STUDIES ON *PSEUDOMONAS METHANICA* (SÖHNGEN) NOV. COMB.[1]

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Ample evidence exists that numerous bacteria can use various gaseous hydrocarbons as a sole source of carbon and energy. From the relatively limited information currently available (ZoBell, 1950; Davis and Updegraff, 1954), it is clear that these organisms show differing specificities in their attack on hydrocarbons.

Considering that methane is the preponderant gaseous hydrocarbon in nature, surprisingly little work has been published on its utilization by bacteria. Methods for isolation and study of methane-utilizing organisms require development and improvement. As it stands, characterization of these bacteria morphologically, culturally and physiologically to meet standards acceptable to modern systematics is a problem almost entirely for the future.

A good illustration is provided by the paucity of information on the prototypic organism among the methane utilizers, *Methanomonas methanica*. Isolated from aquatic plant material by Söhngen in 1906 and named by him *Bacillus methanicus* (later amended by Orla-Jensen (1909) to *Methanomonas methanica*), this is the organism classically associated with methane utilization; its name is perpetuated in most bacteriological textbooks and in all schemes of classification. Curiously enough, the only description available is that furnished 50 years ago by Söhngen. Although some later workers have claimed to have reisolated *M. methanica* from nature, it is extremely questionable whether their isolates were, in fact, identical with Söhngen's *M. methanica*.

Thus, contrary to generally held assumptions, the role of *M. methanica* in the biological utilization of methane in nature is unclear, and will remain so until studies on its distribution in nature are forthcoming. Furthermore, the extent to which other bacteria participate in the utilization of methane in nature is virtually unknown.

A comprehensive study of methane-utilizing bacteria was begun in this laboratory a few years ago. This first report deals with the description and classification of a methane-utilizing bacterium, identical with *M. methanica*, which we have shown to be widely distributed in nature.

MATERIALS AND METHODS

Routine culture of isolated methane-utilizing organisms was carried out in 160 x 22 mm test tubes (cotton plugged or covered with loose aluminum caps) or in small Erlenmeyer flasks fixed in 10-L desiccators which provided a closed gas space. Tubes contained 10 ml of medium and were fixed at a 45° angle in the direction of shaker movement (see below). The desiccator was gassed with a mixture of 50 per cent methane (99.0–99.2 mol per cent[3]; "Pure Grade", Phillips Petroleum Co., Bartlesville, Oklahoma) and 50 per cent air. All gases were passed through a long cotton-stuffed tube attached to the desiccator. Incubation took place at 30 C, either in a stationary condition or on a reciprocating shaker (30 5-in. strokes per min). The liquid medium (Medium D developed in the course of these investigations as best for routine culture of the methane-utilizing organism) had the following composition: $NaNO_3$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4 \cdot 7H_2O$, 1.0 mg; Na_2HPO_4, 0.21 g; KH_2PO_4, 0.09 g; Cu (as $CuSO_4 \cdot 5H_2O$), 50 μg; B (as H_3BO_3), 10 μg; Mn (as $MnSO_4 \cdot 5H_2O$), 10 μg; Zn (as $ZnSO_4 \cdot 7H_2O$), 70 μg; Mo (as MoO_3), 10 μg; Ca pantothenate (filter sterilized), 0.2 g; agar extract (see text below for explanation), 3.0

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[3] Impurities: ethane, 0.26–0.32 mol per cent; carbon dioxide, 0.12–0.16 mol per cent; nitrogen, 0.26–0.32 mol per cent.

ml (90 mg solids); distilled water, 1 L. For a solid medium 20 g of whole agar (Difco) were substituted for the agar extract. Under these conditions, maximal growth, representing about 4×10^8 cells per ml, could be obtained in 2 to 3 days.

For initial primary enrichment cultures the procedure of Söhngen (1906) was followed as closely as possible. Triturated leaves and stems of the aquatic plant *Elodea* obtained from a shallow, fresh water pond were used as inoculum. Approximately 0.1 g of the fresh plant material was placed in a 125-ml Erlenmeyer flask containing 25 ml of Söhngen's medium ($MgNH_4PO_4 \cdot 6H_2O$, 1.0 g; K_2HPO_4, 0.5 g; $CaSO_4$, 0.1 g; distilled water 1 L; pH adjusted to 6.6–6.8). The flask was incubated without shaking at 30 C under an atmosphere of 40 per cent methane and 60 per cent air.

Cells for manometric experiments were obtained from pure cultures in 1 L of medium in a 4.5-L closed suction flask gassed with a methane-air mixture. The flask was incubated for 2–4 days on the shaking machine by which time suitable growth had developed. The cells were harvested and water washed by centrifugation. Manometric techniques were as described by Umbreit *et al* (1949) and were conducted at 31 C.

Quantitative measurement of growth was made turbidimetrically in a Klett-Summerson colorimeter with a 540 mμ filter. Cells were uniformly dispersed before measuring growth by placing the tube in an electric vibrator for a few seconds. Check counts made in a Petroff-Hauser chamber indicate that 1 Klett-Summerson unit represented approximately 10^6 cells per ml.

Gas analyses on a microscale were performed with an instrument designed and constructed by Dr. D. M. Updegraff and Mr. W. B. Huckabay of the Field Research Laboratories, Magnolia Petroleum Co., Dallas, Texas. The determinations are based on successive selective absorptions of the gases under consideration and measurement of the decrease in volume of the gas sample. Carbon dioxide was absorbed by 1 per cent KOH solution, oxygen by alkaline pyrogallol solution. Content of residual methane was determined by difference.

Enzyme studies were done with extracts of sonically disrupted, washed cells. Disruption of the cells in a Raytheon 10-kc sonic oscillator was followed microscopically. The great majority of the cells were broken in 30 to 45 min. Extracts were partially clarified by centrifugation at 4 C at 10,000 rpm for 15 min. The supernatant liquid and the particulate fraction were tested for activity.

Total organic carbon was determined by oxidation with potassium dichromate in a concentrated H_2SO_4-H_3PO_4 mixture. The CO_2 produced was trapped in NaOH solution, precipitated as $BaCO_3$ by the addition of $BaCl_2$ solution, filtered, dried and weighed.

RESULTS

Isolation of the Methane Utilizer

After 7 to 10 days of incubation of the primary enrichment culture described above, there appeared a distinct pinkish pellicle on the surface of the medium, as described by Söhngen (1906). A loopful of the pellicle was then transferred to the same medium and reincubated under the same conditions. In 3 to 5 days, growth in the form of a pellicle again appeared. Subsequent transfers failed to yield significant growth, suggesting a lack of essential nutrients. However, a loopful of the pellicle material from the second enrichment flask streaked on Söhngen's solid medium in petri plates (made from water-washed agar), resulted in the appearance of small, pink colonies after incubation for 5 to 7 days. Serial dilutions of suspensions of pink colonies were successively streaked out until pure cultures were obtained. Criteria adopted for purity were: homogeneous morphology as determined by microscopic examination of living and stained cell preparations; absence of non-pink colonies on streak plates of medium D incubated in methane-air; absence of growth on nutrient agar in air.

