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MICROBIAL CELL DIVISION AND SEPARATION: EFFECT OF CITRATE ON THE GROWTH OF GROUP N STREPTOCOCCI

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Abstract

In the presence of citrate, some strains of lactic streptococci grow as long chains of innumerable cells. The results with citrate-negative and citrate-resistant variants suggest that citrate is involved in the cell separation system of the streptococci. Observations of the long chains under scanning and transmission electron microscopes suggest that citrate inhibits a certain step near the final cell separation, or that citrate can stimulate initiation of cell division at multiple sites, thereby leading to the cells in long chains.

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Introduction

The streptococci represent a group of fastidious microorganisms which exhibit remarkable heterogeneity with respect to their biochemical and genetic characteristics. Group N streptococci occupy an important position as lactic starters in the fields of dairy and food technology. Various strains of these organisms have been used for the flavor enhancement of milk, butter and cream (Mocoquot & Hutel, 1970). However, little is known about the morphology of the lactic streptococci under different growth conditions.

Recently, we observed abnormally long chains of citrate-fermenting lactic streptococci in broth, with the lengths of the chains dependent upon the concentration of citrate. We have attempted to clarify the effect of citrate upon the length of the chains of cells. We believe that our results provide clues that will help to elucidate the mechanism of certain phases of cell division in lactic streptococci.

Materials and Methods

Bacteria and media

All bacterial strains used were group N streptococci. Among them, Streptococcus sp. KSM-1106 was isolated from a sample of cream by S.I., and Streptococcus sp. KSM-1112 from a lactic starter by T.M.. Our strains were of an intermediate type, and were neither typical S. cremoris nor S. lactis. All strains were propagated in 12% reconstituted nonfat dry milk (NFDM, Difco) at 26°C, and incubations were continued in quiescent state until the medium coagulated. The coagulated medium was kept at 4°C by monthly transfers in the NFDM. The protease activity (Prt) of each organism was checked anaerobically by the ability to form clear zones around the colonies on agar plates, which contained 12% NFDM, 1% glucose and 1.5% agar (pH 6.8). The ability to utilize citrate (Cit) was checked by anaerobic growth on the citrate agar plates described by Kempler and McKay (1980). The lactose fermenting ability (Lac) was checked by the growth in a standard medium containing 1% lactose. The standard medium for growth contained the following ingredients (g/liter): glucose or an alternate sugar (10); polypeptone (10, acid-hydrolyzed soy bean digest of Daigo Eiyo Co. Ltd., Japan); yeast extract (5, Difco); and distilled water; the pH was adjusted to 6.8 with NaOH. The medium was sterilized by autoclaving. The citrate medium was prepared by adding disodium citrate at

varied concentrations to the above-mentioned standard medium. Media were supplemented with filter-sterilized inorganic ions, organic acids, amino acids and antibiotics as required.

Isolation of mutants

The gene coding for the Cit phenotype was plasmid-linked in our strains (our laboratory, unpublished data). The Cit phenotype of the KSM-1106 strain was highly unstable and was readily lost when the strain was transferred several times, successively, as a stab in the agar medium of Yashima et al. (1970) at 30°C. The cured cells were incubated in 12% NFDM at 26°C for 3 days and then plated on the citrate agar plates of Kempler and McKay (1980). A citrate-negative (Cit^-) mutant grew up as the white colony on the citrate agar plate and its relevant phenotype was $Lac^+Prt^+Cit^-$ (designated KSM-1106C^-). The Cit^- mutant lacked a citrate permease (unpublished data).

A citrate-resistant (Cit^r) mutant derived from the KSM-1106 strain was prepared as follows. The KSM-1106 strain was grown at 26°C in a 20-ml test tube containing 5 ml of standard medium. Cells were harvested after 16 h of growth, washed twice in chilled saline and then resuspended in 5 ml of chilled deionized water. The resulting cell suspensions were treated with N-methyl-N'-nitro-N-nitrosoguanidine (10 μg/ml) for 90 min at 30°C with gentle agitation. The treated cells were diluted, immediately transferred to 12% NFDM, and incubation was continued without shaking, at 26°C until the medium coagulated. An aliquot was then resuspended in medium that contained 150 mM citrate. After incubation at 26°C for 3 days, the culture was spread plated on the citrate agar plates (Kempler & McKay, 1980) and incubated anaerobically in order to check the phenotype of Cit. Blue colonies grew up, indicative of the Cit^+ phenotype, and were purified. By this procedure, the mutant strain, KSM-1106C^r ($Lac^+Prt^+Cit^+$) was isolated.

