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Amino Acid Requirements of *Streptococcus mutans* and Other Oral Streptococci

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The amino acid requirements of *Streptococcus mutans* strains AHT, OMZ-61, FA-1, BHT, GS-5, JC-2, Ingbritt, At6T, OMZ-176, 6715, *Streptococcus salivarius* HHT, *Streptococcus sanguis* OMZ-9, and strain 72x46 were determined in a chemically defined medium. When grown anaerobically in the presence of sodium carbonate (or bicarbonate for a few strains), few amino acids were required. All strains tested required cystine (or cysteine) as a nutrient. Three strains (*S. mutans* OMZ-176, FA-1, and BHT) required glutamate (and/or glutamine). A third amino acid (lysine for *S. mutans* FA-1 and histidine for *S. mutans* OMZ-176) was required by two of the three strains which required glutamate. The amino acids mentioned above were required for all conditions of incubation (and inoculum) tested. The requirements for several other amino acids were conditional, that is, dependent on the incubation conditions and inoculum used. For example, when carbonate was not added, glutamate was required by *S. mutans* GS-5. Aerobic incubations, with carbonate or bicarbonate added, resulted in requirements for glutamate and leucine by several strains. With these incubation conditions, one strain required isoleucine (*S. mutans* FA-1), another valine (*S. mutans* AHT), and a third tyrosine (72x46). Aerobic incubations in the absence of carbonate or bicarbonate further increased the number of amino acids required by several strains. Furthermore, when stationary-phase cultures replaced exponentially growing cultures as an inoculum, several strains required additional amino acids, presumably for the initiation of growth.

Saliva, and the oral environment in general, provides an ecological niche which, in terms of its physicochemical nature, fluctuates rather widely (1, 12). In this environment, many species and strains of streptococci can grow and metabolize, at least from time to time, and thus seem very much able to survive. The ability of these organisms to extract nutrients from their environment is of considerable interest and importance to the ability of at least some of these strains to colonize and to be involved in the etiology of dental caries. Since most oral streptococci are considered to be non-proteolytic (3, 10), and the oral environment is certainly not rich in free amino acids (8), the manner in which these species obtain or synthesize the variety of amino acids which make up proteins is of importance. There have been several investigations of the amino acid requirements of both cariogenic and non-cariogenic streptococci (4-7, 13).

In general, the presence or absence of oxygen (4-7, 13) was found to influence the number of amino acids required. All of these studies agree in that oral streptococci require a smaller assortment of amino acids than do at least some other lactic acid bacteria (16). For example, eight strains of *Streptococcus mutans* were found (4) to be capable of growing aerobically in a medium containing only glucose and cysteine (or thioglycolate) plus vitamins, uracil, ammonium, and sodium acetates and inorganic salts. Since thioglycolate could replace cysteine, and an ammonium salt was required, the organisms presumably could synthesize all of their amino acids from ammonia. This and other investigations of Carlsson (4-6) used prolonged incubation periods of up to 5 days and up to eight serial subcultures. Similarly, Lawson (13) was able to "adapt" seven strains of oral streptococci to grow in a chemically defined medium.

The chemically defined medium (FMC) described in the accompanying paper (18) yielded reproducible growth rates and final cellular turbidities comparable to those attained in a rich organic medium. In fact, with all strains

tested the culture turbidities observed after 8 to 17 h of either aerobic or anaerobic incubation appear to be comparable to those obtained in Carlsson's study (4) after 5-day incubations. Furthermore, upon transfer to FMC from an organic medium, cultures grew rapidly to high turbidities in the absence of a period of adaptation. We have now examined the requirements for individual amino acids of a number of strains of *S. mutans* of Bratthall serotypes *a* through *d*, two Lancefield group H streptococci (*S. sanguis*), and one strain of *S. salivarius* when grown in FMC. Amino acid requirements were examined in the presence and absence of oxygen and in the presence and absence of added sodium carbonate or bicarbonate.

