Stimulation of Lactic Acid Bacteria by a Micrococcus Isolate: Evidence for Multiple Effects

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Growth of, and rate of acid production by, six cultures of lactic acid bacteria were increased in the presence of Micrococcus isolate F4 or a preparation of its capsular material. Concentrations of hydrogen peroxide found in pure cultures of the lactic acid bacteria were not detectable, or were greatly reduced, in mixed culture with Micrococcus isolate F4. The capsular material was not as effective as whole cells in preventing accumulation of H₂O₂. Catalase stimulated growth of, and the rate of acid production by, the lactic acid bacteria, but not to the same extent as Micrococcus isolate F4 in some cultures. The existence of two mechanisms for micrococcal stimulation of the lactic acid bacteria is postulated. One mechanism involves removal of H₂O₂; the other has not been characterized.

Acid production by lactic acid organisms in milk can be stimulated by the addition of various plant and tissue extracts (3, 4, 9, 12-14, 20, 23). Addition of ferrous ions and catalase to milk also stimulated acid production by certain lactic streptococci, apparently due to the destruction of metabolically produced peroxide which is inhibitory (7). However, in mixed populations there may be many factors which govern the growth of an organism. Some strains of Streptococcus lactis and Streptococcus cremoris produced a heat-labile inhibitor of Lactobacillus casei (1), whereas a peptide from cell extracts of S. lactis stimulated the growth of L. casei (2). Growth of propionibacteria was stimulated by lactobacilli in cheese (17) and by micrococci in lactate agar and Emmental cheese (18, 19). Stimulation of acid production and growth of lactobacilli by capsular material from micrococci has been demonstrated (16). Lactic acid bacteria, important in the production of certain fermented foods, have also been shown to have an antagonistic effect on staphylococci and salmonellae, food-borne pathogens (8), and on a variety of food spoilage organisms (5), including sporeformers (10).

In the present study we have investigated the interaction between a Micrococcus isolate and several lactic acid bacteria. The involvement of hydrogen peroxide is described.

MATERIALS AND METHODS

Cultures. The isolation of Micrococcus F4 used in these experiments was described previously (16). The culture was grown at 30°C on Standard Methods Agar slants (BBL, Cockeysville, Md.) and held at 4°C. Before use, the culture was grown in Trypticase soy broth (BBL) at 30°C on a shaker and transferred daily for at least 2 days. Lactobacilli and streptococci cultures were maintained in sterile litmus milk held frozen at −15°C. Before use these cultures were transferred daily at least three times in litmus milk. Streptococcus lactis 5 (provided by H. B. Naylor, Cornell U.); S. lactis C10 (from E. M. Mikolajczik, Ohio State U.); Streptococcus cremoris Cl (from E. M. Mikolajczik, Ohio State U.), and Lactobacillus casei var. casei (isolated in our laboratory from cheddar cheese) were incubated at 30°C. Streptococcus thermophilus (from H. B. Naylor) and Lactobacillus bulgaricus (from F. V. Kosikowski) were incubated at 45 and 37°C, respectively.

Measurement of growth, peroxide, and acid production. Experiments were carried out in nonfat milk solids reconstituted to 10% and steamed for 30 min. Where indicated, freeze-dried capsular material was added to milk (1 mg/ml) before steaming. The capsular material was prepared from Micrococcus isolate F4 as described previously (16). Catalase (2,000 U per mg; Sigma Chemical Co., St. Louis, Mo.) was filter sterilized at a concentration of 1 mg/ml and added to the milk, where indicated, to a final concentration of 0.01 mg/ml. An equal volume of sterile distilled water was added to control cultures. Experiments were started by inoculating 75 or 100 ml of tempered milk in a 250-ml flask to 10⁴ to 7 × 10⁷ colony-forming units (CFU) per ml with a 16-h litmus milk culture of streptococci or lactobacilli. Micrococcus isolate F4 was collected by centrifugation and suspended in phosphate buffer before addition, where indicated, to a final concentration of 10⁷ to 2 × 10⁷ CFU per ml. Cultures were incubated without shaking.
at the appropriate temperature for growth of the lactic acid bacteria. At intervals, samples of the culture were removed for analyses. CFU were determined by plating on Standard Methods Agar with and without 5% NaCl. The addition of 5% NaCl was found to prevent growth of streptococci but not micrococci during a 24-h incubation period. To measure acid production, 5-ml samples of culture were added to 5 ml of cold (5 C) distilled water and titrated to pH 8.4 with 0.1 N NaOH. The rate of acid production is expressed as the maximum slope of the curve obtained by plotting volume of 0.1 N NaOH required for titration against time (A ml 0.1 N NaOH per h). The enzymatic method of Gilliland (6) was used to determine residual peroxide in 10-ml samples removed from the culture at intervals during incubation.

