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Acidaminococcus gen. n., *Acidaminococcus fermentans* sp. n., Anaerobic Gram-negative Diplococci Using Amino Acids as the Sole Energy Source for Growth

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Acidaminococcus gen. n. and the type species *Acidaminococcus fermentans* sp. n. were described. Amino acids, of which glutamic acid is the most important, could serve as the sole energy source for growth. Acetic and butyric acids and CO_2 were produced; propionic acid and hydrogen were not produced. Amino acid media supporting growth and the amino acid and vitamin requirements were described. Glucose was frequently not fermented or was weakly catabolized. Derivative products from glucose autoclaved in media, but not glucose itself, stimulated or were required for growth in amino acid media. A wide range of polyols and carbohydrates were not attacked. Lactate, fumarate, malate, succinate, citrate, and pyruvate were not used as energy sources for growth. Pyruvate completely suppressed growth. Cytochrome oxidase and benzidine reactions were negative; catalase, indole, acetyl methyl carbinol, and H_2S were not produced; nitrate and sulfonthalein indicators were not reduced; ammonia was produced; gelatin liquefaction was negative or slow and partial; vancomycin (7.5 μg/ml) was resisted. *Acidaminococcus* was different from *Veillonella* in morphology, serology, nutrition, utilization of substrates, and accumulation of products in media supporting growth; *Acidaminococcus* resembled *Peptococcus* in utilization of glutamic acid and accumulation of similar products, but the two genera differed in morphology, gram reaction, serology, guanine plus cytosine content of deoxyribonucleic acid, and nutrition.

Fuller (6) isolated 49 strains of gram-negative anaerobic cocci from the pig alimentary tract. In some cursory morphological and physiological respects, particularly in their reported inability to ferment carbohydrates, polyols, and lactate, these strains appeared to resemble some *Veillonella*, as previously described (1), and also some poorly understood, nonextant "anaerobic neisseria," in the terminology of Prévot (15). In his uncertainty about the status of his isolates, Fuller referred to a suggested classification of *Veillonella* (18), in which all except the lactate-fermenting, clearly identifiable *V. parvula* and *V. alcalescens* (17) would be considered as *nomina incertae sedis*. He concluded: "Under this revised classification group III would no longer be considered as veillonellae. Once again in the absence of an alternative name it is convenient to continue to regard these organisms as *V. reniformis*."

Fifteen of these strains were obtained from Fuller for further studies. My results will demonstrate that these organisms do not have the characteristics of the original description of *Diplococcus reniformis* Cottet (2) syn. *Neisseria reniformis* Prévot (15) syn. *V. reniformis* (1) or any of the veillonellae, and that they are probably unique among previously described gram-negative anaerobic cocci because amino acids serve as the primary energy source for growth and metabolism. Although in this respect they appear to resemble *Peptococcus aerogenes* ATCC 14963, the two groups are distinctive in three significant ways. Therefore, the present strains will be described as a new genus, *Acidaminococcus*.

MATERIALS AND METHODS

Maintenance of cultures. Lyophilized cultures were revived in reinforced clostridial medium (RCM; Oxoid). They were plated in RCM agar and incubated anaerobically at 36 C in jars that contained a cold

catalyst of palladinized alumina and which were flushed with an atmosphere of 95% H_2 plus 5% CO_2. Broth media were dispensed in screw-cap tubes so that the tubes were filled with freshly boiled and cooled media plus 10% (v/v) inocula. Later, medium AHCG (Table 1) was developed for routine transfer and storage of cultures. The acid-hydrolyzed casein used in this medium was a "vitamin-free, salt-free" powder (Nutritional Biochemical Corp., Cleveland, Ohio). It was incorporated directly into media except for investigations of vitamin requirements.

VR salts solution A (Table 1) consisted of Na_2HPO_4, 37.5 g, and KH_2PO_4, 12.5 g, in 500 ml of distilled-water solution. This solution was used at concentrations of 30 ml/liter in all media listed in Table 1 and contributed 2.25 g of Na_2HPO_4 and 0.75 g of KH_2PO_4 per liter. VR salts solution B consisted of: $MgSO_4 \cdot 7H_2O$, 24 g; $CaCl_2 \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$, 0.5 g of each; $ZnSO_4$, $MnSO_4 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, or $Co(C_2H_3O_2)_2 \cdot 4H_2O$, $VSO_4 \cdot 7H_2O$, or NH_4VO_3, and $Na_2MoO_4 \cdot 2H_2O$, 0.25 g of each; and $CuSO_4 \cdot 5H_2O$, 0.125 g. These ingredients were dissolved in about 700 ml of water by applying heat and adding 2 ml of HCl. Separately, and with stirring, 5 g of triglycollamic