MATERIALS AND METHODS

Ten *S. mutans* strains, representative of serotypes *a*, *b*, *c*, and *d* (2), one strain of *S. salivarius* (HHT), and two Lancefield group H strains, obtained as described in the accompanying paper (18), were examined. All cultures were maintained in a lyophilized state and the number of subcultures away from the lyophilized preparations was kept to a minimum. Strains were monitored for colony morphology on mitis-salivarius agar (BBL) and for fermentation of sorbitol and mannitol.

Media. Lyophilized cultures, grown on Todd-Hewitt agar (BBL) plus 2% glucose, were used to inoculate cultures in the chemically defined medium (FMC; 18). The ability of a number of the strains used in this study to grow rapidly to high turbidities in FMC is described in the accompanying paper (18).

Amino acid requirements were determined by growing each strain in FMC media deficient in a single amino acid. Routinely a rump medium (RM) which lacked several amino acids was prepared in a volume 11/12 that of normal strength FMC. The RM was adjusted with NaOH to pH 6.65 ± 0.01 and sterilized through an 0.45-μm membrane filter (Millipore Corp.), and 5.5-ml samples were aseptically distributed into presterilized and calibrated culture tubes (18 by 150 mm) (16) with stainless-steel tops. All the missing amino acids except one were then aseptically added to the tubes containing the RM. For example, the addition of 0.5 ml of a filter-sterilized solution containing arginine, glutamic acid, glutamine, isoleucine, leucine, and valine to 5.5 ml of RM which lacked these amino acids and cystine yielded 6.0 ml of normal-strength FMC lacking cystine. The same method was used to prepare tubes of FMC which individually lacked each of the other 19 amino acids plus control tubes which contained complete FMC. The final pH of all media was 6.55 ± 0.05.

The method outlined was used to simultaneously examine the growth of one or several strains with FMC deficient in a series of single amino acids, using the same fresh batch of RM and solutions of amino acids.

Throughout this study, L-cysteine, sodium carbonate, and sodium bicarbonate were added to media as freshly prepared solutions which were routinely sterilized by filtration through an 0.45-μm filter in a Millipore Swinnex holder. Routinely, cystine (the oxidized form) was added to growth media rather than the reduced form. Differences between these two forms were not observed (see below).

Growth measurements. Culture turbidities were measured as absorbance and expressed as adjusted optical density (AOD) units (16, 18, 19) so that all measurements are proportional to relative bacterial mass.

Cellular turbidities obtained after 24 h of growth of *S. mutans* strains FA-1, GS-5, and OMZ-176 and *S. salivarius* HHT were quantitated by dry weight determinations. Washed cell suspensions were dried to constant weights over H_2SO_4 and P_2O_5 in vacuo. One AOD unit was found to be equivalent to 0.39 μg (cellular dry weight) per ml.

Growth conditions. Although aerated conditions were not employed, growth of cariogenic streptococci growing in culture tubes (18 by 150 mm) containing 6 ml of a medium (depth of 3.5 cm and a surface area of 2.0 cm^2) was considered to be aerobic. All cultures were incubated at 37.8 ± 0.05 C in a water bath or incubator. Anaerobic growth conditions were initially obtained by placing cultures in an anaerobic glove box. An atmosphere of 5% carbon dioxide, 10% hydrogen, and 85% nitrogen was constantly maintained in this glove box without exposure to oxygen. The oxidation/reduction potential of this system was in the range of −50 to −150 mV. Nevertheless, for convenience and accessibility, the GasPak (BBL) anaerobic system was primarily used in this investigation. An oxidation/reduction potential of at least −50 mV was obtained with the GasPak system which was found to yield results identical to those observed in the anaerobic glove box.

