paratyphi A (Banker, 1955), S. dublin (Williams Smith, 1951c), S. enteritidis (Lilleengen, 1950), S. gallinarum & S. pullorum (Lilleengen, 1952) and S. thompson (Williams Smith, 1951a, b) and for other Enterobacteriaceae such as Shigella sonnei (Hammerström, 1949) and Escherichia coli (Nicolle, Le Minor, Buttiaux & Ducrest, 1952; Williams Smith & Crabb, 1956). These schemes differ from the Vi-phage typing method of S. typhosa in that they are based largely or entirely on the principle of different patterns of susceptibility to series of distinct typing phages . This principle of pattern reaction is also the basis of the staphylococcal phage typing scheme (see Wentworth, 1963), which was first placed on a sound footing by Fisk (1942). Other organisms which have been classified by means of phage reactions include Pseudomonas aeruginosa (Sutter, Hurst & Fennell, 1963), mycobacteria (Tokunaga & Murohashi, 1963), Brucellae (van Drimmelen), 1960; Jones, 1960) and Corynebacterium diphtheriae (Fahey, 1952). Most of these typing schemes employ an RTD, usually the highest dilution which produces confluent lysis of the homologous type strain. The use of such dilutions was originally introduced into the Vi-phage typing scheme because all adapted phage typing preparations are adsorbed by all Vi-types of S. typhosa and this adsorption is lethal to cells of a high proportion of heterologous types (Anderson, 1962). Thus with the application of high titre lysates cultures may appear to have undergone phage lysis due to abortive infection with host killing of a restrictive strain. Titration of ten-fold dilutions of the phage on such a strain would reveal a low e.o.p. of the phage, or, in the absence of host-induced modification, result in the abrupt disappearance of

lysis between 2 dilutions without the appearance of individual plaques. Confluent lysis may be produced by concentrated lysates as a result of the presence of a sufficiently high proportion of particles which may undergo host-induced modification by heterologous organisms or host range mutants which are able to multiply in these organisms, (Adams, 1959). In addition concentrated lysates may contain bacteriocins which simulate lysis by inhibition of growth in the drop area (Adams, 1959). This effect also disappears abruptly on dilution. The RTD was introduced into the Staphylococcus typing scheme by Wilson & Atkinson (1945) to obviate similar difficulties which beset the use of undiluted lysates. These include abortive infection (Rountree, 1947; Williams & Rippon, 1952), lysis from without (Ralston, Baer, Lieberman & Krueger, 1957) and lysis of tests strains by host range mutants or by temperate phages derived from strains on which typing phages are propagated. At least 75% of the latter have been shown to be lysogenic (Wentworth, 1963). Concentrated lysates may also cause prophage induction with resultant plaque formation when plated with lysogenic staphylococci (Rountree, 1956).

The attempt made here to classify Lactobacillus fermenti strains by means of phage-typing was complicated by the wide ranges of activity of the phages used and the high incidence of abortive infection encountered. These limitations could be countered to an extent by the use of an RTD which largely eliminated abortive activity and markedly curtailed the spectra of confluent lytic activity. Increasing the RTD had the effect of reducing the number of phage types but resulted in more complex patterns, while decreasing the RTD had the opposite effect. This system of classification is unsatisfactory because of the large number of phages used, the large number of phage types and because it is based purely on the number of phages which attack particular organisms at a particular RTD. Any reduction of the number of phages used for typing resulted in the loss of phage types especially in group C (Table 3) where many of the phages are mono-specific.

Despite repeated attempts no phages which productively lyse Lactobacillus acidophilus or L. salivarius strains have been isolated from sewage, vaginal secretions or saliva nor were any phages primarily isolated on strains of L. plantarum and L. brevis. The possibility that phages may have originated from lysogenic strains of lactobacilli used for enrichment and not from sewage was not entirely excluded and this possibility is investigated in the following Chapter.

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CHAPTER 6

LYSOGENY IN THE GENUS LACTOBACILLUS

SUMMARY

Three hundred and forty-five strains comprising various species of lactobacilli were examined for lysogeny by one or more methods. Two Lactobacillus fermenti strains were found to be lysogenic by ultraviolet induction. This property could be shown only by irradiation of these strains. The host ranges of both phages were confined to L. fermenti strains. Lysogeny was not found among strains of other Lactobacillus spp. tested.

INTRODUCTION

The discovery of bacteriophages was followed within a few years by the observation that their survival was not wholly dependent on the lysis of sensitive host bacteria. Some bacteria were found to carry phage in an intracellular form. Free phage was present in broth cultures of these bacteria and they could not be freed of their carried phages by serial propagation of single cell lines or treatment with anti-phage serum. Such bacteria are called lysogenic (see Bertani, 1953, 1958; Lwoff, 1953; Jacob & Wollman, 1959; Whitfield, 1962; Stent, 1963; Hayes, 1965). That lysogenic bacteria do not carry infectious particles was shown by Burnet & McKie (1929) who noted the absence of such particles on disruption of lysogenic organisms. The concept that lysogeny represents the hereditary potential of all bacteria of a lysogenic strain to produce infectious phage was verified by Lwoff & Gutman (1950). Lysogenic organisms thus carry a non-infectious "anlage" of the phage or prophage which confers on the cell the ability to liberate phage. This capacity is expressed with only a low probability with any particular bacterium and phage liberation occurs through discontinuous lysis of a small but constant fraction of a bacterial population. In lysogenic bacteria host and virus genomes are integrated and replicate as a single unit. Such bacteria are immune to infection with the phage they carry as prophage and to closely related phages (see Lwoff, 1953; Jacob & Wollman, 1959).

The specificity of this immunity (Jacob & Wollman, 1956; Kaiser & Jacob, 1956; Bertani, 1958) is now interpreted in terms of the general theory of the regulation of protein synthesis (Jacob & Monod, 1961). In the lysogenic cell a regulator gene of the prophage codes for a specific cytoplasmic repressor which is able to combine with the operator gene of the viral genome (Ptashne, 1967). This reaction prevents function of the structural genes of the prophage and of any superinfecting phage with the same specificity of operator. Lwoff, Siminovitch & Kjelgaard (1950) showed that exposure of a lysogenic Bacillus megaterium to small doses of ultra-violet light is followed after a latent period by lysis of virtually the entire population accompanied by the liberation of phage particles. Similar inducibility has been shown in lysogenic strains of many other bacterial genera but not all lysogenic bacteria are inducible (Jacob & Wollman, 1957). The type of immunity exhibited by non-inducible strains may differ from that of inducible strains. Induction of a lysogenic culture may also be achieved by a number of inducing agents such as X-rays, gamma rays, nitrogen mustards, hydrogen peroxide, organic peroxides (see Jacob & Wollman, 1959) and Mitomycin C (Otsuji, Sekiguchi, Ijima & Takagi, 1959). There is evidence that the transition of prophage to the vegetative state with ultra-violet light induction results from destruction of the repressor (see Hays, 1965).

Lysogeny has been found in many strains of various bacterial species and genera. In certain species of Salmonella (Burnet, 1932) and in Staphylococcus (Williams Smith, 1948) the majority of strains were found to be lysogenic. Many instances of multiple lysogeny occur and a strain of staphylococcus was found to carry as many as 5 different phages (Rountree, 1949). Attempts to show lysogeny in Pasturella pestis however were unsuccessful (Smith, 1961).

The isolation from sewage of phages active on lactobacilli was reported by Coetzee, de Klerk & Sacks (1960) and de Klerk, Coetzee & Theron (1963). This was done by an enrichment technique in which sewage was incubated with groups of lactobacilli (see Chapter 5). The possibility that some of these phages may have originated from lysogenic strains of lactobacilli and not from sewage was not excluded. The purpose of this investigation was to test this possibility and examine the incidence of lysogeny among strains of lactobacilli in general.

METHODS

Media. The tomato glucose agar and broth (TGA, TGB) described in Chapter 2 were used. Top-layer agar contained 1% (w/v) agar. Plates were incubated in an atmosphere of 100% carbon dioxide. All cultures were incubated at 37^{o}.

Organisms. Three hundred and forty-five strains of lactobacilli were tested for lysogeny by one or more methods. They comprised 92 strains of Lactobacillus acidophilus, 42 of L. salivarius, 30 of L. casei, 72 of L. casei var. rhamnosus, 19 of L. plantarum, 76 of L. fermenti, 12 of L. brevis and one each of L. bulgaricus and L. helveticus. The latter 2 strains, 2 of the L. casei strains and one strain each of the remaining species were type cultures kindly supplied by Dr. M. Elizabeth Sharpe of the National Institute for Research in Dairying, Reading, Berkshire, England. The remainder were locally isolated human oral strains and their isolation and characterization was described in Chapter 3.

Phage techniques. The general phage techniques were those of Adams (1959).

Tests for lysogeny. One hundred and twenty of the lactobacillus strains used for the isolation of phages from sewage active on lactobacilli (see Chapter 5) were again grown together in the same groups of 12 in 100 ml. of TDB. The sewage previously added to the mixtures was replaced by 5 ml. of tap water. Cultures were incubated for 10 days and samples were centrifuged daily and tested for the presence of phage. This was done by spotting the supernatants on toplayer (Adams, 1959) cultures of each of the 12 strains which comprised a mixed culture. Lysogeny was also investigated as follows. Fifteen different strains of lactobacilli comprising 2 strains of Lactobacillus acidophilus, 5 strains of L. casei var. rhamnosus, 6 of L. fermenti and 1 each of L. casei and L. plantarum were grown in all combinations of 2 according to the method of Scholtens (1955). Two drops of overnight cultures of the strains were subcultured into 40 ml. of TDB. These cultures were incubated for 14 days, centrifuged to clarity and the supernatants spotted on to 102 strains of lactobacilli seeded into top layer agar (Adams, 1959). The possible indicators were 31 strains of L. acidophilus, 12 of L. salivarius, 3 of L. casei, 23 of L. casei var. rhamnosus, 5 of L. plantarum and 28 of L. fermenti. A further 15 strains

(2 strains of L. acidophilus, 3 of L. salivarius, 3 of L. casei var. rhamnosus, 5 of L. fermenti and 1 each of L. casei and L. plantarum) were similarly investigated using the same 102 indicator strains. Subsequently, 199 strains were grown singly in 40 ml. of broth for 10 days. These were 59 strains of L. acidophilus, 16 of L. salivarius, 16 of L. casei, 42 of L. casei var. rhamnosus, 13 of L. plantarum, 42 of L. fermenti, 9 of L. brevis and one each of L. bulgaricus and L. helveticus. Cultures were then spun to clarity and the supernatants sterilized with chloroform and spotted on lawns of these 199 strains.

Finally the bacilli in 10 ml. of overnight broth cultures of 50 of the above strains were deposited by centrifugation and resuspended in equal volumes of sterile distilled water in Petri dishes. The suspensions were irradiated with a 30 Watt Hanovia Sterilamp from a distance of 25 cm. The lamp delivers more than 80% of its output at a wave-length of 2357 Å. At intervals of 5, 15 and 30 sec., 3 ml. samples were pipetted into 10 ml. of warm broth and incubated in the dark overnight. The cultures were then centrifuged and the supernatants spotted on lawns of 109 strains of lactobacilli. Plaques were repeatedly picked to young broth cultures of the corresponding indicator strains for purification of the phage and the preparation of high titre lysates.

RESULTS

No phage was isolated from mixed cultures of groups of 12 lactobacilli or from the cultures of pairs of lactobacilli. The Lactobacillus fermenti and L. casei strains against which phages were isolated from sewage (see Chapter 5) were included in one or both of these experiments. From the strains grown singly in broth a number of diffusible antibiotics (de Klerk & Coetzee, 1961) were obtained, but no phage activity was demonstrated. Two of the 50 irradiated strains however, proved lysogenic. They are both strains of L. fermenti (no. 535, 544). The phage liberated by strain 535 forms small clear plaques on L. fermenti strains 222a and 196. The other forms larger turbid plaques on L. fermenti strains F1, 184 and 525. The host range of these 2 phages is confined to L. fermenti strains.

DISCUSSION

Of the 345 strains of lactobacilli tested only 2 strains of Lactobacillus fermenti have proved lysogenic. No previous reference to lysogenic lactobacilli is to be found in the Literature. Both strains were used in all 3 experiments but released phage only on induction with ultra-violet irradiation. This was confirmed by the repetition of the other experiments with the 2 strains. A similar situation exists with some Proteus vulgaris species (Coetzee & Sacks, 1959). The failure of the first 2 methods to show lysogeny in any of the strains supports the conclusion that the lactobacillus phages previously isolated (Coetzee, de Klerk & Sacks, 1960; de Klerk, Coetzee & Theron, 1963) originated from the sewage and not from lysogenic strains used for enrichment. It is possible that a higher incidence of lysogenic strains would have been found if more strains had been irradiated and a wider range of indicators had been used.

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CHAPTER 7

FINE STRUCTURE OF LACTOBACILLUS BACTERIOPHAGES

SUMMARY

Twelve phages isolated from sewage and active on lactobacilli were examined by electron microscopy with a negative-staining technique. Those phages active on Lactobacillus fermenti (heterofermentative) possess icosahedral heads and sheathed tails which end in base-plates and pins. Those phages active on Lactobacillus casei (homofermentative) differ in that their heads are octahedral or icosahedral and they possess collars. The overall length of all the phages is similar and their base-plates remain attached to the sheaths when these contract. No tail fibres were seen. A temperate Lactobacillus fermenti phage was also examined. It has a small hexagonal head and a long unsheathed tail which ends in a star-shaped structure.

INTRODUCTION

Prior to 1959 electron photomicrographs of bacteriophages and other viruses were all of specimens shadowed with various heavy metals. This method, devised by Williams & Wyckoff (1945), reveals only the outer contours of virions and its limitations in the study of the fine structure of viruses are obvious. Williams (1953) shadowed freeze-dried samples to reduce the distortion caused by their drying in air and applied this technique in a study of the structural and functional differentiation of T2 coliphages (Williams & Fraser, 1956).

The development of a negative staining technique (Brenner & Horne, 1959) led to the first detailed description of phage fine structure (Brenner et al. 1959). Variations of this method were investigated by Bradley (1961, 1962) and it has been applied to a variety of phages (Anderson, 1960; Bradley & Kay, 1960; Tromans & Horne, 1961; Bradley, 1963a, b, 1964; Eiserling & Romig, 1962; Kay & Bradley, 1962; Bacq & Horne, 1963; Davison, 1963;

Slayter, Holloway & Hall, 1964) and to many other viruses (see Horne & Wildy, 1961, 1962, 1963). The presence of capsomeres on some phages was revealed by this method (Eiserling & Boy de la Tour, 1965) and its use has led also to proposals for the shape of the heads of the T even coliphages (Bradley, 1965; Moody, 1965).

An electron micrograph of a shadow-cast preparation of a phage active on a strain of Lactobacillus casei was presented in an M.S. thesis (Walter, 1958). De Klerk, Coetzee & Theron (1963) examined shadow-cast preparations of a number of phages active on lactobacilli (see Chapter 5). These phages are morphologically heterogeneous and it was decided to study their fine structure.

METHODS

Media. Media used were MRS broth and agar (de Man, Rogosa & Sharpe, 1960).

