STUDIES ON MARINE AGAR-DIGESTING BACTERIA[1]

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Agar had been in general bacteriological use for almost twenty years before organisms capable of attacking it were first reported. In 1902 Gran, then working in Beijerinck's laboratory, discovered an agar-digesting bacterium quite fortuitously, while engaged in a general study of marine bacteria. Later, by the use of enrichment cultures, he was able to isolate the same organism repeatedly from seawater off the Dutch and Norwegian coasts. Gran showed that the enzyme responsible for agar decomposition was extracellular, and also discovered the iodine reaction, which is the surest test for agar decomposition. In concluding his work on the subject, he wrote:

> Es wäre zu wünschen, dass solche Untersuchungen auch in anderen Meeresgebieten aufgenommen wurden; die Gelasebakterien bilden ebenso wie die Leuchtbakterien eine ganz gut begrenzte Gruppe, die für vergleichend-systematische Untersuchungen ganz wohl geeignet ist.

In the forty years which have elapsed since then, surprisingly little work has been done on the group. It has been shown that terrestrial as well as marine agar-digesting forms exist, a number of new species have been described, in the main rather incompletely, and a few preliminary biochemical studies have been made. Most of the publications in this field have been brief descriptions of organisms isolated more or less accidentally during the course of other work; in fact, only Lundestad (1928) and Gorseline (1933) have made comprehensive studies of a

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number of species. Hence an exhaustive review of the literature seems unnecessary: pertinent work will be cited in the discussion, while for a review of other papers the reader is referred to Lundestad (1928) and Gorseline (1933).

The paucity of work in this field is all the more surprising when one considers the undoubted importance of agar-digesters in the cycle of matter in the ocean, where agar and similar polysaccharides form a large part of the carbohydrate constituents of many marine algae, particularly of the *Rhodophyta*. Indicative of their activity is the report of Bavendamm (1932) that marine sediments collected near the Bahama Islands contained from 50,000 to 200,000 agar-digesters per gram. Waksman and Bavendamm (1931) have put forward the theory, based in part on the earlier work of H. and E. Pringsheim (1910), that a close relation exists in the ocean between agar-digesting and nitrogen-fixing bacteria and algae; they suggest that the monosaccharides released on the hydrolysis of agar and similar polysaccharides may be used as energy sources by nitrogen-fixing bacteria, the nitrogen thus fixed being in turn used by the agar-digesters and the algae. In this way the extensive growth of marine algae in an environment as poor in combined nitrogen as the ocean becomes possible.

The present systematic study was undertaken primarily in order to ascertain the commonly-occurring marine agar-digesting species along the North Pacific coast; apart from the unsatisfactory work of Angst (1929) in which thirteen "new species" were described in such a way as to be completely unrecognizable on reisolation,[2] no previous studies of the agar-digesting flora in this region have been made. Of secondary interest were the ecological problems connected with the group, such as the relationships between agar-digesters of the Atlantic and the Pacific and between terrestrial and marine forms.

SOURCE AND ISOLATION OF ORGANISMS

Over the course of the past two years numerous enrichment cultures for marine agar-digesting bacteria have been prepared.

[2] In this connection, Bavendamm (1932) states "dass sich besonders bei Angst wieder einmal die gefährliche Tendenz zeigt, physiologische Rassen als neue Arten zu beschreiben."

The enrichment medium consisted of a 0.1 per cent agar solution in seawater, either with or without the addition of an inorganic nitrogen source (0.1 per cent KNO_3 or $(NH_4)_2SO_4$). The addition of a nitrogen source appears to be unnecessary, since the agar and inoculum contain enough nitrogenous compounds to ensure a normal development. Flasks containing a shallow layer of the enrichment medium were inoculated with small amounts of seawater or pieces of algal thalli, and incubated at 22°C. Good growth usually occurred after about five days, at which time streaks were made on plates of seawater peptone agar or seawater mineral agar. The former gave uniformly better results; most marine agar-decomposers are unable to grow at all on a medium containing mineral nitrogen alone, and all grow much more rapidly with peptone as a nitrogen source. Usually the ratio of agar-digesters to other bacteria had risen to such an extent in the enrichment culture that the latter, although they could grow on peptone agar, did not interfere effectually with the isolation of the former.

The enrichment culture method has the marked disadvantage that through selective accumulation the organisms obtained by its use are often restricted to a comparatively small number of types. Consequently, isolations were also made by direct streaking of suspensions of fresh or partly decayed algal material in sterile seawater. In this way, species of rare occurrence in enrichment cultures were easily obtained. In addition a number of cultures were picked directly from colonies found on the daily seawater count plates made at the Scripps Institution of Oceanography at La Jolla. One isolation was also made from a seawater cellulose enrichment culture.

All cultures were restreaked at least twice to ensure their purity; in a few cases—namely, with rapid agar-liquefying or rapidly spreading strains—more repeated platings were necessary.

METHODS

In the preparation of stock media, seawater was used when it was readily available; otherwise 3 per cent NaCl in tapwater was substituted with satisfactory results. For synthetic media, "aged" seawater (i.e., seawater which had been allowed to stand

under aerobic conditions until the organic materials had undergone mineralization) was used.

The stock medium for maintaining cultures was peptone seawater agar with the addition of 2 per cent $CaCO_3$. A number of strains, particularly the rapid agar-liquefiers, die off rapidly due to the production of acid if the $CaCO_3$ is omitted.

To test carbohydrate utilization, a medium of the following composition was used:

Aged seawater	100 ml.
Bactopeptone	0.5 g.
K_2HPO_4	0.2 g.
Carbohydrate	0.8 g.

After growth had occurred, sugar analyses were made by Luff's method (Schoorl, 1929) and pH change was determined colorimetrically.

The decomposition of starch, chitin, and alginic acid (used as Na alginate) was studied on seawater peptone agar plates containing 1 per cent of these materials. Starch hydrolysis was determined by flooding the plates with iodine, chitin and alginic acid hydrolyses were observable by a clearing of the surrounding area. For studying cellulose decomposition, three liquid media were used containing respectively 1 per cent peptone, 0.2 per cent peptone + 10.2 per cent KNO_3, and 0.2 per cent peptone + 0.2 per cent $(NH_4)_2SO_4$ in seawater, in which strips of filter paper were partly immersed.

The nitrogen requirements were determined with two series of media using aged seawater as a base. In the first series the nitrogen compounds were used alone in a concentration of 0.5 per cent; in the second, they were used in a concentration of 0.2 per cent, together with 0.5 per cent glucose. In testing salt requirements a basal medium containing 1 per cent peptone in distilled water was used, to which various concentrations of NaCl were added.

All cultures were incubated at 22°C. unless otherwise stated.

Morphology was generally studied in wet mounts. It is difficult to obtain good stained preparations of marine bacteria; if

smears are made with tap or distilled water, the organisms become distorted, while smears with 3 per cent NaCl or seawater undergo equally undesirable plasmolytic changes on drying. On the whole, negative staining with a saturated aqueous nigrosin solution gave the best results of any method tried, although the *Cytophaga* species were often considerably distorted in preparations of this sort (see plate I, fig. 3). Capsule stains were made by the method of Muir and Ritchie. For flagella stains, both the methods of Zettnow and of Plimmer and Paine were tried. The former gave the better results, but under no circumstances were the flagella stains really satisfactory, probably for the reasons mentioned above.