Description of the Pink Isolate

The organism is a gram negative rod, usually occurring singly, but sometimes in pairs or in chains up to four cells in length (figures 1 and 2). The cells, when stained with basic dyes, are characterized by the presence of an unevenly staining intracellular material giving the cells a mottled appearance. This resembles very closely the description and photographs given by Söhngen (1910). Dimensions of cells 7 days old were 0.6 x 1.0 μ. Younger cells (36 hr) were somewhat

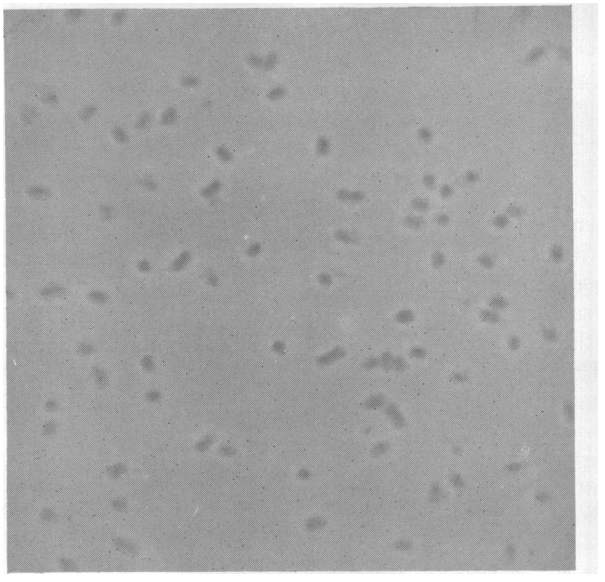


Figure 1. Dark field phase microscopy

thinner and longer, 0.4 x 1.5 μ. The cells are highly motile in young (36 hr) or old (7 days) liquid cultures, possessing a single, polar flagellum (figure 3).

Colonies on agar media usually were pink, the shade varying in different experiments. They are raised, whole, circular, convex, smooth and range from 1 to 3 mm in diameter after a few days (figure 4) becoming considerably larger with prolonged incubation. Although the colonies have a mucoid appearance, they have a tacky consistency and after one to two weeks incubation, become difficult to disrupt with a needle. The pinkness tends to be more concentrated in the central portion of the colony and with increasing age the newer peripheral growth has considerably less pigment; the latter apparently contains a large amount of slime matter. Both slime formation and pink pigmentation coincide with the description of *M. methanica* (Söhngen, 1910).

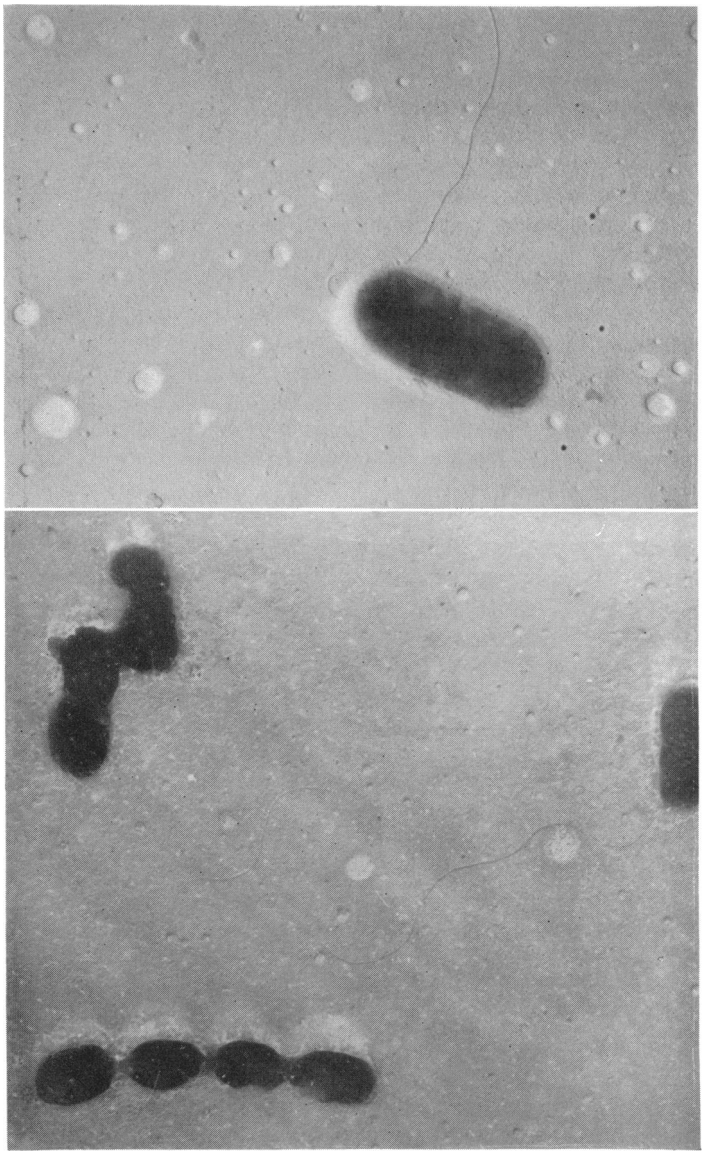
Growth in liquid media shaken during incubation was either dispersed or clumpy (figure 5). Seemingly, those cultures containing a large enough amount of slime assumed a uniformly turbid, highly viscous character, whereas those producing only enough slime to coat the cells resulted in clumped or agglutinated growth.

In unshaken cultures, the freshly isolated organism grew either as a thin, reddish-pink membrane on the surface of the medium, or as a pink turbidity throughout the medium, with accumulation of sedimented cells as the culture aged. Apparently a balance of conditions determines the type of growth. A tendency toward dispersed growth was noted after the organism had been maintained in the laboratory for some time.

Pigment Production

The methane utilizer, under most conditions of growth, produces an intracellular pink pigment. Pigmentation is subject to environmental influences since the pinkness was not always evident, either in colonies or in liquid cultures, nor was the pigmentation always proportional to the amount of growth. This was especially true when the medium was deficient in organic growth adjuncts and also in young cultures in a "complete" medium. Frequently, pigmentation was not striking in well-grown shake cultures, but its presence was unmistakable when the cells became concentrated by settling out through clumping or by centrifugation.