Phages. These were isolated from sewage (see Chapter 5) and were as follows: Nos. 41, 69, 206, 222, 222a, 315, 514, and 517 active on Lactobacillus fermenti strains; 300, 316, 356 and 780 active on strains of L. casei (de Klerk, et al. 1963). The temperate phage 535/222a (Coetzee & de Klerk, 1962) was also examined.

Electron microscopy. Lysates of the sewage phages were prepared, purified and concentrated by differential centrifugation as described in Chapter 5 (de Klerk et al. 1963). High-titre lysates of the temperate phage were prepared by a double agar layer method (Hershey, Kalmanson & Bronfenbrenner, 1943). The purified phages (plaque-forming titres about 1×10^{12}/ml.) were suspended in 0.1 M-ammonium acetate (pH 7.2). The negative staining method of Brenner & Horne (1959) was used. Perforated carbon films were prepared on Veco 400 mesh/in. support grids (Sjöstrand, 1956); these were freed from formvar and oil by immersion in redistilled chloroform. The specimens were mounted by the spreading technique (Bradley, 1962) and examined with a Philips EM 200 electron microscope.

RESULTS

Phages active on Lactobacillus fermenti. Plate 1, figs. 1-5 show Lactobacillus fermenti phages 206, 222a, 315 and 514. Their heads are composed of capsomeres. Some of the latter show a central core filled with phosphotungstate and are presumably hollow. The arrangement of the capsomeres has not been determined but the shapes of the heads are consistent with that of a regular icosahedron. The tails are composed of thin central cores surrounded by contractile sheaths composed of subunits in a helical arrangement. No collars are present and the terminal tail structures are rosette-like when the sheaths are extended. When the sheaths contract the central cores are exposed. The tail structures then show base-plates carrying no more than six tail pins, attached to the sheaths. No tail fibres were seen. The dimensions of these L. fermenti phages are presented in Table 1. The other sewage phages active on L. fermenti organisms which were examined had identical features and similar dimensions.'

Temperate phage. The temperate phage 535/222a possesses a different morphology (Pl. 2, figs. 6, 7). Its head is hexagonal and capsomeres were not seen. The tail is long, hollow and unsheathed and terminates in a star-shaped blob. The dimensions are presented in Table 1.

Phages active on Lactobacillus casei. Plate 2, figs. 8-10, show Lactobacillus casei phages 300 and 356. The heads are composed of hollow capsomeres. The shape of the heads appears to be octahedral but few structural details have been discerned, and the head shape of phage 356 may be icosahedral. These phages also differ from those active on heterofermentative lactobacilli in that collars are present which have the same diameters as the tail sheaths. The remaining tail structures are similar to those of the phages active on heterofermentative lactobacilli. The dimensions of these phages are given in Table 1. Phages 316 and 780 revealed an identical morphology and similar dimensions. The periodicity of the tail sheaths of both groups of sewage phages in the contracted state was 36 Å and 28 Å when extended.

Table 1. Dimensions (Ångström units) of Lactobacillus bacteriophages

Phage	Head*	Tail length	Sheath width		Core width	Overall length
			Uncontracted	Contracted		
206	720≠	1380	-	180	55	2100
222a	690	1380	160	200	55	2070
315	720	1480	160	-	-	2200
514	720	1380	-	180	60	2100
300	820	1230	150	190	70	2050
356	820	1270	160	-	-	2120
535/222a	500	1820	-	-	80	2320

* Dimension from apex to tail joint. ≠ Figures are the mean of 25 to 30 measurements.

DISCUSSION

A clear distinction is possible between sewage phages active on heterofermentative and homofermentative lactobacilli. Not only do they differ serologically (de Klerk et al. 1963) but the latter have collars and probably octahedral heads while the former possess icosahedral heads and lack collars. The collars are thicker than those of coliphage T4 (Bradley, 1963) and resemble the collar of Bacillus subtilis phage SP3 (Eiserling & Romig, 1962). The overall length of the two groups of phages is similar, although the heads of those active on homofermentative organisms are larger. Their dimensions are similar to those of many other phages (Shirling, 1956; Bradley & Kay, 1960; Davison, 1963). The sheaths of both groups of phages, when contracted, remain attached to the base-plates. However, methods to produce contraction which may dissociate the base-plate from the contracted sheath (Kellenberger & Arber, 1955) were not used. The tail endings of these phages in the uncontracted state resemble that of B. subtilis phage SP8 (Davison, 1963), which is described as a mass of fibres and pins. However, no tail fibres or fibrous network around the sheaths of these phages were detected. This is not exceptional, for tail fibres and the fibrous networks detected in coliphages T2 and T4 have not been described in phages active on Gram-positive organisms (Bradley & Kay, 1960; Eiserling & Romig, 1962; Davison, 1963). The temperate lactobacillus phage differs from the sewage phages in having a smaller head and a longer and unsheathed tail which is distinctly broader than the cores of the other phages. No tail pins or fibres have been identified in the rosette-shaped tail ending of this phage, which is similar to the temperate B. cereus phages described by Dawson & Smillie (1962).

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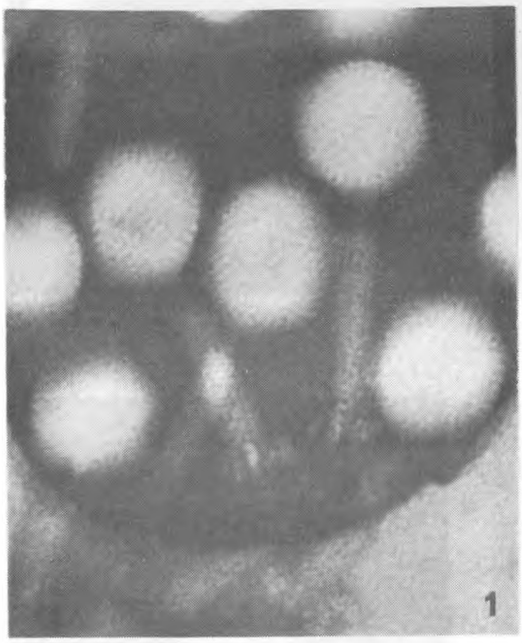
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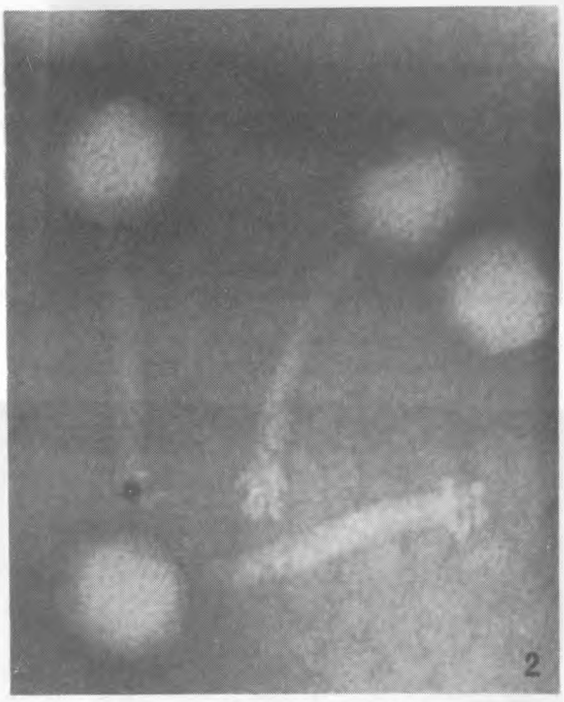
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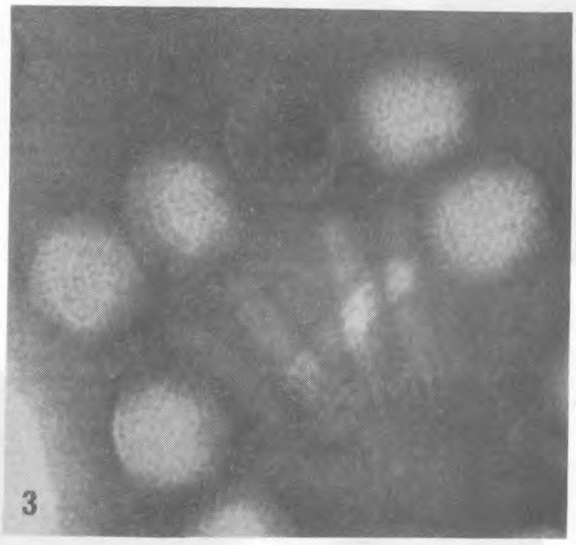
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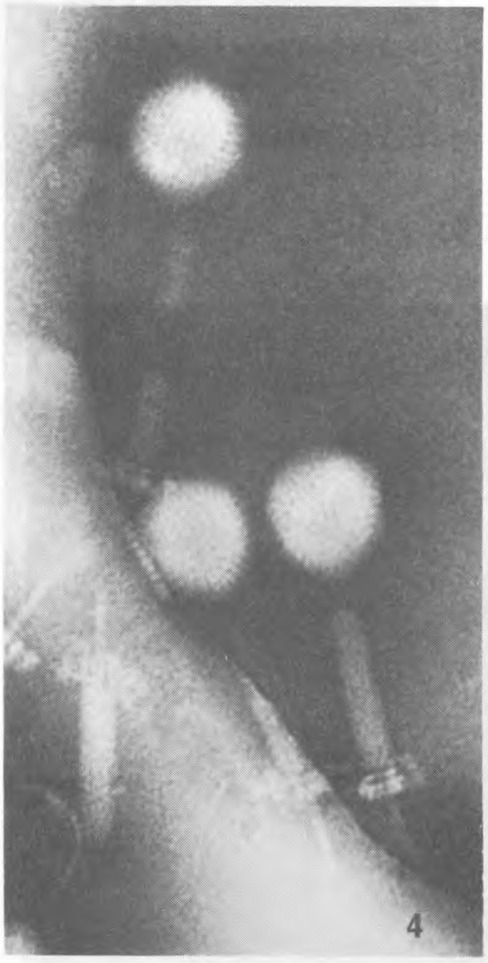
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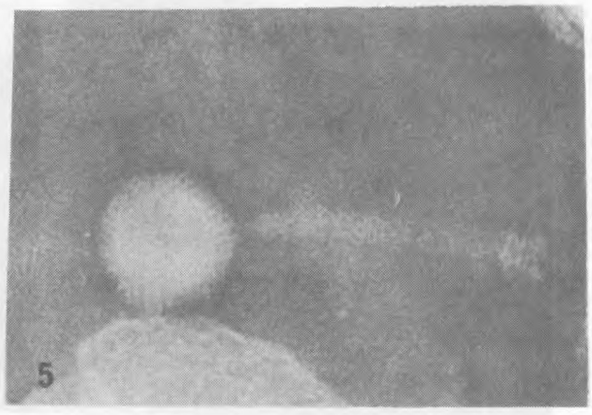
Plate 1





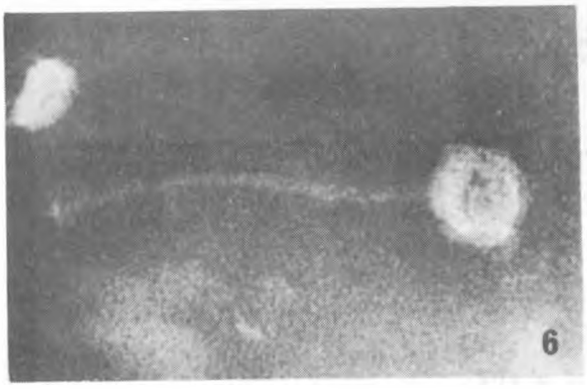






1000 Å

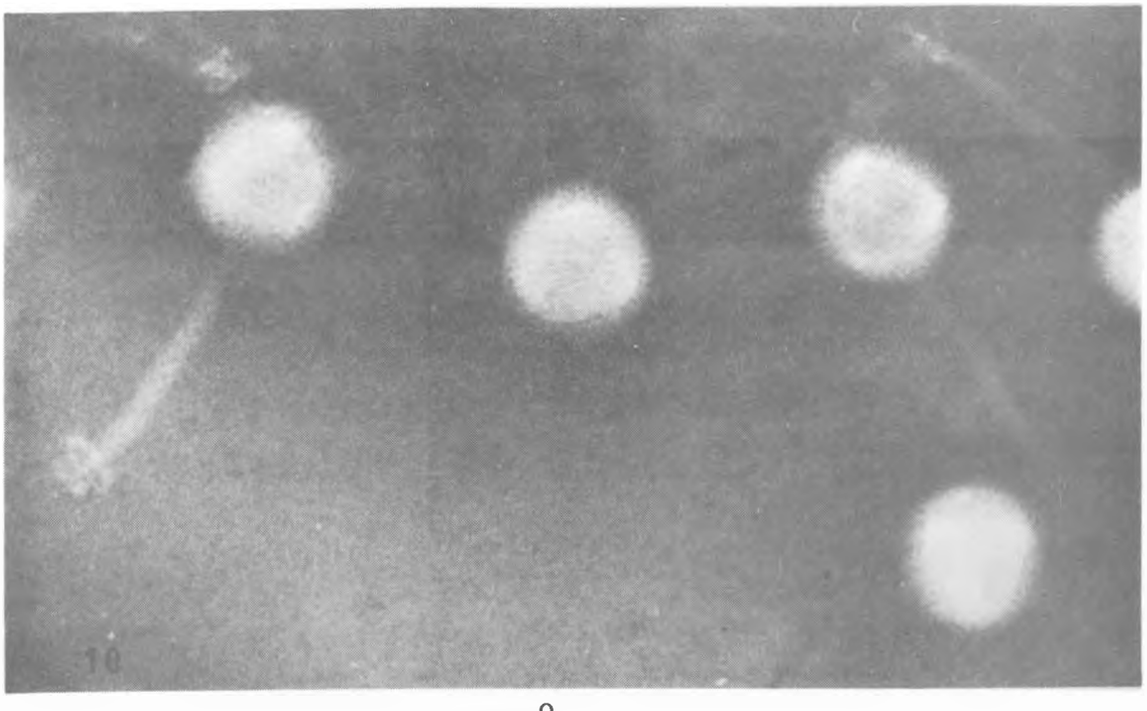
Plate 2











1000 Å

EXPLANATION OF PLATES

The magnification in all figures is x 276,000. All phages in ammonium acetate and phosphotungstate.

Plate 1

Fig. 1	Phage 206.
Figs. 2, 3	Phage 222a.
Fig. 4	Phage 514.
Fig. 5	Phage 315.

Plate 2

Figs. 6, 7	Phage 535/222a.
Figs. 8, 9	Phage 300.
Fig. 10	Phage 356.

CHAPTER 8

BACTERIOCINOGENY IN THE GENUS LACTOBACILLUS

SUMMARY

The supernatants of 10-day-old broth cultures of 11 strains of Lactobacillus acidophilus and one strain of L. fermenti of 199 strains of various Lactobacillus spp. tested cause inhibition of growth when spotted on lawns of the same strains. Of 418 homofermentative and 136 heterofermentative lactobacilli tested a total of 127 strains was susceptible to this inhibitory activity. The properties of active supernatants differ from those of phage suspensions in that the activity is heat stable, diffusable in agar and not serially transmissible. They do not give rise to individual plaques on dilution. The inhibition is also not due to acid production or the accumulation of hydrogen peroxide. Activity can be concentrated by precipitation and is not diffusible through dialysis tubing. The action of the inhibitory agents is bactericidal and restricted to members of the family Lactobacteriaceae. Re-investigation of 121 strains of L. fermenti using solid media revealed a higher incidence of inhibitory activity. Twenty-five inhibitory strains of this species were found with the same spectra of activity. Inhibitory activity of L. fermenti strains could be shown in fluid media. This activity, when concentrated, exhibited the same properties as the inhibitory supernatants initially isolated. The properties of all these inhibitory agents indicate that they are bacteriocins.