GENERAL CHARACTERIZATION OF THE ISOLATED STRAINS

Morphology. The isolated strains could be divided into three main groups on the basis of morphology: gram-negative, curved, polarly flagellated rods; gram-negative, large, flexible, slightly pointed rods showing creeping motility on solid media; and gram-negative, straight, immotile rods. Spore formation was not found in any of the strains studied.

Physiology. All species were strict aerobes, showing little if any growth at reduced oxygen tensions.

The optimum temperature lay between 20° and 25°C., only one species (*Pseudomonas droebachense*) being able to grow above 30°C. Several species showed fair growth as low as 7°. Growth was best in all cases under neutral or alkaline conditions, that of most species being inhibited at a pH of slightly below 6.5. Some of the organisms (notably the *Cytophaga* species and *Vibrio granii*) died off rapidly in cultures which became acid.

As is commonly the case with marine bacteria, all strains required the addition of salt to the medium for growth. There was a broad general optimum NaCl concentration at 2.0–4.0 per cent, but at concentrations above and below this the various species behaved differently. *Vibrio beijerinckii* and *Pseudomonas iridescens* had the widest ranges, growing at concentrations of from 0.25–6.0 per cent (the highest used). On the other hand, the *Cytophaga* species were sharply restricted within the range

1.5–5.0 per cent. The salt range appeared to be rather characteristic for each species. Since it seemed possible that this character might be somewhat variable, repeated attempts were made to adapt the various species to concentrations lower than the first observed minima. All species were carried through a series of transfers in media with the lowest salt concentration at which growth had originally been obtained, transfers being made from time to time into media with salt concentrations 0.25 to 0.5 per cent lower. In no case was the first observed minimum for any species reduced by this treatment. This finding is in contradiction with many reports in the literature (e.g.,

TABLE 1

Growth of the agar-digesters in peptone water with varying concentrations of NaCl

+++, excellent growth; ++, good growth; +, fair growth; ±, scanty growth; −, no growth.

ORGANISM	NaCl CONCENTRATIONS, PER CENT									
	0.0	0.25	0.5	1.0	1.5	2.0	3.0	4.0	5.0	6.0
V. granii	−	−	−	++	++	+++	+++	++	+	−
V. beijerinckii	−	+	++	++	+++	+++	+++	+++	+++	+++
V. fuscus	−	−	−	++	++	+++	+++	+++	±	−
Ps. iridescens	−	+	++	+++	+++	+++	+++	+++	+	±
Ps. droebachense	−	−	−	+	++	+++	+++	+++	±	−
C. diffluens	−	−	−	−	++	+++	+++	+++	++	−
C. krzemieniewskae	−	−	−	−	++	+++	+++	+++	+	−

Baars, 1927; ZoBell and Rittenberg, 1938). However, the experiments were carried out only after the various organisms had been in culture in seawater media for many months, so it is possible that they had become stabilized. Salt ranges for the different species are given in table 1.

Nutritional requirements. All strains attacked a wide range of carbohydrates in addition to agar. As would be expected from the occurrence of these two sugars in agar, glucose and galactose were used by all strains, and in addition many could use pentoses and disaccharides.

It should be mentioned at this point that in the determination of sugar utilization with these, as with most other strict aerobes,

a simple determination of acid or acid and gas production by conventional standard methods is liable to yield very misleading results. These standard methods have been evolved in work with facultative anaerobes, such as the *coli-aerogenes* group, which ferment carbohydrates usually with the production of relatively large amounts of acid or acid and gas. Strict aerobes, on the other hand, (with rare exceptions like the *Acetobacter* species) oxidize carbohydrates completely to carbon dioxide and water with the result that very little if any acid is produced even when relatively large amounts of the sugar are decomposed. In addition, many aerobic bacteria are ammonifiers, so that a peptone-sugar medium may become markedly alkaline in spite of the fact that extensive carbohydrate utilization has occurred. Although the oxidation of sugars without concomitant acid production was clearly shown by Merrill (1930) in studies on the carbohydrate metabolism of the genus *Mycobacterium*, it does not seem to have been generally recognized. For example, ZoBell and Rittenberg (1938) studying the carbohydrate metabolism of aerobic chitin-decomposing bacteria by the conventional standard methods, report organisms decomposing starch, chitin, sucrose and lactose, but not glucose, or even attacking starch and chitin but none of the other sugars tested. Such anomalous results are clearly due to the use of inadequate methods. Conclusive results can only be obtained either by using carbohydrate media with an inorganic nitrogen source and determining utilization by growth, or by determining carbohydrate disappearance by quantitative methods.

Since most of the organisms studied in the present work were unable to grow with inorganic nitrogen alone, the latter method had to be adopted. For the sake of comparison, a colorimetric determination of pH change was also made.

Table 2 shows some typical results for three of the species.

The data for *V. beijerinckii* are particularly interesting. This organism is a strong ammonifier; in media where the carbohydrate was not attacked, the pH rose from 7.1 to as high as 9.0, while in media where carbohydrate decomposition took place, the pH remained virtually unchanged, the slight acid production which

occurred being masked completely by the simultaneous formation of ammonia from the peptone. In all these cultures ammonia could readily be detected. By "standard" methods for determining carbohydrate utilization, this organism would have been judged unable to attack any of the simple sugars tested.

All species with the exception of *V. beijerinckii* attacked cellulose although in some cases (particularly *V. granii* and *P. droebachense*) the action was slow. The two *Cytophaga* species were the most rapid cellulose-decomposers, disintegrating the filter paper at the air-liquid interface in approximately 10 days.

TABLE 2

Sugar utilization by three species of agar-decomposing bacteria as determined by sugar analyses and pH change on 5 week cultures in peptone carbohydrate broth

CARBOHYDRATE	CONTROL		V. BEIJERINCKII			V. FUSCUS			PS. DROEBACHENSE		
	Sugar	pH	Sugar	pH	Utilization	Sugar	pH	Utilization	Sugar	pH	Utilization
	mg./ml.		*mg./ml.*			*mg./ml.*			*mg./ml.*		
Arabinose*	8.4	7.1	8.2	9.0	−	8.4	8.2	−	7.0	5.5	+
Xylose*	9.2	7.1	9.0	9.0	−	3.4	6.8	+	3.8	6.2	+
Glucose	8.0	7.1	3.4	7.1	+	2.7	6.2	+	2.9	6.3	+
Galactose	8.4	7.1	3.6	6.8	+	3.6	6.4	+	4.4	6.3	+
Lactose	9.5	7.1	4.8	7.0	+	3.6	6.4	+	7.4	6.3	+
Sucrose†	8.6	7.1	8.6	8.2	−	4.8	6.4	+	4.1	6.2	+
Cellobiose†	8.8	7.1	3.6	7.1	+	4.0	6.4	+	3.6	6.5	+

* Calculated from the glucose tables.

† Determined after hydrolysis with acid.