TABLE 1. *Concentrations of additives per liter of media employed*

Additive[a]	Media								
	AHCG	AHC	AA	AAG18	AA11	AAG11	AAG11-1	AAG11-2	AAG11-P
Acid hydrolyzed casein (g)	20	20							
DL-Tryptophan (mg)	100	100	120	100	120	120	100	100	120
Guanine, uracil, hypoxanthine (mg)	10	10	10	10	10	10	10	10	
Glucose (g)	5		5	5		5	5	5	5
Sodium-L-glutamate (g)			2.24	4.5	5	5	4.5	4.5	5
L-Tyrosine (mg)			630	150	150	150	75	150	150
DL-Phenylalanine (mg)			500	270	250	250	135	270	250
L-Arginine hydrochloride (mg)			410	180	400	400	90	180	400
L-Histidine hydrochloride·H_2O (mg)			310	180	300	300	90	180	300
Glycine (mg)			200	60	200	200	30	60	200
DL-Valine (mg)			720	750	400	400	375	750	400
DL-Serine (mg)			630	180	2,000	2,000	90	180	2,000
DL-Isoleucine (mg)			610	220	125	125	110	220	125
DL-Alanine (mg)			320	150					
DL-Leucine (mg)			920	220					
L-Proline (mg)			1,060	100					
DL-Methionine (mg)			280	250					
L-Lysine hydrochloride (mg)			820	150					
DL-Aspartic acid (mg)			710	220					
DL-Threonine (mg)			490	200					
L-Cysteine hydrochloride (mg)	350	350	340	350	350	350	350	350	350
L-Asparagine (mg)			160						
VR salts A (ml)	30	30	30	30	30	30	30	30	30
VR salts B (ml)	4	4	4	4	4	4	4	4	4
Pyridoxal hydrochloride and calcium pantothenate, each (μg)	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Thiamine hydrochloride, niacin, riboflavin, each (μg)	50	50	50	50	50	50	50	50	50
p-aminobenzoic acid (μg)	10	10	10	10	10	10	10	10	10
Biotin (μg)	2	2	2	2	2	2	2	2	2
Folic acid, vitamin B_{12}, each (μg)	1	1	1	1	1	1	1	1	1
Total amino nitrogen (mg)	2,411	2,411	1,225	769	897	897	513	617	897

[a] Units of measure in which concentrations of additives are expressed are shown parenthetically.

acid (nitriloacetic acid; Sigma Chemical Co., St. Louis, Mo.) was added to 300 ml of distilled water; 10 N NaOH was added until the *p*H value tended to stabilize (7.0 to 7.4) and solution occurred. This was slowly added to the stirred 700 ml of mixed salts solution until it became fairly clear; the volume was adjusted to 1 liter, and the solution was filtered through paper and refrigerated. VR salts solution B was used at the rate of 4 ml/liter. Solutions of vitamins, guanine, uracil, and hypoxanthine were prepared and added as previously described (19). Media were dispensed in the same way as is described for RCM media, autoclaved at 121 C for 3 min, and inoculated as already described. The cultures were incubated at 36 C for 18 to 24 hr and stored at 5 C. They were lyophilized successfully suspended in skim milk. Source cultures for experiments were transferred daily and were generally 16- to 18-hr old. Experimental media as described in the tables were freshly prepared and inoculated the same day.

Biochemical tests. Glucose and carbohydrate utilization, identification of CO_2 and H_2, nitrate and indicator reductions, oxidase and benzidine reactions, production of catalase, indole, ammonia, and hydrogen sulfide, gelatin liquefaction, and other routine tests were performed as previously described (17). Carbon dioxide and hydrogen were also detected by gas chromatography (10). The lower fatty acid homologues (C_2–C_6) were quantitatively analyzed by means of gas chromatography using a hydrogen flame detector. Aqueous–culture supernatant fluids were directly injected, and the column packing was a single-phase solid polyaromatic resin consisting of polystyrene cross-linked with divinylbenzene (23).

RESULTS

Morphology. Colonies on RCM agar were generally about 0.1 to 0.2 mm in diameter and were round, entire, slightly raised, and whitish gray or nearly transparent. The cells were gram-negative or variable cocci, frequently appearing as diplococci, and were sometimes oval or kidney-shaped. They were usually 0.6 to 1.0 μm in diameter and were larger than cells of *V. parvula* and *V. alcalescens*. This agrees with Fuller's (6) original description. Motility or flagella were never detected. A typical electron micrograph of thin sections of these gram-negative organisms, together with a comparative micrograph of the gram-positive *P. aerogenes* (strain 228 received from H. Whiteley; Fig. 1), shows the multilayered outer cell structures characteristic of gram-negative bacteria. Also, the Shwarzman reaction was elicited in rabbits by using cell extracts prepared with a French Pressure Cell. In contrast, *P. aerogenes* had the dense outer structure typical of gram-positive bacteria, and cell extracts did not elicit the Shwarzman reaction.

Temperature relations. Growth was good at 30 to 37 C and poor or negative at 25 and 45 C. Cells did not survive heating at 60 C for 30 min.