Culture conditions and measurement of growth

Usually, cells used for experiments were prepared from the cultures in the early stationary phase of growth, reached after 14 to 16 h of incubation at 26°C in standard medium. The cultures, containing an inoculum of a 1:100 dilution of the same preculture, were grown anaerobically on standard medium in capped test tubes. At intervals, samples with appropriate dilution were transferred into 3.0 ml cuvettes and absorbance at 590 nm (A_{590}) was measured, using a Hitachi 220 spectrophotometer.

Determination of chain length

The test strains were harvested all in the early stationary phase of growth for counting cell numbers per each chain. The cultures, after 14 to 16 h of growth at 26°C, were mixed by gently inverting the capped test tube twice. A loopful of the culture was placed on a glass slide and covering it with a coverslip. The cells were observed under a light microscope with an oil immersion lens (phase contrast optics, x 1,000 magnification). The cell numbers in 100 to 150 chains were counted and the average chain length, in number of cells/chain, was calculated.

Scanning electron microscopy (SEM)

For SEM, cell pastes were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C for 2 h. Specimens were postfixed in OsO_4, buffered with cacodylate as above, at 4°C for 1 h, washed with cacodylate buffer and then dehydrated by two immersions for 5 to 10 min each in 50, 60, 70, 80, 90, 95 and 100% ethanol. Specimens were then put in isoamyl alcohol for 10 min and critical-point dried in liquid CO_2. Dried specimens were mounted on stubs and coated with gold for 4 to 5 min by vacuum evaporation. A JSM-35C electron microscope (JEOL Ltd., Japan) was operated at 10 or 15 kV.

Transmission electron microscopy (TEM)

Specimens were fixed with glutaraldehyde and postfixed with OsO_4, as described above. Fixed specimens were dehydrated in a graded alcohol series (3 times each in 50, 70, 90 and then in 100% ethanol). Specimens were washed twice with propylene oxide for 10 min and infiltrated with 1:1, 3:1 and 7:1 portions of epoxy resin (Epon 812):propylene oxide for 2 h each. Specimens were infiltrated with 100% resin overnight and then embedded in fresh resin. Thin sections (50 to 80 nm in thickness) were stained with uranyl acetate and Reynolds' lead acetate solutions (Reynolds, 1963) for 10 min and examined under a JEM-100CX electron microscope (JEOL Ltd., Japan) operated at 80 kV.

SEM by Robinson backscattered electron detector (WET-SEM)

The culture, after 14 to 16 h of growth at 26°C, was transferred onto a cover glass and observed directly in a WET-SEM WS-250 (Akashi Seisakusho Ltd., Japan) (Shimakura & Inoue, 1985) equipped with the wide angle backscattered electron detector developed by Robinson (1975). The accelerating voltage used was 15 kV in a vacuum of from 0.2 to 0.3 Torr.

Results

Citrate-induced growth of chains of lactic streptococci

In general, group N streptococci grow as pairs or, in some cultures, as short chains (Teuber & Geis, 1981). During studies on the biochemistry and genetics of the catabolism of citrate, we found that Streptococcus sp. KSM-1106 grew as very long chains in the presence of citrate, as seen by WET-SEM. This strain grew predominantly as pairs of cells or as short chains in standard medium (Fig. 1). The chain length of the organism increased with increasing concentration of citrate. When the concentration of citrate reached 15 mM, the chains of cells became tangled clumps in which the numbers of cells were too great to be counted (Figs. 2 and 3). When the culture was transferred to fresh standard medium, the chain length was reduced again to 2 or 4 cells per chain, indicating a reversion to the more usual pattern of growth.

If one is solely interested in visualizing "untreated" specimens of the chains of streptococci, the WET-SEM method may be the fastest and simplest method since it involves the fewest manipulative procedures. For information that relates to surface structure or ultrastructure, experiments that employ SEM and TEM are necessary, as described below.