Considerable variation exists in the degree of agar decomposition exhibited by the different species. Some bacteria cause liquefaction, others only depression. A valuable aid in studying this is the iodine reaction discovered by Gran (1902). If one pours an I-KI solution over a plate on which agar-digesting bacteria have been growing, the areas surrounding the colonies take on at most a light straw color, whereas the unattacked agar stains a reddish-violet, the intensity of the coloration depending on the age of the plate. Recently-melted agar colors almost as strongly with iodine as does starch, whereas agar which has been

solid for several weeks is hardly colored at all. A more detailed report on the agar-iodine reaction will be made elsewhere.

V. beijerinckii never causes a liquefaction of the agar, the area surrounding growth merely becoming softened, cleared and slightly sunken. Nevertheless, the gelase fields produced by this organism, as shown by the iodine reaction, are wider than those of any other species, with very sharply defined edges. *P. iridescens* characteristically produces small gelase fields with rather hazy edges, but the agar immediately beneath the colonies is sharply sunken so that they lie in concave depressions. *P. droebachense* attacks agar in a manner similar to *V. beijerinckii*, except that the gelase fields are smaller and less clearly defined. The two *Cytophaga* species are agar-liquefiers. Due to their spreading growth, the whole surface of the plate becomes covered with a thin layer of organisms in a few days (see plate II, figs. 1 and 2). The agar beneath becomes softened and sunken and ultimately liquefied but the gelase field rarely extends beyond the area of growth. *V. granii* is a rapid agar-liquefier; vigorous strains completely destroy a slant in 4 to 5 days. Wide, sharply defined gelase fields are formed.

The particular manner of attacking agar seems to be constant for any given species. A diminution or loss of the ability to liquefy agar has never been observed in strains possessing this property, some of which have been under laboratory cultivation for over two years. Conversely, agar-softening strains have never been observed to liquefy agar, even under the most favorable conditions. These observations are opposed to the results of van der Lek (1929) who reported a diminution in the agar-liquefying powers of the soil species *Vibrio agar-liquefaciens* on prolonged cultivation.

The nitrogen requirements of most species are not fulfilled by ammonia or nitrates, *V. beijerinckii* being the only species which was able to grow with these as sole nitrogen sources. The growth of several other species was supported by various single amino-acids (notably glutamic acid and leucine), either alone or in combination with glucose. The *Cytophaga* species grew only with peptone and yeast extract, which points to the necessity of growth

factors for at least these two species. It is possible that in reality several other species also require growth factors, the growth with such amino-acids as leucine being due to the presence of these as impurities. Results are given in tables 3 and 4.

TABLE 3

Utilization of nitrogen compounds as sole sources of carbon and nitrogen

+++, excellent growth; ++, good growth; +, fair growth; ±, poor growth; −, no growth.

ORGANISM	YEAST EXTRACT	PEPTONE	GLUTAMIC ACID	ASPARTIC ACID	ASPARAGIN	HIPPURIC ACID	TYROSINE	LEUCINE	ALANINE	GLYCINE	CONTROL (NO NITROGEN)
V. granii	+++	+++	++	±	−	−	−	+	−	−	−
V. beijerinckii	+++	+++	++	++	+	−	−	+	+	−	−
V. fuscus	+++	+++	±	−	−	−	±	++	−	−	−
Ps. iridescens	+++	+++	++	±	−	−	−	−	−	−	−
Ps. droebachense	+++	+++	+	+	−	−	−	−	−	−	−
C. diffluens	+++	+++	−	−	−	−	−	−	−	−	−
C. krzemieniewskae	+++	+++	−	−	−	−	−	−	−	−	−

TABLE 4

Utilization of nitrogen compounds in the presence of glucose

+++, excellent growth; ++, good growth; +, fair growth; ±, poor growth; −, no growth.

ORGANISM	YEAST EXTRACT	PEPTONE	GLUTAMIC ACID	ASPARTIC ACID	ASPARAGIN	HIPPURIC ACID	TYROSINE	LEUCINE	ALANINE	GLYCINE	$(NH_4)_2SO_4$	KNO_2	KNO_3	CONTROL (NO NITROGEN)
V. granii	+++	+++	++	±	−	−	−	+	−	−	−	−	−	−
V. beijerinckii	+++	+++	+++	+++	+++	+	±	+++	++	−	++	−	++	−
V. fuscus	+++	+++	+++	−	−	−	±	++	−	−	−	−	−	−
Ps. iridescens	+++	+++	++	±	−	+	−	−	−	−	−	−	−	−
Ps. droebachense	+++	+++	+++	++	−	−	−	−	−	−	−	−	−	−
C. diffluens	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−
C. krzemieniewskae	+++	+++	−	−	−	−	−	−	−	−	−	−	−	−

CLASSIFICATION AND DESCRIPTION OF CULTURES

Group I. Vibrio species

These organisms are typical vibrios in young cultures, although after a few days they tend to lose their comma shape and then

occur chiefly as straight rods. Lundestad, who first isolated the organism described here as *Vibrio granii*, characterized it as a *Bacterium*; my strains agree so closely with his rather incomplete description of this remarkable organism that there can be no doubt of their identity. In the present work *Vibrio granii* has been encountered only rarely in enrichment cultures; most of the strains studied were obtained from seawater count plates at the Scripps Institution.

V. beijerinckii, the second organism in this group, was first isolated by Beijerinck (1911) and described by him briefly but unmistakably:

> Tyrosine microbes are small vibrios, chiefly occurring in the sea, and during the winter months present in the plankton. Fresh water is not, however, quite devoid of them, and without much trouble they may be isolated from sewage water. The forms living in the sea produce, at least as regards the stronger varieties, besides tyrosinase also tyrosine, and as this takes place from peptone they can be recognized by the black stains which their colonies produce on broth-agar plates. . . . It is remarkable that these tyrosine-vibrios of the sea can be accumulated in seawater with the addition of agar as the sole source of carbon, ammonium chloride as nitrogenous food and potassium phosphate. In this respect they show analogy to the gelase vibrios which secrete the enzyme gelase by which agar is changed into sugar, and which are also easily produced in this manner.

Numerous strains of the marine form have been isolated during the present work. It appears to be the commonest species of marine agar-digester along the coast of California. All strains were characterized by their striking black or brown pigment production; this was strongest however, not in peptone media, but in media containing certain simple carbohydrates and nitrate nitrogen (see plate II, fig. 3), and on mineral agar. Beijerinck named the freshwater species *Microspira* (*Vibrio*) *tyrosinatica*. It seems likely from his descriptions that the marine form differs from the freshwater one in a number of respects, particularly in its ability to attack agar, and that it should be regarded as a separate species. Consequently the name *V. beijerinckii* n. sp.

is proposed for it, *V. tyrosinaticus* being reserved for the freshwater species.

V. fuscus n. sp., the third organism in this group, was isolated once from a marine cellulose enrichment culture, but has never been obtained from other sources.

Vibrio granii nov. comb.

Synonyms: *Bacterium granii* Lundestad, 1928: *Achromobacter granii* Bergey *et al.* 1934.

Morphology. Small curved rods, usually single, but sometimes occurring in chains of two or more. In cultures older than 3–4 days, most of the cells are straight. Length 2–6μ; average 4μ. Width 0.5μ. Very actively motile by means of polar flagella. Encapsulated, non-sporeforming, gram-negative.