INTRODUCTION

Since the observation of antibacterial activity of strains of Psuedomonas aeruginosa by Emmerich and Löw in 1899 (see Topley & Wilson, 1964) ascribed by these workers to a substance which they called pyocyanase, the inhibition of one bacterial strain by another has been observed many times. The intraspecific inhibition of Escherichia coli Ø by E. Coli V described by Gratia in 1925 led to the intensive investigation of the inhibitory substances produced by this species and by other members of the family Enterobacteriaceae. To these substances the name colicin was given by Gratia

& Frédéricq (1946). Studies which have embraced the distribution, classification, physico-chemical properties, mode of action and the genetic determinants of colicins have been reviewed in detail by Adams (1959), Frédéricq (1948, 1957, 1963a), Reeves (1965) and Nomura (1967). These studies have revealed many unique features of colicins which emphasize their close relationships to bacteriophages and to F factors.

Antibiotics with properties broadly similar to those of the colicians are produced by a wide variety of other species of bacteria (see Frédéricq, 1963b; Hamon, 1964; Ivanovics, 1962, 1965; Reeves, 1965). Among the most recent reports of bacteriocinogeny have been those in strains of Proteus hauseri (Cradock-Watson, 1965), P. morganii and Providence strains (Coetzee, 1967) and P. vulgaris (Coetzee, de Klerk, Coetzee & Smit, 1968). Many of these bacteriocins are as yet insufficiently characterized and it is uncertain whether all of them possess unique features similar to those of the colicins. The general term bacteriocin was proposed to include colicins and other antibiotics which are produced by bacteria, are protein in nature, whose production is lethal and whose action is restricted to strains of the same or closely related species and is dependent on the presence of specific receptors, (Jacob, Lwoff, Siminovitch & Wollman, 1953). These properties distinguish the bacteriocins from all other types of antibiotic. In addition to the characteristics named above the uniqueness of colicins is reflected in the nature of their genetic determinants. In E. coli bacteriocinogeny is determined by genetic factors called colicinogenic or col factors. Col factors may be transmitted from cell to cell by direct contact (Frédéricq, 1954) and may then act as sex factors in that they effect chromosomal transfer between bacterial cells (Ozeki & Howarth, 1961; Smith & Stocker, 1962). The col factors are considered to be plasmids (Lederberg, 1952; Nomura, 1967)..

Anti-bacterial activity associated with cultures of various homofermentative Lactobacillus spp. were reported by Grossowics, Kaplan & Schneerson (1947), White & Hill (1949), Wheather, Hirsch & Mattick (1951) and Polonskaya (1952). In an investigation of lysogeny in lactobacilli (Coetzee & de Klerk, 1962) the supernatants of broth cultures of a number of strains of these organisms were spotted on lawns of the same strains. No phage activity was demonstrated but some supernatants produced clear areas of inhibition of growth. No reports of bacteriocinogenic lactobacilli appear in

the Literature and it was decided to investigate the nature of this inhibition.

METHODS

Media. Initially the tomato glucose agar and broth (TGA, TGB) described in Chapter 2 were used. In subsequent work these were replaced by the solid and fluid MRS media of de Man, Rogosa & Sharpe (1960). Top layer agar of both types contained 1% (w/v) of agar. When necessary agar was buffered to pH 7 with 0.1 M-sodium phosphate buffer or supplemented with 10% (v/v) fresh rabbit serum. Plates were incubated in an atmosphere of 100% carbon dioxide. All cultures were incubated at 37°.

Organisms. The 199 strains first tested for bacteriocinogeny comprised 59 strains of Lactobacillus acidophilus, 16 of L. salivarius, 16 of L. casei, 42 of L. casei var. rhamnosus, 13 of L. plantarum, 42 of L. fermenti, 9 of L. brevis and one each of L. bulgaricus and L. helveticus. Two of the L. casei strains and one each of the other species were type cultures kindly supplied by Dr. Elizabeth Sharpe of the National Institute for Research in Dairying, Reading, Berkshire, England. The origin of the other strains has been described (see Chapter 3). These strains and an additional 156 locally isolated strains of L. acidophilus, 27 of L. salivarius, 49 of L. casei, 95 of L. casei var. rhamnosus, 13 of L. plantarum, 79 of L. fermenti and 7 of L. brevis were used as possible indicators of bacteriocin activity. Subsequently the 121 strains of L. fermenti were re-tested for bacteriocinogeny and used as possible indicators.

Bacteriocin production. In early experiments this was tested as follows: Supernatants of 10-day TGB cultures were sterilized by shaking with chloroform (1% v/v). After all traces of chloroform had been evaporated, drops of these supernatants were spotted on TGA covered with top layer seeded with possible indicator strains. Plates were incubated overnight and examined for zones of inhibition. The Lactobacillus fermenti strains were tested for bacteriocinogeny in subsequent experiments by spotting the supernatants of overnight MRS broth cultures as described above and by stabbing single colonies of the 121 strains into MRS agar. After overnight incubation the plates were sterilized with chloroform vapour, layered with soft MRS agar seeded with an indicator

strain and re-incubated overnight. Fragments of agar from clear areas of inhibition were transferred to fresh broth and incubated overnight, and also suspended in a small volume of broth and re-spotted on fresh lawns of the original indicator organism. Inhibitory activity was assayed by spotting drops of two-fold serial dilutions of supernatants in broth onto plates seeded with an indicator strain.

Microscopy. Small drops of suitably diluted broth cultures of indicator strains were placed on microscope slides covered with a thin layer of TGA. A drop of supernatant from a broth culture of an inhibitory strain or a drop of fresh broth was added and the preparations covered with a cover glass. Fields containing 150 to 200 organisms were selected and the organisms counted at 30 min. intervals during 8 hr of incubation.

Concentration of inhibitory activity: This was done by precipitation with equal volumes of saturated ammonium sulphate or ethanol. Precipitates were centrifuged drained of supernatant and resuspended in small volumes of broth. Supernatants were also dialysed (Visking Co., Chicago, Ill., U.S.A.) against 30% (w/v) polyethylene glycol (carbowax 6000).

Diffusability. This was tested by filling a sealed hole in agar with concentrated supernatant from a broth culture of an inhibitory strain and allowing diffusion to occur overnight in the cold. Plates were then covered with soft agar containing the indicator organism and incubated overnight. Inhibitory strains were also streaked on agar, cross-streaked with sensitive strains and incubated.

Hydrogen peroxide sensitivity of strains. The hydrogen peroxide sensitivity of several inhibitory and indicator strains was determined by the method of Coulthard et al. (1945).

RESULTS

The supernatants of 12 broth cultures of the 199 lactobacillus strains initially tested produced clear areas of inhibition of growth. These were from 11 Lactobacillus acidophilus and 1 L. fermenti strains. The results are summarized in Table 1. Three of the supernatants (nos. 253, 713 and 838) have identical spectra of activity. The remainder show very little overlapping and a

Table 1 Inhibitory spectra of supernatants of broth cultures of Lactobacilli

Supernatants producing zones of inhibition	Number of susceptible strains *				
	A (215)	B (1)	S (43)	H (1)	F (121)
Strain no. 35 A	2	1	1	1	1
Strain no. 93 A	6	1	1	1	15
Strain no.124 A	0	0	1	0	1
Strain no. 185 A	6	1	1	1	9
Strain no. 253 A	15	1	1	1	20
Strain no. 303 A	14	1	1	1	19
Strain no. 496 A	11	1	1	1	17
Strain no. 558 A	20	1	1	1	21
Strain no. 571 A	3	1	0	1	3
Strain no. 713 A	15	1	1	1	20
Strain no. 838 A	15	1	1	1	20
Strain no.466 F	*2*	*0*	*0*	*0*	7
Total number of different susceptible strains	51	1	1	1	73

* = No activity demonstrated on 49 strains of L. casei, 95 strains of L. casei var. rhamnosus, 13 strains of L. plantarum and 16 strains of L. brevis.

Figures in brackets indicate number of strains tested.

A = L. acidophilus

B = L. bulgaricus (Reading Strain)

S = L. salivarius

H = L. helveticus (Reading Strain)

F = L. fermenti

total of 127 strains of lactobacilli are killed by the 12 supernatants. The maximum dilution in which supernatants were active was 1/10. Cultures from which active supernatants were derived are also inhibitory as indicated in Plate 1, fig. 1. On occasions a few colonies composed of resistant variants of the indicator strain were present in the zone of clearing of inhibitory L. acidophilus strains. Sub-cultures of pieces of agar cut from clear areas never showed growth and the inhibitory activity could not be serially transmitted to fresh lawns of the original indicator organisms. Serial dilution of active supernatants always resulted in the abrupt disappearance of inhibitory areas without the appearance of individual plaques. The pH values of supernatants varied between 3.6 and 4.1 but activity was unaffected by adjusting to pH 7 and/or conducting the tests on buffered agar. In subsequent work the pH of supernatants was adjusted to 6.8. Activity is not extractable with ether but is precipitated by ammonium sulphate and it is possible to increase the titre of active supernatants considerably by these methods and by dialysis against polyethylene glycol. The activity is diffusable in agar (Pl. 1, fig. 2) and bactericidal titres are not affected by boiling for 15 min. None of the inhibitory strains tested is more resistant to hydrogen peroxide than its indicator lactobacilli and it is sometimes more sensitive. Furthermore, inhibitory activity is not diminished by pre-incubating the supernatants with 0.1% (w/v) catalase (British Drug Houses) or using indicator strains seeded on agar supplemented with 10% (v/v) fresh rabbit serum. Microscopical observation of sensitive cultures treated with active supernatants showed that the bactericidal action is not due to bacteriolysis. The antibacterial spectrum of the 12 supernatants (concentrated by ammonium sulphate precipitation) was tested on other members of the family Lactobacteriaceae, various enterobacteria and staphylococci. A few strains of Diplococcus pneumoniae and many isolates of enterococci are susceptible to a number of these antibiotics. Enterobacteria and staphylococci appear to be immune. Subsequent experiments showed that inhibitory activity often attains maximal bactericidal titres in the supernatants of overnight cultures. Titres may decline on further incubation.

Re-investigation of inhibitory activity among 121 strains of L. fermenti on solid media showed that 25 of these strains produced clear rings of inhibition of indicator organisms 2-3 mm. wide. This inhibition was also bactericidal

and not serially transmissible. All 25 strains inhibit the same 44 L. fermenti indicators. Sixty-four homofermentative strains of lactobacillus were then tested for susceptibility to the inhibitory action of the L. fermenti strains on agar and 5 strains of L. acidophilus were inhibited by all 25 L. fermenti strains. Subsequently inhibitory activity in undiluted supernatants of overnight broth cultures of the inhibitory L. fermenti strains was demonstrated by the techniques previously used. This activity was variable and could frequently be discerned only as a hazy spot of inhibition in the indicator lawn. The inhibitory activity of supernatants of L. fermenti broth cultures could be concentrated like that of L. acidophilus strains and showed the same diffusability in agar. No resistant mutants of L. fermenti indicator strains were obtained.

DISCUSSION

The properties of active supernatants differ from those of phage suspensions in that the activity is not serially transmissible, no individual plaques are ever produced, activity is diffusable in agar and bactericidal titres are heat-resistant. The inhibition is also not simply due to the production of acid in the supernatants of broth cultures or the agar surrounding stab cultures. Particular attention was paid to avoiding the pitfall of mistaking this inhibition of catalase-negative organisms for the action of hydrogen peroxide which may have accumulated in the supernatants (Wheater, Hirsch & Mattick, 1952). The inhibitory activity is non-dialysable and could be concentrated by precipitation with ammonium sulphate. These properties support the conclusion that these inhibitory agents are bacteriocins (Frédéricq, 1957; Reeves, 1965). The bactericidal action of the bacteriocins described here does not involve lysis of the susceptible organisms and may correspond to the mode of action of colicins (see Adams, 1959). The action also appears to be restricted to certain members of the family Lactobacteriaceae in much the same way as colicin activity is confined to the Enterobacteriaceae. Vincint, Veomett & Riley (1959) have considered the claims of some workers who reported antibiotic effects produced by various homofermentative lactobacilli. They conclude that the hydrogen peroxide effect can only be excluded in the report of Grossowics, Kaplan & Schneerson (1947) and in their own work. The anti-

biotic reported by Grossowics et al. (1947) is produced by an unidentified homofermentative lactobacillus and is soluble in ether. It is active on a wide range of Gram-negative and Gram-positive organisms but no activity against lactobacilli is indicated. The antibiotic described by Vincent et al. (1959) is produced by strains of L. acidophilus. It is produced only by aged agar cultures, is inactivated by ethanol and possesses a wide spectrum of activity which includes certain L. acidophilus strains. Neither of these inhibitory substances nor that described by Sabine (1963) fall within the definition of bacteriocins (Reeves, 1965) and they differ in many respects from the bacteriocins described here.

The production of bacteriocins often depends on growth conditions (see Reeves, 1965) and strains which pproduce bacteriocin on agar may show little or no activity in broth (Lachowicz, 1965). Because the methods initially used may not have been optimal (Reeves, 1965) the detection of 12 bacteriocinogenic strains with the use of 10 day-old supernatants must be regarded as a minimal finding. The re-investigationof bacteriocinogeny among Lactobacillus fermenti strains has confirmed this and the use of solid media has increased the incidence of bacteriocin production in these strains from 2.4% to 15.5%.

The typing of organisms by their susceptibility to and/or production of bacteriocins may be taxonomically and epidemiologically useful. Such typing schemes based on bacteriocin production have been proposed for Shigella sonnei (Abbot & Shannon, 1958; Gillies, 1964), Psuedomonas pyocyanea (Darrell & Wahba, 1964) and Proteus mirabilis (Cradock-Watson, 1965). The spectra of activity of the bacteriocins produced by Lactobacillus acidophilus strains differ and reactions to them may serve as a useful complement to phage-typing schemes of lactobacilli. The bacteriocins of L. fermenti strains form an apparently homogeneous group which in the absence of resistant mutants could not be distinguished from one another by the use of cross-resistance tests (Frédéricq, 1948). The identity of their spectra of activity limits the use of these bacteriocins in the classification of strains of L. fermenti.