Lactic streptococci with the ability to grow as long chains were identified by growth at 30°C for 2 days in medium that contained citrate. Of 26 strains examined, 5, including strain KSM-1106, grew as long chains in the presence of citrate, i.e., Streptococcus sp. KSM-1112, S. diacetylactis DRC-2, S. diacetylactis D-16 and S. cremoris AHU 1083. In contrast, taxonomic strains of S. lactis ATCC 19435 ($Lac^+Prt^+Cit^+$) and S. cremoris ATCC 19257 ($Lac^+Prt^+Cit^-$) occurred in pairs or short chains (about 4 cells/chain) whether citrate was present or absent. Hereafter, Streptococcus sp. KSM-1106 and its

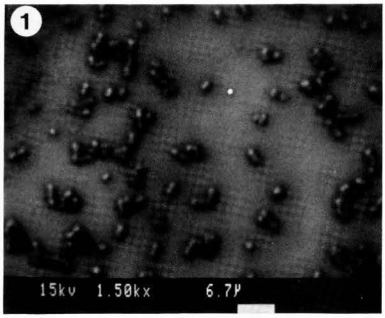
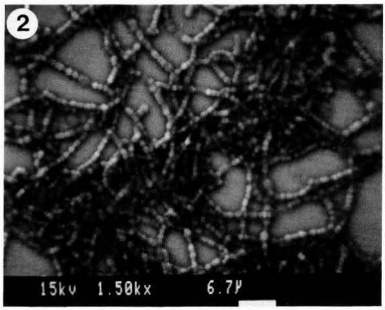
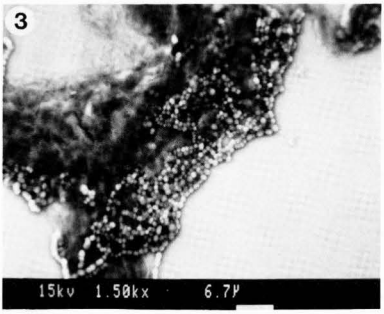


Fig. 1. WET-SEM micrograph of Streptococcus sp. KSM-1106, grown in standard medium. Bar=6.7μm.





Figs. 2 and 3. WET-SEM micrographs of the cells, grown in medium supplemented with 15 mM citrate. Bar=6.7μm.

mutant stains were used extensively.

Effects of growth conditions on the chain length

Effect of several additives were examined on the chain length of Streptococcus sp. KSM-1106, as summarized in Table 1.

Supplementation of standard medium with various organic acids (15 mM or 30 mM) allowed the growth of the test strain; the compounds tested were citrate, oxalacetate, succinate, malate, fumarate, cis-aconitate, isocitrate, tartarate and itaconate. These naturally-occurring organic acids could not replace citrate as a trigger for the formation of long chains. The possibility existed that citrate might act to chelate inorganic ions present in standard medium, thereby potentiating the formation of long chains. This possibility was evaluated by growing the test strain with powerful chelating agents, such as EDTA, tripolyphosphate (STPP) (Cutler, 1972) or Zeolite (Schwuger & Smolka, 1976). These chelating agents were added to give over 50% inhibition of the growth after 16 h of incubation, but no sign of chain elongation was observed by use of these additives. D-Cycloserine, bacitracin, ampicillin, cephazolin, vancomycin and ristocetin, antibiotics inhibitory against the synthesis of bacterial cell walls, did not potentiate the formation of long chains by the strain. Glycine (Gly) and/or isoleucine (Ileu), which are known to alter the bacterial cell wall (Miyashiro et al., 1980), also had no effect upon chain length. In addition, the growth of cells as long chains, induced by 10 mM citrate, was not arrested by addition of the building blocks of bacterial cell wall, such as lysine (Lys), diaminopimelic acid (DAP), glutamic acid (Glu), alanyl-alanine (Ala-Ala), glucosamine ($GluNH_2$) and N-acetyl-glucosamine (N-Ac-$GluNH_2$) (for instance, Johnson & McDonald (1974), who used S. cremoris HP).

The chain length of the test strain did not change when the temperature of incubation was varied between 14 and 34°C. At 37.9°C, the upper limit for growth, the chains (in standard medium, in the absence of citrate) were slightly longer (6 to 8 cells/chain). Variation of the initial pH of standard medium from 4.5 to 7.7 did not cause the formation of long chains, nor did the substitution of any sugars, added in place of glucose, such as fructose, galactose, lactose, maltose, trehalose or salicin. In the presence of citrate, the organism grew as long chains, independently of added sugars.