Cultural characteristics. Colony formation. Seawater peptone agar plate. Pinpoint colonies visible after 48 hours; round, smooth, glistening, flat, entire, white, of slimy consistency and sharply sunken in the agar. After a week, the colonies have increased in diameter to several millimeters, and are usually sunken to the floor of the petri dish, with wide, sharply bordered, surrounding gelase fields. If the plate was thickly seeded, there was a tendency for the colonies to become confluent, in which case the medium became rapidly liquefied.

Sw. peptone agar slant. After 48 hours, smooth, glistening, filiform growth, sharply sunken in the agar and showing a bluish translucency. In some strains the surface becomes wrinkled and membranous as growth proceeds, in others it remains smooth. Considerable liquid collects at the butt of the slant, which usually becomes completely liquefied in 10 to 14 days. Acid is liberated during the decomposition of the agar, and unless the medium is buffered with $CaCO_3$ liquefaction is slow and incomplete and the organisms soon die off.

Sw. nutrient gelatin slant. Filiform, smooth, glistening, sunken growth after 48 hours. Slight liquefaction, never becoming complete.

Sw. nutrient gelatin stab. Fair growth after 48 hours: filiform, best at surface. Slight crateriform liquefaction after a few

days. In some strains, the growth along the stab tends to diffuse out into the surrounding gelatin.

Sw. peptone broth. Good growth after 48 hours. All strains produce a heavy white pellicle, accompanied in some strains by strong turbidity, while in others the liquid is at first clear. After a week, the medium becomes turbid and slimy; the growth can be drawn out into long threads.

Physiological characteristics. Aerobic. Temperature range 5–30°C.; optimum 20–25°C. Salt range 1.0–5.0 per cent; optimum 2.0–3.0 per cent.

Biochemical characteristics. Catalase positive. Non-pigmented in young cultures, produces slight light-brown pigment in old ones. Nitrates reduced to nitrites. Indole negative. Urease negative. H_2S not produced.

Action on carbohydrates: Attacks arabinose, xylose, glucose, galactose, lactose, maltose, sucrose and cellobiose, usually with slight acid production. Most strains do not decompose starch, a few do so rather weakly. Cellulose is slowly attacked by all strains. Agar is rapidly liquefied with considerable acid production. Chitin and alginic acid are not attacked.

Vibrio beijerinckii n. sp.

Synonym: Tyrosine vibrio of the sea, Beijerinck, 1911.

Morphology. Small curved rods, usually single, sometimes occurring in short chains, in older cultures occurring mostly as straight rods. Length 2–6μ; average 4μ. Width 0.4–1.0μ. Actively motile by means of polar flagella. Encapsulated, non-sporeforming, gram-negative.

Cultural characteristics. Colony formation. Sw. peptone agar plate. After 24 hours, colonies are 3–4 mm. in diameter, round, smooth, glistening, mucoid, with an entire edge and varying in color from white to grey. The agar is slightly softened, sunken and cleared for a distance of approximately 3–5 mm. from the edge of the colony, the outer edge of the gelase field being very sharply defined. The colonies later increase in diameter to as much as 10 mm., the gelase fields to 2–3 cm.

Colony formation. Sw. nitrate agar plate. Growth is slower than on peptone, but pigment production is much more marked. After 48 hours, colonies are 1 mm. in diameter, with a dark brown to black center and a colorless periphery. Pigmented granules can be seen lying among the cells.

Sw. peptone agar slant. Abundant growth after 24 hours, spreading, slightly raised, smooth, glistening, mucoid, dirty white to dark grey in color. Agar digestion is evidenced only by a general softening of the slant. After several days, a pale brown, diffusible pigment is produced by some strains.

Sw. nutrient gelatin slant. Good filiform grey growth after 24 hours with considerable liquefaction. Slant completely liquefied after 1 week.

Sw. nutrient gelatin stab. Fair filiform growth after 24 hours, best at surface. Napiform liquefaction, complete after 7 to 10 days.

Sw. peptone broth. Heavily turbid after 24 hours with grey pellicle and flocculent grey sediment. Later a light brown, soluble pigment is produced.

Physiological characteristics. Aerobic. Temperature range 5–30°C.; optimum 23°C. Salt range 0.25–6.0 per cent; optimum 2–4 per cent.

Biochemical characteristics. Catalase positive. A pigment varying in color from grey through light brown to black is produced. Nitrates reduced to nitrites. Indole negative. Urease negative. H_2S not produced. Can utilize nitrates and ammonia as sole sources of nitrogen.

Action on carbohydrates: Attacks glucose, galactose, lactose, maltose, and cellobiose with very slight or no acid production, but not arabinose, xylose or sucrose. Of the polysaccharides, agar is extensively softened but not liquefied; starch is rapidly decomposed. Cellulose, chitin and alginic acid are not attacked.

Vibrio fuscus n. sp.

Morphology. Small slightly curved rods, usually single, sometimes in short chains. Length 1.5–5.0 μ; average 3.0μ. Width

0.7μ. Very actively motile by means of polar flagella. Not encapsulated, non-sporeforming, gram-negative.

Cultural characteristics. Colony formation. Sw. peptone agar plate. After 48 hours, colonies are 1 mm. in diameter, round, smooth, glistening, translucent, entire, very pale yellow in color and slightly sunken in the agar. After 1 week, the colonies increase in diameter to several millimeters, becoming at first bright yellow and later pale brown in color. They are sharply sunken in the agar and surrounded by rather narrow, sharply defined gelase fields. Except on very heavily seeded plates, liquefaction does not occur.

Sw. peptone agar slant. Fair growth after 48 hours, filiform, smooth, glistening, translucent, pale yellow, slightly sunken in the agar. Later a pale yellow diffusible pigment may be produced, and the streak tends to become light brown in color. On old slants the agar is slightly liquefied.

Sw. nutrient gelatin slant. Filiform, smooth, pale yellow growth after 48 hours, with slight liquefaction. After 1 week, liquefaction is almost complete.

Sw. nutrient gelatin stab. Filiform growth after 48 hours, with slight liquefaction, colorless, best at the surface. Later the liquefaction becomes stratiform and is ultimately almost complete.

Sw. peptone broth. Good growth after 48 hours, turbid, with a granular sediment and a yellow pellicle.

Physiological characteristics. Aerobic. Temperature range 5–30°C.; optimum 20–25°C. Salt range 1.0–5.0 per cent; optimum 2.0–4.0 per cent.

Biochemical characteristics. Catalase positive. A pigment varying in color from pale yellow to light brown is produced. Nitrates reduced to nitrites. Indole negative. Urease negative. H_2S not produced.

Action on carbohydrates: Attacks xylose, glucose, galactose, lactose, maltose, sucrose and cellobiose, but not arabinose. Starch, chitin and alginic acid are not attacked. Cellulose is attacked to a slight extent and agar is softened and sometimes liquefied.

Group II. Pseudomonas species

The genus *Pseudomonas* is used in the sense of Kluyver and van Niel (1936):

Ellipsoidal to rod-shaped bacteria, either immotile or motile by means of cephalotrichous flagella. No endospores formed. Chemoheterotrophic, oxidizing various organic compounds. Gram-negative. Adapted to life in neutral to slightly alkaline media.

