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Isolation, Cultivation and Characterization of Flexibacteria

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SUMMARY

Eighty-five strains of aerobic microbes capable of gliding on solid substrata have been isolated from samples of marine and freshwater mud and sand collected in various localities around the world. Each strain has been characterized in about 40 tests by a number of morphological, nutritional, physiological and biochemical features.

INTRODUCTION

This is one of a series of papers on the general subject of the flexibacteria*, a group of common but generally neglected micro-organisms (see Soriano & Lewin, 1965). They are characterized by flexuous filaments, ranging from approximately 5 μm. to an indefinite length and approximately 0·5 to 1 μm. in width. They are capable of gliding on solid substrata, and thus are usually recognizable by the fimbriate margins of their colonies on agar. Members of this group can be distinguished from eubacteria by the relative flexibility of the cell walls, and by the absence of flagella and of endospores. They are apparently all Gram-negative. The flexibacteria are distinguishable from the filamentous blue-green algae—photosynthetic prokaryonts which they resemble in many ways—by the absence of chlorophyll and of phycobilin pigments. All strains of flexibacteria so far studied are heterotrophic. They are not, however, colourless: all of our strains, when grown in mass, can be seen to have a pink, orange, or yellow coloration attributable to the presence of one or more carotenoids.

Their taxonomy has, hitherto, been very unsatisfactory. In an attempt to remedy this in some measure, we collected a number of such gliding microbes, all aerobes, and studied their form, pigmentation, and other physiological and biochemical features in pure laboratory cultures. Our data are presented in this paper, summarized in Tables 2 to 6.

Mandel & Lewin (1969) obtained GC values (molar percentages of guanine+cytosine) for deoxyribonucleic acids extracted from these organisms. Their data, together with those presented in this paper, have been used by Fager (1969) for a recurrent group analysis, on the basis of which a classification of these gliding microbes has been proposed (Lewin, 1969*b*). The data have been independently evaluated by Colwell (1969), who reaches somewhat different taxonomic conclusions.

* These five papers on flexibacteria are published simultaneously. Nomenclature is dealt with in the paper by Lewin (1969) and all questions of priority in regard to names refer to that paper.

Origin and isolation of strains

Eighty-five strains were studied, of which 62 came from marine shores and 23 from freshwater habitats; ten were pure cultures kindly made available by various colleagues; the rest were isolated in this laboratory, or in the Department of Microbiology, University of Costa Rica, without prior enrichment, in order to decrease the selectivity of the media employed.

Thirty-five of the strains were isolated from samplings of nearby streams, ponds, hot springs, and various marine shores, usually streaked on agar within a few hours of collection when this was possible. Fifty other strains were isolated from samples of wet littoral mud or sand from seashores around the world, forwarded to us in small polyethylene bags by colleagues (see Table 1 and Acknowledgements). Most of the samples were received within 3 to 7 days of their dispatch. Upon receipt, the contents of each package were emptied into 5 ml. of sterile sea water in a capped test-tube, left for 2 to 4 days in dim (room) light at room temperatures (*c.* 24°), and then shaken and streaked on 1% agar medium containing 0·02% Tryptone. To suppress the growth and spreading of amoebae, myxomycetes and diatoms, Actidione (Upjohn Company, Kalamazoo, Michigan; 100 mg./l.) was usually incorporated into the media used for first plating, after it had been established that this concentration had no apparent inhibitory effect on any of the 30 flexibacterial strains then in our culture collection. (Use of this agent was suggested by the work of Zehnder & Hughes (1958), who indicated that it is much less inhibitory to Cyanophyta than to eukaryotic algae.) Plates were examined after 3 or 4 days, and every 2 or 3 days thereafter for 2 or 3 weeks. Organisms producing spreading colonies or showing other indications of gliding movement (Lautrop, 1965–67) were isolated by repeated streaking, usually from the margin of each colony, until a pure culture was obtained. (Colonies of this form have been well illustrated by Carlson & Pacha (1968), who regarded such organisms as myxobacteria). The isolation procedure was described in detail by Lewin (1965*b*). No precautions were taken to exclude, or on the other hand to supplement, the ambient light of the laboratories in which the isolations and characterization tests were made. Living cells from the colony margins were examined by phase-contrast microscopy at high magnification (×400). Only gliding microbes were selected and isolated in this way: all flagellate bacteria were rejected.

From many samples of mud, sand, or littoral debris we were unable to isolate any gliding microbe, probably in part because of adverse conditions of temperature and aeration during the postal journey. From some we isolated one or more. As far as possible we tried to avoid isolating duplicates from any one source. Many strains, detected on the original streaked plates, were lost before they could be isolated. Others died before we could develop a suitable medium and regime for maintaining them in culture; some were lost on the journey from Costa Rica to La Jolla. Certain strains were isolated too late to be conveniently subjected to some of the diagnostic tests; hence data on them are incomplete. However, we included for study as many of our isolates as we had available, and excluded only the single sporulating form mentioned below, and a few fastidious strains of *Saprospira albida* and *S. flammula* which proved too delicate for routine handling with the others (see Lewin, 1965*a*). The origins of all strains tested are listed in Table 1, on p. 158.

METHODS

After a considerable amount of preliminary experimentation, the following two media (1, 2) were generally found to be most satisfactory for the maintenance of marine and freshwater cultures respectively.

Medium 1 (for marine stock cultures). Filtered sea water, 1000 ml.; KNO_3, 0·5 g.; Na glycerophosphate, 0·1 g.; trace-element solution, 1·0 ml.; tris buffer (Sigma), 1·0 g.; Bacto-Tryptone (Difco), 5·0 g.; Bacto-Yeast Extract (Difco), 5·0 g.; adjusted to approximately pH 7·0 agar, if required, 10·0 g.

Medium 2 (for freshwater stock cultures). Distilled water, 1000 ml.; $MgSO_4.7H_2O$, 0·1 g.; KNO_3, 0·1 g.; $CaCl_2.2H_2O$, 0·1 g.; Na glycerophosphate, 0·1 g.; trace-element solution, 1·0 ml.; tris buffer (Sigma), 1·0 g.; thiamine, 1·0 mg.; cobalamin, 1·0 μg; casamino acids (Difco), 1·0 g.; glucose (added aseptically after autoclaving), 1·0 g.; adjusted to approximately pH 7·5 agar, if required, 10·0 g.

Filtered sea water could be replaced by a commercial sea-water substitute, such as 'Rila mix' (Utility Chemical Co., Paterson, New Jersey) or by a mixture of the following salts (g./l.): NaCl, 25·0; $MgSO_4.7H_2O$, 5.0; $CaCl_2.2H_2O$, 1·0; KCl, 1·0. The following trace-element solution was found satisfactory: soluble salts of B, Fe, Mn—each (as element) 0·5 mg./ml.; soluble salts of Co, Cu, Mo, Zn—each (as element) 0·01 mg./ml. Final concentrations of each element in the nutrient media were thus 0·5 and 0·01 mg./l.

In these media, growth rates and yields were satisfactory, and cultures retained viable cells for at least 2 or 3 weeks. Stock cultures, both in liquid media and on agar slants, were grown and kept at room temperatures. Higher temperatures (e.g. 30° or 35°) often promoted more rapid growth, but also caused earlier death and lysis, to which flexibacteria seem generally prone. Our cultures were maintained alive by weekly transfers incubated at room temperature, in medium 1 (for marine strains) or 2 (for freshwater strains). Since living cells of certain strains, at least, may be recovered after lyophilization, one set of cultures was lyophilized in the American Type Culture Collection, and another set was frozen in liquid nitrogen; see Sanfilippo & Lewin (1969).

Nutrition was studied in experiments as described by Lewin (1965*b*). Test-tubes, containing 3 ml. of medium, were supported in slanted racks gently shaken at about 75 oscillations/min. at an amplitude of 4 cm. Experimental cultures were ordinarily incubated in a thermostatic room at 30°. The nutrition of two strains (CR-124 and PSY), which initially did not grow at this temperature, was studied at 25°. Other incubation temperatures were used in certain experiments, as indicated below.

Details of various other discriminatory tests follow. In tests 5 to 10, 12, 19, 20 and 22, the media were inoculated by applying a droplet of suspension to the surface; in 11 and 13 to 15, they were inoculated by stabbing. They were incubated at 30° unless otherwise specified (as in tests 1 to 3, 8, 11, 17, 18 and 21). Results from tests 27 to 31 were not used in the evaluation of relationships, since they could not be easily interpreted or regularly reproduced, or since they seemed to reveal no distinctions of taxonomic value because all our strains behaved similarly.

1. *GC value.* Mole percentage of guanine+cytosine in the deoxyribonucleic acid. (See Mandel & Lewin, 1969.)

2. *Morphological examination.* Cultures were grown in liquid without shaking, and were examined approximately 48 hr after inoculation while still in or near the

exponential phase of growth. Morphology was generally studied with a Wild phase-contrast microscope at magnifications of 600 or 800 diameters.

No attempt was made to confirm the absence of flagella by specific staining techniques. However, no swimming forms have ever been seen by phase-contrast microscopy of wet mounts of any gliding microbe in this study.

3. *Electron-microscope examination of lysates.* Two-day-old cultures of each strain were examined by Dr R. E. Reichle for the presence of rhapidosomes (Lewin, 1963), using a simplified form of the procedure described by Reichle & Lewin (1968).

4. *Pigmentation.* Organisms were generally grown in darkness either in Petri plates on 2% agar medium (1 or 2 as appropriate) and collected at the peak of growth by scraping, or in shaken flasks of liquid medium and harvested by centrifugation. Pigments were extracted in ethanol at $-20°$ under nitrogen, in darkness, for at least 24 hr. Extracts were filtered through 'celite' (diatomite) on sintered glass filters. After evaporation of the ethanol in a slow current of nitrogen over a warm bath (approximately 50°), the pigments were transferred to a small volume of *n*-hexane. The crude extracts were examined spectrophotometrically in a Bausch and Lomb Spectronic 505 recording spectrophotometer. Six types of curves were distinguished (see Fig. 1 to 6).

For the purposes of diagnosis, the pigments can be separated and provisionally identified by their differential migration rates in 1-dimensional chromatograms on (*a*) alumina paper (Schleicher and Schuell No. 667 or 288), using as solvent a mixture of benzene + ethyl acetate + isopropanol (10 + 10 + 1, v/v/v; see Fig. 7) and (*b*) silica paper (S. and S. No. 287 or 966), using as solvent a mixture of acetone and petrol ether (9 + 1 v/v; see Fig. 8).

5. *Penicillin tolerance.* Penicillin G (Calbiochem), sterilized by filtration, was incorporated at various concentrations into 1% agar preparations of medium 1 or 2 in Petri dishes. Two to seven days after inoculation the plates were examined for evidence of growth or inhibition.

6. *Lauryl sulphate tolerance.* Plates were prepared with medium 1 or 2 containing 1% agar with or without 0·01% Na lauryl sulphate (Quadling, Cook & Colwell, 1964), and 2 to 7 days after inoculation were examined for evidence of growth or inhibition.

7. *Salinity tolerance.* Plates of 1% agar medium containing KNO_3 0·5 g./l., Na glycerophosphate 0·1 g./l., tris buffer 1·0 g./l., trace-element solution 1·0 ml./l., Tryptone 1·0 g./l., and yeast extract 1·0 g./l., were prepared with distilled water, half-strength sea water, normal sea water, or double-strength sea water, adjusted to pH 7·2. Two to seven days after inoculation they were examined for evidence of growth or inhibition.

8. *Temperature limits for growth.* Replicate plates of a 1% agar medium, 1 or 2 as appropriate, were inoculated and incubated at room temperature (about 24°), at 35°, and at 40°. Growth was recorded after 4 to 7 days.

9. *Survival at +3°.* One-week-old cultures of each strain, grown at room temperatures, were kept refrigerated at +3°, and were tested for viability at intervals of 0, 1, 4 and 7 days by spotting on to medium 1 or 2, as appropriate. Those strains surviving at +3° for 1 week were recorded as positive.

10. *Survival at −196°.* A 1-ml. sample of each strain, grown for 2 weeks in its normal culture medium and adapted in 10% glycerol for 1 day, was immersed in liquid nitrogen (−196°) for 2 hr. It was then thawed and spotted on to medium 1 or 2, as

appropriate. Survival was revealed by visible growth appearing within 1 week (Sanfilippo & Lewin, 1969).

11. *Cellulose and carboxymethylcellulose digestion.* None of our strains clearly softened or otherwise digested native cellulose in the form of filter paper, powdered cellulose, or cellophan. (Strips of cigarette paper partly submerged in liquid mineral medium (1 or 2 as appropriate), showed partial or complete disintegration at the level of the meniscus by certain strains (notably B–A) not included in this survey, showing that the paper was potentially digestible.)

Three per cent sodium carboxymethyl cellulose (high-viscosity: General Biochemicals, Chagrin Falls, Ohio), dissolved in medium 1 or 2, and sterilized by autoclaving, set on cooling to about the firmness of 1 % agar. Separate tubes were inoculated with each strain, incubated at room temperature, and observed for 1 week. Liquefaction was taken as evidence of cellulase activity.

12. *Starch hydrolysis.* Plates were prepared containing 2·0 g. soluble starch/l. and 1 % agar in medium 1 or 2, sterilized by autoclaving. One to three days after inoculation, when the colonies showed appreciable growth, they were flooded with Lugol solution (iodine 0·3 %, KI 0·7 %). A pale halo provided evidence for extracellular amylase.

13. *Agar liquefaction.* One per cent agar 'deeps' of medium 1 or 2 were inoculated and incubated for 3 days at 30°. Agar liquefaction occurred in some cases; in certain others there was only appreciable softening and/or pitting of the surface.

14. *Alginate liquefaction.* Medium 1 or 2, without agar, supplemented with 3 % potassium alginate (Grade IKR, Kelco, San Diego, California), was dispensed in tubes; after autoclaving it set to a sloppy gel. Individual tubes were inoculated with each strain. Liquefaction, observed after about 7 days, was recorded as evidence for alginase activity.

15. *Gelatin liquefaction.* Medium 1 or 2, prepared with 10 % Difco gelatin, without agar, set after autoclaving to a gel. Individual tubes were inoculated with each strain. After a growth period of 3 to 5 days, the tubes were refrigerated at +3°. Failure to solidify indicated proteolytic activity.

16. *Litmus milk media.* Media were prepared with a mixture of skimmed milk (final concentration 50 %) and litmus (final concentration 1 %), each sterilized separately. To preclude coagulation, the milk was autoclaved at 120° for only 5 min. For the marine strains, the medium was supplemented with Rila 'sea-salt mixture', final concentration 4 %. Before inoculation, the tubes were incubated at 30° for a week to ensure that they were not contaminated. After inoculation, they were returned to the 30° incubator and examined at intervals for 2 weeks, observations being made for clotting, subsequent dissolution of the clots, acidification (indicated by the litmus turning pink) and reduction of the litmus (indicated by bleaching below the surface).

17. *Catalase production.* One-week-old agar slant cultures, on medium 1 or 2 as appropriate, were flooded with 10 % hydrogen peroxide. Copious evolution of bubbles was evidence for catalase activity.

18. *Hydrogen sulphide production.* Tubes containing 0·1 g. L-cysteine hydrochloride/l. (sterilized by filtration) in medium 1 or 2 were inoculated with the various strains. Strips of filter paper impregnated with lead acetate were folded over the lip of the tube, which was covered as usual with a glass 'Cencap' (Central Scientific Co.). Blackening of the paper strip, usually in less than a week, indicated H_2S production.

19. *Tyrosine degradation.* Plates were prepared containing a 1 % agar medium, 1 or 2, to which had been added 5·0 g. powdered L-tyrosine/l. (The medium was cloudy white due to undissolved tyrosine.) Three to seven days after inoculation the plates were examined for growth, for production of a coloured halo, varying from pink through red to black (evidence for a tyrosine oxidase), and for clearing of the opalescence.

20. *DOPA oxidase.* DL-3,4-dihydroxyphenylalanine (Calbiochem) (0·1 g./l.) was added to 1 % agar medium 1 or 2; after being autoclaved, the medium became grey. Poured Petri dishes were inoculated, incubated and examined after 4 to 7 days for growth and for the appearance of a darker halo and/or clearing around each colony site.

21. *Nitrate reduction.* The test was a modification of that described in the Difco Manual (1965), media being based on formula 1 or 2 as appropriate. To a week-old slant culture a few drops each of sulphanilic acid and α-naphthylamine reagent were added. A distinct pink or red colour, appearing within a few minutes, indicated the presence of nitrite, presumably formed by the reduction of nitrate.

22. *Carbon sources for growth.* The experimental procedures for flexibacteria are somewhat more complicated than they are for less fastidious bacterial genera such as *Pseudomonas.* The interpretation of many of the data on the utilization of carbon sources is open to question, since the majority of the organisms in the present investigation require other organic factors for growth, and for a number of strains these factors, present in Tryptone or yeast extract, have not yet been defined. It was, therefore, necessary to add low concentrations of complex organic growth supplements to the basal media (0·2 g. Casamino acids/l. for freshwater strains; 0·2 g./l. each of Tryptone and yeast extract for marine strains), and to seek evidence for stimulation of growth, beyond that shown in the controls, when various non-nitrogenous carbon compounds were added. The tests were made on agar medium in Petri plates. Sugars were autoclaved separately. We used at each substrate tested 1·0 g./l. and 5·0 g./l., and assessed growth stimulation subjectively 4 to 7 days after inoculation. In some cases, the higher concentration proved inhibitory to growth.

23. *Nitrogen sources.* The freshwater strains were tested in separate tubes, each containing 5 ml. of liquid medium of the composition given for medium 1 (except for the KNO_3 and Casamino acids), to which one of the following was added (1 g./l.): Tryptone, Casamino acids, sodium glutamate, potassium nitrate. Control tubes did not contain an added source of nitrogen. After inoculation, the cultures were constantly shaken at 30°. The marine strains were similarly studied in a synthetic-sea water medium (NaCl 25·0 g./l., $MgSO_4.7H_2O$ 5·0 g./l., $CaCl_2.2H_2O$ 1·0 g./l., and KCl 1·0 g./l.) supplemented with tris buffer (pH 7·2) 1·0 g./l., trace-element solution 1·0 ml./l., thiamine 1·0 mg./l., cobalamin 1·0 μg./l., and glucose (autoclaved separately) 1·0 g./l. Growth after 2 to 5 days was compared with that in the appropriate control tube, from which a fresh inoculation was made into a similar set of media. These were grown and evaluated as before, the object of this second subculture being to minimize the effects of carry-over of nitrogenous substances with the original inoculum.

24. *Amino acid requirements.* The specific amino acid requirements of certain selected strains were investigated as described for *Saprospira thermalis* (Lewin, 1965*b*).

25. *Vitamin requirements.* The specific vitamin requirements of certain selected strains were studied as previously described (Lewin, 1965*b*).

26. *Nucleic acid factor(s).* Most species of *Saprospira grandis,* when grown in other-

wise defined nutrient media without Tryptone or yeast extract, required the addition of a small quantity (*c.* 10 to 30 mg./l.) of a nucleic-acid hydrolysate, containing growth factor(s) not yet identified. More detailed studies of the nutrition of *S. grandis* will be described elsewhere.

27. *Anaerobic growth.* Stabs of each strain were made in semi-solid (0·25% agar) medium, 1 or 2 as appropriate, and incubated at 30°. Observations were made at intervals for about 2 weeks, to determine at where, at or below the surface, growth was best.

28. *Chitin.* A 2% suspension of chitin (Eastman P 2064) in molten 1% agar medium 1 or 2 as appropriate was poured into plates and cooled. Cultures were spotted on the surface and incubated at room temperatures for 1 to 2 weeks. Clearing of the turbidity indicated digestion of the chitin.

29. *Indole.* Kovacs reagent, 0·2 ml. (Difco Manual, 1965), was added to 4 ml. of a 24 hr culture growing in medium 1 or 2, as appropriate, containing Tryptone (10·0 g./l.). A red colour at the interface indicated the formation of indole.

30. '*Vibriostat*'. A 0·9% aqueous solution of 'Vibriostat' (pteridine compound 0/129, kindly supplied by Dr G. L. Bullock, U.S. Fish and Wildlife Service, Kearnysville, W. Va., U.S.A.; see Shewan, Hodgkiss & Liston, 1954) was sterilized by filtration and added to sterile 1% agar preparations of medium 1 or 2, as appropriate, to give the following final concentrations (mg./ml.): 0·9, 0·09, 0·009, 0. After each strain was spotted on the agar, the plates were incubated at 30° and examined at intervals for about a week for signs of growth.

31. *Ammonia production.* On a white tile one drop of a 48-hr culture was mixed with one drop of Nessler solution. A deep yellow colour would have indicated ammonia production by the organisms. (See Peltier, Georgi & Lindgren, 1959.)

Light microscope observations

As prime and sole requirements for consideration in this survey, all of the strains were capable of some degree of motility by gliding but were devoid of flagella (*cf.* Lautrop, 1965–67). In other words, they were motile, though they did not swim: they may be described as *repent* but not *natant*. The more elongate forms were also capable of active bending. The filaments of all strains are about 0·5 to 1 μm. in width, and ranged from five to many hundreds of μm. in length. (Tabulated lengths are only approximate, because of the considerable range of filament lengths encountered even within a single culture.) In some strains the longer filaments consisted of many cellular units, delimited by cross walls, but unmarked by constrictions like those in certain colourless types such as *Vitreoscilla* spp. No extended tapering, no attachment holdfast, and no other cellular differentiation was observed in these strains, which thus differed from *Leucothrix* and *Thiothrix*. (In some strains the cells were seen to taper somewhat at the ends; this was evident even in living mounts examined by phase-contrast microscopy. However, since it is difficult to distinguish the strains as 'tapered' or 'not tapered', and since this feature seemed to vary even within a single culture as it aged, tapering at the ends of the cells has not been counted among the distinctive features of taxonomic value.) They were generally less refractile than most eubacteria (*cf.* Stanier 1947). Under some circumstances most strains tended to produce lateral inflations or spherical bodies, probably akin to sphaeroplasts; these were especially common in old cultures, and probably resulted from unfavourable or deteriorating cultural conditions.

None of the strains forms true endospores. (One of the isolates (B–A) formed abundant refractile microcysts in every colony examined, though without any apparent differentiation of a fruiting structure; it was capable of digesting cellulose paper; and it was the only one of the strains in this survey which apparently lacked a carotenoid pigment. The GC value, 56·5% (Mandel & Lewin, 1969) was much larger than that of any of the other strains examined here. For these reasons we did not consider it further in this survey.)

RESULTS

All of the flexibacteria studied here appear to be obligately aerobic, growth in stab cultures being restricted to the uppermost 1 to 3 mm. All strains are Gram-negative. All are some shade of yellow, orange, pink or red, and all contain one or more carotenoid pigments. Four major pigment groups have been distinguished on the basis of their absorption spectra and their behaviour on Celite diatomite columns. All but three of our strains can be allocated to one or another of these groups. They have been characterized by Fox & Lewin (1963) and by Aasen & Jensen (1966*a*,*b*,*c*). Two strains (assignable to one species) produce pigment of a fifth type, and one strain a sixth type. Absorption curves of the crude total lipid extracts in *n*-hexane, containing the carotenoid pigments, are given for representatives of each of these six pigment types (Fig. 1–6). Their absorption maxima in *n*-hexane are shown with other data in Table 7, on p. 170.

The results of the specific discriminatory tests are summarized in Tables 2 to 6. For convenience, the organisms have been subdivided in separate tables according to the genera in which we ultimately placed them: *Cytophaga*, *Flexibacter*, *Microscilla*, *Flexithrix*, *Saprospira* and *Herpetosiphon* (Lewin, 1969*b*). The tables summarize various physical and physiological features, enzymic activities, and nutritional characteristics, in that order. In each table, the columns are numbered to correspond with the tests described in the Methods section. Explanatory notes for each column of the tables precede the tables.

Other tests, briefly described in the Methods section, revealed no significant differences among the strains or were equivocal for experimental reasons. They included tests 27 (anaerobiosis), 28 (chitin digestion) and 29 (indole formation), which were all negative, and 30, in which growth of all strains was inhibited by Vibriostat at a concentration of 0·9 mg./l. The results of test 31 (ammonia production) were variable and not reproducible in the various nutrient media used. Dr P. Grilione (personal communication) tested several of our strains for precipitation in the presence of rabbit antisera prepared against *Myxococcus fulvus*, *M. virescens*, *M. xanthus*, *Archangium* sp. and *Polyangium* sp. No positive reactions were detected under conditions in which homologous antigens were precipitated (Grilione, 1968). Flexibacteria tested included: *Flexibacter aurantiacus* (CR-134, DWO); *F. roseolus* (CR-141, CR-155); *F. elegans* (NZ-1); *F. flexilis* (BA-24, CR-63, CR-81, FLE); *F. giganteus* (CR-103, CR-104); *F. ruber* (GEY); *F. sancti* (BA-3, BA-23, MIC); *Herpetosiphon geysericolus* (GC-42); *Saprospira thermalis* (BEG).

DISCUSSION

There was a close correlation between the salinity of the original habitats and the salt tolerance of the strains in culture: few marine species tolerated transfer to fresh-

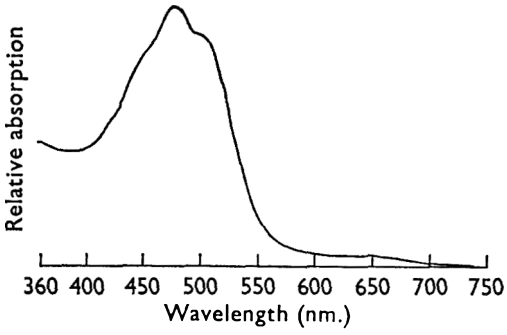

Fig. 1

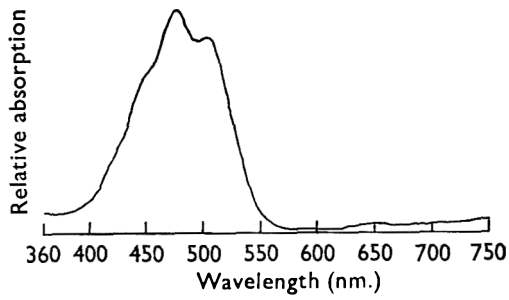

Fig. 2

Fig. 1. Absorption curve of pigment extract, type I, from *Flexibacter ruber* (strain GEY), in *n*-hexane.

Fig. 2. Absorption curve of pigment extract, type II, from *Saprospira thermalis* (strain BEG), in *n*-hexane.

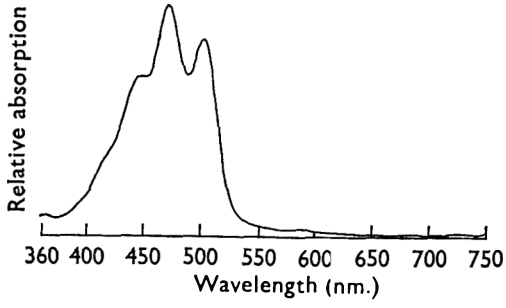

Fig. 3

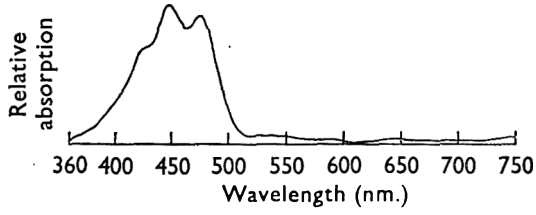

Fig. 4

Fig. 3. Absorption curve of pigment extract, type III, from *Flexibacter elegans* (strain NZ-1), in *n*-hexane.

Fig. 4. Absorption curve of pigment extract, type IV, from *Cytophaga lytica* (strain B-9), in *n*-hexane.

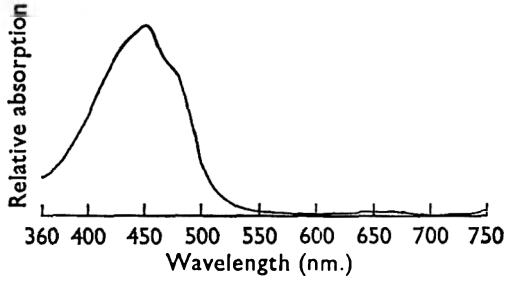

Fig. 5

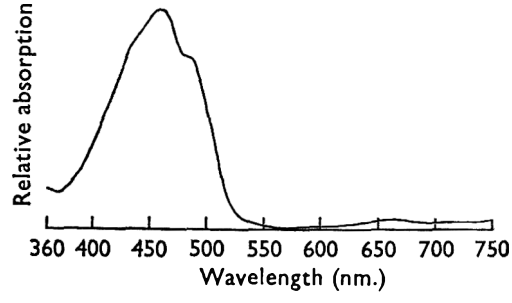

Fig. 6

Fig. 5. Absorption curve of pigment extract, type V, from *Flexibacter aurantiacus* (strain DWO), in *n*-hexane.

Fig. 6. Absorption curve of pigment extract, type VI, from *Cytophaga latercula* (strain SIO-1), in *n*-hexane.

water media, and few freshwater species grew in media prepared with sea water, though there were some exceptions in each direction. The strains isolated from hot springs were not markedly thermophilic, probably because we had made no attempt to isolate them at elevated temperatures. It is doubtful whether other valid correlations can be made between the locality of origin and the nature of the strains isolated, in view of the vicissitudes of the journeys between the points of collection and isolation. Those isolated from the vicinities of the Scripps Institution of Oceanography, California, and the Department of Microbiology at San José, Costa Rica probably include certain less robust forms which might not have survived several days in the postal systems across the world; but we have no experimental information beyond that presented here in the tabulation of physiological data.

It is of interest that a number of the strains examined in this survey proved to require certain amino acids in the nutrient medium. The presence of such compounds in natural waters has been reported by various investigators, but in concentrations considerably lower than those we used. Thus Briggs (1962) reported total free amino acids in lake waters to be in the region of a few micrograms per litre; Degens, Reuter & Shaw (1964) found as much as 3 mg./g. (dry weight) in marine sedimentary mud. It would be interesting to have determinations for hot-spring waters, but no reference was found to such data. (Sarbutt, 1964, recorded only inorganic solutes.) In media containing a limiting amino acid at concentrations less than 5 to 10 mg./l., growth of most auxotrophic strains was not appreciably better than in the controls without the essential factor. We may conclude that the concentrations of free amino acids found in natural waters would not suffice for appreciable growth of such fastidious flexibacteria unless the organisms are able to accumulate them effectively. One is, therefore, led to conclude that the majority of the strains requiring specific amino acids for growth obtain these compounds by the action of extracellular enzymes on proteins in their environment. Their proteolytic activities have been demonstrated in the present study (see Tables 3 to 6, in which are summarized data on gelatin digestion, milk clotting and clot lysis, etc.). Correlated with this must be the ecological roles of such strains. Thus *Chondrococcus columnaris*, a myxobacterial pathogen of fish (Anacker & Ordal, 1959) is presumably able to break down tissues in sites of infection. Certain myxobacteria have also been recorded as capable of digesting algae (Geitler, 1924) or other bacteria (Kononenko, 1937; Snieszko, McAllister & Hitchner, 1942; Singh, 1947).

Dworkin (1966) suggested the interesting possibility that gliding motility might be of selective advantage to organisms dependent on the location and digestion of insoluble substrates for their nutrition.

The colour of the organisms seen in masses seems to be a relatively constant character. In the course of laboratory subculturing for several years, we have not observed any case of a major change in the general colour of any strain, though the intensity of pigmentation is in many strains under the influence of light (see Lewin, 1965*b*, 1969*a*; *cf. Myxococcus xanthus*, Burchard & Dworkin, 1966) or nutritional factors (Lewin, 1965*b*).

It is worth emphasizing that none of our strains was tinged with green, although greenish myxobacterium-like organisms have been reported by Winogradsky (e.g. *Cytophaga tenuissima*, Winogradsky, 1929) and by Imshenetsky & Solntseva (1936). It seems to us likely that the reported greenish colour was in some cases produced by

a combination of yellow carotenoid pigments with greyish extracellular melanins, and, in others a consequence of refraction by patches of aligned cells. If, in fact, real green pigmentation occurs among these gliding forms, we should envisage the possibility that it may be attributable to chlorophylls, or even to mixtures of carotenoids with biliproteins. Photosynthetic bacteria, which contain bacteriochlorophylls and do not evolve oxygen during photosynthesis, are normally flagellate and presumably eubacterial. Blue-green algae, which typically contain chlorophyll and a red or blue bilin pigment, evolve oxygen in photosynthesis. Intermediates are unknown; but they might be worth looking for among the green gliding myxobacteria, if such exist.

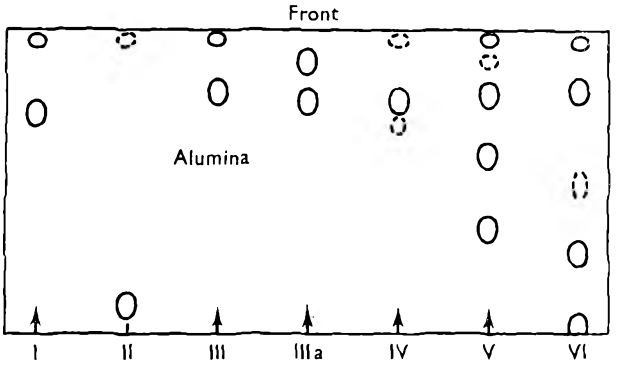

Fig. 7. 1-dimensional chromatograms of crude carotenoid extracts on alumina paper (Schleicher and Schuell no. 288), using as solvent a mixture of benzene+ethyl actate+ *iso*propanol (10+10+1 v/v). The following representative types were selected:

Pigment type	Species (see Lewin, 1969*b*)	Strain
I	*Flexibacter roseolus*	CR-141
II	*Saprospira thermalis*	BEG
III	*S. grandis*	WH
IIIa	*S. toviformis*	A-1
IV	*Flexithrix dorotheae*	QQ-3
V	*F. aurantiacus*	DWO
VI	*Cytophaga latercula*	SIO-1

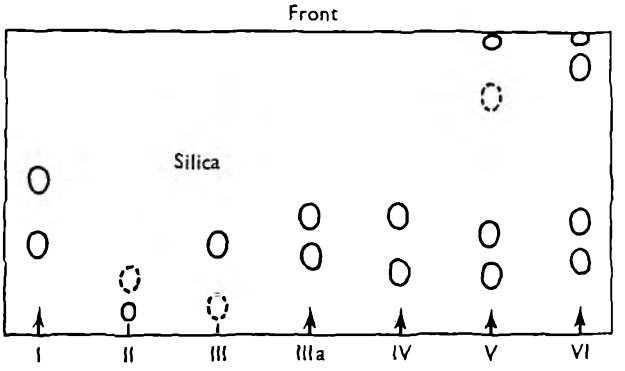

Fig. 8. 1-dimensional chromatograms of crude carotenoid extracts on silica paper (Schleicher and Schuell no. 966), using as solvent a mixture of acetone and petrol ether (30–60° b.p.) (9+1 v/v). For key to pigment types, etc., see legend to Fig. 7.

We acknowledge with thanks the kindness of the many persons who supplied cultures or mud samples, from a large proportion of which we were able to isolate fresh strains for this study (see Table 1). Dr Santos Soriano, of the University of Buenos Aires, offered valuable suggestions on the initial choice and design of the discriminatory tests. Mrs A. Sanfilippo helped with routine maintenance of the stock cultures and with many of the nutritional experiments throughout these studies. Dr R. E. Reichle examined the lysates for the presence of rhapidosomes.

Most of the preliminary studies on the carotenoid pigments were undertaken by Mrs Dorothy White, whose untimely death was a sad loss. Dr D. L. Fox and Mr G. Crozier provided expert guidance and assistance with the extraction and spectrophotometric analyses of these pigments, and Dr F. T. Haxo permitted us to use the Carey spectrophotometer in his laboratory. Dr S. L. Jensen and Mr A. J. Aasen, of the Tekniske Høgskole, Trondheim, isolated and chemically characterized certain carotenoids from mass cultures grown at the Karolinska Institute, Stockholm, by Miss Margareta Fall under the direction of Dr C.-G. Hedén. Dr Jensen also suggested the two paper-chromatographic systems used for preliminary determinations of carotenoids.

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This paper is a Contribution from the Scripps Institution of Oceanography.

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Table 1. *Code designations, names as proposed in another paper of this series (Lewin, 1969b), and sources of strains of flexibacteria studied*

The genera are alphabetically arranged. Within each genus the species are ordered as in the conspectus presented elsewhere (Lewin, 1969*b*; Table 1).

Notes to Table 1

[1] Deposited as ATCC 15854, NCMB 292.
[2] Supplied as '*Cytophaga aurantiaca*'.
[3] Supplied as '*Cytophaga psychrophila*'.
[4] Supplied as '*Flexoscilla* Strain 8Q'.
[5] Supplied as '*Microscilla* sp'.
[6] All strains of *Cytophaga* spp., *Microscilla* spp., *Flexibacter aurantiacus* var. *copepodarum*, *Flexithrix dorotheae*, *Herpetosiphon cohaerens*, *H. persicus*, *H. nigricans*, *Saprospira grandis* and *S. toviformis* were isolated from marine shores.
[7] All pure cultures were isolated by R. A. L. except for those indicated by a superscript as follows:
[8] Isolated by R. A. MacLeod.
[9] Isolated by W. F. Blankley.
[10] Isolated by A. Sanfilippo.
[11] Isolated by M. Dworkin.
[12] Isolated by J. G. Holt.
[13] Isolated by S. Soriano.
[14] Isolated by A. Cataldi.
* Strain designated as type or neotype.

Strain	Name	Location	Habitat[6]	Collected by[7]
	Cytophaga			
SIO-1	**C. latercula*	La Jolla, Calif.	Sea-water aquarium outflow	R. A. Lewin
B-9[1]	*C. lytica*	B.C., Canada	Sea water	R. A. MacLeod[8]
WFB-21	*C. lytica*	La Jolla, Calif.	Sea-water aquarium outflow	W. F. Blankley[9]
BON	*C. lytica*	La Jolla, Calif.	Sea-water aquarium outflow	D. D. Bonnett
ENS	*C. lytica*	Ensenada, B.C., Mexico	Silt	R. A. Lewin
LIM-21	**C. lytica*	Limón, Costa Rica	Mud	R. A. Lewin
DD-1	*C. diffluens* (?)	Bermuda	White sand	J. Beers
DUB-4	*C. d.* var. *aprica*	Dubrovnik, Yugoslavia	Mud	R. A. Lewin[10]
ST-1	*C. d.* var. *aprica*	Bimini, B.W.I.	Sand under 1 metre depth	E. Koschen
JL-4	**C. d.* var. *aprica*	Kailua, Hawaii	Rocky sand	R. W. Grigg
Q-1	**C. d.* var. *carnea*	Split, Yugoslavia	Coarse, grey-brown sand	Director, Oceanogr. Institute
Y-1	*C. diffluens*	Zanaiban, Nigeria	Grey, silty sand	Director, E.A.F.R.O.
GOL-12	*C. diffluens*	Golfito, Costa Rica	Mud	R. A. Lewin
N-3	*C. diffluens*	Nosy Bé, Madagascar	Sand	M. Angot, R. Planke
NN-3	*C. diffluens*	Canoe Beach, Tema, Ghana	Coarse, greenish-brown sand	E. Kwei, J. Teya
B-1	**C. diffluens*	Bombay, India	Black sandy mud, lower littoral	Y. Freitas
LIM-1	*C. diffluens*	Limón, Costa Rica	Mud	R. A. Lewin
O-2	*C. diffluens*	Kaneohe Bay, Oahu, Hawaii	Fine sand, upper littoral	R. Hiatt
	Flexibacter			
SIO-4	**F. litoralis*	La Jolla, Calif.	Sea-water aquarium outflow	R. A. Lewin
DWO[2]	**F. aurantiacus*	Minneapolis, Minn.	Garden soil	M. Dworkin[11]
PSY[3]	*F. aurantiacus*	—	—	E. Ordal
COP	**F. a.* var. *copepodarum*	La Jolla, Calif.	Offshore copepod	B. T. Lang

Table 1 (*cont.*)

Strain	Name	Location	Habitat[6]	Collected by[7]
CR-103	*F. giganteus*	San José, Costa Rica	Scum on rivulet	R. A. Lewin
CR-104	**F. giganteus*	San José, Costa Rica	Scum on rivulet	R. A. Lewin
CR-124	*F. giganteus*	San José, Costa Rica	Rivulet, university	R. A. Lewin
CR-134	**F. a.* var. *excathedrus*	Cartago, Costa Rica	Pool in cathedral	R. A. Lewin
CR-141	*F. roseolus*	Agua Caliente, Costa Rica	Hot spring	R. A. Lewin
CR-155	**F. roseolus*	Agua Caliente, Costa Rica	Hot spring	R. A. Lewin
GEY	**F. ruber*	Geysir, Iceland	Hot spring	R. A. Lewin
FLE[4]	**F. flexilis* var. *pelliculosus*	Birch Lake, Minn.	Lakeshore	E. E. Jeffers [12]
CR-63	**F. flexilis*	San José, Costa Rica	Lily pond, university	R. A. Lewin
CR-81	*F. flexilis*	San José, Costa Rica	Rivulet, university	R. A. Lewin
A-52	*F. flexilis*	Buenos Aires, Arg.	—	A. Cataldi[13]
WAR-5	*F. flexilis*	Warner Hot Springs, Calif.	Hot spring	S. Soriano[13]
BA-24	**F. f.* var. *iolanthae*	Buenos Aires, Arg.	—	A. Cataldi[14]
BA-3	**F. sancti*	Buenos Aires, Arg.	—	A. Cataldi[14]
BA-23	*F. sancti*	Buenos Aires, Arg.	—	A. Cataldi[14]
MIC[5]	*F. sancti*	Ames, Iowa	Soil in feed lot	J. G. Holt[12]
NZ-1	**F. elegans*	Rotorua, N.Z.	Hot spring	G. R. Fish
	Flexithrix			
QQ-3	**F. dorotheae*	Ernakulum, India	Brown silt	K. N. Sankolli
	Herpetosiphon			
II-2	**H. cohaerens*	Biarritz, France	Mid-littoral	Director, Sta. Biol. Mar.
T-3	**H. persicus*	Muigh Inis, Carna Co., Galway, Ireland	Brown mud	C. O'hEocha, M. de Valera
SS-2	**H. nigricans*	Lagos, Nigeria	Brown sand	E. O. Bayagbona
	Microscilla			
HJ-1	**M. arenaria*	Norse Beach, Puerto Peñasco, Son., Mexico	Brown sand	R. W. Hoshaw
HI-3	**M. aggregans* var. *catalatica*	Auke Bay, Alaska	Under frozen sand, upper littoral	J. Quast, R. Haight
Q-3	*M. aggregans*	Split, Yugoslavia	Fine, light-brown sand	Director, Oceanogr. Institute
QQ-1	*M. aggregans*	Ernakulum, India	Brown sand	K. N. Sankolli
QQ-11	*M. aggregans*	Ernakulum, India	Brown sand	K. N. Sankolli
JL-13	*M. aggregans*	Kailua, Hawaii	Coarse white sand, upper littoral	R. W. Grigg
NN-13	**M. aggregans*	Canoe Beach, Tema, Ghana	Green-brown sand	E. Kwei, J. Teya
EE-13	*M. tractuosa*	Heligoland, Germany	Red-brown mud	W. Gunkel, H. Trekel
EG-13	*M. tractuosa*	Normandy, Orne Estuary, France	Brown, fine mud	P. Gayral
GH-1	*M. tractuosa*	Penang, Malaysia	Brown, silty sand	Ong Kah Sin
GH-2	*M. tractuosa*	Penang, Malaysia	Brown, silty sand	Ong Kah Sin
HI-15	*M. tractuosa*	Auke Bay, Alaska	Under frozen sand, upper littoral	J. Quast, R. Haight
H-43	**M. tractuosa*	Nhatrang, Vietnam	Sand	N. Thach
JK-11	*M. tractuosa*	Moreton Bay, Australia	Brown sand	W. Stephenson
T-13	*M. tractuosa*	Muigh Inis, Carna Co., Galway, Ireland	Brown mud	C. O'hEocha, M. de Valera

Table 1 (*cont.*)

Strain	Name	Location	Habitat[8]	Collected by[7]
SIO-7	**M. sericea*	La Jolla, Calif.	Marine aquarium outflow	R. A. Lewin
SIO-9	*M. sericea*	La Jolla, Calif.	Marine aquarium outflow	R. A. Lewin
SIO-8	**M. marina*	La Jolla, Calif.	Marine aquarium outflow	R. A. Lewin
TV-2	**M. furvescens*	Samoa	Brown sand	M. Wilcox
	Saprospira			
BEG	**S. thermalis*	Kotlafjørdur, Iceland	Hot spring	R. A. Lewin
CR-123	*S. thermalis*	San José, Costa Rica	Rivulet, university	R. A. Lewin
CR-125	*S. thermalis*	San José, Costa Rica	Rivulet, university	R. A. Lewin
A-1	**S. toviformis*	Campbell's Bay, N.Z.	Sand at tidepool edge	V. Cassie
WH	**S. grandis*	Woods Hole, Mass.	Rockpool, upper littoral	R. A. Lewin
PA-1	*S. grandis*	Port Aransas, Texas	Decaying algae	C. H. Oppenheimer
PA-2	*S. grandis*	Port Aransas, Texas	Decaying algae	C. H. Oppenheimer
PA-3	*S. grandis*	Port Aransas, Texas	Decaying algae	C. H. Oppenheimer
LJA	*S. grandis*	La Jolla, Calif.	Eggs of *Dendraster*	D. Mazia
E-1	*S. grandis*	Ensenada, B.C., Mexico	Mud	R. A. Lewin
E-2	*S. grandis*	Ensenada, B.C., Mexico	Mud	R. A. Lewin
M-1	*S. grandis*	Mazatlán, Sin., Mexico	Mud	R. A. Lewin
M-2	*S. grandis*	Mazatlán, Sin., Mexico	Mud	R. A. Lewin
ROB	*S. grandis*	Robles, Costa Rica	Mud	R. A. Lewin
SG-1	*S. grandis*	Golfito, Costa Rica	Mud	R. A. Lewin
SG-2	*S. grandis*	Golfito, Costa Rica	Mud	R. A. Lewin
DAW-1	*S. grandis*	Galápagos Island	Mud among mangroves	E. Y. Dawson
DAW-2	*S. grandis*	Galápagos Island	Mud among mangroves	E. Y. Dawson
DUB-2	*S. grandis*	Dubrovnik, Yugoslavia	Mud	R. A. Lewin[10]
DUB-3	*S. grandis*	Dubrovnik, Yugoslavia	Mud	R. A. Lewin[10]
A-2	*S. grandis*	Castor Bay, N.Z.	Sand	V. Cassie
A-3	*S. grandis*	Castor Bay, N.Z.	Sand, mid-littoral	V. Cassie
L-1	*S. grandis*	Kailua, Hawaii	White sand with green algae	R. W. Grigg

Tables 2–6

Specific names are those proposed by Lewin (1969*b*).

Column headings and other explanatory notes

(1) GC% Molar % guanine+cytosine in DNA, determined by CsCl density-gradient method.

(2) Leng. Length of most cells or filaments in active culture, in μm. In many strains, the lengths were very variable, considerably influenced by such factors as shaking, aeration, and age of culture.

(3) Rhap. Rhapidosomes detected in lysate of old culture. A minus sign does not absolutely preclude the possibility of their being present in another lysate of the same strain.

(4) Pig. Pigment type; here Arabic replace Roman numerals (see Table 7). Figs. 7 and 8 illustrate 1-dimensional chromatograms of crude pigment extracts from representative types, on different qualities of paper and with different solvent systems (as suggested by Dr S. L. Jensen—personal communication).

(5) Pen. Negative $\log_{10}$ of highest concentration of penicillin permitting growth.

(6) NaLS Growth in sodium lauryl sulphate (100 mg./l.).

(7) Sal. Salinities permitting growth; F=freshwater medium, ½S, 1S, 2S=half, normal, double-strength sea-water media, respectively.

(8) Temp. Highest temperature (tested in 5° steps) permitting growth.

(9) +3°. Survival for 1 week at +3°.

(10) −196° Survival for 2 hr in liquid nitrogen.

(11) CMC Depolymerization of Na carboxymethyl cellulose (3%).

(12) Star. Digestion of starch (2%).

(13) Agar Liquefaction or softening of agar (1%).

(14) Algin Liquefaction of K alginate (3%).

(15) Gel. Liquefaction of gelatin (10%).

(16) Lit-Mil. Reactions in media containing milk and litmus: C=clotting, R=reduction of dye (varied with age of culture), A=acidification (litmus turned pink: varied with age of culture), P=proteolysis (redigestion of curd).

(17) Cat. Catalase activity (effervescence with H_2O_2).

(18) H_2S Evolution of H_2S (darkening of lead-acetate paper).

(19) Tyr. Growth (+ or −) and degradation of tyrosine (5 g./l.): C=clearing (dissolution of crystals), R=red or pink halo, B=black or grey halo.

(20) DOPA Growth (+ or −) and degradation of dihydroxyphenyl alanine: C=clearing, D=black or grey halo.

(21) Nit. Reduction of nitrate (formation of nitrite); results not always reproducible.

(22) Carbon sources apparently stimulating growth, when present at indicated concentrations of 5·0 or 1·0 g./l.; results not always reproducible (cf. Dworkin, 1966; Mayer, 1967). A dash indicates no perceptible stimulation over control. Ace.=Na acetate, Lac.=Na lactate, Gly.=glycerol, Glu.=glucose, Gal.=galactose, Suc.=sucrose.

(23) Nitrogen sources permitting growth, each at a concentration of 1·0 g./l.: Tryp.=Tryptone, CAA=Casamino acids, Glut.=Na glutamate, NO_3^-=K nitrate.

(24) L-Amino acids essential for growth: A=arginine, As=aspartic acid, G=glycine, H=histidine, I=isoleucine, L=leucine, Ly=lysine, M=methionine, P=phenylalanine or tyrosine, T=threonine, Tr=tryptophan, V=valine.

(25) Vit. Vitamins essential for growth: T=thiamine, C=cobalamin. Experiments to demonstrate a requirement for cobalamin were not always reproducible, possibly because of contamination of glassware or of the distilled water used for nutrient media, or perhaps because of adaptation to vitamin independence. In the table (c) indicates that, in at least some experiments, cobalamin appeared to be essential for growth.

(26) Nuc. Some unidentified factor, present in hydrolysates of yeast nucleic acid, required for growth.

Tables 2*a,b,c*. *Cytophaga species: experimental data for all strains*

For explanation of columns 1–26 see p. 161.

Table 2*a*.

