THE DIFFERENTIATION OF *STREPTOCOCCUS CREMORIS* AND *STREPTOCOCCUS LACTIS* BY MEANS OF BACTERIOPHAGE ACTION

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Many pure cultures of lactic streptococci have been isolated at this Institute during investigations of the various problems associated with the behaviour of cheese starters in commercial factories. These cultures have been derived mainly from commercial starters consisting of mixtures of several types of streptococci. The strains most suitable for use as 'single-strain starters' have proved to be those which do not ferment maltose and dextrin, which form long chains when grown in milk for 6–7 hr. at 37° C., and show little involution at this temperature. Consideration of these characteristics, along with other general physiological properties, has led us to classify these streptococci as strains of *Str. cremoris* (Orla Jensen).

Recent isolations of streptococci from samples of sour milk have given an entirely different picture, characterized by the almost constant occurrence of predominantly diplococcal and short-chained types which ferment maltose and dextrin, and conform in a general way to the standard descriptions of the species *Str. lactis*. Thus at this Institute we have come to regard these two races as separate and distinct species.

This division of species, however, has not been generally accepted by many investigators in the same field, and a review of the literature shows that some confusion exists as to the true identity of the lactic streptococci and their relationships to other groups or species of the genus *Streptococcus*. Hammer & Baker (1926), for example, do not consider *Str. cremoris* as a separate species and include in the '*Str. lactis* group', 'any organism coagulating litmus milk rapidly or fairly rapidly with reduction of the litmus but without digestion or formation of gas, and which appears in strains from milk as a Gram-positive coccus arranged in chains or in pairs, the pairs rarely being grouped in clumps'. Hammer (1936) is further inclined to the view that divisions within the group should be made from a practical standpoint, e.g. the production of a malty flavour, ropiness, etc., rather than on morphological differences or fermentation reactions. Again, many of the reactions given by the 'milk-souring' streptococci are similar to those given by such closely related organisms as *Str. faecalis* and the Lancefield group D streptococci, sometimes termed enterococci, and consequently some difficulty arises in segregating the organisms into clearly defined species or groups. Sherman (1937) has drawn attention to these difficulties in an excellent review, and has suggested an approach to the classification of the streptococci as a whole. Recently, Shattock & Mattick (1943) have brought forward evidence on serological and biochemical grounds to show that *Str. lactis* can be clearly differentiated from *Str. faecalis* and other members of the group D streptococci. They have shown that *Str. faecalis* falls into Lancefield group D, and have assigned *Str. lactis*, along with *Str. cremoris*, to a new serological group 'N'. Without presenting a historical survey of the literature, it may be fairly stated that, although there appears to be little doubt that *Str. lactis* is a distinct species, the claim for *Str. cremoris* remains unsettled.

Having the opportunity to collect different phage races in connexion with cheese-starter problems in New Zealand, it seemed of value, in view of the general uncertainty of the relationship of the various species of streptococci, to ascertain whether phage reactions could help in the definition of species or groups.

ISOLATION OF LACTIC STREPTOCOCCI

Although a few cultures which were already in use as cheese starters were available, it was necessary for the purpose of this investigation to isolate others from commercial mixed cultures and from soured milk. The streptococci obtained from as widely different sources as possible were arbitrarily divided into two groups on the following basic characteristics.

Group I: considered as *Str. cremoris* types.

(1) Characteristic long-chain formation in milk, (2) marked inhibition of growth and acid production at 37° C., (3) growth at 10° C. but not at 40° C., (4) inability to ferment maltose and dextrin.

Group II: considered as *Str. lactis* types.

(1) Predominantly diplococci and short chains in milk, (2) rapid growth and acid production at 37° C., (3) growth at 10 and 40° C. but not at 45° C., (4) ability to ferment maltose and dextrin.

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Other biochemical tests which have been proposed by various workers for the differentiation of streptococci do not appear to carry one much farther forward in defining the position of any given organism isolated from milk. Organisms falling into the two groups defined above did give in general the reactions which have been described for *Str. lactis* and *Str. cremoris* in tests such as fermentation of certain sugars (Davis, 1936; Sherman, 1937), *p*H range of growth, type of growth and final *p*H in dextrose broth, tolerance to methylene blue, reduction in litmus milk and the production of a smooth clot, heat resistance, final acidity produced in milk, growth in the presence of various concentration of NaCl, type of growth in gelatin, hydrolysis of sodium hippurate, power to split esculin, and production of ammonia from peptone; but these tests were not generally used. The simple reactions described above served to give two groups which in the author's view exhibited sufficient differences to justify their being considered distinct.