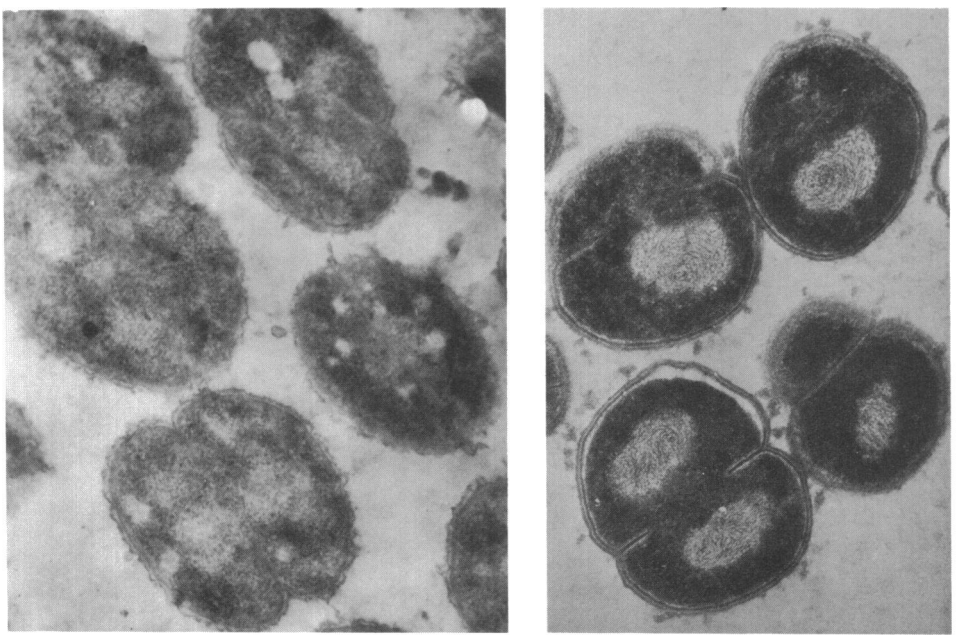

FIG. 1. *Electron micrographs of thin sections of A. fermentans strain VR11 (left) and P. aerogenes strain 228 (right). In Acidaminococcus, the outer layer has a tendency to strip away from the cell as in many other gram-negative organisms. The denser outer layer of P. aerogenes is relatively intact as in many other gram-positive organisms.* × *24,000.*

General cultural and physiological characteristics. All strains were negative in tests for cytochrome oxidase, catalase, acetylmethylcarbinol, benzidine reaction, indole production, and nitrate reduction. H_2S was not produced. Ammonia was produced. Gelatin was generally not liquified, but 21% of the strains partially or very slowly liquified it. Sulfonthalein indicators like phenol red and bromothymol blue were not reduced. The organisms were resistant to vancomycin (7.5 μg/ml).

Aerobic growth on the surface of a variety of suitable agar media did not occur. Even under anaerobic conditions, growth of many strains was first observed at the very bottom of the tube in liquid media, particularly from reasonably small inocula. This was true even when L-cysteine hydrochloride (350 mg/liter) or reduced gluthathione (750 mg/liter) was present. Growth occurred in various media at initial *p*H values between 6.2 and 7.5.

Lactate media favoring the growth of *V. parvula* and *V. alcalescens* (19, 21) failed to support growth in the first transfer or some early passage.

In RCM-agar deeps in screw-cap tubes, gas was produced by 81% of the strains. Since RCM is a highly complex medium containing glucose and lactate, it was essential to investigate the general nutrition of these organisms and to devise a more nearly defined medium for metabolic studies. Eventually, it was found that medium AHCG (Table 1) supported excellent growth routinely. In this medium supplemented with 1.5% agar, sufficient gas was produced by all strains to crack or disturb the agar. Once this medium was established, experiments were performed with known amino acids replacing the acid-hydrolyzed casein.

Amino acids as growth requirements and substrates. Initially, a medium was constructed similar to medium AHCG, in which the acid-hydrolyzed casein was replaced by a mixture of amino acids in the proportions present in 1% casein (29). In this and other amino acid media, D- and L-enantiomorphs were assumed to have the same functional capacities, and, consequently, DL-mixtures were added in the same relative proportions as the L-isomers. The exogenous amino acid requirements for growth were determined by measuring growth when each was omitted singly from medium AA (Table 1); the results of a representative experiment are summarized in Table 2. Single omissions of the following amino acids did not adversely influence growth of any of the 15 strains tested: alanine, leucine, proline, threonine, methionine, lysine, and aspartic acid. Asparagine was also completely dispensable. Indeed, individual omissions of proline, lysine, aspartic acid, or asparagine often improved growth. For instance, increases in growth were observed with all but one strain, and in this case growth was equivalent, when proline was omitted. The omission of glycine reduced growth with one strain only. The presence of valine, phenylalanine, tyrosine, serine, cysteine, arginine, and histidine was necessary for continued good growth of 15, 8, 12, 8, 15, and 14 of the 15 strains, respectively. Tryptophan or glutamate deficiencies drastically reduced growth so that all strains were nonviable in one or two passages. On the basis of such results and the glutamate titrations described below, medium AAG18, which supported good growth through repeated transfers, was devised. The irregular growth which sometimes occurred in medium AA was eliminated in medium AAG18 because of its lesser content of proline, lysine, and aspartic acid (inhibiting in the proportional quantities present in 1% casein) and the omission of inhibiting asparagine. On further investigation, 11 amino acids were selected for the basal medium AA11 and the growth medium AAG11 (Table 1). Al-

TABLE 2. *Growth response to omissions of amino acids from medium AA*[a]

Omissions	Absorbance at 650 nm	
	Range	Mean
None	0.45 to 0.70	0.56
DL-Alanine	0.47 to 0.73	0.57
Glycine	0.23 to 0.72	0.55
DL-Valine	0.03 to 0.52	0.17
DL-Leucine	0.49 to 0.07	0.59
DL-Isoleucine	0.43 to 0.71	0.54
L-Proline	0.53 to 0.77	0.65
DL-Phenylalanine	0.08 to 0.51	0.41
L-Tyrosine	0.03 to 0.70	0.30
DL-Tryptophan	0.01 to 0.16	0.09
DL-Serine	0.35 to 0.49	0.44
DL-Threonine	0.47 to 0.65	0.56
L-Cysteine hydrochloride	0.19 to 0.89	0.41
DL-Methionine	0.43 to 0.77	0.57
L-Arginine hydrochloride	0.26 to 0.46	0.33
L-Histidine hydrochloride	0.38 to 0.70	0.48
L-Lysine hydrochloride	0.48 to 0.89	0.59
DL-Aspartic	0.47 to 1.00	0.67
L-Sodium glutamate	0 to 0.26	0.07
L-Asparagine	0.47 to 0.96	0.63

