Bicarbonate and Potassium Regulation of the Shape of Streptococcus mutans NCTC 10449S

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Morphological changes of S. mutans NCTC 10449S associated with growth in modified Jordan medium and FMC medium (Terlecki et al., Infect. Immun. 11:649–655, 1975) were studied by scanning electron microscopy. The cells were bacillary in Jordan medium, but coccoid and of unequal size in FMC. Transfer of the cells from Jordan medium to FMC and vice versa reversed their shapes, as did salt exchange between these media. Morphological changes could not be ascribed to either medium pH, concentration of P, or Na⁺/K⁺ ratio. However, they were growth dependent, since the changes did not occur when the cells were suspended in salt components alone or in media supplemented with protein synthesis inhibitors. Only a high bicarbonate/K⁺ ratio, as in FMC, produced spherical cells, whereas cells remained bacillary in medium with a low bicarbonate/K⁺ ratio, as in Jordan medium. Manipulating this ratio in other media resulted in similar shape changes. Thus, the shape of S. mutans 10449S can be dictated by the ratio of bicarbonate to K⁺ in the growth medium.

Extensive evidence implicates Streptococcus mutans as the prime causative agent in dental caries in experimental animals and humans (4, 5, 9, 13, 18, 20). This microorganism was first described in 1924 by Clark (2), who named it “mutans” because he thought it a mutant of other streptococci due to its commonly observed bacillary form. The determinants of this bacillary shape have not been described, although many have recognized it (3, 6).

In the course of other studies of this microorganism in our laboratory we observed that its shape in a complex medium was that of a short rod, but in a defined medium its shape was coccoid. In this paper we describe studies which have revealed that the morphology of the typical S. mutans strain, NCTC 10449S, is a reflection of the ratio of bicarbonate to K⁺ ions in the culture medium.

MATERIALS AND METHODS

Microorganism. S. mutans NCTC 10449S (6, 19) was maintained frozen in a modified fluid thioglycollate medium (19). For experiments, it was adapted to various media as described below.

Growth media. The complex medium of Jordan et al. (10) modified to contain 50 mg of Na₂CO₃ per liter (JM) (19), Todd-Hewitt broth (TH; BBL Microbiology Systems, Cockeysville, Md.), brain heart infusion broth (BHI; BBL), and the defined medium designated FMC (21) were used, all supplemented with glucose to 10.0%. In addition, media were further modified by exchange, addition, or deletion of ingredients as detailed below with respect to individual experiments.

Morphological studies. Cells were examined by Gram stain with heat-fixed smears, by Nomarski interference microscopy (model BH; Olympus Optical Co., Tokyo, Japan) with unfixed smears, and by scanning electron microscopy with cells fixed in 2% glutaraldehyde in 100 mM cacodylate buffer (pH 7.2). For scanning electron microscopy, 5 ml of culture was harvested by centrifugation, and the cell pellet was suspended in 1 ml of the fixative. A drop of cell suspension fixed for 2 h at 4°C was then placed on a 13-mm filter (0.22-μm pore diameter; Millipore Corp., Bedford, Mass.) and incubated for 5 min at room temperature. The filter was washed three times with the buffer and then dehydrated stepwise in 50, 70, 90, and 100% ethanol for 15 min each. The filters with adherent organisms were then critical point dried in liquid CO₂ in a DCP-1 critical point drying apparatus (Denton High Vacuum, Cherry Hill, N.J.), mounted on stubs, and coated with gold to a thickness of 5 to 10 nm. Specimens were examined and photographed with a Hitachi H300 electron microscope with H3010 scanning attachment (Hitachi Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV.

Growth protocols. For most experiments bacteria were grown anaerobically with alkaline pyrogallol seals (8) in media adjusted to pH 7.2. Three adaptive transfers in any medium were made before study of cell morphology. Samples were studied during the early log, midlog, and stationary phases and after prolonged maintenance in the stationary phase (more than 24 h). pH was measured before harvesting cells for morphological evaluation, and growth rate (optical density at 600 nm) was followed turbidimetrically with a Bausch & Lomb model 710 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). In addition, experiments were conducted in a pH stat, maintaining pH at 7.0. The pH stat consisted of a pH meter (PHM 82), automatic titrator (TTT 80), and autoburette (ABU 80) (all from Radiometer, Copenhagen, Denmark) and a spinner flask (Bellco, Vineland, N.J.). For all experiments, culture purity was checked by gram stain and by plating on mitis salivarius agar to detect the typical colony shape of strain 10449S and on blood agar incubated both aerobically and anaerobically to detect extraneous contaminants.

