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Clostridium formicoaceticum nov. spec. Isolation, Description and Distinction from *C. aceticum* and *C. thermoaceticum*

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Zusammenfassung. *Clostridium formicoaceticum* wird als eine neue Art beschrieben. Es vergärt Fructose und eine Reihe von Hexon- und Hexuronsäuren zu Acetat und Formiat. Während des Wachstums wird hauptsächlich Acetat gebildet; größere Mengen von Formiat erscheinen unter den Gärungsendprodukten erst in der stationären Wachstumsphase.

C. formicoaceticum ist mesophil. Es unterscheidet sich von *C. aceticum* durch sein Unvermögen, mit Wasserstoff und Kohlendioxid zu wachsen. Weiterhin kann molekularer Wasserstoff auch in Gegenwart einer organischen Energiequelle von *C. formicoaceticum* nicht für die Acetatsynthese genutzt werden.

Summary. A new species—*Clostridium formicoaceticum*—is described. It ferments fructose and several hexonic and hexuronic acids to acetate and formate. During active growth acetate is the main product of the fermentation. Considerable quantities of formate appear among the fermentation products in the stationary growth phase.

C. formicoaceticum is mesophilic. It differs from *C. aceticum* in its inability to grow on hydrogen plus carbon dioxide. Furthermore, hydrogen gas does not stimulate acetate production even in the presence of organic energy sources.

In 1906 Söhngen observed that in enrichment cultures of methane bacteria a small percentage of the hydrogen gas and of the carbon dioxide consumed was converted to non-gaseous fermentation products. The formation of acetate in such enrichment cultures was reported by Fischer *et al.* (1931). When Söhngen's experiments were repeated by Wieringa (1936) under slightly modified conditions, several enrichments were obtained that consumed large amounts of hydrogen and carbon dioxide without any formation of methane. The end product of this type of fermentation was acetate. The microorganism, which produced acetate from hydrogen and carbon dioxide, was isolated and named *Clostridium aceticum* (Wieringa, 1940). It grew on molecular hydrogen and carbon

Abbreviations: K-PO_4 = mixture of KH_2PO_4 and K_2HPO_4; TEA = triethanolamine; Y_{ATP} = molar growth yield (g dry weight/mole ATP).

dioxide only in the presence of mud extract and was able to ferment sugars to acetate. The nutritional requirements of *C. aceticum* were investigated in more detail by Karlsson *et al.* (1948). With glucose as energy source *C. aceticum* could be grown in a synthetic medium that in addition to minerals contained glutamate, biotin, pyridoxamine, and pantothenate. The strain of *C. aceticum* isolated by Wieringa was lost and no further work has been done on this microorganism.

In this laboratory, El Ghazzawi (1967) isolated a clostridial strain from mud by enrichment culture under an atmosphere of 75% H_2 and 25% CO_2. Like *C. aceticum*, this strain ferments carbohydrates to acetate and does not produce hydrogen during the fermentation. It differs from *C. aceticum* Wieringa by its inability to utilize hydrogen as reducing agent for the formation of acetate from CO_2 in pure culture. Furthermore, it forms considerable amounts of formate during growth and does not ferment glucose (El Ghazzawi and Schmidt, 1967). The isolated strain is clearly distinguished from *C. thermoaceticum*, the optimum growth temperature of the former being 37° C and of the latter 55° C. It was considered by El Ghazzawi (1967) to name the isolate *C. formicoaceticum*.

The aim of the work presented in this paper was to isolate clostridial strains of different sources by the procedure of Wieringa (1936) and El Ghazzawi (1967) and to study the isolates with respect to their ability to form acetate from H_2 and CO_2 and to accumulate formate besides acetate. The isolated strains ferment fructose producing acetate and formate. They are able to synthesize acetate from CO_2, but are incapable of using H_2 as reducing agent. Therefore, the designation of these strains as *C. formicoaceticum* Andreesen, Gottschalk and Schlegel is proposed and a description of this species is given.

Methods

Culture Methods. The enrichment culture medium of *C. formicoaceticum* had the following composition: 1. NH_4Cl, 1 g; K_2HPO_4, 2 g; $CaSO_4$, 0,01 g in 600 ml H_2O; 2. $MgSO_4 \cdot 7H_2O$, 0,2 g; $Co(NO_3)_2$, 19,7 mg; casamino acids, 0.3 g in 100 ml H_2O; 3. $NaHCO_3$, 20 g in 276 ml H_2O; trace metal solution—SL 4 (Pfennig and Lippert, 1966), 10 ml; vitamin solution, 14 ml, containing thiamine, nicotinic acid, pantothenic acid, pyridoxol, B_{12}, p-aminobenzoic acid, and biotin in final concentrations according to Karlsson *et al.* (1948).