Function of inorganic ions

Various cations, added as sulfates or chlorides, were tested for their effects on the chain length of Streptococcus sp. KSM-1106. In standard medium that contained 15 mM citrate (over 50 cells/chain), a dramatic destabilization of the chains of cells was observed upon addition of divalent cations, in the order: $Mn^{2+} > Ca^{2+} > Mg^{2+} > Fe^{2+} > Zn^{2+}$. At 15 mM citrate, the shortest chain length of cells of strain KSM-1106 was observed at 5 mM Mn^{2+} (5.0 ± 0.3 cells/chain) and at 15 mM Ca^{2+} (8.8 ± 0.8 cells/chain). Al^{3+}, Co^{2+}, Na^{+}, K^{+} and NH_4^{+} could not interrupt the formation of long chains by citrate (over 50 cells/chain).

Growth characteristics of Cit variants

When the concentration of citrate was raised from zero to 15 mM, the yield of growth of a mutant strain KSM-1106C^{-}, after 16 h of incubation at 26°C, was increased up to 130% of the control yield (in standard medium, in the absence of citrate), but above 15 mM

Table 1. Effect of additives on the chain length of Streptococcus sp. KSM-1106

Exogenous additives	A_{590} after 16 h	PCLC* (%)	ANCC**
In standard medium			
No addition	0.89	52	4.0 ± 0.1
Citrate (15 mM)	1.05	8.3	> 50
Oxalacetate (15 mM)	0.64	56	4.2 ± 0.3
Succinate (30 mM)	0.59	57	3.8 ± 0.7
Malate (30 mM)	0.59	53	3.9 ± 0.9
Fumarate (15 mM)	0.52	52	3.8 ± 0.6
cis-Aconitate (15 mM)	0.59	58	4.2 ± 0.3
DL-Isocitrate (30 mM)	0.64	51	4.2 ± 0.3
Pyruvate (15 mM)	0.52	49	4.6 ± 0.3
Tartarate (15 mM)	0.48	55	4.3 ± 0.3
Itaconate (15 mM)	0.62	49	4.4 ± 0.2
Gly (20 mM)	0.88	56	3.8 ± 0.2
Leu (20 mM)	0.84	54	4.1 ± 0.1
Ileu (20 mM)	0.66	49	4.0 ± 0.2
Gly(10 mM)+Ileu(10 mM)	0.48	53	4.0 ± 0.4
D-Cycloserine (0.1 mM)	0.44	58	4.0 ± 0.1
Bacitracin (0.05 mM)	0.52	44	3.8 ± 0.4
Ampicillin (0.9 mM)	0.44	47	4.2 ± 0.2
Cefazolin (0.5 mM)	0.46	52	4.0 ± 0.1
Vancomycin (0.1 mM)	0.39	53	3.6 ± 0.3
Ristocetin (0.1 mM)	0.44	52	3.8 ± 0.4
EDTA (0.5 mM)	0.42	55	4.6 ± 0.6
STPP*** (20 mg/ml)	0.43	51	4.3 ± 0.1
Zeolite 4A (120 mg/ml)	0.37	48	4.7 ± 0.2
In standard medium containing 10 mM citrate			
No addition (10 mM citrate only)	0.98	9.2	38 ± 2.6
Asp (20 mM)	0.92	8.2	35 ± 4.3
L-Lys (20 mM)	0.96	5.2	36 ± 2.1
DAP (30 mM)	0.88	9.1	38 ± 1.8
Glu (20 mM)	0.98	8.4	36 ± 3.0
L-Ala (20 mM)	0.96	8.0	39 ± 1.6
D-Ala (20 mM)	0.90	7.7	39 ± 2.4
Ala-Ala (20 mM)	0.88	6.9	37 ± 3.2
N-Ac-$GluNH_2$ (20 mM)	0.94	8.2	36 ± 1.5
$GluNH_2$ (20 mM)	0.84	7.8	36 ± 1.9
$GalNH_2$ (10 mM)	0.91	6.8	38 ± 2.4

*% of chains with 4 or less cells. **Average number cells/chain. ***Sodium tripolyphosphate.

growth was retarded. Concentrations of citrate greater than 40 mM completely inhibited the growth of this mutant (and also of the parent). In contrast, another mutant strain KSM-1106C^r could grow in standard medium containing 220 mM citrate. The yield of growth of this mutant, after 16 h of incubation at 26°C, was higher between 5 and 170 mM citrate, and the maximum yield was observed between 30 and 50 mM citrate at which concentrations the yield was 160% of the control yield without citrate. However, the susceptibilities to EDTA, STPP and Zeolite were the same among Cit^- and Cit^r strains.

