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Studies upon the methane-producing bacteria.

By

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With 13 figures in the text.

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There is probably no important group of bacteria so poorly known as that group which is made up of methane-producing organisms. Although the formation of methane is a phenomenon of common and wide spread occurrence in nature and is of increasing importance in sewage purification and for the disposal of waste products with the simultaneous production of fuel (*Buswell* and *Neave*, 1930), the existing knowledge of the causative agents of this fermentation is still very slight, as is evident from the fact that not a single representative of the group is listed in the standard works on bacterial taxonomy (*Bergey*, 1934; *Lehmann* and *Neumann*, 1927).

The reason for this deficiency evidently is that the *methane bacteria* possess properties which make them exceedingly difficult to isolate and to study by ordinary methods. The difficulty in isolating these organisms is, in fact, so great that it has been impossible to obtain pure cultures and even attempts to separate the various members of the group from one another have until now met with little or no success.

Although there is no precise information concerning *methane bacteria* such as can only be obtained by a study of pure cultures, there are several observations upon the organisms which occur in methane-producing enrichment cultures. All these bacteria have been found to be non-spore-forming with the possible exception of those active in the methane fermentation of cellulose (*Omelianski*, 1904). The latter organisms will not be considered in the present paper.

Söhngen (1906) described two organisms which were responsible for the methane fermentation of the calcium salts of some of the lower fatty acids One was an immotile, rod-shaped *bacterium* which was particularly abundant in fermentations of the salts of butyric, caproic, caprylic and capric acids. but also was observed in acetate fermentations. This organism apparently

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could not ferment fatty acids with an odd number of carbon atoms except perhaps formic acid. It was about 5—7 μ long and 0.3—0.5 μ thick. The individual cells appear from the photomicrograph of a stained preparation to be more or less bent. They are described (though not shown) as being often joined together to form long threads which are commonly grouped into bundles or loose, ballshaped aggregates. The bundles are particularly characteristic for this bacterium. Spore formation was never observed. The organism was easily stained with the ordinary basic dyes but was not colored by the *Gram* stain. This methane bacterium of *Söhngen* appears not to have been identified with certainty nor redescribed by any later investigator although *Groenewege* (1920) and *Buswell* and *Neave* (1930) mentioned observing a rod-shaped bacterium which differed only slightly from the organism described by *Söhngen*. *Kluyver* and *van Niel* (1936) in their recent paper upon bacterial taxonomy have created the genus *Methanobacterium* for *Söhngen*'s methane bacterium.

Söhngen's second methane-producing organism is a large and conspicuous *Sarcina* which predominates in fermentations of calcium acetate. It is described as being an immotile, non-sporeforming, coccus-shaped organism, the individual cells of which are grouped into more or less cubical sarcina packets. The edges of the packets are 8—10 μ long. Like the methane bacterium, the *sarcina* was found to be *Gram*-negative. *Söhngen* believed this *sarcina* to be able to ferment formic acid in addition to acetic acid; whether it is also able to ferment the higher fatty acids remains uncertain from his work. Several later investigators have observed the *Söhngen sarcina* both in acetate enrichment cultures and in sewage sludge (*Groenewege*, 1920; *Buswell* and *Neave*, 1930; *Smit*, 1933). *Smit* mentions the possibility that the *sarcina* observed by him is identical with *Sarcina paludosa Schröter* but nevertheless he includes the organism with other anaerobic fermenting sarcinae in his new genus *Zymosarcina* under the name of *Zymosarcina methanica*. *Kluyver* and *van Niel* have, however, pointed out with good reason that the anaerobic sarcinae, though morphologically much alike, are too dissimilar in their physiological characteristics to be grouped together in one genus. They have, therefore, created the genus *Methanosarcina* for the methane-producing sarcina.

A third, perhaps distinct, type of non-spore-forming, methane-producing *bacterium* has been described by *Omelianski* (1916). This organism was found to be responsible for the methane fermentation of ethyl alcohol. In this fermentation it developed to the practical exclusion of all other types. This bacterium is a long, thin and usually bent rod which appeared to be permanently immotile. *Omelianski* does not state whether or not it is capable of fermenting substrates other than ethyl alcohol. In fact, his description is inadequate to make it certain that this organism is different from *Söhngen*'s *methane bacterium*. A comparison of the photomicrographs of the two organisms, however, suggests that they are not identical. The same organism is probably responsible for the methane fermentation of acetone (*Mazé*, 1915).

Another type of alcohol-fermenting *methane bacterium* has been described by *Groenewege* (1920). This organism is claimed to be able to ferment methyl and butyl alcohols and acetone in addition to ethyl alcohol. In cultures with the latter substrate especially it was observed to overgrow all other types of *methane bacteria*. The organism is described as a small *micrococcus*. The individual cells usually stick together to form considerable

masses. Intense staining with carbol fuchsin showed that the cells are imbedded in a slimy matrix. For this organism *Kluyver* and *van Niel* have created the genus *Methanococcus*.

Another coccus-shaped *bacterium*, which on the basis of its physiological characters may well be different from *Groenewege*'s organism, has been described in some detail by *Mazé* (1903, 1915) and has also been observed by *Omelianski* (1906). *Mazé* first noticed this organism, which he called a "*pseudosarcina*", in decaying leaves. He later found that the same organism developed in cultures in which acetone was undergoing a methane fermentation. He regarded the *pseudosarcina* as causing the decomposition of acetone but his interpretation is possibly incorrect, as the organism only appeared after the fermentation had been in progress for a considerable time. It is more probable that the *pseudosarcina* was developing upon some fatty acid formed in the primary decomposition of the acetone. *Mazé* described the organism as being somewhat polymorphic. It occured either as isolated, coccus-shaped cells or in small groups of cells or even in large masses in the form of a mulberry. These different groupings of the organism are clearly shown in a drawing. There can be no doubt, however, that some of the organisms thought by *Mazé* to be modifications of his *pseudosarcina* were actually sarcinae of the type described by *Söhngen*.

