JOURNAL OF BACTERIOLOGY, Sept. 1978, p. 858-867 Vol. 135, No. 3
0021-9193/78/0135-0858$02.00/0
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Nutritional Requirements of Anaerobic Coryneforms

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Received for publication 13 March 1978

The nutritional requirements of three species of anaerobic coryneforms and their serotypes (*Propionibacterium acnes* types I and II, *P. avidum* types I and II, and *P. granulosum*) were determined. Strains of *P. avidum* would consistently grow to a transmittance of 1 to 3% at 560 nm in a basal salts medium supplemented with glucose, pantothenate, biotin, thiamine, and 12 amino acids (alanine, arginine, cysteine, glutamine, glycine, histidine, isoleucine, methionine, phenylalanine, serine, tyrosine, and tryptophan). Strains of *P. acnes* and *P. granulosum*, however, failed to grow in this medium unless six additional amino acids were present (asparagine, leucine, lysine, proline, threonine, and valine). All three species grew equally well whether the 18 amino acids were supplied in the form of a casein hydrolysate supplemented with tryptophan or were added separately. Nicotinamide enhanced growth of *P. acnes* but had no effect on growth of *P. avidum* and *P. granulosum*. Other nutrients which were not absolute requirements, but which significantly improved growth of these species, included the purines guanine and/or adenine, Tween 80, which served as a source of oleic acid, sodium L-lactate, α-ketoglutarate, and pyruvate. Strains (86) comprising all five groups grew well in the defined medium, except four strains of *P. acnes* type II (29 tested), which failed to grow unless heme and vitamin K were added to the medium. One strain of *P. granulosum* (22 tested) failed to grow in any defined medium, suggesting an additional growth factor requirement.

The anaerobic coryneforms differ in a number of major characteristics from the classical corynebacteria as exemplified by *Corynebacterium diphtheriae* (15), and in the latest edition of *Bergey's Manual of Determinative Bacteriology* (16) they have been transferred to the genus *Propionibacterium*. Three common species can be recognized in the group, *Propionibacterium acnes, P. avidum*, and *P. granulosum* (11).

It has been shown that vaccines produced from anaerobic coryneform organisms have the ability to stimulate the reticuloendothelial system (18), and such vaccines have considerable potential value in cancer therapy (2, 10, 13, 14, 26). In the course of investigating the nature of the active substance in these vaccines, we have developed a completely defined medium which supports the growth of anaerobic coryneforms at a rate approaching that obtained in complex media.

The reticulo-stimulating ability of anaerobic coryneforms is generally attributed to strains referred to as *Corynebacterium parvum*. However, the majority of these strains are indistinguishable biochemically, serologically, and by DNA homology from *P. acnes* (4), and, in fact, a number of the strains included in the present investigations were originally described as *C. parvum* (see Table 1).

MATERIALS AND METHODS

Strains. The origins of the five reference strains (*P. acnes* type I, VPI 3706; *P. acnes* type II, VPI 0162; *P. avidum* type I, VPI 0575; *P. avidum* type II, VPI 0589; and *P. granulosum*, VPI 0507) have been reported previously (3). These strains were used to produce antisera for serological typing and have hence been used as the basic test strains in determining the nutritional requirements of the different groups of anaerobic coryneforms. However, preliminary tests showed that strain 0162 was more exacting nutritionally than most other type II strains of *P. acnes* (see below). Another type II strain, VPI 4792, was therefore used as a representative strain of this type in most of the tests. A list of the other strains used in these experiments is given in Table 1.

Media. The basal medium contained a salt solution (100×) based on that of Pittman and Bryant (17). The final concentrations of salts in the medium were as follows: $CaCl_2 \cdot 2H_2O$, 2.62 μg/ml; $MgCl_2 \cdot 6H_2O$, 2.0 μg/ml; KH_2PO_4, 90 μg/ml; NaCl, 90 μg/ml; $(NH_4)_2SO_4$, 1 mg/ml; $FeSO_4 \cdot H_2O$, 0.1 μg/ml (Fisher Chemical Co., Fair Lawn, N.J.). To this were added the redox indicator resazurin (1 μg/ml; Difco Laboratories, Detroit, Mich.), Tween 80 (sorbitan mono-oleate, 0.1% [vol/vol]), and the reducing agent sodium formaldehyde-sulfoxylate (0.03% [wt/vol]; Fisher Chemical