Strain	1 GC%	2 Leng.	3 Rhap.	4 Pig.	5 Pen.	6 NaLS	7 Sal.	8 Temp.	9 +3°	10 −196°
Cytophaga latercula										
SIO-1	34·5	5	—	6	7	—	1 S	30	+	+
C. lytica										
B-9	33·5	> 10	−	4	6	−	½–2 S	30	+	+
WFB-21	34·5	> 15	−	4	.	.	F–2 S	40	+	+
BON	33·5*	> 10	−	4	.	.	½–2 S	35	+	+
ENS	34	> 10		4	6	−	½–2 S	40	+	+
LIM-21	34	> 20	+	4	6	−	½–2 S	35	+	+
C. diffluens (?)										
DD-1	35	> 20	+	3	7	−	½–2 S	30	+	+
C. diffluens var. *aprica*										
DUB-4	37	> 50	−	3	7	+	½–2 S	30	−	+
ST-1	36·5	> 15	−	3	6	+	½–1 S	35	+	.
JL-4	35·5	> 30	+	3	6	+	½–2 S	35	−	.
C. diffluens var. *carnea*										
Q-1	37·5	> 30	−	3	.	−	½–2 S	30	+	+
C. diffluens										
Y-1	41	5–40	−	3	6	−	½–2 S	30	+	+
GOL-12	41	5–10	+	3	6	−	½–2 S	30	+	+
N-3	41	> 20	−	3	.	−	½–2 S	30	+	+
NN-3	40	> 20	−	3	7	−	½–2 S	35	−	+
B-1	42	> 10	−	3	7	−	½–2 S	40	+	+
LIM-1	43	> 30	−	3	6	−	½–2 S	30	−	+
O-2	42	> 10	+	3	7	−	½–2 S	35	+	+

* Satellite GC = 57·5%.

Table 2*b*.

Strain	11 CMC	12 Star.	13 Agar	14 Algin	15 Gel.	16 Lit-Mil.	17 Cat.	18 H_2S	19 Tyr.	20 DOPA	21 Nit.
Cytophaga latercula											
SIO-1	+	−	+	+	+	+CRA	−	+	+C	−	−
C. lytica											
B-9	+	+	+	+	+	+CPR	+	−	+RO	+DC	−
WFB-21	+	+	+	+	+	+CR	+	+	+RC	+	−
BON	+	+	+	+		+CR	+	−	+RC	+	−
ENS	+	+	+	+	+	+CR	+	−	+RC	+	−
LIM-21	+	+	+	+	+	+CR	+	+	+RO	+DC	−
C. diffluens (?)											
DD-1	+	+	+	+	+	+RA	−	+	+	+C	−
C. diffluens var. *aprica*											
DUB-4	+	+	+	+	+	+CRA	−	+	+	−	−
ST-1	+	+	+	+	+	+CRA	−	+	+	+	+
JL-4	+	+	+	+	+	+RA	−	+	+	−	−
C. diffluens var. *carnea*											
Q-1	+	−	+	+	+	−	−	−	+	−	+
C. diffluens											
Y-1	+	+	+	+	+	+CRA	−	−	+	+C	−
GOL-12	+	+	+	+	+	.	−	−	+	−	+
N-3	+	+	+	+	+	+CRA	−	+	+(R)	+C	+
NN-3	+	+	+	+	+	+RA	−	+	+	+C	−
B-1	+	+	+	+	+	+CRA	−	+	+	+C	−
LIM-1	+	+	+	+	+	+CR	−	+	+	−	−
O-2	+	+	+	+	+	+CPRA	−	+	+	+C	+

Table 2*c*.

	22						23				24	25	26
Strain	Ace.	Lac.	Gly.	Glu.	Gal.	Suc.	Tryp.	CAA	Glut.	NO_3^-	Essential AAs	Vit.	Nuc.
Cytophaga latercula													
SIO-1	−	−	−	5,I	5,I	5,I	+	+	+	+	−	−	−
C. lytica													
B-9	5,I	5	5,I	−	−	−	+	+	+	+	−	−	−
WFB-21	−	−	5,I	5,I	5,I	5,I	+	+	+	+	−	−	−
BON	−	−	5,I	5,I	5,I	5,I	+	+	+	+	−	−	−
ENS	−	−	5,I	5,I	5,I	5,I	+	+	+	+	−	−	−
LIM-21	5,I	5,I	5,I	5,I	5,I	5,I	+	+	+	−	−	−	−
C. diffluens (?)													
DD-1	−	−	−	−	I	−	+	+	.	.	.	.	−
C. diffluens var. *aprica*													
DUB-4	5,I	−	−	I	5,I	−	+	.	.	.	.	.	−
ST-1	5,I	−	−	I	5,I	5,I	+	+	.	.	.	.	−
JL-4	I	−	5	−	−	5	+	+	+	−	−	−	−
C. diffluens var. *carnea*													
Q-1	−	−	−	−	−	5,I	+	+	+	+	−	.	−
C. diffluens													
Y-1	I	−	−	−	5,I	−	+	+	.	.	.	.	−
GOL-12	5,I	−	−	−	−	−	+	.	.	.	.	.	−
N-3	−	−	−	−	I	−	+	.	.	.	.	.	−
NN-3	5,I	−	−	−	−	5	+	.	.	.	.	.	−
B-1	5,I	−	−	−	I	−	+	+	+	−	−	.	−
LIM-1	5,I	−	−	I	I	5,I	+	.	.	.	.	.	−
O-2	5,I	−	−	5,I	I	5	+	+	+	+	−	−	−

Tables 3*a*,*b*,*c*. *Flexibacter species: experimental data for all strains*

For explanations of columns 1–26, see p. 161.

Table 3*a*.

Strain	1 GC%	2 Leng.	3 Rhap.	4 Pig.	5 Pen.	6 NaLS	7 Sal.	8 Temp.	9 +3°	10 −196°
Flexibacter litoralis										
SIO-4	31	> 180	−	1	6	−	½–2 S	30	+	+
F. aurantiacus										
DWO	31·5	5–20	−	5	6	+	F–1 S	30	+	+
PSY	32	5–20	−	5	6	+	F	30	+	+
F. aurantiacus var. *copepodarum*										
COP	33	5	−	4	6	−	1 S	30	+	−
F. giganteus										
CR-103	32	> 50	−	2	9	−	F	35	−	−
CR-104	32	> 50	−	2	8	−	F	35	−	−
CR-124	32	> 50	−	2	9	−	F	30	−	−
F. aurantiacus var. *excathedrus*										
CR-134	34·5	10	−	4	7	−	F	40	+	+
F. roseolus										
CR-141	34·5	> 50	−	1	9	−	F–2 S	40	+	+
CR-155	38	> 50	−	1	9	−	F–1 S	40	+	+
F. ruber										
GEY	37	> 50	−	1	8	−	F	40	+	+
F. flexilis var. *pelliculosus*										
FLE	39·5	10–30	−	3	6	−	F	35	+	+
F. flexilis										
CR-63	41	10–50	−	3	6	−	F	40	+	+
CR-81	43	10–50	−	3	8	−	F	35	+	+
A-52	43	10–50	−	3	6	−	F	40	+	+
WAR-5	40·5	10–50	−	3	6	−	F	40	+	+
F. flexilis var. *iolanthae*										
BA-24	41·3	10–30	−	3	6	−	F	35	−	−
F. sancti										
BA-3	46	5–15	−	4	7	−	F	35	+	−
BA-23	47	5–15	−	4	6	−	F	35	+	+
MIC	46·5	10–50	−	4	6	−	F	35	+	+
F. elegans										
NZ-1	47·5	> 50	−	3	8	−	F–1 S	40	+	+

Table 3*b*.

Strain	11 CMC	12 Star.	13 Agar	14 Algin	15 Gel.	16 Lit-Mil.	17 Cat.	18 H_2S	19 Tyr.	20 DOPA	21 Nit.
Flexibacter litoralis											
SIO-4	−	+	−	−	+	+C	−	−	+RC	−	−
F. aurantiacus											
DWO	+	+	−	−	+	+R	+	−	+C	+C	−
PSY	+	−	−	−	+	+	+	−	+C	+C	−
F. aurantiacus var. *copepodarum*											
COP	−	−	−	−	+	+CA	−	−	+C	−	−
F. giganteus											
CR-103	−	+	−	−	+	+CP	−	+	±	−	−
CR-104	−	+	−	−	+	+	−	+	±	−	−
CR-124	−	+	−	−	+	+	−	−	±	−	−
F. aurantiacus var. *excathedrus*											
CR-134	−	+	−	−	+	+CPR	−	−	+BC	+DC	−
F. roseolus											
CR-141	−	−	−	−	+	+	−	−	−	−	−
CR-155	−	−	−	−	+	+	−	−	−	−	−
F. ruber											
GEY	−	+	−	−	+	+	−	−	±	−	+
F. flexilis var. *pelliculosus*											
FLE	+	+	−	−	+	+R	−	+	+R	+DC	+
F. flexilis											
CR-63	−	−	−	−	+	+R	−	+	+	+	−
CR-81	−	−	−	−	+	+R	−	+	+	+	−
A-52	−	+	−	−	+	+R	−	+	+	+	−
WAR-5	−	−	−	−	+	+R	−	+	+	+	−
F. flexilis var. *iolanthae*											
BA-24	−	−	−	−	+	+	−	−	+	+	−
F. sancti											
BA-3	+	+	−	−	+	+AR	−	−	+C	+	+
BA-23	+	−	−	−	+	+	−	−	+C	+	+
MIC	+	+	−	−	+	+R	−	+	+C	+DC	−
F. elegans											
NZ-1	−	−	−	−	+	+	−	−	+C	+	−

Table 3*c*.

	22						23				24	25	26
Strain	Ace.	Lac.	Gly.	Glu.	Gal.	Suc.	Tryp.	CAA	Glut.	NO_3^-	Essential AAs	Vit.	Nuc.
Flexibacter litoralis													
SIO-4	–	–	–	–	–	–	+	–	–	–	AsHILMPHTrV	T	–
F. aurantiacus													
DWO	5,I	–	–	5,I	5,I	5,I	+	+	+	+	–	–	–
PSY	–	–	–	–	–	–	+	+	+	+	–	–	–
F. aurantiacus var. *copepodarum*													
COP	–	–	–	–	–	–	+	+	+	–	–	–	.
F. giganteus													
CR-103	–	–	–	5,I	5,I	–	+	+	–	–	GILMPV	T	–
CR-104	–	–	–	I	–	–	+	+	–	–	GILMPV	T	–
CR-124	–	–	–	I	–	–	+	+	–	–	ILMPV	T (c)	–
F. aurantiacus var. *excathedrus*													
CR-134	–	–	–	–	–	–	+	+	+	–	–	–	–
F. roseolus													
CR-141	5	–	–	–	–	–	+	+	–	–	AILMPTV	T	–
CR-155	–	I	–	–	–	–	+	+	–	–	AGILMPTV	T (c)	–
F. ruber													
GEY	5,I	5,I	–	5,I	5,I	5,I	+	+	+	+	–	T	–
F. flexilis var. *pelliculosus*													
FLE	–	–	–	5,I	5,I	5,I	+	+	+	+	–	–	–
F. flexilis													
CR-63	–	–	–	–	–	5,I	+	+	–	–	AILMTV	T	–
CR-81	–	–	–	–	–	5,I	+	+	–	–	AILMTV	T	–
A-52	5,I	–	–	5,I	–	5,I	+	+	–	–	AILMTV	T	–
WAR-5	–	–	–	5,I	5,I	5,I	+	+	–	–	AILMTV	T	–
F. flexilis var. *iolanthae*													
BA-24	5	–	–	–	–	–	+	.	–	–	.	.	–
F. sancti													
BA-3	–	–	5,I	5,I	5,I	5,I	+	+	–	+	–	–	–
BA-23	–	–	5,I	5,I	5,I	5,I	+	+	–	+	–	–	–
MIC	–	–	–	5,I	5,I	5,I	+	+	+	+	–	–	–
F. elegans													
NZ-1	–	–	–	–	–	–	+	+	–	–	T	.	–

Tables 4*a*,*b*,*c*. *Microscilla species and Flexithrix dorotheae, experimental data for all strains*

For explanations of columns 1–26, see p. 161.

Table 4*a*.

Strain	1 GC%	2 Leng.	3 Rhap.	4 Pig.	5 Pen.	6 NaLS	7 Sal.	8 Temp.	9 +3°	10 −196°
Microscilla arenaria										
HJ-1	32·5	> 20	—	3	7	−	½–2 S	30	−	+
M. aggregans var. *catalatica*										
HI-3	35	> 20	−	4	6	−	½–2 S	30	+	+
M. aggregans										
Q-3	41·5	> 100	−	4	6	−	½–2 S	40	+	+
QQ-1	39·5	> 30	−	4	6	−	½–2 S	40	+	+
QQ-11	40·5	> 30	.	4	6	−	½–2 S	35	+	+
JL-13	36·5	> 20	−	4	8	−	½–2 S	40	+	+
NN-13	36·5	> 70	−	4	6	−	½–2 S	40	+	+
M. tractuosa										
EE-13	39·5	> 15	−	3	7	−	F–2 S	35	+	+
EG-13	37	> 40	+	3	7	−	½–2 S	30	+	+
GH-1	34·5	> 50	−	3	6	+	½–2 S	40	+	+
GH-2	35·5	> 50	−	3	7	−	½–2 S	40	+	+
HI-15	36	5–10	−	3	.	−	F–2 S	−	+	.
H-43	36·5	> 15	−	3	7	−	F–2 S	35	+	+
JK-11	37	> 20	−	3	7	−	F–2 S	30	+	+
T-13	38	> 10	−	3	7	−	F–2 S	30	+	+
M. sericea										
SIO-7	39	> 30	−	3	.	−	½–2 S	30	+	+
SIO-9	38	> 100	+	3	7	−	½–2 S	30	−	+
M. marina										
SIO-8	42	> 150	+	3	6	−	1–2 S	30	+	+
M. furvescens										
TV-2	44	10–50	−	3	.	−	.	.	+	+

Table 4*b*.

Strain	11 CMC	12 Star.	13 Agar	14 Algin	15 Gel.	16 Lit-Mil.	17 Cat.	18 H_2S	19 Tyr.	20 DOPA	21 Nit.
Microscilla arenaria											
HJ-1	+	+	−	+	−	+RA	−	+	+	+	−
M. aggregans var. *catalatica*											
HI-3	−	−	−	−	.	+	+	+	+	+	−
M. aggregans											
Q-3	−	−	−	−	−	+	−	−	+	+	−
QQ-1	+	−	−	−	−	+	−	−	+	+	−
QQ-11	−	−	−	−	−	+CR	−	−	+	+	−
JL-13	+	−	−	−	−	+	−	−	+	+	−
NN-13	+	−	−	−	−	+	−	−	+	+	−
M. tractuosa											
EE-13	−	−	−	−	+	+CR	−	+	+	+	−
EG-13	−	+	−	−	+	+CR	−	−	+	+	−
GH-1	−	+	−	−	+	+CPR	−	−	+	+	−
GH-2	−	+	−	−	+	+R	−	+	+	+	−
HI-15	−	+	−	−	+	+CR	−	+	+	.	+
H-43	−	+	−	−	+	+CPRA	−	−	+	+	−
JK-11	−	−	−	−	−	+CR	−	−	+	+	.
T-13	−	−	−	−	+	+CPR	−	+	+	+	+
M. sericea											
SIO-7	−	+	−	+	−	+R	−	−	+	+	−
SIO-9	−	+	−	+	.	+	−	−	+C	+	−
M. marina											
SIO-8	−	−	−	−	+	+C	−	−	+RC	−	−
M. furvescens											
TV-2	+	+	+	+	+	+	−	−	+B	+	−

Table 4*c*.

	22						23				24	25	26
Strain	Ace.	Lac.	Gly.	Glu.	Gal.	Suc.	Tryp.	CAA	Glut.	NO_3^-	Essential AAs	Vit.	Nuc.
Microscilla arenaria													
HJ-1	I	I	–	5,I	5,I	5	+	+	.	.	.	.	–
M. aggregans var. *catalatica*													
HI-3	–	–	–	–	–	–	+	–	–	–	.	.	–
M. aggregans													
Q-3	–	–	–	5,I	5,I	5,I	+	+	+	+	–	.	–
QQ-I	5,I	5,I	5,I	5,I	5,I	5,I	+	+	+	+	–	–	–
QQ-II	–	–	–	5,I	5,I	5,I	+	+	+	–	–	.	–
JL-13	–	–	–	5,I	5,I	5,I	+	+	+	+	–	–	–
NN-13	–	–	–	5,I	5,I	5,I	+	+	+	–	–	–	–
M. tractuosa													
EE-13	–	–	–	5,I	–	–	+	.	.	.	.	.	–
EG-13	–	–	–	5,I	–	5,I	+	.	.	.	.	.	–
GH-I	–	–	–	5,I	5	–	+	+	.	.	.	.	–
GH-2	I	–	–	I	5	–	+	+	.	.	–	–	–
HI-15	–	–	–	.	–	–	+	+	+	–	–	–	–
H-43	–	–	5,I	5,I	5,I	5,I	+	+	.	.	.	.	–
JK-II	–	–	5,I	5,I	I	–	+	+	.	.	.	.	–
T-13	–	–	5,I	5,I	5	5,I	+	+	.	.	.	.	–
M. sericea													
SIO-7	–	–	5,I	5,I	5,I	5,I	+	–	–	–	.	.	–
SIO-9	–	–	–	–	–	5,I	+	–	–	–	.	.	–
M. marina													
SIO-8	–	5	–	–	–	–	+	±	–	–	.	.	–
M. furvescens													
TV-2	–	–	–	5,I	5,I	5,I	+	+	+	+	–	–	.

Tables 5*a*, *b*, *c*. *Saprospira species: experimental data for all strains*

For explanations of columns 1–26, see p. 161.

Table 5*a*.

Strain	1 GC%	2 Leng.	3 Rhap.	4 Pig.	5 Pen.	6 NaLS	7 Sal.	8 Temp.	9 +3°	10 −196°
Saprospira thermalis										
BEG	36·5	> 50	–	2	8	–	F	40	–	–
CR-123	34·5	150	–	2	9	–	F	40	–	–
CR-125	37·2	> 50	–	2	9	–	F	40	–	–
S. toviformis										
A-I	38	10–50	+	3*a**	> 9	–	F–2 S	30	+	–
S. grandis										
WH	47·5	> 50	+	3	8	–	½–2 S	40	–	–
PA-I	47·5	> 50	+	3	9	–	½–2 S	40	+	–
PA-2	46·5	> 50	+	3	> 9	–	½–2 S	40	–	–
PA-3	46·5	> 50	+	3	8	–	½–2 S	35	–	+
LJA	46·5	> 50	+	3	9	–	½–2 S	30	–	+
E-I	46·5	> 50	+	3	8	–	½–1 S	35	–	+
E-2	48	> 50	+	3	8	–	½–2 S	40	–	+
M-I	48	> 50	+	3	8	–	½–2 S	40	–	+
M-2	47·5	> 50	+	3	8	–	½–1 S	40	–	–
ROB	48	> 50	+	3	8	–	½–2 S	40	–	–
SG-I	47	> 50	+	3	8	–	½–2 S	40	–	+
SG-2	47	> 50	+	3	8	–	½–1 S	40	–	–
DAW-I	47·3	> 50	+	3	8	–	½–2 S	35	–	–
DAW-2	47	> 50	+	3	> 9	–	1–2 S	40	–	+
DUB-2	47	> 50	+	3	8	–	½–2 S	35	+	–
DUB-3	48	> 50	+	3	9	–	½–2 S	40	–	–
A-2	46	> 50	+	3	8	–	F–2 S	30	+	–
A-3	46	> 50	+	3	8	–	F–2 S	40	+	–
JL-I	47·5	> 50	+	3	8	–	F–2 S	40	–	.

* See Table 7.

Table 5*b*.

Strain	11 CMC	12 Star.	13 Agar	14 Algin	15 Gel.	16 Lit-Mil.	17 Cat.	18 H_2S	19 Tyr.	20 DOPA	21 Nit.
Saprospira thermalis											
BEG	−	+	−	−	+	+	−	+	+	±	−
CR-123	−	+	−	−	+	+	−	+	+	±	−
CR-125	−	+	−	−	+	+	−	+	+	±	−
S. toviformis											
A-I	+	−	−	−	+	+C	−	−	+	−	−
S. grandis											
WH	−	−	−	−	+	+C	−	−	+R	−	−
PA-I	−	−	−	−	+	+C	−	−	+R	−	−
PA-2	−	−	−	−	+	+C	−	−	+R	−	−
PA-3	−	−	−	−	+	+C	−	−	+R	−	−
LJA	−	−	−	−	+	+C	−	−	+R	−	−
E-I	−	−	−	−	+	+C	−	−	+R	−	−
E-2	−	−	−	−	+	+C	−	−	+R	−	−
M-I	−	−	−	−	+	+C	−	−	+R	−	−
M-2	−	−	−	−	+	+C	−	−	+R	−	−
ROB	−	−	−	−	+	+C	−	−	+R	−	−
SG-I	−	−	−	−	+	+C	−	−	+R	−	−
SG-2	−	−	−	−	+	+C	−	−	+R	−	−
DAW-I	−	−	−	−	+	+C	−	−	+R	−	−
DAW-2	−	−	−	−	+	+C	−	−	+R	−	−
DUB-2	−	−	−	−	+	+CR	−	−	+R	−	−
DUB-3	−	−	−	−	+	+C	−	−	+R	−	−
A-2	−	−	−	−	+	+C	−	−	+R	−	−
A-3	−	−	−	−	+	+CR	−	−	+R	−	−
JL-I	−	−	−	−	+	+CPR	−	−	+R	−	−

Table 5*c*.

	22						23				24	25	26
Strain	Ace.	Lac.	Gly.	Glu.	Gal.	Suc.	Tryp.	CAA	Glut.	NO_3^-	Essential AAs	Vit.	Nuc.
Saprospira thermalis													
BEG	−	−	−	5,I	−	I	+	+	−	−	ILV	CT	−
CR-123	−	−	−	5,I	−	.	+	+	−	−	ILV	CT	−
CR-125	−	−	−	5,I	−	I	+	+	−	−	ILV	CT	−
S. toviformis													
A-I	5,I	5,I	−	−	−	−	+	+	−	−	AGHILLyMPTV		−
S. grandis													
WH	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
PA-I	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
PA-2	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
PA-3	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
LJA	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
E-I	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
E-2	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
M-I	5,I	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
M-2	5,I	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
ROB	5,I	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
SG-I	5,I	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
SG-2	5	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
DAW-I	−	−	−	5,I	5,I	5,I	+	−	−	−	AAsHILLyMPTTrV		+
DAW-2	5	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
DUB-2	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
DUB-3	I	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
A-2	−	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
A-3	I	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+
JL-I	I	−	−	−	−	−	+	−	−	−	AAsHILLyMPTTrV		+

Tables 6*a*,*b*,*c*. *Herpetosiphon species: experimental data for all strains*

For explanations of columns 1–26, see p. 161.

Table 6*a*.

Strain	1 GC%	2 Leng.	3 Rhap.	4 Pig.	5 Pen.	6 NaLS	7 Sal.	8 Temp.	9 +3°	10 −196°
Herpetosiphon cohaerens										
II-2	44	> 50	−	3	8	−	½–2 S	30	+	.
H. persicus										
T-3	52·5	> 50	−	3	6	−	½–2 S	30	+	.
H. nigricans										
SS-2	53	> 50	−	4	6	−	½–2 S	35	+	+

Table 6*b*.

Strain	11 CMC	12 Star.	13 Agar	14 Algin	15 Gel.	16 Lit-Mil.	17 Cat.	18 H_2S	19 Tyr.	20 DOPA	21 Nit.
Herpetosiphon cohaerens											
II-2	−	−	−	−	+	+CR	−	−	+	+	−
H. persicus											
T-3	−	−	−	−	+	+CR	−	−	+	+	−
H. nigricans											
SS-2	−	−	−	−	+	+CPR	−	−	+B	+	−

Table 6*c*.

	22						23				24	25	26
Strain	Ace.	Lac.	Gly.	Glu.	Gal.	Suc.	Tryp.	CAA	Glut.	NO_3^-	Essential AAs	Vit.	Nuc
Herpetosiphon cohaerens													
II-2	−	−	−	5,1	−	5,1	+	.	.	.	−	.	.
H. persicus													
T-3	−	−	−	5,1	1	5	+	.	+	+	−	.	.
H. nigricans													
SS-2	−	−	−	5,1	5,1	5,1	+	+	+	+	−	−	.

Table 7. *Pigment types of flexibacteria*

Pigment type	Colour of cells	Example (species* and strain)	Absorption maxima of crude extract in *n*-hexane (nm.)	Main pigment(s)	Ref. (Aasen & Jensen)
I	Red	*Flexibacter roseolus* (CR-141)	450, *475*, 505	Flexixanthin and deoxyflexixanthin	1966*b*
II	Pink	*Saprospira thermalis* (BEG)	450, *478*, 505	'S.t. 483'	1966*c*
III	Orange	*S. grandis* (WH)	425, 447, *471*, 505†	Saproxanthin	1966*a*
III*a*	Or.-yell.	*S. toviformis* (A-1)	425, 447, *471*, 505‡	?	—
IV	Yellow	*Flexithrix dorotheae* (QQ-3)	425, *450*, 478	Zeaxanthin	1966c
V	Yellow	*F. aurantiacus* (DWO)	423, *453*, 480	?	—
VI	Red	*Cytophaga latercula* (SIO-1)	440, *465*, 488	?	—

* As proposed by Lewin (1969*b*).
† 447 peak below 505 peak.
‡ 447 peak above 505 peak.

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Deoxyribonucleic Acid Base Composition of Flexibacteria

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SUMMARY

The base compositions of the deoxyribonucleic acids extracted from 93 cultures of flexibacteria classified in the genera *Cytophaga*, *Flexibacter*, *Microscilla*, *Saprospira*, *Herpetosiphon*, *Vitreoscilla* and *Beggiatoa* have been determined. The data demonstrate that the guanine plus cytosine contents (GC values) of the representative cultures ranged from 30 to 53 %. A number of the recently proposed nomen species are shown to contain representatives with different GC values.

INTRODUCTION

Marmur, Falkow & Mandel (1963) reviewed the arguments for considering comparative analyses of DNA base compositions as a cardinal criterion in microbial taxonomy. In brief, they concluded that a necessary, but not sufficient, condition for substantial genetic homology between pairs of organisms is an overall similarity in DNA base composition. As the difference in composition increases, so does the likelihood of less homology, and hence greater evolutionary divergence. A critical parameter in this regard is the 'GC value', the proportion of guanine + cytosine in the deoxyribonucleic acid, expressed as a molar percentage of total bases.

In this paper we present GC values obtained by density-gradient determinations for DNA of 90 of the strains of flexibacteria isolated and characterized by Lewin & Lounsbery (1969), and for three other organisms. These data were used, together with other biochemical and physiological information, in a computer analysis designed to associate these microbes in groups which could serve as a basis for their classification (Fager, 1969). Largely on the basis of Fager's analysis Lewin (1969) assigned these strains to a number of named genera and species. In the present paper, we also discuss the status of Lewin's proposed taxa as viewed solely from the standpoint of their GC values.

METHODS

Organisms were harvested by centrifugation of cultures in liquid media when they had attained maximum growth as described by Lewin & Lounsbery (1969). The organisms were suspended in a small volume of 0·15 M-NaCl containing 0·1 M-Na_2 ethylenediamine tetra-acetate (EDTA) at pH 8·0 (saline EDTA) and were lysed by the addition of sodium dodecyl (lauryl) sulphate to a final concentration of 2 % (w/v). The lysate was immediately deproteinized with fresh phenol saturated with saline-EDTA at neutral pH, the aqueous phase separated by centrifugation, and the DNA precipitated from it by the

addition of two volumes of 99% (v/v) ethanol was 'spooled' on a clean glass rod. Spools prepared in California were shipped in ethanol to Texas for analysis. The spool was freed of phenol by successive immersions in two changes of 70% (v/v) ethanol in water, drained, and then dissolved in 0·15 M-NaCl + 0·015 M-Na_3 citrate at pH 7·0 (solution SSC). When samples were to be analysed by thermal denaturation, highly purified DNA was prepared from the lysates by the procedure of Marmur (1961). All samples were stored over $CHCl_3$ at 5° until they were analysed.

Table 1. *Buoyant density and GC content of DNA of flexibacteria*

Values given for mean density are averages of at least two determinations of the buoyant density in CsCl at 44,770 and 42,040 rev./min. Specific epithets and strain numbers correspond to the designations of Lewin (1969).

Name	Strain	Mean density (g./cm.^{3})	GC (mole %)
Cytophaga latercula	SIO-1	1·6935	34·2
C. lytica	B-9	1·6925	33·2
	WFB-21	1·6935	34·2
	BON	1·693 (1·7165*)	33·7 (57·7*)
	ENS	1·6925	33·2
	LIM-21	1·693	33·2
C. diffluens (?)	DD-1	1·694	34·7
C. diffluens var. *aprica*	DUB-4	1·696	36·7
	ST-1	1·6965	37·2
	JL-4	1·6945	35·2
C. diffluens var. *carnea*	Q-1	1·6965	37·2
C. diffluens	Y-1	1·700	40·8
	GOL-12	1·700	40·8
	N-3	1·6995	40·3
	NN-3	1·6995	40·3
	B-1	1·7015	42·3
	LIM-1	1·7015	42·3
	O-2	1·7007	41·5
Flexibacter litoralis	SIO-4	1·6897	30·3
F. aurantiacus	DWO	1·6905	31·1
	PSY	1·6910	31·6
F. aurantiacus var. *copepodarus*	COP	1·691	31·6
F. aurantiacus var. *excathedrus*	CR-134	1·6935	34·2
F. giganteus	CR-103	1·6925	33·2
	CR-104	1·691	31·6
	CR-124	1·6906	31·2
F. roseolus	CR-141	1.6935	34·2
	CR-155	1·698	38·8
F. ruber	GEY	1·696	36·7
F. flexilis	CR-63	1·700	40·8
	CR-81	1·702	42·9
	A-52	1·7015	42·3
	WAR-5	1·6995	40·3
F. flexilis var. *pelliculosus*	FLE	1·6985	39·3
F. flexilis var. *iolanthae*	BA-24	1·7005	41·3
F. sancti	BA-3	1·705	45·9
	BA-23	1·706	46·9
	MIC	1·705	45·9
F. elegans	NZ-1	1·7065	47·4

(Table 1 *cont.*)

Name	Strain	Mean density (g./cm.^{3})	GC (mole %)
Microscilla arenaria	HJ-1	1·6915	32·1
M. aggregans	Q-3	1·7015	42·3
	QQ-1	1·6985	39·3
	QQ-11	1·6993	40·1
	JL-13	1·695	35·7
	NN-13	1·696	36·7
M. aggregans var. *catalatica*	HI-3	1·694	34·7
(*Flexithrix dorotheae*)	QQ-3	1·6965	37·2
Microscilla tractuosa	EE-13	1·697	37·8
	EG-13	1·6965	37·2
	GH-1	1·6935	34.2
	GH-2	1·6945	35·2
	HI-15	1·695	35·7
	H-43	1·6955	36·2
	JK-11	1·696	36·7
	T-13	1·6965	37·2
M. sericea	SIO-7	1·698	38·8
	SIO-9	1·698	38·8
M. marina	SIO-8	1·7015	42·3
M. furvescens	TV-2	1·704	44·9
Saprospira thermalis	BEG	1·696	36·7
	CR-123	1·6947	35.4
	CR-125	1·692	32·7
S. toviformis	A-1	1·6976	38·4
S. albida	CAR	1·699	39·8
	HH	1·7015	42·3
	MUR	1·702	42·9
S. grandis	WH	1·7068	47·8
	PA-1	1·7064	47·3
	PA-2	1·7055	46·4
	PA-3	1·7055	46·4
	LJA	1·7058	46·7
	E-1	1·7055	46·4
	E-2	1·707	48·0
S. grandis	M-1	1·7065	47·4
	M-2	1·7065	47·4
	ROB	1·7065	47·4
	SG-1	1·706	46·9
	SG-2	1·707	48·0
	DAW-1	1·707	48·0
	DAW-2	1·706	46·9
	DUB-2	1·706	46·9
	DUB-3	1·705	45·9
	A-2	1·705	45·9
	A-3	1·705	45·9
	JL-1	1·7065	47·4
S. flammula	SNZ	1·7073	48·3
Herpetosiphon cohaerens	II-2	1·704	44·9
H. persicus	T-3	1·7115	52·6
H. nigricans	SS-2	1·712	53·1
H. geysericolus	GC-42	1·7075	48·5

* Second component present in trace amount.

The buoyant density in CsCl was determined as described by Schildkraut, Marmur & Doty (1962). All samples were analysed at least once in each of two different analytical ultracentrifuges, one operating at 44,770 rev./min. and recording photographically, the other operating at 42,040 rev./min. and using a photoelectric multiplier scanning system. (For details of operation, see Mandel, Schildkraut & Marmur, 1968.) Samples of 1 to 2 μg. of the DNA of unknown density were brought to isopycnic equilibrium in CsCl gradients containing as a reference sample 0·5 μg. DNA of bacteriophage SP 8 of a density of 1·742 g./cm.^{3} (calibrated against DNA of *Escherichia coli*, assumed to be of a density of 1·710 g./cm.^{3}). The guanine + cytosine (GC) content of the extracted DNA was estimated from the mean buoyant density as described in the above references. This is the GC value as discussed throughout this paper.

Thermal denaturation was done in solution SSC at a DNA concentration of 20 μg./ml. using a Beckman DU spectrophotometer, as described by Marmur & Doty (1962).

RESULTS

The results are summarized in Tables 1 (all strains examined) and Table 2 (combined data for species).

Cytophaga

The 18 strains assigned to the genus *Cytophaga* had GC values of 33 to 42%. The strain BON, assigned to *Cytophaga lytica*, yielded DNA which displayed also a second minor component at equilibrium in the CsCl gradient. Upon heat denaturation both major and minor bands increased in density by about 15 and 12 mg./cm.^{3} respectively; hence we concluded that each was a double stranded DNA molecule. No obvious contaminating organism has been isolated from this culture, but the possibility of contamination has not been excluded beyond question.

The strains assigned to *C. lytica* were homogeneous with respect to GC values. The standard deviation of the twelve analyses (0·0008 g./cm.^{3}) was not appreciably different from the standard deviation usually encountered when a like number of analyses is performed on a single DNA sample. The single strain assigned to *C. latercula* had a GC value similar to that of *C. lytica*.

The remaining strains assigned to the genus *Cytophaga* can be conveniently divided into two groups (Table 2). The first group, with a mean GC value of about 36%, included the strain DD-1, tentatively assigned to *C. diffluens*, and the varieties *C. diffluens* var. *aprica* and *C. diffluens* var. *carnea*. The standard deviation of the analyses is somewhat large and may indicate a lack of homogeneity in this group. The second group, also assigned to the species *C. diffluens*, had a GC value of about 41% and appears homogeneous.

Flexibacter

Lewin (1969) assigned to the genus *Flexibacter* 21 strains ranging in GC value from 30 to 47% (Table 1). The monotypic *F. litoralis* represented the low extreme, with not very different values observed for the groups designated as *F. aurantiacus* and *F. giganteus*. The combination of *F. aurantiacus* with its varities *copepodarus* and *excathedrus* yielded a standard deviation that appeared uncomfortably large (see Table 2). If strain GR-134 were to be excluded from this group, the S.D. would be appreciably decreased A similar reservation has to be made for the association of the three strains

included under *F. giganteus*. *F. roseolus* is represented by two isolates with significantly different GC values, of the order of 4 to 5% apart, indicating that the organisms may be related but are not likely to be members of the same genospecies in the sense of Ravin (1963). The strain designated as *F. ruber* had a GC value intermediate between the two values for the strains of *F. roseolus*. Six isolates assigned to *F. flexilis* and its varieties *pelliculosus* and *iolanthae* have GC values ranging from 39 to 43%, with a mean of 41·6% and a standard deviation of 1·6%. We conclude that these groups, too, display inhomogeneity in this regard, although there is no obvious way by which an effective division may be made with the data at hand. The three strains assigned to *F. sancti* comprised a uniform assemblage with GC values of 46·2 ± 0·9%. The single strain of *F. elegans* was only slightly but not significantly richer in GC content, its value being 47·4%.

Table 2. *Buoyant density and GC content of DNA calculated for groups of strains of flexibacteria*

Species or group	No. of analyses	Mean value and standard deviation: Density (g./cm.^{3})	Mean value and standard deviation: GC (moles %)
Cytophaga latercula	(2)	1·6935	34·2
C. lytica	(12)	1·6929 ± 0·0008	33·6 ± 0·8
C. diffluens group I	(10)	1·6955 ± 0·0014	36·2 ± 1·4
C. diffluens group II	(16)	1·7000 ± 0·0010	40·8 ± 1·0
Flexibacter litoralis	(4)	1·6897 ± 0·0005	30·3 ± 0·5
F. aurantiacus group	(8)	1·6915 ± 0·0014	32·1 ± 1·4
F. giganteus	(9)	1·6911 ± 0·0013	31·7 ± 1·3
F. roseolus	(4)	1·6958 ± 0·0026	36·5 ± 2·6
F. ruber	(2)	1·696	36·7
F. flexilis group	(14)	1·7004 ± 0·0016	41·6 ± 1·6
F. sancti	(6)	1·7053 ± 0·0009	46·2 ± 0·9
F. elegans	(2)	1·7065	47·4
Microscilla arenaria	(2)	1·6915	32·1
M. aggregans group	(15)	1·6974 ± 0·0025	38·2 ± 2·5
(*Flexithrix dorotheae*)	(2)	1·6965	37·2
M. tractuosa	(16)	1·6956 ± 0·0013	36·3 ± 1·3
M. sericea	(4)	1·698 ± 0·0008	38·8 ± 0·8
M. marina	(2)	1·7015	42·3
M. furvescens	(2)	1·704	44·9
Saprospira thermalis	(10)	1·6947 ± 0·0022	35·4 ± 2·2
S. toviformis	(5)	1·6976 ± 0·0009	38·4 ± 0·9
S. albida	(6)	1·7005 ± 0·0015	41·3 ± 1·5
S. grandis	(49)	1·7061 ± 0·0009	47·0 ± 0·9
S. flammula	(4)	1·7073 ± 0·0010	48·3 ± 1·0
Herpetosiphon cohaerens	(2)	1·704	44·9
H. persicus	(2)	1·7115	52·6
H. nigricans	(2)	1·712	53·1
H. geysericolus	(2)	1·7075	48·5

Microscilla and Flexithrix

In this group of 20 strains, *Microscilla arenaria* was at the lower limit with a GC value of 32%, and *M. furvescens* was at the other extreme with a value of 45%. The *M. aggregans* group appeared to be heterogeneous in this respect, and might be better subdivided into a group of 3 strains, Q-3, QQ-1 and QQ-11, with a mean GC value of 41% and a second group, JL-13, and NN-13, which, together with the variety *catalatica* (HI-3), would have

a mean value of 36%. The strain QQ-3, bearing the provisional epithet *Flexithrix dorotheae*, might have an affinity with this latter group or with members of the *M. tractuosa* group. Members of this group, with an average GC value of $36{\cdot}3 \pm 1{\cdot}3$%, may eventually prove to be divisible into at least two species. The two strains assigned to *M. sericea* had indistinguishable GC values. The remaining strain, designated as *M. marina*, had a GC value of 42%, it was thereby readily distinguished from all other strains of the Microscilla group except the higher GC subgroup of *M. aggregans*.

Saprospira

Of the 27 strains assigned to this genus, 19 are considered to be representative of *Saprospira grandis*; the similarity of their GC values (Table 1) is in agreement with this conclusion. The mean value for this species was $47{\cdot}0 \pm 0{\cdot}9$%. Strain A-1 was originally considered to be a member of this species, but the lower density of its DNA indicated a GC value of about 38%. (Melting curves confirmed this difference: the DNA of A-1 melted at a temperature 4 to 5° below that of the DNA from DAW-3). Further phenotypic characterization led to our decision to distinguish this strain as a separate monotypic species, *Saprospira toviformis* (Lewin & Mandel, 1969).

The representatives of *S. thermalis* had DNA base compositions quite distinct from those of *S. grandis*. The GC value for strain CR-125 (33%) was lower than that obtained for BEG and CR-123 (35 to 37%), which it otherwise closely resembled. (In fact, CR-123 and CR-125 were isolated from the same mud sample).

The strain SNZ, designated as *S. flammula* (Lewin, 1965), did not differ significantly in base composition from *S. grandis*, which it closely resembled also in phenotypic appearance, pigmentation and nutrition. Although originally isolated from a non-saline hot spring, it grew well in marine media, so we cannot regard it as specifically distinct from *Saprospira grandis*.

The three strains designated as *S. albida* had GC values very close to 41%.

Herpetosiphon

Holt & Lewin (1968) described a new genus of sheathed gliding bacteria with the type species *Herpetosiphon aurantiacus*. Three strains of this species were examined and found to have GC values of $48{\cdot}1 \pm 1{\cdot}2$%. Four other isolates of morphologically similar filamentous organisms have been assigned to this genus (Lewin 1969). The strain designated as *H. geysericolus* had a GC value indistinguishable from that of the type species *H. aurantiacus*. The representative of *H. cohaerans* was significantly lower in GC content (45%), while *H. persicus* and *H. nigricans* each had GC values about 53%. The genus is therefore clearly divisible into at least three species on the basis of GC values.

Other apochlorotic gliding microbes

We have determined the DNA base compositions of a few similar organisms other than those included in the study of Lewin & Lounsbery (1969). A Vitreoscilla strain provided by Dr B. J. Bachmann (originally isolated by Professor E. G. Pringsheim) had a DNA of a buoyant density indicating a GC value of $43{\cdot}6$%: it was thus indistinguishable from strain V-1 obtained from Dr C. Hageage. Professor R. Y. Morita provided a cell harvest of *Beggiatoa leptomitiformis*, grown as described by Burton & Morita (1964); we obtained for this sample a GC value of 37%.

DISCUSSION

The GC values obtained for 90 of the cultures of flexibacteria studied by Lewin & Lounsbery (1969) ranged from 31 to 53%.

The 18 cultures assigned to the genus *Cytophaga* all had GC values between 33 and 42%, within the range previously reported for that genus by Mandel & Leadbetter (1965). Among the strains assigned by Lewin (1969) to *C. diffluens*, the range of GC values may be too wide for a single species, and it is to be expected that further studies of this group will lead to its division into at least two, possibly three, species.

The 21 cultures assigned to the genus *Flexibacter* ranged in GC values from 30 to 48%. Since most of the species were represented by no more than a few strains, judgements as to homogeneity of GC values can be only tentative. The GC values obtained for two strains assigned to *F. roseolus* differed by at least 4%, and perhaps strain CR-141 should be set apart as a separate species.

The 20 cultures of the genus *Microscilla* had GC values ranging from 32 to 45% GC. *Microscilla aggregans* and *M. tractuosa* may eventually prove to embrace more than one species.

The genus *Saprospira*, represented by 27 isolates, had members with GC values of 53 to 48%. We have reservations as to the uniformity of the members of *S. thermalis* and *S. albida*. *S. toviformis* and *S. flammula* are monotypic; the latter cannot be separated from *S. grandis* on the basis of base composition.

The genus *Herpetosiphon* had representatives whose GC values ranged from 45 to 53%. Three species are monotypic at present, while in the type species, *H. auranticiacus*, the three strains are nearly identical in DNA base composition, with GC values averaging 48%.

The GC values of the Vitreoscilla cultures and of *Beggiatoa leptomitiformis*, as reported in this paper, were within the range found for the filamentous flexibacteria. Leucothrix strains have been reported with GC values, 47 to 49% in the same range (Brock & Mandel, 1966).

The gliding bacteria may be regarded as apochlorotic relatives of the filamentous Cyanophyta, notably the Oscillatoriales (Pringsheim, 1949; Harold & Stanier, 1955). Edelman *et al.* (1967) found GC values for 29 representatives of the blue-green algae to range from 35 to 71%, and commented upon the overlap of GC values for the filamentous gliding bacteria and the Oscillatoriales. In their survey, the 22 representatives of the order Oscillatoriales had GC values which ranged from 39 to 51%. Zimmerman (1966) reported that a strain of *Anacystis nidulans* had a GC value of 49% (*cf.* the value 56% reported by Edelman *et al.* 1967). This strain, when obtained originally from Kratz *via* Myers, has apparently been incorrectly identified, and should more correctly be called *Phormidium mucicola* (Silva, 1962). Its GC value falls neatly in the range of the Oscillatoriales. The additional data now available for filamentous flexibacteria extend the range of their GC values below those for blue-green algae. The argument for relatedness is strengthened by the coincidence of GC values for the sheathed representatives, *Lyngbya* (47 and 51%) and *Herpetosiphon* (44 to 53%).

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Recurrent Group Analysis in the Classification of Flexibacteria

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SUMMARY

A computer programme was used to group 85 strains of flexibacteria on the basis of similarity in 84 structural, physiological and biochemical characteristics. The procedure gave 19 groups, the members of which were very similar in their characteristics. The groups could be grouped in seven assemblages whose members showed lesser, but still considerable, similarity. The groups may represent taxonomic entities at the species level; the assemblages, those at the generic level.

A computer programme originally developed for identifying communities by determining recurrent groups of invertebrates* on the basis of their frequency of co-occurrence in samples of forest litter, soil, and zoo-plankton (Fager, 1957; Fager & McGowan, 1963) was used, with little change, for grouping the strains of flexibacteria investigated by Lewin & Lounsbery (1969). For each pair of strains, the programme calculated an index of affinity, the geometric mean of the proportion of common characteristics corrected for the number of characteristics recorded for the strains $(J/\sqrt{(A \times B)} - 1/2\sqrt{B}$; where A and B are the total numbers of characteristics recorded for the two strains, $B \geqslant A$, and J is the number of common characteristics, i.e. positive matches). All characteristics were weighted equally. Unlike most grouping procedures (Sneath, 1962), the programme first formed the largest group within which all possible pairs of strains had affinity indices at or above a preset value. The members of the first group were then removed from further consideration and the largest possible group was formed among the remaining strains. This process was continued until all strains were either placed in a group or designated associates of a group. The latter were strains that had affinity indices above the preset level with some, but not all, of the members of a group. Where two or more groups of the same size were possible, the programme selected the one for which the sum of affinity indices for all pairs of species within the group was greatest. When the alternative groups had species in common, this resulted in elimination of one or more groups. When there were no species in common, all groups were eventually selected. It has been found in previous work that values of the index in the range 0·500 to 0·600 give useful groupings. In the present case, the use of 0·575 gave a number of groups of reasonable size without involving very large numbers of alternative groups that would require excessive computer time.

The 84 structural, physiological and biochemical characteristics that were recorded for the strains are given in Table 1. Information on the metabolism of various carbo-

* FORTRAN listings are available for the grouping programme (REGROUP), the programme for determining connections between groups (CONNEX), and the programme for listing the characteristics shared by group members (STATION). These have been programmed for the CDC 3600.

Table 1. *Characteristics recorded, based on the data of Lewin & Lounsberg (1969).*

Guanine+cytosine mole % in DNA

Less than 30 %	40·1 to 42·5
30·0 to 32·5	42·6 to 45·0
32·6 to 35·0	45·1 to 47·5
35·1 to 37·5	47·6 to 50·0
37·6 to 40·0	Greater than 50·0

Morphology, etc.

Less than 10 μ in length	Not helical
10 to 50 μ in length	Rhapidosomes observed
Greater than 50 μ in length	Rhapidosomes not observed
Helical	Sheathed

Pigmentation

Type I—red (flexixanthin)	Type IV—yellow (zeaxanthin)
Type II—pink	Type V—yellow
Type III—orange (saproxanthin)	Type VI—red

Digestive enzymes

Liquefies carboxymethylcellulose	Does not liquefy gelatin
Does not liquefy carboxymethylcellulose	Liquefies agar
Hydrolyses starch	Does not liquefy agar
Does not hydrolyse starch	Liquefies alginate
Liquefies gelatin	Does not liquefy alginate

Oxidation-reduction enzymes

Catalase produced	H_2S evolved
Catalase not produced	H_2S not evolved

Reactions to tyrosine (5 g./l.) and dihydroxyphenylalanine (0·1 g./l.)

No growth on tyrosine	No growth on dihydroxyphenylalanine
Growth, plus colour change	Growth, plus colour change
Growth, no colour change	Growth, no colour change
Growth, plus dissolution	Growth, plus dissolution
Growth, no dissolution	Growth, no dissolution

Effect of inhibitors

Not inhibited by penicillin (10^{-6} g./l.)	Not inhibited by lauryl sulphate (0·1 g./l.)
Inhibited by penicillin (10^{-6} g./l.)	Inhibited by lauryl sulphate (0·1 g./l.)

Vitamin requirements

Thiamine required	Cobalamin not required
Thiamine not required	Other vitamins not required
Cobalamin required	

Temperature effects

Can grow above 40°	Can grow above 30° but not 35°
Can grow above 35° but not 40°	

Salinity tolerances

Can grow in above 2 × sea-water media	Cannot grow in diluted sea-water media
Can grow in sea water but not 2 × sea-water media	Can grow in diluted sea water but not freshwater media
Can grow in $\frac{1}{2}$ × sea water but not sea-water media	Can grow in freshwater media

Table 1 (*cont.*)

Nitrogen requirements

Nitrate alone suffices	Phenylalanine or tyrosine required
Glutamate alone suffices, but not nitrate	Threonine required
Arginine required	Tryptophan required
Aspartic acid required	Valine required
Histidine required	Asparagine required
Glycine required	Growth on Casamino acids alone (specific requirements unknown)
Isoleucine required	Growth on Casamino acids plus yeast nucleic acid hydrolysate
Leucine required	Growth on Tryptone but not on Casamino acids
Lysine required	
Methionine required	

hydrates was also available, but it was decided that the data were too imprecise to use (*cf.* Dworkin, 1966) and they were, therefore, omitted. Details of experimental procedures will be found in Lewin & Lounsbery (1969). The strain designations used in this paper are the ones used by those authors.

Eighty-five strains of flexibacteria were used. Some strains had positive scores for as many as 33 characteristics, others for as few as 14. The different numbers are the result of two things: incomplete information about some strains and an attempt to minimize the amount of redundant information used. As an example of the latter, a strain that could use nitrate as sole nitrogen source was not scored as *not requiring* any of the 13 amino acids tested and was, therefore, recorded only once in regard to the nitrogen requirements, whereas a strain that required specific amino acids could be recorded up to 13 times. These differences may have had some effect on the groupings but every species had enough positive scores to have had affinity (at 0·575) with any of the others.

The grouping procedure gave 19 groups, varying in size from 1 to 19 members. The two single-member groups were strains that showed no affinities with any other strains. Nine strains were close associates but not members of groups. In some cases, their exclusion may have been due to lack of information about these strains.

The groups and associates and their interrelations are shown in Fig. 1. The interrelations are based on determination of the number of pairs of strains that showed affinity between two groups and comparison of this with the possible number. For example, if one group consisted of four strains and another of five, there would be 20 possible between-group pairs of strains and, of these, any number from 0 to 19 might show affinity at or above the preset level. If 20 between-group pairs had shown affinity, the grouping procedure would have put the two groups together in one. As Fig 1. indicates, the groups can be grouped in 7 separate assemblages: groups 1, 12, 18 and 19 by themselves and the rest in three more or less closely connected assemblages of groups. (These assemblages have been designated FA through FG.)

The individual groups are based on similarity of characteristics of their component members and may represent taxonomic entities at the species level. After the groups were determined, the common characteristics of the members of each group were listed. These are shown in Table 2.