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Plate 1

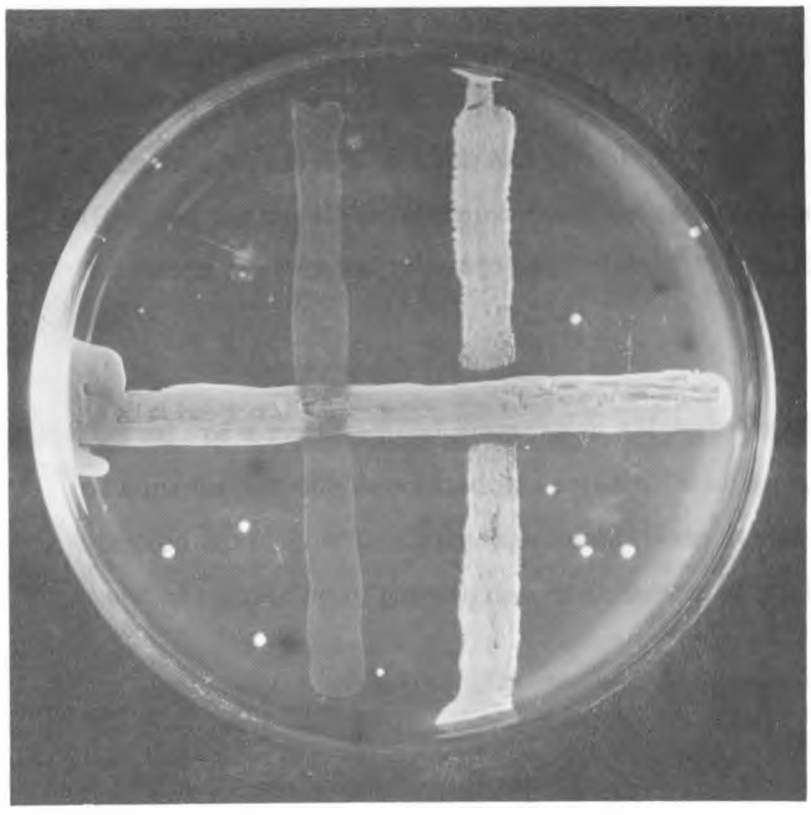


Fig. 1.

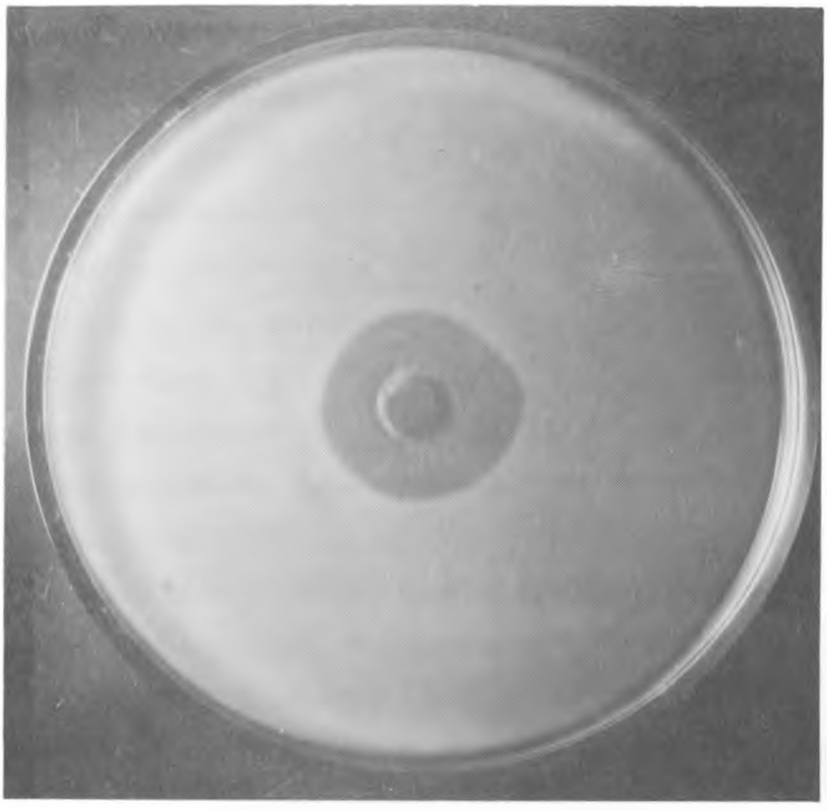


Fig. 2.

EXPLANATION OF PLATE

Plate 1

Fig. 1. Fermenti strain 466 washed and streaked on agar. Strain F 1 (Reading fermenti) and fermenti 206 cross-streaked. Plates incubated anaerobically overnight. Strain F 1 inhibited by strain 466.

Fig. 2. 0.2 ml. of a resuspended ammonium sulphate precipitate (pH 6.8) of supernatant 466 deposited in sealed hole in agar. After 8 hours surface covered with sloppy agar containing strain F 1 and incubated. Area of inhibition of growth due to diffusion of antibiotic.

CHAPTER 9

PROPERTIES OF A LACTOBACILLUS FERMENTI BACTERIOCIN[1]

SUMMARY

The properties of a bacteriocin derived from Lactobacillus fermenti strain 466 were investigated. The bacteriocin was present in low titre in supernatant fluids from overnight broth cultures and was not inducible by ultraviolet radiation. It was purified and concentrated to a titre of 1/1000 by dialysis, chromatography on Sephadex G 100 and calcium phosphate gel columns. The bacteriocin is heat stable, and sensitive to trypsin and pepsin but not to lysozyme. No migration was demonstrated in electrophoretic fields in agar gel. Electron microscopy of the bacteriocin did not show any phage components. The bacteriocin is a macromolecular lipocarbohydrate protein which consists of 16 amino acids, four sugars, hexosamine and phosphorus. The biological activity of this complex is dependent on its structural integrity.

INTRODUCTION

Bacteriocins as defined by Reeves (1965) are produced by homofermentative and heterofermentative species of the genus Lactobacillus (de Klerk & Coetzee, 1961). The incidence of bacteriocinogeny in Lactobacillus fermenti is 15.5% (de Klerk, 1967). All the L. fermenti bacteriocins have an identical host range and attempts to isolate resistant mutants have been unsuccessful. This has precluded the classification of these bacteriocins by cross-resistance tests (Frédéricq, 1948). Investigations of the chemical nature of bacteriocins have shown them to be a heterogeneous group of macromolecular substances. Some are simple proteins, namely megacin 216 (Holland, 1961), a pyocin (Homma & Suzuki, 1964) and colicins E2-P9 and E3-CA38 (Herschman & Helsinki, 1967). Others, colicin E2-CA42 (Reeves, 1963) and bacteriocin MR336 of Proteus morganii (Smit, de Klerk & Coetzee, 1968) are glycoproteins or complexes of protein, carbohydrate and lipid, like colicins K-K235 (Goebel

& Barry, 1958), V-K357 (Hutton & Goebel, 1961, 1962), A-CA31 (Barry, Everhart, Abbot & Graham, 1965), I (Keene, 1966) and the unclassified colicin SG 710 (Nüske, Hösel, Venner & Zinner, 1957). Another group of bacteriocin-like substances consist of particles of high molecular weight which resemble phages (Endo, Ayabe, Amako & Takeya, 1965; Sandoval, Reilly & Tandler, 1965) or parts of phages (Ishii, Nishi & Egami, 1965; Coetzee, de Klerk, Coetzee & Smit, 1968). The specific chemical nature of all other bacteriocins is unknown (see Hindsdill & Goebel, 1964; Reeves, 1965; Nomura, 1967). The chemical nature of the association between lipopolysaccharide and protein in bacteriocins is also unknown but their biological activity is always associated with the protein component (see Nomura, 1967) and it is this protein and not the polysaccharide which is genetically controlled by the bacteriocinogenic factors in Escherichia coli (Hindsdill & Goebel, 1966). The purpose of this study was the purification and chemical analysis of one of the apparently homogeneous group of L. fermenti bacteriocins.

METHODS

Media. The liquid medium used was the MRS broth of de Man, Rogosa & Sharpe (1960) freed from high molecular weight constituents by dialysis (tubing size 18/32, Visking Co., Chicago, Ill., U.S.A.) under negative pressure. Solid medium was MRS broth diffusate + 2% (w/v) agar.

Bacterial strains. The origins of the bacteriocinogenic Lactobacillus fermenti strain 466, the indicator L. fermenti strain F 1 and other indicator strains were described in Chapter 8. Cultures were incubated as described in earlier Chapters.

Preparation of bacteriocin. Crude preparations of bacteriocin were obtained by overnight growth of the bacteriocinogenic strain at 37^{o} in broth diffusate medium. The culture was centrifuged, the supernatant fluid sterilized with chloroform and concentrated in a rotary evaporator at 40^{o}. The concentrate was dialysed against de-ionized water to remove nutrients and small molecular weight bacterial products in the diffusate. The volume of the dialysis residue was further decreased by evaporation and then dialysed against 0.1

M-KCl+0.05 M-tris+HCl buffer (pH 7.5).

Purification of bacteriocin. Crude bacteriocin was purified by gel filtration on Sephadex G 100. Samples (2 ml.) were applied to columns (50 cm. x 2.5 cm.) and eluted with 0.1 M-KCl+0.05 M-tris+HCl buffer (pH 7.5). Column effluents were collected in 5 ml. fractions and assayed for activity and the extinction at 280 mμ determined. High titre fractions were pooled and centrifuged at 200,000 g for 15 min. to remove bacterial debris. The supernatant fluid was concentrated, dialysed against buffer and the dialysis residue filtered through Sephadex or chromatographed on calcium phosphate gel columns.

Adsorption chromatography of bacteriocin on calcium phosphate gel. Precipitated calcium phosphate was prepared according to Tiselius, Hjertén & Levin (1956), suspended in 1 mM-sodium phosphate buffer (pH 6.8) and applied to a chromatography tube (0.8 cm. x 12 cm.). A sample (2 ml. equiv., 10 mg. dry wt) of partly purified bacteriocin was dialysed against 1 mM-sodium phosphate (pH 6.8) and applied to the column. The bacteriocin was eluted with 1 mM-sodium phosphate buffer (pH 6.8) and step-wise elution with 10, 50 and 200 mM-sodium phosphate buffer solutions (pH 6.8; 50 ml. of each concentration) was continued. Fractions (5 ml.) were collected and assayed for activity and the extinction at 280 mμ determined.

Ultraviolet induction of bacteriocin. Lactobacillus fermenti strain 466 organisms from a 20 ml. overnight broth culture were deposited by centrifugation and re-suspended in an equal volume of sterile distilled water in a Petri dish. The suspension was irradiated with a 30 W. Hanovia Sterilamp (wavelength 2537 Å) from a height of 25 cm. At intervals of 5, 10, 15, 25, 40 sec., samples of 4 ml. were pipetted into 20 ml. warmed broth and incubated overnight in the dark. The supernatant fluids of these cultures were sterilized with chloroform and assayed for bacteriocin activity.

Assay of the bacteriocin. This was done by spotting drops of two-fold serial dilutions of the material in MRS broth on to agar plates seeded with the indicator strain and incubating overnight at 37°. The highest inhibitory dilution was the titre.

Analysis of bacteriocin

Purified bacteriocin was dialysed against repeated changes of distilled water and evaporated to constant dry weight under reduced pressure over P_2O_5. The bacteriocin (84.1 mg.) was then dissolved in distilled water (25 ml.).

Samples (0.2 ml.) of bacteriocin preparation were treated with .3 N-NaOH (0.1 ml.) and the protein content measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine albumin (Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) as standard.

Total nitrogen was determined by a micro-Kjeldahl technique (Ballantine, 1957).

Deoxyribonucleic acid and ribonucleic acid were determined by the methods of Burton (1956) and Dische & Borenfreund (1957), respectively.

Bacteriocin (6.728 mg.) was hydrolysed in a sealed tube for 24 hr with 6 N-HCl (5 ml.) at 105°. The acid was evaporated in vacuo over solid P_2O_5 and NaOH, and the amino acid composition determined with a Beckman automatic amino acid analyser, Model 120 B.

Bacteriocin (3 ml.) was treated with 12 N-perchloric acid (0.36 ml.) and left at 0° for 16 hr. The precipitated protein was removed by centrifugation and total phosphate in the supernatant fluid estimated by the method of Gomori (1942).

Estimation of lipids. The total lipid content of bacteriocin solution (10 ml.) was determined according to Salton (1953). The procedure consisted of hydrolysis with 6 N-HCl for 8 hr at 100°, followed by ether extraction of the hydrolysate.

Hexosamine estimation. Samples (2 ml.) of bacteriocin solution were hydrolysed in sealed tubes with 4 N-HCl for 15 hr at 100°. The acid was evaporated in vacuo over solid P_2O_5 and NaOH, and the liberated hexosamine separated from interfering substances (sugars and lipids) by cation exchange chromatography on columns (1 cm. x 10 cm.) of Amberlite IR 120 (H^+-form) resin. The hexosamine was eluted with 2 N-HCl and estimated according to Boas (1953), with glucosamine hydrochloride (British Drug Houses Ltd.; BDH) as standard.

Liberation of carbohydrates. The bacteriocin solution (2.0 ml.) was hydrolysed for 2 hr at 100° in a final concentration of 2 N-H_2SO_4 and the hydrolysate applied to a column (1 cm. x 10 cm.) of Amberlite IR 120 (H^+-form) cation exchange resin (Weibull & Bergström, 1958). Neutral sugars were eluted with water and concentrated under reduced pressure below 40° to a small volume. The sugar solution was transferred quantitatively to a 5 ml. standard flask and made to volume.

Paper chromatography of carbohydrates. One-dimensional descending chromatography was done on Whatman no. 1 filter paper with 2,4,6-trimethylpyridine as solvent (Partridge, 1948). D (+)-Galactose, D(+)-glucose, D(+)-mannose and L(+)-rhamnose (BDH) were used as references. After developing the chromatograms for 40 hr, the solvent was removed by evaporation and the chromatograms sprayed with p-anisidine phosphate in ethanol (Mukherjee & Srivastava, 1952).

Quantitative estimation of carbohydrate components. A sample (0.5 ml.) of the sugar extract was applied as a band (10 cm. wide) in the centre and 8 cm. from the top of Whatman no. 1 filter paper (30 cm. x 57 cm.) together with two guide spots, 5 cm. from each edge, to which 50 µl. of sugar extract were applied. This chromatogram and a blank were developed for 40 hr in 2,4,6-trimethylpyridine and dried for 16 hr at room temperature. The positions of the separated sugars on the guide strips were revealed by the reagent and the corresponding areas excised from the experimental and blank chromatogram. Sugars and blanks were quantitatively eluted from each strip with distilled water into measuring cylinders (5 ml.) according to Dent (1947). Elution was stopped when 2 ml. of solvent had been collected and samples (1 ml.) were assayed for sugar concentration by the anthrone method of Scott & Melvin (1953).

Dissociation of protein and carbohydrate fractions. The phenol method of Goebel & Barry (1958) and hydrolytic techniques (Knox & Hall, 1965) were used in attempts to dissociate protein and carbohydrate components of the bacteriocin respectively.

Sedimentation of bacteriocin. This was done at 56,100 rev./min. at 20° in the AN-D rotor of a Spinco model E ultra-centrifuge. A solution of about 1% purified bacteriocin was used in a 12 mm. standard cell and photographs were

taken at 8 min. intervals, with schlieren optics.

Action of enzymes on bacteriocin

Lysozyme. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of lysozyme (BDH; 100 µg./ml.) in 0.1 M-ammonium acetate for 5 hr.

Pepsin. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of crystalline pepsin (BDH; 1 mg./ml.) in 0.02 N-HCl for 24 hr. After digestion the mixture was adjusted to pH 7.0 with 0.1 N-NaOH.