TABLE 1. *List of strains used*

Type of organism	VPI strain	Original designation	Site of origin (if known)
P. acnes type I	0009	Contaminant of *Actinomyces bovis* 3109C, Prevot	
	0159	ATCC 11827	
	0172	Prevot 3453	
	0208	Prevot 2783	Blood culture
	1152	FTLL BIO2A, Veterinary Science Department, VPI&SU[a]	
	2066	ATCC 15892	
	4532	Emory University Hospital 10R494, Atlanta, Ga.	Leg wound
	6020-A	Emory University Hospital 911-70C, Atlanta, Ga.	Wound swab
	6573	Prevot 1421, *C. parvum*	Subacute bacterial endocarditis
	6630	Prevot 3594, *C. parvum*	Blood culture
	6642	Prevot 2706, *C. parvum*	
	6660	Prevot 1317, *C. parvum*	Blood culture
	0133	Prevot 1574X, *C. parvum*	
	1742	Prevot 3114, *Ristella uniformis* (culture contaminant)	
	3322	Prevot 1328, *C. parvum*	
	3326	Prevot 4191, *C. parvum*	
	6575	Prevot 2721C, *C. parvum*	Pleural fluid
	6622	Prevot 3557, *C. parvum*	
	6647	Prevot 2472, *C. parvum*	
	6651	Prevot 1571, *C. parvum*	
	0863	BBA 12HL88	
	2059	ATCC 15854	
	2742	Roberstad 194, Diphtheroid sp.	Beef liver
	4793	St. Joseph's Hospital, Lexington, Ky., A-288-9	Blood
P. acnes type II	0132	Prevot 1474X, *Catenabacterium lottii*	
	0176	Prevot 355, *C. avidum*	
	0204	Prevot 3298	Dental abscess
	0224	ATCC 13962	
	0225	ATCC 13962C	
	0452-1	Culture contaminant, Anaerobe Laboratory	
	1885	Prevot 2681, *C. parvum*	
	1996	NASA Strain X3	
	3238	Seattle, Wash.	Peritoneal fluid
	4608	10A47, Emory University Hospital, Atlanta, Ga.	Autopsy blood
	4792	A879-9, St. Joseph's Hospital, Lexington, Ky.	Tissue from chest wall
	4995	B505-9, St. Joseph's Hospital, Lexington, Ky.	Blood culture
	5362	B0174, Emory University Hospital, Atlanta, Ga.	Blood culture
	6265	Gundersen Clinic, La Crosse, Wis.	Surgical head wound
	6583	Prevot 476, *C. parvum*	Blood culture
	6623	Prevot 4182, *C. parvum*	Blood culture
	6624	Prevot 3806, *C. parvum*	Blood culture
	6625	Prevot 3456, *C. parvum*	Septicemia
	6626	Prevot 3879, *C. parvum*	Vulvo-vaginitis
	6629	Prevot 3894, *C. parvum*	Septicemia
	6632	Prevot 2355-A, *C. parvum*	Abscess of neck
	6633	Prevot 2500, *C. parvum*	Blood culture
	6635	Prevot "Achard," *C. parvum*	Purulent pleurisy
	6637	Prevot 2508, *C. parvum*	Blood culture
	6641	Prevot 2738	
	6649	Prevot 2501, *C. parvum*	Sinusitis
	6652	Prevot 1904, *C. parvum*	Blood culture
	0426-1	Prevot 655, *Ramibacterium ramosoides*	
P. avidum type I	0667	CDC 3206	Blood
	6451	CDC B7706	Pelvic abscess

TABLE 1.—*Continued*

Type of organism	VPI strain	Original designation	Site of origin (if known)
P. avidum type II	0179	ATCC 25577, Prevot 1689B, *C. avidum*	
	0589	CDC A518	Wound swab
	0670	CDC 6440A	Earlobe cyst
	4388	Prevot 2219, *C. acnes*	
	4982	London Hospital, London, England	Normal skin
	5957	Emory University Hospital, Atlanata, Ga. N-725-70A2	Breast abscess
P. granulosum	5620	J. G. Voss D22	Skin
	6500	Prevot 3085C, *C. parvum*	Canaliculitis
	9549	NCTC 10387	
	9556	Evans 73-710	Normal skin forehead
	9578	Evans 74-423	Acne comedone
	5625	J. G. Voss 17-3	Skin
	5627	J. G. Voss 9-5	Skin
	5630	J. G. Voss 17-6	Skin
	9546	VPI isolate CG7A	Forehead
	9550	Evans 73-533	Skin
	9552	Evans 73-617cr	Skin
	9556	Evans 73-710	Forehead
	9560	Evans 74-162	Forehead
	9561	Evans 74-223	Deltoid
	9568	Evans 74-325	Forehead
	9570	Evans 74-335	Comedone
	9571	Evans 74-400	Forehead
	9573	Evans 74-402	Forehead
	9576	Evans 74-405	Forehead
		Evans 73-354	Forehead
		Evans 74-123	Forehead
		Evans 74-566	Axilla

[a] VPI&SU, Virginia Polytechnic Institute and State University.

Co.). This solution was then heated to drive off oxygen until the resazurin decolorized and then was cooled to room temperature under a steady stream of CO_2. A second reducing agent, L-cysteine-HCl hydrate (500 μg/ml; Sigma Chemical Co., St. Louis, Mo.), and sodium bicarbonate (5 mg/ml; Fisher Chemical Co.) were then added as dry powders. The medium was adjusted to a transitory pH of 7.0 with 12 N NaOH and autoclaved (15 lb/in^2 per 15 min) in 5-ml amounts in stoppered culture tubes in a 100% CO_2 atmosphere. Tubes were placed in a press to hold stoppers in place during autoclaving. The various other consitutents described below, with the exception of glucose, were sterilized by filtration (0.45-μm Metricel filter, Gelman Instrument Co., Ann Arbor, Mich.) and added aseptically under CO_2 to the presterilized and reduced basal medium. The pH of the complete defined medium was 6.4, and this was found to be a good starting pH for these organisms.

Glucose. Glucose (Fisher Chemical Co.) was autoclaved in an aqueous stock solution of 50% (wt/vol) and added aseptically to the autoclaved, prereduced medium under CO_2 to make a final concentration of 10 mg/ml.