Amino acid studies. Cells from Todd-Hewitt agar were inoculated into a culture tube containing 6 to 10 ml of FMC and incubated overnight (15 to 20 h) at 38 C. Depending on the strain used, the turbidities obtained varied from 1,200 to 3,000 AOD. Between 0.1 to 0.2 ml of these stationary-phase cells was then inoculated into a second tube containing 6 to 10 ml of FMC, to yield an initial AOD of 20 to 60. Growth of this culture was followed turbidimetrically, with readings every 30 min after vigorous agitation with a Vortex mixer. The cultures were grown to 400 to 600 AOD, at which time they had attained a constant and expected exponential growth rate for at least the previous 1.5 doublings in turbidity (18). At this time, the exponential-phase cells were chilled to 0 C, harvested by centrifugation (1,500 × *g*, 15 to 20 min, 4 C), washed, and resuspended in a medium which lacked all the amino acids to be tested. Exactly 0.1 ml of this cell suspension was inoculated into each of two tubes containing FMC deficient in a specific amino acid, and the turbidity of the inoculated tubes was recorded. One tube was then incubated aerobically, whereas the other was placed in the GasPak anaerobic system and incubated in a 37 C incubator. All culture tubes were left undisturbed for 20 to 24 h, after which culture turbidities were determined.

In each case where growth occurred in FMC lacking

one amino acid, 50 μl from this culture was inoculated into a second tube containing medium deficient in the same amino acid. If growth occurred in this subculture after 20 to 24 h of incubation, a second subculture was performed using 50 μl of the first subculture as an inoculum. A reasonable level of growth in the third subculture after 20 to 24 h of incubation indicated that the missing amino acid was not essential for the growth of the specific strain tested.

In each case where growth was not obtained in the initial test, the culture tubes were reincubated for another 24 h. The absence of growth after 40 to 48 h of incubation indicated that the missing amino acid was essential for the growth of the streptococcal strain.

Experiments on the growth of each strain in media lacking each of the 20 amino acids were performed at least three times. Growth in the absence of one amino acid was always compared to that in the presence of all amino acids (control culture). The absence of a growth requirement was indicated by, and defined as, the attainment of at least 40% of the turbidity present in the control culture after 20 to 24 h of incubation. An amino acid required for growth was defined as one that yielded growth to 4% or less of the control culture turbidity at 40 to 48 h. In a few cases, partial growth, with turbidities of 4 to 40% of that of control culture at 20 to 24 h, was observed. In these cases, subcultures and/or reincubations were carried out to determine either the essentiality or the nonessentiality of the missing amino acid.

RESULTS

Amino acids required for aerobic and anaerobic growth of S. mutans strain AHT. Table 1 shows the results of a typical series of experiments with one strain, *S. mutans* AHT. When an exponentially growing inoculum was used, aerobic incubations resulted in the absence of significant levels of growth when arginine, cystine, glutamate, and glutamine, leucine, or valine were omitted from the medium (Table 1A). Either glutamate or glutamine satisfied the requirement for this amino acid for this and the other strains tested. Therefore, in all subsequent experiments, both glutamate and its amide were simultaneously withheld. Omission of any of the other amino acids from the medium did not significantly affect the level of turbidity attained at 20 to 24 h. When cultures were inoculated with exponentially growing cultures and incubated anaerobically, only one amino acid, cystine, was required. Note also that, anaerobically, an "intermediate" growth level was observed in the absence of glutamate and glutamine. This type of "intermediate" response is discussed below. Subcultures of tubes in which growth was observed in the absence of an amino acid uniformly yielded growth to comparable levels, indicating that carry-over of these amino acids with inocula was not an important factor.

When inocula from 20-h stationary-phase cultures were used in place of exponential-phase inocula, requirements for two additional amino acids for aerobic growth, isoleucine and aspartate, were detected (Table 1B). In addition, the growth levels observed after 20 h anaerobically in the absence of glutamate/glutamine or of valine were significantly lower.

Cystine (or cysteine) was required for growth of this and *all* of the strains examined under *all* of the conditions of incubation used. The requirement for cystine appears to be a true nutritional requirement rather than a requirement for a compound which can reduce the oxidation/reduction potential of the medium. First, in contrast to the findings of Carlsson (4), thioglycolate failed to replace cystine. Second, the oxidized, S-S form (cystine) satisfied the growth requirement for all strains, even when stationary-phase inocula were incubated aerobically. Third, the concentration of cystine required for half-maximal growth at 20 h of *S. mutans* FA-1 was only 0.25 μg/ml, and the growth response to increasing concentrations of cystine was linear up to 0.4 μg/ml (S. Mattingly, unpublished data). The concentration of cystine required for half-maximal growth of *S. mutans* FA-1 was very similar to that required by *Leuconostoc mesenteroides* P-60 (16).