Solutions 1. and 2. were sterilized separately at 121° C. Solution 3. was gassed for 10 min with CO_2 and filtered afterwards through a Seitz-filter. Solutions 2. and 3. were combined with solution 1. The enrichment cultures were set up in 50- or 500-ml Erlenmeyer flasks, which were filled to one third with the mineral medium. The mud samples were pasteurized before inoculation, and the flasks were incubated under an atmosphere of 20% CO_2 and 80% H_2 at 28° C in desiccators. After microscopical examination and pasteurisation the suspensions were transferred weekly into fresh

medium using a 10% inoculum. The strains of *C. formicoaceticum* were isolated following the procedure of El. Ghazzawi (1967).

C. formicoaceticum was grown heterotrophically in the medium described before (Andreesen and Gottschalk, 1969). In some experiments sodium bicarbonate was omitted or replaced by sodium formate.

The *bacteriological identification* was carried out according to Skerman (1967).

Electron Microscopy. To demonstrate the flagellation, a drop of a dilute cell suspension was placed on a freshly prepared Formvar-coated grid and was allowed to remain for approximately 2 min. At the end of this time, the drop was removed by touching the edge of the grid to absorbent paper. Afterwards it was shadowed with platinum/coal under an angle of 30° C. Thin sections were made after fixation with 1% OsO_4 and 0.5% $K_2Cr_2O_7$ in acetate/veronal buffer, pH 7, for 2 h at 20° C, washing several times with buffer and contrasting with 2% uranyl acetate. Specimens were dehydrated through a graduated series of acetone and embedded in Epon. The sections were stained with 2% uranyl acetate and examined in a Zeiss EM 9 electron microscope.

Bacterial growth was followed by measuring the optical density at 600 nm in a Bausch & Lomb Spectronic 20 or at 758 nm in an Eppendorf photometer.

Dry weight determinations were performed using weight constant membrane filters (M 14, Sartorius GmbH, Göttingen).

Determinations. Separation of the volatile acids from the culture medium was achieved by evaporating samples to small volumes, adjusting the pH with H_3PO_4 or H_2SO_4 to pH 1—2, and by an at least 10 volume steam destillation. For small volumes the apparatus of Markham (1942) was employed. Fatty acids were identified by descending chromatography according to Kennedy and Barker (1951). Amino acids were chromatographed in the solvent system of Hirsch (1963). The total amount of volatile acids was determined by titration with thymolblue as indicator. Formic acid was determined by oxidation with mercury acetate. In case that the acetic acid present in the steam destillate was needed for Schmidt-degradation the oxidizing agent was mercury oxide (Wood and Gest, 1957).

Occasionally formate was assayed with a partially purified formate dehydrogenase from *Pseudomonas oxalaticus* using the procedure of Johnson *et al.* (1964) as modified by Höpner and Knappe (in press).

Acetic acid was determined by converting it with acetokinase, ATP and hydroxylamine into the hydroxamic acid and measuring the latter colorimetrically (Lipmann and Tuttle, 1945). A modification of the method given by Rose (1955) was employed. The assay mixture contained in a volume of 250 µl: TEA-buffer pH 7.4, 36 mM; ATP, 8 mM; $MgCl_2$, 10 mM; $NH_2OH \cdot HCl$ (neutralized), 0.4 M; acetokinase, 50 µg; acetate, 0.1—0.4 µmole. After 1 h incubation at 35° C the reaction was stopped by adding 250 µl 10% trichloracetic acid, and the color was developed with 1.0 ml $FeCl_3$-solution (1.66% $FeCl_3 \cdot 6H_2O$ in 1 N HCl). The measurements were carried out in 1 ml cuvettes at 546 nm in a Zeiss PMQ 2 spectral photometer. The degradation of the radioactive acetate was achieved by the Schmidt-reaction following the procedure given by Sakami (1955).

To separate radioactive acetate from radioactive formate, acetate was converted enzymatically to citrate. The reaction mixture contained in a total volume of 3 ml: TEA-buffer, pH 8.0, 100 mM; oxaloacetate, 10 mM; $MgCl_2$, 3 mM; ATP, 10 mM; CoA, 0.25 mM; acetate kinase, 75 µg; phosphotransacetylase, 10 µg; citrate synthase, 50 µg. The reactions were run for 1 h and stopped by heating at 100° C for 3 min. The solution was then applied to a small Dowex-1-formate column (0.8 × 3 cm), formic acid was removed from the column with 0.1 N formic acid and citric acid with 4 N formic acid.