Vitamins. The vitamins were obtained from Sigma Chemical Co. The following were tested at the concentrations (micrograms per milliliter) indicated: Calcium-D-pantothenate, 2; biotin, 0.05; thiamine-HCl, 2; nicotinamide, 2; riboflavin, 2; *p*-aminobenzoic acid, 0.1; folic acid, 0.05; DL-thioctic acid, 0.05; vitamin B_{12}, 0.02; and pyridoxine-HCl, 2. The vitamins were prepared as a 100× aqueous stock solution. The additional growth factors vitamin K (0.5 μg/ml) and hemin (5 μg/ml) were prepared as described in the *Anaerobe Laboratory Manual* (9).

Amino acids. The amino acids were obtained from Sigma Chemical Co. The aqueous amino acid stock solution (50×) was made by acidification with concentrated HCl and was added to the basal medium to give the final concentrations (micrograms per milliliter) indicated: L-alanine, 200; L-arginine, 200; L-asparagine, 200; L-glutamine, 200; glycine, 100; L-histidine, 200; L-isoleucine, 200; L-leucine, 200; L-lysine, 200; L-methionine, 200; L-phenylalanine, 200; L-proline, 200; L-serine, 200; L-threonine, 200; L-tryptophan, 200; L-tyrosine, 100; and L-valine, 200.

Nucleoside bases. The nucleoside bases were obtained from Sigma Chemical Co. The aqueous purines and pyrimidines (100×) were brought into solution by acidification and added to give the final concentrations (micrograms per milliliter) indicated: adenine, 1 to 20; guanine, 10 to 20; uracil, 10 to 20; cytosine, 10 to 20; and thymine, 10 to 20.

Organic acids. The organic acids were obtained from Sigma Chemical Co. either as the sodium salt or as the free acid (the latter being neutralized with NaOH). The aqueous stock solutions (100×) were added to the basal medium to give the following final concentrations (percent, weight/volume) pyruvic acid, 0.1 to 0.2; citric acid, 0.1 to 0.2; DL-isocitric acid, 0.1 to

0.2; L-malic acid, 0.1 to 0.2; fumaric acid, 0.1 to 0.2; D-, L-, or DL-lactic acid, 0.1 to 0.3; α-ketoglutaric acid, 0.1 to 0.2; and succinic acid, 0.1 to 0.2.

Hydrolyzed casein amino acids. Vitamin-free (salt-free), acid-hydrolyzed casein amino acids from Difco Laboratories (supplemented with 10 mg of L-tryptophan per ml) as an aqueous solution (50×) was added to the medium to make a final concentration of 0.2% (wt/vol). This was used in place of separately added amino acids in some experiments.

Estimation of growth rate. The cultures were grown anaerobically at 37°C in an Aquatherm water bath shaker (New Brunswick Scientific Co., New Brunswick, N.J.). For inoculum, 1 drop from a sterile Pasteur pipette of a late-log/early-stationary culture was added to 5 ml of medium (ca. 1/200 dilution) in a stoppered culture tube (18 by 142 mm). The tubes were immersed to the level of the medium and shaken at low speed to facilitate even heat distribution. We transferred organisms grown in complex medium at least three times in defined medium before using them as an inoculating culture for nutritional studies.

Growth was monitored (1.5-cm light path) with a Spectronic 20 spectrophotometer (Bausch and Lomb, Rochester, N.Y.). Each culture tube (matched to within 1% transmittance) was removed from the water bath, shaken, read, and immediately returned to the incubator to prevent temperature fluctuations. Due to differences in growth rates of the three species, transfers were made at a given turbidity (optical density of approximately 1.3) rather than at a fixed time interval in experiments involving serial passages. A repeatable growth rate, however, was a requirement for determining whether a given strain grew in a particular medium formulation. All experimental points were determined in duplicate or triplicate, and three to eight serial transfers were used to determine the kinetics of growth of the bacteria in a particular medium. The results were expressed as semilog plots of optical density against time.

Determination of vitamin requirements. The vitamin requirements were first examined on defined medium, minus vitamins, solidified with 1.5% agar (Difco Laboratories). The medium containing Tween 80, basal salts, and resazurin, all at concentrations stated above, was buffered at pH 6.4 with 0.1 M potassium phosphate and autoclaved. Filter-sterilized ingredients, including sodium bicarbonate, L-cysteine-HCl, glucose, purines, and casein amino acids at concentrations previously described, were added aseptically to the medium at 48°C just before the plates were poured. The organisms used for inocula were grown anaerobically in peptone-yeast extract-glucose (PYG)-Tween medium (2). The plates of defined medium were inoculated with an overlay of cells washed three times in distilled water. The inoculum (0.2 ml of a suspension containing approximately 10^9 cells per ml) was mixed with 2.5 ml of 0.7% agar at 45°C, and the mixture was immediately poured over the plates, spread by rocking, and allowed to cool. The filter-sterilized vitamins, alone and in various combinations (0.025 ml of 20-μg/ml stock solutions) were added to sterile 6-mm filter paper disks (Whatman, Clifton, N.J.), with six disks in a hexagonal pattern per plate. The plates were then incubated at 37°C in anaerobic jars with GasPaks (Baltimore Biological Laboratory, Cockeysville, Md.) as described in the *Anaerobe Laboratory Manual* (9).

Serological identification of strains. The identities of the various strains were confirmed by precipitin tests, using trichloroacetic acid extracts as described by Cummins (3).

RESULTS

Because it was known that strains of *P. avidum* grew more rapidly in complex media than strains of the other two species, a strain of this group was used in the initial testing for nutritional requirements. The strain chosen was VPI 0589, which is a strain of *P. avidum*, serotype II (3).