Influence of sodium carbonate on the amino acid requirements of S. mutans AHT. When Na_2CO_3 (0.019 M) was added to growth media, *S. mutans* AHT no longer required arginine for aerobic growth and, in tubes lacking other amino acids (e.g., aspartate and isoleucine), growth levels at 20 h were similar to the growth level in control tubes (Table 2). Furthermore, when stationary-phase cultures were used to inoculate media containing carbonate, isoleucine and aspartate were no longer required for growth aerobically (data not shown). The presence of carbonate in anaerobically incubated cultures did not affect the requirement for any single amino acid but did significantly increase growth yields in control tubes and in the absence of certain amino acids (e.g., valine). Since both conditions of anaerobic incubation (anaerobic glove box and GasPak) utilized atmospheres enriched for gaseous CO_2 (about 5 and 10% CO_2, respectively), it was not surprising that added carbonate had little effect on growth or amino acid requirements. Similarly, the addition of carbonate, or for some strains bicarbonate, to the growth medium resulted in requirements for fewer amino acids for a number of other strains (see below).

TABLE 1. *Cellular turbidities after growth of S. mutans strain AHT (serotype a) in FMC upon omission of one of various amino acids*

A. Inoculum of exponential-phase cells[a]

	Aerobic incubation				Anaerobic incubation			
	Initial test		Subcultures		Initial test		Subcultures	
			1	2			1	2
	AOD (20 h)[b]	AOD (40 h)	AOD (21 h)	AOD (23 h)	AOD (20 h)	AOD (40 h)	AOD (21 h)	AOD (23 h)
FMC (control)	2,600	—[c]	2,700	2,800	3,000	—	3,200	3,200
FMC lacking:								
Arginine	18	18	—	—	3,000	—	3,100	3,100
Aspartate	1,700	—	2,500	2,700	2,100	—	3,000	3,200
Cystine	45	45	—	—	100	102	—	—
Glutamate/glutamine	10	15	—	—	900	3,000	1,600	3,000
Isoleucine	2,000	—	2,400	2,500	2,800	—	3,000	3,200
Leucine	10	7	—	—	2,000	—	3,000	3,000
Valine	23	20	—	—	1,400	—	3,000	3,000
B. Inoculum of stationary-phase cells[d]								
FMC (control)	2,200	—	2,400	2,400	1,800	—	3,400	2,500
FMC lacking:								
Arginine	50	45	—	—	1,800	—	3,000	2,500
Aspartate	45	48	—	—	1,800	—	3,000	2,700
Cystine	30	21	—	—	20	25	—	—
Glutamate/glutamine	40	35	—	—	60	1,200	—	—
Isoleucine	60	50	—	—	2,000	—	2,800	2,400
Leucine	21	15	—	—	2,100	—	2,900	2,500
Valine	42	40	—	—	810	—	3,000	2,600
Alanine	2,000	—	2,300	2,300	2,200	—	2,700	2,300
Glycine	2,000	—	2,500	2,400	1,900	—	3,400	2,500
Histidine	2,000	—	2,400	2,400	2,100	—	2,600	2,500
Hydroxyproline	2,100	—	2,500	2,500	2,300	—	3,400	2,600
Lysine	1,800	—	2,200	2,200	2,000	—	2,300	2,400
Methionine	2,200	—	2,400	2,400	2,200	—	3,200	2,500
Phenylalanine	2,100	—	1,100	2,400	2,000	—	3,000	2,500
Proline	2,200	—	1,300	2,400	2,000	—	2,800	2,500
Serine	2,200	—	1,200	2,400	2,000	—	2,900	2,400
Threonine	2,100	—	2,600	2,500	2,000	—	2,900	2,700
Tryptophan	2,000	—	2,400	2,200	1,800	—	2,700	2,400
Tyrosine	2,000	—	2,400	2,400	1,800	—	2,700	2,700

[a] The initial AOD of media inoculated with exponential-phase cultures ranged from 10 to 18 AOD.
[b] Incubation time.
[c] —, Not tested.
[d] Inoculum consisted of 50 μl of a culture (2,800 AOD) grown aerobically in FMC for 20 h. The initial AOD of all inoculated media ranged from 20 to 27 AOD.

