Influence of Incubation Atmosphere on Growth and Amino Acid Requirements of \textit{Streptococcus mutans}

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The growth response of \textit{Streptococcus mutans} representing antigenic type \textit{a} or \textit{d} in a chemically defined medium was influenced by the oxygen concentration of the growth atmosphere. Under controlled aerobic (1.5% \textit{O}_2) conditions these cultures attained a greater density than when the atmosphere contained 0.006% \textit{O}_2 or less. The growth of \textit{S. mutans} strains representing antigenic types \textit{b} or \textit{c} in the defined medium was independent of the oxygen concentration of the growth environment. Under the conditions used in this study, none of the strains tested could utilize ammonium ion as a sole source of nitrogen for growth. The requirement for certain amino acids and inhibition by other amino acids varied with antigenic type and relative oxygen concentration of the growth environment. Under conditions where the atmospheric oxygen was reduced to 0.0006% \textit{O}_2 or less, the amino acid requirements of the cultures became either more numerous or more stringent. \textit{S. mutans} strains of type \textit{c} generally required the least number of amino acids, whereas cultures of type \textit{d} had more numerous requirements. Nearly every culture tested under the anaerobic atmosphere was inhibited by one of the branched-chain amino acids, leucine, valine, or isoleucine. Methionine and lysine were also found to be inhibitory, particularly toward the type \textit{c} strains.

\textit{Streptococcus mutans} has been reported to have more complex amino acid requirements under aerobic culture than under anaerobic conditions of growth. Carlsson (7) reported that \textit{S. mutans} representing antigenic type \textit{c} but not antigenic types \textit{a} or \textit{b} could grow under anaerobic conditions in a minimal medium containing only ammonium sulfate, cysteine, glucose, required vitamins, magnesium sulfate, and phosphate buffer. Under aerobic conditions, none of the cultures tested (six type \textit{c} strains, one type \textit{a} strain, one type \textit{b}) could grow in this medium unless it was supplemented with uracil and either glutamic acid, glutamine, aspartic acid, or asparagine. Lawson (10), noted that seven strains of \textit{S. mutans} could grow anaerobically in a chemically defined medium containing 13 amino acids. Aerobic growth by four strains tested required, in addition to the 13 amino acids, either aspartic acid alone or aspartic acid in combination with either alanine, proline, or threonine. In this study, specific amino acid requirements of the cultures were not determined. In neither of these studies were data presented indicating what constituted aerobic growth conditions.

In preliminary experiments it was found that a number of strains of \textit{S. mutans} representing antigenic types \textit{a}, \textit{b}, \textit{c}, and \textit{d} in our collection failed to grow in the minimal ammonium salts medium described by Carlsson (7), under anaerobic conditions. The present study was therefore undertaken to determine if the specific amino acid requirements were different for antigenic type \textit{a}, \textit{b}, \textit{c}, and \textit{d} of \textit{S. mutans}, and whether these requirements were influenced by the atmosphere of growth.

**MATERIALS AND METHODS**

\textit{S. mutans} strains were selected to represent antigenic types \textit{a}, \textit{b}, \textit{c}, and \textit{d} designated by Bratthall (3). Type \textit{a} strains were AHT-12, 3720, and OMZ-61. Type \textit{b} cultures were FA-1, Q10-5, Q1-7, and BHT-2; whereas type \textit{c} cultures included GS-5, VA-29, VA-29A, VA-29R, PR89-2, and PR25-2. Cultures SL-1, K1-R and sub-strains of K1-R (6715, 6715-19, 6715-49, 6715-58, and 6715-60, all derived from passage in hamsters) which cross-react with AHT antiserum have recently been designated by Bratthall (4) as members of antigenic type \textit{d}. The antigenic type was verified by fluorescence microscopy by using fluorescein-labeled antisera to strain AHT for antigenic type \textit{a}, BHT for antigenic type \textit{b}, and GS-5 for...
antigenic type C according to the procedures of Zinner and Jablon (18).

