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# Study of Plating Efficiency of Bacteriophages of Thermophilic Lactic Acid Bacteria on Different Media

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The qualitative and quantitative abilities of phages of 13 strains of thermophilic lactic bacteria (lactobacilli and streptococci) to produce plaques on six different media were studied. The influence of the addition of calcium, on the one hand, and of incubation conditions (aero-anaerobiosis), on the other hand, was also examined. For the phages of lactobacilli, the best results were obtained with the medium of de Man, Rogosa, and Sharpe (1960), whereas for the phages of streptococci the medium of Hogg and Jago (1970) appeared to be the best. Calcium and incubation conditions play a role which is variable in importance, but rarely negligible.

The thermophilic lactic acid bacteria, especially *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus lactis*, are used for the production of Italian and Swiss cheeses and yogurts. In 10 years, the production of these cheeses in the United States has increased by more than 350% and that of yogurts by 400%. In the same period, the number of factories has greatly decreased, resulting in a concentration of production units, which has in turn led to the rationalization and mechanization of production methods. The success of such large-scale production is highly dependent on the careful preparation and strict control of the starter cultures. Thus, the problem of bacteriophage infections of starters has become very serious.

The extensive utilization of thermophilic lactic acid bacteria is recent, which is probably the reason why the study of the bacteriophages has been until now very limited (6). All the research workers who have isolated and studied these bacteriophages have experienced difficulty in obtaining plaques. The formation of plaques is extremely useful for enumerating and purifying phages and may even be used for isolating mutants. Incidentally, this technique is also essential for studies of phage transduction and plasmids for the development of new strains. The bacteriophages of known thermophilic lactic starters were isolated on different media. In this study we compared their ability to produce plaques on six of the media used.

## MATERIALS AND METHODS

**Bacteriophages and host strains.** The strains are of different origins which are listed in Table 1. The strains and their phages were transferred twice a week into sterilized skim milk enriched with 0.1% yeast extract. Incubation was carried out in a water bath at 40 C. The cultures (in 10-ml tubes) were cooled directly after coagulation (about 3 h for streptococci and 5 to 6 h for the lactobacilli) and stored at + 4 C.

**Phage count.** The technique devised by Valles (8) was used as the standard method of control. Standard resazurin solution (10 ml), prepared by dissolving one tablet of resazurin (BDH) in 50 ml of sterile-distilled water, and a milk culture of the host bacteria (1 ml) are mixed with sterilized skim milk (100 ml) containing 0.1% yeast extract; portions of this suspension (9 ml) are poured into sterile tubes. A series of 10-fold dilutions of the phage suspension is prepared with Ringer solution. Each phage dilution (1 ml) is added to the prepared tubes of inoculated milk containing resazurin and mixed well. Sterile Ringer solution (1 ml) is added to the blank tube instead of a phage dilution. All tubes are incubated in a water bath at 40 C.

Growth of bacteria provokes the reduction of resazurin, which is thus discolored. This reaction begins at the bottom of the tube and spreads upwards; finally the milk coagulates. In the first tubes of the series, containing a large number of phages, the resazurin is not discolored, nor is the milk coagulated. In the following tubes, containing a moderate or small number of phages, the resazurin is at first discolored owing to bacterial growth and then regains its color as the bacteria are progressively lysed. The extent of discoloration depends on the initial number of phages present. In the last tubes of the series, in which the initial number of phages is very small (1 to 100 phages per ml), the culture behaves like the blank and is first discolored and then coagulated. The first three tubes that are coagulated are then tested for presence of phages. The number of phages in the initial suspension is indicated by the dilution factor of the last tube in which presence of phages is confirmed (in this study, $10^6$ to $10^7$ phages/ml).