The assemblages, based on appreciable but lesser similarity between the groups, may represent taxa at the generic level. For example, in assemblage FC, group 7 consisted of strains that had guanine + cytosine values between 32·5 and 35·0%, were not helical, were yellow, liquefied gelatin, carboxymethylcellulose, agar and alginate,

Table 2. *Characteristics shared by members of recurrent groups of strains*

Group 1

Helical
Rhapidosomes observed
Pigment type III—orange (saproxanthin)
Does not hydrolyse starch
Liquefies gelatin
Does not liquefy agar
Catalase not produced
H_2S not evolved
Growth, no dissolution of tyrosine (5 g./l.)
No growth on dihydroxyphenylalanine (0·1 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)

Arginine required
Aspartic acid required
Histidine required
Iso-leucine required
Leucine required
Lysine required
Methionine required
Phenylalanine or tyrosine required
Threonine required
Tryptophan required
Valine required
Asparagine required

Group 2

Not helical
Pigment type III—orange (saproxanthin)
Liquefies carboxymethylcellulose
Hydrolyses starch
Liquefies alginate
Catalase not produced

H_2S evolved
Growth, no colour change on tyrosine (5 g./l.)
Growth, no dissolution of tyrosine (5 g./l.)
Can grow in above 2 × sea-water media
Can grow in diluted sea water but not freshwater media

Group 3

Not helical
Pigment type IV—yellow (zeaxanthin)
Does not hydrolyse starch
Does not liquefy agar
Does not liquefy alginate
Catalase not produced
H_2S not evolved
Growth, no colour change on tyrosine (5 g./l.)

Growth, no dissolution of tyrosine (5 g./l.)
Growth, no colour change on dihydroxyphenylalanine (0·1 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Can grow in above 2 × sea-water media
Can grow in diluted sea water but not freshwater media

Group 4

Greater than 50 μm. in length
Not helical
Rhapidosomes not observed
Does not liquefy carboxymethylcellulose
Liquefies gelatin
Does not liquefy agar
Does not liquefy alginate
Catalase not produced
No growth on tyrosine (5 g./l.)
No growth on dihydroxyphenylalanine (0·1 g./l.)

Inhibited by lauryl sulphate (0·1 g./l.)
Thiamine required
Other vitamins not required
Can grow in freshwater media
Isoleucine required
Leucine required
Methionine required
Phenylalanine or tyrosine required
Valine required

Group 5

Not helical
Does not liquefy agar
Does not liquefy alginate
Catalase not produced
Growth, no colour change on tyrosine (5 g./l.)

Growth, no dissolution of tyrosine (5 g./l.)
Growth, no dissolution of dihydroxyphenylalanine (0·1 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Can grow in above 2 × sea-water media

Group 6

10 to 50 μm. in length
Not helical
Rhapidosomes not observed
Pigment type III—orange (saproxanthin)

Does not liquefy carboxymethyl cellulose
Liquefies gelatin
Does not liquefy agar
Does not liquefy alginate

Table 2 (*cont.*)

Group 6 *cont.*

Catalase not produced
H_2S evolved
Growth, no colour change on tyrosine (5 g./l.)
Growth, no dissolution of tyrosine (5 g./l.)
No growth on dihydroxyphenylalanine (0·1 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Cobalamin not required
Can grow in freshwater media
Isoleucine required
Leucine required
Methionine required
Valine required

Group 7

Guanine + cytosine mole % in DNA, 32·5 to 35·0
Not helical
Pigment type IV—yellow (zeaxanthin)
Liquefies carboxymethylcellulose
Hydrolyses starch
Liquefies agar
Liquefies alginate
Catalase produced
Growth, plus colour change on tyrosine (5 g./l.)
Growth, plus dissolution of tyrosine (5 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Thiamine not required
Cobalamin not required
Other vitamins not required
Can grow in above 2 × sea-water media
Can grow in diluted sea water but not freshwater media
Glutamate, but not nitrate, suffices as nitrogen source

Group 8

10 to 50 μm. in length
Not helical
Rhapidosomes not observed
Liquefies carboxymethylcellulose
Liquefies gelatin
Does not liquefy agar
Does not liquefy alginate
Catalase not produced
Inhibited by lauryl sulphate (0·1 g./l.)
Thiamine not required
Cobalamin not required
Can grow above 35° but not 40°
Can grow in $\frac{1}{2}$ × sea water but not sea-water media
Can grow in freshwater media
Nitrate alone suffices as nitrogen source

Group 9

Greater than 50 μm. in length
Helical
Rhapidosomes not observed
Pigment type II—pink
Does not liquefy carboxymethylcellulose
Hydrolyses starch
Liquefies gelatin
Does not liquefy agar
Does not liquefy alginate
Catalase not produced
H_2S evolved
Growth, no colour change on tyrosine (5 g./l.)
Growth, no dissolution of tyrosine (5 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Cobalamin required
Can grow above 40°
Can grow in $\frac{1}{2}$ × sea water but not sea-water media
Can grow in freshwater media
Aspartic acid required
Isoleucine required
Leucine required
Phenylalanine or tyrosine required
Valine required

Group 10

Guanine + cytosine mole % in DNA, 30·0 to 32·5
10 to 50 μm. in length
Not helical
Rhapidosomes not observed
Pigment type V—yellow
Liquefies carboxymethylcellulose
Liquefies gelatin
Does not liquefy agar
Does not liquefy alginate
Catalase produced
H_2S not evolved
Growth, no colour change on tyrosine (5 g./l.)
Growth, plus dissolution of tyrosine (5 g./l.)
Growth, no colour change on dihydroxyphenylalanine (0·1 g./l.)
Growth, no dissolution on dihydroxyphenylalanine (0·1 g./l.)
Not inhibited by penicillin (10^{-6} g./l.)
Not inhibited by lauryl sulphate (0·1 g./l.)
Thiamine not required
Cobalamin not required
Other vitamins not required
Can grow above 30° but not 35°
Can grow in freshwater media
Nitrate alone suffices as nitrogen source

Table 2 (*cont.*)

Group 11

Greater than 50 μm. in length
Not helical
Sheathed
Pigment type III—orange (saproxanthin)
Does not liquefy carboxymethylcellulose
Does not hydrolyse starch
Does not liquefy agar
Does not liquefy alginate
Catalase not produced
H_2S not evolved
Growth, no colour change on tyrosine (5 g./l.)
Growth, no dissolution on tyrosine (5 g./l.)
Growth, no colour change on dihydroxyphenylalanine (0·1 g./l.)
Growth, no dissolution on dihydroxyphenylalanine (0·1 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Can grow above 30° but not 35°
Can grow in above 2 × sea-water media
Can grow in diluted sea water but not freshwater media

Group 12

Guanine+cytosine mole % in DNA, 37·6 to 40·0
Not helical
Pigment type III—orange (saproxanthin)
Does not liquefy carboxymethylcellulose
Hydrolyses starch
Does not liquefy gelatin
Does not liquefy agar
Liquefies alginate
Catalase not produced
H_2S not evolved
Growth, no colour change on dihydroxyphenylalanine (0·1 g./l.)
Growth, no dissolution on dihydroxyphenylalanine (0·1 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Can grow above 30° but not 35°
Can grow in above 2 × sea-water media
Can grow in diluted sea water but not freshwater media
Growth on Tryptone but not on Casamino acids

Group 13

Guanine+cytosine mole % in DNA, 35·1 to 37·5
10 to 50 μm. in length
Not helical
Pigment type III—orange (saproxanthin)
Does not liquefy carboxymethylcellulose
Hydrolyses starch
Liquefies gelatin
Does not liquefy agar
Does not liquefy alginate
Catalase not produced
H_2S evolved
Growth, no colour change on tyrosine (5 g./l.)
Growth, no dissolution on tyrosine (5 g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Can grow in above 2 × sea-water media

Group 14

Guanine+cytosine mole % in DNA 40·1 to 42·5
Not helical
Pigment type III—orange (saproxanthin)
Liquefies carboxymethylcelullose
Hydrolyses starch
Liquefies gelatin
Liquefies agar
Liquefies alginate
Catalase not produced
H_2S not evolved
Growth, no colour change on tyrosine (5 g./l.)
Growth, no dissolution on tyrosine (5 g./l.)
Not inhibited by penicillin (10^{-6} g./l.)
Inhibited by lauryl sulphate (0·1 g./l.)
Can grow above 30° but not 35°
Can grow in above 2 × sea-water media
Can grow in diluted sea water but not freshwater media

Group 15

10 to 50 μm. in length
Not helical
Rhapidosomes observed
Liquefies carboxymethylcellulose
Hydrolyses starch
Liquefies gelatin
Liquefies agar
Liquefies alginate
H_2S evolved
Growth, no dissolution on tyrosine (5 g./l.)
No growth on dihydroxyphenylalanine (0·1 g./l.)
Not inhibited by penicillin (10^{-6} g./l.)
Thiamine not required
Cobalamin not required
Other vitamins not required
Can grow above 35° but not 40°
Can grow in above 2 × sea-water media
Can grow in diluted sea water but not freshwater media
Glutamate, but not nitrate suffices as nitrogen source

Table 2 (*cont.*)

Group 16

Guanine + cytosine mole % in DNA, 32·6 to 35·0	Does not liquefy alginate
Less than 10 μm. in length	Catalase not produced
Not helical	H_2S not evolved
Rhapidosomes not observed	Growth, plus dissolution on tyrosine (5 g./l.)
Pigment type IV—yellow (zeaxanthin)	No growth on dihydroxyphenylalanine (0·1 g./l.)
Does not liquefy carboxymethylcellulose	Inhibited by lauryl sulphate (0·1 g./l.)
Liquefies gelatin	Thiamine not required
Does not liquefy agar	Cobalamin not required
	Other vitamins not required

Group 17

Not helical	Doos not liquefy alginate
Rhapidosomes not observed	Catalase not produced
Pigment type III—orange (saproxanthin)	H_2S not evolved
Does not liquefy carboxymethylcellulose	Growth, no colour change on tyrosine (5 g./l.)
Does not hydrolyse starch	Growth, no dissolution on tyrosine (5 g./l.)
Liquefies gelatin	Inhibited by lauryl sulphate (0·1 g./l.)
Does not liquefy agar	Can grow in freshwater media

Group 18

Guanine + cytosine mole % in DNA, 40·1 to 42·5	Growth, no colour change on tyrosine (5 g./l.)
Greater than 50 μm. in length	Growth, plus dissolution on tyrosine (5 g./l.)
Not helical	No growth on dihydroxyphenylalanine (0·1 g./l.)
Rhapidosomes observed	Not inhibited by penicillin (10^{-6} g./l.)
Pigment type III—orange (saproxanthin)	Inhibited by lauryl sulphate (0·1 g./l.)
Does not liquefy carboxymethylcellulose	Can grow above 30° but not 35°
Liquefies gelatin	Can grow in above 2 × sea-water media
Does not liquefy agar	Cannot grow in diluted sea-water media
Does not liquefy alginate	Growth on Tryptone but not on Casamino acids
Catalase not produced	
H_2S not evolved	

Group 19

Guanine + cytosine percentage in DNA, 42·6 to 45.0	Liquefies alginate
10 to 50 μm. in length	Inhibited by lauryl sulphate (0·1 g./l.)
Not helical	Thiamine not required
Rhapidosomes observed	Cobalamin not required
Sheathed	Other vitamins not required
Pigment type III—orange (saproxanthin)	Glutamate, but not nitrate, suffices as nitrogen source
Liquefies agar	

hydrolysed starch, grew on tyrosine medium with colour change and dissolution, could use glutamate but not nitrate as nitrogen source, and grew in 2 × to $\frac{1}{2}$ × sea-water media. Group 15 was made up of strains with much the same characteristics except that it contained both orange and yellow strains, had guanine + cytosine values between 32·5 and 37·5%, and growth on tyrosine medium did not result in dissolution and was variable in colour change. The strains in group 14 were similar to those in group 15 but had guanine + cytosine values between 40 and 42·5%, were both orange, and did not produce a colour change on tyrosine medium; no information was available on their nitrogen requirements. Group 2 consisted of strains much like those in group 14 except that they had guanine + cytosine values ranging from 30 to

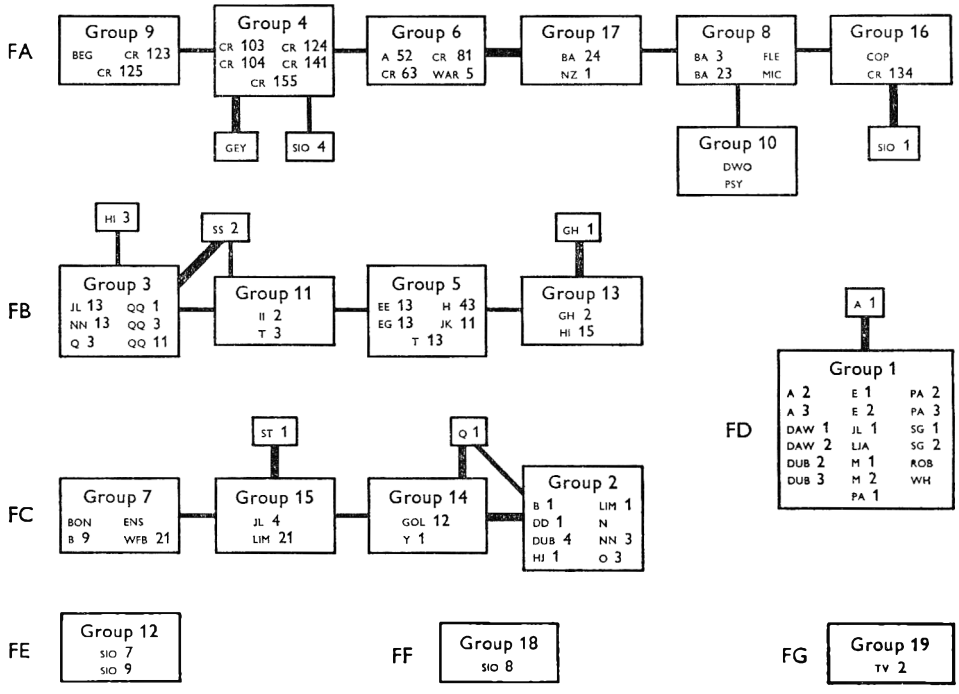

Fig. 1. Groups and interrelations of groups among 85 strains of flexibacteria, as defined by a computer programme for determination of recurrent groups. The groups are numbered 1 through 19. Associates are shown in the small boxes connected to the groups. Assemblages of groups are designated FA through FG. Heavier lines = $\frac{1}{2}$ or more of the possible connections between groups realized; lighter lines = between $\frac{1}{4}$ and $\frac{1}{2}$ of the possible connections realized (see text).

45%, and one strain (HJ 1) which did not liquefy agar or gelatin. The groups in this assemblage (FC) have, therefore, considerable similarity.

The arrangement of the strains in groups and of the groups in assemblages is based on overall similarities using all of the information available. All of the characters were given equal weight. As Sneath (1962) pointed out, this procedure leads to a consistent taxonomy, and is particularly useful when the aim is to form taxa of greatest information content. Although the groups and assemblages suggested by the computer analysis have been used for the most part by Lewin (1969) as a basis for classification, he has in some cases departed from them because he felt that certain characteristics should be given more weight than others in defining species and genera. A detailed discussion of his reasons for the changes is given in his paper.

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A Classification of Flexibacteria

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SUMMARY

About 90 strains of gliding microbes (flexibacteria) have been considered. Data obtained by Lewin & Lounsbery (1969) and Mandel & Lewin (1969) have been used by Fager (1969) in a computer programme designed to indicate affinities and thereby possible relationships. Largely on the basis of Fager's analysis, a classification of these organisms is here proposed.

Including a few species described elsewhere, 27 species and varieties are distinguished, most of them apparently new. A simplified diagnostic key is presented for their identification. They are assigned to six genera, three (*Saprospira*, *Flexithrix* and *Herpetosiphon*) are recognized primarily on morphological grounds; three others (*Cytophaga*, *Flexibacter* and *Microscilla*), here somewhat re-defined, are based largely on other convenient characteristics.

INTRODUCTION

When these studies began, some years ago, the taxonomy of gliding bacteria was in a state of confusion. The Myxobacterales, having recognizably distinct fruiting bodies, were known to a few specialists, though until recently few myxobacteria had been cultured in the laboratory. The gliding microbes which do not form spores were even less well known. *Saprospira* was erroneously included among the spirochaetes. One or two sheathed representatives were associated with the Chlamydobacteriales. Gliding organisms which attack cellulose, and similar organisms capable of digesting a variety of other polysaccharides, were assigned to the genus *Cytophaga*, the delimitation of which has become more and more diffuse in recent years. Other similar microbes were usually unrecognized when material collected from nature was examined directly under the microscope, and tended to be overlooked in bacteriological studies of natural waters since most of the culture media routinely used for such purposes contain concentrations of organic nutrients inhibitory to their growth. Those which did grow, being generally yellow, were usually classed along with yellow eubacteria in the ill-defined genus *Flavobacterium*, and were rarely classified further. Many of these and related taxonomic problems were discussed by Soriano & Lewin (1965). In the present paper we shall first review the descriptions of the genera to which such microbes might be assigned, and then indicate some specific and generic distinctions which we consider to be useful.

A few species, more or less regularly helical, have been set apart in the genus *Saprospira*, comprising *S. grandis* (Lewin, 1962), *S. albida*, *S. flammula* and *S. thermalis* (Lewin, 1965*a*, *b*). (The nutrition of *S. albida* and *S. flammula* presented special problems, and these species were therefore excluded from consideration in this paper.)

Others are distinguished by the presence of a sheath, as in the new genera *Herpetosiphon* (Holt & Lewin, 1968) and *Flexithrix* (Lewin, 1969*a*). In the present article, attention is concentrated chiefly on free-living, aerobic, non-helical species. Some of these form filaments of indefinite length (*Flexibacter* spp. and *Microscilla* spp.); others have either shorter filaments or more or less fusiform cells only a few μm. long. Several of the latter forms have been previously recognized as species of *Cytophaga* (Winogradsky, 1929, as amended by Stanier, 1940), but this genus is clearly in need of further revision. *Cytophaga* and certain other genera have accordingly been somewhat re-defined. (Revision of the genus *Flavobacterium*, which may include similar forms, is even more necessary; Quadling, Cook & Colwell, 1964; Hendrie, Mitchell & Shewan, 1968), but until the nature and necessity of this genus are clarified we prefer to avoid using it.) We have based generic distinctions primarily on morphology, and specific distinctions largely on nutritional and other biochemical characteristics of laboratory cultures. This may not be phylogenetically sound, but it has been commonly accepted practice among bacteriologists. Our recommendations are based largely on the analysis of recurrent groups by Fager (1969), somewhat weighted (or biassed!) by considerations of GC values (Mandel & Lewin 1969). Formal descriptions of the new species are presented in Table 1 of this paper.

Genera and families of flexibactera reviewed and revised

Before considering the various specific groupings indicated by computer analysis we should first define and describe the several genera to which they may be assigned. These are presented, with some historical annotations, in the following review.

Among our most serious problems was that of distinguishing among the genera *Cytophaga*, *Flexibacter* and *Microscilla* (see Soriano & Lewin, 1965). We first considered the practicability of assigning all of these forms, or at least the majority—those with shorter cells or filaments—to the genus *Cytophaga*. As indicated by Imshenetsky & Solntseva (1936), Stanier (1947) and Soriano & Lewin (1965), this genus is in need of a stricter formal definition. The following list of names and references summarizes its history:

1929. *Cytophaga hutchinsonii* Winogradsky. (Also 3 to 4 other spp.) Winogradsky, though he did not obtain pure cultures, recognized that the cells were quite unlike those of eubacteria or spirochaetes. He created the genus on the ability of the cells to digest cellulose, a faculty which he considered to be obligate.

1934. *Cytophaga* spp. Stapp & Bortels. From soil. Gliding ability noted.

1940. *Cytophaga* (em. Stanier, 1940) based on *C. hutchinsonii* Winogradsky. This is the type species of the genus *Cytophaga* as first redefined by Stanier, comprising aerobic, facultatively cellulolytic gliding microbes that do not form microcysts. *C. krzemieniewskae* (note corrected spelling) Stanier and *C. diffluens* Stanier, two new species capable of digesting agar as well as cellulose, were also described.

1942. *Cytophaga* (em. Stanier, 1942) based on *C. hutchinsonii* Winogradsky. The genus was extended in scope to include other aerobic gliding forms recognized 'on purely morphological grounds'; not necessarily able to attack cellulose, but generally capable of digesting other polysaccharides, such as agar. Four species were recognized from soil, and two from marine mud.

1945. *C. columnaris* Garnjobst. A fish pathogen, capable of growth on media containing protein hydrolysates; not cellulolytic, and not requiring any specific polysaccharide for growth.

1946. *C. senstitiva* Humm. Digests agar, apparently used as major carbon source.

1947. *C. johnsonae* Stanier. Digests chitin or cellulose, but can otherwise be grown in protein hydrolysate media. Facultatively anaerobic.

1948. *C. psychrophila* Borg. A fish pathogen; *cf. C. columnaris* Garnjobst, 1945.

1955. *C. fermentans* Bachmann. Utilizes a variety of soluble carbon sources, but not cellulose, agar or chitin. Facultatively anaerobic.

1961. *C. succinicans* Anderson & Ordal. Can grow on protein hydrolysate media; cellulose 'not fermented'. Facultatively anaerobic.

Excludenda (? = *Sporocytophaga myxococcoides* Stanier, 1940).

1919. *Spirochaeta cytophaga* Hutchinson & Clayton.

1930. *Cytophaga myxococcoides* Krzemieniewska.

1934. *Cytophaga globulosa* Stapp & Bortels.

1936. Some *Cytophaga* spp. Imshenetsky & Solntseva.

It may be noted that at least *C. psychrophila*, *C. fermentans* and *C. succinicans* can grow anaerobically as well as aerobically. Though certain obligate anaerobes, such as the gliding species of *Fusobacterium*, could with justification be reassigned to the genus *Cytophaga*, as defined by Stanier in 1942, we feel that this would extend the bounds of the latter genus beyond convenient limits. We consider that it is now necessary to delimit *Cytophaga* more precisely, and propose the following redefinition. (Additional characters, not strictly part of the definition, are added in parentheses.)

Cytophaga Winogradsky (1929) emend. Non-photosynthetic (normally with yellow, orange or red carotenoid pigments); non-flagellate but capable of gliding (and, if sufficiently long, of flexing) on solid substrata; short or elongate rods or filaments (usually 5 to 50 μm. long, 0·3 to 0·7 μm. wide, with rounded or tapered ends); unbranched, unsheathed, not helical; not forming distinct fruiting bodies (though the cells may aggregate in pustular assemblages); not forming either endospores or microcysts (though inflated, more or less spherical cells are commonly formed in some cultures); Gram-negative (generally with a lower refractility than most eubacteria); obligately or facultatively aerobic; obligately heterotrophic; usually capable of digesting (or depolymerizing) several insoluble or macromolecular colloidal polysaccharides such as cellulose (or carboxymethylcellulose), chitin, agar, alginates, etc. The last characteristics, in particular, help to distinguish *Cytophaga* (as redefined here) from *Flexibacter* and *Microscilla* (both redefined below), which have generally more limited extracellular polysaccharase activity.

The ability of certain strains of *Cytophaga* to dissolve and digest cellulose is known to be a labile feature, which tends to be 'lost' in the course of cultivation when the organism is regularly maintained on more readily assimilable organic nutrients. Probably the same is true for the faculty of certain strains to digest other polysaccharides such as agar.

Promyxobacterium, a genus of 'imperfect myxobacteria', was distinguished by Imshenetsky & Solntseva (1936) mainly by the tapered ends of the cells or filaments. However, as these authors indicated, and as Stanier (1947) and we ourselves have confirmed in several clones, this feature is inconstant: the ends may be blunt or tapered according to the state of nutrition and the age of the culture. Stanier (1947) wrote: 'until nonfruiting myxobacteria have been more extensively studied, the genus *Cytophaga* appears to me to provide an adequate taxonomic pigeonhole for all known amicrocystogenous species.' As explained above, we can no longer agree with Stanier completely on this matter; but we, nevertheless, accept his reservations about the genus *Promyxobacterium*. This name may perhaps be conveniently reserved for gliding forms which have sufficiently high GC values to indicate a phylogenetic relationship with the fruiting Myxobacterales. Since cultures were unavailable, we were unable to compare our strains with those non-fruiting myxobacteria described as *Cytophaga* species by Imshenetsky & Solntseva (1936), Fuller & Norman (1943), Humm (1946), Starr (1953) and others referred to in *Bergey's Manual* (1957).

Many bacteriologists have assigned all kinds of yellow, Gram-negative, rod-shaped bacteria to the genus *Flavobacterium*, without regard to other criteria such as flagellation. Some of these can glide, including the generic neotype *F. flavescens* (Taylor's strain B. 2157). Although almost all of the gliding microbes under discussion are yellow or orange (or, more rarely, some shade of red), we cannot yet envisage any causal connection between carotenoid formation and gliding motility. Limitations of time and resources hindered us from any serious attempt to sort out the problems of this 'regrettable genus' (Stanier, 1947). Floodgate & Hayes (1963) and Hayes (1963) have attempted to bring some measure of order to this genus, but we did not compare our isolates with those in other collections by direct examination of the organisms themselves or by consideration of their described features. A comparison on the basis of the published literature would be difficult and relatively profitless, since we had chosen to study a set of characteristics somewhat different from those selected by Hayes and others.

The genus *Flexibacter* was described by Soriano (1945) with the following features (translated from Spanish): 'flexible rods capable of gliding movement, without intracellular sulphur granules, unable to attack cellulose, not forming fruiting bodies on substrate (as do Myxobacteriales), and not spiral in form.' Since his type species, *F. flexilis*, has cells 0·5 to 0·7 μm. wide, 10 to 20 μm. long, and his *F. giganteus* has filaments 100 μm. or more in length, it is clear that Soriano's use of the word 'rods' (= 'bastoncitos') is to be interpreted broadly.

On the basis of this description it is clear that *Flexibacter* cannot be distinguished from *Microscilla* (Pringsheim, 1951; see below), as was pointed out by Soriano & Lewin (1965). Rather than dismiss the later generic name as invalid, we have chosen to redefine both genera as follows, and to use them for our two largest groups of species.

Flexibacter Soriano (1945) emend. (*a*) Characters in common with *Microscilla* (below) include the following: flexible but not helical rods or filaments, usually 1 μm. or less in width; crosswalls not apparent (at magnifications of about $\times$ 1000); not branched or sheathed; without flagella; capable of gliding on solid substrata; Gram-negative; without photosynthetic pigments or intracellular sulphur granules; unable to attack cellulose; reproduction by simple fragmentation; not forming fruiting bodies, spores or microcysts; (*b*) filaments generally

5 to 50 μm. long; colour (seen only in packed masses) pink, red, orange or yellow; liquefying gelatin but not agar or alginate. Habitats: mostly along freshwater banks, hot springs, etc. (However, two of our species were marine).

Microscilla was described by Pringsheim (1951) as one of the new genera of his new family Vitreoscillaceae which he defined as 'colourless, filamentous, gliding micro-organisms, differing from Myxophyceae in lacking assimilatory pigments, and from Myxobacteria by a more pronounced trichome formation and the absence of microcysts'. His brief definition of *Microscilla* was: 'trichomes narrow without perceptible septation. Gliding movements active. Reproduction by division into relatively long daughter trichomes. Type species *M. marina* (trichomes 0·5 to 0·6 μm. wide, up to 100 μm. long...)'

As thus defined, *Microscilla* is not clearly distinguishable from *Flexibacter* (Soriano, 1951; see above). Partially to clarify this distinction, the following amended description is proposed:

Microscilla Pringsheim (1951) emend. (*a*) Characters in common with *Flexibacter* as above; (*b*) filaments usually 20 to 100 μm. or longer; colour (seen only in packed masses) yellow or orange; do not digest cellulose or agar, although some species liquefy alginate and gelatin. Habitat: marine shores.

Vitreoscilla spp. (Pringsheim, 1951) are distinguished by their apparent lack of pigments and by the torulose nature of their filaments, which exhibit marked constrictions at the intercellular nodes that may betray a wholly distinct mode of cell division. They seem less closely related to the other gliding microbes described above and may have closer affinities with the blue-green algae.

The following three flexibacterial genera are characterized by morphological features (helical filaments, simple or branched sheaths) which normally distinguish them from the genera described above.

Saprospira Gross (1911) emend. Lewin (1962). Unbranched, helical, multicellular, filamentous, usually 50 to 500 μm. long. (All of our strains of *S. grandis*, *S. toviformis*, and *S. thermalis* liquefy gelatin but not agar, grow on media containing 5·0 g. tyrosine/l. without clearing (i.e. dissolution of the insoluble excess) or discoloration, and are inhibited by 0·1 g. sodium lauryl sulphate/l.; but we do not consider these necessary prerequisite features for species in this genus.)

Herpetosiphon Holt & Lewin (1968). Unbranched, multicellular, filamentous, *sheathed*, usually 50 to 500 μm. long. (Our marine strains all failed to hydrolyze starch, agar, alginate or carboxymethyl cellulose, produced neither catalase nor H_2S, tolerated 5·0 g. tyrosine/l., grew on medium containing 0·1 g. DOPA/l. without clearing or discoloration, and are inhibited by 0·1 g. sodium lauryl sulphate/l.; but we do not consider these necessary prerequisite features for species in this genus.)

Flexithrix Lewin (1969*a*). Falsely branched (under certain conditions), multicellular, filamentous, up to 500 μm. or more long. The only strain we have isolated was marine, contained the yellow carotenoid zeaxanthin, and in many other respects resembled *Microscilla aggregans* (described below). In the initial survey we observed at least one other dissimilar strain, which was not isolated or studied further. No meaningful generalization can be made at this time about the physiology and biochemistry of species in this genus.

In the light of new information presented in this paper, and the proposed definitions and redefinitions of the genera, it is also appropriate to re-examine here the problems of assigning the genera to higher taxa. This matter was left unresolved in the earlier paper by Soriano & Lewin (1965), in which the literature was reviewed and the arguments discussed. At present, the following modified classification seems to us as convenient as any:

1. Beggiatoaceae	Cylindrical; with sulphur granules
2. Leucotrichaceae	Tapering
3. Simonsiellaceae	Flattened
4. Vitreoscillaceae (excluding *Microscilla*)	Filaments constricted at nodes
5. Cytophagaceae (or Flexibacteraceae)	Cylindrical; with carotenoids
Saprospira	Helical
Flexithrix	Branched
Herpetosiphon	Sheathed
Sporocytophaga	Microcysts
'*Sphaerocytophaga*' (*Fusobacterium* p.p.)	Anaerobic (see Dworkin, 1966)
Cytophaga	Mostly < 20 μm. polysaccharolytic
Flexibacter	Lengths various: mostly from freshwater or mud
Microscilla	Mostly > 20 μm. marine

(*Moraxella* should no longer be considered in this order; see Lautrop, 1965–67).

It is possible that all prokaryotic microbes capable of gliding are phylogenetically related, and that such apochlorotic forms as we have studied here originated from photosynthetic cyanophytes. This possibility was reviewed briefly by Soriano & Lewin (1965). However, it is now the opinion of the present author that, although members of Beggiatoaceae, Leucotrichaceae, Simmonsiellaceae and Vitreoscillaceae could have evolved in this way, the Cytophagaceae, with which this paper is primarily concerned, may have had different origins. New light will be shed on this problem when more biochemical information becomes available.

Preliminary considerations for a revised and expanded classification of flexibacteria

> 'A taxonomy has to be used; groups need to be named and to be capable of recognition...Even the best of numerical classifications must be regarded as an approximation, or a guide to judgement, not a judgement in itself...There is an inalienable region of judgement into which numerical taxonomy cannot enter ...The taxonomist...must have the courage to reallocate in the light of his own experience.' (Selected sentences from Williams, 1967.)

We have confined our attention almost exclusively to our own collection of cultures, which we isolated from nature, under aerobic conditions at room temperature, on agar media containing low concentrations of organic nutrients (Lewin & Lounsbery, 1969). We accepted or rejected them in the first place solely on the basis of a single criterion, the ability to glide, although it is not known to what extent this feature is

phylogenetically fundamental (see Lautrop, 1965–67). Perhaps because of our choice of sources, or our choice of media or physical conditions, we isolated no colourless filamentous types (such as might be recognized as *Vitreoscilla* or *Leucothrix* spp.); only one apparently colourless, spore-forming myxobacterium (which has been excluded from further consideration in this paper), and no fruiting myxobacteria. In attempting to classify these microbial strains, we have tried to reconcile three more or less independent, although interrelated, approaches:

1. *Orthodox or classical taxonomy*, in which daily experience with the organisms and their reactions under a variety of experimental conditions in the laboratory leads to the subjective formulation of groupings based on cultural similarities.

2. *Numerical taxonomy*, in which a number of characters (selected on the subjective bases of experience and expediency) are recorded for each strain, essentially on a yes-or-no basis, and assigned unit values unweighted by prejudice. The data were then processed by a computer programme designed to extract more objectively defined groupings of organisms.

3. *Molecular genetics*, based on the generally accepted theory that, since the biochemical basis of heredity is nucleic acid, organisms genetically similar cannot exhibit very wide divergences in the overall chemical compositions of their nucleic acids. In other words, wide differences indicate an absence of close relationship. For example, all but one of the 23 organisms originally identified on morphological and physiological grounds as *Saprospira grandis* have GC values of $47{\cdot}7 \pm 1{\cdot}1\,\%$, which suggests that this is a reliable distinguishing characteristic. The close morphological, physiological and biochemical resemblances among the various strains identified with this species endorse the soundness of the argument for supporting taxonomy by GC values. The finding of a single aberrant strain, A-1, with a GC value of 38%, throws the argument into question; but we have now detected sufficient minor physiological and cultural characteristics of this strain to warrant distinguishing it as a separate species, to be called *Saprospira toviformis* (Lewin & Mandel, 1969).

It could hardly be expected that we would be able to reconcile perfectly the groupings suggested by these three approaches, but we have tried to eliminate the major points of disagreement; actually, there were few. In such cases, we have chosen to 'split', rather than to 'lump', since this expedient is less likely to be a source of confusion in the future when more relevant information comes to light, but to certain strains we have assigned names of only varietal rank (see below).

We have used for primary generic distinctions such few morphological features as are readily apparent. The four genera or genus complexes under consideration are:

Saprospira (not sheathed; helical): Gross, 1911.

Herpetosiphon (sheathed; unbranched): Holt & Lewin, 1968.

Flexithrix (sheathed; with false branches): Lewin, 1969*a*.

The *Cytophaga* + *Microscilla* + *Flexibacter* complex (not sheathed; not helical).

Although the genera have been distinguished as above we admit that the features we have used are not necessarily constant and invariable. As noted elsewhere (Lewin, 1962), many strains of *Saprospira grandis* tend to lose their helical nature in culture; and we have even picked up in culture a spontaneous bacillary variant, which in the course of laboratory subculture, has now reverted to its original filamentous form. Such changes must occur in nature, too, as the following indicates. From ponds

and ditches in Costa Rica we isolated various pink filamentous forms, of which we called the helical ones *Saprospira* and the non-helical ones *Flexibacter*; yet in almost all other respects certain of these strains were indistiguishable. *Flexithrix* is another debatable case; it may be regarded as a 'form genus'. Though under some circumstances it forms sheathed, falsely branched filaments which clearly distinguish it from other bacteria, it can be grown in liquid as single naked cells which, in their pigmentation and other biochemical and nutritional features, are otherwise indistinguishable from a species of *Microscilla* as defined below. *Flexithrix*, thereby, presents taxonomic problems which are discussed elsewhere (Lewin, 1969*a*).

The classification of the remaining organisms, which constituted the large majority, presented a major problem, and we are especially grateful to Dr E. W. Fager for aid in its solution. Details of his computer analysis are given in a separate paper (Fager, 1969); here we discuss only our treatment of his 19 groups.

Our objective was to sort the organisms of our collection into named species or subspecies. Since we have still insufficient information on the ranges of various characters within our proposed taxa, we considered designating similar strains merely by a code number, rather than a formal binomial, to indicate the provisional nature of our taxa; but we rejected this device since (*a*) it would not conform with the currently accepted International Code of Bacteriological Nomenclature, and (*b*) numbers are less convenient for the human memory than are names. We therefore decided to take a more orthodox taxonomic approach, since we feel that for the gliding bacteria, most of which have at present no formal identity at all, even provisional names are better than none. We are not attempting to impose an immutable nomenclature on these organisms, fully recognizing that the validity of any taxon lasts only as long as it is useful and until something better is proposed. But a start must be made somewhere. We have tried to arrive at a workable classification. Similar strains have been grouped, the groups have been called species, and the species have been assigned names for mnemonic and logistic convenience. Likewise, similar species have been grouped, and each resulting assemblage has been called a genus (this seems the only reasonable definition of a genus), and the genera have been assigned names for the same reasons of convenience.

We have tried to be conservative in the creation of new taxa. Whenever possible we have tried to select existing generic names, with a minimum violation of their original definitions, as indicated in an earlier section of this paper. Thus we have assigned almost all of our isolates to one of the four established genera *Cytophaga*, *Flexibacter*, *Microscilla* and *Saprospira*, though in each case it has been found necessary to modify the original descriptions of these genera At the specific level, we have delimited as species only those in which distinctions were clearly indicated on the basis of such stable characters as GC value, pigment type, experimentally established requirements for particular growth factors, etc. In several cases we have combined more than one of Fager's groups into single species, which we have then subdivided into two or more varieties. Future research or other considerations may indicate the advisability of either submerging these varieties, or of raising them to specific rank.

Proposed classification

Fager (1969) sorted our strains into groups which for the most part seem acceptable to us as useful taxonomic units; and we therefore regard most of them as equivalent to species or varieties, after making certain changes. Each of Fager's groups is discussed in turn below, with proposed changes by which we have tried to make them conform as genera and species in the customary sense. The specific names which we have assigned to the final specific groupings, after the modifications have been made, are formally presented in Table 1, in which their essential distinguishing features are summarized in tabular form.

We have somewhat rearranged the 85 strains, which by Fager's analysis fell into 19 groups, into 24 species (comprising 31 varieties) as follows:

Group 1 (19 strains of *S. grandis*) has been accepted unchanged. An associated member of this group, strain A-1, is recognized as a separate species, *S. toviformis* (Lewin & Mandel, 1969).

Group 2 (N-3, HJ-1, NN-3, DD-1, DUB-4, B-1, O-2, LIM-1) has been considerably modified in our re-evaluation.

Strain HJ-1 has been removed from this group and recognized as a separate species, since it has a GC value (32·5%) lower than that of other group 2 members (35 to 42%) and, unlike them, cannot digest agar or gelatin. We propose for it the new name *Microscilla arenaria.*

Two strains (GOL-12 and Y-1), constituting Fager's group 14, and two others, JL-4 from the group 15 and its associate ST-1, have been included. These changes are justified on the following bases: affinities, as expressed by computer, GC values, pigmentation, agar digestion and other enzymic activities. The resulting 11 strains have then been divided into 2 varieties.

One (DUB-4, JL-4 and ST-1), with a GC value of 35 to 37%, unites the only agar-digesters which tolerate lauryl sulphate; for these we propose the new varietal name *Cytophaga diffluens* var. *aprica.*

The remaining eight strains differ in these features, but are very similar among themselves. Since they digest CMC, we consider that they may be assigned to the species *Cytophaga diffluens* Stanier emend., although we could not demonstrate the digestion of cellulose (cigarette paper) by any of these strains. (*Cytophaga diffluens* Stanier was originally cellulolytic; Stanier, 1942.) Our strains evolve hydrogen sulphide. According to Breed *et al.* (1957), *Cytophaga diffluens* Stanier does not. However, I find no reference to this feature in the original descriptions of this species (Stanier, 1940, 1942).

Groups 3 (QQ-1, QQ-3, Q-3, QQ-11, JL-13, NN-13). Strain QQ-3, which under certain conditions forms sheathed and sometimes branching filaments, has been removed and assigned to a new genus and species, *Flexithrix dorotheae* (Lewin, 1969*a*).

The remaining five strains form a very homogenous group, for which we propose the new specific name *Microscilla aggregans.*

The single strain HI-3, an associate member of group 3, is regarded as a separate variety, since it has complex nitrogen requirements and is catalase positive. For this latter reason we propose to call it *Microscilla aggregans* var. *catalatica.*

Group 4 (CR-103, CR-104, CR-124, CR-141, CR-155). All of these five strains can grow in freshwater media. The first three form pigment of type II and have a GC value of

32%, whereas strains CR-141 and CR-155 are characterized by pigment of type I and GC values of 35·5 and 38% respectively. Strains CR-141 and CR-155 tolerate sea water or even double-strength sea water, whereas the first three strains listed in this group

Table 1. *Conspectus of the major genera and species of flexibacteria, with their chief diagnostic characteristics*

The species or subspecies are listed in order of increasing GC value.

Genus and species	Number of strains	Molar % GC in DNA	Helical	Branched	Sheathed	Length in μm.	Pigment type	Marine/freshwater	Digests CMC	Digests starch	Digests agar	Digests alginate	Digests gelatine	Catalase	H_2S evolved
Cytophaga															
C. latercula	1	34	−	−	−	5	6	M	+	−	+	+	+	−	+
C. lytica	5	33·5–34·5	−	−	−	10–20	4	M	+	+	+	+	+	V	V
C. diffluens (?)	1	35[6]	−	−	−	20	3	M	+	+	+	+	+	−	+
C. diffluens var. *aprica*	3	35·5–37	−	−	−	10–50	3	M	+	+	+	+	+	−	+
C. diffluens var. *carnea*	1	38	−	−	−	30	3	M	+	−	+	+	+	−	−
C. diffluens	7	40–43	−	−	−	5–30	3	M	+	+	+	+	+	−	V
Flexibacter															
F. litoralis	1	30	−	−	−	50	1	M	−	−	−	−	+	−	+
F. aurantiacus[1]	2	31	−	−	−	5–20	5	F	+	V	−	−	+	+	−
F. aurantiacus var. *copepodarum*	1	31	−	−	−	5	4	M	−	−	−	−	+	−	−
F. giganteus[2]	3	31	−	−	−	50	2	F	−	+	−	−	+	−	V
F. aurantiacus var. *excathedrus*	1	34	−	−	−	10	4	F	−	+	−	−	+	−	−
F. roseolus	2	34·5–38	−	−	−	50	1	F	−	−	−	−	+	−	−
F. ruber	1	36	−	−	−	50	1	F	−	+	−	−	+	−	
F. flexilis var. *pelliculosus*	1	39	−	−	−	10–20	3	F	+	+	−	−	+	−	+
F. flexilis	4	40·5–43	−	−	−	10–50	3	F	−	V	−	−	+	−	+
F. flexilis var. *iolanthae*	1	41	−	−	−	10–30	3	F	−	−	−	−	+	−	−
F. sancti	3	46–47	−	−	−	5–50	4	F	+	V	−	−	+	−	V
F. elegans	1	47	−	−	−	50	3	F	−	−	−	−	+	−	−
Flexithrix															
F. dorotheae[3]	1	37	−	+	+		4	M	+	−	−	−	−	−	−
Herpetosiphon															
H. cohaerens	1	44	−	−	+	60–150	3	M	−	−	−	−	+	−	−
H. geysericolus[8]	1	48	−	−	+	10–150	3	F	+	+	−	−	+	+	−
H. persicus	1	52·5	−	−	+	30–150	3	M	−	−	−	−	+	−	−
H. nigricans	1	53	−	−	+	30–50	4	M	−	−	−	−	+	−	−
Microscilla															
M. arenaria	1	32	−	−	−	20	3	M	+	+	−	+	−	−	+
M. aggregans var. *catalatica*	1	34	−	−	−	20	4	M	−	−	−	−	−	+	+
M. aggregans[4]	5	36·5–41·5	−	−	−	30	4	M	V	−	−	−	−	−	−
M. tractuosa	8	34·5–39·5	−	−	−	10–50	3	V	−	V	−	−	V	−	V
M. sericea	2	38–39	−	−	−	30–100	3	M	−	+	−	+	−	−	−
M. marina	1	42	−	−	−	50	3	M	−	−	−	−	+	−	−
M. furvescens	1	44	−	−	−	5–100	3	M	+	+	−	+	+	−	+
Saprospira															
S. thermalis[5]	3	34·5–37·2	+	−	−	50	2	F	−	+	−	−	+	−	+
S. toviformis	1	38	+	−	−	10–50	3[7]	M	+	−	−	−	+	−	−
S. albida[8]	3	41·5–46·5	−	−	−	50	3	F	V	−	−	−	+	−	−
S. grandis	19	46–48	+	−	−	50	3	M	−	−	−	−	+	−	−
S. flammula[8]	1	48	+	−	−	30	3	F/M	−	−	−	+	−	+	−

For footnotes see foot of facing page

will not grow even in half-strength sea water. These two subgroups also differ in their amino-acid requirements.

The characters of CR-103, CR-104 and CR-124 agree with those of *Flexibacter giganteus* Soriano.

CR-141 and CR-155 are separated from those above, and we propose that they represent a new species, *Flexibacter roseolus.*

We have regarded two associate members of this group, SIO-4 and GEY, as two separate species, on the basis of pigment and other features. For strain SIO-4 we propose the new specific name *Flexibacter litoralis*, mentioned provisionally by Fox & Lewin (1963).

We propose GEY as the type strain of the new species *Flexibacter ruber*, also mentioned provisionally by Fox & Lewin (1963), erroneously as '*F. rubrum*'.

Group 5 (T-13, EE-13, EG-13, H-43, JK-11) has been accepted essentially unchanged; but we consider it convenient to combine with it Fager's group 13 and its associate (i.e GH-1, GH-2 and HI-15). For all of these strains we propose the new specific name *Microscilla tractuosa.*

Group 6 (CR-63, CR-81, A-52, WAR-5) has been accepted unchanged; its characters agree with those of *Flexibacter flexilis* Soriano.

Group 7 (B-9, BON, WFB-21, ENS). Strain LIM-21 from group 15 has been added, since it is clearly similar in most respects. For strains of this group we propose the new specific name *Cytophaga lytica.*

Group 8 (BA-3, BA-23, FLE, MIC). Strain FLE has been removed and established as a separate variety, which we propose tocall *Flexibacter flexilis* var. *pelliculosus.* It is distinguished by its orange pigment (type III) and GC value, 39·5%. The others are yellow (pigment type IV) and have a GC value of 47 to 48%.

For the remaining three strains we propose the new specific name *Flexibacter sancti.* In most respects these strains exhibit characteristics similar to those described for *Cytophaga johnsonae* (Stanier, 1947); but since none of them could be shown to digest chitin we have decided not to identify them with the latter species.

Group 9 (BEG, CR-123, CR-125) has been accepted unchanged. BEG has already been established as the type strain of *Saprospira thermalis* Lewin (1965*b*).

Group 10 (DWO, PSY) has been accepted unchanged. Although sent to us under different names, (*Cytophaga aurantiaca* and *Cytophaga psychrophila*), these two strains differ in no major respect as far as our experience goes, and we therefore consider

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Footnote to Table 1

[1] Supplied as *Cytophaga aurantiaca* and *C. psychrophila*, although in our hands neither strain digests cellulose.

[2] Cf. *Saprospira thermalis.* [3] *Flexithrix dorotheae*, cf. *M. aggregans.*

[4] Cf. *Flexithrix dorotheae.* [5] Cf. *F. giganteus.*

[6] GC value lower than that of other strains of this species.

[7] The intact, living cells of A-1 are yellow, whereas those of all *S. grandis* strains are peach coloured. The absorption curve of the pigment extracted from A-1 does not exactly correspond with that of saproxanthin (Aasen & Jensen, 1966; Lewin & Mandel, 1969).

[8] Not in Fager's analysis.

\+ Indicates that all strains tested reacted positively.
− Indicates that all strains tested reacted negatively.
V Indicates variability of reaction among the strains tested.

them conspecific. We note that the latter name is listed by Buchanan, Holt & Lessel (1966) as illegitimate. In our tests neither strain digested cellulose, though they did digest CMC. For this species we propose the new combination *Flexibacter aurantiacus*. This species may be identical or close to an organism described as *Sphaeromyxa xanthochlora* by Bauer (1962).

Group 11 (T-3, II-2), with the addition of its associate SS-2, has been accepted otherwise unchanged. These forms, being sheathed, have been set apart in the genus *Herpetosiphon* (Holt & Lewin, 1968). (The type species of this genus, *H. aurantiacus*, was obtained too late for inclusion in this survey.) We propose to distinguish them as three separate species: *H. cohaerens* (II-2), *H. nigricans* (SS-2) and *H. persicus* (T-3) (Lewin, 1969*b*.)

Group 12 (SIO-7, SIO-9) has been accepted unchanged. We propose for these strains the new specific name *Microscilla sericea*.

Group 13 (GH-2, HI-15), with its associate GH-1, has been combined with group 5 under the new specific name *Microscilla tractuosa* (see above).

Group 14 (GOL-12, Y-1) has been combined with group 2, as explained above, in the species *Cytophaga diffluens* Stanier emend.

Its associate member, Q-1, is regarded as sole representative of a separate variety distinguished in part by its inability to digest starch or to liquefy agar. We propose to call it *Cytophaga diffluens* var. *carnea*.

Group 15 (LIM-21, JL-4) and its associate ST-1 have been distributed between *Cytophaga diffluens* (group 2) and *C. lytica* (group 7).

Group 16 (COP, CR-134) has been separated into two varieties, differing distinctly in their salt tolerances; one is marine, the other is from a freshwater habitat. We propose to call them, respectively, *Flexibacter aurantiacus* var. *copepodarus* and *Flexibacter aurantiacus* var. *excathedrus*.

An associate of this group, strain SIO-1, which digests CMC, agar and alginate, is regarded as sufficiently distinct to warrant its separation and description under a new specific name, *Cytophaga latercula*.

Group 17 (BA-24, NZ-1) has been divided into two separate species on the basis of their considerably different GC values, respectively 41 and 47·5%. For NZ-1, it seems appropriate to use the name *Flexibacter elegans* Soriano. For strain BA-24 we propose the new varietal name *Flexibacter flexilis* var. *iolanthae*.

Group 18 (SIO-8) has been accepted unchanged and assigned to the species *Microscilla marina* Pringsheim (1951), the type species for this genus (Though now lost, the strain which Fox & Lewin (1963) referred to as *Flexibacter marinum* nov. comb. was essentially identical with this species.)

Group 19 (TV-2) has been accepted unchanged, though we had less information on this strain than on any other at the time of Fager's computer analysis. We are naming it *Microscilla furvescens*.

Similarly, we have accepted Fager's seven main assemblages (FA-FG) as bases for genera, again subject to certain changes as explained below:

1. We have combined all helical forms in the genus *Saprospira*, comprising *S. grandis* (assemblage FD, group 1) *S. toviformis* (assemblage FD, associate of group 1) *S. thermalis* (assemblage FA, group 9) and *S. albida*.

2. We have created a separate genus, *Flexithrix* (Lewin, 1969*a*), for branched, sheathed forms, represented here by the single strain QQ-3, which therefore has been

taken out of assemblage FB, group 3. We recognize that this is a matter of convenience, which obscures close relationships between *Flexithrix dorotheae* and some of the yellow, rod-like forms of assemblage FB.