Chemical analyses. Growth media were analyzed for Na⁺ and K⁺ with ion-specific electrode method as suggested by
FIG. 1. Scanning electron micrographs of S. mutans 10449S, which grow (a) as rods in JM, (b) as cocci in FMC, (c) as cocci in JM with salts composition of FMC, and (d) as rods in FMC with salts composition of JM. The background is Millipore filter.

the manufacturer (Beckman Instruments, Inc., Fullerton, Calif.) and for bicarbonate by the phosphoenolpyruvate carboxylase-malate dehydrogenase, NADH₂-coupled reaction (optical density monitored at 340 nm) (15).

RESULTS AND DISCUSSION

Strain 10449S grew with typical bacillary form in JM (Fig. 1a) and exhibited coccoid form in FMC medium (Fig. 1b) (Tables 1 and 2). Shapes did not appreciably change in stationary-phase cultures compared with exponential-phase cultures. These observations were independent of whether cells were grown at a constant pH (7.0) in a pH stat or without pH control, allowing culture pH to fall in response to fermentation. In addition, cells grown in JM (rods) were thinner than those in FMC but, nonetheless, longer. There was some irregularity in the diameter of the cocci in FMC. There was no appreciable difference in growth rate between the two media, with doubling times of about 55 to 60 min.

Initial studies demonstrated that these shape changes could be reversed upon inoculating FMC-grown cells into JM and vice versa. The time required for detection of partial reversal of culture morphology was about four generations (about 4 h). This morphological reversal was complete, as documented by scanning electron microscopy observations, after two successive culture transfers in either medium.

To identify the critical determinants of cell morphology, experiments were conducted in which major ingredients of two media were exchanged. Thus, the vitamin mixture of FMC was exchanged for the yeast extract of JM, the amino acid mixture of FMC was exchanged for the trypticase of JM, or the salts (including buffer electrolytes) of FMC were exchanged for those of JM. These exchange-modified media were then inoculated with log-phase 10449S growing in either normally constituted FMC or JM. Only in the case of salts exchange was the shape change of 10449S reversed (Fig. 1c and d).

To identify the specific components of the salts determining bacillary or coccoid shape, a series of formulations of JM was made (Table 1). Only in the case of simultaneous reduction of K⁺ content and elevation of bicarbonate (added as Na₂CO₃) did cell morphology become coccoid (Fig. 2a).
Notably, deletion of one or more of the other salt ingredients of the typically high-K⁺, low-bicarbonate JM formulation had no effect on the bacillary shape of 10449S. Similarly, change of the Na⁺/K⁺ ratio from 1/20 to 20/1 had no effect on cell shape. Also, inclusion of sodium citrate and sodium acetate at the levels found in FMC was without effect, as was the substitution of NaCl for Na₂CO₃. Notably, reduction of the K⁺ content of the medium without change in the Na₂CO₃ had no effect on the typical bacillary shape of the cells.

JM and FMC have bicarbonate/K⁺ ratios of 0.008 and 2.84, resulting in growth of bacillary and coccoid cells, respectively (Table 2). To test whether this was a general phenomenon, we studied 10449S morphology in BHI and TH broths, both unmodified and modified to have altered compositions with respect to K⁺ and bicarbonate. BHI has a bicarbonate/K⁺ ratio of 0.114 and yields bacillary growth of 10449S. However, altering the bicarbonate/K⁺ ratio to 5.45 resulted in the growth of coccoid cells. By contrast, studies in TH broth, which already contains 2.5 g of Na₂CO₃ per liter but which has a higher K⁺ content than BHI, yielded growth of a mixture of bacillary and coccoid forms (Fig. 2b). The ratio of bicarbonate to K⁺ in this medium was 2.40. However, the addition of K⁺ as 5 g of K₂HPO₄ per liter (as in JM) changed the bicarbonate/K⁺ ratio to 0.36 and the cells to

TABLE 1. Morphological variation of S. mutans 10449S grown in JM modified with different salt combinations and 1% glucose (pH 7.2)