As the concentration of citrate was increased, the Cit^- mutant occurred as long chains with increasing numbers of cells per chain. When the concentration of citrate was brought up to 15 mM, the chains became tangled clumps of innumerable cells, as observed with the parent. In contrast, the Cit^r mutant occurred as short chains (2 to 6 cells/chain) over the range of concentrations of citrate from zero to 40 mM, but above 40 mM it abruptly occurred as long chains.

Observations with the electron microscope

Figures 4 to 8 show SEM micrographs of Streptococcus sp. KSM-1106 and S. cremoris ATCC 19257, grown in the presence or absence of citrate. Cells of Streptococcus sp. KSM-1106 usually occurred in pairs or short chains of ovoid cells, 0.6 to 0.8 by 0.8 to 1.2 μm in size (Fig. 4). Long chains with very many cells were formed in standard medium that contained citrate (Fig. 5). The wall of some cells had burst, demonstrating that the walls of cells in long chains are fragile. In addition, citrate changed the morphology of cells of this strain from ovoid to a mixture of ovoid and rod-shaped cells of irregular size and shape, 0.4 to 1.0 by 1.0 to 1.7 μm. With 5 mM Mn^{2+} present in medium supplemented with 15 mM citrate, the cells of this strain were revealed to be in a well-separated coccoid cell form, about 0.6 to 0.8 μm in diameter (Fig. 6).

Ovoid cells of the typical taxonomic ATCC strain, S. cremoris and S. lactis, grew in pairs or short chains, regardless of the presence or absence of citrate. Normal cells of S. cremoris had many small protrusions on the cell surface (Fig. 7). However, when citrate was present, cracks were often observed in the cell surface (Fig. 8). The cell surface of S. lactis was smooth, independently of the addition of citrate over a range of concentrations.

To gain further insight, we prepared SEM micrographs of the Cit^- and the Cit^r mutants of strain KSM-1106, grown in the presence of 15 mM citrate. The Cit^- strain, lacking a citrate permease, grew as long chains with a mixture of ovoid and rod-shaped cells (Fig. 9). The chain length of the Cit^r strain, grown in standard medium, was very short (about 2 cells/chain) with ovoid and rod-shaped forms (0.6 by 0.6 to 2.2 μm, Fig. 10). Even when grown with 30 mM citrate, this mutant grew as short chains of cells with well-separated coccoid cell form (about 0.6 μm in diameter, Fig. 11).

We next prepared TEM micrographs of thin sections of chaining cells of the KSM-1106, grown in the presence of 15 mM citrate (Figs. 12, 13 and 14). The micrographs obtained suggest apparently that the organism in long chain has undergone a cell division and/or growth cycle without complete formation of cross wall. In fact, the cross wall was not synthesized completely in some cells in the long chain.

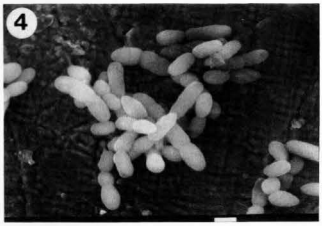


Fig. 4. SEM micrograph of Streptococcus sp. KSM-1106, grown in standard medium. Bar=1.0 μm.

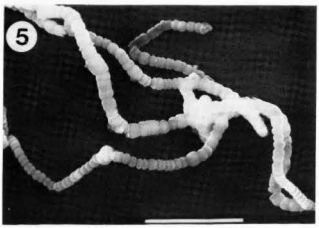


Fig. 5. SEM micrograph of Streptococcus sp. KSM-1106, grown in medium supplemented with 15 mM citrate. Bar=10 μm.

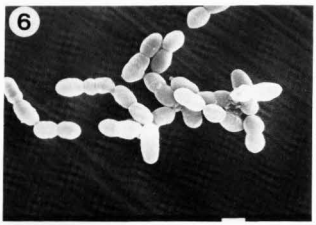


Fig. 6. SEM micrograph of Streptococcus sp. KSM-1106, grown in medium supplemented with 15 mM citrate and 3 mM Mn^{2+}. Bar=1.0 μm.