RESULTS

The rate of acid production by several lactic acid bacteria was increased on the addition of Micrococcus isolate F4 to milk cultures (Table 1). There does not appear to be any correlation between the wide range of rates of acid production (0.06 to 0.74 ml 0.1 N NaOH per h) in pure culture and the extent of the increase in the presence of F4 (15–180%). The effect of F4 on growth also varied widely (Fig. 1 and 2, Table 2) and can account for the increase in acid production. The increase in acid production was directly proportional to the percentage of increase in cell number (Fig. 3) (assuming the cell number is proportional to CFU). F4 added to cultures of S. lactis C10 increased the rate of growth, but not the final cell yield (Fig. 2). Both the final cell yield (Table 2) and the rate of growth was increased when F4 was added to L. casei (Fig. 4) and to S. cremoris Cl. Addition of F4 to S. lactis 5 (Table 2) and S. thermophilus (Fig. 1) increased the final cell yield but not the rate at which the culture grew.

Growth of F4 alone in milk did not result in a change in pH. However, growth of F4 under the conditions of these experiments was usually poor. The growth rate ranged from no growth to 0.4 doublings per h, compared to a range of 0.4 to 4.0 doublings per h for the lactic acid bacteria. In three separate experiments, the rate of growth of S. thermophilus was 4.0 doublings per h. The rate and extent of growth of F4 appeared to depend at least on the rate of acid production by the lactic acid bacteria and the temperature of incubation. At temperatures above 30 C or during rapid acid production, growth of F4 was inhibited.

The amounts of hydrogen peroxide formed by the cultures also varied (Fig. 5). L. casei did not produce detectable quantities of hydrogen peroxide. S. thermophilus, in one of three experiments, produced 0.115 μg of H2O2 per ml at 6 h, but no peroxide was detectable over the remainder of the time interval measured (0–6 h and 25 h). The concentration of H2O2 (0.8–1.8 μg/ml) that occurred in pure cultures of S. lactis 5, S. cremoris Cl, and S. lactis C10 were reduced to less than 0.03 μg of H2O2 per ml (essentially background) in mixed culture with F4. The relatively high concentrations of H2O2 produced by L. bulgaricus were markedly reduced in the

Table 1. Effect of Micrococcus isolate F4 on acid production by some lactic acid bacteria

| Organism        | Rate of acid production* | Increase (%) |
|-----------------|--------------------------|--------------|
|                 | Pure culture          | + F4        |               |
| S. lactis C10   | .74                      | .85          | 15            |
| L. casei var. casei | .08                    | .13          | 62            |
| S. thermophilus | .12                      | .4           | 168           |
| S. cremoris Cl  | .08                      | .12          | 62            |
| S. lactis 5     | .95                      | .17          | 180           |
| L. bulgaricus   | .52                      | 1.02         | 96            |

* A ml 0.1 N NaOH per h measured during the period of maximum rate of acid production.
presence of F4, but remained detectable during the periods of maximum production.

To see if F4 was simply removing H₂O₂ from the medium, the effects of catalase and F4 on growth, acid production, and H₂O₂ production were compared. Except in one case, no H₂O₂ was detectable in medium inoculated with lactic acid bacteria in the presence of 0.01 mg of catalase per ml. In cultures of *L. bulgaricus* to which catalase was added, low levels of H₂O₂ were detectable during the period of maximum production equivalent to the levels found in the presence of F4. Acid production by *L. bulgaricus* was stimulated by catalase to almost the same extent as by addition of F4 (data not shown). Catalase and F4 were equally effective in stimulating acid production by *S. thermophilus* (Fig. 6); however, the final cell yields were higher in flasks containing *Micrococcus* F4 (Fig. 1 and Table 2). Addition of catalase to cultures of *S. lactis* C10 also stimulated acid production by, and growth rate of, the culture to almost the same degree as F4 (data not shown). The final cell yield was not increased by either catalase or F4 (Table 2). Although no H₂O₂ was detected in cultures of *L. casei*, the addition of catalase caused some stimulation both of acid production (Fig. 7) and growth rate (Fig. 4). However, F4 was much more stimulatory. This is seen by comparing increase in acid production, rate of cell growth, and final cell yield (Table 2). These results imply a second means of stimulation by F4 in addition to stimulation by removal of H₂O₂.

