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Growth of *Porphyromonas gingivalis*, *Treponema denticola*, *T. pectinovorum*, *T. socranskii*, and *T. vincentii* in a Chemically Defined Medium

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A chemically defined medium, OMIZ (Oral Microbiology and Immunology, Zürich)-W1 was developed. Medium OMIZ-W1 supports the long-term proliferation of a wide range of oral anaerobes, including representative strains of four *Treponema* species and *Porphyromonas gingivalis*. High concentrations of ascorbic acid and ammonium ions proved to be important for the growth of these organisms. *T. denticola* CD-1 grew in the absence of polyamines and long-chain fatty acids, *T. pectinovorum* and *T. socranskii* required polyamines, whereas *T. vincentii* depended on both polyamines and lecithin for growth. Specific requirements for purines and/or pyrimidines were detected, and these requirements could be used to distinguish *Haemophilus-Actinobacillus* group organisms. Some strains of *P. gingivalis* grew without vitamin K, while others were not satisfied by menadione but required its precursor 1,4-dihydroxy-2-naphthoic acid. Protoporphyrin IX or hemin equally satisfied the porphyrin requirements of *P. gingivalis* and *Bacteroides forsythus*, whereas ferrous sulfate was more efficiently used as a source of iron than was hemin. The cellular cohesiveness of *P. gingivalis* increased with high concentrations of hemin in the growth medium. *Prevotella intermedia*, *B. forsythus*, and several strains of *P. gingivalis* were more fastidious and required a protein or serum supplement to grow in medium OMIZ-W1.

Dental plaque, an integral though variable part of the healthy human oral cavity, can become the center of pathological developments, leading to various dental and periodontal diseases (4). The relative abundances of bacterial species in plaque change by several orders of magnitude between periodontally healthy and diseased states. Since proliferation of resident species is the principal mechanism of plaque growth and maintenance, information on the nutritional requirements of oral bacteria is crucial for understanding and possibly controlling such shifts in population structure. The enormous spectrum of bacterial species represented in plaque is surprisingly constant, irrespective of plaque mass or the local periodontal health status. So far no single-acting pathogen has been identified as the specific cause of one or another form of periodontal disease (4); however, recognizable groups of oral bacteria, in particular, the large spirochetes (5) and the recently described pathogen-related oral spirochetes (8), have not been adequately evaluated because they cannot be cultivated. Improved understanding of the nutritional requirements of the other bacteria that grow in the plaque habitat should assist with future efforts to cultivate these particularly fastidious organisms as well.

This report describes a chemically defined medium, OMIZ (Oral Microbiology and Immunology Zürich)-W1, and the importance of some of its ingredients for the long-term proliferation of a wide range of species of oral anaerobes. Medium OMIZ-W1 supports representative strains of *Porphyromonas gingivalis*, *Treponema denticola*, *T. pectinovorum*, *T. socranskii*, and *T. vincentii*, none of which was hitherto cultivated in a defined medium.