Amino acids required for growth of all 13 strains of oral streptococci. Tables 3 and 4 summarize the results obtained for those amino acids whose omission results in the absence of growth (defined as less than 4% of the control turbidity at 40 to 48 h) when exponentially growing cultures were used as an inoculum for cultures incubated under the conditions specified. In Table 3, amino acid requirements are presented for each species or strain, whereas in Table 4 the strains which require each amino acid are presented. In both Tables the listings are presented in order of conditions which are more restrictive, that is, which result in an increase in the number of amino acids required. For example, the smallest number of amino acids were required when cultures were incubated anaerobically in the presence of carbonate (or bicarbonate). In all cases the amino acids required under these conditions were also

TABLE 2. *Cellular turbidities after growth of strain AHT in FMC containing Na_2CO_3[a] upon omission of one of various amino acids*

	Aerobic incubation				Anaerobic incubation			
	Initial test		Subcultures		Initial test		Subcultures	
			1	2			1	2
	AOD (20 h)[b]	AOD (40 h)	AOD (21 h)	AOD (23 h)	AOD (20 h)	AOD (40 h)	AOD (21 h)	AOD (23 h)
FMC (control)	3,300	—[c]	3,800	3,800	3,800	—	3,800	3,800
FMC lacking:								
Arginine	3,600	—	3,800	3,800	3,800	—	3,400	3,400
Aspartate	3,400	—	3,800	3,800	3,700	—	3,800	3,800
Cystine	45	45	—	—	28	32	—	—
Glutamate/glutamine	15	21	—	—	1,400[d]	3,200	3,400	3,800
Isoleucine	3,400	—	3,600	3,600	3,800	—	3,800	3,800
Leucine	13	18	—	—	2,000	—	3,400	3,700
Valine	18	28	—	—	3,500	—	3,600	3,600

[a] Sodium carbonate (0.019 M) was present in all media.
[b] Incubation time.
[c] —, Not tested.
[d] When a stationary-phase inoculum was used to inoculate glutamate/glutamine-deficient medium, no anaerobic growth of AHT was observed at 20 h, but a turbidity of 3,000 AOD was obtained after 40 h of anaerobic incubation.

required when other incubation conditions were used. Only one strain, *S. mutans* GS-5, had a requirement for one additional amino acid, glutamine and/or glutamate, for anaerobic growth in the absence of added carbonate. It should be noted that although this strain failed to grow in the absence of glutamate and/or glutamine after 20 h of anaerobic incubation plus carbonate (60 AOD), it did grow out, to an AOD of 3,000, after 40 h. Again, since the anaerobic incubation atmosphere contains gaseous CO_2, the significance of these observations requires further careful investigation.

For all of the experiments summarized in Tables 3 and 4, exponential-phase inocula were used. In a few experiments, amino acid requirements were determined using stationary-phase cultures as an inoculum. Although a complete comparison of requirements for exponential-phase and stationary-phase inocula was not undertaken, the limited number of observations made did reveal examples of requirements for additional amino acids for (perhaps the initiation of) aerobic growth from stationary-phase inocula. For example, from stationary-phase inocula, the following strains failed to grow in the absence of the respective amino acid: *S. salivarius* HHT in the absence of arginine; *S. mutans* strains 6715 and OMZ-176 in the absence of valine; *S. mutans* IB in the absence of isoleucine; and *S. mutans* At6T in the absence of isoleucine or leucine.