Addition of dilute acid decolorized the pigment. The pigment was insoluble in water, soluble in *n*-hexane, chloroform and ether. The absorption spectrum of a chloroform extract of dried cells showed a clear cut main peak at 503 mμ. Properties of the pigment resemble the general characteristics of the carotenoid pigments (Goodwin, 1952).



Figures 2 and 3. Electron microscopy

Figure 4. Colonies (with millimeter scale)

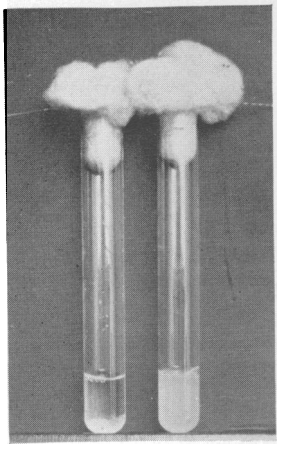


Figure 5. Clumpy (left) and dispersed (right) growth in liquid medium shaken continuously during incubation.

Growth Factor Requirements

Although colonies developed fairly well on Söhngen's salts-washed agar medium, the organism grew very poorly in the same medium without agar, and would not grow from small inocula. The possibility of the presence in agar of an essential growth factor presented itself. This was supported by the fact that the use of 6 per cent carboxy-methylcellulose as a substitute solidifying agent failed to result in growth. An active factor could be extracted from agar and concentrated in the following manner: (1) 20 g of agar (Difco) were spread in a thin layer in a pan, covered with 400 ml of cold deionized water and stored at 4 C for about 12 hr (low temperature was intended to decrease the solubilization of the agar as well as to prevent bacterial growth); (2) the suspension was then filtered through paper and the filtrate concentrated *in vacuo* on a steam bath to 20 ml. One ml of the concentrate now represented the extract of 1 g of agar and contained approximately 30 mg of dry weight material. This concentrate was added in graded doses to Medium D (without pantothenate or agar extract) and its ability to support growth of the bacterium observed. Clearly it was required for growth (table 1). It is also evident from table 1 that agar was not the active factor. The failure of the organism to develop in a corresponding series of tubes incubated in 50 per cent air without methane demonstrated that the agar extract was not functioning as a source of carbon.

TABLE 1

*Comparison of agar extract and whole agar in supporting growth of the pink methane-utilizing bacterium in liquid medium**

Supplement	Amount Added	Growth
	μg/ml	*Klett-Summerson units*
Agar extract	0	0
	2.5	12
	7.5	14
	75	92
	250	100
	750	197
	1500	90
Whole agar	2.5	0
	15	0
	750	0
	1500	4

* Medium D in 50 per cent methane in air; culture 36 hr old.

The extraction procedure described above did not remove all the active factor from the agar. Thus, extracted agar used as a solidifying agent (2 per cent) supported some development of the colonies but was less suitable than unextracted agar. Under these conditions, pigmentation of the colonies was considerably reduced.

Certain properties of the active factor(s) were next examined. Methanol or ether extracts of whole, dry agar were devoid of activity. The insoluble residues, when subsequently extracted with water as previously outlined, yielded the active material. Dialysis of the agar extract in the cold for 24 hr against 25 volumes of distilled water resulted in only a slight loss of activity. Autoclaving at pH 2 (with HCl) for 15 min at 120 C resulted in complete loss of activity. Two ml of the concentrate was treated with 20 ml of cold acetone, the precipitate collected and made

to 2 ml with distilled water. All the activity was recovered in the precipitate.

The foregoing data suggest that the active material may be either polysaccharidic in nature, or bound to a fairly large molecular weight polysaccharide. Accordingly, a number of naturally occurring polysaccharides were tested and found to be ineffective in replacing the agar extract: potato starch, gum camphor, gum arabic, gum mastic, gum guaic, and gum ghatti. In addition, neither gelatin, egg albumin nor the slime produced by the methane utilizer was active.

Next, the following complex materials were tested (100 μg per ml of medium) for their ability to replace agar extract as a source of the growth factor: casamino acids (Difco), yeast extract (Difco), basamine (Busch), liver (Difco), corn steep liquor (Staley), peptone (Difco), brain-heart infusion (Difco) and water-extracted agar. None displayed significant activity as compared to agar extract under these conditions.

A survey of known vitamins showed that calcium pantothenate was capable of partially replacing the agar extract. It is noteworthy, however, that the concentration of calcium pantothenate required for maximal effect, i. e. 10 to 100 μg per ml, was considerably higher than ordinary for this vitamin. The effect was not attributable to a requirement for calcium as shown by the ineffectiveness of calcium chloride. In the absence of methane but in the presence of air, no growth was obtained, thereby eliminating the possibility that the vitamin, at high levels, was used as a source of carbon and energy. β-Alanine and pantoyl lactone were tested individually and together at levels up to 300 μg per ml and were inactive. When agar extract and calcium pantothenate were used conjointly, the combination in appropriate concentrations led to more growth than did either alone.

The active material in agar is not likely to be pantothenic acid since 250 μg of agar extract (representing 8.3 mg whole agar) per ml of medium was required for maximal growth, whereas 10 μg or more per ml of calcium pantothenate was required to achieve nearly the same response. Thus, the agar extract solids would have to contain 4 per cent pantothenic acid and whole agar 0.12 per cent.

One of the more attractive interpretations of these findings, especially because there are similar precedents in the case of other bacteria, is based on the assumption that the methane utilizer is highly deficient in its ability to convert pantothenic acid to the functional form, coenzyme A. Thus, pantethine and pantethine-4′-phosphate are much more active than pantothenic acid for growth of *Lactobacillus bulgaricus* and of *Acetobacter suboxydans*, respectively (Snell *et al.*, 1950; King *et al.*, 1949; Baddiley *et al.*, 1953). It may be that agar extract contains a form of pantothenic acid more active than the free vitamin. It is unique in its apparent conjugation with a high molecular weight component(s) in the extract. This situation is under study and will be reported separately.

Five additional strains of the pink bacterium have been isolated by Mr. E. Leadbetter from various sources; each requires the same organic growth factors as does the original isolate.

Physiological Studies

Phosphate concentration. The methane utilizer exhibited maximal growth in a rather narrow range of concentrations of phosphate, 0.002 M phosphate being the optimum.

Nitrogen source. Nitrate appeared to be the best source of nitrogen. $(NH_4)_2SO_4$ and NH_4Cl were utilized poorly while $NaNO_2$ and asparagine could not be utilized at all.

pH Optima. This was determined in media with different sources of nitrogen, e.g., $(NH_4)_2SO_4$ or $NaNO_3$. In the case of ammonium sulfate, growth was best over a narrow, slightly acidic pH range, pH 6.0–6.6. With $NaNO_3$ the range was considerably wider, spreading from pH 6.6 to at least pH 8.0. pH measurements on the cultures after growth showed no significant shift from the initial pH values.

