Casein as a Necessary Factor in the Production of Stimulatory Material for Associative Growth of Lactic Streptococci

S. L. KOTHARI1 AND V. K. N. NAMBUDRIPAD

Dairy Microbiology Division, National Dairy Research Institute, Karnal (Haryana), India

Received for publication 24 July 1972

Strains of *Streptococcus cremoris* KH and HC produced material that was stimulatory for *S. cremoris* R₄ in milk and in the dialyzable fraction of milk, but not in the dialysate fraction of milk, lactic acid whey, or lactose broth. The addition of casein to these latter media permitted the production of this stimulatory material to occur. Tryptone, peptone, and yeast extract could not be substituted for casein in producing the stimulatory material or in initiating associative growth in the lactic acid whey. The minimum concentration of casein required appeared to be from 2.0 to 2.5%.

The occurrence of associative or symbiotic activity, or both, among lactic acid starter cultures determines the final quality of the product (1, 5–8, 10, 14). A preliminary study has indicated that increased lactic acid yields can be obtained in milk with *Streptococcus cremoris* R₄ when strain KH or HC is also present (12). The stimulatory material produced by these strains appears to be guanine or a guanine-like substance (11). The present study suggests that the constituents present in milk are involved in the production of the stimulatory material by strains KH or HC.

**MATERIALS AND METHODS**

**Test organisms.** Two strains of *S. cremoris*, KH and HC, were used for the production of the stimulatory material. A third strain, R₄, acted as the sensitive organism for estimating this activity. All cultures were grown at 30°C. Titratible acidity was determined by titrating the medium against 0.11 N NaOH and using phenolphthalein and bromothymol blue as indicators in milk and broth, respectively.

Sixteen-hour milk cultures were used, except where specified, for preparing the cell-free filtrate as well as for evaluating the stimulatory activity (12).

**Preparation of milk fractions:** (i) casein and lactic acid whey. A 100-ml amount of skim milk was heated to 45 to 50°C in a water bath. To this, 6 ml of 10% lactic acid (v/v) was added with constant stirring. The material was kept undisturbed for 30 min and filtered. Precipitated casein was washed several times with glass-distilled water. The filtrate, i.e., lactic acid whey, was neutralized with 0.11 N NaOH to pH 7.0 and Seitz-filtered.

(ii) Dialyzed and dialysate fraction. Sterilized skimmed milk was dialyzed against distilled water with cellophane at 5 to 8°C for 3 days by using six changes of water. Toluene was the preservative. The dialyzed and dialysate fractions were concentrated to their original volumes under vacuum at 50°C.

**Media.** Milk: Fresh skimmed cow's milk was sterilized at 15 psi for 1 min and steamed the following day for 30 min.

Lactic acid whey: As described above.

Lactic acid whey supplemented with (i) peptone, 1%; (ii) tryptone, 1%; (iii) yeast extract, 1%; (iv) tryptone and yeast extract, 0.5% each; and (v) casein, 3%.

Dialyzed and dialysate fractions: As described above. To the dialysate fraction, 2.5% laboratory prepared casein was also added.

Lactose broth (13): (i) Half-strength broth: tryptone, 1.5%; yeast extract and beef extract, 0.5% each; lactose, 1%; casein, 1.5% (when supplemented). (ii) Quarter-strength broth: tryptone, 0.75%; yeast and beef extract, 0.25% each; lactose, 1%; and casein, 2.25% (when supplemented). Media were adjusted to pH 7.0 with 0.11 N NaOH and sterilized at 15 psi for 5 min.

**RESULTS**

The lactic acid whey supplemented with peptone, tryptone, yeast extract, or casein. The data
indicate that the paired strains produced more acid in milk, in the dialyzed fraction of milk, and in lactic acid whey to which casein had been added; but not in the dialysate fraction of milk, in lactic acid whey alone, or in lactic acid whey supplemented with peptone, tryptone, or yeast extract (Table 1). The seemingly higher lactic acid production in the whey + casein medium than in the control milk is caused by the longer incubation period (24 versus 8 hr).

Cell-free filtrates of strains KH and HC brought about increased production of lactic acid by strain R, only when casein was incorporated in milk or in the dialyzed fraction (Table 2). This indicates that casein (which remains in the dialyzable fraction) is an important factor in the production of the stimulatory material and the symbiotic activity of strains.