Following the classification of Bergey's Manual (1939) the two forms included here should be placed in the genus *Flavobacterium* or *Cellulomonas*; however, it is inacceptable to me to use genera based on such superficial characteristics as pigmentation or ability to attack cellulose. Admittedly the immotility of *P. iridescens* and *P. droebachense* makes it impossible to assign them with certainty to the genus *Pseudomonas*, but the difficulty of placing non-motile forms is inherent in a system which lays weight on the type of flagellation.

Both species are long, gram-negative, non-sporeforming, immotile rods. *P. iridescens n. sp.* possesses the peculiarity of exhibiting beautiful iridescence by *reflected* light at certain stages of growth on solid media. Pijper (1919, 1923, 1925) has described and explained the iridescence of young cultures of many bacteria on solid media when examined by *transmitted* light. He showed that this phenomenon was due to a highly regular arrangement of the individual cells, which grow in such a way as to produce (in thin layers) a natural diffraction grating. The iridescence of *P. iridescens* is different from this rather common type in that it is never apparent when cultures are examined by transmitted light, and occurs only in isolated, microscopically visible patches or bands of the colony or streak. Attempts to observe a regular arrangement of the cells in these regions have been unsuccessful, although the iridescence is still clearly visible at a magnification of 300 diameters. However, the phenomenon is undoubtedly due to a mass regularity of the cells acting as a diffraction grating of the reflecting type; any disturbance of the colony, as by touching it with the tip of a needle, causes the instant disappearance of iridescence.

P. iridescens is one of the commonest species along the coast of the North Pacific; it is often encountered in enrichment cultures, and occurs very frequently on the surface of red algae.

P. droebachense, a species similar in many ways to *P. iridescens*, but without the latter's characteristic iridescence, can also be found commonly on the surface of algae, but occurs only rarely in enrichment cultures. It was first isolated by Lundestad (1928) but not described very completely.

Ps. iridescens n. sp.

Morphology. Slender, single rods, of variable length. Length 1.5–7.0μ; average 5–6μ. Width 0.2–0.3μ. Capsules not formed, non-sporeforming, non-motile, gram-negative.

Cultural characteristics. Colony formation. Sw. peptone agar plate. After 48 hours, colonies are 2–3 mm. in diameter, concave, sunken, smooth, glistening, translucent, pale yellow, with an irregular edge. After 2 to 3 days a marked iridescence becomes noticeable which persists for about a week. Later the colonies become differentiated into a rather rough, opaque, bright yellow, sharply sunken central portion and a flatter nearly colorless translucent periphery.

Sw. peptone agar slant. Fair growth after 48 hours spreading, smooth, glistening, translucent, slightly sunken, pale yellow, iridescent, of a butyrous consistency. After about a week the surface becomes warty and the iridescence decreases and disappears.

Sw. nutrient gelatin slant. After 48 hours, filiform, colorless growth. Some strains do not liquefy the gelatin, others produce a slow liquefaction.

Sw. nutrient gelatin stab. After 48 hours, filiform, growth, best at surface. Some strains cause liquefaction in 7–10 days.

Sw. peptone broth. Slight even turbidity after 24 hours, later becoming heavily turbid and "silky" with a light yellow granular pellicle.

Physiological characteristics. Aerobic. Temperature range 5–30°C.; optimum 23°C. Salt range 0.25–6.0 per cent; optimum 1.0–4.0 per cent.

Biochemical characteristics. Catalase positive. A pigment

varying in color from pale to bright yellow is produced. Nitrates not reduced. Indole negative. Urease negative. H_2S not produced.

Action on carbohydrates: Attacks xylose, glucose, galactose, lactose, maltose, sucrose and cellobiose with slight acid production, but not arabinose. Starch and cellulose are attacked, but not chitin and alginic acid. Agar is softened but not liquefied.

Ps. droebachense nov. comb.

Synonyms: *Bacterium droebachense* Lundestad, 1928: *Flavobacterium droebachense* Bergey *et al.*, 1934.

Morphology. Slender, single rods, variable in length. Length 1.0–7.0μ; average 5–6μ. Width 0.1–0.3μ. Not encapsulated, non-sporeforming, non-motile, gram-negative.

Cultural characteristics. Colony formation. Sw. peptone agar plate. After 48 hours, colonies are 1–2 mm. in diameter, slightly raised, smooth, glistening, pale yellow with an irregular edge. After a few days the colonies become highly membranous and adherent, and the color changes to an orange-brown.

Sw. peptone agar slant. Fair growth after 48 hours, filiform, smooth, glistening, opaque, yellow. In older cultures the streak becomes differentiated into an orange-brown center and a buff-colored periphery. Growth is membranous and slightly sunken in the agar.

Sw. nutrient gelatin slant. Scanty, filiform, yellow growth after 48 hours: some strains show slight liquefaction which becomes more pronounced later. Other strains do not liquefy gelatin.

Sw. nutrient gelatin stab Slight filiform growth after 2 to 3 days, best at surface. Some strains produce crateriform liquefaction which becomes complete in 10 to 14 days.

Sw. peptone broth. After 48 hours, turbid, "silky" growth. After 3 to 4 days, a yellow membranous pellicle forms, which later becomes brown.

Physiological characteristics. Aerobic. Temperature range 5–35°C.; optimum 25°C. Salt range 1.0–5.0 per cent; optimum 2–4 per cent.

Biochemical characteristics. Catalase positive. Orange-brown

chromogenesis. Nitrates reduced to nitrites. Indole negative Urease negative. H_2S not produced.

Action on carbohydrates: Attacks arabinose, xylose, glucose, galactose, lactose, maltose, sucrose and cellobiose, usually with very slight acid production. Starch is attacked by strains which liquefy gelatin. Cellulose and alginic acid are attacked, but not chitin. Agar is softened but not liquefied, small, hazily-defined gelase fields being produced.

Group III. Cytophaga species

Although the physiological characteristics of *C. diffluens* and *C. krazemieniewskae*[3] are in many respects different from those of the previously described members of this genus, their morphological resemblances are unmistakable. Both organisms are flexible rods, not possessing a rigid cell wall and showing creeping motility on solid media; apart from a few rotatory or flexing movements, motility does not occur in hanging drops and wet mounts. Cell division is by constriction. Characteristic for both species is the rapidly spreading growth on solid media (due to the creeping motility) which precludes the possibility of obtaining isolated colonies. Neither species has ever been obtained from enrichment cultures or by the direct streaking of algal material; all the strains studied have been found on the daily seawater count plates made at the Scripps Institution, on which these forms occur quite commonly at certain times of year. Their inability to develop in enrichment cultures is probably due to their complex nitrogen (or growth factor) requirements.

The two species are culturally and biochemically very similar, although morphologically strikingly different. (See plate I, figs. 1–4.) Their morphology can only be studied properly in wet mounts; the fragile cells often become considerably distorted when smears are made. Among the artifacts occurring in stained preparations (particularly of *C. krzemieniewskae*), the "spiro-

[3] As pointed out by Dr. R. E. Buchanan (personal communication), the generic designation "krzemieniewskii" used by Stanier (1940) is incorrect, since in Polish the surnames of women have a feminine ending; hence the correct adjectival form is "krzemieniewskae."

chaetal" forms, which are never observable in living cells, are specially noteworthy (see plate I, fig. 3). It should be recalled in this connection that Hutchinson and Clayton (1919), who first isolated a representative of this group, characterized it as a spirochaete; it is probable that they were led to this conclusion largely through the observation of similar artifacts.