**Maintenance medium.** The cultures were routinely propagated in Trypticase-glucose broth containing per liter; Trypticase (BBL), 20 g; glucose, 10 g; sodium carbonate, 0.5 g; yeast extract, 4.0 g. These ingredients were solubilized in 200 ml of deionized water and the pH was adjusted to 7.0. The final volume was brought to 1 liter with 0.1 M sodium phosphate buffer, pH 7.0. The medium was dispensed in 7.0-ml quantities and autoclaved for 5.0 min at 121 C. Prior to use, 0.1 ml of filter-sterilized freshly prepared cysteine-hydrochloride, pH 7.0, (5.0 mg of stock per ml) was added to each tube. Unless otherwise stated, the inoculated medium was incubated for 15 h at 35 C under an atmosphere containing 90% N2-10% CO2. Stock cultures were transferred twice weekly into the Trypticase-glucose broth containing 0.5% calcium carbonate, and between transfers the cultures were stored at 5 C.

**Reference defined medium.** A chemically defined medium described by Reiter and Oram (14) for the growth of lactic streptococci was modified to serve as the experimental reference growth medium in this study. The medium contained glucose instead of lactose, and di-ammonium hydrogen carbonate, guanine, folic acid, and vanadium sulfate were omitted. A 1,000-fold concentrated stock solution of the vitamins in the medium was prepared and sterilized by filtration through a 0.2-μm Nalgene membrane-filter apparatus. Between uses, this vitamin solution was stored at 5 C. A fresh stock solution was prepared every 3 weeks. Minerals were prepared as a stock solution as described by Reiter and Oram (14), and after adjustment to pH 7.0, they were autoclaved 5.0 min and stored at 5 C.

The ingredients of the basal portion of the medium excepting the vitamins and minerals were solubilized in 200 ml of distilled water, and the pH was adjusted to 7.0. A double-strength solution of these constituents was prepared by adjusting the volume to 489 ml, after which the solution was autoclaved for 5.0 min at 121 C. After cooling, 1 ml of stock vitamin solution, 10 ml of stock mineral solution, and 500 ml of an amino acid mixture (L-forms) were added to give the completed chemically defined reference medium. The resulting medium prepared daily was dispensed in 5.0-ml quantities into sterile screw-capped test tubes.

The amino acids and the concentration of each (mg/100 ml of medium) in the defined reference medium are given in Table 1. The concentration of each amino acid was based on the free amino acid content of milk (9,14), the major protein source of experimental animal diets used in carries research (12). The purity of each amino acid was verified by using a Beckman 118-H amino acid analyzer. The amino acid mixtures were preweighed and kept in sealed vials, and when used the mixtures were solubilized in 300 ml of deionized water, the solution was adjusted to pH 7.0 with 0.1 N NaOH, and the volume was adjusted to 500 ml. This amino acid solution was autoclaved for 5.0 min at 121 C, and when cooled was combined with the basal constituents of the medium.

**Minimal medium.** The minimal medium of Carls-

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**Table 1. Amino acid composition of chemically defined medium I and group deletion media VII and VIII**

| Amino acid | Concn (mg/100 ml) | I | VII | VIII |
|------------|------------------|---|-----|-----|
| Glutamic   | 320              | + | -   | +   |
| Histidine  | 7                | + | -   | +   |
| Methionine | 7                | + | +   | +   |
| Phenylalanine | 10         | + | -   | +   |
| Proline    | 54               | + | -   | +   |
| Aspartic   | 150              | + | +   | +   |
| Arginine   | 45               | + | -   | +   |
| Lysine     | 81               | + | +   | +   |
| Tryptophan | 10               | + | -   | +   |
| Tyrosine   | 4                | + | -   | +   |
| Cysteine   | 50               | + | +   | +   |
| Valine     | 15               | + | -   | +   |
| Leucine    | 10               | + | -   | +   |
| Isoleucine | 10               | + | -   | +   |

* A + indicates medium contains the designated amino acid at the concentration listed; a - indicates medium does not contain the designated amino acid.
reference-defined medium were similar under either the 70% N₂-10% CO₂-20% H₂ or 90% N₂-10% CO₂ (0.006% O₂) atmosphere.