Growth could be obtained consistently on repeated transfers in a medium which contained basal salts, glucose, vitamins, and a source of amino acids. In fact, the organisms also grew repeatably in the absence of added amino acids, provided they were first accommodated by at least one transfer into the above defined medium minus amino acids but including the purine bases and Tween 80. Initially, growth in the medium without amino acids was very poor, but after two transfers the growth of the accommodated strain was almost as good as in the complete medium. During this process of selection the strain needed purine bases and Tween 80, but it could ultimately be grown in a simple medium composed of salts, glucose, and vitamins. The accommodated organism was shown to be identical to the normal strain by biochemical, chromatographic, and serological tests (3, 9). *P. avidum* strains, however, were the only ones of the three species which would grow in the absence of amino acids.

Factors which were not required but which significantly improved the rate of growth and/or the final cell yield of this strain included the purines guanine and/or adenine, sodium L-lactate, pyruvate, and α-ketoglutarate, and to a lesser extent oleic acid (Tween 80).

When the vitamin requirements of the five representative strains were tested by the disk method on solid media (see Materials and Methods), growth occurred only around those disks which contained calcium-D-pantothenate. No growth was seen in the absence of pantothenate, even if all other nine vitamins were present.

Quantitative growth measurements in tubed medium later showed that pantothenate would support growth of these organisms, albeit at a much slower rate than if the complete vitamin mixture were present. The addition of the other nine vitamins in various combinations with pantothenate showed that biotin and thiamine were sufficient to produce growth rates identical to

those produced with the complete vitamin mixture for *P. avidum* types I and II (Fig. 1, Table 2) and for *P. granulosum* (Fig. 4, Table II) but that nicotinamide was an additional growth factor for strains of *P. acnes* types I and II (see Table 2).

The addition of the nucleoside bases to defined medium resulted in a substantial increase in the growth rates and a shortening of the lag phase of these organisms (Fig. 1). It was found that purines were solely responsible for the stimulation and that pyrimidines were without effect. The growth rates of *P. avidum* strains were improved by the addition of either adenine or guanine, whereas adenine (10 μg/ml) alone was stimulatory for strains of *P. acnes* and *P. granulosum*. By varying the proportions of guanine and adenine in the medium, it was found that a combination of 20 μg of guanine and 1 μg of adenine per ml gave good growth rates for all groups. None of the nucleoside bases up to concentrations of 20 μg/ml showed any inhibition of growth.

When Tween 80, as a source of soluble oleic acid, was added to defined medium, it consistently resulted in greater cell yields for strains of

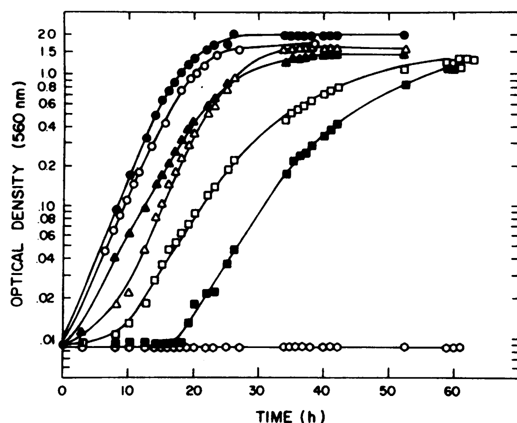

FIG. 1. *Growth response of P. avidum serotype II (VPI strain 0589) in PYG-Tween 80 medium and in defined medium plus 0.2% (wt/vol) acid hydrolysate of casein (salt-free, vitamin-free) with various omissions. Symbols: PYG-Tween 80 medium (●); defined medium (basal salts, glucose [1%, wt/vol], calcium D-pantothenate [2 μg/ml], biotin [0.05 μg/ml], thiamine-HCl [2 μg/ml], nicotinamide [2 μg/ml], guanine [20 μg/ml], adenine [1 μg/ml], Tween 80 [0.1%, vol/vol], sodium L-actate [0.2%, wt/vol], sodium pyruvate [0.1%, wt/vol] and sodium α-ketoglutarate [0.1%, wt/vol]) (○); defined medium minus the organic acid salts (△); defined medium minus Tween 80 and the organic acid salts (▲); defined medium minus the purine bases, Tween 80, and the organic acid salts (□); defined medium minus the purine bases and the organic acid salts (■); defined medium minus vitamins and the organic acid salts (⬡).*

P. acnes I and II and *P. granulosum* (Table 2). Tween 80, however, somewhat lengthened the lag phase and only slightly increased the growth rates for *P. avidum* strains (Fig. 1). A Tween 80 concentration of 0.005% (vol/vol) resulted in the fastest growth rates for *P. avidum* strains, whereas 0.1% Tween 80 gave the best growth characteristics for *P. acnes* and *P. granulosum*.

Intermediary metabolites in the form of the sodium salts of citric, isocitric, fumaric, malic, lactic, pyruvic, succinic, and α-ketoglutaric acids were added in various substrate concentrations to determine their effect on growth. Of these only L-lactate, pyruvate, α-ketoglutarate, and succinate stimulated growth, whereas citrate showed a distinct inhibition. The stimulatory effect of lactate was stereospecific, since D-lactate had no effect on growth whereas the L and DL acid salts both caused increased growth rates. A combination of 0.2% (wt/vol) sodium L-lactate and 0.1% (wt/vol) each pyruvate and α-ketoglutarate gave maximum growth rate increases for the three species. These effects were additive, and the combined growth stimulation could not be duplicated by adding an excess of any one of the three acids. The addition of these compounds resulted in a shortening of the lag phase and slightly increased the growth rate during early log phase, but the final cell yields were not increased (Fig. 1 and 2). Adding succinate (0.2%, wt/vol), on the other hand, resulted in greater cell yields for *P. acnes* strains, whereas it had no apparent effect on the growth of *P. avidum* and *P. granulosum* organisms nor did it improve growth rates or shorten lag phase in any of the three species.