TABLE 1. *Strains of different origins*

| Organisms | Source[a] | Isolated by: |
|---|---|---|
| Lactobacilli | | |
| *L. bulgaricus* 448 | INRA | Chevalier |
| *L. bulgaricus* 449 | INRA | Chevalier |
| *L. helveticus* L 112 | LIN | Sozzi-Maret |
| *L. helveticus* 450 | INRA | Chevalier |
| *L. helveticus* 15807 | ATCC | Kiuru-Tybeck |
| *L. lactis* A | LIN | Sozzi |
| *L. lactis* F | LIN | Sozzi |
| *L. lactis* 15808 | ATCC | Kiuru-Tybeck |
| Streptococci | | |
| *S. thermophilus* L 12 | LIN | Sozzi |
| *S. thermophilus* S 113 | LIN | Sozzi |
| *S. thermophilus* S 265 | LIN | Sozzi-Maret |
| *S. thermophilus* 440 | INRA | Chevalier |
| *S. thermophilus* 19987 | ATCC | Kiuru-Tybeck |

[a] Abbreviations: INRA, Institut National de la Recherche Agronomique Jouy-en-Josas—France; LIN, Laboratoire Industriel Nestle, Orbe—Switzerland; ATCC, American Type Culture Collection.

**Media.** After a preliminary selection, the following six cultures of media were chosen for study.

(i) MRS (De Man, Rogosa, and Sharpe [1], lactobacilli MRS broth, Difco Laboratories, Detroit, Mich.); (ii) HJ (Hogg and Jago [3]), broth containing 3% tryptone, 1% yeast extract, 0.2% beef extract, 0.5% lactose, and 0.5% $KH_2PO_4$, final pH 6.5; (iii) MRT (Murata [5]), composed of 1% D-(+)-glucose, 1% polypeptone, 1% sodium acetate, 0.5% yeast extract, 0.3% beef extract, 0.1% NaCl, 0.02% $MgSO_4$, 0.001% $MnSO_4$, and 0.0001% $FeSO_4$, final pH 6.0; (iv) RR (Reinbold and Reddy [6]), containing 3% tryptic soy broth (Difco), 0.5% yeast extract, and 0.2% L-cystine, final pH 6.5; (v) DO (Douglas et al. [2]), composed of 1% tryptone, 0.3% yeast extract, 0.2% glucose, 1% sodium glycerophosphate (BDH), 0.1% tris(hydroxymethyl)aminomethane (Sigma Chemical Co., St. Louis, Mo.), and 0.033% $CaCl_2 \cdot 2H_2O$, adjusted to a final pH of 7.8 with lactic acid; (vi) KT (Kiuru and Tybek [4]), containing 1.5% nutrient broth (Difco), 10% (vol/vol) tomato juice, 20% (vol/vol) autolyzed yeasts, and 0.5% lactose, final pH 6.5. For solid media, 1.5% agar (Difco) is added. All the media are prepared with distilled water and biological grade chemicals and are sterilized for 15 min at 121 C. Extra calcium, when used, is added as $CaCl_2 \cdot 6H_2O$, 0.1% for liquid media and 0.7% in the second layer only (top agar) for agarized media.

All percentages are given as weight/volume, except where otherwise mentioned.

For the comparison of agar media, the plaque-forming units were counted by the following double-layer plating method. The phage solution is centrifuged at 3,000 × *g* during 30 min and filtered on a membrane filter (Millipore Corp., pore diameter 0.45 μm). Dilutions of 1:10 are made with sterile Ringer solution. The agar medium (10 ml) is poured into a petri dish (diameter, 10 cm) and then cooled. After 1 h, 3 ml of the following mixture is poured on to the agar: 2.5 ml of Ringer solution or a 2.5% solution of $CaCl_2 \cdot 6H_2O$ plus 3 ml of bacteriophage dilution plus 1 ml of culture of the host strain, grown for 6 h at 40 C on the medium to be tested, plus (after 15 min to allow adsorption) 2.5 ml of agar medium. All the preparations are kept at 45 C before plating. After the second layer has solidified (2 h), the petri dishes are incubated for 16 h at 40 C. Incubation of the samples was effected in air, since preliminary assays of incubating the petri dishes in three different types of atmosphere, air, air with 10% $CO_2$ added, and complete anaerobic atmosphere, showed that incubation in air, although not always the best, was satisfactory for most of the strains tested. Thus, air incubation was used, except for strains that produced positive results only when incubated in anaerobic or $CO_2$ atmospheres.