3. We have adopted another separate genus, *Herpetosiphon* (Holt & Lewin, 1968), for unbranched, sheathed forms; and we have, therefore, removed group 11, comprising strains T-3, II-2 and the associate SS-2, from assemblage FB. The new species are described by Lewin (1969*b*.)

4. Assemblage FA has been accepted almost unchanged as the basis for a genus, comprising almost all freshwater species studied in this survey. (We have excluded only group 9, for reasons given in paragraph 1 above, and strain SIO-1, which by reason of its various carbohydrase activities is better assigned to the genus *Cytophaga*.) For most members of this assemblage we propose to use the generic name *Flexibacter* (Soriano, 1945), as redescribed above.

In separating organisms of group 9 from the rest of assemblage FA, we recognize that, on the basis of a single structural feature, helicity, we are separating *Saprospira thermalis* from organisms with which it shares a number of common physiological and biochemical features, and associating it with those of assemblage FD (chiefly *S. grandis*) with which it has fewer common characteristics. This, nevertheless, seems to us to be justifiable, as we have already explained, because it is generally convenient to distinguish genera by readily recognizable features.

5. Assemblage FB has been accepted almost unchanged as the basis for a genus, comprising marine species which do not digest agar and digest few or no other polysaccharides. We have excluded strain QQ-3 (from group 3) and strains T-3, II-2, and SS-2 (i.e group 11 and its associate) for reasons given in paragraphs 2 and 3 above. We propose to use the generic name *Microscilla* (Pringsheim, 1951), as redescribed above. (The type species, *M. marina*, is not in this assemblage; however, see 7 below.)

In dissociating organisms of group 11 from the rest of assemblage FB, we recognize that on the basis of a single structural feature, sheaths, we are separating *Herpetosiphon* spp. from organisms which they resemble in several physiological and biochemical features. This, too, seems justifiable, because the presence of a sheath is a readily recognizable feature.

6. Assemblage FC has been accepted unchanged as the basis for a genus, comprising marine species which digest cellulose or CMC, agar and alginate. We propose to use for these forms the generic name *Cytophaga* as redescribed above.

7. In the interest of nomenclatural economy, the three smallest groups, which by Fager's analyses appear unrelated to any of his four major assemblages, have nevertheless been assigned, as separate species, to the nearest appropriate genus of those defined above, which for each of these strains seems to be *Microscilla*. Strains in group 12 (SIO-7, SIO-9) have been named *M. sericea* nov. sp.; group 18 (SIO-8) has been identified with *M. marina* Pringsheim (the type species); and group 19 (TV-2) has been designated by the new name *M. furvescens*.

All of the above changes are summarized schematically in Fig. 1, for comparison with Fig. 1 in the paper by Fager (1969).

Diagnostic keys

Having allocated the organisms to such specific groupings, we then wished to make our classification useful and usable by other workers handling different but possibly

related strains. To this end we have constructed a diagnostic dichotomous key. Since man classifies, in the first place, largely by appearance, we have chosen to use as primary features those which could be seen fairly easily. For convenience, one or two features of high constancy have been selected to 'define' our species and genera. We have given special consideration to the ease with which these discriminatory tests can be done, even in laboratories with limited equipment and facilities. However, it should be emphasized that our distinctions have been made not solely on the basis

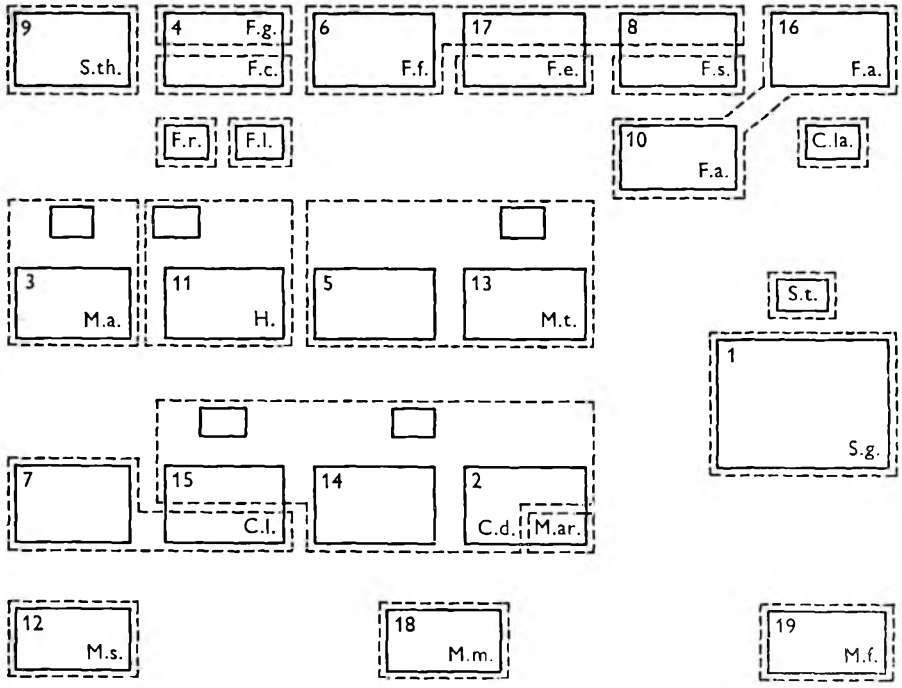

Fig. 1. Genera and species of flexibacteria, in relation to groups and inter-relations of groups as defined by a computer programme; *cf.* Fager (1969), Fig. 1.
Rectangles in unbroken lines indicate Fager's 19 numbered groups and their associates (unnumbered). Dashed lines delimit species as defined in this paper. (Varietal names are not indicated here.)

Key:

C.l.–*Cytophaga lytica*
C.la.–*C. latercula*
C.d.–*C. diffluens*
F.a.–*Flexibacter aurantiacus*
F.c.–*F. roseolus*
F.e.–*F. elegans*
F.f.–*F. flexilis*
F.g.–*F. giganteus*
F.l.–*F. litoralis*
F.r.–*F. ruber*
F.s.–*F. sancti*
H.–*Herpetosiphon* spp.
M.a.–*Microscilla aggregans*
M.ar.–*M. arenaria*
M.f.–*M. furvescens*
M.m.–*M. marina*
M.s.–*M. sericea*
M.t.–*M. tractuosa*
S.g.–*Saprospira grandis*
S.t.–*S. toviformis*
S.th.–*S. thermalis*

of such key characteristics, since we have also used a number of other differences, often less obvious, most of which have tended to reinforce the distinction. The key characters should not be regarded as necessarily the most 'important' or phylogenetically fundamental, but merely as the most convenient in our scheme. In fact,

some, such as pigmentation of packed living cells or filaments, not of medium or extract, halophily, and starch digestion are similar to those used by Stanier (1957) in his key to *Cytophaga* (*sensu lato*).

The use of 'marine', in the following keys, may require some justification. In our experience, almost all flexibacteria from marine sources grow in media based on sea water or a saline equivalent, but not in freshwater media; whereas those strains which we isolated from freshwater sources did not tolerate sea-water media. Origin is, therefore, a good indication of the osmotic or ionic tolerance range of a strain (*cf.* MacLeod, 1965).

Provisional diagnostic key to the genera of flexibacteria which we examined

1*a* At least partly sheathed under some conditions		2
1*b* Sheaths never present; all cells capable of gliding		3
2*a* Filaments with false branches	*Flexithrix*	
2*b* Filaments unbranched	*Herpetosiphon*	H. 1
3*a* More or less regularly helical	*Saprospira*	S. 1
3*b* Not regularly helical		4
4*a* Digests cellulose or CMC, agar and alginate	*Cytophaga*	C. 1
4*b* Does not digest these carbohydrates		5
5*a* Marine (key includes also 2 *Flexibacter* species and one *Cytophaga* species)	*Microscilla*	M. 1
5*b* Freshwater or from soil (key includes also 3 marine species)	*Flexibacter*	F. 1

Provisional diagnostic key to the species of Herpetosiphon

H. 1*a* Freshwater or from soil		H. 2
H. 1*b* Marine		H. 3
H. 2*a* From soil	*H. aurantiacus*†	
H. 2*b* From hot spring	*H. geysericolus**	
H. 3*a* Darkens Tryptone agar	*H. nigricans**	
H. 3*b* Does not darken Tryptone agar		H. 4
H. 4*a* Colonies on agar coherent	*H. cohaerens**	
H. 4*b* Colonies on agar not coherent	*H. persicus**	

† Holt & Lewin, 1968. * Lewin, 1969*b*.

Provisional diagnostic key to the species of Saprospira which we examined

S. 1*a* Marine		S. 2
S. 1*b* Freshwater or from soil		S. 3
S. 2*a* Peach-coloured; gliding about 1 μm. sec.	*S. grandis*	
S. 2*b* Yellow; gliding about 0·2 μm./sec. or imperceptibly	*S. toviformis*§	
S. 3*a* Bright orange	*S. flammula*	
S. 3*b* Pink		S. 4
S. 4*a* Abundant growth in synthetic media containing leu isoleu val. as sole essential amino acids	*S. thermalis*†	
S. 4*b* Slow and slight growth even in complex nutrient media	*S. albida*†	

§ Lewin & Mandel, 1969. † Lewin, 1965*a*.

Provisional diagnostic key to the species of Cytophaga which we examined

C. 1*a* Red	*C. latercula*	
C. 1*b* Yellow or orange		C. 2
C. 2*a* Yellow; softens agar; catalase+	*C. lytica*	
C. 2*b* Orange; catalase−		C. 3
C. 3*a* Pale orange; softens agar	*C. diffluens* var. *carnea*	
C. 3*b* Bright orange; liquefies agar		C. 4
C. 4*a* Grows on 0·01 % sodium lauryl sulphate	*C. diffluens* var. *aprica*	
C. 4*b* Does not grow on 0·01 % sodium lauryl sulphate	*C. diffluens*	

Provisional diagnostic key to the species of Microscilla (plus 3 other species from marine sources) which we examined

M. 1*a* Digests alginate		M. 2
M. 1*b* Does not digest alginate		M. 4
M. 2*a* Red (digests agar)	*C. latercula*	
M. 2*b* Orange		M. 3
M. 3*a* Digests CMC	*M. arenaria*	
M. 3*b* Does not digest CMC	*M. sericea*	
M. 4*a* Yellow		M. 5
M. 4*b* Orange, red or pink		M. 7
M. 5*a* Catalase+	*M. aggregans* var. *catalatica*	
M. 5*b* Catalase−		M. 6
M. 6*a* On 5 g. tyrosine/l., growth with 'clearing'	*F. aurantiacus* var. *copepodarum*	
M. 6*b* On 5 g. tyrosine/l., growth without 'clearing'	*M. aggregans*	
M. 7*a* On 5 g. tyrosine/l., growth without discoloration or 'clearing'	*M. tractuosa*	
M. 7*b* On 5 g. tyrosine/l., growth with ring and/or 'clearing'		M. 8
M. 8*a* On 5 g. tyrosine/l., growth with red ring and 'clearing'		M. 9
M. 8*b* Growth with grey ring	*M. furvescens*	
M. 9*a* Pink	*F. litoralis*	
M. 9*b* Orange	*M. marina*	

Provisional diagnostic key to the species of Flexibacter (including also Cytophaga latercula) which we examined

F. 1*a* Yellow		F. 2
F. 1*b* Pink, orange or red		F. 5
F. 2*a* Catalase+	*F. aurantiacus*	
F. 2*b* Catalase−		F. 3
F. 3*a* Digests CMC	*F. sancti*	
F. 3*b* Does not digest CMC		F. 4
F. 4*a* Marine	*F. aurantiacus* var. *copepodarus*	
F. 4*b* Freshwater or from soil	*F. aurantiacus* var. *excathedrus*	
F. 5*a* Digests CMC		F. 6
F. 5*b* Does not digest CMC		F. 7
F. 6*a* Marine	*C. latercula*	
F. 6*b* Freshwater or from soil	*F. flexilis* var. *pelliculosus*	
F. 7*a* Mucilaginous		F. 8
F. 7*b* Not mucilaginous		F. 9
F. 8*a* Marine	*F. litoralis*	
F. 8*b* Freshwater or from soil	*F. flexilis*	
F. 9*a* Can also grow on SW media		F. 10
F. 9*b* Cannot grow on SW media		F. 11
F. 10*a* Red	*F. roseolus*	
F. 10*b* Orange	*F. elegans*	
F. 11*a* Red	*F. ruber*	
F. 11*b* Pink or orange		F. 12
F. 12*a* Pink	*F. giganteus*	
F. 12*b* Peach or orange	*F. flexilis* var. *iolanthae*	

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Numerical Taxonomy of the Flexibacteria

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SUMMARY

A method of Adansonian taxonomy was applied to a set of flexuous, gliding bacteria. A clustering of strains which corresponded to *Saprospira grandis*, *Flexibacter flexilis*, *Saprospira thermalis*, *Flexibacter giganteus* and *Flexibacter roseolus* was observed from the computed data. High similarity values (> 80%) were found for strains assigned to the genera *Cytophaga*, *Flexibacter* and *Microscilla*.

INTRODUCTION

The classification of the gliding bacteria is relatively confusing. Lewin (1962), Soriano & Lewin (1965) and Lewin (1969) have attempted to clarify the situation. The few fragmentary diagnostic schemes for the flexibacteria proposed hitherto, have relied heavily on morphological criteria for classification and identification. The work of Lewin & Lounsbery (1969) represents a departure from the historical approach. Morphological, physiological, biochemical and nutritional characteristics were included in their analysis of nearly a hundred strains of flexibacteria.

The study reported in this paper was undertaken in order to apply the principles of Adansonian analysis and computer methods of taxonomic analysis to the problem of the classification and identification of these strains. Aside from the studies of Hayes (1963) and Floodgate & Hayes (1963) on strains of marine flavobacteria, some of which glide, no numerical taxonomic analysis of the taxonomic data of the flexibacteria or closely related forms has previously been attempted. The bacterial strains, isolated and characterized by Lewin & Lounsbery (1969), represent a group of organisms capable of gliding on solid media and apparently lacking rigid cell walls, flagella and endospores. The strains studied were all heterotrophic and included both marine and freshwater strains. Data for a total of 98 strains of flexuous, gliding bacteria were subjected to computer analysis.

Although the accumulated information for the strains included in this study is somewhat restricted and represents a minimum degree of information suitable for Adansonian methods (Sokal & Sneath, 1963), such an analysis should offer some insight into the relationships amongst the strains, thereby serving to focus attention on areas requiring additional study. Furthermore, the data, having been recorded and stored in the computer, are available for future reference and a more extended analysis with additional data for these or other strains of like bacteria.

METHODS

In June 1965, the work on Adansonian analysis of the flexibacteria began when raw data for 55 strains of gliding bacteria were received from Dr R. A. Lewin's laboratory. Eighty-two features were coded for the 55 strains, and established procedures were used for a preliminary analysis by high-speed computer (Colwell & Liston, 1961; Colwell, 1964). By the 'highest link' sorting (Sokal & Sneath, 1963), three major clusters were noted, with all strains clustering at levels of $\geqslant 65\%$ S. Since the initial results were encouraging, subsequent computer analyses of 98 strains were done as more data became available. The strains used are listed in Table 1. For information about sources, methods, etc., see Lewin (1962) and Lewin & Lounsbery (1969). The features scored for the strains are given in Table 2.

Table 1. *List of strains included in the final computer study*

Computer identification no.	Laboratory strain no.	Computer identification no.	Laboratory strain no.	Computer identification no.	Laboratory strain no.
1	SIO-1	34	ENS	67	SNZ
2	SIO-4	35	H-43	68	FLE
3	SIO-7	36	HI-3	69	MIC
4	SIO-8	37	HI-15	70	WAR-5
5	SIO-9	38	II-2	71	DWO
6	DAW-3	39	JK-11	72	PSY
7	COP	40	JC-13	73	WH
8	LIM-1	41	N-3	74	PA-1
9	21	42	NN-13	75	PA-2
10	GOL-12	43	TV-2	76	PA-3
11	DUB-4	44	B-A	77	LJA
12	B-9	45	BON	78	LJB
13	WFB-21	46	I-52	79	LJC
14	B-1	47	L-3	80	S-23
15	DD-1	48	R-52	81	E-1
16	GH-1	49	R-53	82	E-2
17	GH-2	50	OL-4	83	M-1
18	HJ-1	51	CR-63	84	M-2
19	JL-4	52	CR-81	85	ROB
20	NN-3	53	CR-103	86	SG-1
21	O-2	54	CR-104	87	SG-2
22	Q-1	55	124	88	DAW-1
23	Q-3	56	131	89	DAW-2
24	QQ-1	57	141	90	DUB-2
25	QQ-3	58	155	91	DUB-3
26	QQ-11	59	A-52	92	A-1
27	SS-2	60	BA-3	93	A-2
28	ST-1	61	BA-23	94	A-3
29	T-3	62	BA-24	95	JL-1
30	T-13	63	VC-2	96	BEG
31	Y-1	64	V-1	97	CR 123
32	EE-13	65	EGP	98	CR 125
33	EG-13	66	GEY		

The laboratory data sheets were transcribed for keypunching, scoring '0' for a feature absent or negative, '1' for a feature present or positive and '3' for a feature not tested or a test not applicable. Negative matches were not included in the computation of S (Sokal & Sneath, 1963). The card file has been kept expansible in order to

accommodate new data as received. The present matrix for the working computer programmes can accommodate 10,000 strains × 1000 features.

During the course of the study a series of programmes was used. Programmes GTP-1 to GTP-5 (GTP = Georgetown Taxonomy Programme) were developed for the IBM

Table 2. *Coded features employed in the final computer analysis of the flexibacteria**

Computer feature code no.	Feature description	Computer feature code no.	Feature description	
1	< 10 μm. length	31	L-Arginine	Amino acid requirement
2	10–50 μm. length	32	L-Aspargine	
3	> 50 μm. length	33	L-Histidine	
4	Helical	34	L-Glycine	
5	Rhapidosomes observed	35	L-Isoleucine	
6	Slight coiling	36	L-Leucine	
7	Pigment I—red (flexixanthin)	37	L-Lysine	
8	Pigment II—pink	38	L-Methionine	
9	Pigment III—orange (saproxanthin)	39	L-Phenylalanine	
10	Pigment IV—yellow (zeaxanthin)	40	L-Threonine	
11	Pigment V—yellow	41	L-Tryptophane	
12	Pigment VI—red	42	L-Valine	
13	Cellulose digested	43	Vitamin B_1 required	
14	Starch hydrolyzed	44	Vitamin B_{12} required	
15	Gelatin hydrolyzed	45	Other vitamin requirement	
16	Agar digested	46	Freshwater	Salinity tolerance
17	Catalase present	47	½ × sea water	
18	Tyrosine 5 g./l.—growth	48	Sea water	
19	Tyrosine 5 g./l.—ring	49	2 × sea water	
20	Tyrosine 5 g./l.—clearing	50	35 °	Growth temperatures
21	H_2S produced	51	40 °	
22	0·1 g./l. DOPA—growth	52	Tryptone	Nitrogen source utilized
23	0·1 g./l. DOPA—ring	53	Casamino acids	
24	0·1 g./l. DOPA—clearing	54	Na glutamate 1 g./l.	
25	0·001 g./l. penicillin tolerated	55	Na nitrate 0·3 g./l.	
26	0·0001 g./l. penicillin tolerated	56	Acetate 5 g./l.	Carbon source utilized
27	0·00001 g./l. penicillin tolerated	57	Acetate 1 g./l.	
28	0·000001 g./l. penicillin tolerated	58	Lactate 5 g./l.	
29	Sodium lauryl sulphate—sensitive	59	Lactate 1 g./l.	
30	Alginate degraded	60	Glycerol 5 g./l.	
		61	Glycerol 1 g./l.	
		62	Glucose 5 g./l.	
		63	Glucose 1 g./l.	
		64	Galactose 5 g./l.	
		65	Galactose 1 g./l.	
		66	Sucrose 5 g./l.	
		67	Sucrose 1 g./l.	

* See Lewin & Lounsbery (1969) for details of media, tests, etc.

1620 and the IBM 360/40 computer systems from programmes written during the earlier work (Colwell & Liston, 1961; Colwell, 1964). Essentially, these programmes provide a full S value matrix (GTP-1), a modified link sort and clustering at decrements of similarity values (GTP-2) (R. R. Colwell, to be published), feature frequency sort (GTP-3) (Colwell, 1964), median organism calculation (GTP-4) (Liston, Wiebe & Colwell, 1963) and an S-value triangle chart (GTP-2-chart).

Since September 1967, programmes GTP-1 to GTP-5 have been consolidated into

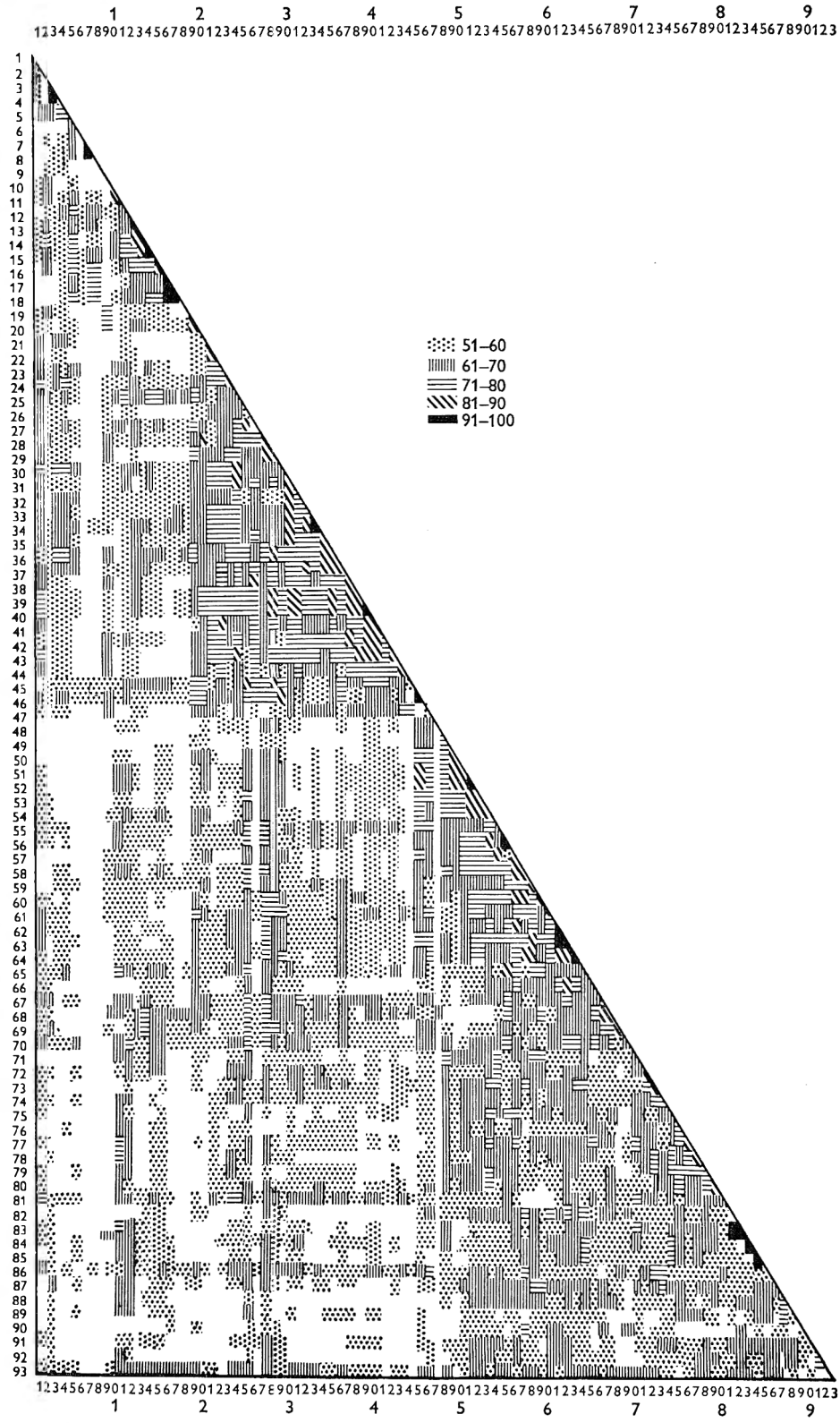

Fig. 2. Total S-value output for 93 strains of flexibacteria.

a master programme for the IBM Model 360/40 System. The IBM 360/40 System used in this study contains 131,000 bytes of core storage, four disk drives and four magnetic tape units.

RESULTS

The sorted output from the computer analysis of 93 strains is presented in Fig. 1. On the basis of the data encoded for the strains, all grouped at > 70% S by the highest linkage sorting technique. A large cluster, A-3 to HI-3, and two subclusters, A-3 to DAW-2 and DD-1 to HI-3, were obtained (see Fig. 1). The full S-value matrix is given in Fig. 2, in which the corresponding strain numbers are 19 to 82, 19 to 47 and 48 to 82, respectively. A third and less homogeneous cluster, SIO 4 to CR-125 (1 to 18 in Fig. 2) was also indicated. In general, except for the more closely related clusters, highly similar in over-all features, the groupings which may be regarded as possible species are: BA-24, CR-155, CR-141; CR-124, CR-104, CR-103; CR-81, CR-63, WAR-5, A-52, BEG, CR-123, CR-125; A-3 to DAW-2 (see Fig. 1) and N-3 to HI-3 (see Fig. 1). From Fig. 2 no completely unequivocal separations into clearly unrelated, although certain internally homogeneous, groups were obvious.

The strains, arranged in order as in Fig. 1 and 2, approximate reasonably well the groupings on the basis of over-all DNA base compositions. For the most part, the features for strains from freshwater and marine habitats and for strains capable of cellulose digestion (Table 3) correlate with other group features; but they would not be suitable key characters in a monothetic taxonomy (Lockhart & Hartman, 1963) since some variation in the frequency of occurrence was observed.

With the full complement of feature data for the flexibacteria strains, listed in Table 2, a final analysis was run, including the full data set (Lewin & Lounsbery, 1969) received after completion of the initial analyses. From this analysis, the 90-, 80- and 75-phenons (Sokal & Sneath, 1963) and the final ordering of strains are given in Table 3. Of the 98 strains examined, 74 were grouped at degrees of similarity of $\geqslant 80\%$. At 75% S, strains T-13 and T-3 joined the major cluster and A-52 joined a group of unidentified strains; strains GEY, CR-103 and CR-104 joined BEG, CR-123 and CR-125; and a new group appeared consisting of the two strains CR-141 and CR-155. At 75-S, 90 of the 98 strains (Table 1) were sorted into groups.

The 90-, 80- and 75-phenons approximate to the species groups of Lewin (1969).

DISCUSSION

A review of the present status of the taxonomy of gliding microbes was presented by Soriano & Lewin (1965). There can be little doubt that major revisions of the flexibacteria are required. The strains selected by Lewin & Lounsbery (1969) for study and included in the Adansonian analysis reported here represent a sampling of flexibacteria, from an analysis of which some suggestions for the taxonomy of these forms can be made. The results of Adansonian analyses, in general, indicate a relatively high over-all similarity among the strains studied, though with some distinguishable subgroupings. The lack of very marked group distinctions may be due to the small number of characters used in the study. The minimum standard error, for example, for the S-value calculations was about $\pm 6\%$.

In comparing the taxonomic decisions of Lewin (1969) based on analysis by Fager

Table 3. *Selected data for strains included in the final analysis of the study*

The ordering of the strains is as established by the computer sorting. Specific epithets and strain numbers correspond to the designations of Lewin (1969).

Laboratory strain no.	Nomenclature assigned by Lewin, 1969	GC content† (moles %)	Pigment category	Cellulose digestion	Habitat (freshwater or marine)
COP	*Flexibacter aurantiacus* var. *copepodarum*	32	IV	–	M
CR-131	.	.	.	.	.
SIO-8	*Microscilla marina*	42	III	–	M
A-1	*Saprospira toviformis*	38	III-a	+	F/M
CR-81*	*F. flexilis*	43	III	–	F
CR-63	*F. flexilis*	41	III	–	F
WAR-5	*F. flexilis*	40	III	–	F
A-52	*F. flexilis*	42	III	–	F
V-1	.	.	.	–	F
VC-2	.	.	.	–	F
EGP	.	44	.	–	F
HI-3	*Microscilla aggregans* var. *catalatica*	35	IV	–	M
SIO-9	*M. sericea*	39	III	–	M
T-13	*M. tractuosa*	37	III	–	F/M
SS-2	*Herpetosiphon nigricans*	53	IV	–	M
NN-13	*M. aggregans*	37	IV	+	M
Q-3	*M. aggregans*	42	IV	–	M
QQ-3	*Flexithrix dorotheae*	37	IV	+	M
ST-1	*Cytophaga diffluens* var. *aprica*	37	III	+	M
H-43	*M. tractuosa*	36	III	–	F/M
ENS	*C. lytica*	33	IV	+	M
MIC	*F. sancti*	46	IV	+	F
HI-15	*M. tractuosa*	36	III	–	F/M
EE-13	*M. tractuosa*	38	III	–	F/M
JK-11	*M. tractuosa*	37	III	–	F/M
QQ-1	*M. aggregans*	39	IV	+	M
GH-2	*M. tractuosa*	35	III	–	M
WFB-21	*C. lytica*	34	IV	+	F/M
B-9	*C. lytica*	33	IV	+	M
DUB-4	*C. diffluens* var. *aprica*	37	III	+	M
NN-3	*C. diffluens*	40	III	+	M
JL-4	*C. diffluens* var. *aprica*	35	III	+	M
SIO-7	*M. sericea*	39	III	–	M
LIM-1	*C. diffluens*	42	III	+	M
TV-2	*M. furvescens*	45	.	.	.
N-3	*C. diffluens*	40	III	+	M
HJ-1	*M. arenaria*	32	III	+	M
GH-1	*M. tractuosa*	34	III	–	M
Q-1	*C. diffluens* var. *carnea*	37	III	+	M
QQ-11	*M. aggregans*	40	IV	–	M
JL-13	*M. aggregans*	36	IV	+	M
EG-13	*M. tractuosa*	37	III	–	M
BA-3	*F. sancti*	46	IV	+	F
FLE	*F. flexilis* var. *pelliculosus*	39	III	+	F
BA-24	*F. flexilis* var. *iolanthae*	41	III	–	F
PSY	*F. aurantiacus*	32	V	+	F
DWO	*F. aurantiacus*	31	V	+	F/M
BON	*C. lytica*	34	IV	+	M
O-2	*C. diffluens*	42	III	+	M

Table 3 (*cont.*)

Laboratory strain no.	Nomenclature assigned by Lewin 1969	GC content† (moles %)	Pigment category	Cellulose digestion	Habitat (freshwater or marine)
DD-1	*C. diffluens* (?)	35	III	+	M
B-1	*C. diffluens*	42	III	+	M
GOL-12	*C. diffluens*	41	III	+	M
Y-1	*C. diffluens*	41	III	+	M
SIO-1	*C. latercula*	34	VI	+	M
T-3	*H. persica*	53	III	–	M
GEY	*F. ruber*	37	I	–	F
BEG	*S. thermalis*	37	II	–	F
CR-125	*S. thermalis*	33	II	–	F
CR-123	*S. thermalis*	35	II	–	F
CR-103	*F. giganteus*	33	II	–	F
CR-104	*F. giganteus*	32	II	–	F
CR-124	*F. giganteus*	31	II	–	F
SIO-4	*F. litoralis*	31	I	–	M
II-2	*H. cohaerens*	45	III	–	M
A-2	*S. grandis*	46	III	–	F/M
DUB-2	*S. grandis*	47	III	–	M
DAW-1	*S. grandis*	48	III	–	M
LJA	*S. grandis*	47	III	–	M
LJB	*S. grandis*	48	III	–	.
WH	*S. grandis*	48	III	–	M
S-23	*S. grandis*	48	III	–	.
E-1	*S. grandis*	46	III	–	M
PA-3	*S. grandis*	46	III	–	M
ROB	*S. grandis*	47	III	–	M
SG-2	*S. grandis*	48	III	–	M
M-2	*S. grandis*	47	III	–	M
SG-1	*S. grandis*	47	III	–	M
M-1	*S. grandis*	47	III	–	M
E-2	*S. grandis*	48	III	–	M
DUB-3	*S. grandis*	46	III	–	M
DAW-3	*S. grandis*	48	.	–	M
PA-1	*S. grandis*	47	III	–	M
LJC	*S. grandis*	48	III	–	.
DAW-2	*S. grandis*	47	III	–	M
PA 2	*S. grandis*	46	III	–	M
JL 1	*S. grandis*	47	III	–	F/M
A-3	*S. grandis*	46	III	–	F/M
SNZ	*S. flammula*	48	III	–	F/M
CR-155	*F. roseolus*	39	I	–	F/M
CR-141	*F. roseolus*	34	I	–	F/M

* Legend: ———, 90-phenon; — — —, 80-phenon; – – –, 75-phenon.
† Mandel & Lewin (1969).

(1969) with the Adansonian conclusions presented here, good correlations were observed in general. There are, however, disagreements on positioning of individual strains and in the selection of certain strains for elevation to generic rank, viz. *Herpetosiphon* species, *Microscilla* species and *Flexithrix dorotheae*.

The *Saprospira grandis* group of strains is clearly distinguishable from most of the other organisms examined; there appears to be no difficulty in identifying members of this group. Of the 24 strains so identified, 16 clustered at $\geqslant 90\%$ S and all 24 at $\geqslant 80\%$ S. The DNA base composition range (46 to 48 moles % GC over-all) is

narrow and offers firm evidence for considering the strains as a homogeneous species. Other species named by Lewin (1969) were *Flexibacter flexilis*, *Saprospira thermalis*, and *Flexibacter giganteus*, which corresponded to groupings in this analysis. The *Cytophaga* and *Flexibacter* species appear to be related to *Saprospira grandis* through *Saprospira thermalis*. The strains COP, CR-131, SIO-8, A-1, HI-3, SIO-9, T-13, T-3, GEY, CR-124, and II-2 did not cluster together or with other strains at degrees of similarity > 75%. Two strains, CR-155 and CR-141, classified as *Flexibacter roseolus* by Lewin, share a 75% degree of over-all similarity.

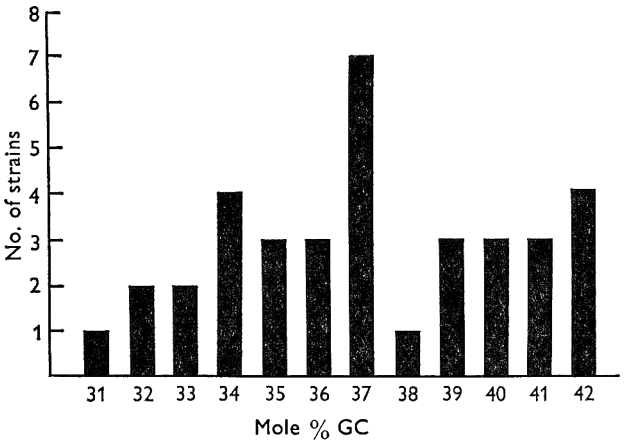

Fig. 3. Distribution of mole % GC within the *Cytophaga/Flexibacter/Microscilla* 80-phenon.

The greatest difficulty in interpreting these data is associated with the strains NN-13 to SIO-1, listed in Table 3, classified by Lewin (1969) as representing four genera, twelve species and four varieties. These strains share an over-all similarity of $\geqslant$ 80% S and represent a GC range of 31 to 42%. Thus it is difficult from these data to make 20 taxonomic distinctions.

Because a minimum of coded characteristics was available for the flexibacteria in cluded in this study, firm conclusions about the identification and classification of these strains must be avoided. Nevertheless, the following comments appear reasonable. There are apparently two major divisions of micro-organisms which show gliding, flexuous motility: the *Saprospira grandis* group and the *Cytophaga/Flexibacter/Microscilla* group. The characters used for describing these strains from nature appear to cross specific and probably, generic lines. The relatively separate and homogeneous clusters of strains identified as *Saprospira grandis*, *Flexibacter flexilis*, *Saprospira thermalis*, *Flexibacter giganteus* and *Flexibacter roseolus* by Lewin (1969) may be considered, as a working hypothesis, roughly equivalent to species. The large *Cytophaga/Flexibacter/Microscilla* aggregation, however, should be examined further for subclustering of strains. The range of DNA base compositions, 31 to 42 moles% GC (see Fig. 3) would indicate, on purely molecular genetic grounds, at least three and perhaps more subgroupings. The high phenotypic similarities within the latter cluster suggest either a convergence of phenotype due to environmental selection, or, more likely, insufficient information at the present time for unequivocal identification of groups.

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NOTE ADDED IN PROOF

Examination of developing microcolonies produced microscopical evidence of general morphological agreement with the groupings obtained in this analysis. There were, however, two morphological types within the major phenon: a long cell form (12 μm.), relatively rapidly moving; and a short flexible rod (12 μm.), rather sluggishly motile. The two morphological types respectively matched closely the *Cytophaga*/*Microscilla* groupings as differentiated in the 'Comprehensive key to genera', sections F 23 and F 24, in V. B. D. Skerman (1967), *A Guide to the Identification of the Genera of Bacteria*, 2nd ed. (Baltimore: Williams and Wilkins, 303 pp.). These observations were made by Professor V. B. D. Skerman during a visit to Georgetown University and the American Type Culture Collection, Washington, D.C., U.S.A.

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Cell Wall Analysis of Oral Filamentous Bacteria

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SUMMARY

The cell wall compositions of 41 strains of oral filamentous bacteria belonging to the genera *Fusobacterium*, *Leptotrichia* and *Bacterionema* were examined by paper chromatography. *Leptotrichia* walls showed principally alanine, glutamic acid, DAP, glycine and aspartic acid. The genus *Bacterionema* had the same amino acids but aspartic acid appeared to be a minor component and was not detected in the mucopeptide fraction of the cell walls. The strains of Fusobacterium gave an amino acid pattern characteristic of Gram-negative bacteria; however, lysine was a major component in the mucopeptide. The genus *Leptotrichia* is thus distinct from the other two genera and should include two species, *Leptotrichia buccalis* and another organism termed 'Anaerobic filaments'.

INTRODUCTION

The human oral cavity supports the growth of a wide variety of micro-organisms including several different Gram-positive filamentous types, some of which are often classified in the genus *Leptotrichia*. Members of this genus are primarily oral inhabitants which are micro-aerophilic or anaerobic. Despite numerous investigations (Thjøtta, Hartman & Bøe, 1939; Baird-Parker, 1959; Theilade & Gilmour, 1961; Sibal, Kroeger, Kumarich & Meyer, 1962; Hofstad, 1967*a*), there is still much confusion concerning the nomenclature and classification of these micro-organisms. For example, the name *Leptotrichia buccalis* has been used synonymously for two completely different oral bacteria and it appears that the type species of the genus *Fusobacterium*, *F. fusiforme*, is synonymous with *L. buccalis* (Jackins & Barker, 1951). Recent reports (Theilade & Gilmour, 1961; Kasai, 1965; Hofstad, 1967*a*) have indicated that there are other filamentous bacteria which may or may not be related to members of the genus *Leptotrichia*.

In the present study the cell wall compositions of 41 strains of oral filamentous bacteria was examined by paper chromatography with the hope that the information might be of some taxonomic value in the classification of these micro-organisms.

METHODS

Organisms. The strains used and their sources are listed in Table 1. The method employed for isolating and characterizing fresh isolates has been previously described

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(Baboolal, 1968). Unless otherwise stated *Bacterionema matruchotii* strains were grown aerobically, while strains of *Leptotrichia buccalis*, *Fusobacterium nucleatum* and the 'Anaerobic filaments' were grown anaerobically in McIntosh and Fildes jars in an atmosphere containing 10 % (v/v) CO_2 in N_2.

Table 1. *Source of strains used in this study*

Micro-organism	Laboratory designation	Source
Leptotrichia buccalis	ATCC 14201	American Type Culture Collection
	ATCC 19616, L 19	M. Gilmour, Rochester, New York
	Z 18, L 20	G. J. Kasai, Chicago
	L 11	Tor Hofstad, Bergen, Norway
	GP 1, GP 2, T 4, T 5, T 7, T 15	G. L. Slack & G. H. Bowden, isolated from experimental plaque
	A 4, A 5	Chronic marginal gingivitis
	A 17, A 19, B 18, B 17, G 103, A 14	Dental plaque
	B 4, V 2	Acute ulcerative gingivitis (Vincent's disease)
	N 3	Carious lesion
'Anaerobic filaments'	L 21, L 44, L 52	Tor Hofstad, Bergen, Norway
	CW 1	E. Theilade, Denmark
	T 2	G. L. Slack & G. H. Bowden, isolated from experimental plaque
	T 12, T 19	Dental plaque
Bacterionema matruchotii	ATCC 14266	American Type Culture Collection
	NCTC 10206	National Collection of Type Cultures
	18, 207	M. Gilmour, Rochester, New York
	G 132	G. J. Kasai, Chicago
	LD 1	Dental plaque
Fusobacterium nucleatum	V 1, F 1	Acute ulcerative gingivitis (Vincent's disease)
	C 3, F 2, F 3	Dental plaque

Biochemical tests. The basal medium for carbohydrate fermentation and other physiological tests was that of Kasai (1965). The sugars (10 % w/v, sterilized by filtration) were added to the basal medium (previously sterilized) to 1 %. Nitrate, gelatin and starch were added to the basal medium to 0·1, 12 and 2 % w/v respectively. The various tests were inoculated with a culture prepared from single colonies selected from blood agar and grown in Medium B (below) for 24 or 48 hr. Since CO_2 lowers the pH of test substances in the anaerobic jars, uninoculated controls were included and the results were read relative to the controls. Tests were conducted according to the procedures outlined in the Manual of Microbiological Methods (1957). H_2S production was detected with lead acetate paper strips.

Medium B contained (%, w/v): Proteose peptone (Oxoid) 1·0; Yeast extract (Oxoid) 0·1; 'Lab Lemco' beef extract (Oxoid) 0·3; Soluble starch 0·2; $Na_2HPO_4 . 12H_2O$ 0·5; Sodium nitrate 0·1; L-Cysteine HCl 0·05; Sodium bicarbonate 0·1; the pH value was 7·6.

Preparations of cell walls. One l. medium B was dispensed in a 1·5 l. flask and 50 ml. liquid paraffin were added to provide semi-anaerobic conditions. The facultative strains of *Bacterionema matruchotii* were grown aerobically in the same medium for 72 hr. at 37°. Cultures were harvested by centrifugation and the cells were heated at 75° for 5 min., washed once in distilled water and twice in physiological saline. The

washed cells were resuspended in 8 ml. of distilled water, one drop of iso-octanol added and the suspension shaken in a Mickle Tissue Disintegrator at room temperature with an equal volume of ballotini glass beads (Jencon's Grade 12). Disruption was considered complete when no intact organism could be seen in Gram-stained smears. The disintegrated cells were separated from the glass beads, centrifuged 10 min. at 2000 *g* to remove any unbroken cells and the supernatant centrifuged 30 min. at 20,000 *g* to deposit the cell walls. The pellet was washed twice with molar saline, resuspended in 6 ml. M/15 phosphate buffer, pH 8, containing 1 mg./ml. crystalline trypsin, incubated 6 hr. at 37° and centrifuged 30 min. at 20,000 *g*. The trypsinized cell walls were washed several times in distilled water and finally freeze-dried. A few cell-wall preparations were checked for purity by electron microscopy.

Preparation of mucopeptide from cell walls. The method employed was essentially similar to that described by Perkins (1965). Freeze-dried cell walls (100 mg.) was extracted with 15 ml. formamide at 160° with constant stirring in an oil bath. The mixture was cooled and 30 ml. acid ethanol were added. The residue was recovered by centrifugation, washed with acid alcohol, ethanol, ether and twice with distilled water. After drying *in vacuo* over P_2O_5 the residue was weighed. The experiment was repeated three times and the proportion of mucopeptide (from the weight of the final residue) in the various cell wall preparations are included in Table 5. The supernatant was treated with 5 vol. acetone and stood 1 hr at room temperature. The white precipitate was redissolved in distilled water, reprecipitated with acetone and further purified by repeated dialysis against distilled water. The quantity of material recovered from the supernatant varied considerably owing to loss during the purification stages. In a typical experiment employing 100 mg. of *Leptotrichia buccalis* cell wall 40 mg. was recovered as mucopeptide and 30 mg. from acetone precipitation of the supernatant.

Lipid estimation. Ether extractable material from cell walls was estimated by a modification of Salton's method (1953). Cell walls were suspended in 2 N-HCl, treated 2 hr at 100° and extracted with ether continuously for 5 days. The ether was removed by evacuation and the residue redissolved in ether. The latter procedure was repeated twice and the final residue was evacuated to dryness before weighing.

Hydrolysis of cell wall mucopeptide and polysaccharide. Hydrolysates for detection of reducing sugars were prepared by heating samples 2 hr with 2 N-HCl in sealed tubes at 100°. They were filtered and the HCl removed by evacuating to dryness *in vacuo* over P_2O_5. For amino acids and hexosamines, samples were hydrolysed 20 hr with 6 N-HCl at 100° and the excess HCl removed as described previously. The final residues were redissolved in 10 % isopropanol and stored at 4°.

Chromatography. Amino acids were repeated on two dimensional ascending chromatograms of Whatman No. 1 10 × 10 in., filter paper irrigated 18 hr each with ethanol + *n*-butanol + water + propionic acid (10 + 10 + 5 + 2 v/v) and, after drying, with *n*-butanol + acetone + water + dicyclohexylamine (10 + 10 + 5 + 5 v/v) as described by Hardy, Holland & Naylor (1955), before developing. The amount of cell walls subjected to chromatography was equivalent to 1 mg. dry weight. Separation of DD-or DL- and LL-diaminopimelic acid (DAP) was similar in all essential details to the method described by Hoare & Work (1957); since the DL- and DD- isomers cannot be separated by paper chromatography the results are recorded as DL-DAP or LL-DAP.

Reducing sugars were separated on Whatman No. 1 filter paper irrigated 20 hr with *n*-butanol + pyridine + water (6 + 4 + 3 v/v) (Jeanes, Wine & Dunbar, 1951). Amino

sugars were separated by one dimensional descending chromatography on Whatman No.1 paper, previously treated with 0·1 M-$BaCl_2$ (Heyworth, Perkins & Walker, 1961) and irrigated 36 hr in the solvent system used for reducing sugars.

Detection of components. Amino acid chromatograms were dried, sprayed with 0·25 % ninhydrin in acetone plus 7 % glacial acetic acid and heated about 5 min. at 75°. The identity of some spots was confirmed by ionophoresis. Reducing sugars were detected with aniline hydrogen phthalate (Partridge, 1949) and their identity confirmed by running standard mixtures on the chromatograms. Amino sugars were detected by the Elson-Morgan reaction as modified by Partridge & Westall (1948).

RESULTS

The micro-organisms were characterized by their colonial and cellular morphology and the tests shown in Table 2. Strains of *Leptotrichia buccalis* and the 'Anaerobic filaments' showed some similarity in a number of tests. Both groups were saccharolytic, failed to produce catalase, H_2S and reduce nitrate. Some variation was found with respect to carbohydrate fermentation among strains of *L. buccalis*, but a number of other investigators (Kasai, 1965; Takazoe & Frostell, 1960; Hamilton & Zahler, 1957) have reported similar observations.

Cell wall analyses of the various groups of filamentous bacteria examined are shown in Table 3. The twenty-three strains of *Leptotrichia buccalis* gave identical cell-wall patterns with respect to their amino acids, amino sugars and reducing sugars. A slow running component which gave a weak positive Elson-Morgan reaction was detected in most hydrolysates; it ran slower than muramic acid in the solvent system used. Glucose was the principal reducing sugar with galactose as a minor component. The principal amino sugars were muramic acid and glucosamine, but galactosamine was also detected in all the strains examined. The major amino acids were alanine, glutamic acid, DAP, aspartic acid, and glycine, with valine as a minor component. Serine, lysine, ornithine and leucine were detected in trace amounts. The occurrence of aspartic acid as a major component in the cell walls of *L. buccalis* and the 'Anaerobic filaments' indicated that this substance might be associated with the mucopeptide. Preliminary experiments, using cell walls extracted with 0·5 % KOH in ethanol (Cummins & Harris, 1958) gave a cell-wall pattern of amino acids similar to that of non-extracted walls, with aspartic acid as a major component. However, if cell walls were extracted with formamide and the insoluble residue analysed, aspartic acid was detected (Table 5). It appears that the mucopeptide fractions of the cell walls of *L. buccalis* and the 'Anaerobic filaments' contained alanine, glutamic acid, DAP, glycine and aspartic acid. *L. buccalis* and the 'Anaerobic filaments' then had similar amino acids in their wall and mucopeptide, and similar proportions of mucopeptide in the cell walls. However, the reducing sugar patterns were different in that the walls of *L. buccalis* had glucose and galactose while the 'Anaerobic filaments' contained glucose and rhamnose.

The principal amino acids of the cell walls of *Bacterionema matruchotii* were in general similar to those of *Leptotrichia buccalis*, but aspartic acid appeared to be a minor component, and the absence of this amino acid in the mucopeptide was considered significant (Table 5). Arabinose was a major component in the walls of *B. matruchotii*; this sugar is commonly found among members of the genera *Corynebacterium*, *Nocardia* and *Mycobacterium* (Cummins, 1962).

Table 2. *Summary of biochemical and physiological tests*

Strain	*Leptotrichia buccalis*										*Anaerobic filaments*			*Bacterionema matruchotii*		*Fusobacterium nucleatum*
	*	A 4	G 103, Z 18, ATCC 14201	L 11	A 17	N 3, V 2	T 5	B 18	T 15, T 7	B 4, GP 2	T 12	L 44, L 52 T 2	L 21, CW 1, T 19	LD 1, 207, G 132	18, ATCC 14266, NCTC 10206	F 1, V 1, C 3, F 2, F 3
Glucose	+	+	+	+	+	+	+	+	+	+	+G	+G	+G	+	+	pH 6·2
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Raffinose	−	−	+	+	+	+	+	+	+	−	+	+	+	−	−	−
Xylose	−	+	+	−	−	+	+	+	−	−	+	+	+	−	+	−
Cellobiose	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
Mannitol	−	+	−	−	+	+	+	−	+	+	−	−	−	+	+	−
Salicin	+	+	+	+	+	+	−	−	−	−	−	−	−	+	+	−
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+
Catalase	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
Methyl red	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Indole	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	+
Voges-Proskauer	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−
H_2S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Reduction of NO_3	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+

* T 4, L 19, NCTC 19616, B 17, L 20, GP 1, A 5, A 19, A 14.
+ = positive; +G = acid and gas; − = negative.