In the matter of morphology, the need for the adoption of a standardized procedure was clearly indicated. Examination of cultures at the time of clotting as suggested by Davis (1936) was not considered satisfactory, since many of the streptococci tend to show a break-up of the chains when the *p*H of the milk is markedly lowered and clotting takes place. A clearer picture was obtained by microscopic examination after growth in milk for 4–6 hr. at 30 and 37° C. when organisms of group I showed long chains with almost complete absence of diplococcal forms, while those of group II occurred mainly as diplococci, sometimes with a few chains of varying length. Furthermore, many of the so-called *Str. cremoris* strains could be distinguished from one another by the shape and arrangement assumed by the cocci after growth at 37° C. for 6 hr. in sterilized milk. Growth at a temperature near the maximum for the organism is necessary for the appearance of these differences in involution form. When the incubation temperature in this morphology test was raised to 40° C. the group I strains were completely inhibited and the group II strains now tended to show much more chain formation. It appears, therefore, that chain formation depends to a large extent on the temperature of incubation, and that the closer the temperature used is to the maximum growth temperature of the organism the greater is the tendency to chain formation. It so happens that the temperatures most commonly used in the culture of lactic streptococci are nearer the maximum for *Str. cremoris* than for *Str. lactis*, and thus they show more chain formation. The effectiveness of the test for differentiating groups I and II depends on the selection of 37° C. as the incubation temperature.

Temperature of incubation is, of course, not the sole factor conditioning chain formation. Cultures of group II which are transferred in milk daily over long periods, and those which are plated regularly and the most active colonies picked for starter use, show a greater tendency to form chains. The former phenomenon has been noted by Hammer (1936). The difference between the groups in the matter of chain formation is therefore by no means clear-cut, but under the conditions specified (i.e. milk cultures at 37° C. examined after 4–6 hr.) the majority of the active acid-forming streptococci fall fairly definitely into one group or the other.

Tests for sugar fermentation were carried out in 0·5% nitrogen casein digest broth (Orla Jensen, 1919) as well as in sugar broth (Davis, 1936). No difference in result was observed except that the growth, especially of group I, was much better in the casein broth.

It should be emphasized that the isolations of lactic streptococci were made partly with a view to obtaining cultures which could be used as cheese starters, and hence the majority of the selected organisms possessed the power to produce acid rapidly in milk and bring about clotting within 24 hr. at 22° C. Further, they were only slightly affected in their growth and morphology (as regards involution) when exposed to a temperature of 37° C. for a period of 4–6 hr. This arbitrary selection of strains of streptococci on the basis of acid production may be criticized on the ground that many of the weaker acid-forming types excluded from consideration in this work might not fit in with the general division into two groups such as have been described. For example, some of them might even correspond more to *faecalis* types. The criticism cannot be entirely countered, but there is reason to believe that most of the feeble acid formers are merely variants of the more active types. Thus it has been found necessary to plate many of the selected cultures at intervals in order to maintain their activity. It has been observed that without frequent plating and picking of the more active colonies, the power of a culture to produce acid rapidly is sometimes lost on constant subculture in milk. The finding (Harriman & Hammer, 1930; Hunter, 1939) that active and weakened individuals appear to be in a state of flux in pure culture, and possibly also in nature, suggests that the exclusion of weak acid-producing strains does not seriously affect the general picture.

Two cultures of group I required 36–48 hr. to clot milk at 22° C., but the remainder were active as cheese starters. All except two group II strains clotted milk at 22° C. within 24 hr., and many of them proved to be sufficiently active to be of use as starters with the usual 1–1½% added to the cheese milk. During daily subculture, a few strains of both groups acquired and lost the property of forming

slime in milk, while a feature peculiar to group II was the high proportion (25%) which produced a 'caramel' flavour and odour in milk. These strains corresponded in type with *Str. lacticus* var. *maltigenus* (Hammer & Cordes, 1921).

Fourteen cultures and one stock strain of *Str. cremoris* (obtained from Kiel in 1928) fell into group I, twenty-eight, including one authentic *Str. lactis* strain (from Kiel), into group II. In addition, seven strains of group D (Lancefield) streptococci supplied by the National Institute for Research in Dairying, Reading, were examined for comparison.