<table>
<thead>
<tr>
<th>Salt combinations (meq/liter of medium)</th>
<th>Avg length/width</th>
</tr>
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<tbody>
<tr>
<td>Selected salts</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄ (57.4) + Na₂CO₃ (1) + JSS*</td>
<td>2.4</td>
</tr>
<tr>
<td>K₂HPO₄ (57.4) + Na₂CO₃ (1)</td>
<td>2.4</td>
</tr>
<tr>
<td>K₂HPO₄ (57.4) + JSS</td>
<td>2.4</td>
</tr>
<tr>
<td>K₂HPO₄ (57.4)</td>
<td>2.4</td>
</tr>
<tr>
<td>Varied Na/K ratio</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄ (60) + Na₂HPO₄ (3) (Na/K, 1:20)</td>
<td>2.4</td>
</tr>
<tr>
<td>K₂HPO₄ (30) + Na₂HPO₄ (30) (Na/K, 1:1)</td>
<td>2.4</td>
</tr>
<tr>
<td>K₂HPO₄ (3) + Na₂HPO₄ (60) (Na/K, 20:1)</td>
<td>2.4</td>
</tr>
<tr>
<td>Varied anions (Na/K ratio of 20)</td>
<td></td>
</tr>
<tr>
<td>K₂HPO₄ (3) + Na₂CO₃ (60)</td>
<td>1.0*</td>
</tr>
<tr>
<td>K₂HPO₄ (3) + NaCl (60)</td>
<td>2.4</td>
</tr>
<tr>
<td>K₂HPO₄ (3.8) + sodium citrate (2.8) + sodium acetate (73.2)</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Jordan salt solution: 0.5 meq of MgSO₄, 0.1 meq of MnSO₄, and 0.025 meq of FeSO₄ per liter.
* Length, 1.2 μm; width, 0.5 μm.
* Length or width: 0.6 - 1.0 μm.
baccillary shape (Fig. 2c). By contrast, the addition of 1.8 g of NaHCO₃ per liter (as in FMC) changed the bicarbonate/K⁺ ratio to 4.50 and the cells to coccoid form (Fig. 2d). The absolute amounts of Na⁺, K⁺, phosphate, and bicarbonate in these media varied and did not correlate with cell morphology; only the bicarbonate/K⁺ ratio correlated with cell shape; high ratios were associated with coccoid forms, and low ratios were associated with baccillary forms.

When cells were washed in buffers containing the bicarbonate/K⁺ ratio and content typical of either JM or FMC, they did not change shape from that which resulted when bacteria were grown in either of those media. In addition, when cells were inoculated into JM or FMC to which growth-inhibiting quantities of actinomycin D and puromycin had been added, neither growth nor change of cell shape occurred.

There are no other data pertaining to the regulation of streptococcal shape. However, variations in bacterial shape have been studied for members of the gram-positive genera Arthrobacter and Bacillus (16). Arthrobacter species change from rods to spheres, and vice versa, under conditions of varied nutrition (7) or growth rate (14). The polysaccharide backbones of the cell wall peptidoglycans of spherical Arthrobacter crystallopoietes were reported to be shorter than those of the rods of the same cells (12), but cell wall autolytic activities were higher in the cocci (11). Studies of rod mutants of Bacillus sp. did not confirm this relationship. One mutant type of Bacillus subtilis, rodA, is bacillar at 30°C but coccoidal at 45°C and is distinguished by derepression of peptidoglycan synthesis, inhibition of teichoic acid synthesis, and loss of autolysins when bacteria are grown at the higher temperature (1). Another mutant, rodB, evidences none of these biochemical changes but, unlike rodA, is affected in its shape and growth rate by the concentration in the growth medium of Mg²⁺ and certain anions, especially Cl⁻, as well as by temperature (10). Other types of B. subtilis Rod⁺ mutants have been reported (16).

The exact mechanisms of morphological variation among these A. crystallopoietes and Rod⁺ mutants of B. subtilis are still obscure. However, it is possible that there exist different mechanisms for different bacteria. The relationship between these data from diverse genera to the present data from S. mutans 10449S is unclear.

The sphere-rod change of S. mutans NCTC 10449S offers a new and different model system for studies of bacterial morphogenesis in which the bicarbonate/K⁺ ratio appears to participate in the determination of cell shape.

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**LITERATURE CITED**