MATERIALS AND METHODS

Bacteria. The following laboratory strains of oral bacteria were used throughout this investigation (unless indicated otherwise, the origins of the strains have been described previously [12, 13]): *Actinobacillus actinomycetemcomitans* FDC Y4, FDC 511, FDC 650, FDC 652, ATCC 29523, ATCC 29524, JP2 (M. Listgarten, University of Pennsylvania, Philadelphia), NCTC 9710, OMZ 302, OMZ 303, OMZ 341, OMZ 346, and OMZ 377; *Bacteroides forsythus* FDC 331, ATCC 43037, OMZ 408, OMZ 471, and OMZ 472; *Bacteroides gracilis* FDC 1084; *Campylobacter concisus* FDC 484 and FDC 569; *Campylobacter sputorum* NCTC 10355; *Capnocytophaga gingivalis* ATCC 33624; *Capnocytophaga ochracea* ATCC 27872; *Capnocytophaga sputigena* ATCC 33612; *Eikenella corrodens* OMZ 342; *Eubacterium brachy* D6B.23 (G. Sundqvist, University of Umea, Umea, Sweden); *Eubacterium nodatum* ATCC 33099; *Eubacterium timidum* ATCC 33094; *Fusobacterium nucleatum* FDC 364 and FDC 373; *Haemophilus aphrophilus* HK 310, HK 315, HK 322, HK 329, NCTC 55906, OMZ 304, OMZ 307, and OMZ 384; *Haemophilus parainfluenzae* HK 47 and HK 95; *Haemophilus paraphrophilus* HK 159 and OMZ 545; *Porphyromonas gingivalis* W83, W50, B262 (G. Sundqvist), 274 (G. Sundqvist), OMZ 409, OMZ 470, OMZ 479, and OMZ 482; *Prevotella intermedia* OMZ 248; *Selenomonas sputigena* OMZ 317, FDC D19B-28, and FDC 1304; *Treponema denticola* CD-1, LL 2513, LL 2519, and 51B2 (all *T. denticola* strains were from M. Listgarten); *Treponema pectinovorum* ATCC 33768; *Treponema socranskii* ssp. *buccale* ATCC 35534; *Treponema socranskii* ssp. *paredis* ATCC 35535; *Treponema socranskii* ssp. *socranskii* ATCC 35536; *Treponema vincentii* LA-1, N9, and Ritz A (all *T. vincentii* strains were from M. Listgarten); *Wolinella curva* Ba 13a-g (G. Sundqvist) and OMZ 453; and *Wolinella recta* ATCC 33238 and OMZ 448. The bacteria were maintained in liquid OMIZ-W1 (or its precursors) by 1:1,000 dilution with fresh medium at least once per week; when necessary, medium was supplemented with 5% fetal calf serum (FCS; GIBCO,

Basel, Switzerland) or 100 mg of asialofetuin (AsF; Sigma, St. Louis, Mo.) per liter.