"Intermediate" growth responses. Summarized in Tables 3 and 4 are results for those amino acids whose absence resulted in culture turbidities of less than 4% of that seen in complete FMC after 40 to 48 h of incubation. In a number of cases, typified by the example of anaerobic growth of *S. mutans* AHT in the absence of glutamate and glutamine mentioned above (Table 2), "intermediate" growth responses were reproducibly observed. In some cases, low levels of turbidity (between 4 and 40% of the control) were seen at 20 to 24 h, followed by increases in turbidity to control levels at 40 to 48 h. In other cases, less than 4% of the turbidity of the control was seen at 20 to 24 h, followed by further growth to control levels at 40 to 48 h. In all cases, subculture resulted in growth comparable to controls at 20 to 24 h. "Intermediate" growth responses were most frequent for certain amino acids (e.g., glutamate, leucine, and valine) and for amino acids which were required when more restrictive conditions of incubation were used. For example, *S. mutans* AHT required glutamate/glutamine for aerobic growth even in the presence of carbonate (Table 2). When incubated anaerobically without carbonate, an AOD of only 900 was reached at 20 h (Table 2); when incubated anaerobically with carbonate, an AOD of 1,400 was reached at 20 h (Table 2); and, when stationary-phase cells were used as an inoculum, an AOD of only 60 was attained

TABLE 3. *Requirement of various streptococcal strains for amino acids*[a]

Conditions of incubation	*S. salivarius* HHT	*S. sanguis* OMZ-9[c]	72x46[b]	*S. mutans*									
				a[c]		*b*[c]		*c*[c]				*d*[c]	
				AHT	OMZ-61[b]	BHT	FA-1[b]	GS-5	IB	At6T	JC-2	6715	OMZ-176
1. Anaerobic a. Plus carbonate (Cys, Glu/Gln, Lys, His)	Cys	Cys	Cys	Cys	Cys	Cys Glu	Cys Glu Lys	Cys	Cys	Cys	Cys	Cys	Cys Glu His
b. No carbonate added (all requirements under 1a plus: Glu/Gln)		[d]	[d]				[d]	Glu	[e]	[e]	[e]		
2. Aerobic a. Plus carbonate (all requirements under 1a and 1b plus: Glu/Gln, Leu, Val, Iso, Tyr)	Glu Leu		Glu Tyr	Glu Leu Val			Leu Iso	Leu				Glu Leu	Leu
b. No carbonate added (all requirements under 1a, 1b and 2a plus: Glu/Gln, Arg, Leu, Iso, Val, Lys, Asp)		[d]	[d]	Arg	Glu Arg Leu Iso Lys	Arg Leu Iso	[d]	Arg Iso Val Asp	Glu Leu		Glu	Arg	

[a] None of the strains tested required methionine, glycine, alanine, serine, threonine, proline, hydroxyproline, phenylalanine, or tryptophan even when tested for growth aerobically in the absence of carbonate or bicarbonate.

[b] Incubations with bicarbonate instead of carbonate.

[c] The letters indicate the Bratthall serotype of the respective *S. mutans* strains.

[d] Strains OMZ-9, 72x46, and FA-1 were not tested in the absence of carbonate (or bicarbonate, for strain FA-1).

[e] Not tested for amino acid requirements anaerobically with no carbonate.

TABLE 4. *Amino acids required by the various streptococcal strains*[a]

Conditions of incubation	Glu/Gln	Leu	Arg	Iso	Val	Lys	Asp	His	Tyr
1. Anaerobic									
a. Plus carbonate	BHT FA-1 OMZ-176					FA-1		OMZ-176	
b. No carbonate added (all strains under 1a plus):	GS-5								
2. Aerobic									
a. Plus carbonate (all strains under 1a and 1b plus):	HHT 72x46	HHT							72x46
	AHT 6715	AHT FA-1 GS-5 6715		FA-1	AHT				
b. No carbonate added (all strains under 1a, 1b and 2a plus):	OMZ-61 JC-2	OMZ-61 BHT IB	AHT OMZ-61 BHT GS-5 6715	OMZ-61 BHT GS-5	GS-5	OMZ-61	GS-5		

[a] All strains required cystine (or cysteine) under all incubation conditions. For *S. mutans* OMZ-61 and FA-1 sodium bicarbonate was used.

at 20 h (Table 1, stationary phase). In all cases the turbidity increased with further incubation. Possible explanations for these "intermediate" growth responses are discussed below.