The culture medium was checked for the presence of lactate by the method of Holzer and Söling (1962) and for the presence of ethanol by liquid gas chromatography.

CO_2 was determined gravimetrically and fructose according to Dische (1962). Anaerobic conditions were achieved as described by El Ghazzawi (1967) and Schoberth and Gottschalk (1969). Radioactive measurements and preparation of cell-free extracts were done as described before (Andreesen and Gottschalk, 1969). The protein content was determined by the method of Beisenherz *et al.* (1953).

Enzyme Assays. Since extracts of *C. formicoaceticum* contained a very active $NADH_2$-oxidase, the reaction mixture was freed from oxygen by flushing with either moistened nitrogen or argon for 20 min. The reaction was started by the addition of the enzyme(s).

The enzymes of the Embden-Meyerhof pathway, lactate dehydrogenase and glucose-6-phosphate dehydrogenase were tested according to Biochemica Informations (Boehringer Mannheim GmbH, Mannheim, Germany), 6-phosphogluconate dehydrogenase according to Glock (1964).

Chemicals and Enzymes. Acetylphosphate, 3-acetylpyridine-adenine-dinucleotide, ADP, ATP, fructose-1-phosphate, glucose-6-phosphate, glutathione (reduced), NAD, NADH, NADPH, oxaloacetic acid, phosphoenolpyruvate, 6-phosphogluconate, 2-phosphoglycerate, 3-phosphoglycerate, 2,3-diphosphoglycerate, triosephosphate ester solution, acetokinase (170 U/mg), aldolase (9 U/mg), enolase (27 U/mg), glucose-6-phosphate dehydrogenase (140 U/mg), glyceraldehyde-3-phosphate dehydrogenase (70 U/mg), glycerol-1-phosphate dehydrogenase (40 U/mg), lactate dehydrogenase (360 U/mg), 6-phosphogluconate dehydrogenase (12 U/mg), phosphoglucose isomerase (350 U/mg), 3-phosphoglycerate kinase (400 U/mg), phosphoglycerate mutase (500 U/mg), phosphotransacetylase (1,000 U/mg), pyruvate kinase (150 U/mg), triose phosphate isomerase (2,400 U/mg) were purchased from Boehringer Mannheim GmbH, Mannheim (Germany), fructose-1,6-diphosphate, fructose-6-phosphate and NADP were preparations of the British Drug Houses Ltd.; glycyl-glycine, protamine sulfate and resazurine were obtained from Serva, Heidelberg (Germany). ^{14}C compounds were purchased from the Radiochemical Centre, Amersham (England).

Results

Enrichment and Isolation of Clostridial Strains under an Atmosphere of Hydrogen and Carbon Dioxide

45 samples of mud from sewers and ditches around Göttingen and East Frisia were pasteurized and incubated under an atmosphere of 75% H_2 and 25% CO_2 in a sterile mineral medium supplemented with vitamins and a trace metal solution. Visible growth started after a few days and the cultures were transferred weekly into fresh medium. After three transfers 16 cultures failed to grow. An examination of the fermentation products of the remaining samples revealed that acetate was the main end product in 11 cultures. Growth of these enrichments ceased after 6 transfers, but continued after the addition of 0.03% casein hydrolysate. Since from this step on growth of the cultures was dependent on the presence of an organic compound as energy source, agar shake

cultures were prepared which contained glucose, fructose and peptone as energy and carbon sources. 8 strains were isolated, they readily fermented fructose without the formation of hydrogen during growth.

End Products of Fermentation

The 8 clostridial strains were grown in a fructose medium and the organic acids formed during fermentation were separated by steam destillation and identified by paper chromatography. Only acetate and formate could be detected. Ethanol and butylene glycol were not formed and only traces of lactate were present in the culture medium. The amounts of acetate and formate synthesized from fructose during growth of the clostridial strains are given in Table 1.