ISOLATION OF PHAGE RACES

The sources from which phage races were obtained included lysed or partially lysed starter cultures, cheese whey, curd, cheese, and factory water supplies. Most of the phages encountered were present in strong concentration in these original materials, with the exception of water, in which case it was usually necessary to 'build up' the strength by transfers on the appropriate organism growing in milk. It has been found that all pure cultures so far introduced into commercial dairy factories have, sooner or later, suffered attack by an appropriate phage.

Cheese proved to be the most convenient source of many of the phages recently isolated. Small pieces were ground, in the proportion of 1 g. cheese to 10 ml. sterile distilled water, in a sterilized mortar, and the resulting emulsion filtered through a sterile paper funnel and finally through a Seitz pad. Isolated plaques, obtained by the agar-plate technique from dilutions of the filtrate (or of other fluid-containing phage) on a mat of the susceptible *Streptococcus*, formed the starting-point for the isolation of the phage by the usual procedure (Whitehead & Hunter, 1939). All the phage preparations were purified at least twice and adjusted to approximately equal titre (10^{-10}) before use in comparative studies.

Except for the range of streptococcal strains on which the various races acted, or in some instances their reaction to changes in incubation temperature (Hunter, 1943), no means of distinguishing phages from one another was available. The size of plaques produced by different phages varied in diameter from 0·25 to 1·0 mm., but no correlation of plaque size with other general characteristics of the phages was evident.

METHODS OF INVESTIGATION

The range of activity of the phage races was determined both in milk and on the surface of lactose-yeast extract-agar, while in order to make the tests equally sensitive for all races, the reactions were observed at several different temperatures.

10 ml. quantities of sterilized skim milk, previously warmed to the required temperature, and seeded with two drops (0·1 ml.) of an 18–20 hr. clotted-milk culture and one drop (0·05 ml.) of neat or diluted phage preparation, were kept under observation by frequent examination of stained smears under the microscope until lysis occurred. When clotting of the milk took place before dissolution of the organisms, the cultures were transferred into fresh tubes of milk and examined for one further generation. For routine purposes, a negative result was recorded if the phage failed to lyse the streptococci after two generations in milk. In the plate test for phage sensitivity, a thick mat of the organisms was spread on the agar medium and drops from a set of serial dilutions of the phage were placed on the surface.

Phage action proved to be sensitive to the conditions under which the tests were carried out, and consequently difficulty was sometimes experienced in defining the limits of the range of activity of a given phage on certain organisms, due principally to differences observed between the action in milk and on agar. These differences were especially evident with phages which attacked group II organisms. Complete agreement occurred in the majority of the tests, while in some the difference appeared to be merely one of degree, i.e. although a strong reaction occurred on the solid medium, the action in milk did not manifest itself until the second generation. In a small percentage of tests, however, although a strong positive result was obtained on a plate, no corresponding activity could be induced in milk. Since a positive result is definite while a negative may merely indicate that conditions were not suitable for phage action, the results obtained on agar were recorded in such cases. The activity of phages in milk happens to have a great practical importance in the dairy industry, but in the matter of classification of the bacteria the reaction between organism and phage, no matter what the medium, is the crucial point. There were no instances where the results were positive in milk but negative on agar.

As has been found in previous work (Hunter, 1943) the various phages showed differences in optimum temperature of action. These differences are not recorded here; the result is given as positive if phage action occurred at any temperature at which the organism would grow.

RESULTS

For reasons of space, it is not possible to record in table form all the data derived from some six to seven thousand single tests. Table 1 gives a representative picture of phage-organism relationships, the salient features of which are as follows:

Table 1. *Phage-Organism Relationships*

Group	Representative strain	No. of strains showing identical sensitivity	Phages isolated on organisms of group I										Phages isolated on organisms of group II											
			3.9.18	2	4	6	7	8.22	12	11	10	34	16	23	24	27	28	25	26	29	30	32	31	33
I	HP	1	−	+	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	K	1	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	R_1	1	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	R_6	1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	FH	1	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	Cr	1	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	RW	1	+	−	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	UD_5	1	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	RB_1	1	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	J	1	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	R_8	1	+	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	B	3	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
II	I	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
	II	2	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−	+	+	−	+	−
	III	2	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+	+
	IV	1	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−
	V	1	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	+	+	+	+	−	+
	VI	1	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	+	+	+	+	+	+	+
	VII	5	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	+	+	+	+	+	+
	X	1	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+	+	+	+	+
	XIII	1	−	−	−	−	−	−	−	−	+	−	+	+	−	+	+	−	+	+	+	+	+	+
	XV	1	−	−	−	−	−	−	−	−	+	−	+	+	−	+	−	−	+	−	−	+	+	−
	XVII	1	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	−	−
	XVIII	1	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
	XIX	1	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	+	+	+	+	+	−
	XX	1	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−	+	−	−	−	+	+
	XXI	1	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	+	+	+	+	+	+
	XXIII	1	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	+	+	+	+	+	−
	XXVII	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
	XXII	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	IX	3	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	XXV	1	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−