Composition of gas phase. In studies on bacterial utilization of gaseous hydrocarbons, gas concentrations are frequently reported in a manner too haphazard to permit exact duplication of conditions. Both percentage composition of the gas phase and the volume of the closed gas space need to be specified. Maximal growth might be limited if the total amount of a particular gas available is relatively small, even though the initial concentration was optimal with respect to rate of growth. Also, most of the information in the literature (reviewed in Beerstecher, 1954) dealing with this particular aspect is unreliable inasmuch as all the experiments were performed under inhomogeneous physiological conditions,

TABLE 2

Substrates not supporting growth of the methane-utilizing bacterium

Substrate*	Concentration Tested
	%
Glucose	0.1, 0.3, 1.0
Fructose	0.1, 0.3, 1.0
Galactose	0.1, 0.3, 1.0
Xylose	0.1, 0.3, 1.0
Sucrose	0.1, 0.3, 1.0
Maltose	0.1, 0.3, 1.0
Lactose	0.1, 0.3, 1.0
Ribose	0.1, 0.3, 1.0
Glycerol	0.3
Ethanol	0.3
Formaldehyde	0.01, 0.5
Sodium formate	0.1, 0.3, 1.0
Organic acids†	
Nutrient broth	0.1
Corn steep liquor (solids)	0.3
Basamin (Busch)	0.1, 0.3, 1.0
Peptone (Difco)	0.3
Yeast extract (Difco)	0.1, 0.3, 1.0
Beef extract (Difco)	0.3
Casamino acids (Difco)	0.1, 0.3, 1.0
Alanine	0.1, 0.3, 1.0
Glycine	0.1, 0.3, 1.0
Starch	0.3
Albumin	0.3

* The sugars, ethanol, formaldehyde and formic acid were filter sterilized. Corn steep liquor, peptone and beef extract were autoclaved separately. Albumin was not sterilized.

† Medium contained 0.1 per cent of Na salts of these acids: succinic, malic, fumaric, oxalacetic, citric, α-Ketoglutaric, *cis*-aconitic, tartaric, pyruvic, acetic.

e. g., in stationary cultures. Paradoxically, the apparatus devised by Söhngen (1906) and frequently used by others, and advocated as the most efficient equipment for this type of investigation (Beerstecher, 1954) actually is one of the worst for studies of gas metabolism. Not only are these cultures stationary, but they are characterized by surface-volume ratios extremely unfavorable for diffusion of gases as compared to the rate of consumption of the gases by the bacterium (Finn, 1954). True gas concentration effects and maximal growth rates are obtainable only under homogeneous conditions where uniform exposure of all cells at all times to a given gas phase is assured, and when the maximal gas demand of the culture is exceeded, or not limited by the rate of solution of gases. In the laboratory, these circumstances are most readily approached in short time experiments with liquid cultures in thin layers vigorously shaken continuously (for larger volumes see Finn, 1954). Growth rates far exceeding those hitherto reported for methane utilizing bacteria have thereby been achieved routinely.

(*a*) *Oxygen.* Under the conditions of the test, oxygen was apparently toxic at the concentration at which it exists in air (21 per cent and higher). An atmosphere containing approximately 15 per cent oxygen was optimal.

(*b*) *Methane.* Concentrations of methane from 10 to 90 per cent resulted in equally good and approximately maximal growth rates while 3 per cent was decidedly below the optimum.

(*c*) *Carbon dioxide.* There seemed to be a definite requirement for exogenous carbon dioxide, the optimal initial concentration in the gas phase being 0.3 per cent. The fairly low initial CO_2 requirement is not unexpected in view of the fact that as soon as the bacteria commenced to grow, metabolic CO_2 would quickly furnish adequate amounts for optimal growth.

Test of other substances as sources of carbon for growth. Of a fairly representative variety of organic materials tested in liquid medium D (table 2), only methane and methanol proved capable of supporting growth of the pink organism. Very likely methanol is an intermediate in the oxidation of methane by the bacteria, and if this be considered an example of simultaneous adaptation (Stanier, 1950), it follows that as far as the testing has gone, this organism is uniquely dependent on methane for growth. The substances listed in table 2 were tested in 100 per cent air and in 50 per cent air and 50 per cent helium or nitrogen. The culture was also found to be incapable of utilizing hydrogen gas (gas phase composition: CO_2, 10; H_2, 50; air, 40 per cent, respectively), ethane under conditions optimal for several ethane-utilizing bacteria (ethane, 40; air 60 per cent, respectively) or ethylene (ethylene, 50; air, 50 per cent, respectively). Also tested, with negative results, both in 100 per cent air atmosphere and in 50 per cent air, 50 per cent nitrogen, were *n*-hexane, *n*-heptane and *n*-hexadecane (all at 0.2–1.0 per cent). In addition, *n*-propane, *n*-butane and *n*-pentane each failed to support growth when present in a gas mixture consisting of 50 per cent hydrocarbon and 50 per cent air.

Influence of other substances on growth of the methane utilizer in methane. Various complex organic extracts had a marked inhibitory effect on growth of the pink bacterium in medium D in the presence of a 50 per cent methane, 50 per cent air gas phase (table 3). A control series run in the absence of methane, showed that those concentrations allowing growth in the presence of methane supported no growth in its absence. Neither glucose nor alanine was inhibitory. In fact, glucose appeared to stimulate growth in the presence of methane. Whether the increase in the Klett-Summerson reading reflected increased cell numbers is not certain, for cultures containing glucose apparently produced more slime as evidenced by their extreme viscosity.

Factors influencing development of enrichment cultures of the pink methane bacterium from natural sources. This study was undertaken to facilitate isolation of the organism from nature via liquid enrichment procedures. Medium D was employed with variations as described below. The gas phase consisted of 35 per cent methane and 65 per cent air. Incubation was carried out in 10 L desiccators. Assuming the presence of definitely pinkish growth in the primary and secondary enrichments to indicate the presence of the desired organism, the following conclusions may be drawn from this rather extensive survey employing many permutations of the treatments listed.

(*a*) *Phosphate concentration.* Although concentrations of phosphate from 0.002 M to 0.021 M gave varying degrees of positive results, abundant growth was observed most frequently when the phosphate concentration was 0.007 M.