Medium OMIZ-W1. The composition of the chemically defined medium OMIZ-W1 (in milligrams per liter, unless indicated otherwise [for the four volatile fatty acids]) was as follows: L-alanine, 45; L-arginine, 174; L-asparagine, 150; L-aspartic acid, 133; L-cysteine hydrochloride, 352; L-glutamine, 680; L-glutamic acid, 294; glycine, 75; L-histidine, 620; L-isoleucine, 131; L-leucine, 131; L-lysine hydrochloride, 182; L-methionine, 149; L-ornithine hydrochloride, 168; L-phenylalanine, 165; L-proline, 115; L-serine, 525; L-threonine, 119; L-tryptophan, 102; L-tyrosine, 90; L-valine, 117; $CaCl_2 \cdot H_2O$, 147; $NaHCO_3$, 1,000; KCl, 968; $MgSO_4 \cdot 7H_2O$, 250; $NaH_2PO_4 \cdot H_2O$, 140; NH_4Cl, 1,600; $CuSO_4$, 0.000798; $MnSO_4 \cdot H_2O$, 0.0169; $ZnSO_4 \cdot 7H_2O$, 0.287; $FeSO_4 \cdot 7 H_2O$, 2,780; Na_2SeO_3, 0.0173; $NiSO_4 \cdot 6H_2O$, 0.000131; $SnCl_2 \cdot 2H_2O$, 0.000118; $NaVO_3$, 0.000610; $(NH_4)_6 Mo_7O_{24} \cdot H_2O$, 0.0124; cholesterol, 1; lecithin [L-α-phosphatidylcholine(β-oleoyl-γ-palmitoyl)], 10; calcium D-(+)-pantothenate, 5; choline chloride, 50; *myo*-inositol, 50; thiamine dichloride, 5; thiamine pyrophosphate, 25; pyridoxal hydrochloride, 5; pyridoxal phosphate, 5; D-(+)-biotin, 0.05; folic acid, 0.05; folinic acid, calcium salt, 1; nicotinamide, 0.5; nicotinic acid, 1; riboflavine, 0.01; vitamin B_{12}, 0.05; β-nicotinamide adenine dinucleotide, 1; coenzyme A, sodium salt, 1; flavin adenine dinucleotide, 1; 2-mercaptoethanesulfonic acid, sodium salt, 10; D,L-α-lipoic acid, 1; hemin, 0.125; 1,4-dihydroxy-2-naphthoic acid (DHNA), 0.408; D-glucose, 2,000; D-fructose, 800; D-maltose, 800; ascorbic acid, 1,000; *N*-acetylmuramic acid, 25; citric acid, trisodium salt, 200; *N*-acetylglucosamine, 200; D-mannitol, 800; D-glucuronic acid, sodium salt, 800; D-galacturonic acid, 800; isobutyric acid, 10 μl/liter; 2-methylbutyric acid, 10 μl/liter; valeric acid, 10 μl/liter; isovaleric acid, 10 μl/liter; pyruvic acid, sodium salt, 550; fumaric acid, disodium salt, 500; formic acid, sodium salt, 300; D,L-lactic acid, sodium salt, 560; hypoxanthine, 1.4; uracil, 1.1; thymidine, 2.4; *N*-(2-acetamido)-2-aminoethanesulfonic acid, 1,822; D,L-carnitine, 200; putrescine 2 HCl, 5; phenol red, sodium salt, 10; and L-valyl-L-lysine hydrochloride, 2.8. Analytical-grade chemicals were obtained from Aldrich (Milwaukee, Wis.), Bachem AG (Bubendorf, BL, Switzerland), Fluka (Buchs, SG, Switzerland), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), and Sigma. Medium OMIZ-W1 was prepared in a double concentration without the following compounds: $NaHCO_3$, ascorbic acid, $FeSO_4$, cholesterol, lecithin [L-α-phosphatidylcholine(β-oleoyl-γ-palmitoyl); Serva]; hemin, and DHNA (Aldrich). All other compounds, with the exceptions of $CaCl_2$, trace metals, vitamins, and volatile fatty acids, were weighed out in the order listed above, dissolved in double-distilled water, and adjusted to pH 6.9 with NaOH. To this solution were added $CaCl_2$ (dissolved in a small amount of water); trace metals (from the following three 1,000× stock solutions in 10 mM HCl: Cu-Mn-Zn; Se; V-Mo-Ni-Sn), the four volatile fatty acids, and mixed vitamins (1,000× neutral aqueous stock solution) and α-lipoic acid (10,000× stock solution in 2-mercaptoethanol–ethanol [1:9]). After the addition of water to volume, the 2× medium (still without $NaHCO_3$, ascorbic acid, $FeSO_4$, cholesterol, lecithin, hemin, and DHNA) was filter sterilized by nitrogen gas pressure through 0.22-μm-pore-size GA-8-Supor membrane filters (Gelman, Ann Arbor, Mich.) and was stored in closed bottles at 4°C. The 2× medium was stable for at least 6 months. Working medium was made by diluting the 2× medium with 1 volume of water; this was followed by the addition of the following missing ingredients from their respective stock solutions: $NaHCO_3$ (1% of a 10% solution), ascorbic acid (1% of a 10% solution, neutralized with NaOH before filtration), $FeSO_4$ (0.1% of a 10 mM solution in 10 mM HCl), cholesterol (0.1% of a 1-mg/ml ethanolic solution), lecithin (0.1% of a 10-mg/ml ethanolic solution), hemin (0.1% of a 0.2 mM solution in 10 mM NaOH), and DHNA (0.1% of a 2 mM ethanolic solution). Stock solutions of $NaHCO_3$ and $FeSO_4$ were kept at room temperature and 4°C, respectively; all other stock solutions were stored at −20°C. Complete OMIZ-W1 medium could be kept in closed bottles (under air) at 4°C for at least 4 weeks.