Table 3. *Cell-wall composition results in strains of Leptotrichia buccalis and other filamentous types*

	L. buccalis 23 strains	'Anaerobic filaments' 7 strains	*Bacterionema matruchotii* 6 strains	*Fusobacterium nucleatum* 5 strains
Glucose	+++	+++	+++	+++
Galactose	+	−	+	++
Arabinose	−	−	+++	−
Rhamnose	−	++	−	−
Glucosamine	+++	+++	+++	+++
Galactosamine	+	+	+	+
Muramic acid	++	++	++	+
LL-Diamino-pimelic acid	−	−	−	−
DL-Diamino-pimelic acid	+++	+++	+++	−
Alanine	+++	+++	+++	+++
Glutamic acid	+++	+++	+++	+++
Aspartic acid	+++	+++	+	++
Glycine	++	++	++	+++
Ornithine	tr	tr	−	++
Leucine	tr	tr	tr	+
Valine	+	+	tr	++
Serine	tr	tr	−	+
Cysteine	−	−	−	+
Lysine	tr	tr	tr	+++

+++, Major component; +, minor component; −, not detected; ++, moderate amount; tr, trace amount.

Table 4. *Comparative patterns of the principal cell-wall components of certain groups of bacteria*

	Leptotrichia buccalis	Anaerobic filaments	*Bacterionema matruchotii*	*Actinomyces israelii**	*Nocardia asteroides**
Arabinose	−	−	+++	−	+++
Galactose	+	−	+	++	++
Glucose	+++	+++	+++	−	+
Rhamnose	−	++	−	−	−
Muramic acid	++	++	++	+	+
Glucosamine	+++	+++	+++	+	+
Galactosamine	+	+	+	−	−
Alanine	+++	+++	+++	+++	+++
Glutamic acid	+++	+++	+++	+++	+++
Diaminopimelic acid					
DL	+++	+++	+++	−	+++
LL	−	−	−	−	−
Lysine	−	−	−	+++	−
Glycine	++	++	++	−	−
Aspartic acid	+++	+++	†	−	−

* Data taken from Cummins (1962).
† Not detected in mucopeptide fraction of the cell walls.

The Fusobacterium strains gave amino acid patterns which were considered typical of Gram-negative bacteria and the small amount of mucopeptide present in their cell walls substantiated this conclusion. Muramic acid and glucosamine were detected in all mucopeptide preparations and analysis of the polysaccharide material obtained by formamide extraction suggested that the galactosamine detected in cell-wall hydrolysates of *Leptotrichia buccalis*, *Bacterionema matruchotii* and the 'Anaerobic filaments' is perhaps associated with the polysaccharide (Table 6).

Table 5. *The quantitative analyses of lipid and mucopeptide from cell walls and the components detected in the mucopeptide fraction*

	Leptotrichia buccalis	Anaerobic filaments	*Bacterionema matruchotii*	*Fusobacterium nucleatum*
Mucopeptide (%)	40	43	58	6
Lipid (%)	12	10	1·5	20
Alanine	+++	+++	+++	+++
Glutamic acid	+++	+++	+++	+++
Diaminopimelic acid	+++	+++	+++	−
Glycine	++	++	++	+
Aspartic acid	++	++	−	−
Lysine	−	−	−	++
Muramic acid	++	++	++	++
Glucosamine	+++	+++	+++	+++
Galactosame	−	−	−	−

+++, Major component; +, minor component; ++, moderate amount; −, not detected.

Table 6. *Qualitative analyses of cell-wall polysaccharides*

	Leptotrichia buccalis	Anaerobic filaments	*Bacterionema matruchotii*
Glucose	+	+	+
Galactose	+	−	+
Arabinose	−	−	+
Rhamnose	−	+	−
Galactosamine	+	+	+

+, Detected; −, not detected.

The amounts of lipid (ether extractable material) in the walls of *Leptotrichia buccalis* and the 'Anaerobic filaments' (10 to 12 % of the weight of the walls, Table 5) were much lower than that of *Fusobacterium nucleatum* but considerably higher than that of *Bacterionema matruchotii* (1·5 %). Walls of *L. buccalis* and the 'Anaerobic filaments' apparently contain substantial amounts of lipid.

DISCUSSION

Chemical analysis of bacterial cell walls appears to be a useful taxonomic tool; Cummins & Harris (1956) suggested that the pattern of amino acids of the cell walls characterizes the various genera, or taxa higher than species, and that the nature of the sugars characterizes species. The 23 strains of *Leptotrichia buccalis* examined form a homogenous group in their cell-wall patterns and no differences were found between strains isolated from Vincent's infection and those obtained from other sources.

The results recorded in Tables 3 and 5 show that *Leptotrichia buccalis* and the 'Anaerobic filaments' have the same amino acids in their cell walls and mucopeptide. From the taxonomic point of view this would indicate that they are probably related if cell-wall composition can be taken as a suitable criterion. Serological data (Baboolal, 1968; Hofstad, 1967*a*) suggest that there is no relationship between these two groups of micro-organisms and other taxonomic characteristics such as biochemical and phhysiological studies (Table 2, Hofstad, 1967*a*) tend to support the results of serological studies.

The taxonomic conclusions drawn from cell-wall analysis and serological studies appear contradictory. This, however, is not surprising since the major cell-wall antigens of these two groups of micro-organisms are two chemically different polysaccharides (Baboolal, 1969). Kasai (1965) and Takazoe & Frostell (1960) have described filamentous types of leptotrichia which the present author believes might be 'Anaerobic filaments'. The cellular and colonial morphology of the 'Anaerobic filaments' and some of their biochemical and physiological properties such as absence of catalase and sensitivity to oxygen would indicate that they are probably related to *Leptotrichia*.

The results presented in Table 3 differ from those of Hofstad (1967*b*) and Davis & Baird-Parker (1959) in that aspartic acid was found as a major component in the cell walls and mucopeptide of *Leptotrichia buccalis* and the 'Anaerobic filaments'. The latter authors used a one dimensional system and the former employed circular paper chromatography, so the difference in the results presented here might be due to the techniques employed.

The amounts of lipid found in the cell walls of these organisms appear to be relatively high for Gram-positive bacteria, but by no means exceptional, since higher quantities have been found in *Corynebacterium diphtheriae* (Salton, 1964). The isolation of lipopolysaccharide from whole cells of *Leptotrichia buccalis* (Gustafson, Kroeger, Gustafson & Vaichulis, 1966) and the detection of heptoses (Hofstad, 1967*b*) in the walls of the 'Anaerobic filaments' suggest that these organisms have an unusual and complicated cell wall atypical of Gram-positive bacteria, since aldoheptoses have so far been demonstrated only in Gram-positive bacteria. However, the presence of large amounts of mucopeptide (Table 5) and the detection of few amino acids after trypsinization of cell walls are characteristic of Gram-positive bacteria (Salton, 1964). The presence of lipopolysaccharide reported in the cell walls of these organisms is an interesting finding and it has also been implicated as a component in the walls of *Clostridium welchii* (Pickering, 1967).

The absence of aspartic acid in the mucopeptide fraction of *Bacterionema matruchotii* and its presence in similar preparations of *Leptotrichia buccalis* and the 'Anerobic filaments' indicates that this difference might be of some taxonomic value. The cell-wall components of *B. matruchottii* suggest that it is perhaps related to *Nocardia* because of the presence of arabinose as a major sugar. Schmidt & Richardson (1961) found that antisera against *B. matruchotii* cross-reacted in complement fixation test with organisms of the genera *Mycobacterium*, *Nocardia*, *Actinomyces* and *Corynebacterium* and *vice versa*. However, the amino acid pattern is different from that reported for these genera by Cummins (1962) (Table 4) and its inclusion in a separate genus in the family Actinomycetaceae as proposed by Gilmour, Howell & Bibby (1961) is supported by the results of cell-wall studies.

Old cultures of *Leptotrichia buccalis* appear 'fusiform'-like and Gram-negative with Gram-positive granules in the cytoplasm. These characters seem to have resulted in the inclusion of this organism in the genus *Fusobacterium* under the name *F. fusiforme* which is the type species of this genus. The description of *F. fusiforme* given in the 7th edition of Bergey's Manual corresponds closely to the descriptions reported for *L. buccalis* by many investigators. *F. fusiforme* has been shown to be invalid (Baird-Parker, 1959) on the grounds that it corresponds to a previously described organism *L. buccalis* (Trevisan). It appears, therefore, that although the genus *Leptotrichia* is omitted from the 7th edition of Bergey's Manual, the type species of this genus is classified as the type species of the genus *Fusobacterium* under the name *F. fusiforme*. The cell-wall analysis data presented clearly indicate that the genus *Fusobacterium* have cell walls characteristic of Gram-negative organisms and are distinct from *Leptotrichia* species. It is interesting to note that lysine is a major component in the mucopeptide fractions of *Fusobacterium* as this amino acid is not usually found in such preparations from Gram-negative organisms.

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Surface Appendages Similar to Fimbriae (Pili) on Pseudomonas Species

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SUMMARY

Twenty-two strains of Pseudomonas, representing 15 species, were examined by electron microscopy, utilizing the techniques of negative contrast staining and to a lesser extent shadowing and ultrathin sectioning. Fimbria-like appendages were found on cells of 12 strains, representing 8 species. Polar, fimbria-like filaments were observed on *Pseudomonas aeruginosa*, *P. acidovorans*, *P. testosteroni*, *P. maltophilia*, *P. alcaligenes* and *P. solanacearum*. Peritrichous filaments were found on *P. multivorans* and *P. fragi*.

INTRODUCTION

Bacterial fimbriae or pili are filamentous appendages of bacterial cells which differ from flagella in a number of ways. Fimbriae do not exhibit the sinuous form characteristic of bacterial flagella and they are apparently not related to the motility of flagellated bacteria (Brinton, 1965; Duguid, Anderson & Campbell, 1966). Fimbriae are often thinner than flagella and may differ from flagella in detailed fine structure (Duguid, Smith, Dempster & Edmunds, 1955; Brinton, 1967). Like flagella, fimbriae may be of polar or peritrichous distribution, depending on the genus or species of bacterium examined (Houwink & van Iterson, 1950). There is no convincing evidence at present of the occurrence of fimbriae in other than Gram-negative bacteria.

Much recent work has been concentrated on fimbriae of the enterobacteria and, with notable exceptions (e.g. Schmidt, 1966; Tweedy, Park & Hodgkiss, 1968), the polar flagellates have been relatively neglected. Fimbriae have been reported on only four Pseudomonas species; *Pseudomonas aeruginosa* (Houwink & van Iterson, 1950; Bradley, 1965, 1966); *P. echinoides* (Marx & Heumann, 1962; Heumann & Marx, 1964), *P. piscicida* (Hansen, Ingebritsen & Weeks, 1963) and *P. multivorans* (Tweedy *et al.* 1968). It would seem that the majority of Pseudomonas species have not been examined systematically using appropriate methods. In order to extend existing knowledge of the occurrence of fimbriae in Pseudomonas, fifteen physiologically diverse species of the genus were examined by electron microscopy as part of a survey of the filamentous appendages found on the surface of pseudomonad cells. The techniques of negative contrast staining and to a lesser extent shadowing and ultrathin sectioning were used.

Like Tweedy *et al.* (1968), we do not wish to imply through use of the term fimbriae for filamentous appendages other than flagella on pseudomonads that such appendages are comparable in every respect to the fimbriae of other organisms. We also use

'fimbriae' (Duguid *et al.* 1955) rather than 'pili' because it has priority and because we accept the other arguments (Duguid, 1966; Duguid & Anderson, 1967) in favour of this term.

METHODS

Organisms and media. The organisms examined in this study, as listed in Table 1, were grown in either peptone yeast extract broth (Difco bacto peptone, 10·0 g.; Difco bacto yeast extract, 5·0 g.; NaCl, 5·0 g.; distilled water 1 l.; pH 7·2), sucrose peptone broth (sucrose, 20·0 g.; Difco bacto peptone, 5·0 g.; $MgSO_4.7H_2O$, 0·25 g.; K_2HPO_4, 0·5 g.; distilled water 1 l.; pH 7·0 to 7·2), 0·1 % lactate mineral salts medium (lactic acid, 1 g./l.; standard mineral base of Stanier, Palleroni & Doudoroff, 1966), or 0·1 % lactate mineral salts medium supplemented with 0·025 % DL-methionine (for *Pseudomonas maltophilia* only).

Table 1. *Pseudomonas species examined for fimbriae*

Strain no.	Organism	Culture collection no. or source
85*	*P. aeruginosa*	ATCC 14216; NCIB 9685
80*	*P. aeruginosa*	ATCC 10145; CCEB 481; NCIB 8295; NCTC 10332
67*	*P. fluorescens*	CCEB 488-A; ATCC 15456; NCIB 9886
62*	*P. fluorescens*	ATCC 13525; NCIB 9046; NCTC 10038
64*	*P. chlororaphis*	NCIB 9392; ATCC 9446; NRRL B-560
70*	*P. aureofaciens*	NCIB 9030; ATCC 13985; NRRL B-1576; Delft E. III. 9.25.1
86*	*P. putida*	CCEB 520; NCIB 9887
83*	*P. putrefaciens*	NCIB 8615
72*	*P. acidovorans*	Den Dooren De Jong strain 7; NCIB 9681; ATCC 15668
81*	*P. testosteroni*	NCIB 8955; ATCC 11996; NRRL B-2611
75*	*P. oleovorans*	ATCC 8062; NCIB 6576
58*	*P. alcaligenes*	ATCC 14909; NCTC 10367
0346	*P. multivorans*	Isolated from forest-litter, Mt. Nebo, Queensland, Australia, in May 1968
036B	*P. multivorans*	Isolated from tomato stems from Cairns, Queensland, Australia in October 1965
56*	*P. fragi*	ATCC 4973; CCEB 387; NCIB 8542; NRRL B-25
0147	*P. maltophilia*	From Commonwealth Serum Laboratories, Parkville, Victoria
61*	*P. maltophilia*	ATCC 13637; NCIB 9203; NCTC 10257
017A	*P. solanacearum*	From bacterial wilt of potato, isolated, April 1965. Biotype II
002A	*P. solanacearum*	From bacterial wilt of tomato, April 1965. Biotype III
003A	*P. solanacearum*	From bacterial wilt of ginger, April 1965. Biotype IV
450	*P. stizolobii*	NCPPB 450; W. H. Burkholder PS 23 isolated from *Trifolium repens* in U.S.A., 1954
0268A	*P. stizolobii*	Isolated from blackened stems of *Vicia sativa* cultivar Golden Tares, April 1967. From Wollongbar Agric. Res. Station, N.S.W. Dept Agric.

* Culture obtained from the Pseudomonas Working Party.

Organisms to be examined by negative contrast staining or shadowing in the survey for fimbriae were grown through various serial subcultures in $6 \times \frac{1}{2}$ in. test tubes containing 5 or 10 ml. medium and incubated aerobically at 28°. Cells for ultrathin sectioning were grown in 100 ml. medical flats containing 10 ml. of 0·1 % lactate mineral salts medium and incubated aerobically at 28° on their wide sides. The exact ages of each culture examined are listed in the 'Explanation of Plates'. Initial inocula in all cases were from stock agar slope cultures. Purity checks were performed on all

cultures examined by plating out from the culture itself or from the washed cell suspension prepared from the culture (see below).

Negative contrast staining. Cells to be examined were harvested by centrifugation, washed once in sterile distilled water before final resuspension in the wash fluid. A 1 % solution of sodium phosphotungstate (pH 6·0, 7·0 or 7·4) containing bovine plasma albumin to 0·03 % was employed as a negative contrast stain. Negative contrast preparations were usually made by applying a drop of washed bacterial suspension to a carbon-coated copper grid. After allowing up to 3 min. for adherence of cells to the carbon film to take place, excess fluid was removed with filter paper. A drop of negative contrast stain was then applied to the grid and excess fluid again removed. The grid was then transferred to the electron microscope specimen chamber where final drying was allowed to take place. In some cases the negative contrast agent was applied to the grid first (see below).

Shadowing. A drop of washed bacterial suspension (prepared in the same manner as for negative contrast staining, with the exception that in some cases 0·1 % formalin was used as a wash fluid) was applied to a carbon-coated grid and excess fluid removed. The preparation was then shadowed with platinum/carbon. The angle of shadowing was not determined.

Ultrathin sectioning. After 26 hr growth in 100 ml. medical flats containing 10 ml. of 0·1 % lactate mineral salts medium incubated aerobically at 28°, cells were harvested by centrifugation. Pelleted cells were fixed with 4 % (w/v) glutaraldehyde buffered to pH 6·2 with 0·1 M-sodium cacodylate (Sabatini, Bensch & Barrnett, 1963). Fixation was allowed to proceed for 18 hr at room temperature. Cells were then washed in 0·2 M-sucrose solution buffered to pH 6·2 in 0·1 M-cacodylate buffer and postfixation was carried out in osmium tetroxide overnight. After postfixation, cells were washed in 0·5 % uranyl acetate for 1 hr. Dehydration was performed via a series of ethanol solutions and the pellet was embedded in Epon 812 by a method similar to that of Luft (1961). Sections were cut from the resulting block on a Porter-Blum ultramicrotome. Sections were poststained with 5 % (w/v) uranyl acetate for 15 min. and lead citrate (Reynolds, 1963) for 2 min.

Electron microscopy. A Siemens Elmiskop IA was used at accelerating voltages of 80 kV or 100 kV.

Standard procedures for obtaining statistically analysable fimbrial diameter measurements. Organisms were grown for 48 hr in 6 × ½ in. tubes containing 5 ml. of peptone yeast extract broth and incubated aerobically at 28°. Cells were harvested by a standard method and purity checks performed. All preparations were negatively stained with 1 % sodium phosphotungstate (pH 7·0) containing 0·03 % bovine plasma albumin, applied by a standardized procedure. All micrographs of standard preparations were taken at an instrumental magnification of × 80,000. Measurement of fimbrial diameters was made from prints enlarged × 2, using a Peak Scale Lupe 7 x (Kellner type) micrometer with a linear graticule. For determination of means and standard deviations, thirty diameters were measured in each case.

RESULTS

Survey for fimbria-like appendages in Pseudomonas species. Negatively stained preparations of all the strains listed in Table 1 were examined exhaustively in the

electron microscope for fimbriated cells. Among the criteria used to distinguish fimbriae were: (i) position (i.e. surface appendages); (ii) shape (i.e. showing no regular undulations); (iii) diameter (i.e. thinner than flagella); (iv) substructure (i.e. a substructure differing from that of flagella of the same species); (v) distribution (only if fimbrial distribution differed from the flagellar distribution of the same species). Results of the survey are summarized in Table 2. At least 12 out of the 22 strains examined, representing 8 of 15 species, definitely possessed fimbria-like appendages. Appendages thinner than flagella were seen on one strain of *Pseudomonas stizolobii* (strain 0268 A) but these filaments were of variable diameter and seemed to taper in some cases. They were only seen in very old cultures and there is a definite possibility that they are an artifact resulting from autolytic effects or represent slime material.

Table 2. *Occurrence of fimbriae in Pseudomonas species examined by negative contrast staining*

Bacterial strain	Fimbriae observed	Distribution
P. aeruginosa (85)	+	Polar
P. aeruginosa (80)	+	Polar
P. fluorescens (67)	−	.
P. fluorescens (62)	−	.
P. chlororaphis (64)	−	.
P. aureofaciens (70)	−	.
P. putida (86)	−	.
P. putrefaciens (83)	−	.
P. acidovorans (72)	+	Polar
P. testosteroni (81)	+	Polar
P. oleovorans (75)	−	.
P. alcaligenes (58)	+	Polar
P. multivorans (0346)	+	Peritrichous
P. multivorans (036B)	+	Peritrichous
P. fragi (56)	+	Peritrichous
P. maltophilia (0147)	+	Polar
P. maltophilia (61)	−	.
P. solanacearum		
biotype II (017A)	+	Polar
biotype III (002A)	+	Polar
biotype IV (003A)	+	Polar
P. stizolobii (0268A)	?	?
P. stizolobii (450)	−	.

In a number of strains, attempts to demonstrate fimbriae gave negative results, even after examination of cells grown by several different subculturing procedures and/or in different media. Negative results such as those obtained must be interpreted cautiously for a number of reasons, however (see Discussion), and do not necessarily mean that the organisms are genotypically incapable of synthesizing fimbriae. Among those strains in which fimbriae were found, fimbriation of all cells in a sample was never observed. Usually the majority of cells examined in a preparation were not fimbriated. This perhaps emphasizes the dangers of misinterpreting negative results and the necessity for exhaustive scanning of preparations in a survey for fimbriation.

Polar and peritrichous distributions of fimbriae on individual cells were observed. Where distribution was polar, fimbriae were found on the flagellated pole only, except in the case of strain 85 of *Pseudomonas aeruginosa* and possibly strain 002 A of

P. solanacearum. Bipolar fimbriae were sometimes found on cells of strain 85 of *P. aeruginosa*. Distribution of fimbriae on pseudomonad cells cannot be related in any predictable way to flagellar distribution, apparently, since all the species of Pseudomonas examined possessed polar flagella while cells of at least two species possessed peritrichous fimbriae.

In no case did fimbriae on Pseudomonas species display a sinuous outline with regular undulations. Fimbriae (especially those of polar distribution) were sometimes seen to be slightly curved or irregularly bent in certain portions. It may be that these filaments are flexible rather than rigidly straight; distortion may result from stress during drying in the course of preparation.

Table 3. *Diameters of fimbriae found on Pseudomonas species by negative contrast staining*

P. aeruginosa (85)	54 ± 14 Å
P. aeruginosa (80)	52 ± 14 Å
P. multivorans (0346)	62 ± 5 Å
P. solanacearum (017 A)	49 ± 9 Å
P. solanacearum (002 A)	55 ± 10 Å
P. solanacearum (003 A)	60 ± 9 Å
P. testosteroni (81)	40 ± 8 Å
P. alcaligenes (58)	65 ± 20 Å

Values for measured diameters of fimbriae (30 diameters measured for each value) are given as the mean ± the standard deviation. Measurements for these values were of fimbriae on cells grown and negatively stained by a standard procedure (see Methods).

Although there may be error in determination of the maximum number and length of fimbriae on individual cells, due to possible breakage of fimbriae, some general observations can perhaps be made. The maximum number of fimbriae observed on either strain of *Pseudomonas multivorans* was greater than 100/cell. About 30 fimbriae/cell was the maximum observed on *P. fragi*. Among those strains with polar fimbriae, however, more than 10 fimbriae/cell were never observed (10/cell were only observed on strain 85 of *P. aeruginosa*). The maximum fimbrial length observed never exceeded 1·7 μ on any strain.

The diameters of fimbriae seen in negative contrast preparations are given for several strains in Table 3. They are all below the diameters observed for the flagella of these strains, and are also below the range of published diameters of unsheathed flagella, i.e. 120 Å (Hoeniger, 1965) to 220 Å (Richter & Kress, 1967).

Substructure of fimbriae seen in negative contrast preparations. Fimbrial substructure was clearly discerned in only a few species. In most cases substructure was not visible or very obscure.

The fimbriae of *Pseudomonas testosteroni* and *P. fragi* exhibited substructures of a periodic nature suggesting that they are composed of repeating subunits of some kind (Pl. 1, fig. 2, Pl. 5, fig. 10).

Dark central lines were observed in the fimbriae of *Pseudomonas multivorans* (Pl. 3, fig. 6) and *P. fragi* (Pl. 5, fig. 10), when seen in negative contrast preparations. The central regions were 13 to 19 Å wide in both species. It is perhaps significant that dark central lines were never seen in the polar fimbriae of other species. Bradley (1966) stated that the fimbriae of *P. aeruginosa* which he observed in negative contrast

preparations appeared to be hollow in places. This was not observed in the fimbriae of either strain of *P. aeruginosa* examined in this study,

The flagella of the Pseudomonas species examined were either sheathed or unsheathed. Sheathed flagella were found only on strains of *P. stizolobii* (Fuerst & Hayward, 1969). Where substructure of unsheathed flagella was visible, it usually consisted of an aggregate of globular subunits (e.g. Pl. 5, fig. 10) or a number of dark longitudinal lines alternating with light ones (e.g. Pl. 1, fig. 1), perhaps corresponding to the A and B forms of Lowy & Hanson (1965).

Table 4. *Published reports of fimbriae on bacteria with polar flagella*

Bacterial strain	*Diameter	Distribution	References
Vibrio cholerae (*sic*)	72 Å	Uniform	Tweedy, Park & Hodgkiss (1968)
V. eltor	79 Å	Uniform	Barua & Chatterjee (1964); Tweedy *et al.* (1968)
Caulobacter spp.	40 Å	Polar	Schmidt (1966)
Aeromonas hydrophila	.	Peritrichous	Page (1962)
A. liquefaciens	79 Å	Peritrichous	Tweedy *et al.* (1968)
Photobacterium splendidum	.	Peritrichous	Houwink & van Iterson (1950)
Pseudomonas pyocyanea (*P. aeruginosa*)	.	Polar	Houwink & van Iterson (1950)
P. aeruginosa	45 Å	Polar	Bradley (1965, 1966)
P. echinoides	50 Å (shadowed)	Polar	Marx & Heumann (1962) Heumann & Marx (1964)
P. multivorans	86 Å (shadowed)	Peritrichous	Tweedy *et al.* (1968)
P. piscicida	.	Peritrichous	Hansen, Ingebritsen & Weeks (1963)

* All diameter measurements are from negative contrast preparations unless otherwise stated.

Results of shadowing and ultrathin sectioning. Shadowed preparations were made using both strains of *Pseudomonas multivorans*. Results with respect to distribution of fimbriae confirmed those from negative contrast preparations. However, dimensions and appearances of the filaments were different from those in negative contrast preparations because of the evaporated metal deposit and perhaps because of drying *in vacuo*. Dimensions of fimbriae are above those which would be expected by extrapolation from negative staining results according to the predictions of Thornley & Horne (1962) and Tweedy *et al.* (1968). However, in this study the shadowing material used was different from those used by those workers and the shadowing angle may likewise have been different.

Plate 4, fig. 8 shows cells of strain 0346 of *Pseudomonas multivorans* in what appears to be transverse section. Extending from the periphery of one of the cells are about 15 straight or slightly curved filamentous structures, 31 to 78 Å wide. Another cell in the same micrograph appears to possess similar appendages. These filaments should not be confused with a finger-like extension on one of the cells which does not appear to be a rigid structure and is 94 to 235 Å wide. It may represent a 'bleb' of cell wall or membrane material seen in thin section. The thin filaments occur all around the periphery of one of the cells seen in section, and may be appendages which are peritrichously distributed on the unsectioned cell. Similar filaments were seen on other sectioned cells of the same strain. Penetration of the filaments into the cell sections was obscured in most cases. The filaments did not display clearly definable substructure in these sections. It would seem likely that these filaments represent the same type of

appendage as the peritrichous fimbriae seen in negative contrast and shadowed preparations of this strain.

DISCUSSION

Earlier reports of fimbriae on polarly flagellated bacteria are given in Table 4; there are records of fimbriae on only four species of Pseudomonas, two of which have been re-examined here. In this study, fimbriae were found on eight species of Pseudomonas, six of which had not been examined before by negative contrast staining. Tweedy *et al.* (1968) state that it has been the experience of one of them in electron microscopic work that fimbriae are possessed by only a few species of bacteria with polar flagella. However, the number of species examined to reach this conclusion was not stated. The results presented here indicate that the ability to produce fimbriae may be a more common feature of pseudomonads than had previously been realized.

Any consideration of the occurrence of fimbriation among different bacterial species must include the interpretation of negative results. The absence of fimbriae on cells of a bacterial strain is a result which may be interpreted in several ways. Not all strains of a species may be genotypically capable of producing fimbriae. Even if they were genotypically fimbriate, organisms may not have been grown under conditions enhancing fimbriation or selecting for fimbriated cells. Another important factor may be examination at a stage of growth when the proportion of fimbriated cells is highest. However, consistently negative results among closely related strains may have some significance. For instance, fimbriae were not observed on *Pseudomonas fluorescens* (strains 62, 67), *P. chlororaphis*, or *P. aureofaciens*. Stanier *et al.* (1966) consider that *P. chlororaphis* and *P. aureofaciens* should be incorporated into the species *P. fluorescens* as biotypes. However, confirmation of these results using other strains is required.

No attempt was made in this study to correlate the presence of fimbriae with haemagglutinating activity. Haemagglutinating activity is not necessarily associated with fimbriae and there are some types of fimbriae without haemagglutinating properties of any kind (Duguid *et al.* (1966). It is also possible that some types of fimbriae may have adhesive properties but because of restricted distribution on the cell (e.g. monopolar) are unable to confer haemagglutinating properties (Heumann & Marx, 1964). Because of these difficulties most attention was given to electron microscopy in this study. However, preliminary work on haemagglutinating properties of pseudomonads has confirmed the results of Tweedy *et al.* (1968) with respect to the mannose-sensitive haemagglutinating properties of *P. multivorans*.

The results of Houwink & van Iterson (1950) on the distribution of fimbriae of *Pseudomonas pyocyanea* (*P. aeruginosa*), which were obtained from shadowed preparations, were confirmed in this study by negative contrast staining. Bradley (1966) could not observe the fimbriae of *P. aeruginosa* by negative contrast methods unless cells examined had been treated with a specific RNA bacteriophage. In this study fimbriae were found readily by negative contrast staining of *P. aeruginosa* which had not been deliberately infected with phage. Bradley (1966) found that the diameter of fimbriae of *P. aeruginosa* observed in phage-infected preparations was about 45 Å, which is similar to the fimbrial diameter measurements for both strains of *P. aeruginosa* examined in this study (i.e. 54 ± 14 Å and 52 ± 14 Å).

Tweedy *et al.* (1968) found numerous, short, straight fimbriae on cells of *Pseudomonas multivorans* NCIB 9691 by shadowing. We have observed numerous peritrichous

fimbriae on two other strains of *P. multivorans* (i.e. 036 B and 0346) by both negative contrast and shadowing methods. In the case of strain 0346 these appendages have also been observed in ultrathin sections. Tweedy *et al.* (1968) predicted from shadowed preparations of *P. multivorans* NCIB 9691 that the diameter of the fimbriae of this strain in negative contrast preparations should be about 60 Å. The fimbrial diameter measurement for strain 0346 in this study (i.e. 62 ± 5 Å) is very close to that predicted by Tweedy *et al.* (1968).

The occurrence of polar fimbriae in three biotypes of *Pseudomonas solanacearum* is of interest; the only other plant pathogenic bacteria so far reported to possess fimbriae are some species of *Agrobacterium* (De Ley, Bernaerts, Rassel & Guilmot, 1966). The presence of polar fimbriae on cells of both *P. acidovorans* and *P. testosteroni* is also noteworthy, since these species are considered to be closely related (Stanier *et al.* 1966).

The fimbriae of *Pseudomonas testosteroni* and *P. fragi* appear to exhibit a periodic substructure suggesting that they are composed of repeating subunits of some kind. Regular periodic substructure has been observed before in the fimbriae of several bacteria (e.g. Thornley & Horne, 1962; Meynell & Lawn, 1967), including *P. echinoides* (Heumann & Marx, 1964). The appearance of the filaments of *P. testosteroni* seen in Pl. 1, fig. 2 is similar to that of F-actin filaments from muscle seen in negative contrast preparations (Hanson & Lowy, 1963; Haggis, 1966). The model of F-actin substructure constructed by Hanson & Lowy (1963) consisting of two helically wound strands composed of globular subunits might also apply to fimbriae of similar appearance.

Dark central regions, 13 to 19 Å wide, were seen in fimbriae of strain 0346 of *Pseudomonas multivorans* and *P. fragi* when these were prepared by negative contrast staining. Lawn (1966) has observed central dense lines of maximum diameter 15 Å in both common fimbriae and F-fimbriae of *Escherichia coli* when negatively stained. Brinton (1967) states that an axial hole 20 to 25 Å wide occurs in the Type 1 fimbriae of *E. coli* while the axial hole of F-fimbriae is 25 to 30 Å wide. The central regions seen in the fimbriae of *P. multivorans* and *P. fragi* in this study may indicate that the fimbriae possess axial holes along which negative contrast stain can penetrate, similar to those postulated to exist in Type 1 fimbriae and F-fimbriae of *E. coli*.

Present classifications of fimbriae are in our opinion artificial and premature. They have already given rise to confusion (Duguid & Anderson, 1967). No attempt has therefore been made to identify fimbriae of pseudomonads with any of the existing 'Types' in the existing classification schemes.

We wish to stress the possible value of the occurrence and distribution of fimbria-like appendages as taxonomic criteria. With the advent of improved negative contrast staining techniques and the more general use of electron microscopy, such features may be of use in the characterization and identification of bacteria. However, the reproducibility of electron microscopic results and the consistency of fimbriation throughout a particular taxonomic group must be considered. For instance, with respect to the former problem, it is important to know the conditions which are optimal for the production of fimbriae by pseudomonads. Studies on these aspects are proceeding in this laboratory.

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EXPLANATION OF PLATES

Abbreviations in figures: F, flagellum; P fimbria.

PLATE 1

Fig. 1. *Pseudomonas aeruginosa* ATCC 14216 after 2 × 24 hr + 1 × 22 hr serial subcultures in lactate mineral salts medium incubated at 28°. At least two fimbriae arise from one of the cells. Negatively stained with sodium phosphotungstate, pH 7·4. × 160,000.

Fig. 2. *P. testosteroni* ATCC 11996 after growth for 48 hr in peptone yeast extract broth incubated at 28°. Five fimbrial filaments are visible. Negatively stained with sodium phosphotungstate, pH 7·0. × 160,000.

PLATE 2

Fig. 3. *P. acidovorans* ATCC 15668 after 1 × 48 hr + 1 × 72 hr + 1 × 88 hr serial subcultures in lactate mineral salts medium incubated at 28°. Two fimbriae are visible. Negatively stained with sodium phosphotungstate, pH 7·0. × 80,000.

Fig. 4. *P. alcaligenes* ATCC 14909 after growth for 88 hr in peptone yeast extract broth incubated at 28°. One long fimbria is visible, folded across the flagellum in some parts. Negatively stained with sodium phosphotungstate, pH 7·0. × 80,000.

Fig. 5. *P. multivorans* strain 0346 after 2 × 24 hr + 1 × 20 hr serial subcultures in lactate mineral salts medium incubated at 28°. Numerous peritrichous straight fimbriae are visible. Negatively stained with sodium phosphotungstate, pH 7·4. × 36,000.

PLATE 3

Fig. 6. *P. multivorans* strain 0346 after 2 × 24 hr + 1 × 22 hr serial subcultures in lactate mineral salts medium incubated at 28°. Several fimbriae are visible, some of which display dark central regions. Negatively stained with sodium phosphotungstate. pH 7·4. × 160,000.

Fig. 7. *P. multivorans* strain 0346 after growth for 22 hr in peptone yeast extract broth incubated at 28°. Numerous peritrichous appendages are visible. Shadowed with platinum/carbon. × 44,000.

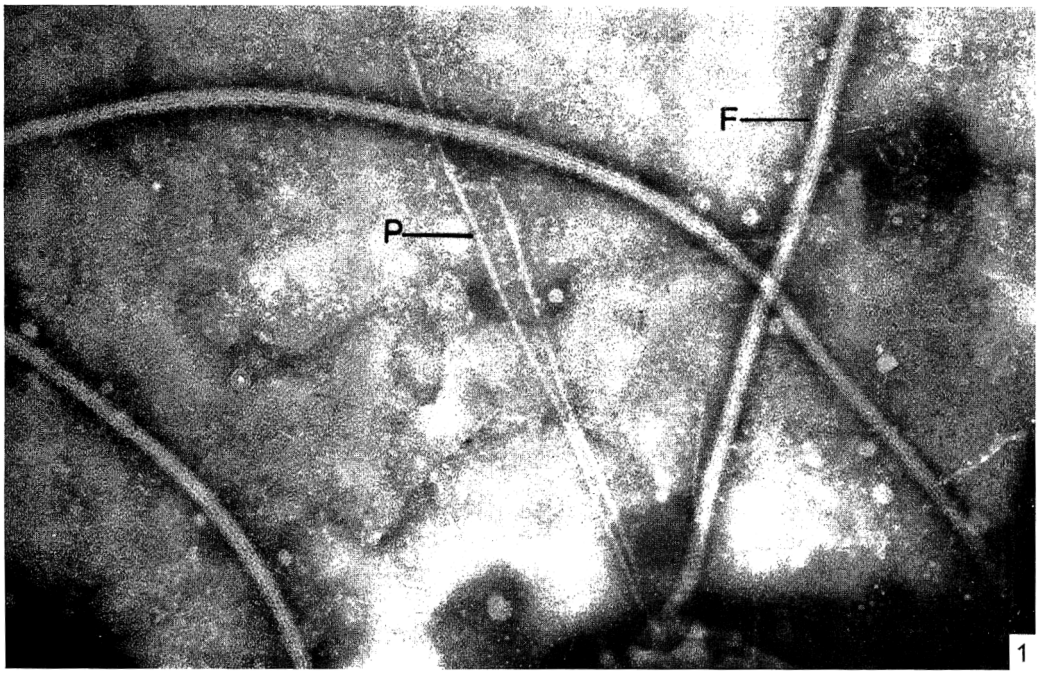
PLATE 4

Fig. 8. *P. multivorans* strain 0346 after growth for 26 hr in lactate mineral salts medium incubated at 28°. Numerous filamentous appendages extend from the periphery of sectioned cells. Glutaraldehyde-osmium tetroxide fixation. × 128,000.

Fig. 9. *P. fragi* ATCC 4973 after 1 × 24 hr + 1 × 39 hr serial subcultures in lactate mineral salts medium. Numerous peritrichous fimbriae are visible. Negatively stained with sodium phosphotungstate, pH 7·0. × 52,000.

PLATE 5

Fig. 10. *P. fragi* ATCC 4973 after 2 × 24 hr + 1 × 22 hr serial subcultures in lactate mineral salts medium at 28°. Straight fimbriae displaying dark central regions are visible. Negatively stained with sodium phosphotungstate, pH 7·0. × 272,000.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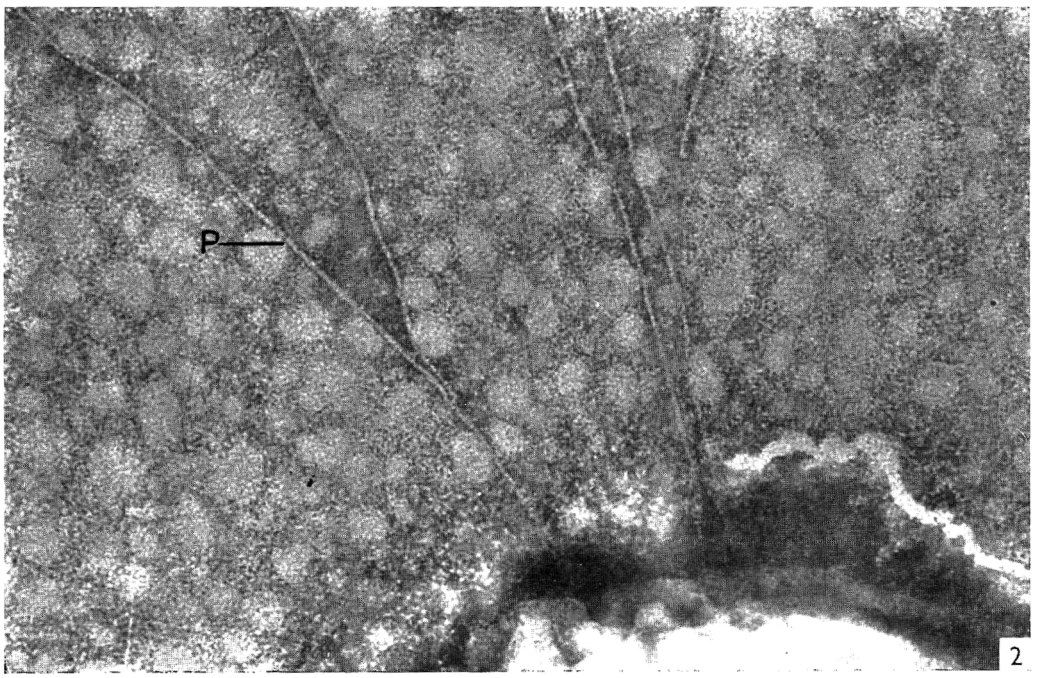
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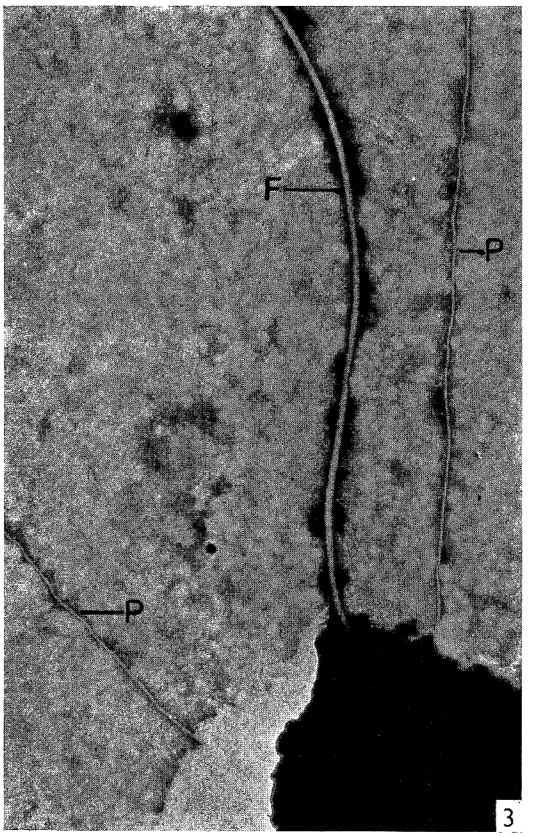

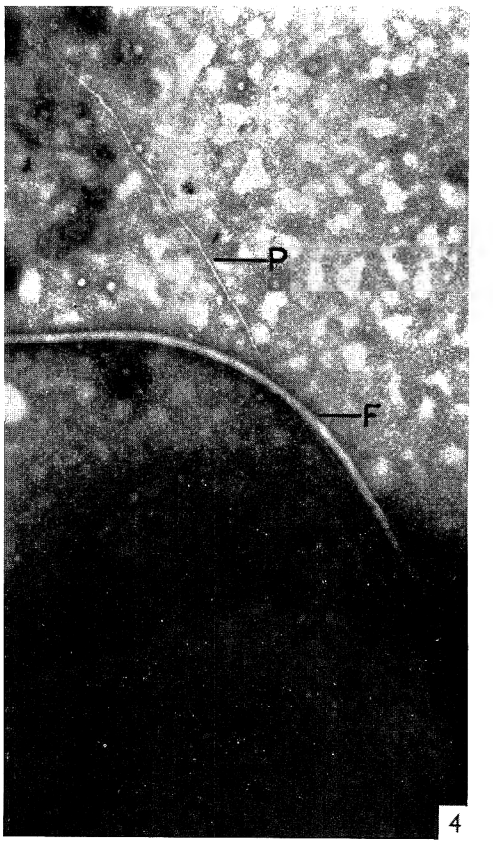

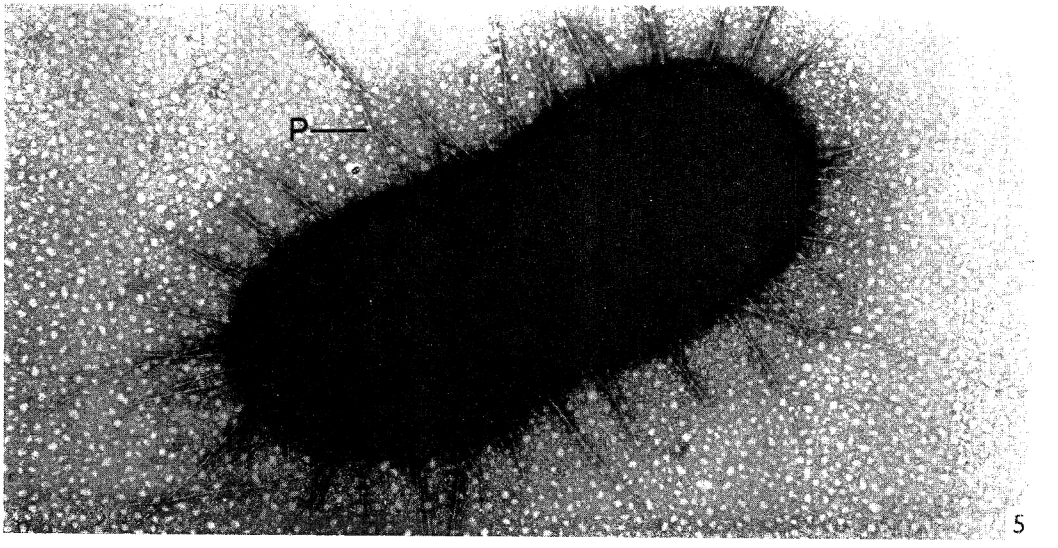

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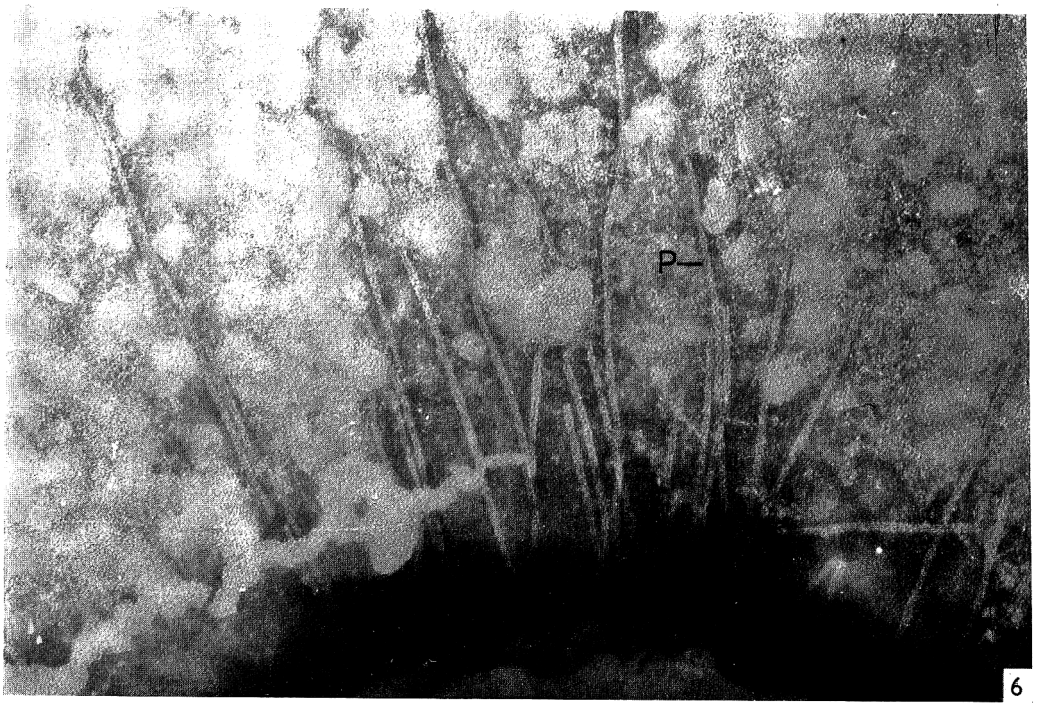
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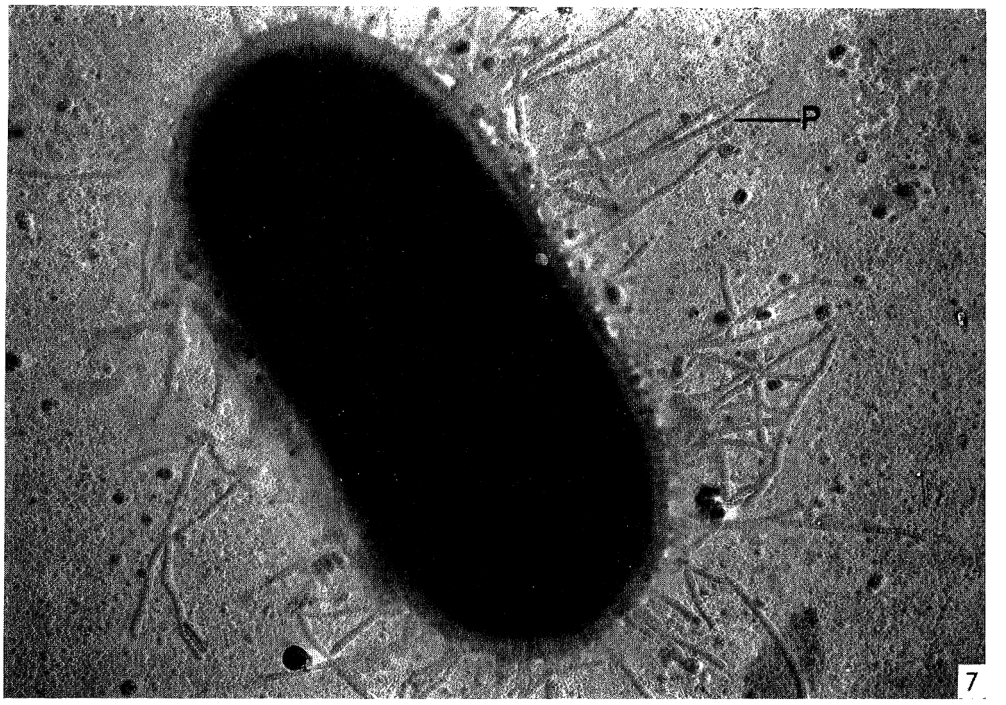
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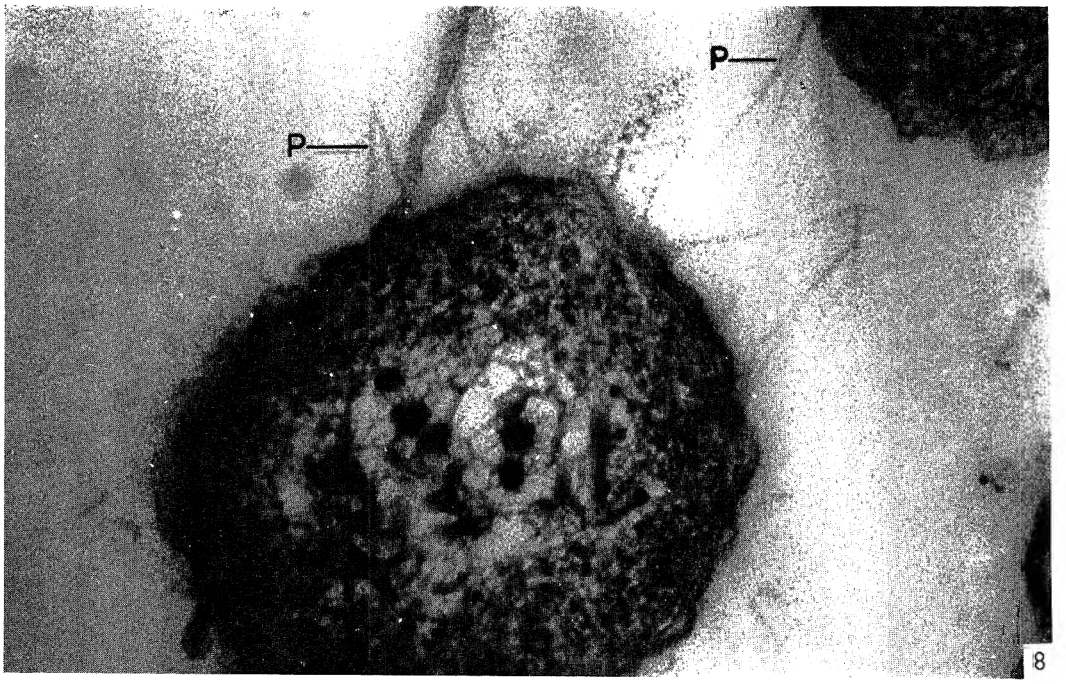
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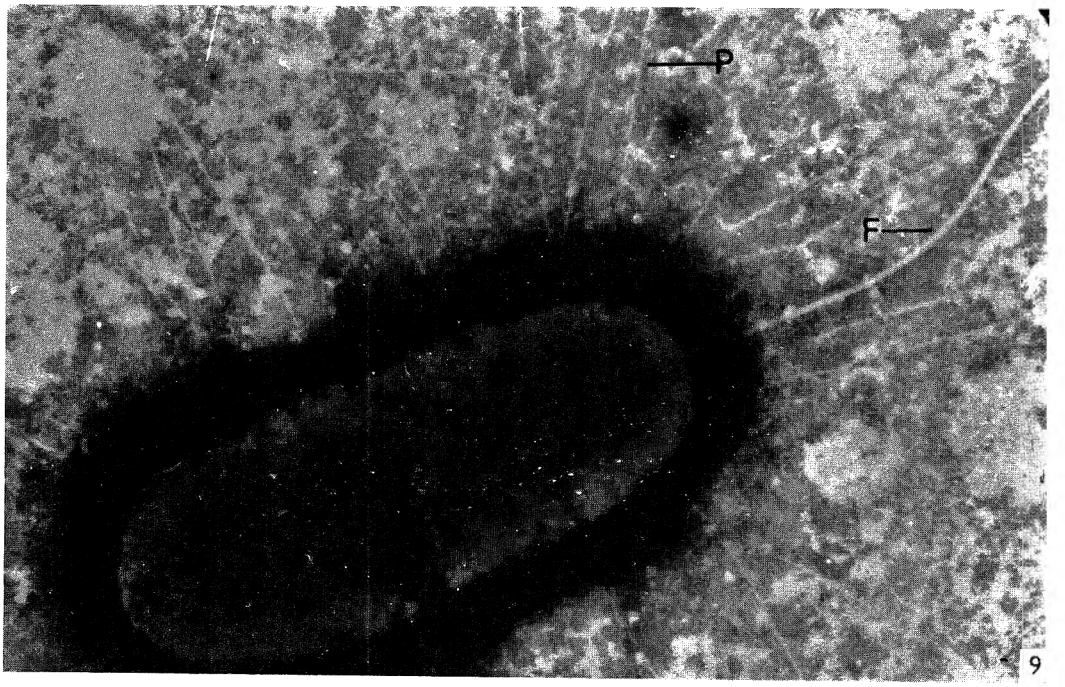
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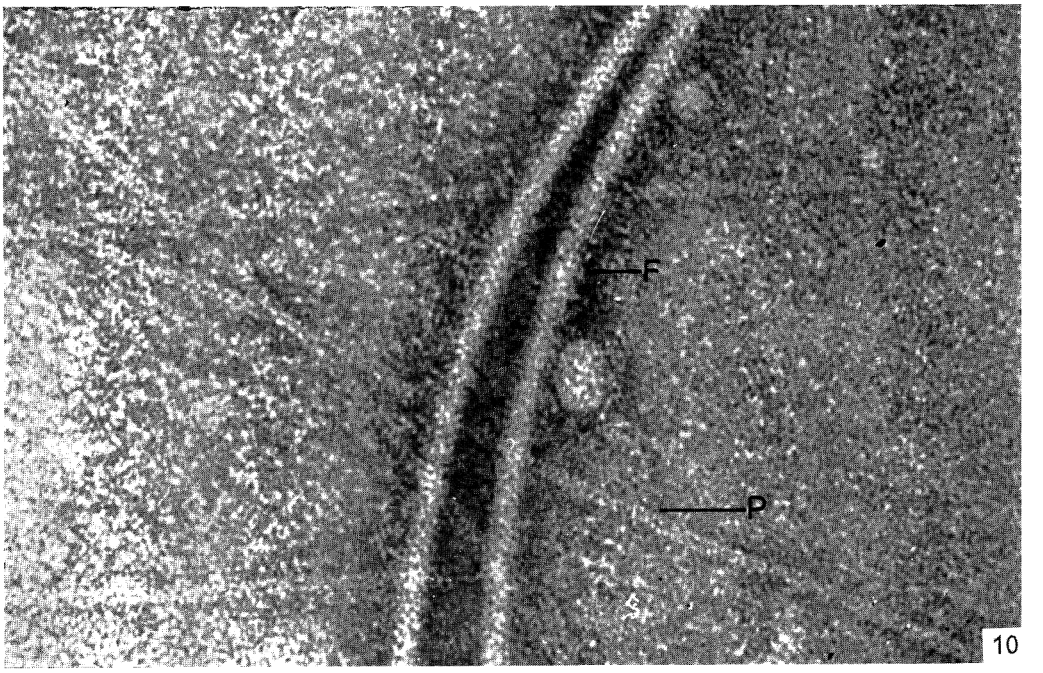