(*b*) *Growth factors.* The presence of agar extract and calcium pantothenate (as in medium D) decidedly enhanced pink growth in primary enrichments from all sources studied and under all conditions studied. In certain treatments, raw sewage and pond mud yielded no sign of pink growth unless the organic adjuncts were added. Similarly, where aquatic plant material and pond mud would produce some slight pink growth in the absence of adjuncts, the presence of the latter markedly stimulated the rate of appearance and total amount of pinkness. Development of the pink methane utilizer in enrichment cultures to which no organic adjuncts were added presumably had as an explanation either a syntrophic relationship between it and other organisms present or the inoculum may have contained the essential growth factor(s).

TABLE 3

*Effect of various substances on growth of the methane-utilizing bacterium in the presence of methane**

Supplement	Concentration	Growth
	%	*Klett-Summerson units*
None		300
Yeast extract (Difco)	0.3	0
	0.1	260
	0.03	271
	0.01	268
Corn steep liquor† (Staley)	0.3	7
	0.1	6
	0.03	140
	0.01	152
Peptone (Difco)	0.03	140
Beef extract	0.3	0
L-Alanine	0.1	263
Glucose	0.3	415

* Gas phase = 50 per cent methane in air.
† Solids.

(*c*) *Source of inoculum.* As found by Söhngen (1906) for *M. methanica*, fresh water pond mud and aquatic plant material were excellent sources of the pink organism; raw sewage and garden soil were inferior. It remains to be seen whether this can be attributed to elaboration of methane by the anaerobic methane-producing bacteria in mud, to the presence in certain inocula of larger amounts of the essential growth factor(s) or to the absence of competitive and antagonistic influences of other microorganisms.

(*d*) *Shake versus stationary conditions.* Stationary cultures proved superior to shake cultures for the primary enrichments. The surface pellicle which is characteristic of the pink organism in crude enrichments in unshaken cultures may well confer on it a selective advantage under those conditions. Non-pellicular organisms would be at a disadvantage as far as availability of oxygen and methane is concerned.

Quantitative Studies of Methane Utilization

Manometric experiment. It was essential to verify, as did Söhngen (1906), that the methane-utilizing bacterium could not have been growing exclusively at the expense of impurities in the methane. A washed suspension of cells was placed in a Warburg respirometer vessel. Gas volume in the system was approximately 18,000 μL and KOH was in the center well. The system was flushed with a mixture of 50 per cent methane and 50 per cent air. According to the manufacturer's analysis,[3] the total hydrocarbon impurity present in the system calculated as ethane could not be in excess of 90 μL (4 μmoles). Oxidation of ethane entirely to CO_2 and H_2O requires 3.5 moles of O_2 per mole of ethane. Total gas uptake (ethane plus O_2) from the amount of ethane which could be present as an impurity in this experiment is 403 μL (18 μmoles). In 320 minutes the bacterial suspension took up 2,484 μL (110 μmoles) of gas in excess of endogenous, with no diminution of rate apparent at that time. Hence, methane must have been utilized.

Carbon balance of a growth culture. A 4-L suction flask containing 250 ml of medium D was inoculated with the methane-utilizing culture. A 10-ml sample was immediately withdrawn for the determination of the organic carbon initially present. After the flask was closed with a sterile rubber stopper and all rubber-glass contacts sealed with sealing compound, the flask was gassed through the side arm with a mixture of methane, oxygen and carbon dioxide. A small gas sample was withdrawn immediately in a syringe containing 0.1 N H_2SO by piercing the rubber connecting tube with the hypodermic needle. Analysis of this sample gave the exact initial composition of the gas phase. At the end of 7 days' incubation on the shaker the flask was allowed to equilibrate to room temperature, and the contents acidified by addition of H_2SO_4 through a separatory funnel penetrating the rubber stopper. Bound CO_2 was thereby released, and the gas pressure within the closed flask recorded manometrically. The gas within the flask was then analyzed. Total organic carbon present in the culture was also determined.

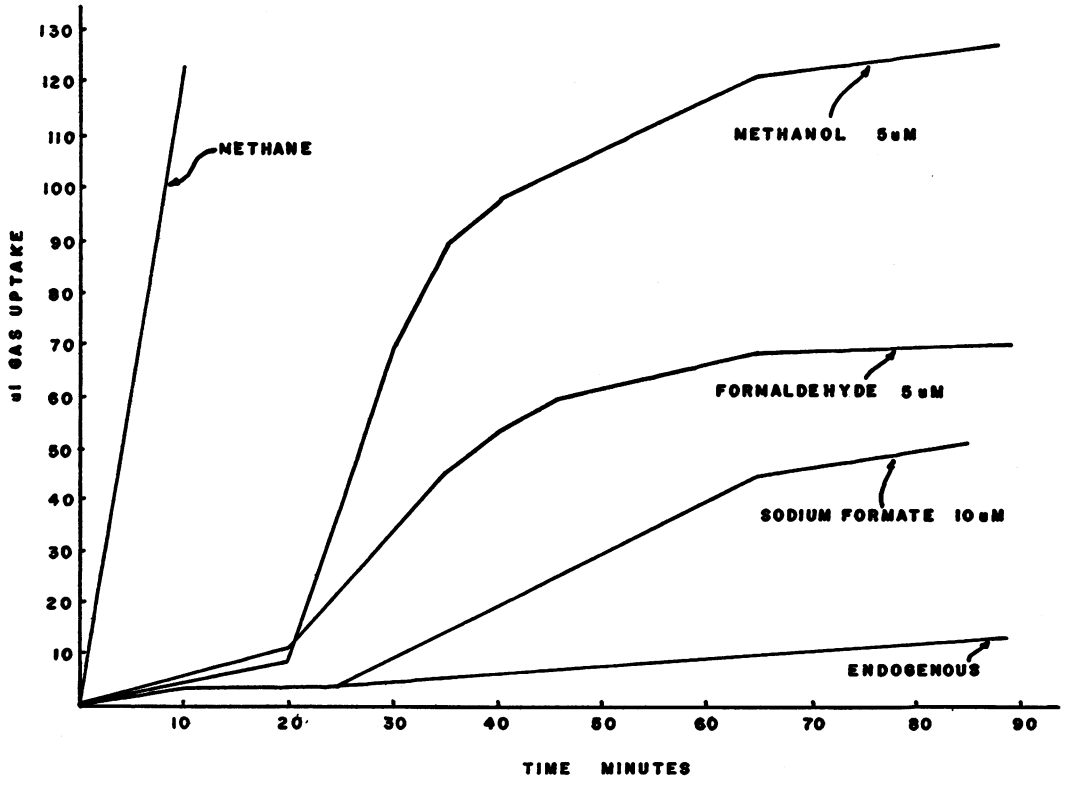


Figure 6. Oxidation of one-carbon compounds by methane-utilizing bacterium

In the first experiment, the following data were obtained: methane consumed, 31.5 mM; oxygen consumed, 16.0 mM; carbon dioxide produced, 7.2 mM; net increase of fixed carbon in the medium (including cells), 22.6 mA. A repetition gave similar results. The conversion of the methane carbon consumed to fixed carbon is unexpectedly high: namely 72 per cent.