Most experiments were performed in semisolid cultures in 24-well multidishes (Nunc, Roskilde, Denmark). Each well was preloaded with two 25-μl additions of test compounds at 40-fold concentrations, to which was then added 1 ml of test medium containing the relevant bacteria. Test medium was prepared at 42°C with 0.5% agarose (Fluka) in lieu of water to bring the 2× medium to the working concentration. The relevant test compounds were omitted or added during preparation of the test medium. Medium preparation and inoculations were performed in ambient air in a laminar flow hood. After gelling of the agarose, cultures were incubated anaerobically at 36°C in BBL GasPak System jars, and growth was estimated visually with respect to colony number, colony size, and colony appearance by using a dissecting microscope.

RESULTS

The chemically defined medium OMIZ-W1 supported the long-term proliferation of most of the strains listed in Materials and Methods (see also below). Not all of these strains, however, were tested at all stages of the development of OMIZ-W1, which was a continuation of the medium used previously (13). Many changes in medium composition that were found to expand the spectrum of oral anaerobes that could grow under defined conditions were made sequentially and were not comprehensively reevaluated in the final medium. The high concentrations of ascorbic acid and ammonium ions proved critical for the growth of *P. gingivalis*, *T. denticola*, *T. pectinovorum*, *T. socranskii*, and *T. vincentii* and were not inhibitory toward other bacteria. The addition of glucuronic and galacturonic acids was required for the proliferation of *T. pectinovorum* (10), maltose promoted the growth of *T. socranskii*, lactic acid stimulated proliferation of *P. gingivalis*, flavin adenine dinucleotide improved the growth of *T. denticola* and *T. vincentii*, citric acid promoted the growth of *F. nucleatum*, pyridoxal phosphate was required for the growth of *T. pectinovorum*, and lecithin was essential for the growth of *T. vincentii*. Coenzyme A eliminated the requirement for *N*-acetylated carbohydrates of *T. vincentii* but not the *N*-acetylmuramic acid requirement of *B. forsythus* (14); among the test strains, the requirement for hypoxanthine, thymidine, and uracil differed widely, with *T. vincentii* requiring all of these compounds (Table 1).

Serum and AsF. A number of laboratory strains of oral anaerobes were still unable to proliferate in OMIZ-W1, and they required supplementation with FCS for growth (Table 2). For many other strains, FCS was highly stimulatory but not essential. In contrast, growth of all three strains of *T. socranskii* was completely inhibited by FCS as well as by several batches of horse serum and human serum at concentrations of as low as 0.5%. While searching for a less complex substitute for FCS, it was observed that 100 mg of AsF per liter promoted the growth of *B. forsythus* at least as

TABLE 1. Requirements for hemin, NAD, polyamines, thymidine, uracil, and hypoxanthine of 11 species of oral anaerobes

Species	No. of strains	Requirement for the following compound[a]					
		Hemin	NAD	Polyamine[b]	Thymidine	Uracil	Hypoxanthine
A. actinomycetemcomitans	13	−	−	?	−	+	+
B. forsythus[c]	4	+	?	−	−	−	−
H. aphrophilus	8	−	−	?	−	+	−
H. paraphrophilus	2	−	+	?	−	−	−
H. parainfluenzae	2	−	+	?	−	+	−
P. gingivalis	4	+	?	−	−	−	−
P. intermedia[c]	1	+	?	−	−	+	+
T. denticola	1	−	?	−	−	−	−
T. pectinovorum	1	−	?	+	−	−	−
T. socranskii	3	−	?	+	−	−	+
T. vincentii	1	−	?	+	+	+	+

[a] The compounds were tested by elimination of each one from OMIZ-W1. −, compound not required for colony formation; +, compound essential for colony formation; ?, specific requirement for compound not tested.
[b] Polyamine is usually putrescine, as in OMIZ-W1, but in some earlier studies spermine at 5 mg/liter was also included.
[c] The organisms were grown in the presence of 100 mg of AsF per liter.