In this study, for comparative purposes only, the 90% N₂-10% CO₂ (1.5% O₂) system was designated a “controlled aerobic” atmosphere; whereas the 70% N₂-10% CO₂-20% H₂ or 90% N₂-10% CO₂ (0.006% O₂) atmospheres were considered anaerobic.

**Effect of atmosphere on growth.** Initially, 12 daily sequential transfers into fresh reference-defined medium were made to verify that the medium satisfied the nutritional requirements of each strain. The influence of the atmosphere of incubation on the growth response of the cultures was then tested. Reference-defined medium (5-ml amounts) were inoculated with 1 drop of a 15-h Trypticase-glucose broth culture and were incubated for 24 h at 35 C under the 90% N₂-10% CO₂ (1.5% O₂) or 70% N₂-10% CO₂-20% H₂ atmosphere. For experimental comparisons the cultures were routinely transferred three times into the reference medium. After the third transfer, the growth density (absorbance at 660 nm, 1.0-cm light path) attained by each culture in 24 h under each atmosphere was recorded. In repetitive comparisons the growth density of a given culture after the third transfer did not vary by more than 10%.

**Amino acid requirements.** All glassware used in the nutritional aspects of this study was acid cleaned. Media used in the determination of amino acid requirements were inoculated and incubated as described for the growth studies in the reference-defined medium except cultures transferred once into the reference medium served as the source of inoculum. Different versions of the reference medium were formulated to eliminate selected groups of amino acids as an aid in later determination of specific requirements. The composition of the group-deletion media applicable to this study is given in Table 1. After three consecutive transfers into each group-deletion medium and the reference-defined medium, a given deletion medium was considered to satisfy the growth requirements of a culture if a growth response of 75% or better was obtained relative to the reference medium.

The specific amino acid requirements of each strain were established by single or multiple omission of amino acids from the simplest group-deletion medium which supported satisfactory growth of that culture. When a culture failed to grow in an omission medium in the first 24 h, it was reincubated an additional 24 h to verify that the amino acid omitted was essential. All tests were repeated three times with comparable results. In each test, the culture was routinely transferred sequentially three times into each omission medium. (Periodically, a culture was transferred in an omission medium at least 12 times as verification that the omitted amino acid was not required.) After the third transfer, the growth response was expressed as a percentage of that obtained in the reference medium. Based on this value the omitted amino acid was designated: (i) essential, growth 50% or less of the reference medium after 48 h; (ii) non-essential, growth 75 to 125% of the reference; (iii) stimulatory, growth 55 to 74% of reference or, (iv) inhibitory, growth in excess of 127% of reference.

**RESULTS**

The growth response of *S. mutans* strains representing type a or d in the reference-defined medium was influenced by the incubation atmosphere (Fig. 1). The observed disparity of growth of these cultures was maintained even after 48 h of incubation. Inoculum size was apparently not a contributory factor to the disparity, since media inoculated with the same numbers of viable cells continued to exhibit less growth under the 70% N₂-10% CO₂-20% H₂ atmosphere.

Selected cultures were then grown in the reference medium with incubation under 90% N₂-10% CO₂ (1.5% O₂), 90% N₂-10% CO₂ (0.006% O₂), or the 70% N₂-10% CO₂-20% H₂ atmospheres. Growth of cultures K1-R, 6715, and 6715-49 under the controlled aerobic atmosphere was considerably better than that observed under either of the anaerobic atmospheres (Fig. 2). The growth responses of these cultures under the two anaerobic atmospheres were similar. Conversely, the growth response of strains FA-1 (type b) or VA-29R (type c) was not greatly different under the three incubation atmospheres.