Trypsin. Bacteriocin solution (3 mg./ml.) was incubated at 37° with an equal volume of crystalline trypsin (BDH; 0.5 mg./ml.) in 200 mM-sodium phosphate buffer (pH 7.5) for 3 hr. Control experiments were done without enzymes and bacteriocin titres determined.

Other investigations

Action of urea on bacteriocin. Bacteriocin was dissolved in a final concentration of 6 M-urea. Controls consisted of bacteriocin in water and a 6 M-urea solution. After 16 hr at 37° the solutions were assayed.

Chromatography on ion-exchange resins. This was done on Amberlite IR 410 (Cl^--form) anion and Amberlite IR 120 (H^+-form) cation exchange resins.

Agar electrophoresis. Agar electrophoresis of purified bacteriocin at pH 5.2 and pH 8.5 was done by the method of Maré, Coetzee & de Klerk (1964).

Electron microscopy. Purified bacteriocin dissolved in distilled water was negatively stained with neutral potassium phosphotungstate (Brenner & Horne, 1959). Samples were mounted on carbon support films by a spreading technique (Bradley, 1962) and examined with a Philips EM 200 electron microscope.

RESULTS

Isolation of pure bacteriocin

Supernatant fluids of overnight cultures of Lactobacillus fermenti strain 466 in the MRS diffusate medium had inhibitory titres of 1/4. This medium did not contain molecules with a weight exceeding 10,000 (Leggett Bailey, 1962) and facilitated the separation of the bacteriocin which was not diffusible through the Visking tubing (de Klerk & Coetzee, 1961). Bacteriocin production was not enhanced by ultraviolet irradiation. One litre of crude bacteriocin was purified and concentrated to 2 ml. with a titre of 1/1000, this represented a recovery of 40-60%. The primary separation of fractions on Sephadex (Fig. 1) and further purification on calcium phosphate gel (Fig. 2) are shown. In both graphs two peaks are present. The first had an absorption maximum of 277 mμ and contained all the activity. The second peak represented bacterial protein and broth constituents, and was virtually absent after repassage through either column. Ultracentrifugation of the purified bacteriocin revealed a single peak with an S_{20w}, value of 2.8 (Plate 1).

Chemical composition

The chemical composition of the bacteriocin is shown in Table 1. It consisted mainly of carbohydrate, protein and lipid, with lesser amounts of hexosamine and phosphorus, and was free from nucleic acids. The carbohydrate fraction (Table 2) contained four sugars; the mannose content being 56.7%. The protein fraction contained 16 amino acids (Table 3). Alanine, glycine, serine and the acidic amino acids were present in the highest concentrations.

Properties of the bacteriocin

Purified bacteriocin was a fluffy, white amorphous powder freely soluble in water. Aqueous solutions kept at room temperature for several

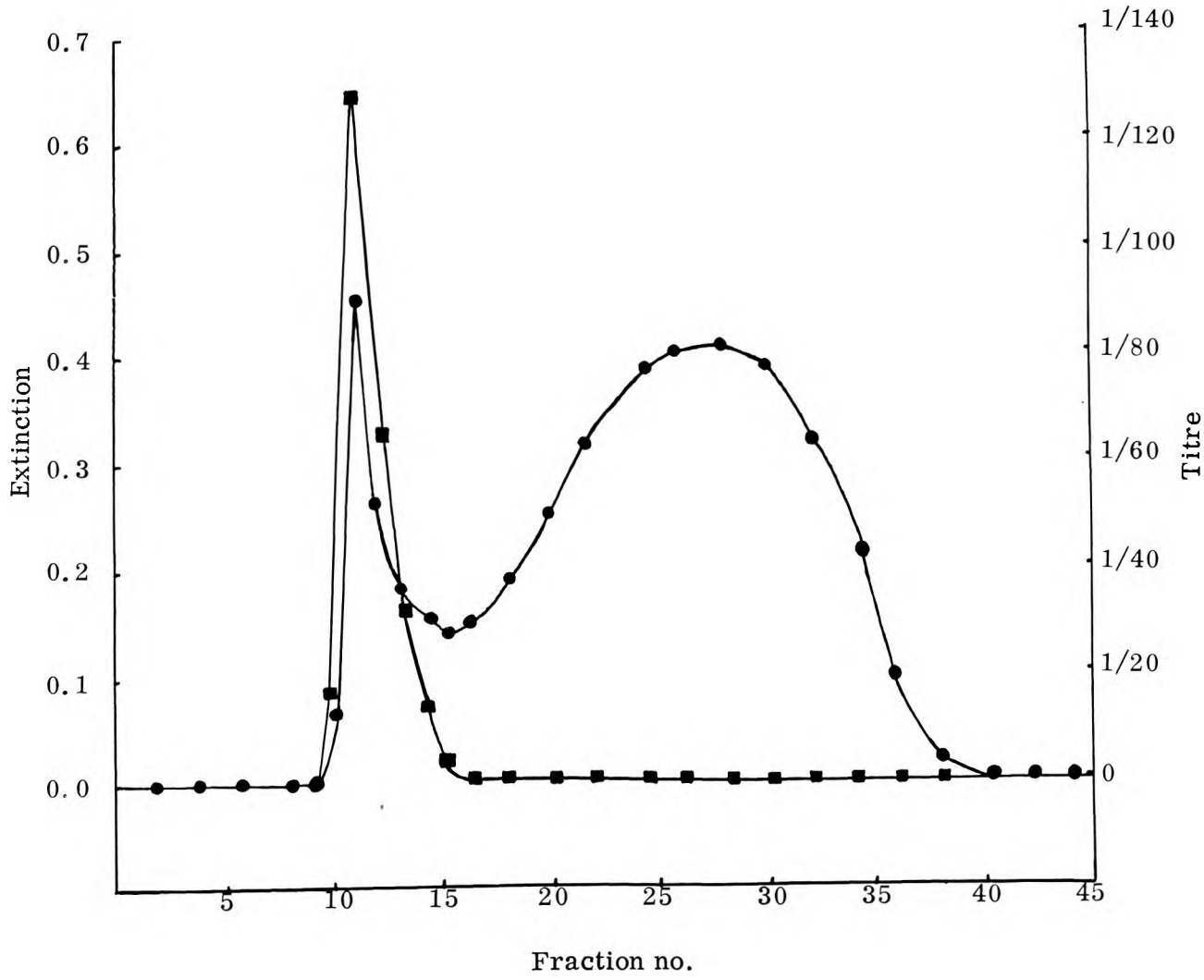


Fig. 1. Chromatography of crude bacteriocin of Lactobacillus fermenti 466 on Sephadex-G-100. A 2 ml. sample (titre: 1/1000) was applied to the column and eluted with 0.1 m-KCl+0.05M-tris+HCl buffer (pH 7.5). Fractions (5 ml.) were collected and assayed for:

● ———— ●, extinction; and ■ ———— ■, inhibitory activity.

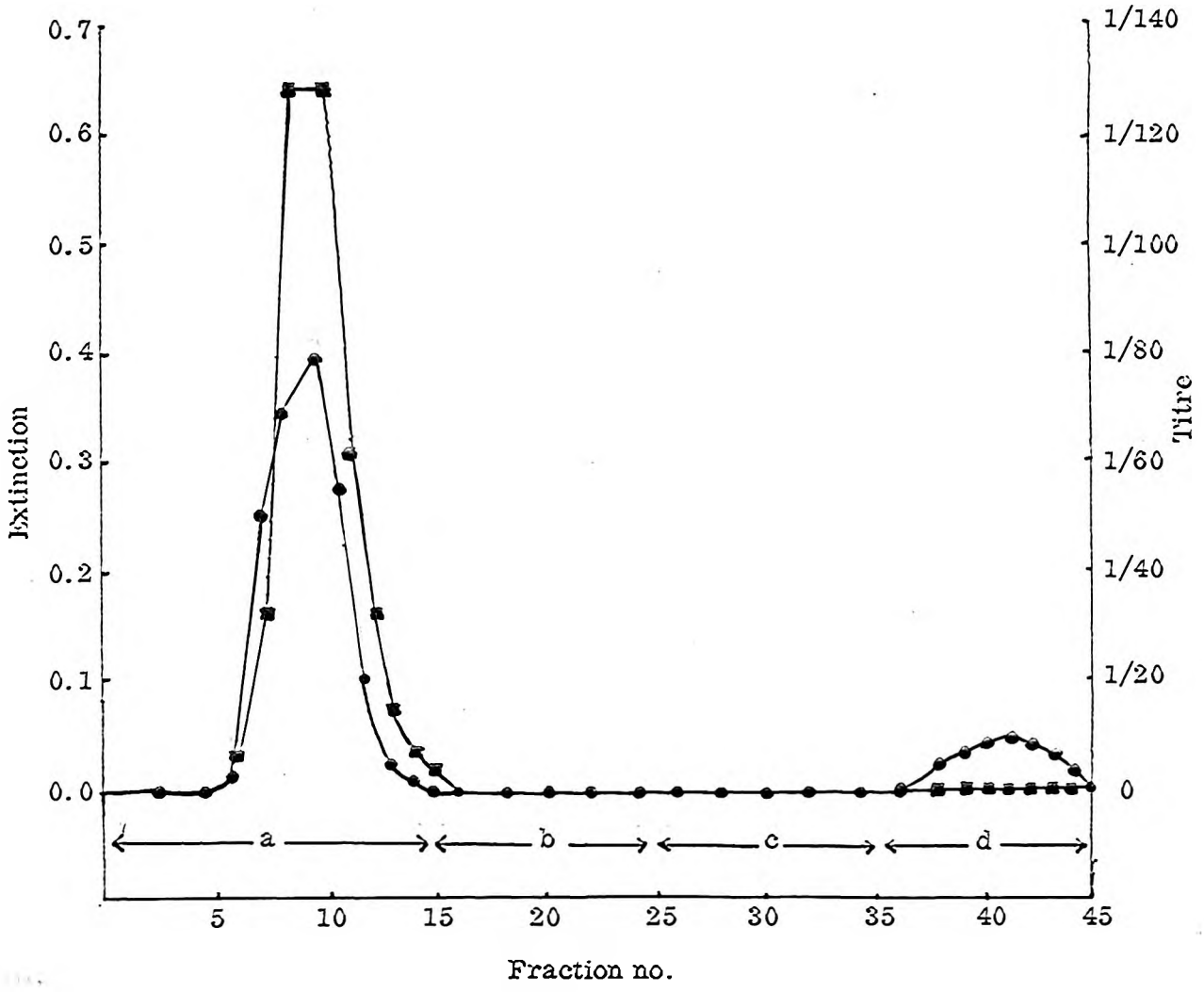


Fig. 2. Chromatography of bacteriocin of Lactobacillus fermenti 466 on calcium phosphate gel. Partially purified bacteriocin was concentrated and dialysed against 1 mM-sodium phosphate buffer (pH 6.8). A 2 ml. sample (titre: 1/1000) was applied to the column and stepwise elution done with sodium phosphate buffer (pH 6.8) at concentrations of 1, 10, 50 and 200 mM. Fractions (5 ml.) were collected and assayed for: o _____ o, extinction; and ▪ _____ ▪, inhibitory activity.

(a) 1mM, (b) 10mM, (c) 50mM, (d) 200 mM-sodium phosphate.

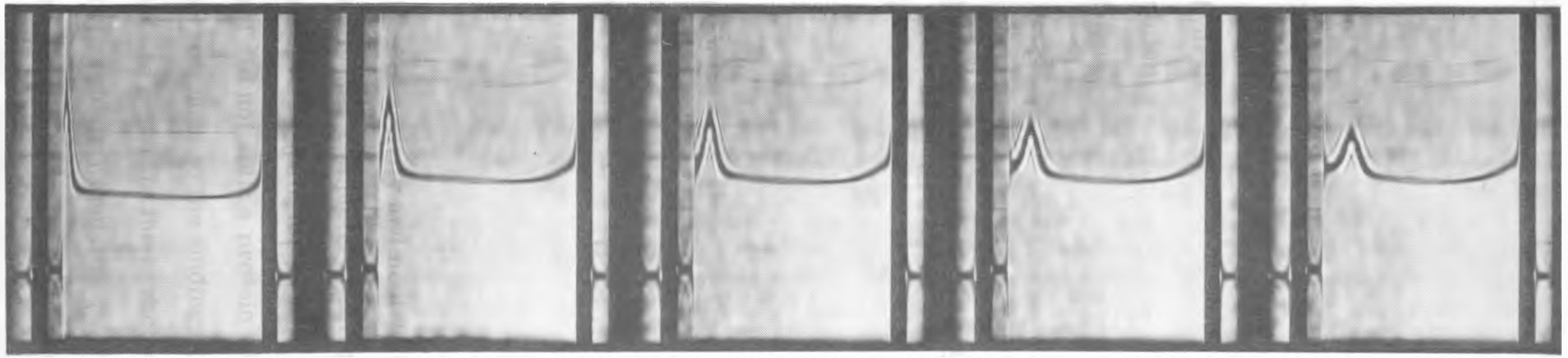


Plate 1. Sedimentation pattern of a c. 1% (w/v) solution of purified bacteriocin of Lactobacillus fermenti strain 466 in 0.1 M-KCl recorded at 8 min. intervals at 56,100 rev./min. Sedimentation was to the right.

Table 1. Chemical composition of bacteriocin of Lactobacillus fermenti strain 466

	(%)
Nitrogen	4.96
Protein	23.80
Lipid	20.80
Carbohydrate	53.20
Hexosamine	0.80
Phosphorus	0.30

Table 2. Carbohydrate composition of bacteriocin of Lactobacillus fermenti strain 466

	(%)
Galactose	19.4
Glucose	16.7
Mannose	56.7
Rhamnose	7.2

days showed no loss of activity and neutral solutions withstood heating at 96° for 30 min. Activity was completely destroyed by trypsin and was decreased to half when treated with pepsin for 24 hr. The bacteriocin was unaffected by lysozyme. Phenol did not split a protein with bacteriocin activity from the complex; this corresponds to the findings of Barry et al. (1965) with colicin A-CA31. Attempts to separate the carbohydrate moiety by hydrolysis with hot 0.1 N-sulphuric acid or warm 10% (w/v) trichloroacetic acid resulted in total inactivation. Extraction of a bacteriocin solution with chloroform or ether removed no lipid material and did not affect activity. The bacteriocin activity was also unaffected by treatment with urea. Agar electrophoresis at different pH values showed no migration of the bacteriocin when several diffe-

Table 3. Amino acid composition of the protein fraction of bacteriocin of Lactobacillus fermenti strain 466.

Amino acid	Residues/1000 total amino acid residues
Lysine	57.3
Histidine	18.9
Arginine	29.0
Aspartate	100.1
Threonine*	87.6
Serine*	101.0
Glutamate	92.3
Proline	47.2
Glycine	111.2
Alanine	134.3
Valine	56.0
Methionine	4.5
Isoleucine	37.6
Leucine	64.0
Tyrosine	30.7
Phenylalanine	28.3

* Corrected for loss during hydrolysis

rent Lactobacillus fermenti strains were used as indicators. Anion and cation exchange resins did not retain the bacteriocin. Electron microscopic examination showed only amorphous material.