TABLE 3. Effects of different vitamin K precursors on the proliferation of different strains of *P. gingivalis*

Strain	Growth in medium OMIZ-W1 without DHNA, supplemented with[a]:				
	Water[b]	Menadione[c]	DHNA[d]	1,4-Naphthoquinone[e]	Shikimic acid[f]
W83[g]	+	+	++	+	+
W50,[g] OMZ 482[g]	+	+	+	+	+
OMZ 470[g]	−	−	++	+	−
OMZ 470[h]	−	−	++	+	−
OMZ 409[h]	−	+	++	+	−
B 262,[i] 274[i]	−	+	++	+	−

[a] Growth was rated from no growth (−) to maximal growth (++) according to colony size and number.
[b] Water control.
[c] Menadione was used at 3×10^{-6} M.
[d] DHNA was used at 2×10^{-6} M.
[e] 1,4-Naphthoquinone was used at 3×10^{-6} M.
[f] Shikimic acid was used at 3×10^{-6} M.
[g] In OMIZ-W1 without DHNA.
[h] In OMIZ-W1 without DHNA but supplemented with 100 mg of AsF per liter.
[i] In OMIZ-W1 without DHNA but supplemented with 5% FCS.

well as 5% FCS did (15), but for other strains AsF was not an adequate replacement for FCS (Table 2).

Vitamin K. Menadione is routinely added to culture media for black-pigmented *Bacteroides* spp. since many strains within this heterogeneous group of oral anaerobes are vitamin K dependent. However, this requirement for vitamin K seems not only to differ between strains but also to depend on the composition of the medium (6), whereas it does not correlate with virulence (3). The results summarized in Table 3 confirmed the differences in vitamin K responsiveness between different strains of *P. gingivalis*; however, no relaxation of the vitamin K requirement was detected when OMIZ-W1 was supplemented with AsF, FCS, succinic acid (5 mM), and/or estradiol (10^{-5} M). The vitamin K precursor DHNA, but not 1,4-naphthoquinone, was considerably more active in promoting the growth of *P. gingivalis* than was menadione, while shikimic acid was completely inactive in promoting the growth of *P. gingivalis*. The stimulatory effect of DHNA on *P. gingivalis* W83 was principally an increase in colony number rather than colony size. The pronounced DHNA requirement of *P. gingivalis* OMZ 470 limited its growth even on conventional blood agar plates. Since its isolation from mixed colonies with an unidentified species, *P. gingivalis* OMZ 470 has been maintained by cocultivation on blood agar plates with *B. forsythus* OMZ 408, under which conditions it grows slowly and with delayed pigmentation (12). Supplementation of blood agar plates with 10^{-5} M DHNA allowed rapid growth and pigment formation by OMZ 470 in pure culture (data not shown).

TABLE 2. Effects of AsF and FCS on the proliferation of some anaerobes in medium OMIZ-W1

Strain tested for growth	Growth in medium OMIZ-W1 supplemented with[a]:		
	OMIZ-W1[b]	AsF[c]	FCS[d]
B. forsythus FDC 331, ATCC 43037, OMZ 408, OMZ 471, and OMZ 472	−	+	+
E. brachy D6B.23	+	+	+++
E. nodatum ATCC 33099	+	+	+++
E. timidum ATCC 33094	+	+	+++
P. gingivalis W50, W83, OMZ 470 and OMZ 482	+	++	+++
P. gingivalis OMZ 409 & 479	−	+	++
P. gingivalis B 262, 274	−	−	+++
P. intermedia OMZ 248	−	+	+++
T. denticola CD-1	+	+	++
T. pectinovorum ATCC 33768	+	+	+
T. socranskii ssp. *buccale* ATCC 35534	+	+	−
T. socranskii ssp. *paredis* ATCC 35535	+	+	−
T. socranskii ssp. *socranskii* ATCC 35536	+	+	−
T. vincentii LA-1	+	+	++
W. recta ATCC 33238	+	+	+

[a] Growth was rated from no growth (−) to maximal growth (+++) according to the number and sizes of the colonies formed from the same inoculum.
[b] Medium OMIZ-W1 without supplement.
[c] Medium OMIZ-W1 with 100 mg AsF per liter.
[d] Medium OMIZ-W1 with 5% FCS.