The amino acids were supplied either by adding a vitamin-free casein hydrolysate or by addition of the individual acids. Experiments with different combinations of the various amino acids resulted in a defined medium for *P. avidum* strains that contained the following 12 amino acids: L-alanine, L-arginine, L-glutamine, L-histidine, L-isoleucine, L-methionine, L-phenylalanine, glycine, L-tryptophan, L-serine, and L-tyrosine (Fig. 2). L-Cysteine was always present in the medium as a reducing agent. This combination, however, was not sufficient to support growth of *P. acnes* and *P. granulosum* strains, even with the addition of the organic acids (Fig. 3 and 4). These strains required the complete complement of 18 amino acids, which could be supplied either by the casein hydrolysate supplemented with L-tryptophan or by the addition of the 18 individual amino acids. The growth rates for the representative strains in completely defined medium were virtually equal to that in medium with casein amino acids and approached the growth rates in complex media

TABLE 2. *Effects of selected nutrients on the growth of anaerobic coryneforms*

	Vitamins[1]				Nucleoside Bases			Organic Acids[3]							
	Panto-thenate	Biotin	Thia-mine	Nicotin-amide	Pyrim-idines	Purines[2] Guanine	Purines[2] Adenine	Lactate isomers L-	Lactate isomers D-	Pyru-vate	α-keto-glutarate	Succi-nate		Tween 80 Oleate	Amino[4] acids
Propionibacterium acnes serotypes I, II	R	S+	S	S	N	N	S+	S+	N	S+	S	S		S+	R
P. avidum serotypes I, II	R	S+	S	N	N	S+	S+	S+	N	S+	S	N		S	S+
P. granulosum	R	S+	S	N	N	N	S+	S+	N	S+	S	N		S+	R

[1] Riboflavin, p-amino-benzoate, folic acid, thiotic acid, vitamin B_{12}, pyridoxine-HCl, vitamin K and heme have no effect on growth with the exception of four strains of *P. acnes* II which require these vitamins for growth.

[2] Guanine and adenine (in 20/1 ratio) give best growth rates for the three species of *Propionibacterium*.

[3] Isocitrate, malate and fumarate do not affect growth, while citrate inhibits growth.

[4] *P. acnes* and *P. granulosum* strains require all 18 amino acids for growth while *P. avidum* strains will grow with no added amino acids although they are very stimulatory when present in the medium.

R = required for growth; S = stimulatory; S^+ = very stimulatory (increase growth rate >25%); N = no effect on growth.

such as PYG with 0.1% Tween 80 (PYG-Tween) (Fig. 1 through 4).

In preparing the complete medium, the method of sterilizing the various constitutents was found to be important because glucose would caramelize unless it was autoclaved separately from the other medium constituents. The vitamins and amino acids had to be filter sterilized because autoclaving them resulted in poorer growth rates. The organic acids were routinely filter sterilized, but the effects of autoclaving were not studied. Autoclaving the purine bases with the medium constituents had no apparent effect on growth rates, so they need not be filter sterilized and added separately.

Table 2 outlines the nutritional requirements of the three species of anaerobic coryneforms. Doubling or halving the concentration of the nutrients described above had no effect on the growth of these organisms.

When 86 strains comprising the five major groups of the propionibacteria were tested for growth in the complete, prereduced defined medium with casein amino acids, 94% grew anaerobically at constant rates to a percent transmittance of ≤5% on three successive homologous transfers, using a 1/200 inoculum. The breakdown by groups was: *P. acnes* I, 25 of 25; *P. acnes* II, 25 of 29; *P. avidum* I, 3 of 3; *P. avidum* II, 6 of 6; and *P. granulosum*, 21 of 22; representing the number of strains growing through three transfers of the total tested. The strains are listed by group in Table 1. Those which failed to grow through three transfers were *P. acnes* II strains 6637, 0162, 6633, and 6649 and *P. granulosum* strain 9552.

It was subsequently found that by supplementing the complete defined medium with hemin and vitamin K and/or the full complement of 10 vitamins, poor, but consistent, growth of the four exacting *P. acnes* II strains occurred through three successive transfers, using a 1/100 inoculum. Combining these additions resulted in the greatest growth stimulation in these four strains, whereas it neither stimulated nor inhibited the growth of strains which would grow in their absence.

Figure 5 shows growth curves of *P. acnes* II strains 6624 and 0162 and compares growth rates in complex medium with those in complete defined medium and in the complete defined medium supplemented with complete vitamins and vitamin K plus hemin. The growth curves represent the second serial transfers and illustrates the failure of strain 0162 to grow on repeated transfer in the defined medium without vitamin K, hemin, and 10 vitamins.

P. granulosum strain 9552 failed to grow in any defined medium tested and appears to require an additional growth factor.

Table 3 outlines the procedure for preparing 1 liter of the prereduced medium.