[a] Cultures were grown 17 hr in medium AHCG and centrifuged; the cells were suspended in 5 ml of water. Inocula were 0.1 ml per 15 ml. A second serial transfer (0.1 ml) was made, and readings are of these second transfers after 21 hr incubation at 36 C. The mean values, optical densities (OD), in this and other tables are the mean results with 15 strains.

though isoleucine was not necessary for growth, it was retained in media AA11 and AAG11 because of its structural similarity to the essential valine and because in some experiments occasional growth irregularity was eliminated by including isoleucine with valine in the proportions shown in Table 1. Purines and pyrimidines, such as adenine, guanine, xanthine, hypoxanthine, and uracil were dispensable. However, occasional strains were sometimes slightly stimulated by a combination of guanine, uracil, and hypoxanthine (Table 1).

We have presented evidence (Table 2) that glutamic acid is indispensable for growth. Table 3 gives further evidence, from two experimental approaches, of the importance of glutamate as an energy source at high substrate levels. In one experiment, acid-hydrolyzed casein was used at levels of 2.5, 5.0, and 10.0 g per liter in a medium otherwise identical with medium AHCG. To these media, glutamate was added so that the concentration of total calculated glutamate, including that contributed by the acid-hydrolyzed casein (22.4% glutamic acid), was constant at 4.5 g/liter. Good growth was obtained even when the contribution of the remaining amino acids by the acid-hydrolyzed casein (2.5 g/liter) was minimal. Doubling the concentration of acid-hydrolyzed casein from 2.5 to 5.0 g per liter, with the total glutamate constant, only slightly increased early growth. But the final growth yield was approximately the same. Fourfold increases of acid-hydrolyzed casein enhanced growth slightly more. Thus, the amino acids, other than glutamic acid, do not appear to be required at the same high level as glutamate for maximal growth; e.g., with the indispensable amino acid tryptophan, there was very little if any difference in growth when 50 mg of the DL-enantiomorph was used rather than the usual 100 to 120 mg per liter. Tryptophan is generally representative of required or stimulating amino acids except for glutamate. In a medium containing 11 amino acids including glutamate (medium AAG11), there was a steady increase of growth with added glutamate (Table 3). If growth response was plotted as a function of glutamate concentration, the response was sharply ascending from 0 to 1.25 g and increased without substrate saturation often to at least 5 g of glutamate per liter.

The amino acid media AAG11-1, AAG11-2, and AAG18 (Table 1) were identical in their cysteine, tryptophan, and glutamate concentrations. They were also identical in all ingredients other than amino acids. The content of eight amino acids, namely, tyrosine, phenylalanine, arginine, histidine, glycine, valine, serine, and isoleucine was twice as great in media AAG11-2 and AAG18 as in medium AAG11-1. In addition, medium AAG18 had seven additional amino acids to make a full complement of 18 amino acids. The amino nitrogen content of these three media was 513, 617, and 769 mg per liter, respectively.

TABLE 3. *Growth response to glutamate in amino acid and acid-hydrolyzed casein media.*[a]

Additions		Absorbance at 650 nm			
		21 hr		29 hr	
Media	Glutamate	Range	Mean	Range	Mean
Acid-hydrolyzed casein					
2.5	3.94	0.53 to 0.70	0.60	0.62 to 0.85	0.71
5.0	3.38	0.59 to 0.80	0.71	0.56 to 0.85	0.68
10.0	2.26	0.66 to 0.96	0.78	0.70 to 0.96	0.80
Medium AAG11 (without glutamate)					
	5.000	0.51 to 0.96	0.73		
	3.622	0.33 to 0.77	0.61		
	2.500	0.35 to 0.66	0.51		
	1.250	0.26 to 0.57	0.41		
	0.625	0.17 to 0.41	0.30		
	0.156	0.08 to 0.37	0.20		
	0	0 to 0.32	0.01		

[a] Inocula and growth readings in the first serial transfer were as shown in Table 2. The acid-hydrolyzed casein was incorporated in the amounts shown, in grams per liter, in basal medium AHCG, so that the total glutamate contributed by acid-hydrolyzed casein plus added glutamate remained constant at 4.5 g per liter.

Relative growth in each of these media and the effect of serine on growth were tested (Table 4). Doubling the concentration of the group of eight amino acids in medium AAG11-2, where a total of 11 amino acids was present, increased mean growth of the strains by approximately 33%. Further addition of the seven amino acids, alanine, leucine, proline, threonine, methionine, lysine, and aspartic acid, to make a total of 18 amino acids, increased growth a relatively minor 8%. Increasing the concentrations of the single amino acid, serine, from 3.10 to 24.74 mM in a medium otherwise identical with medium AAG11-2 increased final growth 37% and reduced lag time and initial irregularities. Indeed, with serine added in the latter concentration to the 10 other amino acids in medium AAG11-2, cell yields were greater than with any other quantity of serine and were greater than in medium AAG18, where a complete group of 18 amino acids was present.

Vitamin requirements. Vitamin requirements were investigated (Table 5) by using medium AAG11 without purines and pyrimidine (~AAG11-P, because of the well-known role of folic acid, vitamin B_{12}, and related analogues in their biosynthesis (7). Putrescine, folic acid, folinic acid, thiamine, niacin, and riboflavin were not required, and their absence did not significantly limit the growth of any strain. Vitamin B_{12}, pyridoxal, pantothenate, and biotin were indispensable; *p*-aminobenzoic acid (PABA) was indispensable or highly stimulatory.