Finally, there is the *thermophilic methane bacterium* of *Coolhaas* (1928). This bacterium was claimed to cause a fermentation of formic, acetic, isobutyric and other acids but not of propionic acid or normal butyric acid. Its optimum temperature for development is 63° C.; its maximum, about 70° C. The organism is a moderately long (3—6 μ), thin, immotile rod which is usually somewhat bent and forms long threads and bundles of threads. *Coolhaas* says that it occasionally forms spores but the evidence for this appears to be exceedingly slight. No spores are visible in the photomicrograph of the bacterium. Since *Coolhaas* worked only with crude enrichment cultures, it seems probable that the occasional spores which he observed were those of a different organism. In general appearance *Coolhaas*' thermophilic bacterium certainly shows a striking resemblance to the other methane-producing rods. The high optimum temperature is not in itself a sufficient criterion to separate this organism from related mesophilic forms, for as *Kluyver* and *Baars* (1932) have proved with *thermophilic sulfate-reducing bacteria*, the ability of an organism to grow in a given temperature range is an adaptive rather than a fixed character.

This completes the list of the non-spore-forming organisms which have been described as the agents responsible for the formation of methane. It will be seen at once that all the organisms are similar in being permanently immotile and that in other respects they belong to one of two morphological types. One type has coccus-shaped cells and, therefore, belongs to the family of the *Micrococcaceae* (*Kluyver* and *van Niel*). To this group belong the *Sarcina* of *Söhngen*, the *pseudosarcina* of *Mazé* and *Omelianski*, and the *Micrococcus* of *Groenewege*. The other type has rod-shaped cells which are generally rather long, thin, more or less bent, and with a tendency to the formation of threads or filaments. This type should probably be included in the family of the *Mycobacteriaceae*. It is represented by the *butyrate-fermenting bacterium*

of *Söhngen*, the *alcohol-* and *acetone-fermenting bacteria* of *Omelianski* and *Mazé*, and the *thermophilic acetate-fermenting organism* of *Coolhaas*. Further discussion of the relation of these various organisms to one another can best be left until we have described our own observations.

Essential physiological characters of methane bacteria.

Before describing the methods which we have used for the culture of *methane bacteria* it will be well to briefly mention their more important physiological characters upon which any rational culture method of these organisms must be based.

Methane bacteria are obligate anaerobes which ferment a great variety of organic compounds with a production of methane. Their sensitivity towards oxygen seems to be greater than that of most other anaerobic bacteria. This fact when considered together with their lack of ability to produce resistant endospores accounts already to a considerable degree for the difficulty which has been experienced in culturing these organisms.

However, in addition there are several other characters which are undoubtedly very important in this connection. One of these is the necessity of carbon dioxide for the metabolism of the *methane bacteria*. This requirement has until recently been completely overlooked because of the inadequate knowledge which existed concerning the mechanism of methane formation. We have shown elsewhere (*Barker*, 1936) that the hypothesis of *van Niel* that the methane fermentation is a process of carbonate or, perhaps better, of carbon dioxide reduction is undoubtedly correct. This process is strictly analogous to the process of sulfate reduction (*Baars*, 1930) in that the molecules of the organic substrate act only as hydrogen donators. In sulfate reduction sulfate acts as the only hydrogen acceptor and is reduced to hydrogen sulfide; in the methane fermentation carbon dioxide is the hydrogen acceptor and is reduced to methane. This analogy is expressed by the general equations:

$$4\,H_2A + H_2SO_4 \rightarrow 4\,A + SH_2 + 4\,H_2O, \quad (1)$$

$$4\,H_2A + H_2CO_3 \rightarrow 4\,A + CH_4 + 3\,H_2O. \quad (2)$$

Another character of the *methane bacteria* which has made their culture difficult is their very low rate of development. This character is undoubtedly an expression of their extraordinary dissimilation process which yields, for each unit of substrate fermented, only a small amount of energy for growth. Conversely, this means that each cell must convert a relatively large quantity of substrate in order to be able to synthesize a small amount of cell material and, therefore, development will be slow.

The nitrogen requirements of the *methane bacteria* are satisfied with ammonia nitrogen.

To sum up, in developing a culture method for the *methane bacteria* it must be remembered that they are strict anaerobes which do not form spores, that in addition to a hydrogen donator (usually an organic compound) they require carbon dioxide as a hydrogen acceptor, that they grow slowly, and that they require only inorganic nitrogen sources.

Methods for the isolation of methane bacteria.

By the aid of the enrichment culture method it is easy to obtain vigorously fermenting cultures of various types of *methane bacteria*. Ordinary garden soil may be used as an inoculum for such cultures, but more rapid development of the desired bacteria is usually obtained when black mud containing hydrogen sulfide is used, because such mud contains greater numbers of non-spore-forming obligate anaerobes. The feces of herbivorous animals seem also to be a good source of *methane bacteria*. An inoculum of several grams per 100 ccm of culture medium is desirable.