Cytophaga diffluens Stanier, 1940

Morphology. Pointed, sometimes spindle-shaped, flexible rods. Length 4–10μ; average 6μ. Width 0.5–1.5μ. In old cultures peculiar involution forms, consisting of long, very thin, highly flexible threads are found. Cell division is characteristically by constriction. Creeping motility on solid surfaces; immotile except for a few rotatory and flexing movements in liquids. Non-microcyst forming. Gram-negative.

Cultural characteristics. Sw. peptone agar plate. Colony formation in the ordinary sense has never been observed. On a streaked plate after 2 to 3 days wide, hazy, barely visible patches of growth, shallowly sunken in the agar occur and extend rapidly over the whole surface of the plate. Examination of the edge of the growth over a period of 15 minutes shows clearly the movement of the advancing masses of cells. The color of the growth is at first pink, later becoming orange. As the culture becomes older, the growth sinks irregularly into the agar, its surface having an etched appearance. The gelase field never extends much beyond the outer edge of growth, but after 7 to 10 days, particularly if the medium be buffered with $CaCO_3$, extensive liquefaction takes place.

Sw. peptone agar slant. Thin, diffuse, glistening, pink growth after 48 hours, sunken in the agar. Later the surface of the growth tends to become irregularly pitted, and the color changes to orange. Liquefaction of the agar becomes evident after a week, ultimately almost complete in a buffered medium. In the absence of buffers the medium becomes markedly acid in the course of a few days and growth may cease.

Sw. nutrient gelatin slant. Slight, spreading colorless growth after 48 hours accompanied by considerable liquefaction. Liquefaction complete in from 2 to 5 days.

Sw. nutrient gelatin stab. Filiform growth, best at surface, with rapid crateriform saccate liquefaction which becomes complete in 3 to 4 days.

Sw. peptone broth. Turbid growth with suspended flocs, often with a heavy pink pellicle after 2 days. A pink floccular sediment accumulates later.

Physiological characteristics. Aerobic. Temperature range 15–30°C.; optimum 25°C. Salt range 1.5–5.0 per cent; optimum 2–4 per cent.

Biochemical characteristics. Weakly catalase positive. Pink to orange chromogenesis. Nitrates reduced to nitrites. Indole negative. Urease negative. H_2S not produced.

Action on carbohydrates: Attacks xylose, glucose, galactose, lactose, maltose and cellobiose but not arabinose or sucrose. Cellulose and alginic acid are decomposed, but not chitin and starch. Agar is liquefied.

Cytophaga krzemieniewskae Stanier 1940 emend.

Morphology. Long, flexible rods, sometimes slightly pointed and spindle-shaped. Length 5–20μ; average 12μ. Width 0.5–1.5μ. Cell division by constriction. Creeping motility on solid surfaces: immotile except for rotatory and flexing movements in liquids. Non-microcyst forming. Gram-negative.

Cultural characteristics. Sw. peptone agar plate. Growth is similar in most ways to that of *C. diffluens*. It differs in that after 4 to 5 days the surface assumes a warty appearance due to local accumulations of the cells in drop-like masses having the appearance of immature fruiting bodies (see plate II, fig. 2). These droplets contain perfectly normal vegetative cells, and no evidence of microcyst formation in them has ever been found. The color of the growth is at first pale pink, but after 5 to 7 days this is masked by the production of a brown to black soluble pigment.

Sw. peptone agar slant. Similar to *C. diffluens*, but differing in the color (see above) and warty appearance.

Sw. nutrient gelatin slant and stab. Similar to *C. diffluens*, liquefaction less pronounced.

Sw. peptone broth. After 2 days, cloudy, "silky" growth with

a pink granular sediment. Later the medium becomes dark brown.

Physiological characteristics. Aerobic. Temperature range 15–30°C.; optimum 25°C. Salt range 1.5–5.0 per cent; optimum 2–4 per cent.

Biochemical characteristics. Weakly catalase positive. Produces a pink water-insoluble and a dark brown water soluble pigment. Nitrates reduced to nitrites. Indole negative. Urease negative. H_2S not produced.

Action on carbohydrates: Attacks xylose, glucose, galactose, lactose, maltose and cellobiose with slight acid production, but not arabinose and sucrose. Cellulose, alginic acid and starch are decomposed, but not chitin. Agar is liquefied.

TAXONOMY OF THE AGAR-DIGESTING BACTERIA

Although the majority of the agar-decomposing bacteria so far described have been representatives of the *Vibrio-Pseudomonas* group (*sensu* Kluyver and van Niel), it is evident that not all forms capable of attacking agar aerobically belong here. Panek's (1905) *Bacterium betae viscosum* is undoubtedly a lactic acid bacterium, probably of the heterofermentative type; Gorseline (1933) encountered peritrichously flagellated gram-negative forms; Mulder (personal communication to Dr. van Niel) has isolated an aerobic sporeformer with this property; and in the present work it has been shown that *Cytophaga* species, which are representatives of the *Myxobacteriales* (Imsenecki and Solntzeva, 1936, Stanier 1940), are also to be counted among the agar-decomposers. Nor is this property to be found solely among the *Schizomycetes*; Richter (1903) demonstrated that some diatoms can decompose agar, and Cohen (1940) has also shown it for myxomycetes. It is thus apparent that the ability to hydrolyze this polysaccharide, like the ability to hydrolyze cellulose and starch, is widespread among microörganisms.

In view of this, a physiological genus *Agarbacterium*, as proposed by Angst (1929), is clearly taxonomically unsound. It might still be argued, however, that separate genera should be set aside for agar-decomposing bacteria within the families to

which they morphologically belong, as has been done for the cellulose-decomposers with the genera *Cellvibrio, Cellfalcicula, Cellulomonas* and *Cytophaga*. That such a taxonomic policy would be undesirable is shown clearly enough by the unsatisfactory state of some of the genera of cellulose-decomposers referred to above.

The genus *Cellvibrio* was created in 1929 by Winogradsky for organisms morphologically typical vibrios, which attack cellulose and grow poorly (if at all) on other substrates. Inorganic nitrogen is the preferred source, although one of Winogradsky's species could grow slowly on peptone agar. In 1930, Kalnins described a large number of cellulose-decomposing vibrios which produce abundant growth on "ordinary" media and attack a number of simple carbohydrates; some of them even liquefy gelatin. Another cellulose-decomposer, *V. agar-liquefaciens* of Gray and Chalmers (1924), attacks agar rapidly and grows well in peptone-containing media. Closely related to this is *Vibrio andoi* of Aoi and Orikura (1928), which is, however, unable to attack cellulose, although it attacks various other hemicelluloses in addition to agar. In the present work it has been shown that *V. granii* attacks cellulose, but this form is unable to develop with mineral nitrogen alone, showing abundant growth only with peptone as a nitrogen source. *V. beijerinckii*, on the other hand, does not attack cellulose but grows well with inorganic nitrogen sources. There exists, in fact, a whole series of species, obviously closely related to the typical *Cellvibrios*, yet excluded from the genus by their inability to attack cellulose, or by their abundant growth on "ordinary" media. This being so, the genus *Cellvibrio* had best be abandoned; all the polysaccharide-decomposing species can be placed in the genus *Vibrio*, where their natural relationships can be brought out by the adoption of an intrageneric system of natural grouping like that used by Chester for the treatment of the genus *Bacillus* in the fifth edition of Bergey's Manual.