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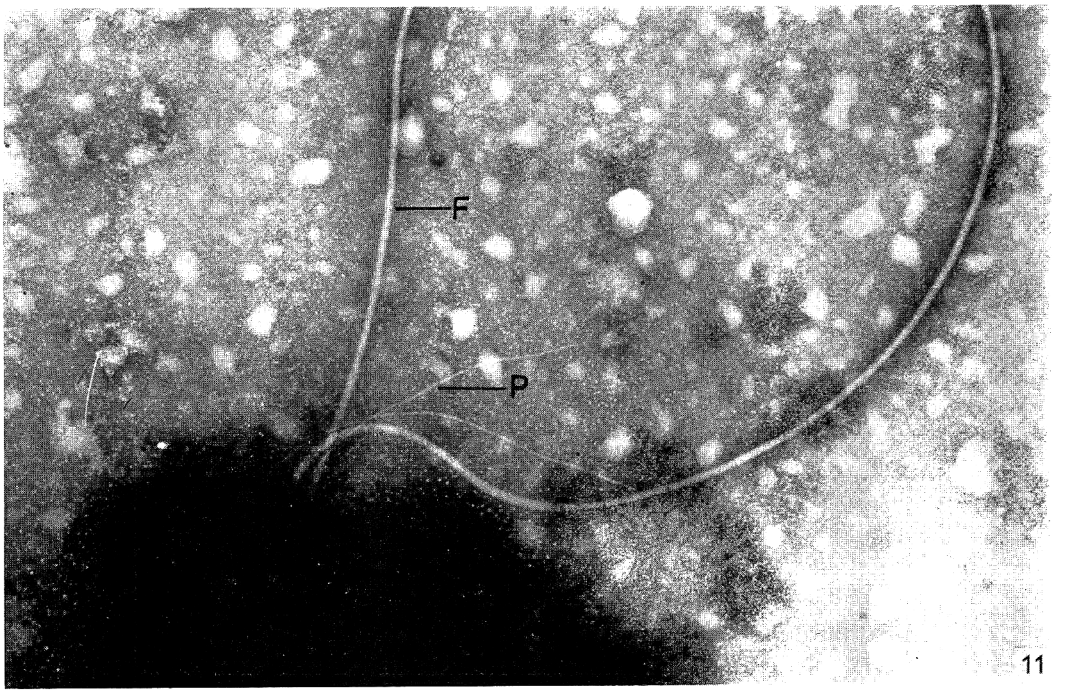

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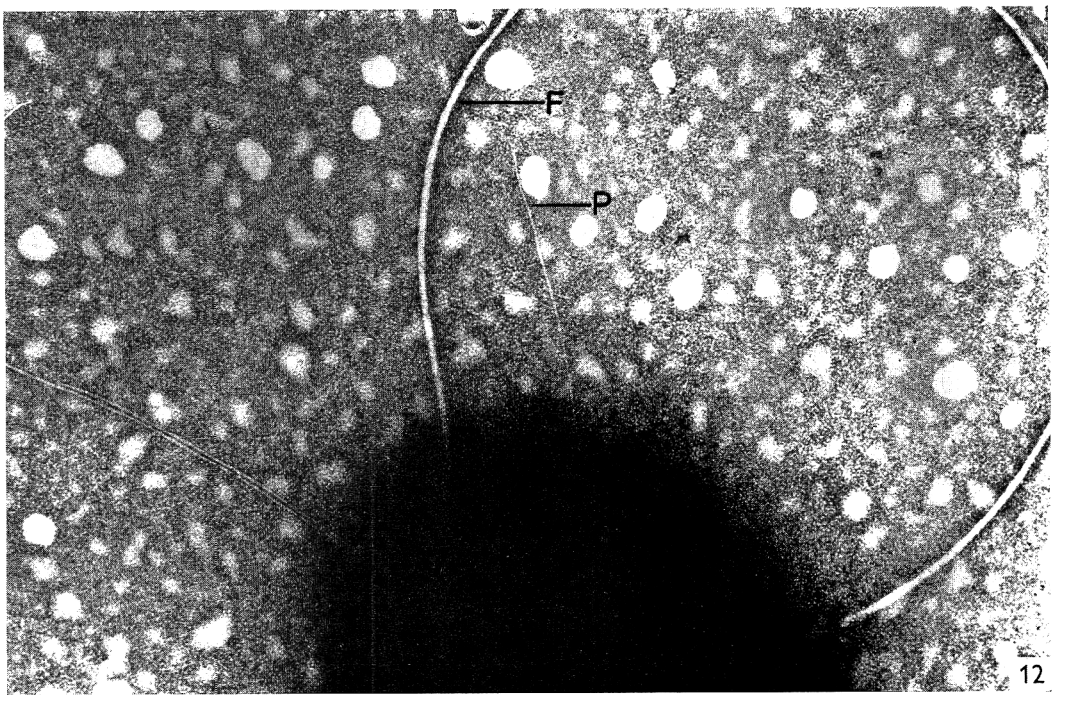
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Fig. 11. *P. maltophilia* strain 0147 after 1 × 48 hr + 1 × 72 hr + 1 × 88 hr serial subcultures in lactate mineral salts medium containing methionine, incubated at 28°. Two curved fimbriae are visible. Negatively stained with sodium phosphotungstate, pH 7·0. × 80,000.

PLATE 6

Fig. 12. *P. solanacearum* strain 017 A, biotype II after growth for 64 hr in sucrose peptone broth incubated at 28°. Three fimbriae extend from a flagellated cell. Negatively stained with sodium phosphotungstate, pH 6·0. × 88,000.

Fig. 13. *P. solanacearum* strain 002 A, biotype III after growth for 48 hr in sucrose peptone broth incubated at 28°. Six fimbriae extend from one pole of the cell. Negatively stained with sodium phosphotungstate, pH 7·0. × 40,000.

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The Sheathed Flagellum of *Pseudomonas stizolobii*

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SUMMARY

Organisms of two strains of *Pseudomonas stizolobii* possessed one polar flagellum of unusual thickness. Negative-contrast staining and ultrathin sectioning indicated that the flagella are sheathed and are comparable in structure to the sheathed flagella described in Vibrio and Bdellovibrio. In some instances, flagella displayed sheath and core structure after negative constrast staining. Distal 'tubules' and 'knobs' apparently consisting entirely of sheath material were also seen. The thickness of the sheath, which in section consisted of an outer dense component and an inner lighter component, was similar to that of the outer double track membrane of the cell wall.

INTRODUCTION

During the examination of a collection of species of Pseudomonas for the presence of fimbria-like appendages (Fuerst & Hayward, 1969), unusual polar flagella were seen on *Pseudomonas stizolobii* (Wolf) Stapp, 1935. This species is a plant pathogen causing a disease of the leaves and stems of mainly leguminous plants (Burkholder, 1957; Rothwell & Hayward, 1964). Whereas flagella of most bacteria are 120 to 150 Å in diameter and, under the electron microscope at high resolution, preparations of negatively stained material exhibit a helical aggregate of globular subunits, (Joys, 1968; Doetsch & Hageage, 1968), the flagella of *P. stizolobii* were of greater thickness and lacked the substructure characteristic of unsheathed bacterial flagella. Our aim was to determine by electron microscopic techniques the nature of the flagellum of *P. stizolobii* and to compare it with the sheathed flagella which are known to occur in a very few genera of Gram-positive and Gram-negative bacteria.

METHODS

Organisms and media. Pseudomonas stizolobii NCPPB 450 and *P. stizolobii* strain 0268 A, as listed by Fuerst & Hayward (1969), were grown at 28° in either motility medium (Difco bacto peptone, 5·0 g.; glycerol, 5·0 g.; $MgSO_4.7H_2O$, 0·25 g.; K_2HPO_4, 0·5 g.; Difco agar, 3·0 g.; distilled water, 1 l.), peptone yeast extract broth (see Fuerst & Hayward, 1969), or glucose peptone yeast extract broth (glucose, 1 g.; peptone yeast extract broth, 100 ml.).

Organisms not to be treated with reagents were carried through two 72 hr serial subcultures in Craigie tubes of motility medium and 5 or 8 serial subcultures of variable duration in $6 \times \frac{1}{2}$ in., tubes containing 5 ml. peptone yeast extract broth. Organisms to be treated with various reagents (including distilled water controls) were

grown in tubes of peptone yeast extract broth for 70 hr or tubes of glucose peptone yeast extract broth for 20 hr. Organisms for ultrathin sectioning were grown in 100 ml. medical flats containing 10 ml. of glucose peptone yeast extract broth and incubated for 26 hr on their wide sides.

Initial inocula in all cases were from stock agar slopes. Purity checks were performed on all cultures examined by plating out from washed cell suspensions.

Negative contrast staining of untreated flagella. Cells were harvested by centrifugation and washed once in sterile distilled water before resuspension in the wash fluid. A 1 % solution of sodium phosphotungstate (pH 7·0 or 7·3) containing bovine plasma albumin to 0·03 % was employed as a negative contrast stain. Negative contrast preparations were made by applying drops of washed suspension and negative contrast stain to carbon-coated copper grids as described by Fuerst & Hayward (1969).

Treatments of flagella with reagents and negative contrast staining following treatment. Flagellated cells were treated with various reagents according to a modification of the procedure of Follett & Gordon (1963). Unlike Follett & Gordon (1963), cells were not fixed with formalin before treatment and ammonium molybdate was not used as a negative contrast stain. In addition Follett & Gordon in all cases treated cells first with a 1 % potassium iodide (KI.) solution before treatment with other reagents. Although a pretreatment with 1 % KI. was included in some cases, this treatment was often omitted because it was not known what effect the potassium iodide itself might have on flagellar structure.

Carbon-coated grids were floated on top of washed cell suspensions. The grids were then floated on a number of wash solutions and reagents. Treatments with N/100 HCl (pH 2·2), 6 M-urea, 10^{-3} M-sodium ethylenediaminetetra-acetic acid (Na_2EDTA) and 0·05 % phenol were interpolated between two distilled water washes. Negative contrast stain was applied by floating grids from the second distilled water wash on top of a 1 % sodium phosphotungstate solution (pH 7·4) containing 0·03 % bovine plasma albumin, removing excess fluid and placing in the microscope specimen chamber while still wet. The time of exposure to 6 M-urea was much shorter than that used by Follett & Gordon (1963) since, if longer times were used, no cells could be found adhering to the grid after such treatment. This may well have affected results and similar qualifications may apply to treatments with phenol and Na_2EDTA, which were also necessarily short.

Ultrathin-sectioning. Procedures used for obtaining ultrathin sections were those used by Fuerst & Hayward (1969).

Electron microscopy. A Siemens Elmiskop IA was used at accelerating voltages of 80 or 100 kV.

RESULTS

Negative contrast staining

Both strains of *Pseudomonas stizolobii* possessed one polar flagellum per cell. The cells were usually bullet shaped, being pointed at the flagellate pole and rounded at the aflagellate pole (Pl. 1, fig. 2). The tip of the flagellum as seen in untreated preparations was most commonly even, but occasionally an irregular appendage appeared to extend from the tip (Pl. 1, fig. 2). The typical appearance of intact flagella is seen in Pl. 1, fig. 1. A cell of strain NCPPB 450 displays a flagellum of diameter 250 to 286 Å. The tip of the flagellum is rounded and no substructure of any kind is evident, although

untreated flagella which displayed core and sheath were seen in some cases. An enlarged portion of a flagellum of strain 0268 A is shown in Pl. 2, fig. 3; the diameter (262 to 311 Å) is similar to that of strain NCPPB 450, the tip is rounded and there is a lack of substructure.

Chemical treatments known to enhance the contrast between the core and sheath of vibrio flagella (Follett & Gordon, 1963) were applied to some preparations of *Pseudomonas stizolobii* prior to negative contrast staining. In other cases cells were treated with solutions of the disodium salt of ethylenediaminetetra-acetic acid (Na_2EDTA) or with phenol, also in the hope of delineating sheath and core. These reagents, among others, were shown by Varon & Shilo (1968) to inhibit the motility of *Bdellovibrio bacteriovorus*, an organism with a single polar sheathed flagellum. They postulated that this inhibition might be connected with the sheathed flagellum structure.

After potassium iodide treatment alone prior to negative staining, delineation of sheath and core structure was seen in some cases, and the flagella were in some instances unusually thick. Thin, irregularly folded 'tubules' were often seen at the distal ends of the flagella. In Pl. 2, fig. 4 an enlarged portion of a flagellum of strain NCPPB 450 is shown after potassium iodide treatment and treatment with N/100 HCl solution prior to negative staining. The flagellum is 225 to 295 Å in diameter and displays core and sheath. Two dark lines traverse the interior of the flagellum. If only the outer light portions on both sides of the dark lines are taken to represent sheath material, then the sheath is 46 to 78 Å and the core is 130 to 155 Å in diameter. Plate 4, fig. 7 shows a flagellum after treatment with urea, and serves as a general example of the 'irregular tubule' and 'knob' appearance. The main portion of the flagellum in this micrograph is 280 to 312 Å in diameter. The 'tubule' at its distal end appears to be continuous with the outer parts of the flagellum, and is 100 to 166 Å in diameter. The round 'knob' appears to be continuous with the 'tubule' and is 425 Å in diameter.

Treatments with urea or hydrochloric acid did not result in appearances similar to those of flagella of Vibrio species (Follett & Gordon, 1963) and *Bdellovibrio bacteriovorus* (Seidler & Starr, 1968) after similar treatments. This may have been due to the different times of exposure to the reagents used in this study, or to differences in relative stabilities of the sheaths of different species.

Core and sheath delineation and distal 'tubules' and 'knobs' were also seen in phenol- and EDTA-treated preparations (see Pl. 3, fig. 5, 6). In Pl. 3, fig. 6 the more proximal part of the flagellum does not show core and sheath structure, but towards the distal portion near to the 'tubule' and 'knob' such a structure is more clearly seen. The core is 125 to 150 Å wide where there is a well-defined core and sheath while the sheath material on either side is 50 to 75 Å in diameter.

Appearance of sheathed flagella in ultrathin sections

Ultrathin sections of cells of *Pseudomonas stizolobii* in certain instances displayed what appeared to be flagella in longitudinal section (Pl. 4, fig. 8, 9). The sheath appears to be composed of at least an outer dense component and an inner light component, and there are indications of another dense line bounding the inner light component, particularly in Pl. 4, fig. 8. In Pl. 4, fig. 8, the sheath is 42 to 58 Å wide while the core is 100 to 133 Å wide. Although no obvious continuity between the outer layers of the cell wall and the sheath could be discerned in these sections, the outer triple-layered

membrane around the sectioned cells is comparable in thickness to the sheath bounding the flagellar core.

DISCUSSION

Sheathed flagella are known to occur in few species of bacteria, including Gram-negative and Gram-positive organisms and organisms with peritrichous as well as those with polar flagella. Present information is summarized in Table 1. The sheathed flagella of species of Vibrio and *Bdellovibrio bacteriovorus* have been most fully studied. The sheathed flagellum of *Pseudomonas stizolobii* resembles those of Vibrio and Bdellovibrio in diameter and structure. It is of interest to determine whether *P. stizolobii* has other characters in common with Vibrio. However, there is little evidence to support such a relationship. A detailed description of the plant disease caused by *P. stizolobii*, and of the characteristics of this species will be reported elsewhere. (Allen, Hayward, Halliday & Fulcher, to be published). *P. stizolobii* differs from the human and animal pathogenic vibrios in which sheathed flagella have been described in having an oxidative metabolism of glucose, a negative oxidase reaction, and in failing to hydrolyse starch or gelatin. *P. stizolobii* is a short straight rod, containing refractile sudanophilic inclusions, which never exhibits the marked degree of cell curvature and of pleomorphism in old cultures which is characteristic of Vibrio (Park & Holding, 1966). *P. stizolobii* is thus the first member of the genus Pseudomonas in which a sheathed flagellum of the kind observed in Vibrio and Bdellovibrio has been identified. In *P. rhodos* sheathed flagella have been reported but they are clearly different in size and organization, being more slender (*cf.* Table 1) and having a sheath consisting of two helically wound ribbons which were easily visible by negative contrast (Lowy & Hanson, 1965); furthermore the fine structure of the core could be seen through the sheath when negative contrast methods were employed. There is therefore good reason to think that the sheath of this type is different in structure and size from those occurring in Vibrio, Bdellovibrio and *P. stizolobii*.

Table 1. *Reports of sheathed flagella in bacteria*

Bacterium	Flagellum diameter	Reference
Bacillus brevis	?	De Robertis & Franchi (1951)
Vibrio metchnikovii	270 ± 20 Å	van Iterson (1953)
		Follett & Gordon (1963)
		Glauert, Kerridge & Horne (1963)
Vibrio cholerae (*sic*)	300 Å	Das & Chatterjee (1966)
Vibrio eltor	270 ± 20 Å	Follett & Gordon (1963)
Vibrio sp.	?	Tweedy, Park & Hodgkiss (1968)
Bdellovibrio bacteriovorus	280–290 Å	Abram & Shilo (1967)
		Seidler & Starr (1968)
		Burger, Drews & Ladwig (1968)
Pseudomonas rhodos	177 Å	Lowy & Hanson (1965)
		Lowy (1965)
Proteus vulgaris	?	Lowy & Hanson (1965)
Spirillum spp.	?	Lockard & Boler (1968)

The value of the occurrence of sheathed flagella as a taxonomic criterion should be stressed. With the advent of improved negative contrast staining and sectioning techniques, and the more general use of electron microscopy, this is a feature of the fine structure of the bacterial cell which, like the occurrence of fimbria-like appendages,

will be of use in the characterization and identification of bacteria. Sheathing appears to be a stable feature of *Pseudomonas stizolobii* since one of the cultures used, NCPPB 450, was isolated in 1954 and had been maintained since then in various culture collections. In a survey of some Pseudomonas species for the presence of fimbria-like appendages, only the two strains of *P. stizolobii* examined in that survey possessed sheathed flagella. The remaining 20 strains representing 14 species all possessed flagella of a diameter and substructure which were normal for the naked flagella of bacteria (Fuerst & Hayward, 1969). However, a much more extensive survey for the presence of sheathed flagella in Pseudomonas is required before general conclusions can be made.

At present nothing is known about the functional significance of the sheath in motile bacteria. A comparison of the motility of Bdellovibrio, Vibrio and *Pseudomonas stizolobii* with the motility of polarly flagellate Gram-negative bacteria with naked flagella, or with sheathless mutants of normally sheathed bacteria, may provide information about function. The sheath may be 'protective' or be an integral part of the flagellum (Doetsch & Hageage, 1968).

'Tubules' and 'knobs' at the distal ends of sheathed flagella were frequently seen in this study. It is not clear whether these structures are an artifact resulting from autolytic effects or the effects of chemical treatment, or whether they occur naturally as a result of a lack of synchrony between synthesis of core and sheath. The 'tubules' were always irregularly folded structures. Follett & Gordon (1963) considered similar structures to be empty sheaths. Abram & Shilo (1967) and Seidler & Starr (1968) have also noted that sheath material often extends beyond the distal end of the flagellar filament. The evidence from the present study seems to confirm the general opinion that these 'tubules' represent sheath material.

It should again be emphasized that at this stage it is not possible to attribute any of the effects on the sheathed flagella seen after various treatments to particular reagents. Distal 'tubules' and delineation of core and sheath were sometimes seen in preparations which had not been treated with potassium iodide or any other reagent. The treatments were undertaken either because other workers had found similar procedures successful or because it seemed possible that they might enhance delineation of sheath and core. One might note that these qualifications may also apply to the results of treatments of sheathed flagella performed by Follett & Gordon (1963) and Seidler & Starr (1968).

Continuity of the outermost envelope of the cell wall with the sheath covering the flagellum has been determined in ultrathin sections of Bdellovibrio by Abram & Shilo (1967) and by Seidler & Starr (1968), and in *Vibrio metchnikovii* by Glauert, Kerridge & Horne (1963). Sections showing a continuity with the cell wall were not obtained in this study, although it was established that the thickness of the sheath was comparable to that of the outer membrane of the cell wall. There is no reason to doubt that more favourable sections would show a continuity between these layers.

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EXPLANATION OF PLATES

Abbreviations used in figures: T, distal 'tubule'; K, distal 'knob'; C, core; S, sheath.

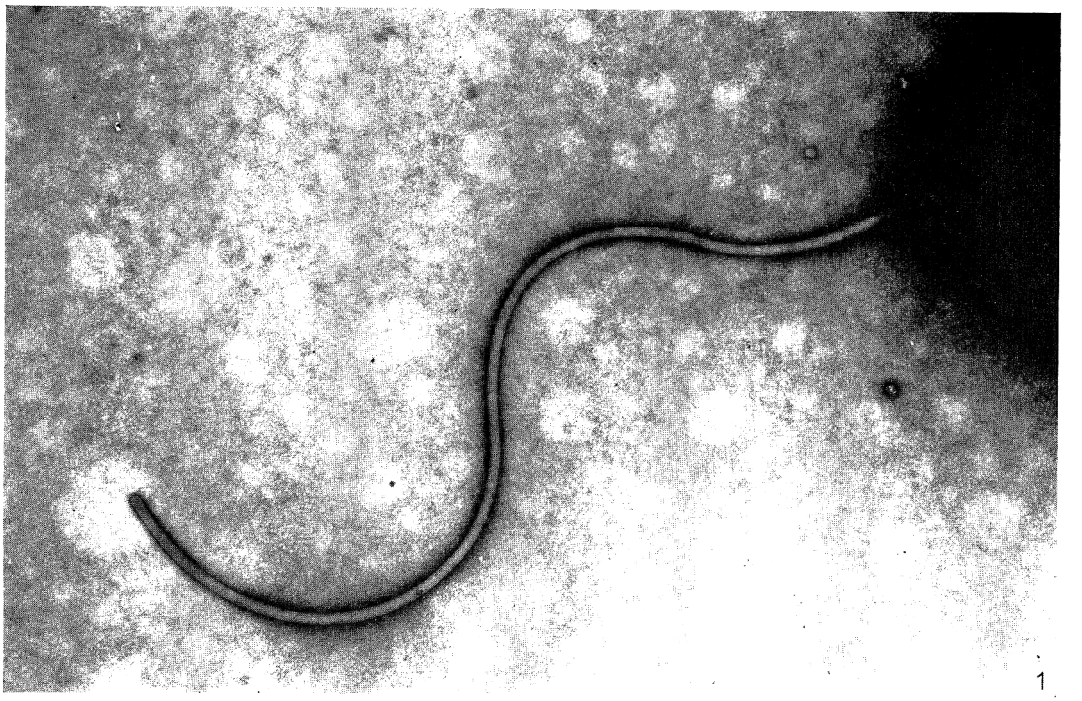
PLATE 1

Fig. 1. *Pseudomonas stizolobii* strain NCPPB 450 after two 72 hr serial subcultures in motility medium + 5 serial subcultures in peptone yeast extract broth incubated at 28°. A single polar flagellum without visible substructure arises from the cell. Negatively stained with sodium phosphotungstate, pH 7·0. × 56,000.

Fig. 2. *P. stizolobii* strain NCPPB 450 after two 72 hr serial subcultures in motility medium + 8 serial subcultures in peptone yeast extract broth incubated at 28°. Several cells with single polar flagella are visible. Negatively stained with sodium phosphotungstate, pH 7·3. × 20,000.

PLATE 2

Fig. 3. *P. stizolobii* strain 0268 A after two 72 hr serial subcultures in motility medium + 8 serial subcultures in peptone yeast extract broth incubated at 28°. An enlarged portion of a sheathed flagellum (note the rounded tip). Negatively stained with sodium phosphotungstate, pH 7·3. × 160,000.



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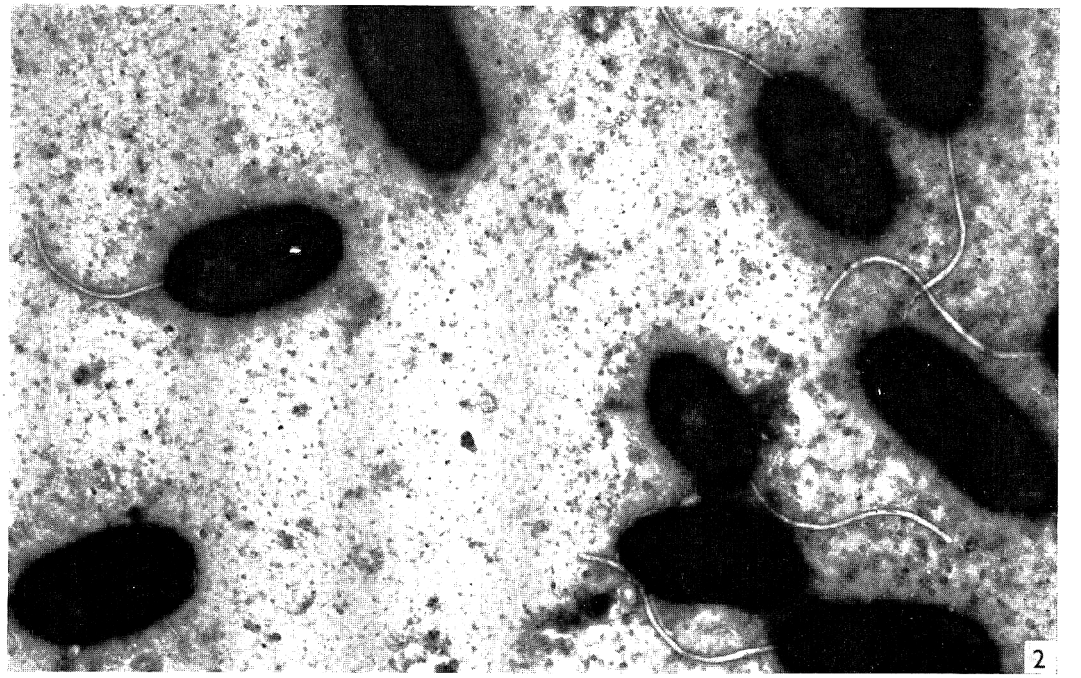

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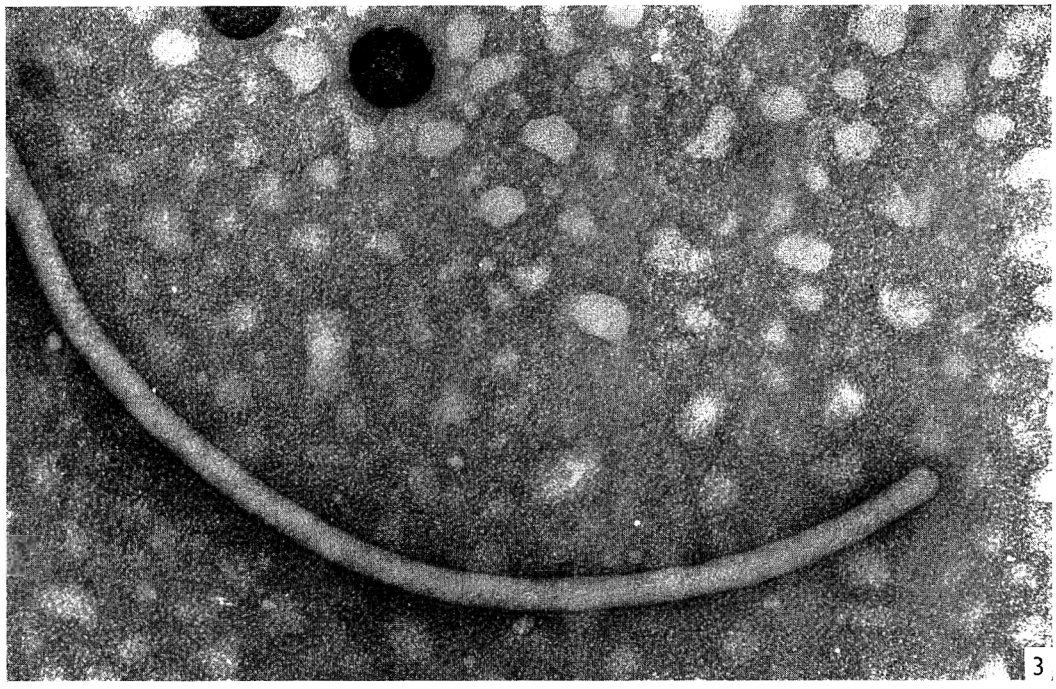
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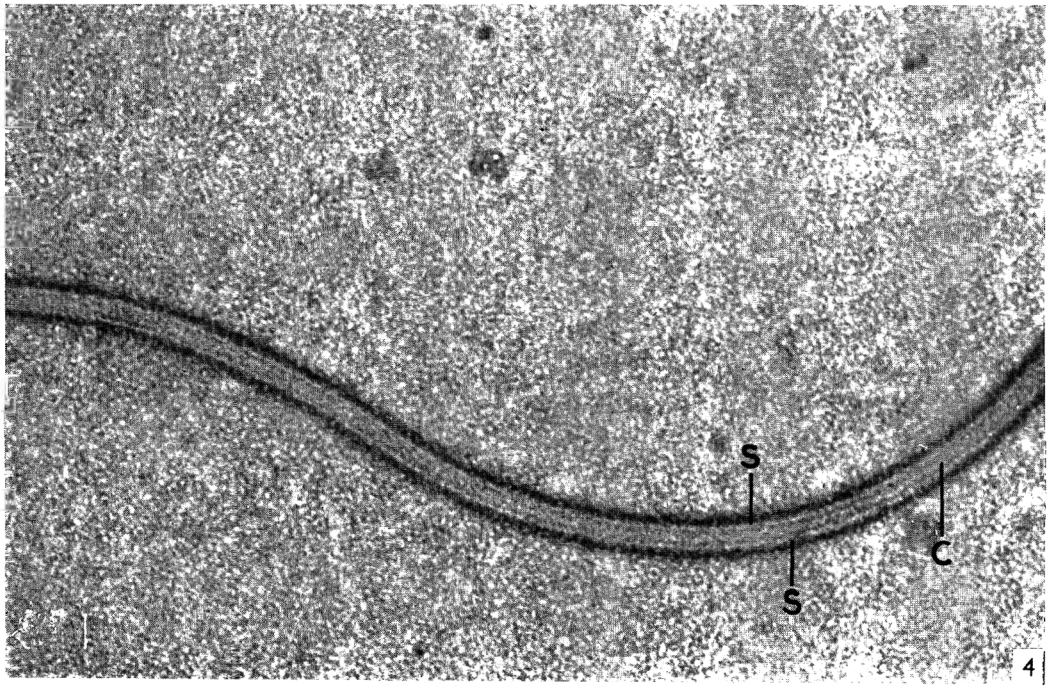
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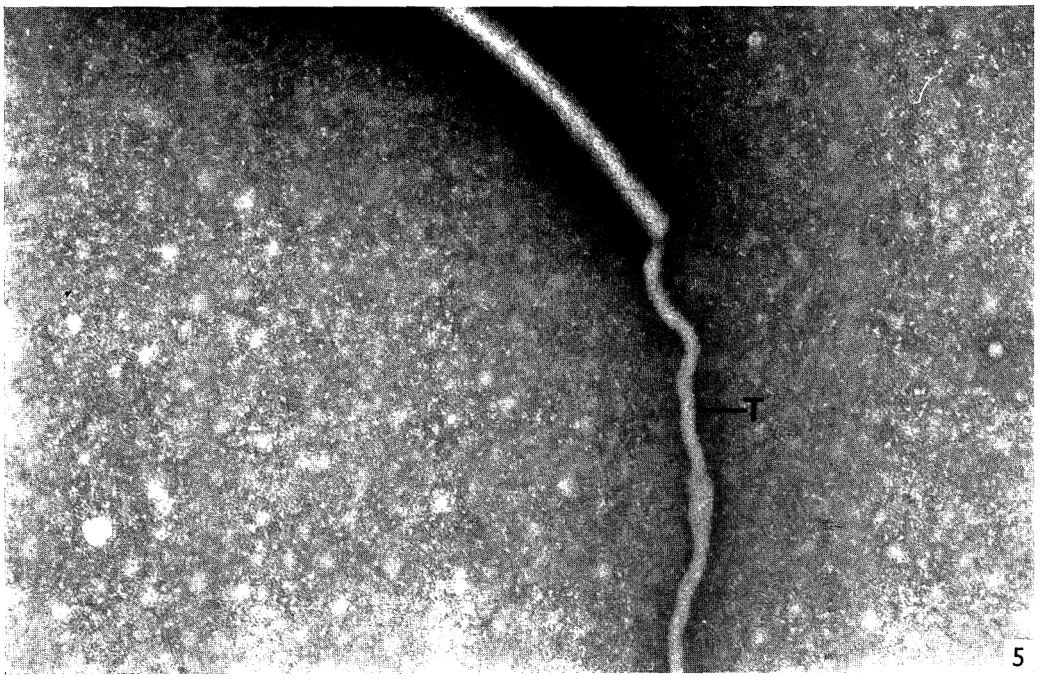
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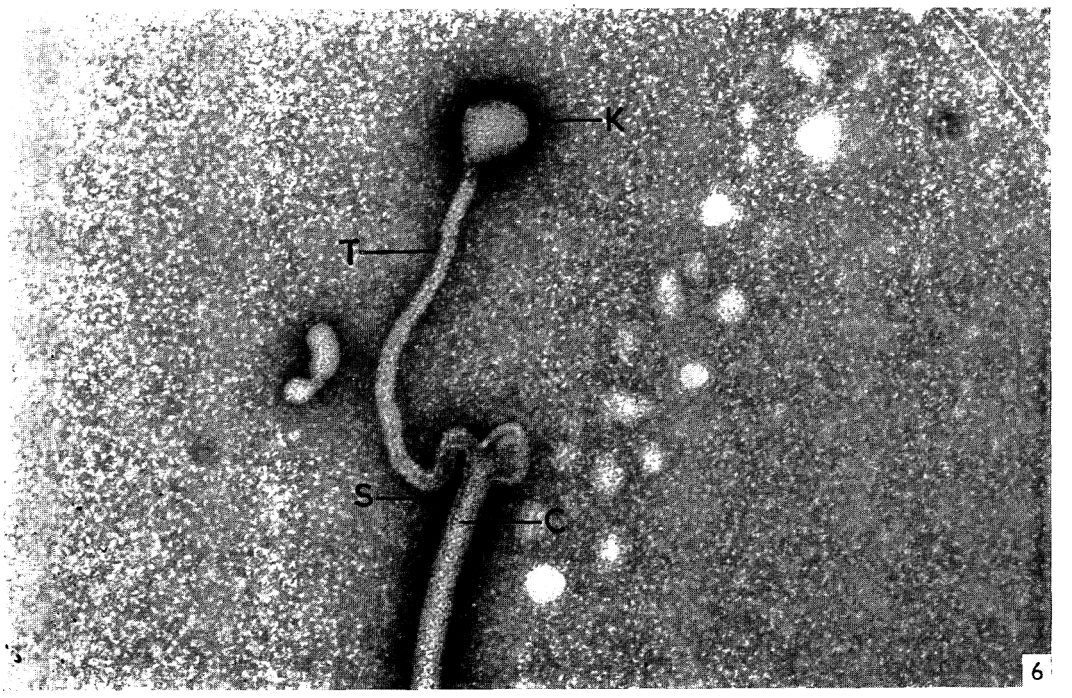
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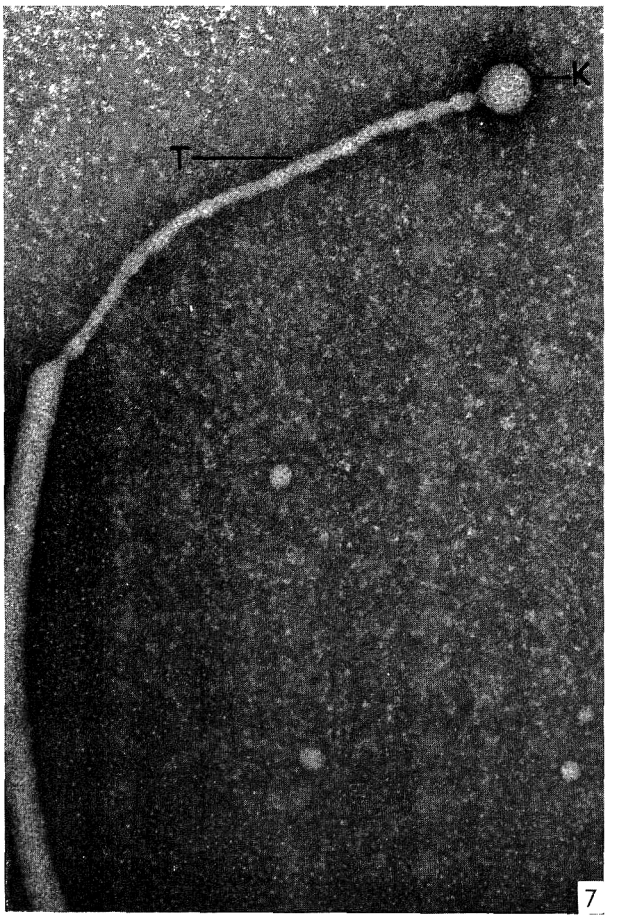
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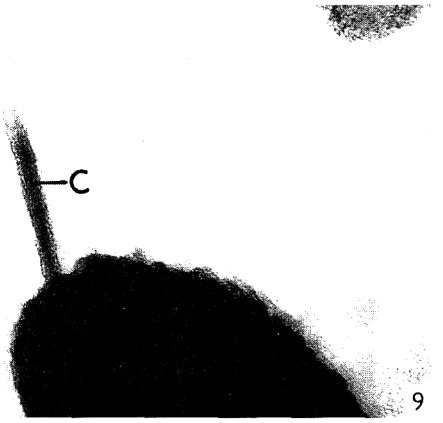
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Fig. 4. *P. stizolobii* strain NCPPB 450 after growth for 70 hr in peptone yeast extract broth incubated at 28° followed by treatment for 10 sec. with 1 % KI. + 10 sec. distilled water + 20 sec. N/100 HCl (pH 2·2) + 10 sec. distilled water. Delineation of core and sheath structure is visible. Negatively stained with sodium phosphotungstate, pH 7·4. × 130,000.

PLATE 3

Fig. 5. *P. stizolobii* strain NCPPB 450 after growth for 20 hr in glucose peptone yeast extract broth incubated at 28°, followed by treatment for 5 sec. with distilled water + 45 sec. 10^{-3} M-Na_2 EDTA + 5 sec. distilled water. An irregularly folded distal 'tubule' extends from an apparently intact flagellum. Negatively stained with sodium phosphotungstate, pH 7·4. × 160,000.

Fig. 6. *P. stizolobii* strain NCPPB 450 after growth for 20 hr in glucose peptone yeast extract broth incubated at 28°, followed by treatment for 5 sec. with distilled water + 12 sec. 0·05 % phenol + 5 sec. distilled water. Core and sheath delineation and distal 'tubule' and 'knob' are visible. Negatively stained with sodium phosphotungstate, pH 7·4. × 160,000.

PLATE 4

Fig. 7. *P. stizolobii* strain NCPPB 450 after growth for 20 hr in glucose peptone yeast extract broth incubated at 28°, followed by treatment for 5 sec. with distilled water + 15 sec. 6 M-urea + 5 sec. distilled water. A distal 'tubule' and 'knob' extend from an apparently intact flagellum. Negatively stained with sodium phosphotungstate, pH 7·4. × 160,000.

Fig. 8. *P. stizolobii* strain NCPPB 450 after growth for 26 hr in glucose peptone yeast extract broth incubated at 28°. Sheath and core structure is visible in the sectioned flagellum. Glutaraldehyde-osmium tetroxide fixation. × 120,000.

Fig. 9. *P. stizolobii* strain NCPPB 450 after growth for 26 hr in glucose peptone yeast extract broth incubated at 28°. Sheath and core structure is visible in the sectioned flagellum. Glutaraldehyde-osmium tetroxide fixation. × 120,000.

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Haematin-dependent Oxidative Phosphorylation in *Streptococcus faecalis*

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SUMMARY

Functional cytochrome(s) and an oxidative phosphorylation system were demonstrated in *Streptococcus faecalis* grown on media supplemented with haematin. The membrane fraction of cell-free extracts coupled the oxidation of reduced NAD to the formation of ATP from ADP and in organic phosphate. This reaction was sensitive to cyanide, azide and Antimycin A. A b_2 type cytochrome was detected in the membrane fraction with absorption maxima at 412, 425, 529 and 559 nm.

INTRODUCTION

Lactic acid bacteria normally lack haematin enzymes in their election transport system (Dolin, 1953) but are able to grow aerobically with oxygen as a terminal electron acceptor (Whittenbury, 1964). Only in one instance has it been claimed that oxygen utilization is coupled to the synthesis of ATP; Gallin & Vandemark (1964) showed oxidative phosphorylation with extracts of *Streptococcus faecalis*. As the organism lacked haematin enzymes Gallin & Vandemark postulated that phosphorylation might occur at the 'DPNH/flavin' level or, alternatively, during the oxidation of napthoquinone, shown to be present in *S. faecalis* by Baum & Dolin (1963). The possibility that some lactic acid bacteria might form functional cytochromes and have conventional oxidative phosphorylation arose from the observation of Whittenbury (1964) that many types of lactic acid bacteria synthesized catalase when provided with haematin and that a few strains also formed cytochromes of the *a* and *b* types. Of those strains possessing cytochromes, only *S. faecalis* seemed to benefit functionally in that colony size was markedly increased on media containing haematin, above colony size on media lacking haematin. Consequently an investigation has been made of *S. faecalis* growing on media supplemented with haematin. Evidence is presented for the presence of a cytochrome electron transport system in haematin-grown *S. faecalis*, resulting in oxidative phosphorylation coupled to the oxidation of reduced pyridine nucleotide.

METHODS

Organism. Streptococcus faecalis 581 (National Collection of Dairy Organisms).

Basal medium. Basal medium contained 0·5 % yeast extract, 0·5 % peptone and 0·5 % Lemco (all w/v) adjusted to pH 6·5, sterilized by autoclaving for 15 min. at 121°.

Haematin medium. Haematin (British Drug Houses Ltd., 0·1 g.) was dissolved in a minimum volume of triethanolamine, made up to 100 ml. with distilled water and sterilized by heating to 100° for 15 min. on two consecutive days. The haematin solution was stored at 4° and freshly prepared every two months; it was added to the basal medium to give a final concentration of 50 μg. haematin/ml.

Partially defined medium. The partially defined medium for *Streptococcus faecalis* described by Bauchop & Elsden (1960) was used for growth yield studies.

Washed suspensions. Organisms from liquid media or washed from slopes were centrifuged, washed twice in buffer and resuspended in buffer as required.

Cell-free extracts. Washed suspensions were treated ultrasonically (M.S.E. 100 W ultrasonic generator) in a tube surrounded by ice. The debris and unbroken cocci were removed by centrifugation at 23,000 ***g*** for 30 min.

Broken-cell suspensions were prepared as above but only centrifuged at 3000 ***g*** for 10 min., so removing any whole organisms but not the cell debris.

The protein content of extracts was measured by the Folin-Ciocalteu phenol reagent as described by Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as a standard.

Cell membrane preparation. Cell membranes were prepared by the method of Shockman, Kolb, Bakay, Conover & Toennies (1963), by using lysozyme, osmotic shock and centrifugation.

Manometry. Oxygen uptake was measured by conventional Warburg manometric techniques (Umbreit, Burris & Stauffer, 1951). The gas phase was air and each flask contained 1 to 5 mg. dry cell wt and 150 μmole potassium phosphate buffer (pH 6·5) in the main compartment; 0·2 ml. of 10 % KOH (w/v) with a filter paper wick in the centre well and 10 μmole of substrate in the side arm, total volume of liquid being made to 2·5 ml. with distilled water.

Enzyme assays. The oxidation of reduced pyridine nucleotides (NADH and NADPH) was followed spectrophotometrically on a Unicam S.P. 800 spectrophotometer at 340 nm. Cuvettes contained 13 μmole glycylglycine buffer (pH 7·2); 3 μmole potassium phosphate buffer (pH 7·2); 6 μmole $MgCl_2$; 3 μmole KF; 0·15 μmole NADH (or NADPH) and cell extracts equivalent to 0·5 mg. protein; total volume made to 2 ml. with distilled water.

The molar extinction coefficient of Horecker & Kornberg (1948) was used to convert extinction at 340 nm. to molar quantities of reduced pyridine nucleotide.

Adenosine triphosphate was measured by the method of Pinchot (1953). Cuvettes containing the reagents listed above plus 0·03 mg. bovine serum albumin; 10 μmole glucose; 0·3 μmole ADP or AMP; with or without 0·15 μmole NADP; 5 units hexokinase (British Drug Houses Ltd., ex-yeast) and 150 units glucose-6-phosphate dehydrogenase were used to detect and measure oxidative phosphorylation coupled to NADH oxidation. ATP was trapped to form glucose-6-phosphate by the enzyme hexokinase and the glucose-6-phosphate was oxidized by glucose-6-phosphate dehydrogenase with a simultaneous reduction of NADP. The rate of reduction of NADP was indicative of the rate of ATP formation. NADP reduction was measured as the difference in the rate of extinction decrease at 340 nm. between cuvettes containing NADP and cuvettes containing no NADP.

In most ATP assays a preparation of glucose-6-phosphate dehydrogenase extracted from *Leuconostoc mesenteroides* grown anaerobically on glucose was used. Ragland,

Kawasaki & Lowenstein (1966) showed that *L. mesenteroides* has a very high degree of glucose-6-phosphate dehydrogenase activity. A cell free extract of *L. mesenteroides* containing 5 to 10 mg. protein/ml. was treated with 0·1 vol. of 0·25 M-$MnCl_2$, shaken and allowed to stand at room temperature for 10 min. The precipitate was removed by centrifugation at 23,000 *g* for 10 min. The supernatant fluid was treated with saturated ammonium sulphate and the fraction precipitated between 45 and 60 % saturation was retained for its glucose-6-phosphate dehydrogenase activity. The precipitate was dissolved in 0·02 M-tris-HCl buffer (pH 7·2) and a sample containing 0·2 mg. protein used in each cuvette for the assay of ATP.