In a separate experiment, the fixed carbon in 1 L of medium was separated by centrifugation into two fractions: washed bacterial cells and soluble slime material. The slime was separated by acetone precipitation, re-solution in water and reprecipitation with acetone, whereupon it was dried. Yields were 92.5 and 104 mg of dry weight cells and slime material, respectively. If the same proportion of slime material held true in the previously described experiment, then 35 per cent of the methane consumed was converted to cell material and 37 per cent to slime material. Hutton and ZoBell (1949), by an indirect method, computed a 10 to 40 per cent conversion of methane to bacterial cell material by *M. carbonatophila.*

Manometric experiments with "resting" cells. Although only methane and methanol supported growth, the organism can also oxidize formaldehyde and Na formate (figure 6). From the data obtained in this experiment, it may be computed that the oxygen utilized per μmole of substrate consumed was 1.0 μmoles, 0.52 μmoles and 0.16 μmoles respectively for methanol, formaldehyde and Na formate (endogenous oxygen uptake subtracted). If each substrate were oxidized entirely to CO_2 and H_2O, the amount of oxygen theoretically required would be 1.5 μmoles, 1.0 μmoles and 0.5 μmoles, respectively. The experimentally obtained values were, therefore, 67, 52 and 32 per cent of theory, respectively. On the assumption that no one-carbon oxidation endproducts other than CO_2 were formed from any of the substrates, it may tentatively be concluded that the difference between observed and theoretical oxygen uptake represents oxidative assimilation. If this is correct, this organism's inability to grow in a medium containing minerals, organic growth adjuncts and formaldehyde or formate may well be a spurious result, possibly attributable to toxicity.

From the foregoing, it is rather likely that methane oxidation by the bacterium proceeds as follows: $CH_4 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$. In all probability, this 1-carbon oxidation series represents an instance of simultaneous adaptation (Stanier, 1950). The oxidation need not necessarily occur via the free 1-carbon compounds—they could well be in coenzymatic or other bound forms. However, in figure 6, the "breaks" in the slopes of curves representing oxidation of methanol and formaldehyde, and especially in the case of the former, suggest that formate accumulated and then was oxidized after the initial substrate was largely depleted.

Cell-free extracts. Attempts to obtain active enzyme preparations from the methane oxidizer were unsuccessful. Neither vacuum-dried whole cells nor the supernatant liquor or debris of cells disrupted in a sonic vibrator showed any oxidative activity toward methane, methanol, formaldehyde or formate. Addition of di- or tri-phosphospyridine nucleotide, cytochrome C, methylene blue, mixtures of B vitamins or mineral supplements, failed to activate the enzyme preparations.

DISCUSSION

Identity of the organism as M. methanica. The new isolate coincides with *M. methanica* of Söhngen (1906, 1910) in all major taxonomic features: morphology, flagellation, methane utilization, slime formation, pink pigmentation. Although further comparison is impossible owing to the lack of details about Söhngen's organism, the more complete description of our organism allows embracement of Söhngen's bacterium, and the limits of the species *M. methanica* are defined by the information furnished in this paper.

One apparent discrepancy warrants clarification, *viz.*, growth factor requirements. In spite of Söhngen's report of growth in a purely mineral medium (plus methane), his organism could well have been auxotrophic if he used large inocula. In our experiments two or three serial liquid transfers were required before the absolute growth factor requirement manifested itself, a result ascribed to carry over of growth factor(s) in a generous inoculum. We are not inclined to the view that Söhngen's sole allusions[4] to this

[4] "Sticht man eine durch wiederholte Überimpfung bekommene Haut, wenn diese noch jung ist, so erhält man schon am zweiten Tage reine Kolonieen des *Bacillus methanicus* . . ." (Söhngen, 1906, p. 515) and "Quand on inocule assez tôt une de ces colonies dans l'appareil décrit, on obtient une novelle membrane de bactéries dans quelques jours." (Söhngen, 1910, p. 270).

point, are specific enough to be taken as constituting proof of prototrophy in this organism. The small colony size on methane-washed agar in one of Söhngen's (1910) photographs might be indicative of a deficiency of growth factors.

Already discussed was the other distinctive feature of Söhngen's description, e. g., pellicle formation. Experiments with our organism, as expected, indicate this is not a constant cultural characteristic. Whether the inconstancy has a phenotypic or a genotypic explanation is uncertain, but pellicle formation was frequently observed when our culture was freshly isolated.

Taxonomic status of the genus Methanomonas. The taxonomic status of the genus *Methanomonas* has not been critically considered since that name was introduced by Orla-Jensen in 1909. It is, therefore, appropriate to re-examine the justification for recognition of methane-oxidizing organisms as a physiological genus.

Creation of the genus *Methanomonas* by Orla-Jensen, who never worked with these organisms, was part of his revolutionary proposal to give physiological characteristics a dominant role in bacterial classification. This extreme position has never been accepted. The consequences of attempting to construct a phylogenetic system on a primarily physiological basis are too numerous to discuss here, and have been cogently presented by van Niel (1946) and Stanier and van Niel (1941). Arguments for a judicious integration of morphology and physiology have been made by Kluyver and van Niel (1936).

Ideally, recognition of a physiological trait at the generic level (or higher) should be restricted to very special instances, for example, where growth is uniquely dependent on, or linked to a particular mode of metabolism. Outstanding illustrations of this type are photosynthetic bacteria, chemoautotrophic bacteria and methane-producing bacteria. Few would question that the physiology involved here is at least as important as the morphology in adequately denoting the natural relationships involved. However, even common possession of admittedly basic metabolic characteristics is no guarantee of logical relationships, for as seen in the case of the sulfur bacteria (van Niel, 1946), recourse to physiology exclusively may lead to an amalgamation of evolutionally strange bedfellows.

The crux of the problem is that not often is there "... a clear understanding of what constitute important physiological characters ..." (Stanier and van Niel, 1941). Here is where application of what Benecke (1912) called "scientific tact" is so vital for bacterial systematics.