## RESULTS

The quality of the different media tested was judged using the following criteria: for liquid media, the amount of bacterial growth and the degree of lysis; for agarized media, the quality of the bacterial lawn and the number and quality, size, and definition of plaques. The influence of added calcium was also recorded.

The best medium for *L. bulgaricus* and its phages was MRS (Fig. 1). MRT and HJ media were also satisfactory but no plaques were formed on HJ. The presence of additional calcium in liquid media promoted more efficient lysis by phages. However, the influence of calcium was probably indirect, by inducing a more active growth of the bacteria, which in turn rendered them more susceptible to the lysing action of phages. The liquid form of KT medium was satisfactory, but no plaques were formed on the agarized medium. RR was even less satisfactory and DO did not even permit sufficient bacterial growth.

MRS, HJ, and MRT media permitted vigorous growth of all three strains of *L. helveticus* and their phages (Fig. 2). Liquid KT was satisfactory, apart from giving only mediocre results with the strain L112. Conversely, this latter strain was the only one to grow, even moderately, on DO, and this only in the presence of added calcium, DO being totally inadequate for the other two strains. Similar, satisfactory results were obtained for all three strains in liquid RR. Agarized RR was only unsuitable for growth of strain 15807, which produced no bacterial lawn. MRT proved the best overall medium for production of plaques by the three strains of *L. helveticus* tested, as consistant high yields of well-defined plaques were obtained. Additional calcium sometimes stimulated and sometimes inhibited growth, but often had no effect at all (particularly in RR medium). It was essential for the growth of L112 on DO and for the production of plaques by phage 450 on MRS, HJ, and MRT. It is noteworthy that phage growth was generally underesti-

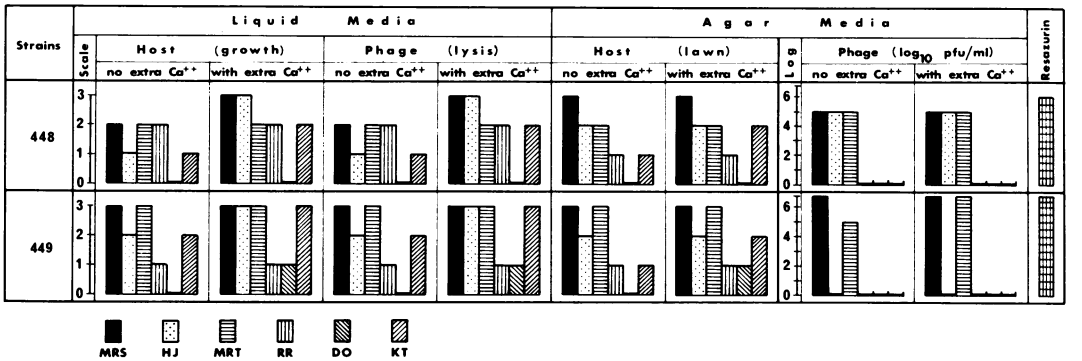


FIG. 1. *Development of strains and phages of L. bulgaricus on different liquid and solid media, with and without addition of calcium chloride. The different media tested were rated qualitatively on a scale of 0 to 3, in which 0 = negative, 1 = poor, 2 = moderate (just satisfactory), 3 = good (entirely satisfactory), based on the following criteria: for the host in liquid media, rate and density of growth; for the phage in liquid media, degree of lysis of the culture; for host on solid media, density and homogeneity of the bacterial lawn. The number of plaque-forming units (pfu) per milliliter appearing as well-defined, easily visible plaques, and the initial concentration of phages determined by the standard resazurin method of control were assessed on the log scale of 0 to 7.*