Lactate dehydrogenase was detected by observing the reduction of 2,3,5-triphenyltetrazolium chloride in the presence of lactate in Thunberg tubes. Each tube contained: 1 mmole potassium phosphate buffer (pH 7·5); 6 μmole NAD; 6 μmole 2,3,5-triphenyltetrazolium chloride; 1·3 μmole *N*-methyl phenazonium methosulphate; 100 μmole DL-sodium lactate and 5 to 10 mg. protein; total volume to 10 ml. with distilled water. Each tube was evacuated and refilled with nitrogen twice. The reaction was started by tipping in the extracts from the side arm.

Analysis of the products of glucose and lactate fermentation. The fermentation experiments were carried out in a conical flask on a shaking water bath at 30°. A stream of either air or oxygen-free nitrogen was passed through a carbon dioxide trap, the conical flask, a cooled U tube to trap any volatile products and finally through two Drechsel bottles, each containing 50 ml. of 0·05 M-$Ba(OH)_2$. Each flask contained 5 mmole potassium phosphate buffer (pH 6·5); 100 μmole of glucose or DL-sodium lactate; 20 mg. dry wt cocci; total volume to 100 ml. with distilled water.

Residual glucose was measured by the method of Fuller, Lampitt & Coton (1955) using the reduction of ferricyanide and titration with 0·01 M-ceric sulphate with xylene cyanol FF as an indicator.

Formic, acetic and lactic acids were separated chromatographically on a silica gel column with a benzene plus butanol gradient solvent (Lessard & McDonald, 1966). The effluent from the column was titrated against 0·01 M-NaOH using a Radiometer (Denmark) automatic titration and recorder assembly.

Carbon dioxide was absorbed in 0·05 M-$Ba(OH)_2$ in Drechsel bottles and measured by subsequent titration against 0·1 M-HCl.

Pyruvate was determined by the method of Friedemann & Haugen (1943), using benzene to extract the phenylhydrazone.

Acetoin was determined by the method of Langlykke & Peterson (1937).

Growth yield experiments. Aerobic growth yield measurements were made in shallow broth cultures in flasks incubated in a shaking incubator. The partially defined medium of Bauchop & Elsden (1960) was used and haematin and substrates were added as filter-sterilized solutions. Four flasks were used at each substrate concentration, three of these being used to follow growth and when growth had ceased the contents of the fourth flask were made up to the original volume with distilled water and the extinction of the culture measured with a Unicam S.P. 600 spectrophotometer at 540 nm., and the bacterial dry weight calculated by reference to a dry weight against extinction curve. All growth yields were measured at several substrate concentrations and the results plotted on a graph. Final growth yield results were then calculated from the gradient of the linear portion of the graph.

RESULTS

Growth yield experiments. Aerobic and anaerobic growth yields of *Streptococcus faecalis* were measured (Table 1) in the presence and absence of haematin. In the absence of haematin, anaerobic growth yields of 16 g. dry wt/mole glucose and 18 g. dry wt/mole fructose fermented were recorded. Assuming a theoretical yield of 2 moles ATP/mole hexose fermented by homolactic fermentation these figures approximate to a yield of 9 g. dry wt/mole ATP.

Aerobically, glucose and fructose each provided the equivalent of 3 moles ATP/mole hexose. If all the pyruvate, normally reduced to lactate, were oxidized to acetate and CO_2, a theoretical yield of 4 mole ATP/mole hexose oxidized would be possible. Chemical analysis of the products of glucose oxidation (Table 8) showed 38·5 % of the glucose was converted to lactate and 17 % to acetoin and, assuming the remainder was oxidized via acetyl phosphate to acetate, a yield of 2·9 moles ATP/mole glucose would be expected, which is very close to the experimental ATP yield recorded.

In the presence of haematin a yield of 6 moles ATP/mole hexose oxidized was recorded. This yield exceeded the theoretical maximum of 4 moles ATP/mole hexose and the extra energy was presumed to be produced by oxidative phosphorylation coupled to a haematin enzyme electron transport system. Haematin did not improve anaerobic growth yields. Protoporphyrin IX, the immediate precursor of haematin, did not stimulate in place of haematin. This agrees with Whittenbury (1961) who found only haematin stimulated haematin enzyme formation in lactic acid bacteria and could not be replaced by protoporphyrin IX or ferrous or ferric salts.

Assuming that energy was obtained from oxidative phosphorylation, a greater growth yield would be expected with lactate than with pyruvate, aerobically in the presence of haematin, because of the extra pair of electrons on lactate. This was found to be the case. No growth was obtained anaerobically with acetate, pyruvate or lactate.

London (1968) grew *Streptococcus faecium* with lactate as sole energy source. The growth yield with lactate for *S. faecalis* under aerobic conditions (Table 1) was slightly greater than the yield of *S. faecium* obtained by London. Aerobic growth on lactate in the absence of haematin is presumably at the expense of energy obtained from pyruvate breakdown following the oxidation of lactate to pyruvate. In the presence of haematin, growth yields of *S. faecalis* obtained aerobically were much greater than in the absence of haematin. The stimulatory effect of haematin again indicated the occurrence of oxidative phosphorylation.

Manometric measurement of oxygen uptake. Streptococcus faecalis grown on media with added haematin took up oxygen more rapidly than did *S. faecalis* grown without haematin (Table 2). The considerable oxygen uptake on pyruvate and lactate by haematin-grown cocci probably accounted for the extra oxygen used on glucose by these same cells.

Since *Streptococcus faecalis* grew readily with lactate as an energy source when incubated aerobically in the presence of haematin, the oxygen uptake of these organisms with lactate and glucose was tested. The results (Table 2) showed that whilst cocci grown with haematin and either glucose or lactate had the same rate of oxygen uptake on glucose, the lactate-grown cocci showed a faster rate of oxygen uptake on lactate than did the glucose-grown cocci, indicating that the lactate-oxidizing enzyme system may be inducible.

From the analysis of the products of glucose and lactate oxidation by *Streptococcus faecalis* (Table 8) it seemed that some of the substrate, particularly glucose, was fermented. Some of the pyruvate from lactate breakdown was not oxidized to acetate but fermented to acetoin. Therefore, the figures for O_2 used/mole of these substrates metabolized oxidatively are lower than if only pathways using oxygen as a terminal electron acceptor had been used. These results again indicate the active participation of haematin enzyme(s) in oxidation.

Table 1. *Molar growth yields obtained with Streptococcus faecalis*

For cultural details see test.

Additions to basal medium...	Incubation conditions: Anaerobic, None		Anaerobic, 50 μg. haematin/ml.		Aerobic, None		Aerobic, 50 μg. haematin/ml.		Aerobic, 50 μg. protoporphyrin IX/ml.	
	G/M*	Y^{**}_{ATP}	G/M	Y_{ATP}	G/M	Y_{ATP}	G/M	Y_{ATP}	G/M	Y_{ATP}
Substrate										
Glucose	16·0	1·8	16·1	1·8	26·8	3·0	55·2	6·1	31·9	3·5
Fructose	18·0	2·0	n.t.		26·2	2·9	55·2	6·1	35·9	4·0
Acetate	0·0	0·0	0·0	0·0	0·0	0·0	2·0	0·2	n.t.	
Pyruvate	0·0	0·0	0·0	0·0	5·0	0·6	5·0	0·6	n.t.	
Lactate	0·0	0·0	0·0	0·0	2·0	0·2	15·6	1·8	n.t.	

* G/M = dry weight cocci in g./mole of substrate.
** Y_{ATP} = mole ATP/mole of substrate, calculated from the G/M figure assuming an average of 9·0 g. dry weight cocci/mole ATP.
n.t. = not tested.

Table 2. *Manometric measurements of oxygen uptake by resting suspensions of Streptococcus faecalis with several substrates*

Growth conditions of cocci...	μl. O_2 uptake/mg. dry wt cocci/hr: Aerobic with glucose	Anaerobic with glucose	Aerobic with glucose + haematin	Aerobic with lactate + haematin
Substrate				
None	0·16	0·15	0·35	0·30
Glucose	44·7	28·6	56·6	58·2
	(1·1)	(0·7)*	(1·4)	(1·4)
Acetate	0·15	n.t.	0·43	n.t.
Pyruvate	0·42	n.t.	0·92	n.t.
Lactate	0·35	n.t.	2·90	12·4

n.t. = not tested.
* Figures in parentheses with glucose as a substrate represent the mole oxygen consumed/mole substrate.

Oxidation of reduced pyridine nucleotides. The figures in Table 3 show that most of the NADH-oxidizing enzymes in cocci grown in the presence of haematin were associated with the cell membranes. Extracts of the haematin-grown cocci had a higher level of NADH oxidases than did extracts of cocci grown without haematin. Also the NADH-oxidizing activity of the extracts of haematin-grown cocci was sensitive to

cyanide. A crudely prepared membrane fraction, containing cell membranes, cell debris and soluble proteins, but with whole cocci removed by centrifugation, was the most convenient fraction for the study of the oxidation of reduced pyridine nucleotides.

Table 3. *NADH and NADPH oxidation by extracts of Streptococcus faecalis grown aerobically with or without haematin*

Preparation	Additions to the growth medium	mμmole reduced pyridine nucleotide oxidized/min./mg. protein: NADPH	NADH	NADH with 10^{-2} M-KCN	NADH with 10^{-2} M-NaN_3	NADH with $1{\cdot}6 \times 10^{-4}$ Antimycin A
Cell-free extract	—	1·15	5·21	4·00	10·00	n.t.
Cell-free extract	50 μg. haematin/ml.	0·76	17·6	3·01	14·56	n.t.
Membrane preparation	50 μg. haematin/ml.	n.t.	505·0	28·8	516·0	468·0

n.t. = not tested.

Table 4. *Control experiments prior to ATP assay during NADH oxidation by extracts of Streptococcus faecalis*

The complete reaction system for the assay of ATP contained 13 μmole glycylglycine buffer, pH 7·2; 3 μmole potassium phosphate buffer, pH 7·2; 6 μmole $MgCl_2$; 3 μmole KF; 0·15 μmole NADH; cocci extract, 0·5 mg. protein; 0·03 mg. bovine serum albumin; with or without 0·15 μmole NADP; 0·3 μmole ADP or AMP; 5 units hexokinase and 150 units glucose-6-phosphate dehydrogenase, total vol. to 2 ml. with distilled water.

System being tested	Added to basic buffer system (concentrations as in the complete ATP assay system shown above)	μmole NAD or NADP reduced/min./mg. protein
Reduction of NADP by *S. faecalis* extract	NADP, extract, glucose	0·0
	NADP, extract, glucose, ADP	6·4
	NADP, extract, glucose, ADP, hexokinase	16·1
Reduction of NAD by *S. faecalis* extract	NAD, extract, glucose	0·0
	NAD, extract, glucose, ADP	0·0
	NAD, extract, glucose, ATP	0·0
	NAD, extract, glucose, ATP, G-6-P dehydrogenase, hexokinase	0·0
	NAD, extract, glucose, ATP, G-6-P dehydrogenase, hexokinase, NADP (−ve control)	41·6
Enzymic ATP assay system controls (with NAD)	NADP, glucose, ADP	0·0
	NADP, glucose, ADP, extract	6·0
Enzymic ATP assay system controls (with NADP)	NAD, extract, ADP, glucose	0·0
	NAD, extract, ATP, glucose	0·0
	NADP, extract, ATP (−ve control), glucose	40·0

Oxidative phosphorylation during oxidation of reduced pyridine nucleotides. Before attempting to detect ATP during NADH oxidation by extracts of *Streptococcus faecalis*, control experiments were made on all components of the reaction mixture (Table 4). Reduction of NADP by the *S. faecalis* extract in the presence of glucose and ADP showed that a myokinase activity generating ATP was present. When excess

ADP was present in the ATP assay system there was a steady ATP production due to myokinase activity for which a correction was made when ATP was formed during NADH oxidation. The ATP assay system did not reduce NAD in the presence of ATP with or without the *S. faecalis* extract in the system.

The G-6-P dehydrogenase preparation derived from *Leuconostoc mesenteroides* extracts gave similar results to commercial (British Drug Houses Ltd.) G-6-P dehydrogenase except for a slight myokinase activity in the former for which a correction was made along with the *S. faecalis* myokinase activity.

Table 5. *Comparison of oxidative phosphorylation in extracts of Streptococcus faecalis grown under various conditions*

Complete reaction system as outlined for Table 4.

	Additions to the basal medium	Reaction system	mμmole/min./mg. protein: NADH oxidized	mμmole/min./mg. protein: NADP reduced	P:O
Broken cell preparations of cocci grown aerobically	Glucose + haematin	Complete	215·0	73·0	0·34
		Complete	189·2	64·4	0·34
		Complete	202·0	64·7	0·32
	Glucose	Complete	90·0	0·0	0·0
		Complete	95·0	0·0	0·0
	Lactate + haematin	Complete	53·2	15·0	0·28
		AMP in place of ADP	48·5	9·7	0·20
		AMP in place of ADP	48·8	7·2	0·15
Broken cell preparations of cocci grown anaerobically	Glucose + haematin	Complete	54·5	0·0	0·0
	Glucose	Complete	57·8	0·0	0·0

Extracts of *Streptococcus faecalis* grown on media containing haematin produced ATP during oxidation of NADH whilst cocci grown in media without haematin did not produce ATP during NADH oxidation (Table 5). A P:O ratio of about 0·3 was found, which decreased to 0·15 to 0·20 when AMP replaced ADP in the reaction mixture. The lack of oxidative phosphorylation by extracts of cocci grown without haematin contrasts with the results obtained by Gallin & Vandemark (1964) who showed ATP production during NADH oxidation by extracts of *S. faecalis* grown without haematin. Extracts prepared from *S. faecalis* grown anaerobically in the presence or absence of haematin showed no detectable phosphorylation coupled to NADH oxidation.

Effects of inhibitors of oxidative phosphorylation on Streptococcus faecalis extracts. Sodium azide, potassium cyanide and Antimycin A were used. With 1×10^{-2} M-cyanide there was a 59 % inhibition of NADH oxidation by extracts of haematin-grown cells and a 74 % decrease in the P:O ratio; with 3×10^{-2} M-cyanide phosphorylation was completely inhibited while NADH oxidation was decreased to 6 % of the original rate (Table 6). With 1×10^{-2} M-NaN_3 there was only an 8 % decrease in NADH oxidation and no decrease in the P:O ratio. Sodium azide at $1{\cdot}5 \times 10^{-2}$ M resulted in an 18 % inhibition of NADH oxidation but a slightly increased P:O ratio. These

results contrast with those obtained by Gallin & Vandemark (1964) who detected no inhibition of oxidative phosphorylation in their *S. faecalis* extracts in presence of cyanide or azide. They used lower concentrations (4×10^{-4} M-KCN, 1×10^{-4} M-NaN_3) which might account for the difference. It would seem that Gallin & Vandemark were measuring a different type of oxidative phosphorylation from the one being assayed in the present work.

Table 6. *Effects of various inhibitors of oxidative phosphorylation on extracts of Streptococcus faecalis grown aerobically on glucose plus haematin*

ATP assay system as described for Table 4.

Extract	Inhibitor	Concentration (molar)	mμmole/min./mg. protein: NADH oxidized	mμmole/min./mg. protein: NADP reduced	P:O
S. faecalis broken-cell preparation	None	—	215·0	73·0	0·34
	KCN	1×10^{-2}	88·2	7·9	0·09
	KCN	3×10^{-2}	12·8	0·0	0·0
	NaN_3	1×10^{-2}	197·0	58·2	0·30
	NaN_3	$1{\cdot}5 \times 10^{-2}$	176·0	74·8	0·42
S. faecalis membrane preparation	None	—	505·0	221·0	0·42
	Antimycin A	$1{\cdot}6 \times 10^{-4}$	468·0	0·0	0·0

Antimycin A ($1{\cdot}6 \times 10^{-4}$ M) completely inhibited oxidative phosphorylation by our *Streptococcus faecalis* extracts although 90% of the NADH-oxidizing activity remained. Gallin & Vandemark (1964) obtained a decreased P:O ratio with 8×10^{-4} M Antimycin A. Some observers (Smith 1954; Breummer, Wilson, Glenn & Crane, 1957; Lightbown & Jackson, 1956) have noted a lack of sensitivity to Antimycin A amongst bacterial oxidative phosphorylation systems. However our *S. faecalis* system was sensitive to this antibiotic and perhaps it is significant here that a cell-free system was used.

The effect of adding haematin to extracts of Streptococcus faecalis grown in the absence of haematin. An experiment was made to test whether there was any preformed enzyme in *S. faecalis* grown without haematin, which, on addition of haematin to an extract, would form the haematin-enzyme NADH oxidase system. Several workers have noted a preformed apoenzyme of catalase in organisms unable to synthesize haematin, the catalase being activated when haematin was added to the cocci (Whittenbury, 1965) or to a cell-free extract (Beljanski & Beljanski, 1957). A broken-cell preparation of *S. faecalis* grown aerobically with glucose was incubated with haematin for 30 min. at 30° and then tested for oxidative phosphorylation during NADH oxidation. None was detected. This experiment served as a control for other tests reported here in that it showed that the activities claimed for haematin-enzyme systems were not attributable to the haematin alone.

Division of oxidative phosphorylation between different fractions of Streptococcus faecalis extracts. Activities of soluble and particulate fractions of *Streptococcus faecalis* extracts were compared to determine in which fractions the haematin enzymes occurred. *S. faecalis* grown aerobically with lactate + haematin were broken ultra-

sonically and whole cocci and debris removed by centrifugation at 3000 *g* for 10 min. Of the NADH oxidizing activity, 88 % was in the supernatant fluid which contained soluble enzymes and cell membranes. This finding agrees with results obtained with cell membranes prepared by treatment of cocci with lysozyme (Table 3).

The soluble fraction + membrane fraction was centrifuged at 23,000 *g* for 30 min. to precipitate the membranes which were resuspended in 0·05 M-potassium phosphate buffer (pH 6·5). The myokinase activity noted earlier was mainly in the soluble fraction so that when ATP production by membrane fractions was studied possible error due to this enzyme was decreased.

Table 7. *Division of NADH oxidizing enzymes between soluble and membrane fractions of Streptococcus faecalis extracts*

Cocci grown on basal medium with	Fraction	mμmole/min./mg. protein: NADH oxidized	mμmole/min./mg. protein: NADP reduced	P:O
Glucose + haematin	Soluble fraction	294·0	77·0	0·26
	Membrane fraction	650·0	195·0	0·30
	Soluble fraction* + membrane fraction	432·0	138·3	0·32
	Soluble fraction (spun at 110,000 *g* for 30 min.)	89·5	14·3	0·16
Lactate + haematin	Soluble fraction	191·0	52·1	0·27
	Membrane fraction	109·0	46·5	0·43
	Soluble fraction* + membrane fraction	201·0	90·5	0·45

* In these determinations the soluble fraction and the membrane fraction were mixed in a ratio of 1·0:0·8 of wt of protein.

Table 7 shows the results of experiments to determine the site of oxidative phosphorylation within the cell. The membrane fraction of cocci grown with glucose + haematin had a higher NADH oxidase activity than did the soluble fraction although the P:O ratio was only slightly greater in the former fraction. Ultracentrifugation (110,000 *g* for 30 min.) further decreased the rate of NADH oxidation by the soluble fraction and decreased the P:O ratio to half the value found for the membrane fraction. The decreased P:O ratio in the soluble fraction suggested that there was an NADH oxidizing system in this fraction not coupled to oxidative phosphorylation. Cocci grown with lactate + haematin gave similar P:O ratios. This preparation was not ultracentrifuged.

Reduction of cytochrome pigments in the extracts. With cyanide (1×10^{-2} M-KCN) and an excess of NADH, peaks of reduced cytochrome were detected in membrane fractions of *Streptococcus faecalis* grown aerobically with lactate or glucose in the presence of haematin. A typical spectrum is shown in Fig. 1, the peaks of which are similar to a '*b*' type cytochrome. There was also an oxidized peak at 412 nm., typical of a cytochrome *b*.

No water-soluble cytochrome was detected and attempts to isolate cytochrome oxidase by using sodium cholate extraction techniques (Yonetani, 1960) were unsuccessful. When haematin (15 μg./ml.) was added to a broken-cell preparation of

cocci grown aerobically with glucose no cytochrome peaks were observed. Haematin itself gave a weak peak at 425 nm. but this was not increased or otherwise altered by cyanide, NADH or *Streptococcus faecalis* enzymes, indicating that the cytochrome peaks were not simply due to haematin from the growth medium.

Oxygen was shown to be necessary for the formation of the cytochromes: extracts of cocci grown anaerobically in the presence of haematin gave no cytochrome peaks.

Oxidation of lactate coupled to ATP production. In an experiment in Thunberg tubes, 2,3,5-triphenyltetrazolium chloride (TTC) was reduced in the presence of lactate only by extracts of haematin-grown cocci; there was no reduction in the absence of lactate.

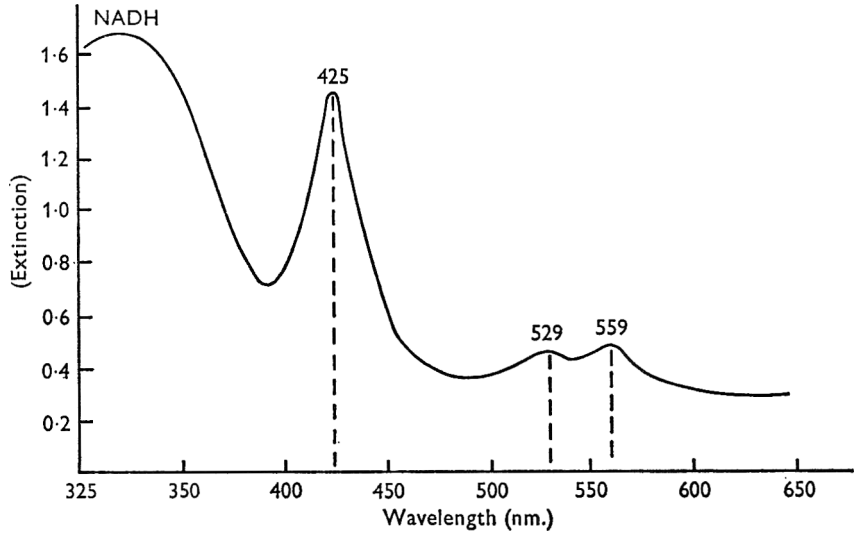

Fig. 1. Difference spectrum of a broken-cell suspension of *Streptococcus faecalis* grown aerobically on glucose+haematin. The sample cuvette contained the broken-cell suspension reduced with excess NADH in the presence of KCN (10^{-2} M) and ADP (1 mM). The reference cuvette contained a broken-cell suspension alone. The spectrum was plotted using an SP 800 recording spectrophotometer at room temperature (21°) and with a light path of 10 mm.

Difficulty was encountered in demonstrating reduction of NAD in the presence of lactate because of the very active NADH oxidases present in the extracts. However, with lactate (5 μmole/ml.) the rate of NADH oxidation by an extract of *Streptococcus faecalis*, prepared from cocci grown aerobically with lactate+haematin was decreased. The rate of NADP reduction was measured in cuvettes containing the ATP assay system, ATP, *S. faecalis* extract (lactate+haematin grown), AMP, NAD and with or without lactate. Without lactate the rate of NADP reduction was 6·5 mμ mole/min. and with lactate present this rate increased to 7·7 mμ mole/min. NADP was not reduced without the ATP assay system. These differences in rate of NADP reduction and NADH oxidation when lactate was present or absent indicate that reduction of NAD to NADH was coupled to lactate oxidation and that the resulting NADH was subsequently oxidized with the formation of ATP from oxidative phosphorylation. The oxidation of lactate in this fashion appeared to require ATP to initiate the reaction, although the final result was a net gain of ATP.

Products of glucose and lactate breakdown. Washed suspensions of *Streptococcus faecalis* grown aerobically without haematin converted 94 % of glucose to lactate under anaerobic conditions, but aerobically only 37·5 % of glucose was converted to lactate (Table 8). The remainder of the pyruvate, which would otherwise have been reduced to lactate, was either oxidized to acetate and CO_2 or fermented to acetoin and CO_2. Cocci grown aerobically on glucose + haematin converted only 14 % of the glucose into lactate. Cocci grown aerobically on lactate plus haematin converted 13 % of glucose to lactate and also oxidized lactate to produce CO_2, acetate and acetoin.

Table 8. *Products of glucose and lactate oxidation by washed Streptococcus faecalis*

All cocci were grown aerobically.

Growth substrate	Incubation conditions in experiment*				
	Anaerobic with glucose	Aerobic with glucose	Aerobic with glucose + haematin	Aerobic with lactate + haematin	Aerobic with lactate + haematin
Oxidation substrates					
Glucose	100	100	100	100	—
Lactate	—	—	—	—	100
Oxidation products					
Lactate	187	77	29	25	—
Acetate	0	64	61	108	38
Formate	0	0	8	4	0
Pyruvate	3	1	3	4	9
CO_2	6	130	171	153	91
Acetoin	—	26	43	10	23
C recovery (%)	96	99	95	101	95

* 100 mole of each substrate were added and the concentrations of the products are expressed as mole/100 mole of substrate.

DISCUSSION

The evidence presented points conclusively to the development of a pathway in *Streptococcus faecalis* grown aerobically with haematin in which the oxidation of NADH is catalysed by haematin enzyme(s) and is coupled to oxidative phosphorylation. Gallin & Vandemark (1964) showed oxidative phosphorylation in *S. faecalis* strain 10C1 and suggested that potential sites for phosphorylation were at the NADH/flavin level and at the point of oxidation of reduced naphthoquinone. Their experiments were concerned with a phosphorylation which was insensitive to cyanide and to azide. The present work, however, has revealed no oxidative phosphorylation of this type in *S. faecalis* grown without haematin.

The P:O ratios observed in the present work were relatively low, the highest value being 0·44, in comparison with the work of Pinchot (1953) who reported a P:O ratio of 0·78 in *Alcaligenes faecalis* and of Hartmann, Brodie & Gray (1957) who reported a P:O ratio approaching 1 in *Azotobacter vinelandii* extracts. The value of the P:O ratio for *Streptococcus faecalis*, and the results of experiments when the soluble and membrane fractions were examined separately and together, suggested that there was only one site of phosphorylation coupled to NADH oxidation in *S. faecalis* extracts. It is known that the preparation of extracts affects the activity of the sites of oxidative

phosphorylation. Racker (1965) noted that the preparation of submitochondrial particles by ultrasonic treatment and mechanical fragmentation decreased the efficiency of phosphorylation sites, and especially of the site coupled to the oxidation of cytochrome *c* and to a lesser extent of the site coupled to the oxidation of NADH by flavoproteins. The third site, coupled to the oxidation of cytochrome *b* was relatively unaffected. Antimycin A inhibits at the level of this last mentioned site (Racker, 1965) and this could well be the site in operation in *S. faecalis* extracts.

Growth yield measurements in experiments with *Streptococcus faecalis* on glucose where oxygen uptake was measured manometrically (with Hg. as a manometer fluid replacing Brodie solution) yielded a figure of 0·16 g. dry wt/g. oxygen used, a low figure in comparison to the 0·853 g. dry wt/g. oxygen used found by Hernandez & Johnson (1967) with *Pseudomonas fluorescens*. The lower growth yield with *S. faecalis* again points to only one phosphorylation site; Hernandez & Johnson suggested that the pseudomonad probably has up to three oxidative phosphorylation sites.

Assuming only one oxidative phosphorylation site it is possible to account for the production of 1 mole ATP/mole NADH oxidized. Ideally, 4 mole ATP are obtainable from glycolysis and pyruvate oxidation. If the 2 mole NADH produced/mole glucose oxidized and the 2 mole of NADH produced/mole pyruvate oxidized are oxidized via the haematin enzymes, a potential yield of 8 mole ATP/mole glucose oxidized can be expected. Experimentally a yield of 6·5 mole ATP/mole glucose oxidized was obtained. This less than maximum yield was partly accounted for by the presence of lactate and acetoin amongst the products of glucose oxidation, showing some competition from fermentative pathways. In addition some NADH is probably oxidized by the *Streptococcus faecalis* flavoprotein/NADH oxidases (Dolin, 1955) as shown by the residual rate of NADH oxidation in the presence of cyanide. This oxidation would also partly account for the low P:O ratio.

The spectrum of the reduced cytochrome pigment of the *Streptococcus faecalis* extract was very similar to cytochrome b_2 of yeast as reported in the yeast lactate dehydrogenase system by Appleby & Morton (1959). A strain of *S. faecalis* (H.69.D5) has been shown to form cytochromes *b*, *a* and a_2 (Whittenbury, 1964). No *a*-type cytochrome was detected in the present study, either in a difference spectrum or by a cholate extraction technique (Yonetani, 1960). The terminal oxidase was cyanide sensitive as with cyanide the cytochrome b_2 was reduced by NADH but not oxidized.

These results raise interesting evolutionary and taxonomic questions. The appearance of residual, either degenerate or undeveloped, aerobic pathways suggests that the dividing line between, say, streptococci and staphylococci may be less than at first appears to be the case.

Even though the presence of haematin stimulates *Streptococcus faecalis* to behave like an aerobe, it still performs inefficiently in that a functional Krebs cycle is lacking and final products of glucose oxidation include lactate, acetate and acetoin. Perhaps apoenzymes of missing enzymes may be present in some strains of *S. faecalis* and in future studies a 'complete' aerobe may be resurrected with the aid of preformed prosthetic groups. On present evidence it would seem that *S. faecalis*, and possibly the other lactic acid bacteria, are metabolic 'cripples' capable of being useful experimental models in the study of the evolution and the mechanism of aerobic metabolism.

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Interactions between Some Aural Aspergillus Species and Bacteria

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SUMMARY

Some interactions of Aspergillus species with *Staphylococcus aureus* and *Pseudomonas aeruginosa*, all of which are concerned in otitis externa, are described. The pigments pyocyanine, 1-hydroxyphenazine and the fluorescent green pigments produced by *P. aeruginosa* inhibited the germination of spores of *Aspergillus terreus*; the most toxic of these pigments was 1-hydroxyphenazine. *P. aeruginosa* did not produce fluorescent pigments in Czapek-Dox medium unless the ferrous sulphate was omitted and asparagine added. Following incubation of the Aspergillus species in this medium, however, *P. aeruginosa* grew and produced fluorescent pigment therein. *S. aureus* produced antifungal toxic material which was capable of diffusing through cellophan and causing distortion of *A. terreus* hyphae.

INTRODUCTION

Yeasts and filamentous fungi, mainly Aspergillus species, have often been recorded as the cause of otitis externa and fungal infections are often associated with bacterial infection especially after mastoidectomy or fenestration operations. Attempts to elucidate the relationship of bacteria and fungi in mixed infections have been unsuccessful (Haley, 1950*a*, *b*; Singer *et al.* 1952; Leshin, 1953; Stuart & Blank, 1955; Powell, English & Duncan, 1962). Whether a fungus can be the primary cause of otitis externa remains undecided. Smyth (1962) and others have suggested that the secretions of the apocrine and sebaceous glands protect the healthy ear from invading organisms and that fungal infection occurs only after these glands have been damaged by bacteria or some other agency or when the host resistance is otherwise diminished; but English (1963) found that both *Aspergillus fumigatus* and *A. terreus* grew on the cerumen produced by these glands, as well as on keratin. English & Stanley (1966) made preliminary observations on the interactions of four Aspergillus species with four common aural bacteria. The present paper reports an examination of some of these interactions.

METHODS

Organisms and general methods. Cultures of *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from cases of otitis externa. The strain of *S. aureus* could not be typed but gave positive

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results in egg yolk and lipase tests. The fungi were maintained on glucose peptone agar and the bacteria on nutrient agar.

All incubations were at 37°. The media used were nutrient broth, nutrient agar, glucose peptone broth, glucose peptone agar (4 % glucose, 1 % peptone), Czapek-Dox liquid and Czapek-Dox agar (Thom & Church, 1926). Filtrates of fungal cultures were obtained by means of a membrane filter of pore size 0·45 μ.

Interaction between bacteria and fungi. An aqueous suspension of the spores (3×10^6 spores/ml.) of one of the species of Aspergillus was prepared from 3 to 5 day agar cultures; 0·1 ml. samples of suspension were added to each of eight 50 ml. conical flasks containing 10 ml. nutrient broth. These were incubated in a shaken water bath. Flasks 1 to 7 were further inoculated, respectively, at 0, 4, 8, 12, 16, 20 and 24 hr after the addition of the fungus spores, with 0·1 ml. of a 1/100 dilution in nutrient broth of a 16 hr nutrient broth culture of the bacterium to be tested. After incubation for 48 hr, fungal inhibition was estimated macroscopically and microscopically. To determine the viability of fungus, drops of the mixed culture were placed on Czapek-Dox agar, a medium which allowed growth of Aspergillus species even in the presence of the bacteria.

Antifungal toxic material of Staphylococcus aureus. Two layers of agar medium separated by sterile cellophan were poured into 9 cm. Petri dishes. The lower layer contained 5 ml. nutrient agar and 0·1 ml. bacterial inoculum; the upper layer contained 5 ml. glucose peptone agar and 0·1 ml. of *Aspergillus terreus* inoculum. The inocula of both organisms were prepared as described above. A bacterial culture filtrate was obtained by means of a membrane filter of pore size 0·45 μ. The bacterial filtrate was tested for fungal toxicity by adding 10 ml. of a 24 hr nutrient broth culture filtrate of *S. aureus* to 10 ml. of nutrient agar seeded with 0·1 ml. of fungal spore suspension. In the control cultures 10 ml. of nutrient broth replaced the bacterial filtrate.

Preparation of bacterial pigment solutions. Solutions of pyocyanine, 1-hydroxyphenazine (both chloroform soluble) and the fluorescent green pigments (water soluble) were prepared from *Pseudomonas aeruginosa* cultures by modifications of the methods of Schoental (1941), Young (1947) and Liu, Abé & Bates (1961). The pigments were extracted from cultures incubated in 10 ml. amounts of nutrient broth + 1 % glycerol for 7 days. Pyocyanine was extracted from 50 ml. of broth culture with successive 10 ml. amount of chloroform; this pigment was then transferred to dilute HCl in which it was stored until required. After neutralization, re-extraction into chloroform and evaporation, the pigment was used in a phosphate buffer solution at pH 7·3.

1-Hydroxyphenazine was present in such small quantities in the cultures that it was prepared from the stock acidic pyocyanine solution (Rodd, 1959), extracted in chloroform, evaporated and dissolved in phosphate buffer solution (pH 7·3). Fluorescent green pigments were obtained by dialysis and concentration of cultures from which the above two pigments and the bacteria had already been removed. Two ml. of each of the pyocyanine and fluorescent solutions thus prepared was equivalent to the amount of these pigments contained in 10 ml. of culture, while 2 ml. of 1-hydroxyphenazine solution was the quantity obtained by converting the pyocyanine present in 10 ml. of culture.

Toxicity of the pigments of Pseudomas aeruginosa to fungi. Two ml. bacterial filtrate

were added to 10 ml. molten nutrient agar containing 0·5 ml. fungal inoculum prepared as before. Microscopic examination for spore germination was made hourly from 8 to 14 hr in lactophenol cotton blue mounts. Spores were considered to have germinated when the germ tube length was equal to the diameter of the spore. Percentage germination was based on counts of 400 spores. Control counts were made on nutrient agar plates without added bacterial filtrate.

RESULTS

Pseudomonas aeruginosa inhibited the growth of both *Aspergillus terreus* and *A. fumigatus* in broth cultures when added within 20 hr but was never fungicidal. In contrast, when *Staphylococcus aureus* was grown in mixed culture with *A. fumigatus* or *A. terreus* the bacterium inhibited growth of the fungi when added within 16 hr; it both decreased

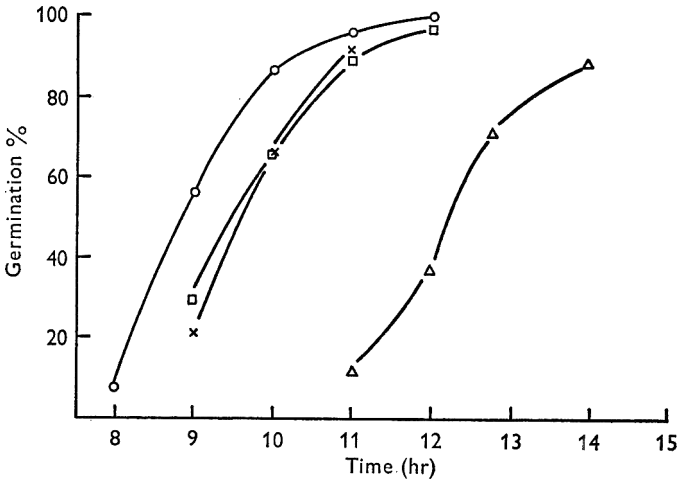

Fig. 1. *Aspergillus terreus* spore germination at 37° in nutrient agar in the presence of *Pseudomonas aeruginosa* pigment solutions. ○, control; □, pyocyanine; △, 1-hydroxyphenazine; ×, fluorescent pigments. A χ square test was performed between the control and both the pycocyanine and fluorescent pigments after 10 hr, and between the control and 1-hydroxyphenazine at 11 hr; in all cases a highly significant effect was detected ($P < 0{\cdot}001$).

their growth rate and induced repeated dichotomy of the hyphal tips, the latter effect being more pronounced with *A. terreus* than with *A. fumigatus*. The growth of *A. fumigatus* and *A. terreus* was compared in pure culture and in the presence of *S. aureus* added after 8 hr growth of the fungus in: (*a*) nutrient broth; (*b*) nutrient broth + 1 % glucose or glycerol; (*c*) glucose peptone broth. The toxic effect of *S. aureus* was greater in media (*b*) and (*c*) than in (*a*). In pure culture no abnormalities of the fungi were observed in any of these media. In the presence of *S. aureus*, *A. fumigatus* exhibited repeated apical dichotomy of the hyphae while *A. terreus* showed also subapical swellings in glucose or glycerol containing media (Pl. 1 *a*, *b*, *c*, *d*).

1-Hydroxyphenazine was much more toxic to spore germination of *Aspergillus terreus* than either of the other two substances (Fig. 1). Abnormal hyphal development was not observed in the presence of any of the three pigments. Spore germination of *A. fumigatus*, *A. flavus* and *A. niger* was also inhibited by 1-hydroxyphenazine.

In mixed cultures *Pseudomonas aeruginosa* grew and produced its fluorescent green pigments on Czapek-Dox agar only when colonies of the Aspergillus species were present. The fungi were only slightly inhibited by *P. aeruginosa* on this medium. *P. aeruginosa* also grew and produced these pigments in filtrates of 24 hr Czapek-Dox liquid cultures of *Aspergillus terreus* and *A. fumigatus*, suggesting that in this medium the fungi caused a change necessary for bacterial growth and pigment production. No pH change took place in the 24 hr broth culture. The addition to Czapek-Dox liquid medium of glucose, glycerol or asparagine (1 %), peptone or casamino acids (0·3 %), nicotinic acid, pyrodoxin, thiamine or riboflavin (10 μg./l.), did not induce pigment production, although glucose permitted good bacterial growth without pigment production. Investigation showed that ferrous sulphate was inhibitory at the normal concentration of 10 mg./l and also at 1 mg/l. and that a medium without added ferrous sulphate still did not support pigment production unless 0·025 % asparagine was also added; then strong pigmentation resulted. Replacement of the asparagine with 1 % peptone allowed only weak pigment production. This suggested that the fungi decreased the iron content of Czapek-Dox agar to a value suitable for bacterial pigment formation and also supplied asparagine or some related substance required by *P. aeruginosa*. However, paper chromatography of the fungal filtrate did not show asparagine, aspartic acid or any other amino acid.

Layered plates of *Aspergillus terreus* and *Staphylococcus aureus* showed that *S. aureus* produced material capable of diffusing through agar and cellophan and causing the same hyphal distortion as in growth of mixed cultures. The addition of a bacterial culture filtrate to nutrient agar seeded with the *A. terreus* resulted in decreased fungal growth rate but no hyphal abnormalities. Investigation of the properties of this filterable antifungal material showed that: (i) incubation for 24 hr was optimum for production of the toxic material; (ii) the amount of toxic material produced by *S. aureus* in nutrient broth was not increased by adding 1 % glucose or glycerol; (iii) the toxic material withstood autoclaving at 116° for 10 min., was adsorbed on activated charcoal by boiling, was not extracted by chloroform or ether and was not precipitated by ethanol or acetone from aqueous solution. Since a concentrated solution of the toxic material was not prepared it was impossible to ascertain whether it was identical with that bacterial product which diffused through cellophan and induced hyphal tip branching in mixed fungal and bacterial culture.

DISCUSSION

Stokes, Pick & Woodword (1942) found that 1-hydroxyphenazine was more active against dermatophytes and *Candida albicans* than pyocyanine while the reverse was true for bacteria. My results show that *Aspergillus terreus* behaved like dermatophytes. Very little is known or the identity or quantity of substances available within the human ear for growth of micro-organisms. If conditions in the ear resemble those in nutrient agar, the toxic material of *Pseudomonas aeruginosa* might be expected to prevent fungal infection, but Singer *et al.* (1952) found *P. aeruginosa* in 50 % of ears infected with Aspergillus species. On the other hand, the interaction might resemble more closely that observed on Czapek-Dox agar where one organism benefited from the presence of the other on the particular substrate. The production of toxins by *Shigella dysenteriae* (Dubos & Geiger, 1946) and *Corynebacterium diphtheriae* (Pappenheimer,

1947) is related to the concentration of iron in the medium and perhaps the inhibitory effect of iron observed in the present work was a complex reaction such as that seen with *C. diphtheriae*.

The filterable antifungal toxic material produced by *Staphylococcus aureus* is not identical with any of the well-known toxins such as the haemolysins, dermonecrotoxins, hyaluronidase and leucocidins produced by this organism (Elek, 1959), since it withstands autoclaving. The addition of glucose to nutrient broth did not increase the amount of filterable antifungal toxic material by *S. aureus* but increased the sensitivity of the fungus to the bacterium in mixed culture. The production of dichotomously branched hyphal tips in the presence of *S. aureus* is similar to the response which may occur in otomycosis and in pulmonary aspergillosis. It is suggested that the hyphal swellings seen in *Aspergillus terreus* are severe expressions of the swelling noted by Robertson (1958) to occur before division of the hyphal tip following any check in growth which lasts 60 seconds or more. Only a small proportion of the *A. terreus* hyphal tips produced these swellings. *A. fumigatus*, which is a faster growing organism than *A. terreus*, showed less sensitivity to *S. aureus* both in nutrient broth and in the glucose or glycerol containing media.

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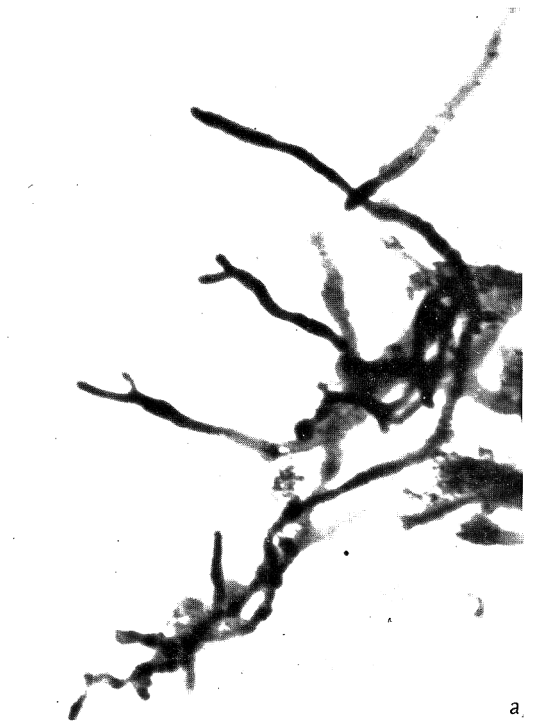
EXPLANATION OF PLATE

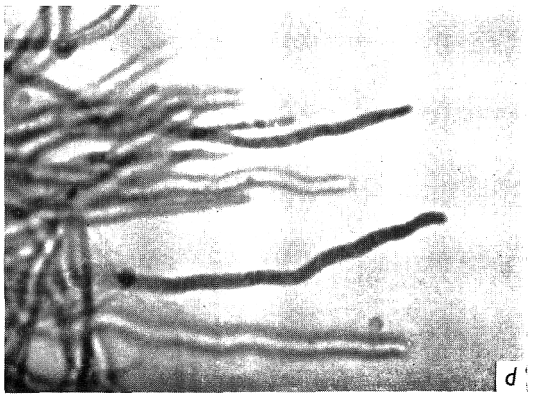
(*a*) *Aspergillus terreus* 20 hr culture in nutrient broth with *Staphylococcus aureus* added after 8 hr. (*b*) *A. terreus* + *S. aureus* as (*a*) in glucose peptone broth. (*c*) *A. fumigatus* + *S. aureus* as (*a*) in glucose peptone broth. (*d*) *A. terreus* 20 hr culture in nutrient broth. Magnification × 800.



a

b

c



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Recombination in *H1*, the Gene Determining the Flagellar Antigen-*i* of *Salmonella typhimurium*; Mapping of *H1* and *fla* Mutations

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SUMMARY

Five *fla* mutations of *Salmonella typhimurium* LT2 have been mapped by transduction. They were isolated from five motile (*fla*$^+$) strains each with a different form of antigen-*i*, caused by mutation of *H1*, the structural gene for phase-1 flagellin. *H1* was cotransduced (frequency 0·1 to 0·5) with each of the *fla* mutations. Three-point crosses using a serological selection technique indicate the following order: *fla*-50—*fla*-58—*fla*-55-*H1*. The position of *fla*-52 was not discovered. If the *fla* complementation groups correspond to the *fla* genes their order is *flaB*—*flaD*—*flaA*—*H1*.

Of the five *H1* mutations four (M5, M20, M11, M12) appear to be very closely linked. The fifth (M6) is outside this cluster. Transductants with functionally and antigenically normal antigen-*i* were obtained, at a very low frequency, in crosses between *i*-curly and *fla* strains: they are attributed to crossing over within *H1* between the 'curly' mutation and the mutations causing alteration in serological character. Some earlier conclusions (Joys & Stocker, 1963) as to gene order, especially within *H1*, now seem unjustified.

INTRODUCTION

The numerous phase-1 antigens of the Salmonella group are determined by different forms of a gene termed *H1* (Lederberg & Edwards, 1953). We have described several altered forms of antigen-*i*, the wild-type phase-1 antigen of *Salmonella typhimurium* strain LT2, obtained by selection of mutants, termed M, able to spread faster than the parent strain through semisolid medium containing enough anti-*i* serum to retard its spreading growth (Joys & Stocker, 1966). Their alterations in serological character presumably result from local changes, probably single amino acid substitutions, in some antigenically active part of the flagellar protein (flagellin); indeed, alterations in the position of a peptide have been detected in the peptide 'maps' of digests of four of the mutant *i* flagellins (McDonough, 1962). It seemed probable that each mutant resulted from alteration of the *H1* gene at only a single point. We here report an attempt to map these mutations in some of the serological mutants.

Non-flagellate mutants, resulting from mutations in *fla* genes closely linked to *H1*, and therefore co-transducible with it, were obtained from each serological mutant,

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so that the *fla* mutations might serve as external markers in crosses between *H1* mutants; for the present experiments five such *fla* mutants, each obtained from a different serological mutant, were chosen from amongst 11 whose isolation and complementation behaviour we have described (Joys & Stocker, 1965). In transductional crosses of some pairs of serological mutants we readily showed recombination within the *H1* gene leading to the production of the ancestral (wild type) gene and antigen. We could not draw any firm inferences about the order of mutations within *H1* and the present more complete investigation does not confirm some earlier tentative conclusions (Joys & Stocker, 1963). We have been able, however, to determine the order of several *fla* genes relative to each other and to *H1*.

Iino (1962) described a strain of *Salmonella typhimurium* which when in phase-1 produced functionally deficient flagella with an abnormally short wavelength ('curly' phenotype). He inferred that the deficiency was due to a mutation which in his crosses could not be separated from *H1;* it now seems probable that this mutation is within the structural gene for phase-1 flagellin. We crossed this mutant with *fla* derivatives of serological mutants with the object of defining more precisely the position of the 'curly' mutation.

METHODS

Bacterial strains. Five mutants with altered forms of antigen *i* and one stable *fla* derivative of each were used: M5 *fla*$^+$ and *fla-50;* M6 *fla*$^+$ and *fla-52;* M10 *fla*$^+$ and *fla-57;* M11 *fla*$^+$ and *fla-55;* M12 *fla*$^+$ and *fla-58* (Joys & Stocker, 1965, 1966). A spontaneous mutant resistant to high concentrations of streptomycin was selected in each *fla* line. Genetically labelled sublines of the *fla*$^+$ strains, for use as donors in transduction experiments and as absorbing antigens in the preparation of specific antisera, were obtained by the isolation of (non-lysogenic) *pur*$^+$ and *pur*$^+$ *pro*$^+$ forms by successive transduction with phage P22.H4 (Yura, 1956). The phase-1-curly strain used was *Salmonella typhimurium* SW 577 (Iino, 1962). All the above strains make flagellins containing *N*-methyllysine (Stocker, McDonough & Ambler, 1961). *S. typhimurium* strain SD7, used in some experiments as *fla*$^+$ donor with a phase-1 antigen unrelated to *i*, is a (P22-sensitive) LT2 transductant in which the *S. typhimurium* wild-type *H1* gene has been replaced by the *H1* gene of *S. abony*, determining antigen-*b* (Spicer & Datta, 1959). The flagellins of this strain lack *N*-methyllysine, because of co-transduction of the *nml* gene of the donor with its *H1* gene (Stocker, McDonough & Ambler, 1961).

Serological methods. Sera specific for each of the mutant forms of antigen-*i*, for use in selection in semisolid medium, were prepared by absorption of antimutant sera with *pur*$^+$ *pro*$^+$ (streptomycin-sensitive) bacteria having the wild-type antigen-*i* (Joys & Stocker, 1966) and sterilized by Seitz filtration. Sera specific for the wild-type form of antigen-*i* were prepared by the absorption of anti-*i* (wild type) serum with mutant forms of the antigen (Joys & Stocker, 1966). The anti-*1,2* serum used, active on the phase-2 antigens of all the strains, was obtained by inoculation of a rabbit with a *Salmonella typhimurium* strain which is non-flagellate when in phase-1 owing to an *ah1* mutation (Iino, 1961); it was absorbed with an *i* (wild type) suspension to remove anti-O and possible anti-*i* activity. The concentration of an absorbed serum used in semisolid medium was that which just sufficed to prevent spreading of *fla*$^+$ transductants with the homologous antigen, and was about double that needed

for agglutination in a micro test (Joys & Stocker, 1966). Tests on the flagellar antigens of clones recovered from spreading swarms confirmed the efficacy and specificity of the serum selections.

Recombinants with wild-type antigen-*i* were sought by selection in semisolid medium containing both serum specific for the mutant *i* antigen of the recipient parent and serum specific for that of the donor; the efficacy of this selective medium was tested in each series of experiments by inoculation of a plate with about 20 motile bacteria producing the wild-type antigen together with about 10^9 *fla* bacteria.