To determine whether acid production by strain R, was increased in the presence of stimulatory material in media lacking casein, the cell-free filtrates of strains KH and HC were added at a 1% level to the dialyzed and dialysate fractions of milk, to the dialysate plus casein, to lactic acid whey alone, and to lactic acid whey supplemented with peptone, tryptone, yeast extract, or casein. Acid production by strain R, in the presence of stimulatory material was not increased in media without Table 2. Production of stimulatory material in various media by Streptococcus cremoris KH and HC as measured by lactic acid production of R, in milk

| Source of cell-free filtrate* | Incubation time (hr) | Lactic acid production by R, in milk with added cell-free filtrates from S. cremoris (%) |
|-----------------------------|-----------------------|--------------------------------------------------------------------------------------------|
| Control:                    |                       |                                                                                           |
| Milk                        | 8                     | 0.39, 0.54, 0.53                                                                         |
| Lactic acid whey* (whey)    | 8                     | 0.39, 0.40, 0.40                                                                         |
| Dialyzed fraction           | 8                     | 0.39, 0.54, 0.54                                                                         |
| Dialysate fraction          | 8                     | 0.38, 0.37, 0.37                                                                         |
| Dialysate + casein, 2.5%    | 8                     | 0.37, 0.50, 0.51                                                                         |
| Whey + peptone, 1%*         | 8                     | 0.36, 0.36, 0.36                                                                         |
| Whey + tryptone, 1%*        | 8                     | 0.37, 0.36, 0.36                                                                         |
| Whey + yeast extract, 1%*   | 8                     | 0.36, 0.36, 0.37                                                                         |
| Whey + yeest extract, 1%*   | 8                     | 0.36, 0.36, 0.37                                                                         |
| Whey + 3% casein            | 8                     | 0.39, 0.53, 0.53                                                                         |

* Cell-free filtrate was prepared after 48 hr of incubation rather than 16 hr.
* One milliliter of uninoculated control whey medium. Each value represents the mean of four trials in duplicate.

Table 1. Lactic acid production by Streptococcus cremoris strains in various media

| Medium | Incubation time (hr) | Initial acidity | Lactic acid production by S. cremoris (%) |
|--------|----------------------|------------------|-------------------------------------------|
|        |                      | KH | KH + R, | R, | HC + R, | HC |
| Milk    | 8                    | 0.16 | (6.60) | 0.42 | (5.50) | 0.54 | (5.10) | 0.40 | (5.10) | 0.55 | (5.70) | 0.35 |
| Dialyzed fraction | 8               | 0.05 | (6.90) | 0.19 | (5.80) | 0.23 | (5.40) | 0.16 | (5.40) | 0.23 | (6.0) | 0.15 |
| Dialysate | 24                | 0.02 | (6.95) | 0.14 | (6.0) | 0.18 | (5.90) | 0.21 | (5.60) | 0.21 | (6.0) | 0.17 |
| Lactic acid whey (whey)  | 24               | 0.01 | (6.95) | 0.20 | (5.90) | 0.20 | (5.90) | 0.19 | (6.00) | 0.19 | (6.00) | 0.20 |
| Whey + peptone, 1%       | 24               | 0.01 | (6.90) | 0.56 | (4.90) | 0.74 | (4.60) | 0.75 | (4.60) | 0.60 | (4.80) | 0.73 |
| Whey + tryptone, 1%      | 24               | 0.01 | (6.90) | 0.60 | (4.70) | 0.80 | (4.70) | 0.79 | (4.70) | 0.66 | (4.70) | 0.78 |
| Whey + yeast extract, 1% | 24               | 0.01 | (6.90) | 0.49 | (4.70) | 0.75 | (4.70) | 0.75 | (4.70) | 0.62 | (4.70) | 0.77 |
| Whey + tryptone, 0.5% + yeast extract, 0.5% | 24 | 0.01 | (6.90) | 0.75 | (4.80) | 0.90 | (4.40) | 0.88 | (4.40) | 0.78 | (4.40) | 0.82 |
| Whey + casein, 3%        | 24               | 0.10 | (6.70) | 0.80 | (4.80) | 0.95 | (4.40) | 0.91 | (4.40) | 0.97 | (4.40) | 0.93 |

* Each value represents the mean of three trials in duplicate.
* Numbers in parentheses represent pH.
casein, but was stimulated in both the dialysate and in lactic acid whey when supplemented with casein (Table 3). This would indicate that casein is essential for the proper utilization by strain R of the stimulatory material produced by strains KH and HC. Since very little acid was produced in media lacking casein, measurements were made after 24 hr whereas, in the other cases, measurements were made after 8 hr.