From the viewpoint of modern microbiology and systematics, generic designation on the basis of the utilization of particular compounds as a sole source of organic nourishment would hardly be tactful. If adopted in the case of all organic compounds arbitrarily regarded as unusual substrates, such a practice would produce taxonomic and nomenclatural chaos. Yet, evidently, this has been the sole *raison d'être* for the genus *Methanomonas* for nearly half a century. Further intimations of the difficulties engendered by the application of the property of methane utilization as a generic characteristic may be had from the realization that this property is by no means limited to the genus *Methanomonas*. Methane utilization is recognized as a specific or even varietal character satisfactorily included in at least one existing morphologically based genus, e. g., *Myocbacterium methanicum* and *Mycobacterium flavum* var. *methanicum* (Nechaeva, 1949). In the same manner would this property fit as a specific characteristic when it belongs to a member of the genus *Pseudomonas*. The alternative, recognition of physiological genera based on methane utilization and parallel to *Mycobacterium* would confer no advantage on systematics or diagnostics, and would imply a phylogenetic distinction more profound than is warranted. Furthermore, it might be a precedent for a whole series of trivial genera in connection with other individual compounds.

Not irrelevant are precedents for obligate dependence on particular compounds as substrates; for example, *Bacillus fastidiosus* (den Dooren de Jong, 1929), *Clostridium acidi-urici* (Karlson and Barker, 1949), *Diplococcus glycinophilus* (Cardon and Barker, 1946) and *Clostridium kluyveri* (Bornstein and Barker, 1948). Relegation of their distinctive properties to an importance secondary to morphology at the generic level fulfills ideally the purposes of classification whether from the point of view of diagnostics or of natural relationships.

For yet another reason the generic name *Methanomonas* should be abolished. Possession of the prefix *Methano-* in common with *Methanobacterium*, *Methanococcus* and *Methanosarcina* implies a homology which does not exist. It is unfortunate that Breed *et al.* (1948), by their

adoption of the generic designations introduced by Kluyver and van Niel (1936) for methane-*producing* bacteria, created a nomenclatural ambiguity.[5] This was not a problem in Kluyver and van Niel's scheme because they dropped *Methanomonas*; organisms of that type fall into their genus *Pseudomonas*.

Taxonomy of the pink, methane-oxidizer. The genus *Pseudomonas* accommodates the methane-utilizing bacterium here under consideration perfectly satisfactorily. It is an aerobic, gram negative, polarly monoflagellated rod. We propose, therefore, to rename Söhngen's organism *Pseudomonas methanica* (Söhngen) nov. comb. The distinctive physiological character of this species is its extremely limited substrate range: it cannot use as sole source of carbon any of a large number of conventional carbon sources and methane is the only saturated gaseous hydrocarbon which it can oxidize.

This, as well as its pink pigmentation, differentiates the species from all other described methane-utilizing pseudomonads. For these other methane-utilizing pseudomonads either the substrate range was not tested (Hasemann, 1927), a variety of non-hydrocarbon compounds could be utilized (Münz, 1915; Tausz and Donath, 1930; Bokova *et al.*, 1947), or gaseous hydrocarbons other than methane could also be utilized (Slavnina, 1948; Münz, 1915; Hutton and ZoBell, 1949). In several cases morphological and cultural characteristics are so incompletely described that it is impossible to evaluate the organism for taxonomic purposes.

Methane-dependent pseudomonads displaying cultural and physiological differences from *P. methanica* probably exist. It would not be surprising to find pigmentless or differently pigmented strains displaying the same obligate requirement as *P. methanica*. The character of methane dependency could be preserved in the names of such new species, and the genotypic variation denoted by applying a suitable prefix to the species term *methanica*.

Within this framework, pseudomonads possessing the ability to utilize other gaseous hydrocarbons in addition to methane, would also represent new species. Such pseudomonads have been described by Hutton and ZoBell (1949, 1953). Although they did not name their isolates, their apparently preliminary conclusions (Hutton, 1948) have led to the introduction into the literature of a new species of *Methanomonas*, *M. carbonatophila* (Beerstecher, 1954). Cited as distinctive to this species is homogeneous growth in liquid media and acceleration of growth by added CO_2. Such characteristics are subject to environmental influences and at best are of dubious value for specific differentiation. Certainly they are meaningless for this purpose unless the authentic species and new isolates are studied simultaneously. Evidently this was appreciated by Hutton and ZoBell for they chose not to report their isolates as a new species (Hutton and ZoBell, 1949). The small size of colonies of their organisms and the very slow rate of growth in liquid, salts-methane medium imply a growth limitation due to a deficiency of growth factors. At any rate, their organisms although resembling *P. methanica*, are distinguishable on the basis of their utilization of gaseous hydrocarbons other than methane. It is this feature which, if the organisms were more thoroughly described, would warrant their designation as a new species.

[5] Illustrative of the confusion is the description of *Methanomonas* in authoritative treatises on bacterial physiology (Frobisher, 1949, p. 614; Lamanna and Mallette, 1953, p. 361–362, 376; Porter, 1946, p. 634, 642; Skerman, 1949, p. 180; and Thimann, 1955, p. 597).

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SUMMARY

A pink methane-utilizing bacterium has been isolated in pure culture. In all respects it is identical with *Methanomonas methanica* Söhngen. Specific enrichment culture conditions have been worked out for its isolation from natural sources. A number of nutritional and physiological characteristics of the pure culture were defined. An absolute requirement for organic growth factor(s) present in a cold water extract of agar (Difco) has been demonstrated. This requirement is partially satisfied by high concentration of calcium pantothenate. Carbon balances have

been made in methane medium. Washed cells also oxidized methanol, formaldehyde and formate. Of a large number of substrates tested, including conventional substrates and gaseous and liquid aliphatic paraffinic hydrocarbons, the organism would grow only on methane and methanol. Reasons are advanced for abandoning the genus *Methanomonas*, and for reclassifying the organism as *Pseudomonas methanica* nov. comb.

ADDENDUM

Shortly before galley proof of this paper was received, a copy of N. L. Söhngen's doctoral dissertation ("Het ontstaan en verdwijnen van waterstof en methaan onder den invloed van het Organische Leven", Delft, 1906) came to hand. In it (p. 136) Söhngen states that *Bacillus methanicus* (*Pseudomonas methanica*) developed very well in media other than that containing methane as the sole source of carbon and energy. These included malt extract, yeast water or peptone; also inorganic salts solution containing asparagine, malate, lactate, citrate, or succinate. Since Söhngen's published papers (1906, 1910) on methane utilization are based on his dissertation, it is difficult to understand why Söhngen did not include this important physiological aspect in those papers, if he considered those findings reliable. In view of the fact that many methane utilizing strains exhibiting basic similarities with Söhngen's organism have been obtained in this laboratory, none of which can utilize conventional bacteriological media, the accuracy of Söhngen's observation is open to question and examination. There was no corroborative evidence in Söhngen's dissertation that the growth in the various organic media was indeed "*Bacillus methanicus.*" Difficulties we experienced in obtaining pure cultures of this organism before we elucidated details of its physiology, and the several literature reports of other methane utilizing bacteria capable of utilizing other organic substrates, information of which Söhngen was unaware, lead us to the possibility that a contaminant existed in Söhngen's culture.