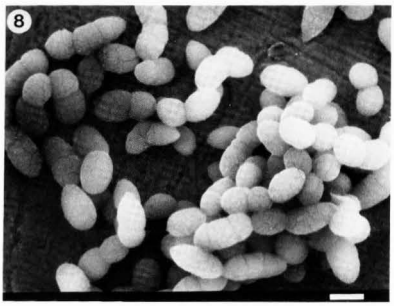


Fig. 7. SEM micrograph of S. cremoris ATCC 19257, grown in standard medium. Bar=1.0 μm.

Fig. 8. SEM micrograph of S. cremoris ATCC 19257, grown in medium supplemented with 15 mM citrate. Bar=1.0 μm.

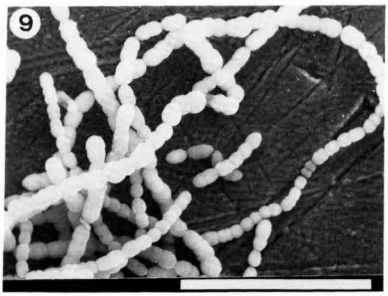
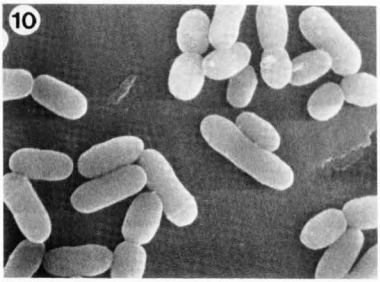
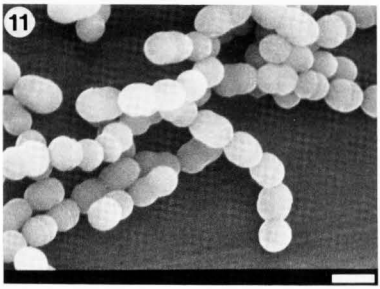
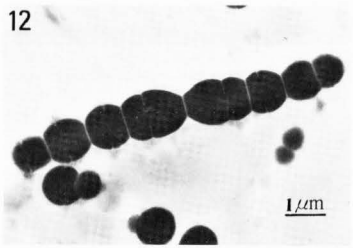
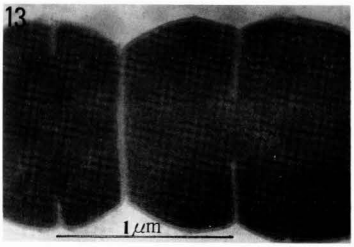


Fig.9. SEM micrograph of the mutant KSM-1106C⁻, grown in medium supplemented with 15 mM citrate. Bar=10 μm.









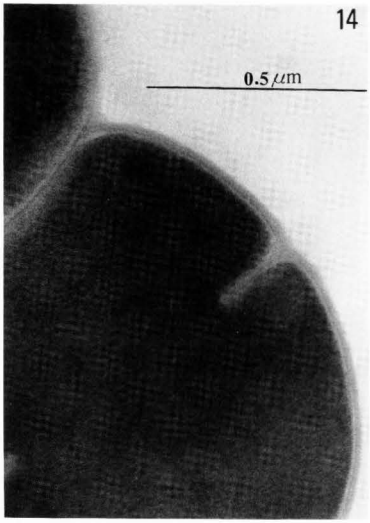


Fig. 10. SEM micrograph of the mutant KSM-1106Cr, grown in standard medium. Bar=1.0 μm.

Fig. 11. SEM micrograph of the resistant mutant cells, grown in medium supplemented with 30 mM citrate. Bar=1.0 μm.

Figs. 12 - 14. TEM micrographs of thin sections of Streptococcus sp. KSM-1106 grown in medium supplemented with 15 mM citrate.

Discussion

SEM and TEM are useful techniques for studying the microstructure of a variety of microorganisms including a group of prokaryotes having no distinct organelle. Experimentally we used WET-SEM for morphological studies of streptococci. This technique is not useful at present for making comparisons of the microstructural appearance of bacterial specimens but can be used to obtain much information about the wet appearance of specimens, free from drying artifacts.

Morphology of bacterial cells has been reported to be changeable under different environmental conditions such as pH (Rhee & Pack, 1980) and temperature (Goel & Marth, 1969). The streptococci characteristically tend to grow as pairs or short chains, but sometimes they grow in chains of variable length, dependent, for instance, on the strain (McDonald, 1971; Shaikh & Steward-Tull, 1975), addition of immune antisera (Ekstedt & Stollerman, 1960) or contact with suramin, a lysozyme inhibitor (Lominski & Gray, 1961). Whitehead and Hunter (1949)

observed so-called "involution" forms of S. cremoris, on cultivation at 37°C, which had very long chains of flattened cocci. McDonald (1971) also isolated long chains of S. cremoris and S. lactis from growths in the orifices of chemostat medium inlet tubes.