The capsular material prepared from *Micrococcus* isolate F4 is a heat-stable substance containing riboflavin, *N*-acetylhexosamine, sialic acid, glutamic acid, and an allo-isoleucine (16). The abilities of capsular material and whole cells to stimulate growth and acid production of *S. lactis* C10 were compared. The stimulation of acid production (Fig. 8) and growth (Fig. 2) by capsular material was as great as that by whole cells. However, the capsular material was not as effective as whole cells in preventing an early accumulation of H₂O₂, although some activity was evident (Fig. 9). Capsular material did not reduce the concentration of H₂O₂ added to uninoculated milk or to 0.01 M acetate, pH 4.5. This suggests that capsular material, and presumably the whole cell, stimulates *S. lactis* C10 by some means in addition to removing H₂O₂.

![Graph](image)

**Fig. 2.** Growth of *S. lactis* C10 in the presence and absence of *Micrococcus* isolate F4 or its capsular material. Symbols: ●, *S. lactis* C10; □, *S. lactis* plus 1 mg of capsular material per ml; ○, *S. lactis* C10 in the presence of *Micrococcus* isolate F4.

**Table 2.** Effect of *Micrococcus* isolate F4, catalase, and capsular material on growth of certain lactic acid bacteria

| Culture          | Final cell yield* | Maximum increase in cell number in the presence of F4 (%)a |
|------------------|------------------|----------------------------------------------------------|
|                  | Pure culture     | Plus *Micrococcus* isolate F4 | Plus catalase | Plus capsular material |                  |
| *S. lactis* C10  | 295              | 245                        | 260           | 240c                  | 66.3             |
| *L. casei* var. casei | 160            | 390                        | 140           |                       | 116              |
| *S. thermophilus* | 20               | 78                         | 40            |                       | 35               |
| *S. cremoris* C1 | 3.9              | 5.5                        |               |                       | 380              |
| *S. lactis* 5    | 25               | 110                        |               |                       |                  |

* Lactic acid bacteria CFU per ml × 10⁻¹.

a Percentage of increase in lactic acid bacteria CFU measured at the time of maximum difference in CFU.

c Separate experiment (Fig. 2).
There appear to be two ways in which the addition of whole micrococcii stimulate cultures of lactic acid bacteria. The presence of F4 cells prevents accumulation of detectable levels of \( \text{H}_2\text{O}_2 \) and the addition of catalase results in stimulation of acid production and growth of the bacteria tested. We conclude, therefore, that the ability of the micrococcii to remove \( \text{H}_2\text{O}_2 \) is at least in part responsible for stimula-

**DISCUSSION**

The data presented indicate that whole cells of *Micrococcus* isolate F4 and its capsular preparation stimulate growth and acid production in several lactic-acid-producing bacteria.
tion. However, we postulate the existence of at least one other mechanism of stimulation to account for the greater stimulatory activity of F4 compared with catalase in cultures of *S. thermophilus* (Fig. 1) and *L. casei* (Fig. 4 and 7). A second means of stimulation is also implicated in experiments with capsular material added to the culture of *S. lactis* C10. In these experiments, increased growth occurs even though the H$_2$O$_2$ accumulation is either not reduced (Fig. 9) or only slightly reduced (data not included). The data also suggest that, in *S. lactis* C10, either removing H$_2$O$_2$ or affecting the unknown target is sufficient to stimulate the culture. It is evident that under certain conditions H$_2$O$_2$ may not be inhibitory. Organisms without catalase that are not particularly sensitive to H$_2$O$_2$ have been reported (15). Gilliland and Speck (7) described a *L. bulgaricus* strain that was not stimulated by catalase even though in the absence of catalase it produced larger quantities of H$_2$O$_2$ than did a *S. lactis* culture which was stimulated by catalase. This is another situation where H$_2$O$_2$ may not be inhibitory. Our culture of *L. bulgaricus*, however, was stimulated by addition of cata-

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**FIG. 7.** Acid production by *L. casei* in the presence and absence of catalase or *Micrococcus* isolate F4. Acid production measured as in Fig. 6. Symbols: ○, *L. casei*; □, *L. casei* plus 20 U of catalase per ml; ●, *L. casei* in the presence of F4.