In making up an enrichment culture medium for *methane bacteria*, advantage is taken of the fact that these organisms have exceedingly simple requirements. It has already been mentioned that ammonium salts suffice as a nitrogen source. The only other inorganic salts that need be added to a medium made up with tap water are potassium phosphate and magnesium chloride. The addition of sulphates should be especially avoided in order to prevent the growth of *sulphate-reducing bacteria*. The following medium is suitable: tap water, NH_4Cl 0.1 %, K_2HPO_4 0.04 %, $MgCl_2$ 0.01 %, organic substrate 1—2 %, p_H 7. The choice of the organic substrate depends upon the type of methane organism that is desired. In general, however, only comparatively simple organic compounds should be used, such as are not fermented under anaerobic conditions by the more common saprophytic bacteria. To obtain the *Sarcina* of *Söhngen* or the *Coccus* of *Mazé* calcium acetate should be used as organic substrate in the above medium. Calcium butyrate causes the abundant development of rod-shaped organisms, similar to or identical with *Söhngen*'s *methane bacterium*, along with a restricted number of cocci. In media containing ethyl alcohol the rod-shaped bacterium of *Omelianski* (1916) develops first to the exclusion of all other forms; only in cultures in which the alcohol has largely been converted into acetic acid do the coccus and to a lesser extent the sarcina appear in appreciable numbers. When alcohols are used as substrates, an equal quantity of calcium carbonate must be added to neutralize the acids which are formed. To obtain the above organisms the enrichment cultures can best be incubated at 30°—37° C. The same or similar organisms will grow at temperatures as high as 45° C. Above 45° C., especially in the region of 55°—65° C., only thermophilic varieties such as that described by *Coolhaas* develop.

An enrichment medium containing any one of the above organic compounds will allow the exclusive development of *methane bacteria* only if it is incubated under strictly anaerobic conditions. In the presence of oxygen many types of aerobic organisms can multiply as well. In order to suppress the growth of the latter, the medium, just before being inoculated, should be boiled to remove dissolved oxygen, and glass stoppered anaerobic

bottles should be used as culture vessels. Small amounts of oxygen which remain in the medium will be of no importance for the first enrichment culture, because the mud or soil used for an inoculum will contain an appreciable quantity of organic matter and large numbers of obligate or facultative aerobic organisms; the respiration of the latter will quickly remove the last traces of oxygen and will also produce the carbon dioxide which is essential for the dissimilation process of the *methane bacteria* (*Barker*, 1936).

Although in initial enrichment cultures the complete removal of oxygen and the production of carbon dioxide can be left to the activities of ordinary saprophytic bacteria present in the inoculum, in further transfers from the initial cultures special care must be taken to remove oxygen and provide carbon dioxide. This can easily be done by adding to the medium described above, after it has been boiled to remove most of the oxygen and then cooled, small amounts of hydrogen sulfide and sodium bicarbonate. For convenience these substances are added in the form of a solution containing 1 % of $Na_2S \cdot 9\,H_2O$ and 5 % of Na_2CO_3. This solution can be sterilized at 120° C without loss of the hydrogen sulfide or carbon dioxide. 3 ccm of this solution are added to each 100 ccm of medium, and then the reaction is readjusted to p_H 7 with a separately sterilized solution of hydrochloric acid. In a medium to which hydrogen sulfide and carbon dioxide have been added in this way, transfers can be made indefinitely from the initial enrichment culture.

It is characteristic of *methane bacteria* that they develop mainly in the sediment of a culture and very little in the upper layers of the liquid. Growth is frequently restricted to the sediment to such an extent that the liquid of an actively fermenting culture remains as clear as ordinary tap water. This fact has led several authors (*Coolhaas*, 1928; *Breden* and *Buswell*, 1933) to believe that some sort of inert sediment is essential for the development of *methane bacteria*. In our experience this belief has not been substantiated, but it is certainly true, in general, that cultures with a considerable sediment are more stable than those without a sediment. As a sediment we have always used either calcium carbonate or shredded asbestos (*Breden* and *Buswell*) or, in some instances, small amounts of sterile mud.

Before attempting to isolate *methane bacteria* in solid media it is desirable to purify the initial enrichment culture by three or four successive transfers. Each culture requires at 35° C. about two weeks to develop sufficiently to be transferred. A few cubic centimeters of sediment (1 ccm is sufficient) are then directly inoculated into a new medium which has been freed of oxygen by the addition of hydrogen sulfide as mentioned above. After four transfers the mud of the original inoculum will have disappeared by dilution and the number of contaminating aerobic and facultative anaerobic bacteria will be comparatively low. Such a purified enrichment culture is suitable as an inoculum for agar shake cultures.

The extreme anaerobic character of the *methane bacteria* only becomes strikingly evident when an attempt is made to grow them upon solid media. Many authors (*Söhngen*, 1906; *Groenewege*, 1920; *Coolhaas*, 1928; *Stephenson* and *Stickland*, 1933; and others) have already tried to isolate these organisms by this means, but only one (*Mazé*, 1915) has reported any success. In fact, the opinion has grown up that for some occult reason the growth of *methane bacteria* in or on solid media is not possible. This is manifestly only a prejudice, for *Mazé* had observed already in 1915 the formation of colonies of

his "*pseudosarcina*" in agar tube cultures. However, it is apparent that it is exceedingly difficult, if not impossible, to obtain growth upon ordinary agar plates, probably because the *methane bacteria* are killed by even a brief exposure to the air. Our own attempts in this direction have so far always resulted in failure.

These facts led us to use a culture method whereby all contact of the organisms with the air could be avoided. For this purpose agar shake cultures in test tubes were employed. The medium used was the same as has been described for the liquid enrichment cultures, only it was solidified with 2% of washed agar, i. e., agar which had been freed from all soluble organic compounds, which might favor the growth of contaminating organisms, by prolonged washing in cold water. The medium was first made up with the essential inorganic salts and the organic substrate and then sterilized. After sterilization the agar medium was cooled to about 45° C. and while it was still liquid the sterile sodium sulfide-sodium carbonate solution and the sterile hydrochloric acid solution were added. The medium was then poured into six or seven sterile test tubes. One tube was inoculated with about 0.5 ccm of sediment from an actively fermenting, purified enrichment culture, and the other tubes were inoculated successively each from the preceding tube so that a series of dilutions resulted. The tubes were then allowed to solidify, covered with a layer of paraffin[1] to prevent evaporation and contact with the air, and placed in an incubator at 35° C.