Similarly, the genus *Cytophaga* was created by Winogradsky for obligate cellulose-decomposing bacteria of a peculiar morphological type. The two species described in the present work are morphologically typical representatives, but although they can

attack cellulose slowly, all their other biochemical characters are at complete variance with the physiological requirements of the genus as defined by Winogradsky. They attack a number of carbohydrates in addition to cellulose; they liquefy gelatin and, far from being inhibited by the presence of complex nitrogenous materials, peptone and yeast extract are the only nitrogen sources with which growth can be obtained. The genus, however, need not be abandoned. It has a sound morphological basis in that its representatives are simple myxobacteria forming neither fruiting bodies nor microcysts, and it should be retained without Winogradsky's reservations as to nitrogen requirements and carbohydrate specificity.

These examples do not imply that the use of biochemical characters in creating genera is always unsound; on the contrary, a division of genera within a morphological group on the basis of *fundamental* katabolism as carried out by Kluyver and van Niel in 1936, can be entirely satisfactory both from the theoretical and the determinative standpoint. As long as one deals with obligate and facultative anaerobes, which exhibit well-defined and sharply separated katabolic types (fermentations) generic divisions on a physiological basis work well. However, at present our knowledge of the mechanism of oxidative katabolism is not extensive enough to decide whether here also a recognition of major "types" will be feasible. Hence, it is impossible to use the same approach for a biochemical subdivision of the strictly aerobic bacteria. To make the divisions on the basis of the ability or inability to hydrolyze certain polysaccharides or to use nitrates or ammonia as the sole nitrogen source is neither theoretically sound nor practically useful.

THE ECOLOGICAL PROBLEMS

In the present work, a study has been made of the commonly-occurring marine agar-decomposing bacteria of the California coast. Several species previously described from European waters—*V. granii*, *V. beijerinckii* and *P. droebachense*—have been regularly encountered. Nevertheless, *Pseudomonas gelatica*, which according to Gran (1902), Lundestad (1928) and Waksman

and Bavendamm (1931) appears to be a very widespread species in the Atlantic has not been found in this region, whereas *P. iridescens*, which in my investigations has occurred frequently in enrichment cultures and direct isolations, has never been described by previous workers. Further studies will be needed to find out whether or not these exceptions have a real ecological importance. The fact that *Cytophaga diffluens*, *C. krzemieniewskae* and *V. fuscus* have not been previously discovered is probably to be ascribed to the impossibility of obtaining them by the usual enrichment culture methods.

One point of great ecological interest does emerge from the investigations made so far on this group of bacteria; namely, that a very clear cut division exists between the marine and the terrestrial agar-decomposers. None of the species described as occurring in soil, manure and other land habitats has ever been found in the sea or *vice versa*. During the present work a number of enrichment cultures for soil agar-digesters were set up in an attempt to obtain further information on this point. A preliminary investigation of the isolated strains showed that they were entirely dissimilar to the marine species. They could be roughly divided into three groups; rapid agar-liquefiers of the *Vibrio agar-liquefaciens-Vibrio andoi* type (this group seems to be rather rare in California soils); agar-softening, non-sporeforming gram negative peritrichous rods showing yellow chromogenesis—possibly similar to the type described by Nicol (1931): and agar-softening non-sporeforming encapsulated gram-negative peritrichous rods similar in their general characters to *Achromobacter pastinator* Gorseline, but with weaker agar-decomposing abilities. The conclusion is unavoidable that the microörganisms active in this part of the carbon cycle in the ocean are specifically different from those playing a similar rôle in the soil.

This appears to me to suggest a new approach to what still remains the central problem of marine microbiology, the question of the existence of specific marine bacteria. Investigators from Fischer (1894) on have expressed opinions (usually affirmative) on this question, but the evidence so far brought forward does not permit an unequivocal answer to be given. (See Benecke (1933)

for an exhaustive review of the literature.) Most of the experiments undertaken in attempting to settle the matter have dealt with the temperature range and salt tolerance of marine bacteria, usually without a realization of the instability of such physiological characters. The work of Baars (1927) shows very clearly the fallaciousness of this approach. Baars was able to interconvert at will *Vibrio desulfuricans*, *V. aestuarii* and *V. thermodesulfuricans*, three varieties of sulfate-reducing bacteria which, purely on a basis of physiology (temperature and salt ranges) and habitat, had previously been regarded as separate species. Many other pertinent examples could be cited. One would naturally expect that bacteria normally living in the ocean would grow better in media containing 3 per cent NaCl than in freshwater media, and would have a low temperature optimum and thermal death point, as has been shown by many workers (see, e.g., ZoBell and Conn, 1940); but merely to establish this fact contributes nothing to the real problem, since the possibility still exists that such organisms are only varieties of similar soil forms, in the same way that *V. aestuarii* is a variety of *V. desulfuricans*. Because the marine organisms studied in this manner have usually been casual isolations about whose biochemical behavior little or nothing is known, comparisons with parallel soil forms have been excluded. However, it is clear that such comparisons are essential, since the existence of specific marine bacteria necessarily implies that organisms carrying on a certain process in the cycle of matter in the ocean differ from those carrying on the same process in the soil. If it can be shown that such differences do in fact exist in a number of microbial processes, the problem will be much closer to solution. What is needed is a series of intensive studies of small, biochemically specialized, bacterial groups whose soil representatives are well known and which are capable of being isolated by strictly-defined enrichment culture methods. As examples, one might mention the various autotrophs, the nitrogen-fixing bacteria and the aerobic cellulose-decomposers.

It might be added that until this ecological problem is settled one way or the other, work on marine bacteriology apart from studies on gross transformations of matter has very little point.

SUMMARY

A study has been made of the marine agar-decomposing bacteria occurring on the California coast.

Seven species have been differentiated and described belonging to the genera *Vibrio*, *Pseudomonas*, and *Cytophaga*, of which five are new species.

The taxonomy of aerobic polysaccharide-decomposing bacteria is discussed in the light of the present work.

The ecology of the agar-decomposing bacteria is considered in connection with the problem of the specificity of marine bacteria.