Hemin. The importance of hemin for the growth as well as the virulence of black-pigmented *Bacteroides* spp. has been amply demonstrated in studies in which complex media are used (6). This is confirmed by the results summarized in Table 1, with representatives of 11 species of oral anaerobes grown in medium OMIZ-W1. In addition to *P. gingivalis* and *P. intermedia*, growth of the nonpigmenting *B. forsythus* was also strictly dependent on hemin. The addition of AsF, which was required in the tests with *B. forsythus*, *P. intermedia*, and some *P. gingivalis* strains, did not alleviate the hemin requirement of those *P. gingivalis* strains (W50, W83, OMZ 470, and OMZ 482) which could be tested in the absence of AsF. However, the addition of hemin was not required for the growth of any strain when OMIZ-W1 was supplemented with FCS.

The specificity of the hemin requirement was further analyzed by using four AsF-independent strains of *P. gingivalis*. These bacteria had an absolute requirement for hemin, such that no microcolony formation (indicative of residual

growth) was observed even when the inoculum (usually no more than 0.01% [vol/vol]) had been grown with 5×10^{-6} M hemin. The addition of succinic acid (5 mM) and/or estradiol (10^{-5} M) or 1% (vol/vol) OMIZ-W1 medium conditioned by the growth of *W. recta* ATCC 33238 did not affect the hemin requirement of *P. gingivalis*. However, protoporphyrin IX, the iron-free precursor of hemin, proved to be a somewhat more efficient growth promoter than hemin; the lowest dose that supported colony formation by *P. gingivalis* (or by *B. forsythus* in the presence of AsF) was 1×10^{-8} M for protoporphyrin IX and 3×10^{-8} M for hemin, and growth was maximally stimulated by both compounds at concentrations of between 1×10^{-7} and 1×10^{-6} M. Concentrations of both porphyrins in excess of 5×10^{-6} M were markedly inhibitory toward these species as well as toward a number of other oral anaerobes tested.

Protoporphyrin IX could mimic the growth-promoting effects of hemin, but only in the presence of at least 10^{-7} M ferrous sulfate. Hemin proved to be a less efficient source of iron than equimolar ferrous sulfate at concentrations of up to 10^{-5} M. At concentrations of hemin exceeding 5×10^{-6} M, *P. gingivalis* growing in soft agar OMIZ-W1 medium formed tight colonies, likely because of increased cell cohesiveness. This effect of hemin was not observed at any concentration of protoporphyrin IX. If it is operative in vivo, it might serve to localize *P. gingivalis* to areas of high inflammation and regular bleeding and may be due to increased fimbriation in the presence of excess hemin (7).

Volatile fatty acids. All oral treponemes included in this study were strictly dependent on either isobutyric acid or 2-methylbutyric acid, but they required no additional volatile fatty acid. To improve the viability in stationary-phase cultures of *T. vincentii* LA-1 and *T. pectinovorum*, valeric and isovaleric acids were also included in the medium.

Polyamines. *T. denticola* CD-1 differed from the other treponemes by its ability to grow in the absence of exogenous polyamine (Table 1). The growth-promoting efficiencies of polyamines (5 mg/liter) toward *T. pectinovorum*, *T. socranskii*, and *T. vincentii* followed the order putrescine > diaminopropane > spermidine and spermine, whereas 5 mg of cadaverine per liter was inhibitory.

DISCUSSION

Disequilibration of plaque growth may lead to the development of periodontal diseases. To maintain periodontal health, it is therefore important to understand and sustain the mechanisms responsible for plaque stability. Such mechanisms are known to include metabolic competition, symbiosis, and antagonism. While the complexity of the plaque ecosystem precludes direct identification of such mechanisms, defined in vitro models could be used to identify candidate coupling pathways.

The development of medium OMIZ-W1 was a long, iterative process during which undefined, complex medium components were progressively replaced by synthetic chemicals and established laboratory strains of oral anaerobes were used as test organisms. Expansion of the range of species supported by the medium was always given priority over optimization of the performance of the medium for a single species and over the development of a minimal medium. Medium performance was evaluated in terms of bacterial proliferation to classify medium parameters as essential, stimulatory, permissive, or inhibitory.