(1) There is a general lack of specificity of phage action in group II, in marked contrast to the definite tendency towards strain specificity in the phage races which attack group I organisms. Thus the number of representative strains lysed by different phages prepared on group II streptococci varies from two to fourteen (average nine), while the maximum number attacked in group I is three; in some instances there appears up to the present to be a strict specificity. Where a phage race acts upon more than one culture in group I, a similarity in morphology of the organisms after growth in milk at 37°C. has been observed.

(2) Phage races 10 and 34 form an intermediate group with wide lytic capacities. First isolated in dextrin is susceptible to attack by very few (sometimes only one) phage races. A short-chained *Streptococcus* which ferments maltose and dextrin is susceptible to attack by a variety of phage races. In passing, it may be mentioned that this has repercussions in dairy practice because there is a big advantage in having available several cheese-starter cultures which are not all attacked by the same phage or phages. *Cremoris* strains are thus preferable to *lactis* strains.

Five cultures in group II have proved immune to all group II phages, but with the exception of one (XXII) they are susceptible to the polyvalent intermediate group phages 10 or 34. Although these strains are similar to all other group II strains in

Table 2. *Phage races appearing on two cultures used as cheese 'starters' in several localities*

Group II: Organisms: Representative strain	No. of strains showing identical sensitivity	Phage races isolated on strain XI: *i*	*r*	*h*	*t*	*p*	*m*	*g*	Group I: Organisms: Strain	Phage races isolated on strain HP: *s*	*i*	*r*	*h*	*t*	*p*	*m*	*g*
XI	3	+	+	+	+	+	+	+	HP	+	+	+	+	+	+	+	+
I	1	−	+	−	−	−	−	−	R_9	−	−	−	−	−	−	−	−
II	2	−	−	+	+	−	+	−	B	−	−	−	−	−	−	−	−
IV	1	−	+	−	−	+	−	−	R_{11}	−	−	−	−	−	−	−	−
V	1	−	+	+	+	+	−	+	K	−	−	−	−	−	−	−	−
VII	8	−	+	+	+	+	+	+	RW	−	−	−	−	−	−	−	−
IX	6	−	−	−	−	−	−	−	R_6	−	−	−	−	−	−	−	−
XV	1	−	+	−	−	+	+	−	R_8	−	−	−	−	−	−	−	−
XVII	1	−	+	+	−	−	−	−	UD_5	−	−	−	−	−	−	−	−
XX	1	−	+	−	−	−	+	+	R_1	−	−	−	−	−	−	−	−
XIX	2	−	+	+	+	+	+	−	FH	−	−	−	−	−	−	−	−
XXVII	1	−	−	+	−	−	−	−	Cr	−	−	−	−	−	−	−	−

connexion with strains of group I, they have generally been considered as '*cremoris*' races. It is of interest to note that if the susceptible strains of group I only are considered, the two races are identical. This, however, is disproved when their action on group II strains is taken into account.

(3) None of the phages isolated in connexion with group II strains are capable of lysing group I organisms.

(4) All *Str. faecalis* cultures and other group D (Lancefield) streptococci so far tested are characterized by complete lack of sensitivity to phages isolated on groups I and II strains.

These results seem to confirm the division of lactic streptococci into *lactis* and *cremoris* species which had originally been made on the basis of morphology and sugar reactions. In general terms, it seems to be true that a long-chained lactic *Streptococcus* which does not ferment maltose and their morphological and biochemical reaction, they occupy an anomalous position at present.

Further evidence that phages for group I streptococci are more specific in action than those for group II was derived from the following experiment. Two representative strains, XI and HP, were sent out during one season to seven commercial factories in widely separated localities for use as cheese starters. After a period, samples of cheese from vats in which the particular cultures had been used were collected, the phages isolated and purified on the appropriate organism, and tested against all available strains of groups I and II. The results are tabulated in Table 2. It is significant that seven phages isolated in connexion with strain XI were all different over a series of organisms, while the seven phages which appeared on strain HP (together with a stock phage) were all of the same type showing strict specificity.