DISCUSSION

Lactobacillus fermenti strain 466 produces a single bacteriocin, which is non-inducible by ultraviolet radiation. The bacteriocin differs in the latter

respect from megacin 216 (Holland, 1961), colicin E2-CA42 (Reeves, 1963) and pyocin (Homma & Suzuki, 1964). It is a lipocarbohydrate protein similar in general composition to colicins (Hinsdill & Goebel, 1964). Attempts to isolate an active protein fraction from the complex were unsuccessful although the phenol method ruptures covalent bonds (Holland, 1961) and an active protein was split from the colicin K-K235 complex in this way (Goebel & Barry, 1958). Mild techniques of hydrolysis which split sugar phosphate bonds to release a carbohydrate fraction (Knox & Hall, 1965) destroyed the biological activity of the bacteriocin. These findings support the conclusion that activity depends on the integrity of the lipocarbohydrate protein complex. The chemical composition of only one of the bacteriocins produced by Gram-positive organisms, megacin 216 (Holland, 1961), is known; this is a protein with traces of phosphorus and carbohydrate. It is exceptional in that it causes lysis of sensitive cells (Ivanovics, Alföldi & Nagy, 1959) and appears to be an enzyme, a phospholipase A (Ozaki et al. 1966). The enterocin of Streptococcus zymogenes (Brock & Davie, 1963) is possibly a protein (Hamon, 1964) with a lipid component. The 16 amino acids of the Lactobacillus fermenti strain 466 bacteriocin correspond to those of megacin 216 (Holland, 1961), but the concentration of acidic amino acids in the latter bacteriocin is higher. The lactobacillus bacteriocin has a trace of methionine whilst cysteine/cystine are absent. This suggests that the three-dimensional structure of the macromolecule does not depend on the presence of disulphide linkages. The activity of this bacteriocin is probably independent of hydrogen bonds since it is not affected by high concentrations of urea.

The absence of migration in an electric field at different pH values was unexpected since the dicarboxylic amino acids, aspartate and glutamate, predominate. The bacteriocin diffused through 0.8% (w/v) agar (de Klerk. 1967) and its electrophoretic immobility did not result from inability to penetrate the agar lattice. The failure of ion-exchange resins to retain the bacteriocin confirms its lack of net ionic charge: its ionic groups may be masked by lipid.

Homma & Suzuki (1964) isolated a cell-wall protein with bacteriocin activity from Pseudomonas aeruginosa. Cell-walls of Lactobacillus fermenti contain galactose, glucose, glucosamine and aspartate, glutamate, alanine and lysine (Ikawa & Snell, 1960). The bacteriocin of L. fermenti strain 466

differs in composition from its cell-wall and is much more complex.

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CHAPTER 10

PROPOSED INVESTIGATIONS ARISING FROM THESE STUDIES

Inevitably unanswered questions and new avenues of investigation have been revealed by these studies and it is my intention to persue some of these. Three main courses will be followed.

The first is the search for a system or systems through which genetic analysis of lactobacilli may eventually be accomplished, and several approaches to this problem are now possible. Markers for genetic studies in lactobacilli are at a premium. Streptomycin resistance is a natural characteristic of most Lactobacillus spp. (de Klerk, & Coetzee, 1962) and this precludes its use for this purpose. The S-R variation in strains of L. casei (see Chapter 4) may provide such a marker. This variation has now been observed in more strains of L. fermenti and attempts will be made to select mutants of lactobacilli with other markers such as resistance to sodium azide and various antibiotics like novobiocin for possible intraspecies transduction (see Hayes, 1965) with temperate phages of L. fermenti. The search for further temperate phages is continuing.

The genetic transfer of hereditary characters by transformation was first shown in pneumococci (Griffith, 1928). This mechanism which operates in other genera (see Hayes, 1965) has also been demonstrated in another member of the family Lactobacteriaceae, the streptococci (Perry & Slade, 1964). Attempts are being made to transform lactobacilli using the S-R variation as the marker. The extraction of suitable deoxyribonucleic acid (DNA) is proving difficult. In conjunction with this work it is proposed to investigate the possibility of transfection of lactobacilli (see Spizizen, Reilly & Evans, 1966) and of lactobacillus protoplasts (see van de Pol, Veldhuizen & Cohen, 1961) with nucleic acids extracted from lactobacillus phages. Although Repaske (1958) failed to produce protoplasts of L. casei using lysozyme, EDTA and Tris it has proved possible to produce them from strains of L. fermenti. A modification of the method of Lederberg (1956) in which penicillin G is replaced by Ampicillin was used.

Another approach to genetic analysis of lactobacilli may lie in the

possible existence of sex factor activity in the genetic determinants of their bacteriocins as in the case of certain colicinogenic factors (see Nomura, 1967). To this end attempts would first be made to show possible infectivity of the genetic determinants by transfer of the bacteriocinogenic property between strains of lactobacilli. This would establish the extra-chromosomal nature of such determinants and these might possibly exhibit sex activity.

The second line of investigation to be followed concerns the characterization of the nucleic acids from lactobacillus phages. Nothing is as yet known of the nature or conformation of their nucleic acids or whether they exhibit such characteristics as cohesive terminal sites as in phage lambda DNA (Hershey & Burgi, 1965) or show terminal redundancy like the DNA of phages T3 and T7 (Ritchie, Thomas, MacHattie & Wensink, 1967).

The final direction in which the extension of these studies may be persued envisages further investigation of the bacteriocins of lactobacilli. Nothing is known of the chemical nature of the heterogeneous group of L. acidophilus bacteriocins and representatives of the group should be analysed. Finally, bacteriocins have been shown to possess a variety of mechanisms of action (see Nomura, 1967) and the mode of action of the lactobacillus bacteriocins deserves attention.

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APPENDIX 1. Lactobacillus counts/ml. saliva* from Nuwerus.

6-Year age group.

Code NO:	Sample No: I	II	III	IV	V
NH. 30	2.0×10^4	6.0×10^1	0		
	1.4×10^4	1.5×10^2	0		
NH. 31		8.9×10^5	1.7×10^5		
	6.1×10^5	8.0×10^5	8.3×10^4		
NH. 32	3.6×10^5	2.9×10^5	2.5×10^5		
	2.9×10^5	3.0×10^5	1.7×10^5		
NH. 33	1.3×10^5		9.4×10^5		
	7.0×10^4		8.4×10^5		
NH. 34	7.6×10^4	6.1×10^4	6.1×10^4		
	5.6×10^4	4.6×10^4	4.0×10^4		
NT. 36	9.1×10^5				
	-				
NH. 37	0				
	-				
NH. 38	3.3×10^3				
	-				
NT. 39	3.4×10^5				
	-				
NT. 40	2.1×10^6				
	-				
NH. 41	8.7×10^3				
	-				
NT. 42	3.6×10^5				
	-				

* In all tables upper figures denote counts in control salivas, lower figures denote counts after oscillation.

APPENDIX 1 cont. Lactobacillus counts/ml. saliva from Nuwerus.

7-Year age group

Code No:	Sample No: I	II	III	IV	V
NH. 1	0	0	0		
	0	3.0×10^2	0		
NH. 2	4.6×10^4	2.5×10^4	0		
	5.0×10^4	2.3×10^4	0		
NT. 3	7.6×10^4	2.6×10^4	3.0×10^3		
	1.0×10^5	2.2×10^4	9.0×10^3		
NT. 4	1.7×10^5	2.4×10^5			
	1.1×10^5	1.9×10^5			
NH. 5	2.8×10^4	5.1×10^4			
	3.1×10^4	3.8×10^4			
NT. 6	5.7×10^5		0		
	5.1×10^5		0		
NH. 30				6.0×10^1	0
				9.0×10^1	
NH. 31				7.2×10^4	3.8×10^4
				3.4×10^4	
NH. 32				3.7×10^5	6.0×10^5
				2.9×10^5	
NH. 33				5.5×10^5	1.5×10^6
				5.2×10^5	
NH. 34				3.7×10^4	2.0×10^5
				2.9×10^4	

APPENDIX 1 cont. Lactobacillus counts/ml. saliva from Nuwerus.

8-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH. 1				6.0×10^1	0
				0	
NH. 2				3.0×10^1	2.9×10^5
				3.0×10^1	
NT. 3				3.0×10^1	5.1×10^3
				0	
NH. 5			0	1.8×10^3	
			9.0×10^1		
NT. 6				4.5×10^3	
				6.0×10^3	
NH. 7	3.0×10^2	6.0×10^1	0		
	1.5×10^2	9.0×10^2	0		
NH. 8	1.8×10^4	4.2×10^4	1.2×10^5		
	2.3×10^4	2.2×10^4	1.0×10^5		
NH. 9			1.9×10^5		
			9.5×10^4		
NH. 10	1.9×10^4	2.7×10^4			
	1.5×10^4	1.2×10^4			
NH. 11	3.1×10^5		1.6×10^5		
	3.3×10^5		1.6×10^5		
NH. 12	0	3.0×10^2	0		
	0	6.0×10^1	6.0×10^2		
NH. 13	1.6×10^4	2.8×10^5			
	2.2×10^4	9.9×10^4			

APPENDIX 1 cont. Lactobacillus counts/ml. saliva from Nuwerus.

9-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH. 7				0 1.8×10^2	0
NH. 8				4.8×10^5 3.1×10^5	3.6×10^6
NH. 9				3.6×10^5 4.0×10^5	0
NH. 10			4.9×10^4 7.8×10^4	1.4×10^5	
NH. 11				9.6×10^3 1.1×10^4	4.3×10^4
NH. 12					0
NH. 13			1.1×10^4 5.4×10^3	4.1×10^5	
NT. 14	1.9×10^3 1.9×10^3	0 6.0×10^1	7.9×10^4 3.5×10^4		
NH. 15	 3.4×10^5	3.0×10^5 4.8×10^5	1.5×10^5 9.5×10^4		
NH. 16	0 0	3.0×10^1 3.0×10^2	0 0		
NH. 17	1.4×10^4 1.4×10^4	9.0×10^2 6.3×10^2	0 0		
NH. 18	7.8×10^2 1.2×10^3	9.0×10^1 9.0×10^1	3.0×10^2 3.0×10^2		
NT. 19	1.7×10^5 2.0×10^5	4.0×10^5 2.6×10^5	9.6×10^4 6.5×10^4		

APPENDIX 1 cont. Lactobacillus counts/ml. saliva from Nuwerus.

10-Year age group.

Code No:	Sample No: I	II	III	IV	V
NT. 14				2.4×10^3 1.7×10^3	4.5×10^3
NH. 15				2.4×10^6 2.4×10^6	3.3×10^3
NH. 16				3.0×10^1 1.2×10^2	0
NH. 17				0	
NH. 18				3.3×10^3 3.0×10^3	8.4×10^4
NT. 19				6.5×10^5 4.4×10^5	2.4×10^5
NH. 20	8.0×10^4 7.4×10^4	3.2×10^4 2.5×10^4	1.7×10^5 7.8×10^4		
NH. 21	1.0×10^5 6.0×10^4	3.8×10^5 3.8×10^5			
NH. 22	0 0	3.0×10^1 3.0×10^1	6.0×10^2 0		
NH. 23	0 0	1.5×10^2 1.5×10^2	9.0×10^2 6.0×10^2		
NH. 24	5.7×10^3 5.4×10^3	6.6×10^4 8.8×10^4	1.1×10^5 6.6×10^4		
NT. 25			1.7×10^4 1.9×10^4		
NH. 26	0 0	0 1.1×10^3	0 0		
NH. 27	0 0	0 1.2×10^2	3.0×10^3 0		
NH. 28	1.3×10^5 5.5×10^4	4.4×10^3 4.5×10^3	2.4×10^4 1.2×10^4		
NH. 29			0 0		

APPENDIX 1 cont. Lactobacillus counts/ml. saliva from Nuwerus.

11-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH. 20				1.3×10^5 1.7×10^5	1.6×10^5
NH. 21			1.1×10^6 7.2×10^5	9.6×10^5	
NH. 22				0 0	0
NH. 23				5.0×10^4 3.2×10^4	6.9×10^3
NH. 24				6.1×10^3 8.4×10^3	8.7×10^3
NT. 25				4.3×10^3 6.3×10^2	1.1×10^5
NH. 26				0 3.0×10^1	0
NH. 27				3.0×10^2 3.0×10^1	9.0×10^2
NH. 28				4.3×10^4 2.5×10^4	4.1×10^4
NH. 29				0 0	0

APPENDIX II Lactobacillus counts/mg. plaque material from Nuwerus.

6-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH.30	0	0	discarded		
NH.31	0	discarded	1.9×10^1		
NH.32	discarded	discarded	1.5×10^2		
NH.33	discarded	3.2×10^1	2.5×10^2		
NH.34	0	discarded	0		

7-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH.1	0	0	0		
NH.2	0	0	0		
NT.3	0	discarded	7.6×10^0		
NT.4	3.2×10^0	1.4×10^2			
NH.5	0	0			
NT.6	6.8×10^1	7.4×10^1	0		
NH.30				0	
NH.31				0	
NH.32				9.1×10^1	
NH.33				4.9×10^3	
NH.34				0	

APPENDIX II cont. Lactobacillus counts/mg. plaque material from Nuwerus.

8-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH. 1				discarded	
NH. 2				0	
NT. 3				0	
NT. 4					
NH. 5			0		
NT. 6				7.4×10^{1}	
NH. 7	0	0	0		
NH. 8	1.7×10^{1}	6.5×10^{1}	7.6×10^{1}		
NH. 9	0	2.0×10^{2}	1.8×10^{0}		
NH. 10	discarded	0			
NH. 11	0	0	1.4×10^{1}		
NH. 12	0	5.5×10^{0}	discarded		
NH. 13	5.8×10^{1}	discarded			

9-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH. 7				4.8×10^{0}	
NH. 8				2.8×10^{2}	
NH. 9				3.7×10^{1}	
NH. 10			0		
NH. 11				0	
NH. 12				0	
NH. 13			1.8×10^{2}		
NT. 14	0	5.5×10^{0}	0		
NH. 15	0	2.0×10^{1}	1.2×10^{1}		
NH. 16	2.7×10^{2}	3.6×10^{0}	0		
NH. 17	6.2×10^{2}	0	discarded		
NH. 18	0	0	discarded		
NT. 19	3.1×10^{1}	2.7×10^{1}	0		

APPENDIX II cont. Lactobacillus counts/mg. plaque material from Nuwerus.

10-Year age group.

Code No:	Sample No: I	II	III	IV	V
NT. 14				0	
NH. 15				6.1×10^{1}	
NH. 16				discarded	
NH. 18				discarded	
NT. 19				1.8×10^{4}	
NH. 20	1.8×10^{1}	0	discarded		
NH. 21	2.6×10^{0}	4.9×10^{2}			
NH. 22	0	0	3.9×10^{1}		
NH. 23	1.7×10^{1}	1.3×10^{1}	1.3×10^{1}		
NH. 24	1.4×10^{1}	discarded	discarded		
NT. 25	6.1×10^{3}	4.2×10^{2}	1.1×10^{1}		
NH. 26	6.0×10^{1}	3.4×10^{0}	0		
NH. 27	0	0	0		
NH. 28	0	0	2.9×10^{1}		
NH. 29	3.5×10^{3}	4.7×10^{1}	0		

11-Year age group.

Code No:	Sample No: I	II	III	IV	V
NH. 20				0	
NH. 21			5.5×10^{1}		
NH. 22				0	
NH. 23				discarded	
NH. 24				4.7×10^{1}	
NT. 25				0	
NH. 26				0	
NH. 27				0	
NH. 28				0	
NH. 29				0	

APPENDIX III. Lactobacillus counts/ml. saliva from Langkloof.