Serum is a major determinant of the plaque milieu and is often used as a physiological substrate for in vitro culture. However, even within a single culture system the effects of serum may be multiple, and it is difficult to distinguish specific growth-promoting effects from anti-inhibitory activities or the effects of specific growth inhibitors from the inhibitory effects that arise from the sequestration of essential medium components. Therefore, the complete inhibition by serum of the proliferation of *T. socranskii* in OMIZ-W1 was unexpected and needs further investigation. In contrast, the serum dependence of *B. forsythus* may be adequately explained by its requirement for specific peptides (15).

As for *T. vincentii*, *T. denticola*, and *P. gingivalis*, it is still unclear what essential role serum played in previously used media (13); inclusion of lecithin and increased concentrations of ascorbic acid and ammonium chloride is needed for the growth of these organisms, although neither ascorbic acid nor ammonium ions are present in FCS in comparable concentrations (1), and lecithin was required only by *T. vincentii*. The lecithin requirement of *T. vincentii* LA-1 was not observed when valyl-lysine was added to the medium at a concentration of 10^{-5} M. Both *T. vincentii* LA-1 and *T. denticola* CD-1 were thus maintained in the absence of long-chain fatty acids, despite their reported strict dependency on long-chain fatty acids in complex media (11). However, after several months, growth of the two treponemes in medium without lecithin but with valyl-lysine suddenly stopped, while proliferation of all other strains under investigation continued. A search for inhibitory contaminants in the water, chemicals, or glassware used for medium preparation failed to yield an explanation. Growth of *T. denticola* was subsequently restored when the glycine concentration was reduced from 10 to 1 mM. *T. vincentii* also showed this hitherto unseen sensitivity to glycine, but in addition, it again required lecithin for growth. This inconsistent growth behavior cannot be explained. In view of these results, however, valyl-lysine was maintained as an ingredient of OMIZ-W1. The metabolic fate of lecithin in cultures of *T. vincentii* has yet to be explored.

Neither *N*-acetylmuramic acid nor vitamin K precursors are synthesized by the human host, while *P. gingivalis* and *B. forsythus* each require one of these substances for growth in vitro. This could form the basis for a symbiotic association between these bacteria. Further analytical work should establish the identity and the producers of the linking metabolites which in vivo satisfy the DHNA requirement of *P. gingivalis* and the *N*-acetylmuramic acid requirement of *B. forsythus*. Moreover, these two organisms must compete for essential nutrients like hemin, which may be provided not only by the human host but also by members of the plaque microflora.

The example of hemin stresses the difficulties of drawing conclusions from results obtained in different experimental systems. *P. gingivalis* belongs to those oral anaerobes which originally attracted attention, because of the black pigment formed in their colonies on blood agar plates (6). Formation of the protoporphyrin-related pigment (9) has been proposed as an explanation for the hemin dependence of *P. gingivalis* (7); however, the proliferation and virulence of *P. gingivalis* were hemin dependent in medium without blood, in which pigment formation was never observed even with an excess of hemin (7). Although no evidence exists as to its in vivo production, the pigment of *P. gingivalis* has also been proposed to function as reservoir of iron and porphyrin (2). The exact concentrations and distribution to different species of iron and porphyrins in the plaque environment, and therefore their importance for community behavior in vivo, are not known. In vitro studies nevertheless serve to esti-

mate the range of metabolic pathways open to microorganisms that live in the oral cavity.

Work is in progress to compare the behaviors of oral anaerobes in pure and mixed cultures in the chemically defined medium OMIZ-W1. Such studies should be extended to recently isolated bacteria, should address the effects of major milieu parameters like pH and redox potential, and should include both the consumption of substrates and the release of metabolites. Eventually, such efforts will lead to the in vitro growth of presently uncultivatable bacteria (5, 8) and an improved understanding of plaque ecology.

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