DISCUSSION

In the experiments described, the requirements for individual amino acids of 10 strains of *S. mutans* of Bratthall serotypes *a* to *d* and three other strains of oral streptococci were determined in a chemically defined medium which supports rapid growth to high culture turbidities and in which all of the strains can be subcultured for at least five transfers (18). In contrast to many other streptococci and lactobacilli (16), all of these inhabitants of the oral cavity were found to require few individual amino acids to grow to high cell densities in 20 to 24 h. In general, our results therefore confirm earlier observations which used prolonged incubations and multiple transfers in chemically defined media (4–6, 13). More importantly, the present studies establish relationships between the availability of a source of CO_2 (carbonate or bicarbonate) and/or the presence of oxygen on the amino acids required by these organisms.

In general (Tables 1 to 4), few amino acids were individually required when anaerobic incubations with carbonate (or bicarbonate) were used, whereas a larger number were required for growth aerobically in the absence of carbonate. For anaerobic incubations, carbonate did not decrease the number of amino acids required except in the case of the glutamate and/or glutamine requirement of *S. mutans* GS-5. This was probably due to the presence of gaseous CO_2 in the atmospheres used for anaerobic incubations. For aerobic incubations, carbonate (or bicarbonate) decreased the number of amino acids required by a variety of *S. mutans* strains of all four serotypes. The order of increasing amino acid requirements (Tables 3 and 4) was (1a) anaerobic plus carbonate; (1b) anaerobic with no carbonate; (2a) aerobic plus carbonate; and (2b) aerobic with no carbonate.

With each strain, all of the amino acids required under condition (1a) was also required under conditions (1b), (2a), and (2b); those required under condition (1b) were also required under conditions (2a) and (2b); and those required under condition (2a) were also required under condition (2b). Thus, both the absence of carbonate and the use of aerobic incubations increased the number of amino acids required.

The amino acids required only when some incubation conditions are used are obviously

"conditionally required." A conditional requirement indicates that the bacterial cells contain sufficient genetic information to code for the synthesis of all of the enzymatic machinery required for the synthesis of that particular amino acid. Under permissive conditions the ability is expressed and the amino acid is synthesized. Under restrictive conditions the capacity is not expressed and the amino acid is required. Thus it is not surprising that the absence of a conditionally required amino acid can, under some circumstances, result in the slow or poor synthesis of that amino acid and the observation of an "intermediate" growth response.

When stationary-phase (20 h), aerobically grown inocula were used in place of exponential-phase inocula, a few additional amino acid requirements for aerobic growth were detected.

Cystine (or cysteine) required by *all* strains for growth under *all* of the incubation used and, as discussed above, is viewed as a true nutritional requirement.

Except for *S. mutans* At6T and *S. sanguis* OMZ-9 (which was not tested under these conditions), glutamate/glutamine was required by all strains for growth aerobically in the absence of carbonate. Three strains of *S. mutans*, strains BHT, FA-1, and OMZ-176, required this amino acid for growth under all of the conditions of incubation used, whereas for the other strains the requirement was conditional and seen only when the more restrictive incubation conditions were used. When not required, glutamate/glutamine frequently "stimulated" growth and, in its absence, an intermediate growth response was observed.

None of the strains tested required glycine, alanine, serine, threonine, methionine, proline, hydroxyproline, phenylalanine, or tryptophan, even when tested for growth aerobically in the absence of carbonate. In the absence of any one of these amino acids, growth to turbidities comparable to those of the appropriate controls at 20 to 24 h was repeatedly observed under all conditions of incubation tested and good growth occurred on serial subculture in the absence of the respective amino acid.

The requirements of the strains for amino acids other than cystine or glutamate/glutamine failed to follow a clearly discernible pattern. For growth under the most permissive conditions used (anaerobic plus carbonate), only two *S. mutans* strains, FA-1 and OMZ-176, each required only one amino acid (lysine and histidine, respectively) in addition to cystine and glutamate/glutamine. All other amino acid requirements were conditional and exhibited only when aerobic incubations were used.