Development of the *methane bacteria* in these shake cultures is indicated by the production of gas which causes a splitting of the agar and a rising of the paraffin plug. The time required for the gas production to become visible in this way in a particular tube depends, of course, upon the size of the inoculum. The greater the dilution of the inoculum, the longer is the time required for the agar to begin to split. The first three dilutions commonly show gas production within two weeks. Fourth dilutions have been observed to show gas in from 8 to 39 days; fifth dilutions in from 13 to 60 days; sixth, from 20 to 110 days; and seventh, from 30 days on up. Some series of cultures never develop in the highest dilutions. It is not entirely clear why there are such large variations in the rate of development of cultures, but most probably such factors as the absolute size and the age of the inoculum and the number and kinds of contaminating organisms can account for the observed differences. In particular, it has been observed that the organisms from very old inoculating cultures are less vigorous than those from young cultures.

The multiplication of the *methane bacteria* though slow nevertheless results in the formation of easily distinguishable colonies. Already at the time when gas production is first evident in tubes with the more highly diluted inocula, an examination of sections of the agar with a low power objective shows colonies 50—100 μ in diameter. Colonies have been observed to reach an ultimate diameter of 500—600 μ which is easily visible with the unaided eye. Photomicrographs of the colonies of various types of *methane bacteria* are shown in the figures.

[1] A mixture of one part of paraffin and one part of paraffin oil was used. The melting point of the mixture should be about 45° C.

A microscopic examination of the colonies which developed in the agar tubes showed that the organisms were in many instances the same as those which predominated in the enrichment culture which had been used as an inoculum for the tubes. Several kinds of common saprophytic bacteria also formed colonies which could, however, readily be distinguished from the colonies of *methane bacteria*. In order to prove more conclusively that the organisms which looked like *methane bacteria* were actually capable of producing methane, the colonies were picked out and inoculated into a suitable liquid medium. Because of the smallness of the colonies they were picked out from thin sections of agar with the aid of a sterile micropipette and a micromanipulator. In order to protect the organisms as much as possible from contact with the air, the micropipette was filled with the hydrogen sulfide containing culture medium and the colonies were drawn up into the pipette and inoculated into the isolation tubes as quickly as possible. For each colony a new sterile pipette was used. The small (12 × 1 cm) tubes into which the colonies were inoculated were filled to a height of about 8 cm at which point there was a constriction. The culture medium was the same as that used for the enrichment cultures. There was a small sediment of asbestos or calcium carbonate, and sterile mud. After inoculation the medium in the tubes was covered with a layer of paraffin. The tubes were, of course, protected from outside contamination by cotton plugs.

Many of these small isolation tubes inoculated with single colonies produced gas after a period of incubation. The length of time required for the fermentation to start was very variable just as with the agar cultures. The extreme variations of the incubation period were from 10 days to 6 months; the average was about 30 days. The proportion of the tubes which produced gas in any single series of inoculations was also extremely variable. Even when all the colonies used for inoculation were undoubtedly those of methane organisms, the proportion which showed a fermentation even after a very long incubation varied from one in ten to nine in ten.

Because of the small size of the isolation tubes, a fermentation once started could only last for 1—2 weeks before the substrate was all decomposed. At the end of the fermentation the tubes were broken open and the contents were transferred under aseptic conditions to special culture tubes holding about 30 ccm of medium.

The special culture tube (see Fig. 1) is a modification of the constricted tube with marble seal designed by *Hall* (1921) for the culture of gas producing anaerobes. The special feature is a short closed side arm situated just above the constriction and so designed that the glass bead, which ordinarily seals the constriction, can be rolled into it. This makes possible the removal of the bead from the constriction and the intro-

duction of a sterile 1 ccm pipette into the tube to remove samples of the sediment for examination or for inoculation into other media. The general advantage of this bead-sealed tube over the ordinary glass-stoppered anaerobic bottle is that gas may escape and nutrients may be added without the liquid coming in contact with the outside of the tube. This greatly lessens the chance of the culture becoming contaminated with air-carried organisms. The glass cap which covers the top of the tube prevents dust from collecting on the cotton plug and also greatly lessens evaporation. In such tubes cultures can be kept indefinitely by adding a sterile, concentrated solution of nutrients from time to time as the medium becomes exhausted.

Fig. 1. Anaerobic culture tube.

We have now completed the description of the general methods used in the isolation and culture of *methane bacteria*. The procedure given may, of course, be repeated several times or modified in various ways, for example, by making successive transfers from one solid medium to another instead of from a solid to a liquid medium. In principle the former procedure is much to be preferred, but we have so far only succeeded in applying it to the isolation of one special organism. Although the methods described still leave much to be desired, they have made possible the isolation[1] of several types of organisms which could be proved beyond any reasonable doubt to be the causative agents in the production of methane.

Observations upon special types of methane bacteria.

As a result of our studies, it has become possible to clearly distinguish four types of non-spore-forming *methane bacteria*, namely, a *sarcina*, a *coccus*, and two kinds of *rod-shaped organisms*. It is not to be implied that these are the only types of methane-producing bacteria. Indeed, it seems highly probable that other species will be recognized when fermentations are studied with substrates and conditions different from those which we have used. But our own experience, as well as a review of the literature, indicates that the four organisms which will be described below are the most common and perhaps the most important of the *methane bacteria*.