Different results, however, were obtained with medium AHCG. The acid-hydrolyzed casein in this medium was a "vitamin-free, salt-free" preparation treated as previously described (19) so that it was free of any of the vitamins tested. As will be shown below, there are two groups of strains, one utilizing glucose and one not utilizing glucose. In medium AHCG, the six glucose-utilizing strains needed no added vitamins, whereas the nine strains not utilizing glucose required biotin, PABA, pantothenate, and pyridoxal, as they had previously done in medium AAG11-P. Omission of guanine, uracil, and hypoxanthine from medium AHCG did not alter these results. Therefore, the presence of these exogenous purines and uracil in medium AHCG could not have exerted a sparing effect on the

TABLE 4. *Growth response to increasing concentrations of amino acids*

Conditions[a]	Absorbance at 650 nm	
	Range	Mean
Medium AAG11-1	0.20 to 0.50	0.38
Medium AG11-2	0.41 to 0.72	0.56
+ 3.10 mM DL-Serine	0.31 to 0.70	0.48
+ 6.19 mM DL-Serine	0.43 to 0.72	0.53
+12.37 mM DL-Serine	0.37 to 0.92	0.62
+24.74 mM DL-Serine	0.60 to 1.00	0.76
+28.55 mM DL-Serine	0.21 to 1.05	0.56
+33.30 mM DL-Serine	0.07 to 1.00	0.39
Medium AAG18	0.44 to 0.82	0.61

[a] Medium AAG11-1 contained glycine (0.400 mM), isoleucine (0.839 mM), phenylalanine (0.817 mM), serine (0.856 mM), arginine (0.427 mM), histidine (0.428 mM), tyrosine (0.414 mM), and valine (3.201 mM). Medium AAG11-2 contained two times the concentrations of these acids. Medium AAG18 contained all these acids at the concentrations present in medium AAG11-2 plus alanine (1.683 mM), leucine (1.677 mM), proline (0.868 mM), threonine (1.678 mM), methionine (1.675 mM), lysine (0.821 mM), and aspartic acid (1.625 mM). Cysteine, tryptophan, glutamate, and all other ingredients were identical in concentration in these media and their complete compositions in weight and volume units are listed in Table 1. Inocula and growth readings in the first serial transfer were as shown in Table 2. The results were essentially the same on further serial transfers.

TABLE 5. *Growth response to omissions of vitamins*[a]

Omissions	Absorbance at 650 nm	
	Range	Mean
None	0.32 to 0.61	0.46
Putrescine	0.30 to 0.57	0.44
Folic acid (FA)	0.37 to 0.62	0.49
Folinic acid (FN)	0.31 to 0.57	0.44
FA + FN	0.39 to 0.65	0.52
Vitamin B_{12}	0	0
p-Aminobenzoic acid (PABA)	0.17 to 0.38	0.28
PABA + FA	0 to 0.16	0.06
PABA + FN	0.14 to 0.34	0.23
PABA + FA + FN	0 to 0.14	0.02
Pyridoxal hydrochloride	0	0
Calcium pantothenate	0	0
Biotin	0	0
Thiamine hydrochloride	0.40 to 0.59	0.47
Niacin	0.39 to 0.63	0.50
Riboflavin	0.28 to 0.70	0.50

[a] Medium AAG11-P (Table 1) was used. Cells from AHCG (Table 1) were washed two times with distilled water; inoculum was 0.1 ml to 17 ml of medium in the first transfer. Subsequent serial transfers (0.1 ml) were made directly from each proceeding transfer. Results shown are OD in the second serial transfer. When guanine, uracil, and hypoxanthine were added to AAG-P, thus resulting in medium AAG11 (Table 1), the range of OD in the complete medium with no vitamin omissions was 0.33 to 0.70 with a mean of 0.50.

vitamin requirements of the strains utilizing glucose. The possibility was investigated that aspartic acid in medium AHCG might act to spare or modify the biotin requirement of the glucose-utilizing strains as it does for certain other organisms (7). This was not the case here because the addition of 175 mg of aspartic acid per liter of medium AAG11 or medium AAG11-P did not modify the biotin requirement.

Glucose utilization and stimulatory effect. Since inability to metabolize glucose is a distinguishing characteristic of unequivocally recognizable *Veillonella* species (17, 18, 22), the present strains were tested for glucose utilization using the glucose-oxidase technique. The strains were tested after 48 hr at 36 C in medium AHCG containing 0.25, 0.5, or 1.0% glucose autoclaved in the medium. Two groups were found. Nine strains did not utilize glucose at any concentration; the analytic recovery of initial glucose ranged from 97 to 104%. The remaining six strains consumed glucose to various extents; in four cases, all of an initial 0.25% glucose disappeared, 22 to 100% (mean = 52%) of an initial concentration of 0.5% and 6 to 43% (mean = 15%) of an initial 1%; the remaining two strains consumed 62 and 40% of the initial 0.25% glucose, but, paradoxically, at 0.5% concentrations of glucose or greater, glucose was attacked feebly, if at all. In medium AHCG, with 0.5% glucose (Table 1), the *p*H of autoclaved uninoculated medium was 7.50. The final *p*H in the case of the strains not utilizing glucose ranged from 6.50 to 6.75; with the glucose metabolizing strains, it ranged from 6.00 to 6.24.