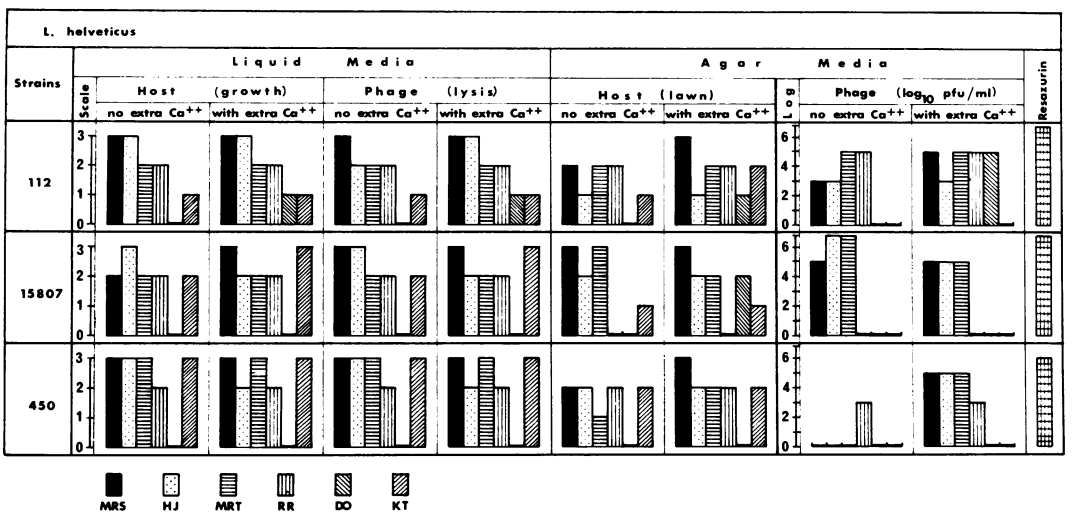


FIG. 2. *Development of strains and phages of L. helveticus on different liquid and solid media, with and without addition of calcium chloride.*

mated by the plaque count method compared with the resazurin test.

MRS and MRT, followed by HJ, were the most successful media overall for culturing strains and phages of *L. lactis* (Fig. 3). MRT was superior to MRS as liquid medium, but much inferior to MRS when agarized. Results on HJ were less regular generally and totally inadequate for plaque production by phage F1. RR was satisfactory when liquid, but unsuitable for plaque production. DO gave poor results, and KT was only suitable for the strain and phage 15808. Addition of calcium favored growth; only one case of inhibition was recorded (strain A in MRT liquid medium). When added calcium in solid media had an influence, it increased production of either the bacterial lawn or the plaques. Although extra calcium was essential for plaque production by the phage of strain A on HJ and MRT, it inhibited their growth in liquid MRT. This anomaly is possibly due to the difference in concentration of calcium chloride in the two types of media: 0.1% in broth and 0.7% in top agar. The com-

plete absence of plaques on RR should also be noted, although two strains (F1 and 15808) produced a satisfactory lawn.

The results obtained with strains and phages of *S. thermophilus* differed appreciably from those of the lactobacilli (Fig. 4). KT broth and HJ agar were the most satisfactory. MRS was generally adequate, except for plaque production by phage 440. Irregular results were obtained with MRT, RR, and DO, and no plaques were produced on the RR and DO. Overall, HJ proved the most satisfactory and consistent. Furthermore, it was the only medium on which plaques were produced by all five strains tested.

The effect of calcium was widely divergent:

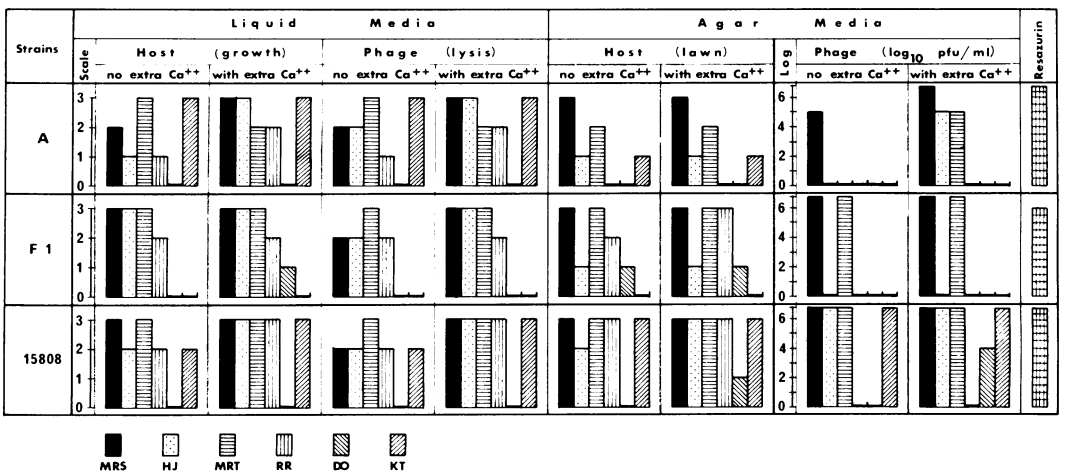


FIG. 3. *Development of strains and phages of L. lactis on different liquid and solid media, with and without addition of calcium chloride.*

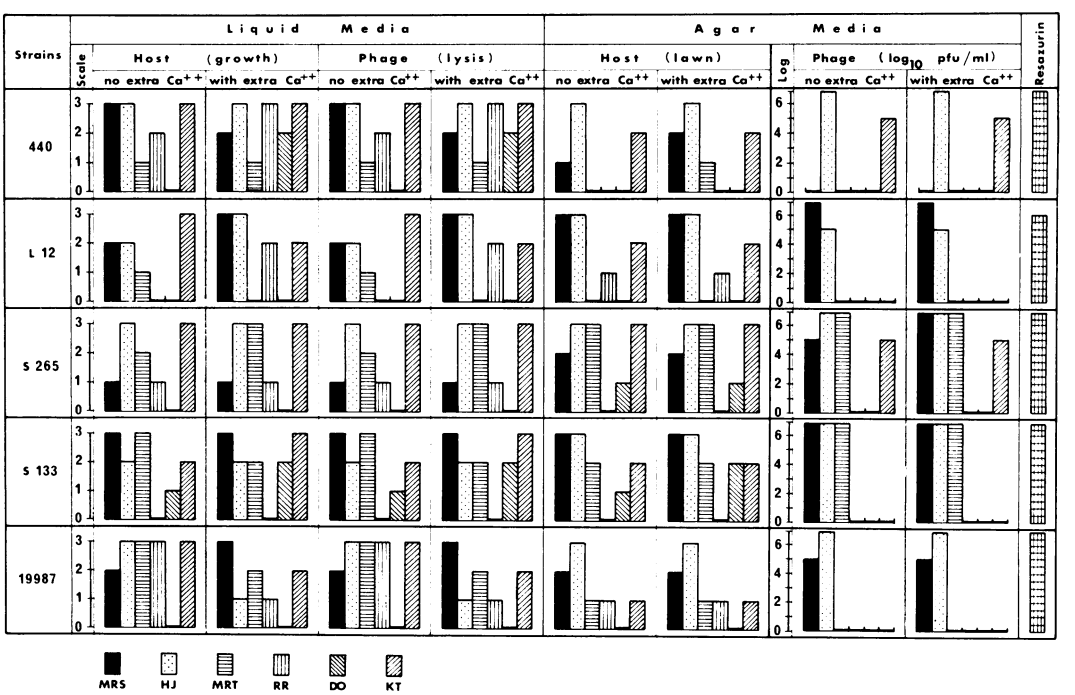


FIG. 4. *Development of strains and phages of S. thermophilus on different liquid and solid media, with and without addition of calcium chloride.*

stimulatory, inhibitory, or negligible, especially in liquid media. As phages and bacteria were affected in the same way, the calcium probably acts basically on the bacteria. In solid media, additional calcium only had an effect in three cases, provoking improvement of the bacterial lawn.

Our results thus indicate that the best agar media for obtaining large numbers of well-defined plaques are MRS for the thermophilic lactobacilli and HJ for the thermophilic streptococci (Table 2).

The majority of strains produced the most distinct plaques when incubated in a totally anaerobic atmosphere, but in several cases the bacterial lawn was so dense that small plaques were hidden, so that yield was underestimated (Fig. 5). However, the plaques developed in an atmosphere enriched with carbon dioxide or simply in air were usually sufficiently well defined and even the smallest were visible. The petri dishes were therefore incubated routinely in air, except for *S. thermophilus* 19987 and 440 and their phages, which could not produce plaques unless incubated under totally anaerobic conditions or in an atmosphere enriched with carbon dioxide.