6-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA. 97	5.3×10^5 3.2×10^5				
LA. 98	2.4×10^5 2.3×10^5				
LC. 99	2.4×10^3 3.0×10^3				
LC. 100	2.0×10^5 1.8×10^5				
LC. 101	5.9×10^4 1.2×10^5				
LC. 102	5.7×10^5 3.5×10^5				
LE. 92	6.7×10^4 5.9×10^4				
LE. 94	0 0				
LB. 113	1.2×10^3 0				
LB. 115	2.8×10^4 2.7×10^4				
LB. 116	0 0				
LB. 117	4.3×10^6 3.0×10^6				
LD. 121	9.6×10^3 7.2×10^3				
LD. 122	6.9×10^4 3.1×10^4				
LF. 108	6.0×10^2 9.0×10^2				
LE. 109	2.8×10^4 1.9×10^4				
LE. 112	5.1×10^4 3.2×10^4				

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

	7-Year age group				
Code No:	Sample No: I	II	III	IV	V
LA.1	0 3.0×10^{3}				
LA.97		7.2×10^{5} 6.4×10^{5}	1.3×10^{6} 7.6×10^{5}		
LA.98		1.1×10^{6} 6.4×10^{5}	3.8×10^{5} 3.1×10^{5}		
LB.10	2.2×10^{6} 1.7×10^{6}				
LB.11	1.8×10^{5} 7.8×10^{4}	9.0×10^{3} 9.0×10^{3}			
LB.12	2.4×10^{5} 1.7×10^{5}				
LB.14	6.9×10^{5} 6.0×10^{5}	7.2×10^{5} 6.5×10^{5}			
LB.15	7.0×10^{4} 1.1×10^{4}				
LC.25	7.8×10^{5} 7.2×10^{5}				
LC.26	0 0				
LC.27	1.4×10^{6} 1.4×10^{6}				
LC.29	2.7×10^{5} 3.8×10^{4}				
LC.30	1.1×10^{5} 7.3×10^{4}				
LC.33	6.7×10^{4} 3.4×10^{4}				
LC.37	8.7×10^{3} 3.0×10^{2}				
LC.99		3.0×10^{1} 3.0×10^{1}	0 0		
LC.100		6.0×10^{1} 0	4.5×10^{3} 9.0×10^{2}		
LC.101		5.2×10^{3} 3.8×10^{3}	3.6×10^{3} 5.1×10^{3}		
LC.102		8.5×10^{4} 7.8×10^{4}	2.8×10^{6} 1.4×10^{6}		

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

7-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LC.103	0 0				
LD.54	0 0				
LD.55	4.3×10^5 2.4×10^5				
LD.56	6.0×10^3				
LD.58	1.7×10^5 1.3×10^5				
LD.59	3.1×10^5 2.3×10^5				
LD.95	8.3×10^5 4.5×10^5				
LE.71	1.4×10^5 1.6×10^5	6.2×10^4 7.1×10^4			
LE.79	1.3×10^4 6.9×10^3	4.5×10^4 3.2×10^4			
LE.92		1.8×10^2 1.2×10^2	0 0		
LE.93	4.5×10^4 2.4×10^4	0 3.0×10^2			
LF.87	4.5×10^5 4.2×10^5				
LF.89	0 0	0 0			
LF.90	1.2×10^5 1.0×10^5	1.2×10^4 4.2×10^3			
LF.91	9.9×10^5 8.2×10^5	1.5×10^6 8.4×10^5			
LB.96	1.0×10^2 7.0×10^2				
LF.104	0 3.0×10^2				
LF.105	2.1×10^5 6.6×10^4				
LF.106	9.0×10^2 6.0×10^2				

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

7-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LF.107	1.2×10^5 8.3×10^4				
LD.118	0 0				
LD.119	7.2×10^3 3.6×10^3				
LD.120	1.5×10^5 1.9×10^5				
LE.110	5.2×10^5 2.5×10^5				
LE.111	3.6×10^5 3.9×10^5				

8-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA.1		1.3×10^3 1.0×10^3	4.8×10^3 0		
LA.4	3.4×10^5 2.7×10^5				
LB.10		0 9.0×10^2			
LB.11			1.5×10^5 1.5×10^5	1.9×10^5 1.6×10^5	
LB.12		2.4×10^5 1.8×10^5	1.8×10^5 6.9×10^4		
LB.13	2.1×10^5 1.8×10^5	1.8×10^4 9.3×10^3			
LB.14			1.1×10^6 6.3×10^5	8.5×10^5 1.5×10^6	
LB.15		6.0×10^4 5.6×10^4			
LB.16	1.1×10^5 1.7×10^5	1.6×10^5 2.0×10^5			

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

8-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LB. 17	0 0	3.0×10^2 3.0×10^2			
LB. 18	1.2×10^4 4.5×10^3	1.5×10^4 1.2×10^4	9.0×10^2 1.2×10^3		
LC. 25		0 0	0 0		
LC. 26		0 0	0 0		
LC. 27		0 0	0 3.0×10^2		
LC. 28	1.5×10^5 5.4×10^4				
LC. 29		2.3×10^4 1.6×10^4	0 1.5×10^3		
LC. 30		1.7×10^5 1.1×10^5	2.4×10^5 7.5×10^4		
LC. 31	1.4×10^4 4.8×10^3				
LC. 32	1.0×10^5 1.1×10^5				
LC. 33		6.7×10^4 4.3×10^4			
LC. 34	6.1×10^5 4.7×10^5				
LC. 35	5.4×10^5 3.6×10^5				
LC. 37		6.0×10^2 1.2×10^2	7.5×10^3 6.3×10^3		
LC. 103		1.8×10^3 1.3×10^3	5.1×10^3 8.7×10^3		
LD. 54		1.8×10^4	1.2×10^4 8.7×10^3		
LD. 55			2.7×10^3 1.3×10^4		
LD. 56		1.8×10^3 9.0×10^2			
LD. 57	2.7×10^5 1.9×10^5				

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

8-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LD. 58		2.0×10^4 1.4×10^4	5.5×10^4 3.6×10^4		
LD. 59		1.5×10^4 1.3×10^4	8.8×10^4 4.7×10^4		
LD. 63	1.2×10^4 4.8×10^3				
LD. 64	1.9×10^6 1.4×10^6				
LD. 95		6.8×10^4 5.5×10^4	4.3×10^5 1.7×10^5		
LE. 71			1.2×10^5 1.0×10^5	5.4×10^4	
LE. 73	0 0	4.9×10^4 4.5×10^4			
LE. 75	4.4×10^5 5.0×10^5	3.6×10^5 4.5×10^5			
LE. 79			9.6×10^4 6.7×10^4	3.9×10^3 3.6×10^3	
LE. 81	1.1×10^6 1.9×10^6	9.0×10^3 6.0×10^3			
LF. 83		6.6×10^4 6.3×10^4			
LC. 38	5.0×10^4 3.0×10^4				

9-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA. 4		4.4×10^4 3.6×10^4	2.9×10^5 5.5×10^5		
LA. 6	6.0×10^3 3.6×10^3				
LA. 7	3.7×10^6 3.6×10^6				
LA. 8	5.6×10^5 4.0×10^5				

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

9-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LB. 13			8.5×10^3 7.0×10^3	3.6×10^5 3.6×10^5	
LB. 16			9.6×10^5 8.0×10^5	6.3×10^4 5.3×10^4	
LB. 20	3.8×10^4 2.1×10^4	1.6×10^5 1.3×10^5			
LB. 21	6.6×10^5 4.3×10^5				
LB. 22	1.4×10^4 1.3×10^4				
LC. 28		1.2×10^4 9.5×10^3	4.5×10^4 3.6×10^4		
LC. 31		2.7×10^5 1.4×10^5	4.0×10^5 2.9×10^5		
LC. 32		3.1×10^4 2.9×10^4			
LC. 34		1.8×10^3 9.0×10^2	1.8×10^4 1.7×10^4		
LC. 35		3.3×10^5 3.0×10^5	8.4×10^3 3.9×10^4		
LC. 36	0 0				
LC. 38		3.5×10^4 2.9×10^4	2.7×10^4 7.8×10^3		
LC. 39	2.5×10^4 0				
LC. 40	2.9×10^4 1.1×10^4				
LC. 45	1.9×10^5 1.4×10^5				
LD. 60	6.3×10^3 3.0×10^3				
LD. 61	4.0×10^4 3.3×10^4				
LD. 62	0 0				
LD. 63		3.2×10^3 3.7×10^3	3.8×10^4 1.4×10^4		

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

9-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LD.64		1.2×10^3 3.6×10^2	1.5×10^5 1.5×10^5		
LE.75			2.3×10^5 1.4×10^5	5.0×10^4 5.6×10^4	
LE.76	6.9×10^4 4.7×10^4				
LE.81			1.4×10^4 1.3×10^4	3.4×10^5 2.6×10^5	
LF.84	9.3×10^3 9.3×10^3	1.8×10^5 1.2×10^5			
LA.2	4.9×10^3 4.1×10^3	3.7×10^4 2.9×10^4			
LB.114	0 6.0×10^2				

10-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA.5	7.6×10^4 7.8×10^4				
LA.6		8.1×10^3 9.9×10^3			
LA.7		0 0			
LA.8		2.6×10^5 3.1×10^5	2.1×10^6 9.8×10^5		
LB.19	7.4×10^4 7.2×10^4	7.8×10^4 5.9×10^4			
LB.20			4.5×10^4 2.8×10^4		
LB.21		7.2×10^5 1.3×10^6			
LB.22		3.0×10^5 1.8×10^5			

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

10-Year age group. cont.

Code No:	Sample No: I	II	III	IV	V
LB. 24	2.4×10^5 3.4×10^5	6.7×10^4 6.3×10^4			
LC. 36		0 0	0 0		
LC. 39		8.2×10^4 7.9×10^4	1.2×10^6 2.2×10^5		
LC. 40		3.9×10^5 3.0×10^5	8.6×10^5 9.0×10^5		
LC. 42	1.5×10^3 3.0×10^2				
LC. 43	3.2×10^4 2.5×10^4				
LC. 44	1.5×10^3 2.1×10^3				
LC. 45		0 3.3×10^2	0 0		
LC. 46	3.2×10^4 1.5×10^4				
LC. 51	2.1×10^5 1.2×10^6				
LC. 53	8.4×10^5 1.2×10^6				
LD. 60		2.7×10^3 9.0×10^2	0 0		
LD. 61		3.8×10^4 3.1×10^4	3.0×10^2 0		
LD. 62		9.0×10^1 3.0×10^1	6.5×10^4 5.5×10^4		
LD. 65	2.3×10^5 2.3×10^4				
LD. 66		2.9×10^4 2.1×10^4	1.4×10^4 1.1×10^4		
LD. 69	3.3×10^5 1.7×10^5				
LE. 72	1.1×10^6 7.8×10^5	1.2×10^6 1.4×10^6			

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

10-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LE.74	1.6×10^4 1.6×10^4	1.2×10^4 1.1×10^4			
LE.76		3.0×10^4 2.0×10^4	5.4×10^3 5.1×10^3		
LE.77		9.0×10^1 1.8×10^2	3.0×10^4 2.6×10^4		
LE.78	8.9×10^4 5.5×10^4	1.3×10^5 1.4×10^5			
LE.80	7.6×10^5 7.7×10^5	8.4×10^4 3.9×10^4			
LE.82	0 0				

11-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA.3	4.2×10^4 2.4×10^4				
LA.5		3.3×10^4 3.1×10^4	2.6×10^4 3.5×10^4		
LA.9	0 0				
LB.19			1.6×10^5 1.3×10^5	6.4×10^4 1.5×10^3	
LB.23	9.0×10^3 3.0×10^3	3.0×10^3 0			
LB.24			3.0×10^2 6.0×10^2		
LC.41	3.4×10^4 8.1×10^3				
LC.42		9.6×10^3 3.7×10^3	1.7×10^4 7.8×10^3		
LC.44		1.5×10^3 3.0×10^2	0 0		
LC.46		2.4×10^5 1.3×10^5	5.0×10^5 4.8×10^5		

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

11-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LC.47	7.9×10^3 6.9×10^3				
LC.48	2.4×10^5 1.6×10^5				
LC.49	3.2×10^5 1.9×10^5				
LC.50	7.5×10^4 8.3×10^4				
LC.51		3.0×10^4 2.6×10^4			
LC.52	5.7×10^5 3.2×10^5				
LC.53		2.0×10^4 1.4×10^4	1.6×10^6 1.2×10^6		
LD.65		5.2×10^3 3.2×10^3	3.0×10^2 0		
LD.67	3.3×10^3 1.8×10^3				
LD.68	1.9×10^5 1.8×10^5				
LD.69		6.3×10^4 4.8×10^4	7.3×10^4 5.1×10^4		
LD.70	2.2×10^4 1.2×10^4				
LE.72			5.6×10^5 4.7×10^5	2.2×10^6 1.4×10^6	
LE.74			1.2×10^4 1.3×10^4	7.5×10^4 4.0×10^4	
LE.78			4.6×10^4 5.0×10^4	1.8×10^3 1.5×10^3	
LE.80			1.8×10^3 1.5×10^3	3.0×10^2 0	
LE.82		0 0	0 0		
LF.86	1.8×10^3 1.2×10^3	4.8×10^3 2.7×10^3			
LF.88	3.0×10^1 0	9.8×10^4 4.0×10^4			

APPENDIX III cont. Lactobacillus counts/ml. saliva from Langkloof.

12 Year age group.

Code No:	Sample No: I	II	III	IV	V
LA.3		2.8×10^5 2.4×10^5			
LA.9		0 2.4×10^3			
LB.23			3.2×10^4 2.5×10^4	1.5×10^4 4.8×10^3	
LC.49		7.9×10^4 7.9×10^4			
LC.50		0 2.4×10^3			
LC.52		1.4×10^5 6.0×10^4			
LD.67		0 0			
LD.68		3.0×10^5 4.1×10^5			
LD.70		1.8×10^5 8.3×10^4			

APPENDIX IV. Lactobacillus counts/mg. plaque material from Langkloof.

6-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA. 97	2.6×10^{1}				
LA. 98	0				
LC. 99	1.1×10^{1}				
LC. 100	discarded				
LC. 101	5.7×10^{0}				
LC. 102	discarded				
LE. 92	5.0×10^{2}				
LE. 94	0				
LB. 113	0				
LB. 115	9.8×10^{0}				
LB. 116	6.0×10^{0}				
LB. 117	7.0×10^{2}				
LF. 108	0				
LD. 121	3.1×10^{1}				
LD. 122	6.7×10^{1}				
LE. 109	discarded				
LE. 112	3.9×10^{0}				

7-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA. 1	0				
LA. 97		5.0×10^{2}	1.6×10^{1}		
LA. 98		1.1×10^{2}	5.1×10^{1}		
LB. 10	0				
LB. 11	0	1.4×10^{3}			
LB. 12	0				
LB. 14	2.0×10^{1}	1.2×10^{2}			
LB. 15	1.2×10^{1}				
LB. 96	0				

APPENDIX IV cont. Lactobacillus counts/mg. plaque material from Langkloof.