Although the incubation atmosphere did not affect the growth of the type b or c strains of *S. mutans*, two of the type b cultures and all of the type c strains exhibited limited growth in the reference medium. Since repetitive transfers of these cultures in this medium have consistently given growth responses comparable to those presented in Fig. 1, the limited growth of the cultures could be due either to nutrient limitation or inhibition by one or more of the medium constituents.

**Growth in minimal medium.** *S. mutans* has been reported (7) to utilize ammonium salts as a nitrogen source for growth under anaerobic conditions. However, none of our cultures grew in filter-sterilized minimal medium of Carlsson (7) under the anaerobic conditions used in this study. The cultures were then inoculated into minimal medium steam heated according to Carlsson (7) and incubated under 70% N₂-10% CO₂-20% H₂. Strains AHT-12 (type a), VA-29 (type c), VA-29A (type c), VA-29R (type c), PR89-2 (type c), 6715 (type d), and 6715-58 (type d) attained very limited growth in the second transfer into the heated medium after 4 days of incubation. Transferable growth was not obtained after a third passage into the medium. These observations indicated that many of the cultures apparently required amino acids for growth and, therefore, the specific amino acid requirements were determined.

**Amino acid requirements of antigenic type**
AMINO ACID REQUIREMENTS OF S. MUTANS

FIG. 1. Influence of atmosphere on growth of S. mutans representing antigenic type a, b, c, and d in reference defined medium after 24 h at 35 C. Cross-hatched bars represent growth under 90% N₂-10% CO₂ (1.5% O₂); open bars represent growth under 70% N₂-10% CO₂-20% H₂.

FIG. 2. Comparative growth of selected strains of S. mutans in reference defined medium. Cultures were incubated for 24 h at 35 C under A, 90% N₂-10% CO₂ (1.5% O₂); B, 70% N₂-10% CO₂-20% H₂; C, 90% N₂-10% CO₂ (0.0006% O₂). T, standard deviation based on five replicate experiments.

The amino acid requirements of the antigenic type a cultures were determined by using group deletion medium VIII (Table 1). Under the controlled aerobic atmosphere the amino acid requirements ranged from very simple for strain 3720 to relatively complex for strain OMZ-61 (Fig. 3). All of the cultures required cysteine, and this requirement could not be replaced by dithiothreitol. Two of the three cultures also required aspartic acid.

Aspartic acid and cysteine were required by all three cultures for growth under anaerobic conditions. Strain AHT-12 which had also required histidine and methionine under the more aerobic atmosphere, required neither of these amino acids for anaerobic growth.

Better growth of the cultures occurred in medium VIII than in the reference medium, particularly under the 70% N₂-10% CO₂-20% H₂ atmosphere. Since the two media differed only in the absence or presence of valine, leucine, and isoleucine, it was possible that one or more of these amino acids was inhibitory. Isoleucine was found to be particularly inhibitory to the cultures under anaerobic conditions. Strain OMZ-61 was also inhibited by leucine under both atmospheric conditions.

FIG. 3. Effect of amino acid deletions on the growth of S. mutans antigenic type a under A, 90% N₂-10% CO₂ (1.5% O₂); or B, 70% N₂-10% CO₂-20% H₂. Symbols: ■, amino acid essential; ●, amino acid stimulatory; ★, amino acid inhibitory; □, amino acid non-essential.
**Amino acid requirements of antigenic type**

**b.** All of the cultures grew in group deletion medium VIII, but strains Q1-7 and Q10-5 also maintained satisfactory growth in the less complex group deletion medium VII (Table 1). The amino acid requirements of these two strains were determined by making omissions from medium VII. Under the "controlled aerobic" atmosphere, aspartic acid and cysteine were required by both cultures (Fig. 4). Strain Q10-5 also required either lysine or methionine, but not both. Glutamic acid could replace the requirement for aspartic acid in both cultures, but omission of both amino acids resulted in failure of the cultures to grow. The two cultures had an absolute requirement for aspartic acid and cysteine for growth under anaerobic conditions. Also, under the anaerobic atmosphere both cultures were inhibited by either lysine of methionine.