Several *S. mutans* strains, notably AHT, OMZ-61, BHT, GS-5, 6715, IB, and JC-2, conditionally required one or more amino acids for aerobic growth only when carbonate or bicarbonate was not added to the growth medium. As mentioned above, the atmospheres used for anaerobic growth contained CO_2. Furthermore, no attempt was made to eliminate CO_2 from aerobically grown cultures. Recently Repaske et al. (15) showed that *S. sanguis*, strain Challis, not only required CO_2 but responded to increasing concentrations of CO_2. Similarly, other species of *Streptococcus* and *Lactobacillus* have been shown to require CO_2 and, in some cases, the presence of CO_2, or a source of CO_2, decreased the number of individual amino acids required (5, 14). CO_2 is undoubtedly a nutrient for these organisms. The efficiency by which various strains can extract CO_2 from their environment probably varies and accounts for the failure of some strains (e.g., *S. sanguis* OMZ-9 and 72x46) to grow, and the poor growth of other strains (e.g., *S. mutans* FA-1 and OMZ-61) in the absence of added carbonate (or bicarbonate).

In the absence of added carbonate (or bicarbonate) aerobically, the requirement of four strains for arginine might be accounted for by a requirement for a substrate (arginine) for arginine dihydrolase, an enzyme which generates CO_2 (9, 11, 17). The requirement for aspartate or glutamate/glutamine of three strains might be accounted for by the absence of a sufficient level of a substrate (CO_2) for the phosphoenolpyruvate carboxylase-catalyzed synthesis of 4-carbon dicarboxylic acids (14, 20). However, the requirements for leucine, isoleucine, valine, and lysine for aerobic growth in the absence of added carbonate (or bicarbonate) are more difficult to explain, especially since the precursor amino acids (e.g., alanine and aspartate) were present in the growth medium, and other amino acids, synthesized by the same or a closely allied metabolic pathway (e.g., methionine and threonine in the case of isoleucine and lysine; leucine and valine for each other), were not required. It is possible that, in the presence of the relatively high concentrations of amino acids present in FMC (100 to 300 μg/ml), feed-back inhibitions and repressions could play a rather selective role. The conditional requirements for these amino acids were not examined in the absence of, or presence of reduced concentrations of, amino acids in the same "family." In our experiments, at no time was a significantly increased level of growth noted when an individual amino acid was

omitted. We did not examine the omission of more than one amino acid or the presence of any single amino acid at a substantially higher or lower concentration than that normally present in the medium. In any event, we did not observe any evidence of inhibition of growth by the presence of leucine, valine, isoleucine, methionine, or lysine such as that observed by Cowman et al. (7). Comparison of our results with those of Cowman et al. is extremely difficult, if not impossible, since these investigators failed to present a quantitative index of the degree of inhibition that they observed. Also, it should be noted that the FMC medium used in these studies yielded cellular turbidities at 24 h estimated to be substantially more than three-fold higher than those observed by Cowman et al. in their "reference-defined medium." It is of interest to note that the amino acids found to be required for aerobic growth of several strains in this study (arginine, glutamate, leucine, isoleucine, and valine) are the same amino acids which were found to be indispensable for growth of *Streptococcus bovis* P-10 in the complete absence of CO_2 (14). It seems possible that, for the strains studied here, additional amino acids may be required for growth upon more complete removal of CO_2, or upon increasing the degree of aerobiosis.

Although it is relatively easy to understand the role of sources of CO_2 in providing an additional nutrient, it is more difficult to understand exactly how the absence of oxygen can provide nutrients. In fact, the opposite tendency might be expected, since metabolic activities associated with the utilization of oxygen, such as the tricarboxylic acid cycle, would favor the synthesis of the 4- and 5-carbon precursors of entire "families" of amino acids. Although citrate was found to stimulate growth of *S. mutans* FA-1, and was routinely added to the growth medium (18), *S. mutans* AHT required the same amino acids for either anaerobic or aerobic growth in the presence or absence of citrate. Although it seems possible that specific enzymatic activities could be inhibited by O_2 (or a higher oxidation/reduction potential), the general trend toward fewer requirements in the absence of oxygen suggests a role for O_2 in influencing regulatory processes.

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