DISCUSSION

Our aim in this work was to develop a medium which was not only completely defined but also capable of supporting growth of these organisms at a rate comparable to that in complex medium. We have therefore included substances that stimulate growth as well as those that are absolute requirements for growth. Our finding that oleate is stimulatory rather than required for growth agrees with findings of other researchers (6, 19), and it can be seen from the results shown in Fig. 1 that *P. avidum* will grow well in the absence of Tween 80. It is possible, as suggested by Puhvel and Reisner (19), that oleate serves

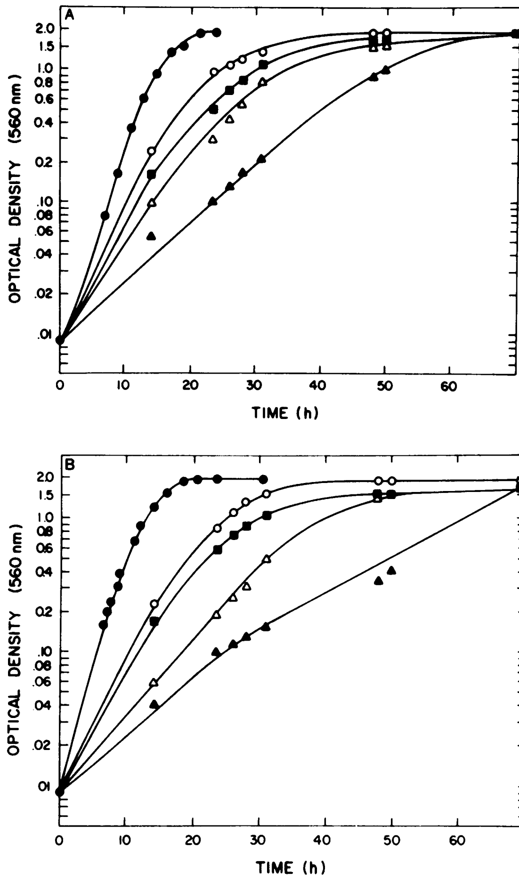

FIG. 2. *Growth responses of P. avidum (A) serotype II, VPI strain 0589, and (B) serotype I, VPI strain 0575, in PYG-Tween 80 medium and in defined medium with various substitutions and omissions. Symbols: PYG-Tween 80 medium (●); defined medium plus 0.2% acid hydrolysate of casein (vitamin-free, salt-free) as in Fig. 1 (○); defined medium with a mixture of 18 pure amino acids (0.34%, wt/vol) substituted for casein hydrolysate and all other ingredients as in Fig. 1 (■); defined medium with a 0.22% (wt/vol) mixture of 12 pure amino acids (minus L-asparagine, L-leucine, L-lysine, L-proline, L-threonine, and L-valine) substituted for the 18 pure amino acids and all other ingredients as in Fig. 1 (△); defined medium with 12 amino acids minus the organic acid salts and all other ingredients as in Fig. 1 (▲).*

as an energy source and that the increase in the lag phase may be caused when the organisms are modifying their metabolism to utilize oleate as a carbon source, or it may simply be incorporated as a cell lipid and thus spare the energy of synthesis.

That oleic acid causes greater growth stimulation of *P. acnes* and *P. granulosum* strains than of *P. avidum* is consistent with the conditions encountered in their natural habitats. *P. acnes* and *P. granulosum* are normally found in the lumen of the pilosebaceous canals of the human skin, whereas *P. avidum* strains are more frequently isolated from wound infections, where they are unlikely to encounter large concentrations of fatty acids (see Table 1).

The stimulation of growth elicited by the three organic acid salts, lactate, pyruvate, and α-ketoglutarate, occurs at substrate concentrations (0.1 to 0.2%). In experiments not shown in detail, it was determined that the stimulation is additive and cannot be mimicked by the addition of excess concentrations of any one acid. Since these anaerobic coryneforms carry out propionic acid fermentation with the production of acetate and propionic acid from pyruvate (9, 15), these compounds are possibly priming the pump by providing preformed substrates. The substantial

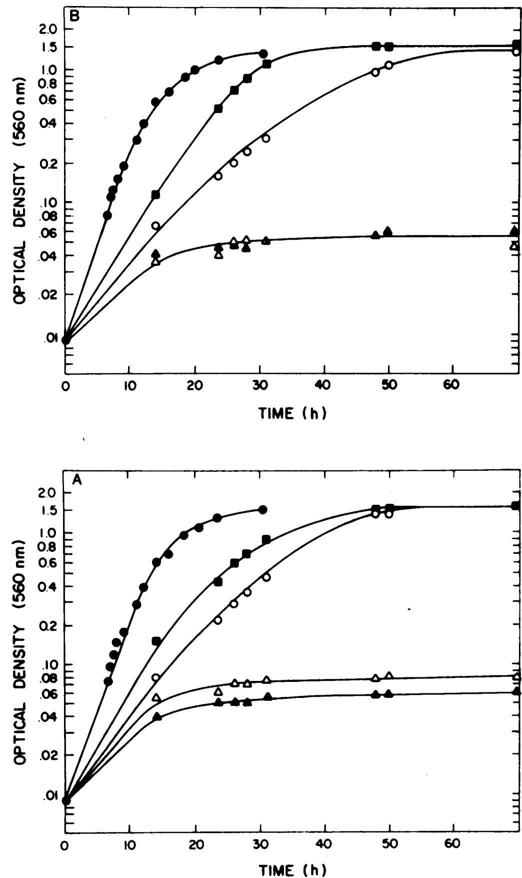

FIG. 3. *Growth responses of P. acnes (A) serotype II, VPI strain 4792, and (B) serotype I, VPI strain 3706, in PYG-Tween 80 medium and in defined medium with various substitutions and omissions. Symbols as in Fig. 2.*