The previous experiment, in which glucose was autoclaved in medium AHCG, was repeated with the same general results. However, when the glucose was added aseptically as an aqueous solution sterilized separately, either by autoclaving or filtration, early growth in AHCG was markedly retarded and final growth was only 50% as great as when glucose was autoclaved in the medium (Table 6). Although growth occurred in AHCG when glucose was absent or had not been autoclaved in the medium, it was often limited, erratic, and characterized by prolonged lag periods. This effect was even more dramatic in medium AAG11 containing 11 essential and stimulatory amino acids; there was no growth or it was negligible and could not be sustained through repeated passages unless the glucose had been autoclaved in the medium (Table 6). Stimulation by glucose autoclaved in media was equally strong with the strains utilizing glucose and the strains not utilizing glucose. Consequently, the growth stimulation must be attributed, not to the glucose, but to derivative products when glucose was autoclaved in the media as little as 5 min at 121 C. Such an effect has been reported previously (4, 13, 14, 16, 28), but it is difficult to identify these mediating substances because of the great multiplicity of possible products (3, 11).

When medium AHGG containing 0.5% glucose was autoclaved at 121 C for 5 min, the mean loss of glucose, as measured by the glucose oxidase assay, was 14.6% or 0.004 moles per liter of the initial glucose. Aseptic additions of sterile filtrates of glucosamine (0.004 M) to medium AA11 or AHC (neither of which contained glucose) did not stimulate growth, indicating that the growth-promoting effect of autoclaved glucose is probably not due to this single possible Maillard reaction product.

Growth enhancement by glucose autoclaved in media has been attributed to derivative production of aldehydes, such as pyruvic aldehyde (methyl glyoxal; 14). Such uninoculated media

TABLE 6. *Growth stimulation by products formed during autoclaving of glucose in media*[a]

Medium and glucose treatment	Absorbance at 650 nm at			
	17 hr		22 hr	
	Range	Mean	Range	Mean
AAG11, autoclaved in medium	0.51 to 0.96	0.73	0.54 to 1.16	0.87
AAG11, autoclaved in water	0 to 0.07	0.01	0 to 0.11	0.04
AAG11, filter-sterilized	0 to 0.13	0.07	0.02 to 0.22	0.09
AHCG, autoclaved in medium	0.85 to 1.05	0.94	0.82 to 1.22	1.00
AHCG, autoclaved in water	0.05 to 0.52	0.27	0.05 to 0.80	0.41
AHCG, filter-sterilized	0.05 to 0.64	0.29	0.11 to 0.82	0.50

[a] The final glucose concentration in all cases was 0.5%. The 17-hr cultures in medium AHCG were washed two times in distilled water and suspended in water, and 0.1 ml per 17 ml of medium was inoculated. Absorbancies were determined on the first serial transfer.

contained substances reacting as pyruvate by the Friedeman-Haugen technique (i.e., substances capable of forming 2,4-dinitrophenyl hydrazones); these substances were equivalent to 2.5% of the total glucose degradation products after autoclaving at 121 C for 10 min. Media without glucose or media containing glucose sterilized by filtration or autoclaved separately in water gave no response for pyruvic-like substances. Therefore, pyruvic aldehyde was tested as a possible growth stimulant (Table 7). When aseptic additions of sterile filtrates of pyruvic aldehyde (0.004 and 0.0135 M) were made to medium AA11, growth was inhibited or suppressed. However, if pyruvic aldehyde was autoclaved in the medium, growth stimulation occurred and growth tended to approach that which occurred when glucose was autoclaved in the medium. This result agrees with previous findings with a number of lactic acid bacteria (14).

Substrate utilization. Since adequately controlled basal media were now available, experiments were performed with lactate (0.032 M), fumarate (0.025 M), malate (0.022 M), combinations of fumarate and malate, pyruvate (0.027 M), succinate (0.011 to 0.022 M), and citrate (0.01 M) as potential substrates. Freshly prepared solutions of these compounds were sterilized by filtration and added aseptically. None of these potential energy sources supported any growth in the defined amino acid medium AA11. Positive controls, whereby growth was obtained when added glucose was autoclaved in the media, were also included.

In unsupplemented medium AHC, growth was fairly good in the first serial transfer. Added succinate slightly inhibited growth but lactate, fumarate, malate, and mixtures of fumarate and malate seriously inhibited early growth and continued to inhibit all strains during prolonged incubation. The effect of pyruvate was drastic. The growth of all 15 strains was suppressed completely. Also, growth stimulation by autoclaving glucose in the medium was again clearly evident.

Fermentation products. Fourteen strains, which had been carried in medium AHCG through 75 transfers, were grown in this medium and medium AHC (with and without glucose) for 72 hr at 36 C. The presence of glucose made no difference in the products. Visible gas bubbles (CO_2) were produced by all strains in both media, and the accumulated CO_2 can account for the poising of the final *p*H at 6.50 to 6.75 with strains not utilizing glucose (17). The only lower fatty acid homologues produced were acetic and butyric acids. In medium AHC, in which amino acids were the only significant sources of carbon and nitrogen, the range of acetic acid produced was from 0.0262 to 0.0304 moles per liter (mean = 0.0277) and that of butyric acid was from 0.0115

TABLE 7. *Effect of pyruvaldehyde (PVA) on growth*[a]