In this paper, we have reported that citrate induces the formation of long chains of cells of group N streptococci. The observations obtained with the SEM may be interpreted to indicate that the formation of long chains occurs as the result of a failure of cells to separate after division and not of a failure of cross wall formation. It also raises a possibility that citrate can stimulate initiation of cell division at multiple sites, resulting in greater fragility of cells in long chains. However, the TEM micrographs of thin sections of chains of cells of the KSM-1106 strain showed that the cross walls were not synthesized completely in several cells in the chain. This result alternatively suggests that citrate inhibits an intermediary step between cross wall formation and cell separation but not the final step of cell separation. As our results have been obtained from cells during an unbalanced growth, it is inadequate at present to elucidate the details by which some strains of group N streptococci grew as long chains in the presence of citrate.

In medium supplemented with citrate, a Cit^r mutant, capable of growing at high concentrations of citrate, grew as very short chains, but Cit^-, a mutant missing a citrate permease, grew as very long chains. These results suggest that citrate must be a specific trigger for the growth of long chains of cocci, but this abnormal morphology may be unrelated to their ability to metabolize citrate. Inhibition of the formation of long chains by divalent cations further suggests that these cations are essential for normal growth as short chains. We hypothesized that the cations in the growth medium might be sequestered by citrate and, in fact, the separation of bacterial cells has been reported to require divalent cations (Webb, 1949; Kojima et al., 1970). Especially, McDonald (1957) has demonstrated that lactic streptococci require divalent cations for growth in the presence of citrate. However, the mechanism of the effect of citrate on chain length cannot readily be explained by its chelating of the essential divalent cations since strong chelating agents, such as EDTA, STPP and Zeolite, exerted no effect on the morphology or chain length of the cells.

Involvement of an enzyme or a set of enzymes (autolysin(s)) responsible for division and separation (dechaining) of streptococcal cells has been studied (Lominski et al., 1968; Shockman et al., 1967; Higgins et al., 1970). Fein and Rogers (1976) presented direct evidence for the mechanism of the formation of long chains of bacterial cells, using an autolysin-deficient strain of Bacillus subtilis. Therefore, the formation of long chains of group N streptococci may be related to inhibition of the dechaining enzyme by citrate. Inhibition of dechaining activities has frequently been reported to induce the formation of long chains of some streptococci (for instance, Soper & Winter (1973)) and other organisms (Chatterjee et al., 1969; Tomasz, 1968; Fan, 1970). Recently, we found a dechaining activity in cell free extract of Streptococcus sp. KSM-1106 (Ito et al., 1984). The dechaining enzyme which was extracted from the parent was inhibited by citrate, but the enzyme obtained from the Cit^r mutant was not affected by citrate. It seems likely that such an enzyme in the Cit^r mutant is one that has been genetically altered to have a lower affinity for citrate. These preliminary results might tend to eliminate the possibility that citrate stimulated initiation of cell division at multiple sites of the test strain, as described earlier in Discussion. We suggest that this dechaining enzyme may have a specific binding or affinity site for citrate and, hence, the essential role of divalent cations in normal growth might be nullified in some way, with the net result that long chains of cells are generated. Recently, we have obtained the data which suggest that the dechaining enzyme of Streptococcus sp. KSM-1106 locates on the outer membrane or in the periplasmic space of the cells, using spheroplasts of the parent and the Cit^- strains. We are now engaged in attempts to purify this enzyme in order to compare it with the previously reported autolysins with respect to catalytic nature and physiological meaning involved in cell division system.

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Discussion with Reviewers

R.A. Holley: Could the surface cracks of S. cremoris be artifacts of preparation?
Authors: It is not clear whether these cracks were artifacts or not. However, the cracks of S. cremoris, induced by citrate, were observed in repeated experiments.

I.J. McDonald: Did the pH of citrate-containing media have any effect on chain length of Streptococcus sp. KSM-1106?
Authors: Any effect was not observed at pH values between 5.0 and 7.5. At these pH values, our strain occurred as long chains in the presence of 30 mM citrate.