To determine the optimal levels of casein required for associative growth, casein was added at levels of 0.5 to 2.5% to lactic acid whey, and the strains were grown both individually and in pairs. Data indicate that 2.0 to 2.5% casein was required for associative growth (Table 4).

The effect of incorporating casein in lactose broth containing suboptimal levels of nutrients (i.e., half-strength and quarter-strength media) indicates that there was a greater increase in associative growth when casein was added to the quarter-strength medium than to the half-strength medium (Table 5).

**DISCUSSION**

These results suggest that casein is an important factor in the production of stimulatory material by *S. cremoris* strains KH and HC for symbiotic growth with strain R. The nature of the components present in casein or casein-rich media that are responsible for this associative growth is still unknown. It has previously been established that lactic acid-producing streptococci are dependent upon an organic nitrogenous source for their supply of essential amino acids needed for growth (9, 15). It has also been shown that *Streptococcus lactis* contains intracellular proteinase, which can function to provide the cell with a mechanism to acquire essential amino acids via the breakdown of milk proteins (2-4). Presumably, these amino acids are not available in their entirety from simpler nitrogenous materials (tryptone, peptone, etc.). Similarly, the proteolytic activity of the endocellular enzymes in *S. lactis* is greatly reduced if the casein in the medium is replaced by tryptone or peptone (16).

**Table 3.** Production of stimulatory material in milk by *Streptococcus cremoris* KH and HC as measured by lactic acid production of R in various media

| Medium                        | Incubation time (hr) | Lactic acid production in various media with added cell-free filtrates from *S. cremoris* (%) | None | KH | HC |
|-------------------------------|----------------------|-----------------------------------------------------------------------------------------------|------|----|----|
| Milk                          |                      |                                                                                               | 0.36 | 0.56 | 0.58 |
| Lactic acid whey (whey)       | 8                    |                                                                                               | 0.36 | 0.19 | 0.19 |
| Dialyzed fraction             | 8                    |                                                                                               | 0.35 | 0.45 | 0.45 |
| Dialysate fraction            | 24                   |                                                                                               | 0.20 | 0.20 | 0.20 |
| Dialysate + casein, 2.5%      | 8                    |                                                                                               | 0.39 | 0.60 | 0.62 |
| Whey + peptone, 1%            | 24                   |                                                                                               | 0.56 | 0.57 | 0.56 |
| Whey + tryptone, 1%           | 24                   |                                                                                               | 0.59 | 0.60 | 0.60 |
| Whey + yeast extract, 1%      | 24                   |                                                                                               | 0.61 | 0.60 | 0.60 |
| Whey + yeast extract, 0.5%    | 24                   |                                                                                               | 0.60 | 0.61 | 0.60 |
| Whey + casein, 3%             | 8                    |                                                                                               | 0.36 | 0.58 | 0.56 |

*Cell-free filtrate was prepared after 16 hr of incubation. Each value represents the mean of four trials in duplicate.

**Table 4.** Effect of casein upon associative growth of *Streptococcus cremoris* cultures in lactic acid whey medium

| Medium                        | Lactic acid produced by strains of *S. cremoris* (%) |
|-------------------------------|------------------------------------------------------|
|                               | KH | KH + R | R | HC + R | HC |
| Lactic acid whey (whey)       | 0.28 | 0.34 | 0.36 | 0.36 | 0.31 |
| Whey + casein, 0.5%           | 0.29 | 0.36 | 0.36 | 0.37 | 0.31 |
| Whey + casein, 1.0%           | 0.30 | 0.37 | 0.35 | 0.38 | 0.33 |
| Whey + casein, 2.0%           | 0.37 | 0.44 | 0.39 | 0.46 | 0.40 |
| Whey + casein, 2.5%           | 0.40 | 0.49 | 0.43 | 0.51 | 0.43 |

* Incubation time was 24 hr. Each value represents the mean of three trials in duplicate.