Transduction methods. Phage P22 grown on a *fla*$^+$ or *fla* donor strain was added to a 1 ml. volume of a 37° overnight shaken broth culture of a *fla* recipient strain (about 10^9 bacteria) to give a phage: bacterium ratio of about 10:1. After 20 min. at 37°, ten-fold dilutions to 10^{-4} were made and 'standard drop' inocula (0·02 ml.) of each dilution, of the undiluted mixture, and of the resuspended deposit (0·1 ml.) from the centrifuged transduction mixture, were placed on four sets of 5 cm. plates of semisolid medium. Appropriate control plates tested the bacterial sterility of sera and phage. Streptomycin (1 mg./ml.), added to the semisolid medium as a precaution against airborne contamination, would have prevented the growth of any viable donor bacteria present in the lysate, or of any viable bacteria of the absorbing strain present in the sera, since these strains were streptomycin-sensitive. One set of plates contained no serum and a second set only anti-*1,2* serum. The approximately equal yields of swarms on these two sets confirmed that the majority of cells in the recipient *fla* culture were, as intended, in latent phase 1, so that *fla*$^+$ transductants expressed their *H1* genes. The highest dilution giving swarms in these two sets gave an estimate of the total number of *fla*$^+$ transductants, most of which would retain the *H1* gene of the recipient since the rate of co-transduction of *fla* and *H1* was $< 0{\cdot}5$ for the *fla* mutants used. A third set contained anti-*1,2* serum and serum specific for the mutant antigen of the recipient; the yield of swarms on these plates showed the number of *fla*$^+$ transductants with the phase-1 antigen of the donor, or with wild-type (recombinant) antigen. A fourth set contained anti-*1,2* serum and two anti-*i* sera, one specific for the mutant antigen of the donor and the other specific for the mutant antigen of the recipient; swarms in these plates indicated the production of *fla*$^+$ transductants with the wild-type antigen-*i*. The absence of *fla*$^+$ revertants was tested in each experiment by inoculation of semisolid medium with the recipient strain treated with broth instead of phage. In an additional control experiment each *fla* recipient was treated with phage grown on its *fla*$^+$ parent, so that all *fla*$^+$ transductants would have the same mutant antigen. The absence of swarms on plates containing serum specific for this antigen confirmed the efficacy of the serum and showed that the population of *fla*$^+$ transductants did not give second-step serological mutants (in particular revertants) able to spread in its presence.

After 48 h of incubation at 37°, the highest dilution yielding spreading growth (i.e. confluent swarms) was recorded for each set of plates and subcultured for determination of flagellar antigens by slide agglutination. Growth from plates selective for the recombinant antigen was tested for lack of agglutination by sera specific for the donor and recipient antigens and for agglutination by anti-*i* (wild type) sera absorbed with the donor antigen or with the recipient antigen. Several cultures which satisfied these criteria were used to absorb an anti-*i* (wild type) serum, and the fully absorbed serum was tested on wild-type *i* agglutinable suspension. A rabbit serum was prepared

against one such recombinant, absorbed with wild-type *i* antigen and tested for agglutinating activity against the inoculated strain. All suspected recombinants were tested for the nutritional characters of the recipient *pur pro* strains.

In experiments in which the donor strain was SW 577, the *Salmonella typhimurium* phase-1-'curly' mutant, only three sets of plates were used, one without antiserum, one with only anti-*1,2* serum and the third with anti-*1,2* serum and serum specific for the mutant antigen-*i* of the *fla* recipient.

RESULTS

Order of fla mutations and H1

Each *fla* strain, derived from a *fla*$^+$ parent with an altered phase-1 antigen, was treated with phage P 22 lysates of the *fla*$^+$ parent and the *fla* derivative of each of the other four mutants with altered antigen-*i*, and the numbers of *fla*$^+$ transductants with the recipient, donor and wild-type (recombinant) forms of antigen-*i* were determined. Table 1 records the results of one set of experiments. Nearly all the crosses were repeated at least once, with essentially similar results. No *fla*$^+$ revertants were detected on the control plates.

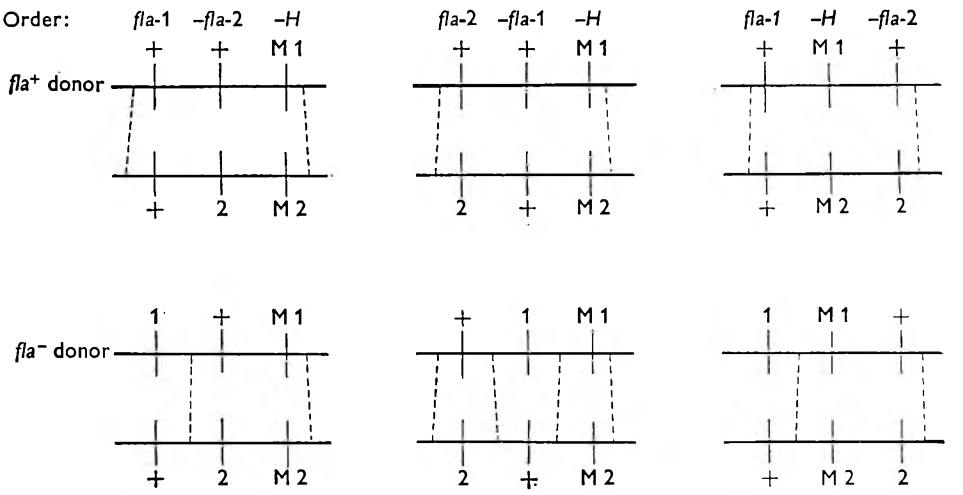

Fig. 1. Mapping of *fla* mutations by transduction. The figure shows the three possible orders of two *fla* mutations with respect to a mutation, M, in *H1*. The upper line in each diagram represents the donor fragment. The broken lines represent the crossovers required to yield a motile M 1 recombinant in each case. We expect that depending on the order of the markers a *fla* donor may yield far fewer motile M 1 recombinants than its *fla*$^+$ analogue.

Consider first the inferences which may be drawn about the order of the *fla* mutations relative to each other and to *H1*. All crosses, except those involving M 6, which is discussed below, yielded very few or no wild-type *i* swarms. Our *H1* mutations must therefore be very close together and may be treated in the genetic analysis as changes at a single site. In this way the *fla* mutations can be ordered with respect to *H1*.

A *fla*$^+$ donor having a mutant *H1* gene (say M 1) will yield motile transductants in a cross against a *fla* recipient. When the recipient has a different *H1* mutation (say

M 2) the transductants will be either M 1 or M 2. The proportion inheriting M 1 will depend on this distance between *H1* and the *fla* mutation (*fla*-2 in Fig. 1). This is between 0·1 and 0·5 for the mutants concerned. A *fla* mutation in the donor (*fla*-1 in Fig. 1) may affect this proportion. Should the order be *fla*-1—*fla*-2—*H1*, a motile M 1 recombinant needs two crossovers and the proportion of this type should be same as in the *M1* → *fla*-2 M 2 cross, although the absolute number of motile transductants may be decreased. If the order is *fla*-1—*H1*—*fla*-2 the proportion of *fla*$^+$ M 1 recombinants may be decreased but by no more than a half (the maximum co-transduction rate of *H1* and *fla* is 0·5). Such a decrease would probably not be detected by the method of quantitation which we used. If, however, the order is *fla*-2—*fla*-1—*H1*, then four crossovers are needed to produce the *fla*$^+$ M 1 recombinant. Their proportion amongst the *fla*$^+$ transductants should therefore be much less when the donor is *fla*-1 than when it is *fla*$^+$.

The pairwise combinations of the *H1* mutants tested (four, since M6 and its *fla* derivative are excluded) provide twelve crosses, in each of which the effect of a *fla* mutation in the donor was tested (Table 1). In crosses 2, 4, 17 and 18 (indicated A in Table 1) the proportion of *fla*$^+$ transductants with the donor *H1* gene was decreased at least a hundredfold when the donor was *fla*. We infer that in these crosses the donor *fla* is between *H1* and the recipient *fla*. The orders thus indicated are: *fla-50*—*fla-57*—*H1*; *fla-50*—*fla-58*—*H1*; *fla-58*—*fla-57*—*H1*; and *fla-58*—*fla-55*—*H1*. Taken together these give: *fla-50*—*fla-58*—(*fla-57*, *fla-55*)—*H1*. In crosses 3 and 10 (indicated B in Table 1), each of which was repeated at least twice, the estimated proportion of donor-type swarms was consistently decreased when the donor was *fla*, but only by a factor of ten. If this decrease results from the need for four crossovers the orders indicated are: *fla-57*—*fla-55*—*H1* and *fla-50*—*fla-55*—*H1*, both compatible with the order already inferred and combining with it to give: *fla-50*—*fla-58*—*fla-57*—*fla-55*—*H1*. In the other six crosses (indicated C in Table 1), which are the reverse crosses of the six so far considered, the *fla* mutation of the donor had no detactable effect on the proportion of donor-type swarms. This was the result expected on the inferred order, since in each of these six crosses the *fla* mutation of the recipient lies between *H1* and that of the donor. Most possible crosses involving M6 *fla-52* were made. The presence of a *fla* mutation in the donor did not consistently cause a great decrease of the proportion of donor-type swarms in any of these crosses; we are therefore unable to draw any firm inferences about the position of *fla-52*.

Recombination in the H1 gene between serological mutants

All the crosses involving M6 (as donor) or M6 *fla-52* (as recipient or as donor) yielded spreading growth on the plates which selected for *fla*$^+$ transductants with the recombinant (wild type) antigen, and in most of these crosses swarms of this sort were abundant. Few crosses not involving M6 yielded swarms on such plates, but a very low proportion were observed in crosses of M 5 with M 10, of M 10 with M 11 and of M 5 with M 11. The serological identity of the phase-1 antigen of these transductants with the parental wild-type antigen was confirmed by the tests described under 'Methods'; and all these transductants had the nutritional characters of their recipient parent. As no swarms were obtained on the control plates which tested for reversion of serological character, we conclude that the *H1* gene of these transductants is wild type, produced by recombination between the parental *H1* genes. Attempts to infer

Table 1. *Crosses between serological and non-flagellate mutants*

Cross no.	Recipient	Donor	Highest dilution* with swarms with antigen *i* of type: Recipient	Donor	Wild type	Inference†
1	M5 *fla-50*	M6 *fla*$^+$	10^{-4}	10^{-3}	10^{-4}	
		fla-52	10^{-3}	10^{-1}	10^{-3}	
2	M5 *fla-50*	M10 *fla*$^+$	10^{-3}	10^{-2}	*neg.*	
		fla-57	10^{-3}	1	*neg.*	A
3	M5 *fla-50*	M10 *fla*$^+$	10^{-3}	10^{-2}	*neg.*	
		fla-55	10^{-3}	10^{-1}	*neg.*	B
4	M5 *fla-50*	M12 *fla*$^+$	10^{-3}	10^{-2}	*neg.*	
		fla-58	10^{-2}	*sed.*	*neg.*	A
5	M6 *fla-52*	M5 *fla*$^+$	10^{-4}	10^{-4}	10^{-4}	
		fla-50	10^{-4}	10^{-4}	10^{-4}	
6	M6 *fla-52*	M10 *fla*$^+$	10^{-4}	10^{-3}	10^{-3}	
		fla-57	10^{-2}	10^{-2}	10^{-2}	
7	M6 *fla-52*	M11 *fla*$^+$	10^{-3}	10^{-2}	10^{-2}	
		fla-55	10^{-2}	10^{-1}	10^{-2}	
8	M10 *fla-57*	M5 *fla*$^+$	10^{-4}	10^{-3}	*sed.*	
		fla-50	10^{-4}	10^{-3}	*neg.*	C
9	M10 *fla-57*	M6 *fla*$^+$	10^{-3}	10^{-2}	10^{-2}	
		fla-52	10^{-2}	10^{-1}	10^{-2}	
10	M10 *fla-57*	M11 *fla*$^+$	10^{-4}	10^{-3}	10^{-1}	
		fla-55	10^{-4}	10^{-2}	1	B
11	M10 *fla-57*	M12 *fla*$^+$	10^{-4}	10^{-3}	*neg.*	
		fla-58	10^{-4}	10^{-3}	*neg.*	C
12	M11 *fla-55*	M5 *fla*$^+$	10^{-3}	10^{-3}	*sed.*	
		fla-50	10^{-3}	10^{-3}	*neg.*	C
13	M11 *fla-55*	M6 *fla*$^+$	10^{-4}	10^{-4}	1	
		fla-52	*sed.*	*sed.*	*sed.*	
14	M11 *fla-55*	M10 *fla*$^+$	10^{-3}	10^{-3}	1	
		fla-57	10^{-3}	10^{-3}	*neg.*	C
15	M11 *fla-55*	M12 *fla*$^+$	10^{-3}	10^{-3}	*neg.*	
		fla-58	10^{-2}	10^{-2}	*neg.*	C
16	M12 *fla-58*	M5 *fla*$^+$	10^{-3}	10^{-2}	*neg.*	
		fla-50	10^{-3}	10^{-2}	*neg.*	C
17	M12 *fla-58*	M10 *fla*$^+$	10^{-4}	10^{-3}	*neg.*	
		fla-57	10^{-3}	*neg.*	*neg.*	A
18	M12 *fla-58*	M11 *fla*$^+$	10^{-4}	10^{-3}	*neg.*	
		fla-55	10^{-3}	*neg.*	*neg.*	A

* Plates of semisolid medium containing appropriate sera (see Methods) were inoculated with standard-drop volumes of tenfold dilutions of transduction mixture, i.e. *fla* recipient and P22 grown on donor (phage: bacterium ratio 10:1). Entries record most dilute inocula giving swarms with indicated form of antigen *i*. (1 = undiluted mixture; *sed.* = mixture concentrated by centrifugation; *neg.* = no swarms of indicated type, even from inoculum of concentrate.)

† *Inferences.* A. *fla* of donor must lie between *H1* and *fla* of recipient, because its presence causes hundredfold or greater reduction in proportion of *fla*$^+$ transductants with donor *H1* gene. B. *fla* of donor probably central, since it causes about a tenfold decrease in donor-type transductants. C. No indication that *fla* of donor is central, since it causes no consistent decrease in yield in donor-type transductants.

the order of mutations within *H1* were unsuccessful, since the argument that recombinants with wild-type antigen probably arose by only two crossovers led to mutually contradictory map orders.

Recombination between a curly mutant and the serological mutants

The five crosses in which the donor carried an *i*-curly mutation and the recipient was a *fla* derivative of a serological mutant all gave similar results (not included in Table 1). Swarms were obtained from high dilutions (e.g. 10^{-3}) of the transduction mixture on plates not containing any anti-*i* serum. Swarms were also obtained on medium containing serum specific for the mutant *i* antigen of the recipient, but only from plates inoculated with the concentrated transduction mixture. Thus *fla*$^+$ transductants with the wild-type *i* antigenic character of the donor, but without its *i*-curly (and therefore phase-1 non-motile) character, formed only a very small proportion (10^{-4} to 10^{-5}) of all *fla*$^+$ transductants in these crosses. This class presumably arose by crossing-over in *H1* between the mutation determining the curly character of the *i* flagellin of the donor and that determining the changed serological properties of the *i* flagellin of the recipient. Because of the rarity of recombinants with wild-type antigen and because a *fla* mutant of the *i*-curly donor was not available no inferences could be drawn about the order within *H1* of the curly and altered-antigen mutations.

Control experiments confirmed the previously reported inseparability of the curly character from the determinant of phase-1 antigenic character in crosses between strains with unrelated phase-1 antigens. The *Salmonella typhimurium* derivative SD 7, with *b* as its phase-1 antigen, was treated with phage P 22 lysates of several strains with antigen-*i*, wild type or mutant in respect of serological character; numerous swarms with the donor phase-1 antigen were obtained on plates containing anti-*b* (and anti-*1,2*) serum. By contrast no *i* swarms were obtained when the donor carried the *i*-curly allele.

DISCUSSION

The mutations which altered the serological character of antigen-*i* (Joys & Stocker, 1963, 1966) presumably altered amino acids at serologically determinant sites in the polypeptide chain of *i* flagellin and we hoped to map the sites of the corresponding mutations within *H1*, the structural gene for *i* flagellin. Motile (*fla*$^+$) transductants with wild-type antigen-*i* were obtained in various crosses in which the recipient was *fla* and each parent carried a mutant *H1* gene determining an altered form of antigen *i*. We consider that the control experiments described prove that the wild-type antigen of these transductants arose by crossing-over within *H1*, not by reversion. (We did not detect any recombinants of the reciprocal type, which would presumably have had the mutant serological specificities of both parents, but such recombinants would probably not have been detected by the method of serum selection used.) Transductants with wild-type antigen-*i* were abundant in most crosses involving M 6, either as donor or recipient, but rare or absent in all other crosses. We infer that the position of the M 6 mutation within *H1* is not very close to those of M 5, M 10, M 11 and M 12, and that these latter mutations are very close to each other. The clustering of most of the serological mutations within a small region of *H1* may be a consequence of the fact that all the mutants were isolated by selection with a single antiserum, which

would presumably tend to select mutants all of which had lost the same antigenic subfactor. Or it may be that only a small part of the polypeptide chain of flagellin is antigenically determinant.

In this system, in which various functionally efficient but serologically distinct forms of a single protein are available, it thus proved feasible to use the antigenic character of the protein for selection (and recognition) of intragenic recombinants. Attempts to infer the sequence of the mutations within *H1* were, however, unsuccessful—because of the small numbers of wild-type *i* recombinants obtained in crosses not involving M6 and because of the equivocal indications as to gene order provided by crosses of M6 *fla*$^+$ and M6 *fla-52*. In an earlier report (Joys & Stocker, 1963) we drew some conclusions about the order of sites within *H1*; we now consider those conclusions to be invalid since they relied on the premise that all quadruple cross-over classes would be very infrequent, a premise which no longer seems tenable, as discussed below.

In some 'three-point' crosses, the presence of a *fla* mutation in the donor strain greatly decreased the proportion of *fla*$^+$ transductants with the donor-type antigen, whereas in other crosses there was no decrease. On the interpretation that the rare class requires four crossovers the data strongly indicate the order: *fla-50—fla-58—fla-57—fla-55—H1*. Complementation tests divide *fla* mutants into groups (Joys & Stocker, 1965; Iino & Enomoto, 1966); *fla-55* and *fla-57* fall in complementation group *A*, *fla-58* in group *D* and *fla-50* in group *B*. If each complementation group corresponds to a gene, the gene order indicated is *flaB—flaD—flaA—H1*, in agreement with that suggested by Iino & Enomoto (1966). In our earlier communication (Joys & Stocker, 1963) we placed *fla-55* and *fla-52* on the opposite side of *H1* from *fla-50*, *fla-57* and *fla-58*. The order now inferred is more plausible, in that it brings together *fla-55* and *fla-57*, both of complementation group *A*, which in the earlier order were separated by *H1*. Our present data do not show the position of *fla-52*, but since this mutant also falls in complementation group *A* its mutation is presumably in *flaA*, and so between *flaD-58* and *H1*, a position indicated by later mapping experiments in which the *nm1* gene served as external marker (U. Pearce & B. A. D. Stocker, unpublished). Our earlier inference that *fla-52* and *fla-55* were separated by *H1* from the other *fla* mutations was based on the premise of extreme rarity of quadruple cross-over recombinants. The more complete results now reported, especially those of crosses in which both parents are *fla*, show that this premise is incorrect, since reliance on it leads to inferred map orders which contradict each other.

The flagellins determined by 'curly' genes differ from normal flagellins by their tendency to form fibres of abnormally short wavelength on polymerization *in vivo* or *in vitro*. In one instance an alteration in the tryptic peptide map of the flagellin (Enomoto & Iino, 1966) proves that its 'curly' character results from mutation of its structural gene. In our crosses of an *i*-curly donor to various *fla* recipients having *H1* genes determining serologically altered forms of antigen-*i* a very small fraction of the *fla*$^+$ transductants produced phase-1 flagellin which was non-curly (as shown by the normal motility of the transductants when in phase 1) and of wild-type *i* antigenic character (as shown by agglutination tests and by the failure of antiserum specific for the mutant *i* antigen of the recipient to immobilize the transductants). The *H1* genes of these transductants must have arisen by recombination, with a crossing-over between the mutation determining the curly character of the donor *H1* gene

and the serologically determinant mutation in the *H1* gene of the recipient. The very small proportion of *fla*$^+$ transductants with the wild-type antigen may reflect merely the infrequency of the required crossover within *H1*—perhaps the curly mutation is close to the serologically determinant area. Alternatively it may be that in some or all the crosses the production of a *fla*$^+$ transductant with wild-type antigen-*i* requires four crossovers, but we do not now consider that the data permit any reliable inference as to the order of the curly and serologically determinant mutations within *H1*. In the control experiment we did not obtain any transductants able to make normal *i* flagellin as a result of recombination of the *i*-curly *H1* (donor) allele with a wild-type *H1* allele determining flagellin of antigenic type *b*. The amino acid composition of *b* flagellin differs substantially from that of *i* flagellin (McDonough, 1965) and the *H1* genes determining these flagellins must differ correspondingly in base sequence. Such non-homology would be expected to make recombination between these *H1* genes less frequent than when the donor and recipient *H1* genes determine almost identical flagellins.

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Note added in proof

Recently, S. Yamguchi & T. Iino (*J. gen. Microbiol.* (1969), **55**, 59) have demonstrated intragenic recombination between genes specifying various forms of the *g* . . antigen complex and have placed some antigenically important areas of the *H1* gene in a linear array.

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Failure of Putative Nitrogen-fixing Bacteria to Fix Nitrogen

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SUMMARY

Analyses, tests with isotopic nitrogen and tests for acetylene and isocyanide reduction, using both continuous and batch cultures, were made with seven strains of putative nitrogen-fixing bacteria and three local isolates. Only two (single strains of *Mycobacterium flavum* and *Pseudomonas azotogensis*) fixed nitrogen; the active pseudomonad differed in several respects from the organism originally reported. Other Pseudomonas, Nocardia and Azotomonas species and the three local isolates did not fix; some simulated nitrogen fixation in cultural tests most impressively, but proved simply to be very efficient scavengers of traces of fixed nitrogen.

INTRODUCTION

Over the past 15 years, several microbial species have been reported to fix nitrogen in addition to the well-known Azotobacteriaceae and the clostridia related to *Clostridium pasteurianum*. Nicholas (1963), L'vov (1963) and Stewart (1966) have listed or reviewed such organisms, which include species of *Nocardia*, *Pseudomonas*, *Azotomonas*, and *Mycobacterium*. Parejko & Wilson (1968) reported complete failure to obtain nitrogen fixation by strains of *Azotomonas insolita*. We have examined a number of putative nitrogen-fixing bacteria obtained from culture collections and, since some strains simulated nitrogen fixation impressively in cultural studies but proved not to fix on close examination, we feel that a report of our tests is desirable.

METHODS

Organisms. The following micro-organisms were obtained from the National Collection of Industrial Bacteria (NCIB; Torry Research Station, Aberdeen, Scotland); their collection number follows their name, together with a reference to a report (if any) of their ability to fix nitrogen: *Nocardia calcarea*, 8863; *Nocardia cellulans*, 8868 (Metcalfe & Brown, 1957); *Pseudomonas azotogensis*, 9277 (Paul & Newton, 1961); *Pseudomonas azotocolligans*, 9391 (Anderson, 1955); *Azotomonas insolita*, 9749 (Stapp, 1940); *Azotomonas fluorescens*, 9884 (Krasil'nikov, 1945); *Derxia gummosa*, 9064 (Jensen, Petersen, De & Bhattacharaya, 1960); *Azotobacter chroococcum*, 8003. Professor J. De Ley kindly provided the original strain of *Pseudomonas azotogensis* (hereafter called 'strain V') isolated by Professor J. P. Voets (Voets & Debacher, 1956); a culture has been deposited with the National Collection of Industrial Bacteria and assigned the number 10,386. Dr T. Kalininskaya kindly provided a culture of *Mycobacterium flavum* 301 (Fedorov & Kalininskaya, 1961). Three local

isolates were examined, 'FINCHLEY' and 'WORCESTER' were rod-shaped facultative aerobes isolated from garden soil from North London and Worcestershire, respectively, by using Belyakov's (Belyakov, 1957) technique for Azotobacter; 'SANIBEL' was a marine aerobic rod from a sea water sample off Sanibel Island, Florida, isolated by enrichment and conventional plating on Burk sucrose medium (Newton, Wilson & Burris, 1953) supplemented with NaCl 2·5 % w/v.

Culture. Stock cultures of all aerobes except *Mycobacterium flavum* were maintained on Burk's sucrose agar or a comparable medium based on mannitol, though the specimens received from elsewhere had been maintained on 'nutrient agar'. Special supplements are described under the individual organisms. *M. flavum* was grown in a medium prescribed by Dr. T. Kalininskaya: K_2HPO_4, 1·74 g.; KH_2PO_4, 0·91 g.; $MgSO_4.7H_2O$, 0·3 g.; $CaCl_2.6H_2O$, 0·1 g.; NaCl, 0·9 g.; $FeCl_3$, 0·01 g.; $NaMoO_4.2H_2O$, 5 mg.; biotin, 10 μg.; yeast extract (Difco) 80 mg.; distilled water to 1 l.; ethanol, 4 ml. In cases where 'adaptation' to nitrogen-fixation seemed desirable, strains were subcultured on nitrogen-deficient media at least four times before testing. Batch cultures were grown at 25 or 30° in stagnant flasks, stirred flasks or stagnant standing test-tubes as appropriate; in prolonged tests the liquid level was marked and readjusted with distilled water to compensate for evaporation before sampling for nitrogen-analysis. All tests included two controls. One was a culture, in a vessel of similar dimensions to the rest, which had been boiled and acidified to about N-H_2SO_4; the acidification was critically important since the control thus scavenged atmospheric ammonia like an efficient nitrogen-starved ammonia-scavenging culture, which a simple boiled control would not have done. The other control was a culture of *Escherichia coli* (NCTC 86) as an authentic non-nitrogen fixer, incubated in conditions in which the growth was limited by the amount of ammonium salt in the medium. The *E. coli* control was also included in tests for acetylene or isocyanide reduction and incorporation of $^{15}N_2$ (below).

Continuous cultures were set up in apparatus of the kind described by Baker (1968). When necessary, they were initiated in media containing fixed nitrogen as yeast extract or an ammonium salt and nitrogen-free medium was pumped in when the culture was established.

Nitrogen analyses. For pilot experiments, Kjeldahl digestion followed directly by Nessler analysis was used (Meynell & Meynell, 1965). For more precise work, culture samples of 5 to 20 ml. were digested, distilled and analysed with Nessler reagent.

Isotopic nitrogen $^{15}N_2$. Nitrogen enriched with 30 atom % of ^{15}N was either prepared by oxidizing 30 atom % enriched $(NH_4)_2SO_4$ with sodium hypobromite, taking care to avoid carry over of unchanged $^{15}NH_3$ (see Sprinson & Rittenberg, 1949) or obtained by diluting 95 atom %-enriched nitrogen gas from M.W. Hardy & Co. (Mercantile) Ltd. (55 Basinghall St., London, E.C.2). Cultures of aerobic bacteria were grown under oxygen + argon mixtures pO_2 = 0·2 or 0·1 atm. with 0·2 atm. of 30 atom % enriched N_2 in closed vessels with magnetic stirring or shaking. To keep the pO_2 at approximately 0·1 atm., 1 ml. gas samples were analysed for oxygen daily by gas chromatography and, when necessary, oxygen was added by injection; however, some cultures became anaerobic (see 'Results'). Burk medium was used but generally contained, in addition, the following trace elements: $FeSO_4.7H_2O$, 0·82 mg.; H_3BO_3, 2·32 mg.; $CoSO_4.7H_2O$, 9·56 mg.; $CaSO_4.5H_2O$, 0·08 mg.; $MnSO_4.H_2O$, 0·08 mg.; $NaMoO_4.2H_2O$, 0·30 mg.; $ZnSO_4.7H_2O$, 1·74 mg. in 1 l.; plus nitrilotriacetic acid,

56 mg./l. Sufficient fixed nitrogen was added to support visible growth but calculated to ensure nitrogen-limited conditions; the fixed nitrogen was added either as NH_4Cl + 1 μg. yeast extract/ml. or as yeast extract alone. After incubation each culture was digested and the ammonia distilled off. Preliminary tests showed that precautions to avoid sequential contamination due to adsorption of $^{15}NH_3$ in the still (Newman, 1966) were unnecessary. The ammonia was converted to N_2 with hypobromite. Gas samples were analysed in an A.E.I. M.S.3 mass spectrometer either locally or by Dr C. W. Crane (Queen Elizabeth Hospital, Birmingham). An increase of 0·015 atom % ^{15}N above reagent $(NH_4)_2SO_4$ or unlabelled N_2 was taken as positive; in fact all positives recorded here exceeded 0·7 atom % ^{15}N.

Isotopic nitrogen, $^{13}N_2$. Through the courtesy of Dr N. E. R. Campbell of the University of Manitoba, *Azotomonas insolita*, *Azotomonas fluorescens*, *Nocardia cellulans*, *Nocardia calcarea*, *Pseudomonas azotogensis* (NCIB 9277), *Pseudomonas azotogensis* (strain V), *Pseudomonas azotocolligans* and strain 'Finchley' were tested for $^{13}N_2$ incorporation (Campbell, Dular, Lees & Standing, 1967). *Azotobacter vinelandii* was used as a positive control.

Acetylene and isocyanide reduction. All unequivocally nitrogen-fixing organisms so far examined reduce acetylene to ethylene (Dilworth, 1966; Schöllhorn & Burris, 1967) and methyl isocyanide to methane (Kelly, 1968). These reactions provide very sensitive tests for the presence of the nitrogenase system since the products can be detected by gas chromatography. For isocyanide reduction tests, concentrated suspensions (equiv. 1 to 3 mg. dry wt/ml.) in growth medium were prepared from growing cultures by centrifugation and exposed to 10^{-3} M-CH_3NC (aqueous solution) in air in stoppered flasks up to 4 hr at 30°. For acetylene reduction three types of experiments were made. (1) 10 ml. cultures were set up in 50 ml. conical flasks and, once growth had started, the cotton-wool plugs were replaced by sterile 'Suba-seal' caps, and acetylene freshly prepared from calcium carbide and water (chromatographically a purer material than commercial acetylene) was injected to about 0·02 atm. The flasks were returned to the incubator and 0·5 ml. gas samples were removed for chromatographic analysis and replaced by O_2 daily. (2) Cultures were grown on slopes of Burk's agar medium in 'universal' containers and, once growth was well established, the screw caps were replaced by 'Suba-seal' caps and acetylene injected to about 0·02 atm. After 1, 4 and 24 hr incubation, 1 ml. gas samples were removed for chromatographic analysis and replaced by air. Control cultures containing no acetylene were tested in parallel. In some experiments the agar contained yeast extract (5 μg./ml.). (3) *Pseudomonas azotogensis* NCIB 9277, *Pseudomonas azotogensis* strain V, *Pseudomonas azotocolligans*, *Nocardia cellulans*, *Nocardia calcarea*, and *Azotomonas fluorescens* were grown in 100 ml. batch cultures in 250 ml. flasks in Burk medium including trace elements + plus nitrilotriacetic acid + NH_4Cl, (50 ug./ml.) + yeast extract, (1 μg./ml.). Where necessary, chalk was present to keep the pH value near 7·0. The cultures were grown aerobically under atmospheres containing $pO_2 = 0{\cdot}1$, $pN_2 = 0{\cdot}2$, $pC_2H_2 = 0{\cdot}01$, $pA = 0{\cdot}69$ and anaerobically under $pN_2 = 0{\cdot}2$, $pC_2H_2 = 0{\cdot}01$, $pA = 0{\cdot}79$; they were incubated on a rotary shaker and 1 ml. gas samples were removed for gas-chromatographic analysis daily. The atmospheres over the aerobic cultures were renewed daily.

For tests with continuous cultures, a portion of the culture was deliberately overflowed and fresh nitrogen-free medium pumped in rapidly, so that the population ceased to be limited by any component of the medium except fixed nitrogen. Acetylene

was then injected into the gas input line, in the non-sterile zone (before the cotton-wool filter), in amounts calculated to give about 0·02 atm. in the growth vessel. After sufficient time had elapsed for the acetylene to be carried into the growth vessel, the gas influent and effluent lines were clipped off so as to isolate the culture vessel, with a modest positive pressure within to allow for sampling. The medium input system was also turned off. The continuous culture thus ceased to be 'continuous' but exposure of the actively growing culture to acetylene gave rapid reduction in positive cases. Samples were removed for gas analysis, using sterile syringes, either through a 'Subaseal' cap in the growth vessel or through a silicone rubber connection on the effluent line; after 2 to 5 hr the test was stopped by opening the gas lines and restarting the flow of medium.

Gas chromatography. Acetylene and ethylene were detected on a Pye 104 gas chromatograph with a 5 ft (152 cm.) 'Porapak R' column of 4 mm. internal diameter at 45° with N_2 as carrier gas at a flow rate of 60 ml./min. using a flame ionization detector; ethylene production greater than 1·7 n-mole C_2H_4/ml. gas sample was accepted as significant provided it increased progressively with incubation. Methane from methyl isocyanide was assayed on a similar but rather less sensitive apparatus and progressive formation of CH_4 in excess of 35 n-mole/ml. gas sample was accepted as positive. Controls without C_2H_2 or CH_3NC were included in all tests. Oxygen over batch cultures was measured with a 5 ft (152 cm.) Molecular 5A sieve (100 to 120 mesh) column of 4 mm. internal diameter at 40° with a gas density balance (filament current 120 mA) as detector. Argon was the carrier gas, at a flow rate of 25 ml./min. on the analytical side and 120 ml./min. on the reference side.

Protein contents of bacteria. Batch cultures in Burk sucrose medium containing growth-limiting amounts of NH_4Cl (40 to 50 μg./ml.) were incubated until the organisms were well into the stationary phase. Dry weights were obtained by filtering 10 to 20 ml. portions of the culture on to weighed filter membranes (Oxoid Ltd.), rinsing with distilled water, drying to constant weight at 80° and reweighing; protein contents were determined by Stickland's (1951) method on centrifuged bacteria resuspended in distilled water.

RESULTS

Azotobacter chroococcum. This organism served as a positive control for our techniques. It grew readily in nitrogen-deficient media and fixed nitrogen; it was established without difficulty in continuous culture and reduced acetylene in the condition described. Suspensions reduced isocyanide readily; $^{15}N_2$ was incorporated during growth.

Derxia gummosa. This organism is a typical awkward nitrogen fixer. On transfer to a new nitrogen-free agar or liquid medium, growth and nitrogen fixation was often delayed for 2 to 3 weeks. On agar plates, background growth would appear but good growth typical of fixation occurred only with the development of yellowish masses in isolated parts of the plates; subcultures from these zones gave a repetition of the same behaviour. This kind of behaviour was described by Jensen *et al.* (1960) in their original description of the organism; it made growth in nitrogen-fixing conditions in batch culture a very unreliable procedure. Various batch culture conditions were tested and reproducible growth was obtained in stagnant plugged conical flasks, with a modified Burk medium, containing K_2HPO_4, 0·64 g.; KH_2PO_4,

0·16 g.; $MgSO_4.7H_2O$, 0·2 g.; NaCl, 0·2 g.; $CaCl_2$, 10 μg.; mannitol, 10 g;. trace elements plus nitrolitriacetic acid, as before; distilled water to 1 l.; adjusted to pH 7·9 before autoclaving. Such flasks could be shaken if the pO_2 were lowered to 0·1 atm. The strain was readily established in continuous culture in air by allowing it to grow in this modified Burk's medium with a little NH_4Cl, then running in nitrogen-deficient medium slowly. The preliminary growth with NH_4Cl was unnecessary when the culture was run from the start at $pO_2 = 0.1$ atm. Once established it grew and fixed nitrogen continuously in air for several months provided vigorous stirring was avoided; it seemed particularly sensitive to inhibition by aeration of the kind reported by Dalton & Postgate (1969) but, once a sufficient population was established to keep down the dissolved oxygen concentration, cultures became stable. Bacteria from the continuous culture fixed $^{15}N_2$ in batch culture, reduced CH_3NC as suspensions, and both batch cultures and the continuous culture reduced acetylene.

Mycobacterium flavum 301. Dr Kalininskaya's medium for this organism contained about 80 mg. yeast extract/l. The organism grew to give a rather low yield in this medium but fixed nitrogen unequivocally; it doubled the nitrogen content of the medium in about 10 days. Batch cultures reduced acetylene about half as fast as *Azotobacter chroococcum* grown in a similar medium; the ability of *M. flavum* to reduce CH_3NC was not tested. The organism grew without difficulty in continuous nitrogen-fixing culture; further data about this organism, including a positive test with $^{15}N_2$, were reported by Biggins & Postgate (1969).

Pseudomononas azotogensis, strain V. This organism gave impressive viscid colonies on nitrogen-deficient agar and did not fix nitrogen nor reduce CH_3NC or C_2H_2 under the aerobic test conditions used for *Pseudomonas azotogensis* NCIB 9277 and *Pseudomonas azotocolligans.* However, acetylene was reduced by growing cultures when initiated with a low concentration of NH_4Cl (50 μg./ml.) in Burk medium (containing additional trace elements, nitrilotriacetic acid (see Methods) and chalk to keep the culture neutral) if, and only if, conditions were anaerobic. Incorporation of $^{15}N_2$ was observed: 2·57 atom % excess ^{15}N in 12 days in a comparable medium at $pO_2 = 0.1$; the culture became anaerobic infrequently during the incubation period and nitrogen fixation probably occurred then. Aerobically grown suspensions of this strain appeared to incorporate $^{13}N_2$ marginally: Dr N. E. R. Campbell recorded ^{13}N counts 0·18 % in excess of the lower limit of significance, 0·2 % of the value obtained with *Azotobacter vinelandii.* Chalk-free batch cultures with sodium gluconate as carbon and energy source remained at about pH 7·0 and, under anaerobic conditions, fixed 0·9 mg.N/g. sodium gluconate consumed. A continuous culture was established in Hino & Wilson's medium (Hino & Wilson, 1958) but containing no chalk and, under anaerobic conditions using nitrogen gas only, the culture fixed sufficient nitrogen (42 μg./ml.) to be measured analytically. Voets & Debacher (1956) described *Pseudomonas azotogensis* as a short rod, 0·5 μ by 2 to 3 μ and Gram-negative, actively motile with 1, 3 or 4 polar flagella. The microbe was described as growing aerobically without gas and acid production on a variety of carbohydrate media. Our strain which, according to Professor De Ley, came originally from Professor Voets, was a Gram-negative rod in our hands (width 0·8 μ, length variable) but had peritrichous flagella. It also grew anaerobically in media containing a fixed nitrogen source, and produced acid and gas when grown in a glucose nutrient agar shake culture. A second culture sent by Professor De Ley had identical properties. These characters suggest the

organism is not a Pseudomonas. De Ley & Park (1966) reported that the DNA base ratio of this strain (G+C=48·1 %) was outside the normal range of *Pseudomonas*.

Other Pseudomonas species. Pseudomonas azotogensis NCIB 9277 and *Pseudomonas azotocolligans* gave impressive viscid colonies on nitrogen-deficient agar but grew only very slowly in liquid media, whether in conical flasks or upright tubes. Fixed nitrogen did accumulate in such cultures, but we attribute it to atmospheric NH_3 since, even in our laboratory where ammonia is banned, control vessels accumulated 4 to 6 μg.N/ml. in 2 or 3 weeks. Methyl isocyanide was not reduced by growing cultures in a liquid medium containing limiting amounts of fixed nitrogen as NH_4Cl (30 μg./ml.). Acetylene was not reduced by growing cultures in any of the following conditions: on Burk medium set with agar, in liquid medium containing 80 μg. yeast extract/ml. in aerobic conditions (in which *Azotobacter chroococcum* and *Mycobacterium flavum* reduced it), in liquid medium containing 50 μg. NH_4Cl/ml. and 1 μg. yeast extract/ml. in aerobic conditions but $pO_2=0{\cdot}1$ atm. and the pH value kept near neutral. *P. azotogensis* 9277 and *P. azotocolligans* did not grow anaerobically with NH_4Cl as fixed nitrogen source. Cultures of both, grown with limiting fixed nitrogen as NH_4Cl (50 μg./ml.) and 1 μg. yeast extract/ml., incorporated no $^{15}N_2$. *P. azotogensis* 9277 did not incorporate $^{13}N_2$, a very small apparant incorporation was obtained with *P. azotocolligans*: Dr N. E. R. Campbell reported a ^{13}N count 36 % above the limit of significance, 3·9 % of the value obtained with *Azotobacter vinelandii*. The organisms were readily established in continuous culture with limiting amounts of fixed nitrogen as NH_4Cl, but always diluted out when nitrogen-free medium was pumped in. A continuous culture of *P. azotocolligans* with limiting fixed nitrogen as yeast extract did not reduce acetylene nor fix nitrogen when grown under nitrogen with either 0·05 or 0·1 atm. O_2; sodium lactate was the carbon and energy source and 5 % CO_2 (v/v) was included in the gas phase to hold the pH value about 7·0. The culture of *P. azotocolligans* we obtained from the NCIB contained two colony types; neither bred true on subculture.

Azotomonas. Azotomonas insolita and *Azotomonas fluorescens* grew poorly even on solid media as compared with the pseudomonads. Similar tests to those just described for the two negative pseudomonads provided no evidence for N_2 fixation, methyl isocyanide reduction or acetylene reduction; continuous cultures diluted out in nitrogen-free media. *A. insolita* did not incorporate $^{15}N_2$ or $^{13}N_2$. Two lots of *A. fluorescens* received from the NCIB appeared to contain two morphologically distinct types of colony and organisms; no attempt was made to separate them. This culture incorporated neither $^{15}N_2$ nor $^{13}N_2$. Experiments to test the effect of stress on these organisms and the pseudomonads are described in the next section.

Nocardia. Nocardia cellulans and *Nocardia calcarea* also grew poorly on solid media and were tested in parallel with the azotomonads, with similar negative results. Metcalfe & Brown (1957) reported that these strains sometimes lost their ability to fix nitrogen in the laboratory, but that it was regained after a period of storage on sterile soil. Both strains of *Nocardia*, *Pseudomonas azotogensis* 9277 and *Azotomonas insolita* were stored for 2 years on sterile soil and reisolated: only *N. cellulans* and *A. insolita* survived and neither had acquired ability to fix nitrogen in our hands. Since drying on soil is a stress and might be mutagenic, three other stresses were imposed on the negative strains. Cultures of the two negative pseudomonads, azotomonads and nocardias, grown in Burk medium with added trace elements and a limiting supply

of ammonia, were exposed separately to (1) 15 to 30 min. at 50°, (2) freezing in liquid nitrogen and thawing, (3) u.v.-irradiation for 2 to 4 min. 90 mm. from a lamp emitting 30 W at 253·7 mμ. At least one stress caused a 50 % decrease in viability, assessed by plate counts, with each strain. None developed ability to reduce acetylene after this treatment when tested on agar slopes containing 5 μg. yeast extract/ml. (see Methods).

'*FINCHLEY*' *and* '*WORCESTER*'. These strains had been isolated by one of us (J. R. P.) while working in a veterinary college, and appeared from analyses to fix nitrogen if provided with small amounts (50 μg./ml.) of purine such as adenine or guanine. On transfer to our present address, no such fixation could be detected. Only one strain ('FINCHLEY') was tested with $^{13}N_2$ and neither was tested in continuous culture. The importance of the site of the analyses will be brought out in the Discussion.

'*SANIBEL*'. This strain produced impressive growth on plates but gave no indication of nitrogen fixation in our tests.

Mixed cultures. Cultures of *Azotobacter chroococcum* were set up and deliberately infected with the negative aerobes to see if take-overs based on parasitism or other interactions might occur. All grew well in association, no sign of take-over by the non-Azotobacteriaceae was observed and azotobacters were the only nitrogen-fixing species we could isolate from such cultures.

DISCUSSION

The field of nitrogen fixation has a history of mysterious differences between the findings of one laboratory and another. Nevertheless, we can assert that, in our hands, there is no evidence whatever that the aerobic Pseudomonas, Azotomonas and Nocardia species, as now available from culture collections, fix nitrogen or reduce acetylene. Acetylene was not reduced even under conditions found favourable for *Mycobacterium flavum*. This is not to say that they did not fix nitrogen when first isolated, but either they have lost that property or they are not the original organisms. Systematic data such as their poly-β-hydroxybutyrate content (Stockdale, Ribbons & Dawes, 1968), DNA base composition (De Ley & Park, 1966) or morphology (De Ley, 1968) cast doubt on their status. The one organism which did fix nitrogen consistently in our hands, *Pseudomonas azotogensis* strain V, provided by Professor De Ley, was already under suspicion of not being a true Pseudomonas and did not correspond to its discoverers' description. In fact it proved only to fix in anaerobic conditions, like *Bacillus polymyxa* or *Klebsiella pneumoniae*.

The question arises why some of these bacteria, particularly *Pseudomonas azotogensis* 9277, give such impressive growth on nitrogen-free agar; their growth on agar resembled that of *Phoma casuariniae*, a non-nitrogen fixing mould, illustrated by Wilson (1952). For example, *P. azotogensis* 9277 on Burk agar formed, after 21 days at 30°, domed colonies 2 to 3 mm. diam. x 1 mm. elevation; a non-nitrogen-fixing *Aerobacter aerogenes* formed flat colonies 1 mm. diam. in similar conditions. Ammonia is present in all environments where people are working, originating as a bodily exudate; even in our laboratory where reagent ammonia solutions are banned, a plugged test tube of N-H_2SO_4 can absorb nitrogen up to 6 μg. N/ml. in 2 to 3 weeks. An agar surface is a superior environment for scavenging atmospheric ammonia (and oxides of nitrogen for which we had no controls). If these organisms are capable of subsisting with bare minima of nitrogenous nutrients they might yield impressive growth on fixed nitrogen

scavenged from the atmosphere. Table 1 shows the protein contents of nitrogen-limited cultures of various strains; since the protein contents of ordinary heterotrophs range from 50 to 70 %, it is clear that the protein contents of several of these strains are remarkably low. It seems likely that these organisms can form cells of unusually low nitrogen content; this view is supported by several circumstantial details. Growth of *Azotomonas insolita* occurred on 'nitrogen-free' media far more readily at the National

Table 1. *Protein contents of putative nitrogen-fixing micro-organisms*

Cultures (100 ml.) of micro-organisms were grown with 40 μg. NH_4Cl/ml. Burk medium with forced aeration and analysed for protein/dry wt micro-organism after 5 days at 30°. For procedures see text.

Organism (NCIB number)	mg. protein/mg. bacteria (%)
Nocardia cellulans (8868)	25
Pseudomonas azotogensis (9277)	36
Pseudomonas azotocolligans (9391)	12
Azotomonas insolita (9749)	10
Azotomonas fluorescens (9884)	25
Strain 'FINCHLEY'	26*
Strain 'WORCESTER'	28*

* Grown in a glycerol medium, with 50 μg. adenine/ml. as fixed nitrogen, for 48 hr.

Collection of Industrial Bacteria (Aberdeen, Scotland) than at the University of Sussex; the Collection is located across an estuary from a fish market and it is thus often subject to ammoniacal components in its atmosphere. In a corroborative experiment at the National Collection, Mr A. R. MacKenzie, who used six reisolates from the original culture of *A. insolita* deposited with the Collection and two reisolates from the American Type Culture Collection's strain, showed that growth on nitrogen-deficient medium was markedly improved when ammonia-generating cultures such as *Proteus* spp. were in the same incubator. Finally, we should record that the original isolations of 'FINCHLEY' and 'WORCESTER' were made in a laboratory situated five floors above stables and animal houses, from which atmospheric NH_3 would unquestionably arise.

These observations show how easy it is to simulate nitrogen fixation by growing aerobic microbes of exceptionally low protein content on agar in air. Such cultural tests, together with analyses, are useful preliminary tests for nitrogen fixation but they should obviously be supplemented by isotopic tests and tests for reduction of acetylene and/or other nitrogen analogues. Scavenging of traces of fixed nitrogen may account for some false positives, but it does not explain the significant enrichment of $^{15}N_2$ obtained with *Pseudomonas azotocolligans* by Anderson (1955). The possibility exists that the original workers on the aerobes were handling mixed cultures of an azotobacter with a parasitic pseudomonad of the kind described by Postgate (1967) and failed to observe the azotobacter; but this is intrinsically unlikely because tests for parasitic activity among the aerobic species were negative.

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SHORT COMMUNICATION

A Note on the Production of Simulacra of Certain Genera of Actinomycetales by Streptomyces Grown on Different Culture Media

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Until 1957 there existed only two recognized genera of non-sporangial, saprophytic mould-like bacteria: *Streptomyces*, bearing spores in chains, and *Micromonospora*, bearing them singly on short branches (*Bergey's Manual*, 1957). Since that time, several further genera have been described on the basis of spore arrangement; *Waksmania* (Lechevalier & Lechevalier, 1957) or *Microbispora* (Nonomura & Ohara, 1957), with paired spores; *Micropolyspora* (Lechevalier, Solotorovsky & McDurmont, 1961; Kalakutskii, 1964; Kosmachev, 1964) with short, branched chains of spores; *Microtetraspora* (Thiemann, Pagani & Beretta, 1968) with spores in chains of four.

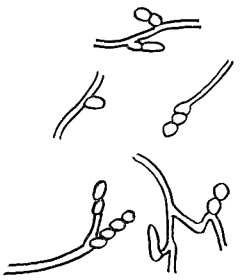

Fig. 1

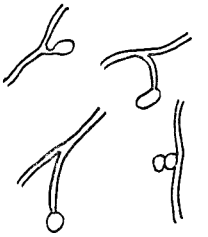

Fig. 2

Fig. 1. *Streptomyces erythraeus*, 2-weeks growth on peptone-yeast-extract-iron agar, morphologically simulating *Micropolyspora*. Drawn by direct observation. × 3000 (approx.)

Fig. 2. *Streptomyces venezuelae*, 2-weeks growth on starch agar, morphologically simulating *Waksmania*. Drawn by direct observation. × 3000 (approx.)

It is common experience that Actinomycetales, especially *Streptomyces*, can vary in the morphology of their sporogenous hyphae under different conditions of culture (Bisset, 1959), and all these descriptions of new genera record the necessity of standardizing the culture medium in order to obtain the typical morphology. At least one new genus, *Chainia* (Thirumalachar, 1955), has since proved to be a variant Streptomyces (Gattani, 1957), and other variants are capable of producing a wide variety of different sporulation appearances in the same culture (Bisset, 1957).

In the course of examination of ten newly-isolated strains of *Streptomyces* from soil, using the range of media recommended for characterization by Shirling &

Gottlieb (1966), I observed repeatedly that exact simulacra of two of the recent genera were produced by growth of certain strains upon appropriate media. For example, three strains classifiable as *Streptomyces erythraeus*, growing with long, branched chains on glycerol-asparagine agar, exactly resembled *Micropolyspora* on peptone-yeast-extract-iron agar (Fig. 1); one, classifiable as *S. venezuelae* on the former medium, resembled *Waksmania* when grown on starch agar with inorganic salts (Fig. 2).

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