Methanosarcina. The *methane sarcina* is already well known from the work of *Söhngen*. This organism can, in general, readily be recognized by its large size and characteristic sarcina packets (see Fig. 2). However, both the size and the grouping of the cells vary considerably with the

[1] By this it is not meant that pure cultures in the absolute sense have been obtained; further details on this point are given below.

environmental conditions. Sometimes also the methane-producing *coccus* forms aggregates of cells which are only with difficulty distinguishable from the sarcina packets.

The *sarcina* is almost always abundant in enrichment cultures containing calcium acetate. Starting from such cultures several series of agar tube cultures were made. The agar medium, like the liquid medium, contained calcium acetate as the only fermentable carbon compound. When examined with a low magnification, the colonies of the *sarcina* are easily recognizable (Fig. 4, S. 430) by the large size of the

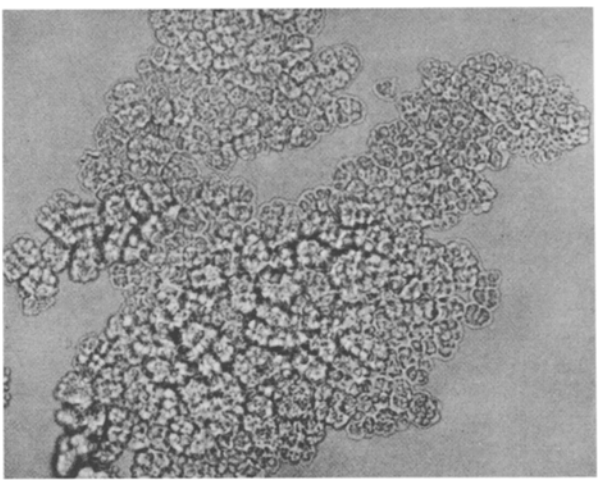

Fig. 2. *Methanosarcina methanica.* Sarcinae in crushed colony in agar. Unstained. × 520.

cells. By inoculating single colonies into isolation tubes, about twenty strains have been isolated. None of these has so far proved to be entirely free of contaminating organisms, although they all contained only the one type of methane-producing bacterium. The cultures were tested for aerobic contaminants by streaking out some of the medium upon peptone agar plates and for anaerobic contaminants by inoculating into anaerobic bottles containing a yeast extract-glucose-calcium carbonate-medium. Both types of test media were incubated at 37° C. Ten of the *sarcina* cultures showed no growth upon the aerobic plates and, therefore, were presumably free of aerobic contaminants; of these ten, three produced no fermentation in the anaerobic test medium. These latter might consequently have been regarded as being pure cultures of the *methane sarcina*. However, continued observation showed that these cultures also were undoubtedly contaminated with small numbers of anaerobic organisms. We cannot, therefore, claim to have isolated any pure cultures of the *methane sarcina*.

We can make only a few additions to the description of the *methane sarcina* given by *Söhngen*. First, the conspicuous variations in the size and appearance of the organism should be emphasized. The large and regular sarcina packets which are so characteristic of this organism (Fig. 2) under some conditions change into much smaller and less regular groups of cells (Fig. 3). Secondly, *Söhngen*, described the *sarcina* as being *Gram* negative. We have observed considerable variation in the behavior of the organism towards this stain so that it could better be described as *Gram* variable. Finally, we have examined the ability of the *sarcina* to ferment ethyl alcohol and have found that this compound is not attacked.

In agreement with *Kluyver* and *van Niel* we feel that the *methane sarcina* should be placed in a separate genus from the other fermenting sarcinae and, therefore, recognize the name *Methanosarcina methanica (Smit) Kluyver et van Niel.*

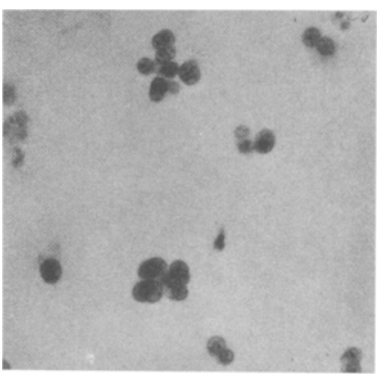

Fig. 3. *Methanosarcina methanica.* Cells in old liquid culture. Unstained. × 820.

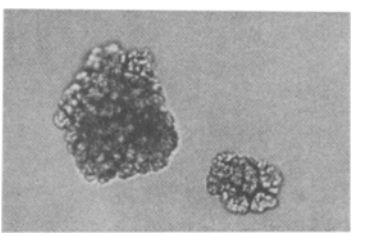

Fig. 4. *Methanosarcina methanica.* Colonies in agar. Unstained. × 200.

Methanococcus. Early in our attempts to isolate the *methane sarcina* we obtained a culture from a single colony which, though it fermented calcium acetate vigorously, did not contain any sarcinae. A closer microscopic examination of the culture showed that it contained large numbers of small *micrococci* which sometimes occurred singly but more usually adhered to one another to form large irregular aggregates. Not infrequently there were observed in addition many sharply contoured bodies ranging in size from 5 μ to 100 μ and in shape from spherical to what *Mazé* described as mulberry-shaped. These bodies commonly appeared to be quite homogeneous but sometimes could be seen to be made up of small round cells. It seemed probable that the micrococci and these larger bodies or cysts represented different aspects of the same organism, especially since they showed a close resemblance to the cyst forming bacteria previously described by *Müller-Thurgau* (1908). An attempt was made, therefore, to isolate the methane-

producing micrococcus in order to prove that it actually was capable of forming the observed cysts.