**Table 5.** Effect of casein upon associative growth of *Streptococcus cremoris* cultures in lactose broth

| Medium                        | Lactic acid produced by strains of *S. cremoris* (%) |
|-------------------------------|------------------------------------------------------|
|                               | KH | KH + R | R | HC + R | HC |
| Control lactose broth         | 0.62 | 0.68 | 0.68 | 0.79 | 0.89 |
| Half-strength broth           | 0.23 | 0.33 | 0.33 | 0.34 | 0.32 |
| Half-strength broth + casein, 1.5% | 0.33 | 0.55 | 0.42 | 0.50 | 0.40 |
| Quarter-strength broth        | 0.08 | 0.11 | 0.11 | 0.22 | 0.22 |
| Quarter-strength broth + casein, 2.25% | 0.12 | 0.48 | 0.36 | 0.47 | 0.33 |

* Incubation time was 24 hr. Each value represents the mean of three trials in duplicate.
The minimal requirements of casein for associative growth of the different strains of *S. cremoris* appear to be between 2.0 and 2.5% (Table 4). Lower concentrations, although able to support growth, were insufficient to induce any associative effect. When semisynthetic media, i.e., lactose broth (13), were used, the lactic acid-producing culture did not show any symbiosis (Table 5). However, when casein was added to replace part of the tryptone, associated growth occurred. This further strengthens the concept that milk proteins are essential for the symbiotic growth of the lactic acid-producing milk cultures. A similar observation, that milk is essential for the production of stimulatory material, has been noted for *Pseudomonas fluorescens* in lactic acid starter cultures (10).

**LITERATURE CITED**

1. Bautista, E. S., R. S. Dahiya, and M. L. Speck. 1966. Identification of compounds causing symbiotic growth of *Streptococcus thermophillus* and *Lactobacillus bulgaricus* in milk. *J. Dairy Res.* 33:299–307.
2. Cowman, R. A., and M. L. Speck. 1967. Proteinase enzyme system of lactic streptococci. I. Isolation and partial characterization. *Appl. Microbiol.* 16:881–886.
3. Cowman, R. A., H. E. Swaisgood, and M. L. Speck. 1967. Proteinase enzyme system of lactic streptococci. II. Role of membrane proteinase in cellular function. *J. Bacteriol.* 94:942–948.
4. Cowman, R. A., S. Yoshimura, and H. E. Swaisgood. 1968. Proteinase enzyme system of lactic streptococci.
5. Dahiya, R. S., and M. L. Speck. 1962. Symbiosis among lactic streptococci. *J. Dairy Sci.* 45:607–612.
6. East, A. 1956. Associative growth of lactic acid bacteria in mixed starter cultures. *Int. Dairy Congr.* 14th 2:161–169.
7. Galesloot, T. E., F. Hassing, and H. A. Veringa. 1968. Symbiosis in yoghurt. I. Stimulation of *Lactobacillus bulgaricus* by a factor produced by *Streptococcus thermophilus*. *Neth. Milk Dairy J.* 22:50–63.
8. Green, V. W. 1959. Interaction among pure strains of lactic streptococci isolated from multiple strain cheese starters. *J. Dairy Sci.* 42:906.
9. Husain, I., and I. J. McDonald. 1957. Amino acids and utilization of sodium caseinate by lactic streptococci. *Can. J. Microbiol.* 3:487–491.
10. Koburger, J. A., and T. J. Claydon. 1961. Identification of substances in milk cultures of *Pseudomonas fluorescens* which stimulate lactic starter culture. *J. Dairy Sci.* 44:1811–1817.
11. Kothari, S. L., and V. K. N. Nambudripad. 1972. Isolation and identification of stimulatory substances involved in the associative growth of cheese cultures. *J. Dairy Sci.* in press.
12. Kothari, S. L., V. K. N. Nambudripad, and H. Laxminarayana. 1971. Studies on the associative growth of some streptococci. *Milchwissenschaft.* 26:415–418.
13. Meanwell, L. J. 1962. The influence of penicillin on cheese starter activity. *J. Appl. Bacteriol.* 25:128–136.
14. Olson, H. C. 1960. Symbiosis in lactic streptococci. *J. Dairy Sci.* 43:459.
15. Reiter, R., and J. Oram. 1962. Nutritional studies on cheese starters. I. Vitamin and amino acid requirements of single strain starters. *J. Dairy Res.* 29:63–77.
16. Vander Zant, W. C., and F. E. Nelson. 1953. Characteristics of an endocellular proteolytic enzyme system of *Streptococcus lactis*. *J. Dairy Sci.* 36:1212–1222.