Single or multiple omissions from group deletion medium VIII were made in ascertaining the requirements for strains FA-1 and BHT-2. Regardless of the incubation atmosphere, both strains required cysteine and either aspartic acid, glutamic acid, or proline (Fig. 4). Only when all three of these amino acids were omitted did the cultures fail to grow. Strain BHT-2 also required arginine under both growth conditions, whereas strain FA-1 also required this amino acid only for anaerobic growth. Strain FA-1 also required lysine for growth under both atmospheres. Valine was inhibitory to both cultures under the controlled aerobic but not under the anaerobic atmosphere.

**Amino acid requirements for antigenic type**

**c.** Under the controlled aerobic atmosphere all six strains tested grew satisfactorily in group-deletion medium VII. In this environment all of the strains required cysteine and either aspartic or glutamic acid, except strain VA-29 which had an absolute requirement for aspartic acid (Fig. 5). Most of the cultures were inhibited by the branched-chain amino acids and/or methionine.

Under anaerobic conditions no additional amino acids were required for growth, but only in strain VA-29A could glutamic acid substitute for aspartic acid. The branched-chain amino acids and/or methionine and lysine generally inhibited growth of these cultures under the anaerobic atmosphere.

**Amino acid requirements of antigenic type**

**d.** Cultures of this antigenic type grew satisfactorily in group-deletion medium VIII, and single omissions were made from this medium. All of the cultures, under the controlled aerobic atmosphere, required cysteine, glutamic acid, and aspartic acid except strain Kl-R which did not require the latter amino acid (Fig. 6). Variations in additional requirements, if any, were attributed to strain differences. The requirement for cysteine could not be replaced by dithiothreitol.

![Amino Acid Deletion Table](image1)

**ANTIGENIC TYPE b STRAIN TESTED**

Fig. 4. Influence of amino acid deletions on the growth of S. mutans antigenic type b under A, 90% N₂-10% CO₂ (1.5% O₂); or B, 70% N₂-10% CO₂-20% H₂. Symbols: ■, amino acid essential; ■, amino acid stimulatory; ■, amino acid non-essential.

![Amino Acid Deletion Table](image2)

**ANTIGENIC TYPE c STRAIN TESTED**

Fig. 5. Effect of amino acid deletions on the growth of S. mutans antigenic type c under A, 90% N₂-10% CO₂ (1.5% O₂); or B, 70% N₂-10% CO₂-20% H₂. Symbols: ■, amino acid essential; ■, amino acid stimulatory; ■, amino acid inhibitory; ■, any one amino acid required; ■, either amino acid required, but not both; ■, amino acid non-essential.
Under anaerobic growth conditions, all of the cultures required at least one additional amino acid not required under the more aerobic condition. Strain 6715 required five additional amino acids.

All of these cultures grew better in deletion medium VIII than in the reference medium under the anaerobic atmosphere. Under this growth condition, all of the cultures were inhibited by isoleucine, whereas no inhibition by this amino acid under the controlled aerobic atmosphere was observed.

DISCUSSION

Studies by Carlsson (7), and Lawson (10) have indicated that the relative anaerobicity of the growth environment can influence the nutritional requirements of *S. mutans*. In the present study, as well, the growth of the various *S. mutans* serotypes in defined media was influenced by the growth atmosphere. That these were not unrelated phenomena was apparent from the data of the present study which showed that the environmental atmosphere affected not only the pattern of amino acid requirements of the microorganisms, but also their inhibition by amino acids. In the studies of both Carlsson (7) and Lawson (10) the aerobic condition of growth was not defined. However, we have shown that the presence of as little as 1.5% O₂ in the gaseous environment can influence the nutritional requirements of various *S. mutans* cultures.