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PLATES

PLATE I

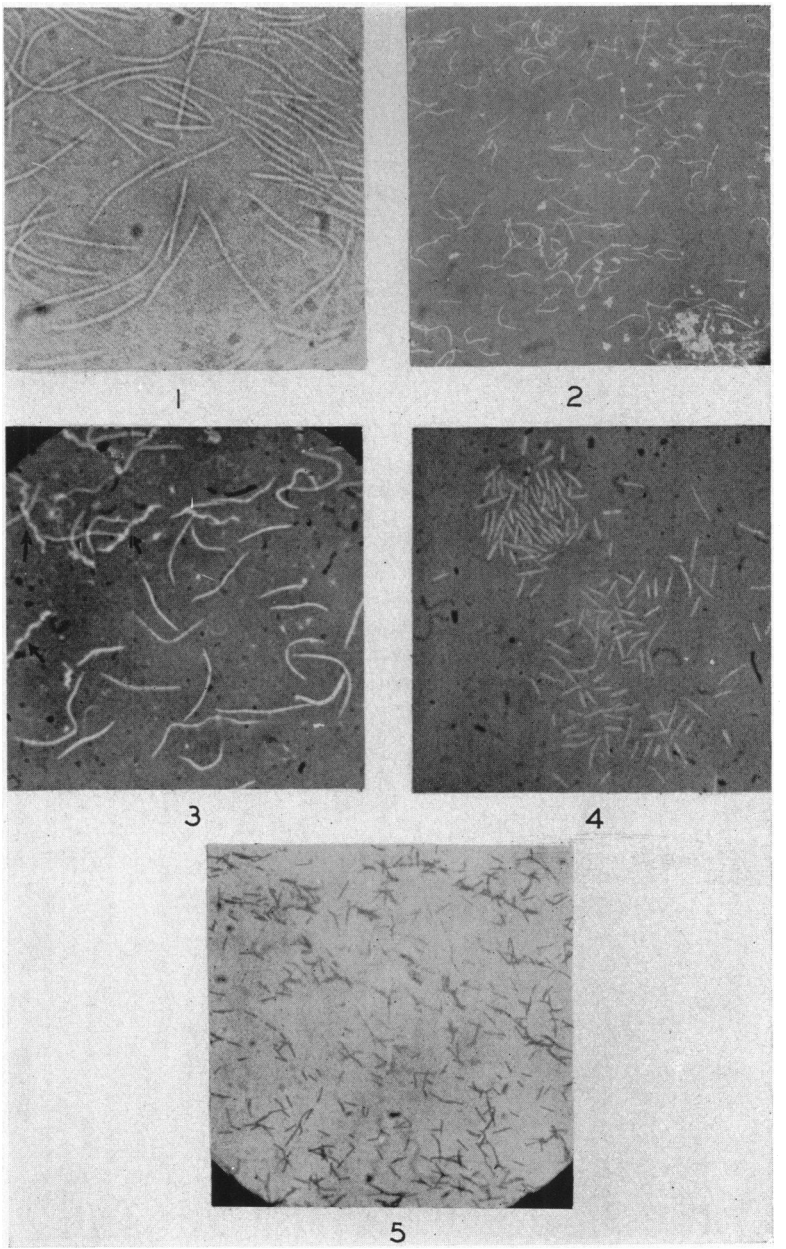
FIG. 1. *C. krzemieniewskae.* Wet mount. × 930.

FIG. 2. *C. krzemieniewskae.* Nigrosin preparation. × 400.

FIG. 3. *C. krzemieniewskae.* Nigrosin preparation. × 875. The arrows point to "spirochaetal" cells, which are artifacts. Compare with figure 1.

FIG. 4. *C. diffluens.* Nigrosin preparation. × 875.

FIG. 5. *Ps. droebachense.* Stained with gentian violet. × 875.



(R. Y. Stanier: Studies on marine agar-digesting bacteria)

PLATE II

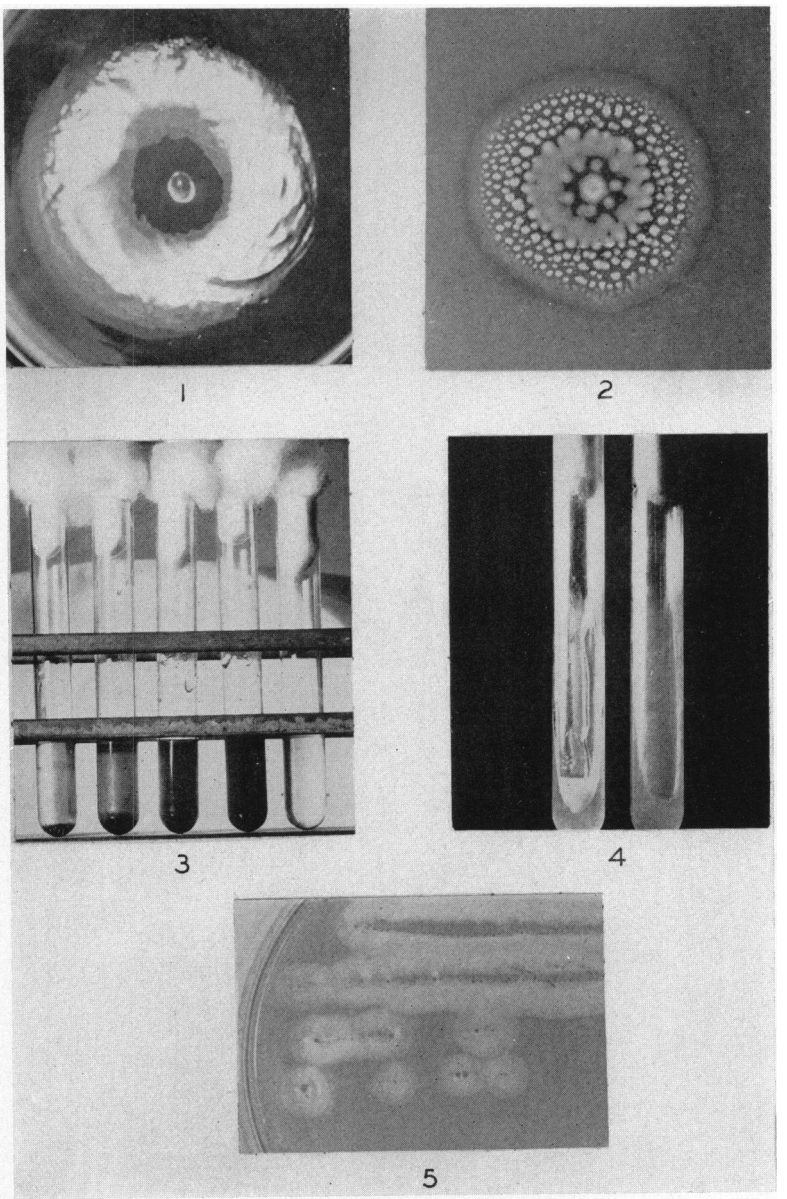
FIG. 1. *C. diffluens*. Growth after 3 days on an agar plate inoculated in the center. Slightly reduced. Photographed by reflected light to show the characteristic etched appearance of the growth due to decomposition of the underlying agar. The central dark area is more deeply sunken than the rest owing to rapid agar decomposition in that region by the inoculum.

FIG. 2. *C. krzemieniewskae*. Growth after 3 days on an agar plate inoculated in the center. × 1. Note the undifferentiated peripheral swarm and the drop-like accumulations of cells in the inner, older region.

FIG. 3. *V. beijerinckii*. Pigment production after 3 weeks in carbohydrate media with $(NH_4)_2SO_4$ as nitrogen source. Left to right: mannitol, maltose, galactose, glucose and sucrose.

FIG. 4. Left: 2 day peptone agar slant culture of a vigorously growing strain of *V. granii*. Right: uninoculated slant.

FIG. 5. *V. granii*. Portion of a streaked plate treated with I-KI solution to show the gelase fields.



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