7-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LC.26	discarded				
LC.27	-				
LC.29	0				
LC.30	0				
LC.33	0				
LC.37	discarded				
LC.99		0	3.1×10^{1}		
LC.100		0	0		
LC.101		6.0×10^{0}	1.2×10^{1}		
LC.102		4.0×10^{3}	1.3×10^{2}		
LC.103	0				
LD.54	1.3×10^{1}				
LD.55	6.6×10^{1}				
LD.56	0				
LD.58	2.7×10^{0}				
LD.59	8.8×10^{1}				
LD.95	2.1×10^{1}				
LE.71	4.6×10^{1}	1.9×10^{2}			
LE.79	discarded	3.2×10^{1}			
LE.92		discarded			
LE.93	discarded	0			
LF.87	discarded				
LF.89	discarded	0			
LF.90	1.6×10^{1}	0			
LF.91	8.9×10^{1}	discarded			
LF.104	0				
LF.105	0				
LF.106	0				
LF.107	discarded				
LD.118	8.8×10^{1}				
LD.119	4.8×10^{0}				

APPENDIX IV cont. Lactobacillus counts/mg. plaque material from Langkloof.

7-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LD.120	8.1×10^{0}				
LE.110	discarded				
LE.111	2.7×10^{0}				

8-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA.1		2.8×10^{0}	discarded		
LA.4	0				
LB.10		0			
LB.11			8.4×10^{0}	2.0×10^{1}	
LB.12		1.1×10^{1}	discarded		
LB.13	0	7.8×10^{1}			
LB.14			2.2×10^{1}	discarded	
LB.15		1.8×10^{1}			
LB.16	1.1×10^{1}	1.4×10^{2}			
LB.17	0				
LB.18	7.7×10^{2}	0			
LC.25	0	2.6×10^{1}			
LC.26		discarded	discarded		
LC.27		0	0		
LC.28	discarded				
LC.29		1.4×10^{4}	8.2×10^{1}		
LC.30		4.6×10^{1}	8.8×10^{0}		
LC.31	0				
LC.32	discarded				
LC.33		0			
LC.34	2.6×10^{1}				
LC.35	5.9×10^{1}				
LC.37		discarded	6.4×10^{0}		

APPENDIX IV cont. Lactobacillus counts/mg. plaque material from Langkloof.

8-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LC.38	0				
LC.103		2.0×10^{0}	3.3×10^{0}		
LD.54		5.5×10^{0}	0		
LD.55		4.3×10^{1}	discarded		
LD.56		discarded			
LD.57	1.5×10^{1}				
LD.58		1.2×10^{0}	2.6×10^{1}		
LD.59		discarded	0		
LD.63	discarded				
LD.64	1.1×10^{1}				
LD.95		discarded	discarded		
LE.71			4.3×10^{1}	2.9×10^{0}	
LE.73	discarded	discarded			
LE.75	discarded	1.1×10^{1}			
LE.79			discarded	9.5×10^{0}	
LE.81	discarded	9.7×10^{1}			
LF.83	1.2×10^{1}	3.9×10^{0}			

9-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA.2	1.3×10^{2}	8.0×10^{0}			
LA.4		2.8×10^{1}	5.2×10^{1}		
LA.6	0				
LA.7	0				
LA.8	2.5×10^{1}				
LB.13			discarded	1.2×10^{3}	
LB.16			2.7×10^{1}	1.4×10^{1}	
LB.17		1.2×10^{1}			
LB.18			2.9×10^{0}		

APPENDIX IV cont. Lactobacillus counts/mg. plaque material from Langkloof.

9-Year age group cont.

Code No:	Sample No: I	II	III	IV	V
LB.20	0	discarded			
LB.21	1.0×10^2				
LB.22	0				
LC.28		discarded	1.6×10^1		
LC.31		0	discarded		
LC.32		8.8×10^1			
LC.34		0	1.8×10^0		
LC.35		1.0×10^2	1.3×10^1		
LB.114	1.2×10^2				
LC.36	0				
LC.38		discarded	2.3×10^0		
LC.39	discarded				
LC.40	discarded				
LC.45	5.1×10^2				
LD.60	4.8×10^1				
LD.61	discarded				
LD.62	1.8×10^1				
LD.63		discarded	0		
LD.64		0	8.8×10^0		
LD.66	1.5×10^2				
LE.73			1.4×10^2	0	
LE.75			1.2×10^1	6.2×10^0	
LE.76	2.9×10^2				
LE.77	0				
LE.81			1.6×10^1	discarded	
LF.84	1.2×10^1	0			

APPENDIX IV cont. Lactobacillus counts/mg. plaque material from Langkloof.

10-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA.5	0				
LA.6		8.3×10^{0}			
LA.7		0			
LA.8		discarded	2.9×10^{1}		
LB.19	4.2×10^{0}	4.1×10^{2}			
LB.20			0		
LB.21		7.9×10^{1}			
LB.22		0			
LB.24	8.8×10^{1}	2.6×10^{4}			
LC.36		0	0		
LC.39		discarded	6.5×10^{0}		
LC.40		1.7×10^{4}	1.8×10^{1}		
LC.42	discarded				
LC.43	4.1×10^{1}				
LC.45		4.4×10^{1}	discarded		
LC.46	0				
LC.51	discarded				
LC.53	1.1×10^{1}				
LD.60		0	2.1×10^{0}		
LD.61		2.1×10^{0}	0		
LD.62		0	0		
LD.65	4.4×10^{2}				
LD.66		discarded	discarded		
LD.69	1.9×10^{1}				
LE.72	discarded	5.4×10^{1}			
LE.74		9.8×10^{0}			
LE.76		discarded	discarded		
LE.77		0	0		
LE.78	1.3×10^{1}	3.1×10^{1}			
LE.80	7.7×10^{3}	7.3×10^{2}			
LE.82	discarded				

APPENDIX IV. Lactobacillus counts/mg. plaque material from Langkloof

11-Year age group

Code No:	Sample No: I	II	III	IV	V
LA.3	0				
LA.5		1.4×10^{1}	1.3×10^{2}		
LA.9	0				
LB.19			3.8×10^{2}	0	
LB.23	3.1×10^{1}	4.4×10^{2}			
LB.24			2.2×10^{0}		
LC.41	0				
LC.42		1.2×10^{2}	0		
LC.44	0	6.6×10^{0}			
LC.46		8.5×10^{1}	7.0×10^{1}		
LC.47	discarded				
LC.48	0				
LC.49	discarded				
LC.50	8.1×10^{0}				
LC.51		2.3×10^{1}			
LC.52	0				
LC.53		0	discarded		
LD.65		0	0		
LD.67	0				
LD.68	discarded				
LD.69		discarded	discarded		
LD.70	discarded				
LE.72			2.9×10^{1}	1.2×10^{4}	
LE.74			9.6×10^{1}	2.4×10^{0}	
LE.78			0	5.4×10^{0}	
LE.80			4.8×10^{0}	discarded	
LE.82		3.9×10^{0}	discarded		
LF.86	1.8×10^{1}	1.3×10^{0}			
LF.88	0	2.7×10^{0}			

APPENDIX IV cont. Lactobacillus counts/mg. plaque material from Langkloof.

12-Year age group.

Code No:	Sample No: I	II	III	IV	V
LA. 3		1.5×10^{1}			
LA. 9		0			
LB. 23			3.2×10^{0}	4.5×10^{0}	
LC. 49		6.4×10^{0}			
LC. 50		0			
LC. 52		3.4×10^{0}			
LD. 67		0			
LD. 68		discarded			
LD. 70		1.1×10^{1}			

APPENDIX V. Children examined at Nuwerus.

Code No.	Name.	Age.
NH. 1	Maatjie Schade	7 years
NH. 2	Henda Coetzee	7 years
NT. 3	Otto Basson	7 years
NT. 4	Johannes de Beer	7 years
NH. 5	Kosie Coetzee	7 years
NT. 6	Albertus van Wyk	7 years
NH. 7	Cecile Coetzee	8 years
NH. 8	Wilna van Eeden	8 years
NH. 9	Hannie v.d. Westhuizen	8 years
NH. 10	Mona Ras	8 years
NH. 11	Coenie Truter	8 years
NH. 12	Gerrit Lochner	8 years
NH. 13	Gert Pool	8 years
NT. 14	Anita Pereira	9 years
NH. 15	Lana Niewoudt	9 years
NH. 16	Annie van Zyl	9 years
NH. 17	Willem Lucas	9 years
NH. 18	Christo Coetzee	9 years
NT. 19	Steenkamp Mostert	9 years
NH. 20	Johanna Visagie	10 years
NH. 21	Gertruide Pool	10 years
NH. 22	Hettie Engelbrecht	10 years
NH. 23	Netta v.d. Westhuizen	10 years
NH. 24	Mariana Loubscher	10 years
NT. 25	Willem Mostert	10 years
NH. 26	Gert Rossouw	10 years
NH. 27	Joos Pool	10 years
NH. 28	Hentie v.d. Westhuizen	10 years
NH. 29	Daniel Kotze	10 years
NH. 30	Anna Mulder	6 years
NH. 31	Johanna Mulder	6 years
NH. 32	Hendrika Pool	6 years
NH. 33	Frederick Emil Krugman	6 years
NH. 34	Ockert Ridgart	6 years
NT. 36	Hendrik Kewny	6 years
NH. 37	Frans van Eeden	6 years
NH. 38	Wilhelmina Coetzee	6 years
NT. 39	Jeanette Mostert	6 years
NT. 40	Maria Ferreira	6 years
NH. 41	Johanna v.d. Westhuizen	6 years
NT. 42	Stella van Zyl	6 years

APPENDIX VI. Children examined at Langkloof.

Code No.	Name.	Age.
LA.1	William Ferreira	7 years
LA.2	Albert Ferreira	9 years
LA.3	Sylvia Ferreira	11 years
LA.4	Barbara Ferreira	8 years
LA.5	Hippert Ferreira	10 years
LA.6	Graham Ferreira	9 years
LA.7	Minnie Barnard	9 years
LA.8	Marieta Ferreira	9 years
LA.9	Andries du Preez	11 years
LA.97	Elsabe Ferreira	6 years
LA.98	Loma du Preez	6 years
LB.10	Wouter van Wyk	7 years
LB.11	Johann Schreiber	7 years
LB.12	Hendrina Schreiber	7 years
LB.13	Emerentia Schreiber	8 years
LB.14	Madelein Olivier	7 years
LB.15	Michael Schreiber	7 years
LB.16	Elianor Olivier	8 years
LB.17	Andrina van Wyk	8 years
LB.18	Ryno Olivier	8 years
LB.19	Elizabeth Strydom	10 years
LB.20	Edion van Wyk	9 years
LB.21	Andre Olivier	9 years
LB.22	Japie Schreiber	9 years
LB.23	Gielie Olivier	11 years
LB.24	Anna-Maria Olivier	10 years
LB.96	Hilda Schreiber	7 years
LB.113	Leon Olivier	6 years
LB.114	Frederick Olivier	9 years
LB.115	Petrus Schreiber	6 years
LB.116	Irene Schreiber	6 years
LB.117	Riena Olivier	6 years
LC.25	Bennie Kritzinger	7 years
LC.26	Denise Lansdell	7 years
LC.27	Anita de Vos	7 years
LC.28	Johannes Boshoff	8 years
LC.29	Pieter van Niekerk	7 years
LC.30	Naas Ferreira	7 years
LC.31	Maga Beer	8 years
LC.32	Maria du Preez	8 years
LC.33	Mariana Schoeman	7 years
LC.34	Fanie Gerber	8 years
LC.35	Tommie Pettit	8 years
LC.36	Jacobus Olivier	9 years
LC.37	Maryna Boshoff	7 years
LC.38	Marie Olivier	8 years
LC.39	Magdalena Oelofse	9 years
LC.40	Elsette Snyman	9 years
LC.41	Harris Schoeman	11 years

APPENDIX VI cont. Children examined at Langkloof.

Code No.	Name.	Age.
LC.42	Francois Kritzinger	10 years
LC.43	Giel Olivier	10 years
LC.44	Johannes Geldenhuys	10 years
LC.45	Charlotte du Preez	9 years
LC.46	Helena Ferreira	10 years
LC.47	Vivian Strydom	11 years
LC.48	Cornelius de Beer	11 years
LC.49	Ockert Olivier	11 years
LC.50	Roelof Potgieter	11 years
LC.51	Maria Kluyts	10 years
LC.52	Leona de Beer	11 years
LC.53	Andrietta Olivier	10 years
LC.99	Lidia Oelofsen	6 years
LC.100	Elsa Oelofsen	6 years
LC.101	Martie Ackerman	6 years
LC.102	S.J. Kritzinger	6 years
LC.103	Eugene Kritzinger	7 years
LD.54	Henville Kemp	7 years
LD.55	Nielen Strydom	7 years
LD.56	Laurie Strydom	7 years
LD.57	Ricus Kemp	8 years
LD.58	Linette Strydom	7 years
LD.59	Leonora Kemp	7 years
LD.60	Jacob Strydom	9 years
LD.61	Marinus Kemp	9 years
LD.62	Deon Strydom	9 years
LD.63	Marlene Kemp	8 years
LD.64	Mariska Strydom	8 years
LD.65	Clifton Strydom	10 years
LD.66	Aletta Vosloo	10 years
LD.67	Thurston Kemp	11 years
LD.68	Daniel Strydom	11 years
LD.69	Sunita Strydom	10 years
LD.70	Cynthia Strydom	11 years
LD.95	Michael Kemp	7 years
LD.118	Theunis Ackerman	7 years
LD.119	Marlene de Jager	7 years
LD.120	Denise Ferreira	7 years
LD.121	Petronella Strydom	6 years
LD.122	Merle Rhoda Haggard	6 years
LE.71	Jannie Kritzinger	7 years
LE.72	Johann Rademeyer	10 years
LE.73	Hennie Rademeyer	8 years
LE.74	Ferreira Strydom	10 years
LE.75	Maryne Strydom	8 years
LE.76	Annette Nortier	9 years
LE.77	Rinette Moore	9 years
LE.78	Beatrix van Tonder	10 years
LE.79	Bennie Meyer	7 years

APPENDIX VI cont. Children examined at Langkloof cont.

Code No.	Name.	Age.
LE.80	Jannie Meyer	10 years
LE.81	Valerie Strydom	8 years
LE.82	Pierre Strydom	10 years
LE.92	Johann Kritzinger	6 years
LE.93	Derick Ferreira	7 years
LE.94	Sarah M.M. Kritzinger	6 years
LE.109	Dennis Ferreira	6 years
LE.110	Handre Strydom	7 years
LE.111	Monika Beer	7 years
LE.112	Salome Ferreira	6 years
LF.83	Cornelia Kritzinger	8 years
LF.84	Geriana Meyer	9 years
LF.86	Rita Kritzinger	11 years
LF.87	Kathleen Howell	7 years
LF.88	Matthys Kritzinger	11 years
LF.89	Lourens Kritzinger	7 years
LF.90	Andre J. de Wit	7 years
LF.91	Amanda Stewart	7 years
LF.104	Johannes Kritzinger	7 years
LF.105	Theodorus Kritzinger	7 years
LF.106	R.S. Baldie	7 years
LF.107	Jacolean Kritzinger	7 years
LF.108	Hester Kritzinger	6 years