Several additional cultures of the *micrococcus* were isolated from calcium acetate enrichment media by the use of agar tube cultures. Typical colonies in agar are shown in Fig. 9. The organism grows and ferments rather more vigorously than the *sarcina* and, therefore, is easier to isolate. None of these original isolation cultures was free from contaminants and, therefore, a second series of agar tube cultures was made from these cultures. But again all the newly isolated cultures proved to be contaminated. Further purification was attempted by transferring single colonies directly from one agar tube culture to another instead of always transferring from a solid medium back into a liquid medium. It should be mentioned that the agar medium of these cultures contained 2 % of a clear mud extract in addition to the usual constituents. The solid medium to solid medium transfers were successful and after an incubation of seven weeks, twelve out of fourteen tubes showed gas formation and contained many colonies of the *micrococcus*. In all tubes, however, there also developed colonies of contaminating organisms. The process of isolation was repeated once more by transferring again to a solid medium but still without complete success. The *methane*

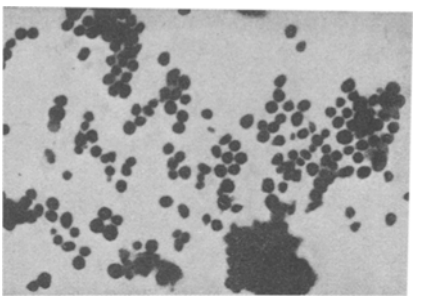

Fig. 5. *Methanococcus Mazei.* Cells in crushed colony in agar. Stained with erythrosine. × 1000.

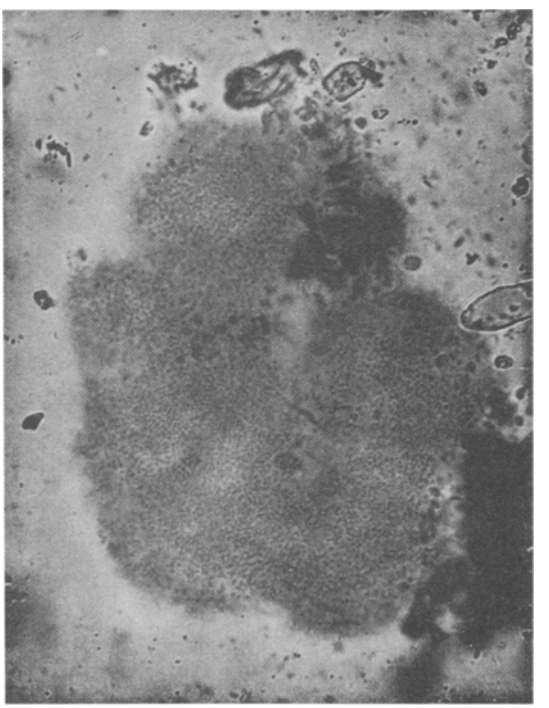

Fig. 6. *Methanococcus Mazei.* Large aggregate of cells in liquid culture. Unstained. × 340.

micrococcus, like the *sarcina*, therefore still remains to be obtained in strictly pure culture.

The isolation of the *micrococcus*, though not completely successful, has been carried far enough to make certain that the isolated coccus-shaped cells (Fig. 5), the large irregular aggregates (Fig. 6), and the sharply defined cysts of various sizes (Fig. 7 and 8) as observed in liquid

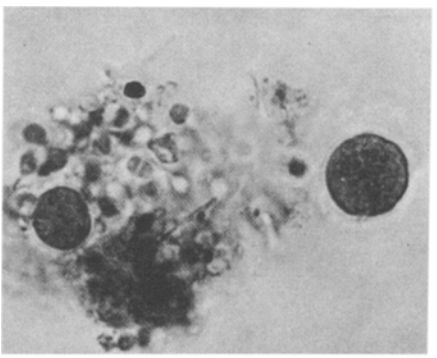

Fig. 7. *Methanococcus Mazei.* Single cells and small cysts in liquid culture. Stained with erythrosine. × 1350.

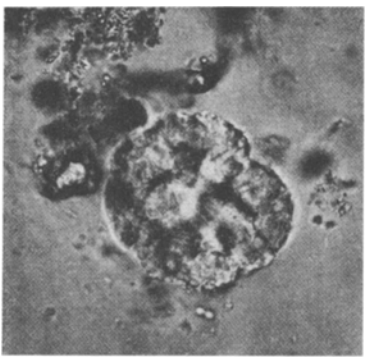

Fig. 8. *Methanococcus Mazei.* Large cyst in liquid culture. Unstained. × 340.

cultures are all forms of one organism. Cultures which have been purified by the repeated isolation of colonies from solid media retain the same heterogeneous appearance as comparatively crude cultures. Further,

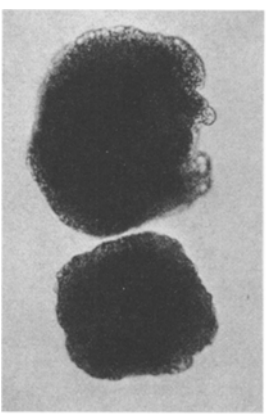

Fig. 9. *Methanococcus Mazei.* Colonies in agar. Unstained. × 200.

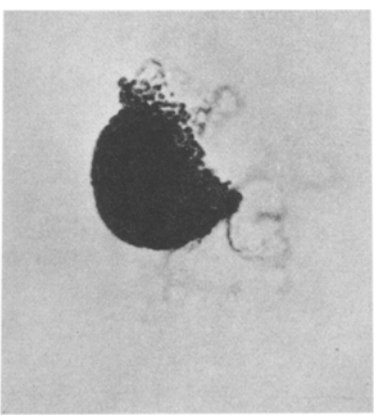

Fig. 10. *Methanococcus Mazei.* Partially broken cyst. Stained with erythrosine. × 1000.

it is frequently possible to see in one culture many stages of transition (Fig. 10) from one type of cell association to the next. Characteristic aggregates and cysts are shown in Fig. 6, 7 and 8.