The apparent oxygen effect on the four serotypes (a, b, c, and d) studied was demonstrably quite different. In the presence of 1.5% O₂ the type a cultures were observed to be more fastidious than under conditions where the oxygen concentration of the environment was 0.0006% or less. Conversely, the type d cultures had more numerous amino acid requirements under conditions in which the oxygen concentration was less than 1.5%. The overall growth of the type b and c cultures in the reference-defined medium was not influenced by the atmospheric oxygen concentrations tested. However, in the presence of 1.5% O₂, 9 of the 10 cultures could utilize either glutamic or aspartic acid for growth, but when the oxygen concentration was reduced to 0.0006% or less, aspartic acid was specifically required by seven of these cultures. The specific effect(s) of oxygen in the environment on the growth and nutritional requirements of the cultures was beyond the scope of this study and is being investigated.

The requirement for aspartic or glutamic acid and other amino acids by the cultures could be correlated with their serological grouping. Aspartic or glutamic acid and cysteine were required by the type c cultures, and in comparison with the other serotypes, this serotype had the simplest requirements. The type b strains differed from type c in that only lysine, methionine, or arginine also was required for growth under aerobic (1.5% O₂) culture. These strains could be differentiated further based on the ability of proline to substitute for the aspartic or glutamic acid requirement. Additional strains are being tested to substantiate such a subdivision of this serotype.

A specific requirement for either glutamic or aspartic acid, or both, under the controlled aerobic atmosphere distinctly characterized the type a or d strains from each other, and from the type b or c strains. The type a cultures required either aspartic or glutamic acid and cysteine. Considerable variation was observed in additional amino acids required by this serotype, but this variation was attributed to strain differences. The type d strains generally represented cultures of *S. mutans* having relatively complex amino acid requirements. All but one of these cultures required both aspartic and glutamic acid under aerobic conditions, whereas both these amino acids were required for growth under the anaerobic atmosphere.

Most other streptococci which have been studied specifically required glutamic acid and not aspartic acid (11, 13-16). Willett et al. (17) found that one strain of *Streptococcus agalactiae* specifically required aspartic acid. *Streptococcus sanguis* (8) has been observed to require glutamic acid, cysteine, valine, leucine,
methionine, and arginine. Smiley et al. (16) reported that Streptococcus salivarius required glutamic acid, leucine, valine, isoleucine, arginine, lysine, and either cysteine or methionine. None of the S. mutans cultures examined in this study required branched-chain amino acids, and only a few required arginine.

Many of the S. mutans strains were, in fact, inhibited by valine, isoleucine, leucine, lysine, or methionine. However, the inhibition of any given strain by any one of these amino acids generally depended on the culture's serotype and occasionally the oxygen concentration of the growth atmosphere. Isoleucine inhibited the greatest number of cultures under our anaerobic growth condition. The branched-chain amino acids as well as lysine and methionine were particularly inhibitory toward the type c cultures. Several of the type d cultures were also inhibited by the branched-chain amino acids under either growth atmosphere, but lysine or methionine did not inhibit any of these cultures. Valine, lysine, and methionine were inhibitory to certain of the type b strains, but only under anaerobic growth.

The presence of such inhibitory amino acids in the reference-defined medium undoubtedly contributed to the limited growth of the type c and affected type b cultures (Q1-7, Q10-5). Also, an inhibitory concentration of isoleucine in the medium could be partially responsible for the lowered growth response of the type a and d cultures under the anaerobic atmosphere. Based on these observations, it appears possible that the inhibition of S. mutans by certain amino acids could influence the ecological status of various serotypes of this organism in the oral environment, and this effect could be modified by the oxygen levels within different locations in the oral cavity.

Similarly, the availability of required amino acids for growth could play a role in the ecology of S. mutans within the oral cavity. The levels of free amino acids, and cysteine in particular, are relatively low in the salivary secretions (2). However, required amino acids could be obtained from salivary proteins (1) and food residues either by the direct action of S. mutans or through the action of other oral microorganisms.

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