Media and supplements	Absorbance at 650 nm at			
	16 hr	20 hr	23 hr	41 hr
AA11	0 to 0.55 (0.18)	0 to 0.59 (0.28)	0 to 0.60 (0.31)	0.03 to 0.82 (0.50)
AA11 + glucose (0.5%) ~ AAG11	0.05 to 0.66 (0.24)	0.16 to 0.70 (0.51)	0.30 to 0.92 (0.63)	0.53 to 0.96 (0.68)
AA11 + PVA (0.004 M), autoclaved	0.07 to 0.74 (0.24)	0.14 to 0.68 (0.40)	0.24 to 0.70 (0.52)	0.30 to 0.85 (0.62)
AA11 + PVA (0.004 M), filtered	0 to 0.11 (0.05)	0 to 0.21 (0.09)	0 to 0.31 (0.13)	0 to 0.70 (0.40)
AA11 + PVA (0.0135 M), autoclaved	0 to 0.77 (0.20)	0.03 to 0.77 (0.30)	0 to 0.82 (0.33)	0.22 to 0.92 (0.56)
AA11 + PVA (0.0135 M), filtered	0 to 0.11 (0.04)	0 to 0.20 (0.06)	0.01 to 0.27 (0.10)	0 to 0.70 (0.44)

[a] The results are ranges and mean values (in parentheses) with 15 strains. The cells from 17-ml cultures were spun down and suspended in 5 ml of distilled water without washing; inoculum was as shown in Table 2. In this experiment, growth in medium AA11 (without glucose) was significantly greater than shown in Table 6 where the cells were washed well twice; this washing effect was replicable, was observed with other test situations, and is commonly observed in the laboratory with other anaerobes. Nevertheless, the stimulating effect of autoclaved glucose was significant; at 23-hr incubation, five strains failed to grow without glucose, and seven other strains grew only 50% as well.

to 0.0145 moles per liter (mean = 0.0132). For the 14 different strains, the molar ratio of C_2 to C_4 acids ranged from 1.94 to 2.20 with a mean ratio of 2.09. Propionic acid was not detected by the hydrogen flame detector under conditions where 0.01 μmole or less could be found. Hydrogen was also not detected and lactate was neither produced nor fermented.

Serological studies. Antisera against 14 strains were prepared and titered for agglutinins as previously described (18). Cross-titrations of these strains with the seven antisera of serological groups of *Veillonella* and of *Veillonella* organisms against the present antisera gave negative reactions even at 1:4 dilutions of serum. Homologous *Veillonella* reactions occurred at 1:2,560 to 1:40,960 serum dilutions; homologous reactions of the present strains occurred at dilutions of 1:640 to 1:2,560. Thus, these organisms and the various serological subspecies of *Veillonella* (18) do not share any detectable antigens. Because of the possible general biochemical relatedness and similar gross microscopic morphology of *Peptococcus* and the present organisms, this type of experiment was also performed with cells and antisera of *P. aerogenes* (ATCC 14963) and strain 228, also with negative results.

Moles per cent guanine plus cytosine (GC) of deoxyribonucleic acid (DNA). The organisms were grown at Bethesda in AHCG medium. After 1 day of growth, all cultures were again carefully checked for purity by a variety of microscopic and cultural techniques. The cultures were centrifuged, washed twice in saline ethylenediaminetetraacetic acid (0.15 M NaCl plus 0.1 M EDTA, *p*H 8.0), resuspended in the same solution, packed in solid carbon dioxide, and shipped air mail special delivery to M. Mandel at the University of Texas, Anderson Hospital and Tumor Institute, Houston, Tex. The frozen cell suspensions (1 g or more) were thawed at Houston and the cells lysed by a combination of treatments with lysozyme, cell wall-digesting enzymes produced by *Streptomyces albidoflavus*, and sodium dodecyl sulfate. The DNA was isolated and duplicate samples from each strain were then analyzed by CsCl density-gradient centrifugation. The GC content was calculated from the mean buoyant density of the DNA duplex by the formula of Schildkraut, Marmur, and Doty (27).

The strains of *Acidaminococcus* not utilizing glucose (VR2, VR3, VR4, VR5, VR6, VR13, VR14, and VR15) contained DNA with mean GC values (moles per cent) of 55.6, 57.1, 56.6, 56.6, 56.1, 56.1, 56.6, and 57.4, respectively. The five strains utilizing glucose, VR7, VR8, VR9, VR11, and VR12, had GC values of 56.6, 56.6, 56.6, 57.1, and 56.1 moles per cent, respectively. The percentage GC mean values for the two clusters were 56.5 ± 0.9 and 56.6 ± 0.5 and were indistinguishable from each other. One strain not utilizing glucose, VR16, had a somewhat different GC value of 54.1. On reexamination, the value was 56.1.

These results were widely different from those with *P. aerogenes* where GC moles per cent were 36.7 ± 0 and 35.7 ± 0 for strains 236 and 228, respectively. *Veillonella* strains were not examined by us. However, Lee, Wahl, and Barbu, cited by Rosypal and Rosypalová (26), found a GC content of 36.5 moles per cent by direct chromatographic analysis. DNA from *Veillonella* has not been analyzed by CsCl gradient centrifugation. In keeping with the distinctive phenotypic characteristics of *Acidaminococcus*, the GC content is also distinct from *Peptococcus*, *Veillonella*, or any other known gram-negative anaerobic coccus.