TABLE 2. *Yield of good quality plaques produced by the six different media tested*[a]

| Medium | *Lactobacillus* | | *Streptococcus* | |
|---|---|---|---|---|
| | Yield of media[a] (maximum, 160 points) | N[b] positive results from 16 experiments | Yield of media[a] (maximum, 100 points) | N[b] positive results from 10 experiments |
| MRS | 129 | 15 | 74 | 8 |
| HJ | 60 | 9 | 96 | 10 |
| MRT | 124 | 14 | 40 | 4 |
| RR | 22 | 4 | 0 | 0 |
| DO | 12 | 2 | 0 | 0 |
| KT | 23 | 3 | 32 | 4 |

[a] The first column shows the overall score of each medium according to an arbitrary point scale of 0 to 10 drawn up on the basis of both quality of bacterial lawn and plaque count (0 = negative, 10 = good). Each medium was thus rated twice for each strain, once with and once without addition of calcium.

[b] The number of experiments in which countable plaques were produced is given in the second column.

## DISCUSSION

The results presented indicate that the optimal medium for formation of plaques is not the same for bacteriophages of thermophilic streptococci as for phages of thermophilic lactobacilli. The streptococcal phages produce the largest plaques on HJ; those of lactobacilli do so on MRS. Although addition of calcium chloride did not always favor formation of plaques, it never once (phage 15808 on HJ and MRT media) exerted an inhibiting action. Its addition may therefore be recommended.

The results obtained on KT medium with phages of *L. helveticus* 15807 and *L. lactis* 15808 confirm those of previous work (4). The phages of the former do not produce visible plaques, whereas those of the latter do. However, the bacteriophages of *S. thermophilus* 19987 did not form plaques on KT medium, probably owing to a modification of certain of its biological characteristics occurring during transfer of the bacterial culture. The negative results of plaque formation on RR medium, which is recommended as the best medium for studying bacteriophages of *S. thermophilus*, are noteworthy. One explanation for this anomaly is that, in our case, the bacteria and phages used in this study were grown up on MRS and HJ media, thus encouraging the selection of mutants growing well in these media. Once transferred to another medium, they grew more slowly, so no plaques were formed. This suggests that the optimal medium for growth of the host microorganism should also be used for production of plaques.

MRS is the medium most frequently used for isolation and maintenance of lactobacilli and, on the basis of our results, may also be recommended for the preparation of plaques by phages of thermophilic lactobacilli.

HJ medium, compared with many other media for transfer of mesophilic and thermophilic streptococci, is the best for growth of both the microorganisms and their phages. Well-defined plaques were always formed on this medium.

The results of this study indicate that the use of MRS medium for lactobacilli and HJ medium for streptococci is advisable. However, the complexity of our results using different media for several strains of the same genus illustrates that no one medium is ideal for obtaining satisfactory plaques for all the bacteriophages of thermophilic lactic acid bacteria.

The results of yield and clarity of plaques produced by the phages of thermophilic lactic acid bacteria incubated in different atmospheres are irregular. Therefore, no one atmosphere can be selected as optimal. However, incubation in an anaerobic atmosphere is slightly better, but as incubation in air gives in most cases satisfactory results, this latter technique is recommended for practical reasons. It is important to choose suitable incubation conditions to obtain a lawn of host bacteria which is neither too weak (in which case visibility of the