Once we had learned to recognize the *micrococcus* we became aware of the fact that it was common in all enrichment cultures containing acetic and butyric acids. This organism also appeared in the late stages of the fermentations of ethyl or butyl alcohols after these substrates had been partly converted into the corresponding acids, and in spontaneous methane fermentations of sewer mud. It would appear that the *micrococcus* is one of the most active methane-producing organisms in nature.

The *micrococcus* readily ferments acetic and butyric acids. Propionic acid may also be slowly fermented but this is not yet certain. Ethyl and butyl alcohols apparently cannot be attacked. The organism stains readily with erythrosine. Its behavior to the *Gram* stain is variable.

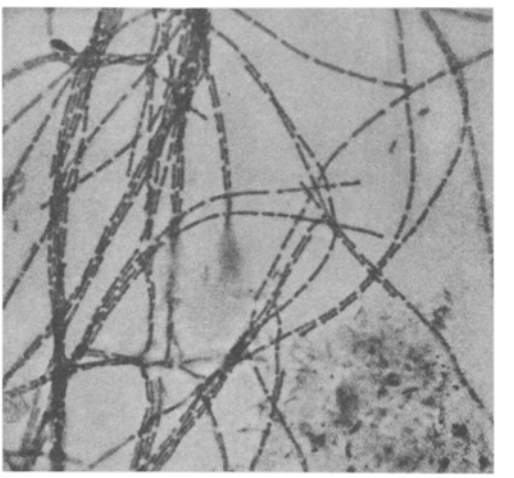

Fig. 11. *Methanobacterium Söhngenii*. Characteristic threads and bundles in liquid medium. Stained with erythrosine. × 1000.

The *micrococcus* which we have studied is undoubtedly the same as that described by *Mazé* (1915). This author did not give the organism any name but referred to it only as a *pseudosarcina*. *Groenewege* (1920) described a morphologically similar organism which differs, however, from our bacterium in its presumed ability to ferment ethyl alcohol. We have never observed a bacterium of this morphological type in alcohol enrichment cultures. *Groenewege* also did not use any name, but on the basis of his description *Kluyver* and *van Niel* have created the genus *Methanococcus*. There can be no doubt but that our bacterium must be placed in this genus. In view of the fact that *Mazé* was the first to give a clearly recognizable description of this type of methane organism, we propose to name our bacterium *Methanococcus Mazei*.

Methanobacterium. The two rod-shaped *methane bacteria* which we have observed are morphologically somewhat similar, but they may be sharply distinguished on the basis of their physiological properties, since one ferments acetic acid and not ethyl alcohol while the other ferments ethyl alcohol and apparently not acetic acid. The acetate-fermenting rod will be considered first.

The fatty acid-fermenting methane bacterium occurs most abundantly in enrichment cultures containing calcium butyrate as the only organic compound though it also is nearly always to be found in cultures containing calcium acetate. The four strains which we have studied were isolated from the latter type of enrichment culture by means of an acetate agar medium. None of these first isolations was entirely free of contaminating bacteria and we have not as yet attempted any further purification.

This organism in liquid cultures at once draws attention by its long bent threads, composed of rods joined end to end, which frequently lie parallel to one another in small bundles (Fig. 11). In other instances

Fig. 12. *Methanobacterium Söhngenii.* Slightly crushed colonies in agar. Stained with erythrosine. × 300.

the arrangement is more irregular and the individual threads are so bent and intertwined as to appear like a loosely tangled ball of yarn. The colonies in solid media are also characteristic (Fig. 12). The center of a colony is compact and with a moderate magnification can be seen to be made up of bundles of threads lying in close contact. At the edges of the colony some of these bundles grow away from the main mass and penetrate into the surrounding agar in all directions for a distance about as great as the diameter of the central compact part. This gives an irregular appearance to the colony as a whole. When the individual threads either from colonies in agar or from liquid cultures are examined without being stained, the spaces between the individual cells are frequently difficult to see. It is only as a result of staining that the composite structure of the threads is clearly evident. The individual rods

are straight or only slightly bent. When the bacterium is stained with erythrosine (Fig. 11), the individual rods can be seen to be most deeply colored at their ends. Frequently there are several more or less regularly spaced regions in the rods which stain only lightly or not at all. The outcome of the *Gram* stain is negative.

The physiological characters of this bacterium are similar to those of *Methanosarcina methanica* and *Methanococcus Mazei*. Acetate and butyrate but not propionate are fermented. Alcohols are not fermented.

It seems highly probable that this organism is identical with *Söhngen*'s *methane bacterium*. Although the photomicrograph which he gives shows a bacterium which could hardly be identified with ours, yet his description of the characteristic thread and bundle formation in liquid cultures leaves little doubt as to the essential similarity of the two organisms. For *Söhngen*'s *methane bacterium Kluyver* and *van Niel* suggested the generic name of *Methanobacterium*. In remembrance of *Söhngen*'s fundamental work upon the methane fermentation, it is appropriate to name the bacterium which we have studied *Methanobacterium Söhngenii*.

The second rod-shaped *methane bacterium* is, as we have already mentioned, distinguished by its ability to ferment alcohols. This bacterium can be readily obtained with enrichment cultures containing ethyl or butyl alcohols and an excess of calcium carbonate. The calcium carbonate is essential not only to neutralize the acids that are formed by a dehydrogenation of the alcohols, but also, as we have shown elsewhere (*Barker*, 1936), to act as an acceptor for the hydrogen resulting from these conversions. Enrichment cultures, particularly with ethyl alcohol, develop rapidly and can be repeatedly transferred without difficulty.