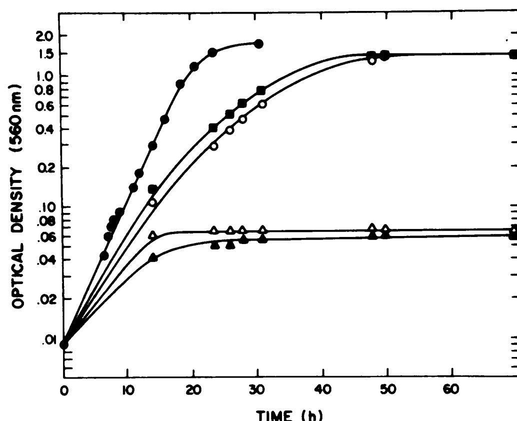

FIG. 4. *Growth response of P. granulosum, VPI strain 0507, in PYG-Tween 80 medium and in defined medium with various substitutions and omissions. Symbols as in Fig. 2.*

shortening of the lag phase in defined medium supplemented with these acids supports this view (Fig. 1 and 2). The major advantage of complex medium over defined medium is the inclusion of many preformed substrates and metabolites which allow the organisms to short-cut certain metabolic pathways, resulting in short lag phases and rapid growth rates. These acids improved the growth characteristics of this defined medium in such a way that the growth rates nearly equalled those in PYG-Tween 80.

The vitamin requirements for the anaerobic coryneforms are consistent with the propionic acid fermentation pathway for carbohydrate found in the classical propionibacteria, which leads to the formation of propionic and acetic acids (1, 5, 7, 8). Chromatography of the end products of fermentation also suggests this pathway for these organisms (9, 15). Pantothenic acid was found to be an absolute requirement for growth of all three species, and this precursor to coenzyme A (CoA) is involved at both the point at which pyruvic acid is decarboxylated to form acetyl CoA, CO_2, and reduced nicotinamide adenine dinucleotide, leading to the formation of acetic acid and ATP, and at the point at which succinic acid is converted to succinyl CoA in the pathway leading to the formation of propionic acid. Biotin, another requirement for optimal growth of these organisms, assists in transferring a carboxyl group from methyl malonyl CoA (formed from the molecular rearrangement of succinyl CoA) to pyruvic acid, resulting in the formation of oxaloacetic acid in the pathway leading to propionate. Thiamine, like biotin, stimulates growth in all three species and, as a precursor to thiamine pyrophosphate, functions in the decarboxylation of pyruvate in association with pyruvate dehydrogenase and CoA to form acetyl CoA, with the eventual production of acetate. Nicotinamide is required for optimal growth of *P. acnes* strains only, so that in these strains the synthesis of nicotinamide adenine dinucleotide must be rate limiting in the absence of the vitamin.

The observation that four strains of *P. acnes* type II failed to grow in the defined medium unless it was supplemented with vitamin K and hemin suggests that these factors may be important in the synthesis of the cytochrome system

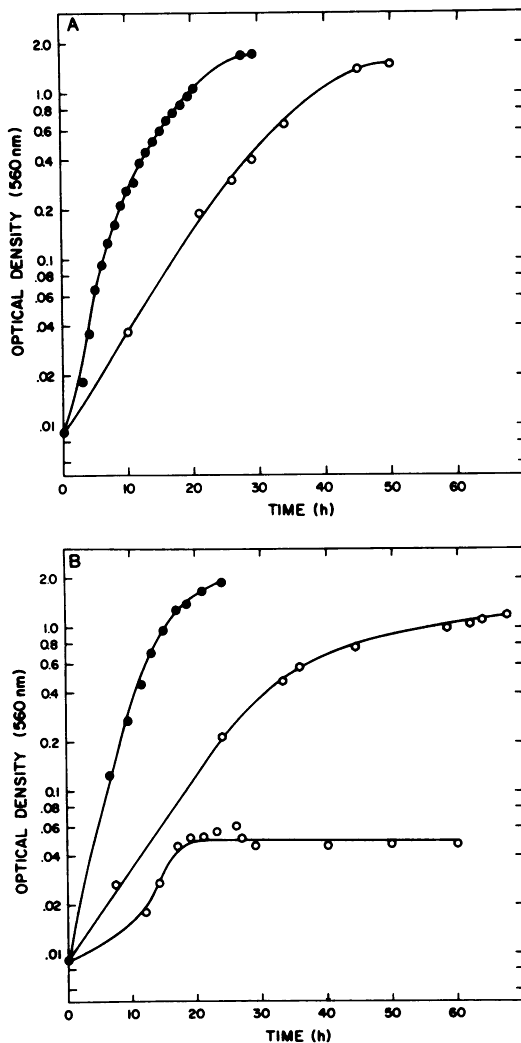

FIG. 5. *Growth responses of (A) a typical (VPI 6624) and (B) a fastidious (VPI 0162) strain of P. acnes serotype II in a second serial transfer in PYG-Tween 80 medium and defined medium. Symbol: Defined medium with 10 vitamins and hemin (5 μg/ml) and vitamin K (0.5 μg/ml) (◻). Other symbols as in Fig. 2.*