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DISCUSSION

The outstanding feature of phage reactions within the two groups comprising 'long-chained' species on the one hand and 'diplococcal' types on the other is the marked difference in specificity of the races which attack the various strains. With few exceptions, a diplococcal and short-chained type is usually susceptible to attack by numerous non-specific phages, while long-chained cocci are liable to attack by only a very few more or less specific phage races. A real distinction is therefore evident, and it would appear that the differentiation of types by biochemical and cultural methods and the consequent admission of *Str. cremoris* to species rank is justified.

Up to the present, no apparent parallelism between susceptibility to phage and other physiological characteristics of an individual 'lactic' *Streptococcus* has been found. Criteria regarded as important for classification purposes may be considered under three headings:

(A) 'Superficial' properties, such as power to produce acid, production of slime or ropiness in milk, and ability to withstand high temperature conditions have been used by some workers to classify species. These properties are known to vary considerably. They bear no relation to phage sensitivity (Hunter, 1939).

(B) Other physiological and biochemical characters, especially the fermentation of various carbohydrates, are frequently sufficiently well defined to permit distinctions between different species. Previous work (Hunter, 1939) has shown, however, that sugar reactions of an organism are capable of change while its susceptibility to phage remains constant. Thus a variant of *Str. cremoris* which failed to ferment lactose but was still susceptible to a specific phage has been encountered.

(C) Antigenic structure appears to be the most constant and deep-seated characteristic of most bacterial species. From data obtained in an investigation of species of Salmonella, Flexner and Staphylococcal groups, Burnet (1930) put forward the view that there is a definite correlation between antigenic structure and phage reactions of a given bacterial strain. Kendrick & Hollon (1931) observed a parallelism between serological and 'bacteriophagic' reactions of the faecal streptococci, and by the introduction of a new technique using certain phage filtrates along with phages in the 'nascent' state, Evans (1936) found that 'phagological' reactions of the haemolytic streptococci were in agreement with serological groupings. Hucker (1932) reported that certain strains designated as *Str. lactis* and *Str. cremoris* appeared to be serologically distinct, but, without further cultural studies, was unable to determine whether the differences were specific or merely varietal. Preliminary studies of Shattock & Mattick (1943) indicate that *Str. cremoris* and *Str. lactis* fall into the same serological group.

The phage reactions of the lactic streptococci as detailed in the present paper seem to give finer distinctions between organisms which are only doubtfully and with difficulty to be distinguished on serological grounds. The species, *Str. cremoris* and *Str. lactis*, appear to the present author to be quite definite entities even although there may be intermediate forms as there frequently are between other species. The results suggest also that phage sensitivity is an exceedingly 'deep-seated' property of the bacterial body, more so perhaps than the antigenic structure, or at any rate showing finer differences from strain to strain. Phage sensitivity among the lactic streptococci is apparently subject to change only through the action of phage itself when a resistant form is produced similar to the parent form in all respects except phage sensitivity. This seems to be a very special form of variation, but its mechanism is completely obscure at present.

The use of a phage sensitivity test for routine classification of lactic streptococci would be unwieldy because trial of a whole series of phages on an unknown organism would be necessary. In the author's opinion the support which the results of phage reactions give to differentiation by biochemical tests make the simple criteria detailed earlier in the paper all that is necessary to distinguish between *Str. lactis* and *Str. cremoris*.

SUMMARY

1. A series of lactic streptococci which were almost all capable of clotting milk in 24 hr. at 22° C. were divided into two groups on the basis of a few simple biochemical and morphological tests. The two groups corresponded to what some workers consider to be the species *Str. lactis* and *Str. cremoris*.

2. A series of phage races were tested for their action on the streptococci under various temperature and growth conditions.

3. The characteristics of phage attack on the organisms tended to confirm the division into *Str. lactis* and *Str. cremoris* species. Each of the strains of the long-chained *cremoris* type was attacked by only a few phages at the most, sometimes by a single phage only, whereas each of the *lactis* (short-chained) strains was attacked by a variety of phages. Furthermore, with two exceptions, the phage attacking the *cremoris* types on the one hand and the *lactis* types on the other formed quite separate and distinct groups.

4. None of the phages acted upon the several available strains of *Str. faecalis* and other group D (Lancefield) streptococci.

5. The evidence obtained from the phage sensi-

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tivity tests seems to justify the division of the lactic streptococci into two species, *Str. lactis* and *Str. cremoris*.

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