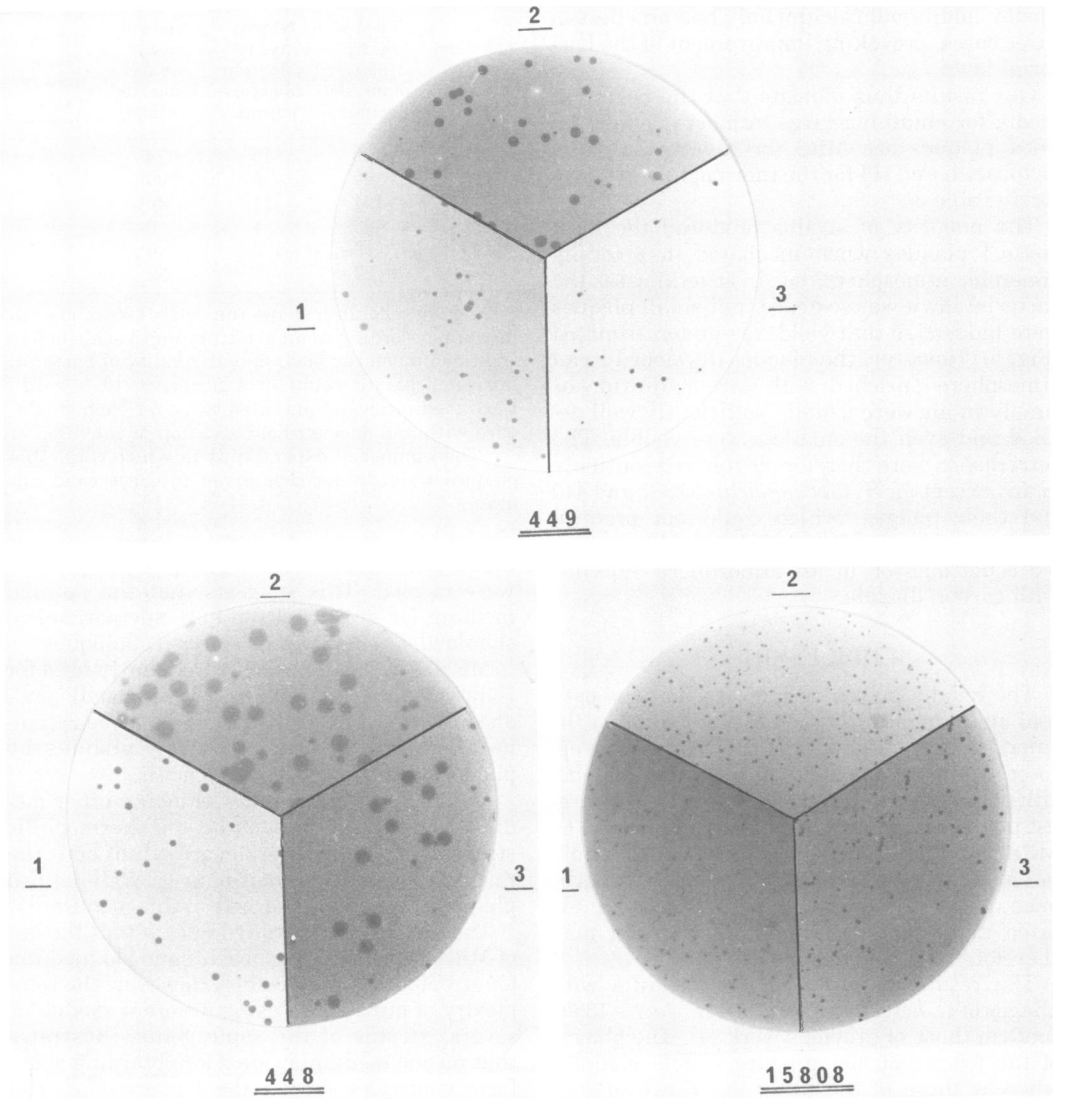


FIG. 5. *Petri plate sections from phage platings of three strains of lactobacilli, incubated in different atmospheres, as follows: section 1, aerobiosis (normal air); section 2, anaerobiosis (no oxygen; 10% carbon dioxide) (obtained by using BBL GasPak Systems); section 3, 10% carbon dioxide (added to normal air) (obtained by using BBL GasPak Systems). Best results are those giving a well-grown bacterial lawn, but not too dense, to allow the best visibility together with the greatest count of plaques: section 2 for both strains 448 and 449 (L. bulgaricus); section 3 for strain 15808 (L. lactis); 448 (1) and 449 (3) show the masking effect on plaques count of a too dense lawn.*

plaques will be poor) nor too dense (in this case the smallest plaques may not be visible).

ACKNOWLEDGMENTS

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