TABLE 3. *Preparation of prereduced defined medium for growth of anaerobic coryneforms (1 liter)*

To a 2,000-ml Erlenmeyer flask with stirring bar:

1. Add:

Basal salts solution (100×; see text)	10 ml
Ammonium sulfate solution (100 g/liter)	10 ml
Ferrous sulfate solution (0.1 g/liter)	1 ml
Guanine	20 mg
Adenine	1 mg
Tween 80 (10%, vol/vol)	10 ml
Resazurin (22 mg/80 ml)	4 ml
Sodium formaldehyde sulfoxylate	0.3 g
Distilled water	up to 930 ml

2. Heat with stirring until resazurin turns colorless.
3. Cool to room temperature under a constant stream of CO_2 in an ice bath.
4. Add: L-cysteine hydrochloride .... 0.5 g
5. Autoclave with rubber stopper clamped in place and with both cotton-plugged inlet tube and slit rubber outlet tube unclamped (30 min at 15 lb/in^2).
6. Immediately upon removal from the autoclave, attach the inlet tube to a CO_2 cylinder with a reducing valve and let a slow stream of gas pass through the flask for about 5 min. Then clamp off the outlet tube, leaving the inlet open, reduce gas pressure to about 0.5 to 1 lb/in^2, and allow to cool to room temperature under slight positive pressure of CO_2. When cool, clamp off inlet tube and remove from gas supply. The medium should remain colorless and fully reduced.

For use, the Basal salts medium needs to have added to it amino acids, vitamins, glucose, organic acids, and sodium bicarbonate. To add the components, the stopper is loosened, the inlet tube is connected to a CO_2 supply, and the neck of the flask is continually flushed out with a slow stream of sterile CO_2 while the various solutions are poured in with sterile precautions. To make the necessary additions in the most convenient way, the components can be combined in the following solutions:

A. *Amino acids.* These can be added either as (i) a filter-sterilized 10% solution of vitamin-free Casamino Acids (Difco) or (ii) a filter-sterilized mixture of 18 individual amino acids (see text for composition of suitable mixture which contains glycine and L-tyrosine at 0.5% and all other amino acids at 1%). A 20-ml amount of either (i) or (ii) is added per liter of medium.

B. *Vitamins and organic acids.*

Calcium-D-pantothenate	200 μg/ml
Biotin	5 μg/ml
Thiamine hydrochloride	200 μg/ml
Nicotinamide	200 μg/ml
Sodium L-lactate	20% (wt/vol)
Sodium pyruvate	10% (wt/vol)
Sodium α-ketoglutarate	10% (wt/vol)

Solution is filter-sterilized, and 10 ml is added per liter of medium.

C. *Glucose solution, 50% (wt/vol).*
This solution is sterilized by autoclaving in stoppered tubes at 15 lb/in^2 for 15 min.

D. *Sodium bicarbonate.*
This is sterilized as dry powder by autoclaving in stoppered tube at 15 lb/in^2 for 15 min; 5 g is added per liter of medium.

E. *Vitamin K-hemin solution.*
This solution is prepared as described in reference 2, and 10 ml is added per liter of medium.

The pH of the final medium should be 6.4. The flasks should be incubated at 37°C overnight to check sterility and allow the medium to become fully reduced after the various additions.

which perhaps functions in the steps between the reduction of fumarate to form succinate, resulting in the formation of ATP in the classical propionic acid pathway. These factors, however, do not seem to be a general requirement for these organisms.

Because of differences in classification, it is difficult to compare our results with those of Ushijima (22–25), and for this reason it is impossible to be sure where his strains of *C. avidum, C. granulosum,* and *C. diphtheroides* would fit into the scheme of classification we have used. His growth requirements for *C. liquefaciens,* which is considered to be identical to *C. acnes* (*P. acnes*) by Zierdt et al. (27) and Smith (20), are the same as for our strains except for the amino acids. In our hands, only *P. avidum* would grow (at much reduced rates) in the amino acids mixture specified by Ushijima (25), whereas representative strains of *P. acnes* and *P. granulosum* failed to show growth even after 5 days of incubation at 37°C. Our results agree with those of Ushijima (25) and Smith (20) in showing that purines stimulate the growth of

the anaerobic coryneforms, but unlike Smith and in agreement with Ushijima, our strains of *P. acnes* are more generally stimulated by adenine rather than by guanine.

We were able to achieve repeatable growth in completely defined media for 85 of the 86 strains tested (99%), and, of these, 94% gave growth rates approaching those in complex media. The *P. avidum* strains were relatively unaffected by the presence of oxygen, whereas *P. acnes* and *P. granulosum* strains were significantly inhibited in their growth. *P. avidum* strains were also much less fastidious in their nutritional requirements than the latter two species. These properties may again reflect the differences in habitat of these organisms, since a primary source of *P. acnes* and *P. granulosum* appears to be the sebaceous glands, where they may encounter many preformed metabolites such as amino and fatty acids.

The results reported here show that the anaerobic coryneforms and the classical propionibacteria are very similar in their nutritional requirements and in the products of their metabolic pathways (5, 7-9, 12, 21, 28). This provides further support for their transfer from the genus *Corynebacterium* to the genus *Propionibacterium*.

ACKNOWLEDGMENTS

The work reported in this paper was made possible by grants from the J. S. Abercrombie Foundation and the Public Health Service National Cancer Institute (CA17790).

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