In agar tube cultures made up with ethyl alcohol and calcium carbonate or sodium bicarbonate and inoculated from the enrichment cultures, colonies develop and gas is produced frequently to the seventh dilution. As yet we have made only a few attempts to start new cultures by transferring single colonies to liquid media. Of the isolation cultures which have been started, only one so far has developed a methane fermentation of ethyl alcohol. Our description of the alcohol-fermenting organism is, therefore, largely based upon the examination of purified enrichment cultures.

In liquid media these *methane bacteria* appear as rather long, thin, immotile rods which are typically somewhat bent (Fig. 13). The length of the rods and the degree of bending are variable. The rods are more delicate than those of *M. Söhngenii* and they never are joined together to form the long threads so characteristic of the latter. Spore formation has never been observed. This organism is *Gram* negative.

The known physiological characteristics of this methane-producing organism may be briefly summed up by saying that it is able to dehydrogenate ethyl alcohol to acetic acid, butyl alcohol to butyric acid, and perhaps also butyric acid to acetic acid. Apparently it cannot attack acetic acid, since this substance accumulates to a considerable concentration when ethyl alcohol is being fermented; the acetic acid only disappears in alcohol enrichment cultures after other types of methane organisms have become numerous.

This alcohol fermenting organism is sufficiently similar morphologically to *Methanobacterium Söhngenii* to be included in the same genus. Since it is clearly very closely related to, if not identical with, the alcohol fermenting rod described and pictured by *Omelianski* (1916), we propose to name this organism *Methanobacterium Omelianskii*.

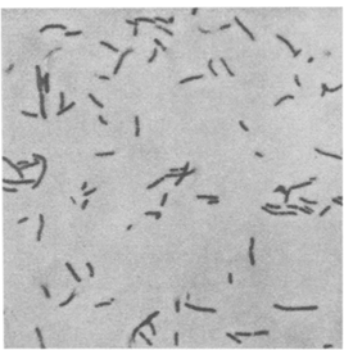

Fig. 13. *Methanobacterium Omelianskii.* Cells in liquid culture. Stained with erythrosine. × 1000.

Of the various non-spore-forming *methane bacteria* which have been mentioned in the literature and in the introduction to this paper, there are only two which cannot be more or less definitely identified with the four organisms described above.

One of these is the thermophilic, fatty acid-fermenting, rodshaped bacterium of *Coolhaas*. Morphologically this bacterium is very similar to *Methanobacterium Söhngenii*. Indeed, it only differs from the latter by being thermophilic. For reasons we have already given, this character does not seem sufficient to justify the creation of a separate species. Until further information is available regarding this organism it can, therefore, best be considered as a thermophilic variety of *Methanobacterium Söhngenii*.

The other methane organism which seems not to belong to any of the above species is the *micrococcus* described by *Groenewege* (1920). This micrococcus must belong to the genus *Methanococcus*, but it differs from *Methanococcus Mazei* in its supposed ability to ferment alcohols. However, this character does not seem to us to have been established beyond reasonable doubt. *Groenewege*'s observations were made upon sediments containing organisms which had been grown primarily upon acetate containing media. Only after a heavy development of various kinds of methane bacteria was ethyl alcohol added to the cultures. It was observed then that the fermentation of the alcohol was accompanied by an increase in the number of micrococci. *Groenewege* interpreted this

as meaning that the micrococcus fermented the alcohol, but it seems quite conceivable to us that actually the alcohol was converted into acetic acid by another organism and that the micrococcus developed on the acid. We have observed just this phenomenon in many alcohol enrichment cultures. The existence of an alcohol-fermenting micrococcus must, therefore, be considered doubtful for the present.

In conclusion, the known morphological and physiological characters of the methane bacteria which have been described may be summarized as follows:

Methanosarcina methanica (Smit) Kluyver et van Niel. Large spherical cells characteristically grouped to form cubical sarcina packets. Tetrads and, more rarely, diplococci also are formed under some conditions. Permanently immotile and non-spore-forming. *Gram* variable. Acetic acid and possibly also butyric acid fermented with a production of methane. Ethyl alcohol is almost certainly not fermented. The availability of other substrates is unknown.

Methanococcus Mazei n. sp. Small spherical cells occur singly, in small groups, or in large, irregular and somewhat slimy aggregates. Very characteristic also are sharply contoured cysts of various sizes and forms. Permanently immotile and non-spore-forming. *Gram* variable. Acetic and butyric acids are fermented with a production of methane. Ethyl and butyl alcohols are not fermented.

Methanobacterium Söhngenii n. sp. Rod-shaped, permanently immotile cells. Non-spore-forming. The rods are moderately long, and straight to slightly bent. They are characteristically joined into long threads which lie parallel to one another so as to form bundles. *Gram* negative. Acetic and butyric acids are fermented with production of methane. Ethyl alcohol is not fermented.

Methanobacterium Omelianskii n. sp. Thin, frequently bent rods. The rods single or joined to form short threads. Permanently immotile and non-spore-forming. *Gram* negative. Ethyl alcohol is fermented to acetic acid and butyl alcohol to butyric acid with methane formation. The same organism probably also ferments butyric acid to acetic acid. Acetic acid is almost certainly not fermented.

Summary.

1. A survey has been given of the non-spore-forming methane-producing organisms on the basis of the rare descriptions scattered throughout the literature.

2. The reasons for the great difficulty of isolating these organisms have been discussed and as a result methods have been developed which make possible the growth of various representatives of this group in solid media.

3. For the first time highly purified cultures of four different types have been obtained by transferring single colonies into appropriate culture media but it has not as yet been possible to isolate strictly pure cultures.

4. Nevertheless, the purified cultures allowed of a positive identification on the basis of morphological and physiological characters of four clearly distinct types of methane-producing organisms which have been described under the names of *Methanosarcina methanica*, *Methanococcus Mazei*, *Methanobacterium Söhngenii*, and *Methanobacterium Omelianskii*.

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