**FIG. 8.** Acid production by *S. lactis* C10 in the presence and absence of *Micrococcus* isolate F4 or its capsular material. Acid production measured as in Fig. 6. Symbols: □, *S. lactis* C10; ○, *S. lactis* C10 plus 1 mg of capsular material per ml; ●, *S. lactis* C10 in the presence of F4.

**FIG. 9.** Hydrogen peroxide production by *S. lactis* C10 in the presence and absence of *Micrococcus* isolate F4 or its capsular material. Symbols: ●, *S. lactis* C10; □, *S. lactis* C10 plus 1 mg of capsular material per ml; ○, *S. lactis* C10 in the presence of F4.
lase. It may be pointed out here that stimulation of acid production by addition of catalase does not necessarily mean that the lower rates of acid production were due to an inhibitory concentration of H₂O₂. The peroxidatic activity of catalase has been demonstrated with substrates such as ethanol acting as hydrogen donor (11). It is possible that increased growth is due not to removal of inhibitory concentrations of H₂O₂, but to secondary effects involving utilization of H₂O₂ to oxidize another compound and render it stimulatory or to effects involving utilization of H₂O₂ to remove a different inhibitory compound by oxidation. However, demonstration of the inhibition of lactic acid bacteria by added H₂O₂ in several laboratories (21, 22) including our own supports our conclusion that stimulation results at least in part from removal of an inhibitory concentration of H₂O₂. Also, a culture of L. bulgaricus to which both F₄ and H₂O₂ were added was not stimulated to a greater extent than one to which F₄ alone was added (data not shown). Greater stimulation might be expected if both factors were involved in the formation of a stimulatory compound.

Ranges of sensitivity to H₂O₂ have been reported from 5 μg/ml for single-strain lactic streptococci and commercial lactic starter cultures (21) to 100 to 200 μg/ml for strains of L. lactis (22). In our experiments, cultures of L. casei and S. thermophilus did not produce concentrations of H₂O₂ detectable by our assay (<0.1 μg/ml), but were stimulated by catalase. Again, stimulation in this case may have been due to peroxidatic activity of catalase. In the experiments of Keilin and Hartree (11) high concentrations of H₂O₂ were decomposed to water and oxygen, whereas low concentrations could be used to oxidize ethanol, catalase functioning as a peroxidase under these conditions. Stimulation by catalase could also be accounted for if a small amount of H₂O₂, capable of inhibiting the L. casei and S. thermophilus cultures, is masked to the assay by a component of the milk. It has been demonstrated with the assay used for this work that H₂O₂ added to milk cannot be totally recovered as measured by the assay (6). This may be due to masking of the peroxide as well as to decomposition by organic matter. In addition, the data could be explained if the small amounts of H₂O₂ responsible for inhibition, and accessible to catalase in the growing culture, are bound in some way to the cells and removed with them during centrifugation before assay, or are decomposed during this procedure.

The concentration of catalase (0.01 mg/ml or 20 Sigma U/ml) added to cultures of L. bulgaricus did not remove all the H₂O₂ formed. This concentration is expected to decompose 680 μg of H₂O₂ per ml per min at pH 7 and 25°C. The milk system was at pH 6 to 5 at 37°C and contained about 2 μg of H₂O₂ per ml, compared to approximately 340 μg of H₂O₂ per ml present during estimation of activity of the enzyme preparation. Perhaps these differences in conditions are responsible for the residual H₂O₂ in spite of the very high concentration of catalase.

To insure that catalase concentration was not limiting stimulation of L. casei, the effect of a threefold increase in concentration of catalase was determined. The higher concentration did not result in greater stimulation of either growth or acid production, indicating that sufficient or excess catalase was present for maximum stimulation by this means.

Our data agree with the results of Gilliland and Speck (7) that indicate that the lactic streptococci can produce significant quantities of H₂O₂. In their experiments, maximum accumulation of H₂O₂ occurred during the time of most rapid decrease in pH and dropped before the rate of acid production (measured as pH change) decreased. In the experiments described here, where detectable, the H₂O₂ levels increased or remained constant as cell growth began to slow down.

The second means by which micrococci stimulate lactic acid bacteria is not known. The capсуlar material has limited ability to remove H₂O₂ and therefore might be acting on this second target. More specific characterization of the composition of this material is underway. This may give some indication of how it functions.

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