DISCUSSION

These gas-forming organisms do not conform to the original descriptions of *D. reniformis* (2) syn. *Micrococcus reniformis* (12) syn. *N. reniformis* (15) syn. *V. reniformis* (1), because the most extensive description of *N. reniformis* (15) states that gas was not produced in complex media and thus suggests basic metabolic differences. The evidence also indicates that the present organisms differ from *V. parvula* and *V. alcalescens* in morphology, serology, nutrition, and the distribution of fermentation end products from the utilization of different substrates. Where exogenous vitamins are required for growth, such other aerogenic organisms as veillonellae and heterofermentative lactobacilli (19, 20, 24, 25) require thiamine. Such is not the case here.

V. parvula and *V. alcalescens* grow with lactate and pyruvate as substrates (17, 18, 19). For every 2 moles of lactate catabolized, generally 1 mole each of acetic and propionic acids, CO_2, and H_2 are produced. In contrast, the present organisms could not grow at the expense of these substrates. Furthermore, when lactate or pyruvate were added to growth-promoting media, growth was markedly inhibited or suppressed. Therefore, since saccharoclastic activity was absent or weak, these organisms must derive their energy for growth chiefly from the dissimilation of amino acids, of which glutamate is especially significant. The end products from the amino acid fermentations were acetic and butyric acids, in a molar ratio of 2:1, and CO_2. Hydrogen and propionic acid were not detected. Since a multiplicity of amino acids were present during the accumula-

tion of the lower fatty acid homologues and CO_2, it is clearly impossible to formulate a balance equation for such a complex system. In this situation, information is obviously needed concerning the metabolism of individual amino acids by nongrowing cells or cell extracts. General amino acid metabolism of these organisms may bear some similarity to that of *P. aerogenes* syn. *M. aerogenes* (5), which also ferments glutamic acid in high concentration, to acetic and butyric acids and CO_2 (8, 9, 30, 31, 32). Whether the intermediary metabolism of glutamic acid is also similar in the two groups of organisms only future studies can determine.

Despite these overall metabolic similarities differences in the Gram reaction, cell structure, lipopolysaccharide and GC content, and nutritional behavior clearly serve to differentiate the two. *P. aerogenes* (three strains) could not grow in any of the media listed in Table 1 but required complex media containing large amounts of peptones and yeast extract.

Therefore, the characteristics of the presently studied strains are sufficiently different from known, adequately described, gram-negative anaerobic cocci to justify the recognition of a new genus. The nomenclature and description are as follows:

Family **NEISSERIACEAE** Prévot 1933

Genus **Acidaminococcus** gen n. organisms are anaerobic, gram-negative, nonsporulating cocci, 0.6 to 1.0 μm in diameter, frequently appearing as oval or kidney-shaped diplococci. Growth is good at 30 to 37 C, poor or absent at 25 and 45 C. Cells do not survive 60 C for 30 min. They have multiple nutritional requirements. No growth occurs on surface of agar media incubated in air; no growth occurs in lactate media which support growth of *Veillonella*. Growth occurs at initial *p*H values between 6.2 and 7.5, although best growth occurs at a neutral reaction; final *p*H values in media initially at 7.5 range from about 6.1 to 6.7. Amino acids, of which glutamic acid is the most important, can serve as the sole energy source for growth. In such media, acetic and butyric acids accumulate in a molar ratio of 2:1, and CO_2 is also formed; hydrogen and propionic acid are not detectable. Glucose is not fermented by about 60% of strains and only weakly catabolized by 40% of strains. The genus is characterized by, at best, weak saccharoclastic activity. Lactate, fumarate, malate, succinate, citrate, and pyruvate are not used as energy sources for growth. Pyruvate suppresses growth completely. Derivative products from glucose autoclaved in amino acid media are necessary or highly stimulatory for growth. Tryptophan, glutamic acid, valine, and arginine are required; 93% of strains require cysteine and histidine; 50% require phenylalanine and serine; 79% require tyrosine; glycine is sometimes stimulatory; and alanine, leucine, isoleucine, proline, threonine, methionine, lysine, and aspartic acid are not required for growth. In amino acid media, vitamin B_{12}, pyridoxal, panthothenate, and biotin are indispensable for growth; *p*-aminobenzoic acid is essential or highly stimulatory; exogenous putrescine, folic acid, folinic acid, thiamine, niacin, and riboflavin are completely dispensable. There are no serological cross-reactions between strains of *Acidaminococcus* and either *Veillonella* serotypes or *P. aerogenes*. Cytochrome oxidase, catalase, benzidine, indole, and nitrate reduction tests are all negative. H_2S is not produced. Ammonia is produced. Sulfonthalein indicators are not reduced. Gelatin is generally not liquified, although slow and partial liquefaction may sometimes occur. Resistant to vancomycin (7.5 μg/ml). The type species is *Acidaminococcus fermentans* sp. Its description is as follows:

Polyols including adonitol, dulcitol, glycerol, inositol, mannitol, sorbitol, and erythritol are not attacked; amygdalin, arabinose, galactose, inulin, fructose, maltose, mannose, melezitose, α-methyl-D-glucoside, α-methyl-D-mannoside, raffinose, salicin, sorbose, sucrose, trehalose, xylose, erythrose, and aesculin are also not attacked; ambiguous, extremely weak, or negative reactions occur with cellobiose, lactose, melibiose, rhamnose, ribose, and fucose. Otherwise the type species is identical with the already presented description of the genus *Acidaminococcus*.

The type strain is *A. fermentans*, strain VR4, ATCC 25085. This strain does not ferment glucose. Additional strains deposited in the American Type Culture Collection are strain VR14, ATCC 25088 (also not fermenting glucose), and strains VR7, ATCC 25086, and VR11, ATCC 25087, (the latter two fermenting glucose weakly).

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