In spite of this discrepancy in the claim of identity of our organism and Söhngen's, it would appear that the interests of taxonomy are served better by the information and viewpoint in this paper.

REFERENCES

BADDILEY, J., THAIN, E. M., NOVELLI, G. D. AND LIPMANN, F. 1953 Structure of Coenzyme A. Nature, **171**, 76.

BEERSTECHER, E. 1954 *Petroleum microbiology*, 375 pp. Elsevier Press, Inc., N. Y.

BENECKE, W. 1912 *Bau und Leben der Bakterien*, p. 212; 650 pp. B. G. Teubner, Leipzig und Berlin.

BOKOVA, E. N., KUZNETSOVA, V. A. AND KUZNETSOV, S. I. 1947 Oxidation of gaseous hydrocarbons by bacteria as a basis of microbiological prospecting for petroleum. Doklady Akad. Nauk. S.S.S.R., **56**, 755–757.

BORNSTEIN, B. T. AND BARKER, H. A. 1948 The nutrition of *Clostridium kluyveri*. J. Bacteriol., **55**, 223–230.

BREED, R. S., MURRAY, E. G. D. AND HITCHENS, A. P. 1948 *Bergey's manual of determinative bacteriology*, 6th ed. The Williams & Wilkins Co., Baltimore.

CARDON, B. P., AND BARKER, H. A. 1946 Two new amino-acid-fermenting bacteria, *Clostridium propionicum* and *Diplococcus glycinophilus*. J. Bacteriol., **52**, 629–634.

DAVIS, J. B. AND UPDEGRAFF, D. M. 1954 Microbiology in the petroleum industry. Bacteriol. Revs., **18**, 215–238.

DEN DOOREN DE JONG, L. E. 1929 Über *Bacillus fastidiosus*. Centr. Bakteriol. Parasitenk., II, **79**, 344–353.

FINN, R. K. 1954 Agitation-aeration in the laboratory and in industry. Bacteriol. Revs., **18**, 254–274.

FROBISHER, M. 1949 *Fundamentals of bacteriology*, 4th ed., 936 pp. W. B. Saunders Co., Philadelphia.

GOODWIN, T. W. 1952 *The comparative biochemistry of the carotenoids*, 356 pp. Chapman and Hall, Ltd., London.

HASEMANN, W. 1927 Zersetzung von Leuchtgas und Kohlenoxyd durch Bakterien. Biochem. Z., **184**, 147–171.

HUTTON, W. E. 1948 Studies on bacteria which oxidize methane. Thesis, Univ. of Calif., Los Angeles, Calif.

HUTTON, W. E. AND ZOBELL, C. E. 1949 The occurrence and characteristics of methane-oxidizing bacteria in marine sediments. J. Bacteriol., **58**, 463–473.

HUTTON, W. E. AND ZOBELL, C. E. 1953 Production of nitrite from ammonia by methane-oxidizing bacteria. J. Bacteriol., **65**, 216–219.

KARLSSON, J. L. AND BARKER, H. A. 1949 Tracer experiments on the mechanism of uric acid decomposition and acetic acid synthesis by

Clostridium acidi-urici. J. Biol. Chem., **178**, 891–902.

KING, T. E., FELS, I. G. AND CHELDELIN, V. H. 1949 Pantothenic acid studies. VI. A biologically active conjugate of pantothenic acid. J. Am. Chem. Soc., **71**, 131–135.

KLUYVER, A. J. AND VAN NIEL, C. B. 1936 Prospects for a natural system of classification of bacteria. Centr. Bakt. Parasitenk., II, **94**, 369–403.

LAMANNA, C. AND MALLETTE, M. F. 1953 *Basic bacteriology*, 677 pp. The Williams & Wilkins Co., Baltimore.

MÜNZ, E. 1915 Zur Physiologie der Methanbakterien. Thesis, Friedrichs Universitat, Halle. Abstract in Centr. Bakteriol. Parasitenk., II, **51**, 380 (1920).

NECHAEVA, N. B. 1949 Two species of methane-oxidizing mycobacteria. Mikrobiologiya, **18**, 310–317. (English translation: Associated Technical Services, East Orange, N. J.)

ORLA-JENSEN, S. 1909 Die Hauptlinien des Natürlichen Bakteriensystems. Centr. Bakt. Parasitenk., Abt. II, **22**, 305–346.

PORTER, J. R. 1946 *Bacterial chemistry and physiology*, 1073 pp. Wiley and Sons, Inc., New York.

SLAVNINA, G. P. 1948 Fluorescent method applied to the study of some hydrocarbon oxidizing bacteria. Mikrobiologiya, **17**, 76–81. Chem. Abstr., **42**, 7834.

SKERMAN, V. B. D. 1949 A mechanical key for the generic identification of bacteria. Bacteriol. Revs., **13**, 175–188.

SNELL, E. E., BROWN, G. M., PETERS, V. J., CRAIG, J. A., WITTLE, E. L., MOORE, J. A., MCGLOHON, V. M. AND BIRD, A. D. 1950 Chemical nature and synthesis of the *Lactobacillus bulgaricus* factor. J. Am. Chem. Soc., **72**, 5349–50.

SÖHNGEN, N. L. 1906 Über Bakterien, welche Methan als Kohlenstoffnahrung und Energiequelle gebrauchen. Centr. Bakt. Parasitenk., Abt. II, **15**, 513–517.

SÖHNGEN, N. L. 1910 Sur le rôle du methane dans la vie organique. Rec. trav. chim., **29**, 238–274.

STANIER, R. Y. AND VAN NIEL, C. B. 1941 The main outlines of bacterial classification. J. Bacteriol., **42**, 437–466.

STANIER, R. Y. 1950 Problems in bacterial oxidative metabolism. Bacteriol. Revs., **14**, 179–191.

TAUSZ, J. AND DONATH, P. 1930 Über die Oxydation des Wasserstoffs und der Kohlenwasserstoffe mittels Bakterien. Z. Physiol. Chem., **190**, 141–168.

THIMANN, K. V. 1955 *The life of bacteria*, 775 pp. Macmillan Co., New York.

UMBREIT, W. W., BURRIS, R. H. AND STAUFFER, J. F. 1949 *Manometric methods and tissue metabolism*. Burgess Publ. Co., Minneapolis.

VAN NIEL, C. B. 1946 The classification and natural relationships of bacteria. Cold Spring Harbor Symposia Quant. Biol., **11**, 285–301.

ZOBELL, C. E. 1950 Assimilation of hydrocarbons by microorganisms. Advances in Enzymol., **10**, 443–486.