Table 1. *Acid production from fructose during growth of strains of* C. formicoaceticum

Strain	Source	Fructose consumed	Acetic acid produced	Formic acid produced
A 1	Sewage plant in Göttingen	20.0	41.6	15.6
5(1)b	Ditch near Ossenfeld	24.0	49.4	15.2
12(1)c	Ditch near Ossenfeld	24.8	54.2	12.6
12(3)b	Ditch near Ossenfeld	20.2	39.8	15.0
26(1)a	Lake near Seeburg	24.6	45.1	19.8
29(2)b	Stagnant tributary of the Weser	21.2	39.5	18.4
32(1)b	Alder swamp near Bernshausen	24.2	42.0	19.0
34(1)a	Ditch near Ossenfeld 2nd sample	24.2	46.2	19.0

The cultures were grown at 37° C in 100 ml screw capped bottles, harvested after 41 h, and analyzed as described under Methods.

Growing cultures of the isolated strains produced about two moles of acetate and less than one mole of formate per mole of fructose. Since in these growth experiments fructose was partially incorporated into cellular material this high amount of acids produced already indicated that acetate might have been in part synthesized from carbon dioxide.

Bicarbonate promotes the growth of the isolated strains. Fig. 1 demonstrates the dependence of growth of strain *A 1* on the amount of bicarbonate added to the culture medium. In the absence or in the presence of only small amounts of bicarbonate the lag-phase is prolonged

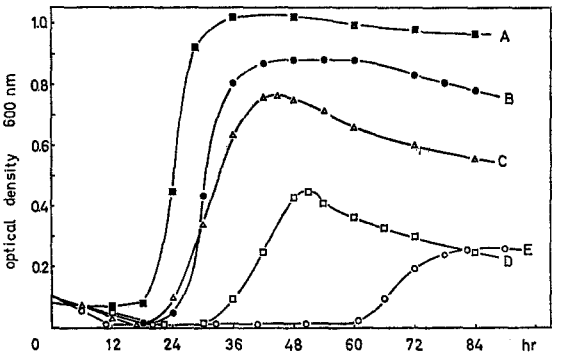

Fig. 1. Effect of Na-bicarbonate on growth of *C. formicoaceticum A 1.* The sterile growth medium was gassed with N_2 and the desired amount of $NaHCO_3$ was added. The cells used as inoculum were washed twice with 0.05 M K-PO_4 buffer, pH 7.0. Anaerobic conditions were established by a K_2CO_3/pyrogallol seal (curve *E*, KOH/pyrogallol). The cells were grown in test tubes and after suspending the sedimented cells the optical density was measured at 600 nm. Bicarbonate concentration: *A* 240 mM; *B* 60 mM; *C* 12 mM; *D* mM; *E* no bicarbonate

and poor growth yields are obtained. Like strain *A 1* the remaining strains require also the addition of 0.5 to 2% sodium bicarbonate to the culture medium for optimum growth.

Incorporation of Carbon Dioxide into Acetate and Formate

C. aceticum Wieringa was shown to produce acetate from bicarbonate and gaseous hydrogen (Wieringa, 1940). When cells of *C. aceticum* grown in a glucose or glutamate medium were suspended in a yeast-malt extract medium and exposed to an atmosphere containing H_2 and CO_2, they were able to produce acetate from H_2 and CO_2 (Wieringa, 1940; Karlsson *et al.*, 1948). *C. thermoaceticum*—another bacterium which produces only acetate as fermentation product—has been shown to incorporate CO_2 into acetate during growth on glucose. From the results given in Table 1 and Fig. 1 and from the nature of the fermentation products it could be expected that the isolated clostridial strains were also able to synthesize acetate and formate from CO_2. In the experiments described in Tables 2 and 3 this and the ability of gaseous hydrogen to serve as reductant in acetate and formate synthesis was tested. The amount of radioactive CO_2 incorporated into volatile acids under argon and under hydrogen was measured in the presence and in the absence of an energy source. It is evident from Table 2 that with strain *A 1* the amount of $^{14}CO_2$ incorporated into volatile acids depended on the presence of fructose as energy source. Without fructose, the radioactivity in formate plus acetate con-

Table 2. *Incorporation of $^{14}CO_2$ into volatile acids by washed cells of* C. formicoaceticum *strain* A 1

Additions	Total radioactivity in organic acids (cpm)	
	Atmosphere	
	Hydrogen	Argon
1. —	77,360	73,970
2. Vitamins, trace metals, 0.03% casamino acids	86,740	80,910
3. Vitamins, trace metals, 0.2% yeast extract	108,440	98,880
4. as 3. plus 0.3% malt extract	140,740	147,500
5. as 4. plus minerals and 1% fructose	4,542,000	4,578,000

The cells were grown under an atmosphere of H_2/CO_2 in the medium described by Karlsson *et al.* (1948), except that fructose instead of glucose was the energy source. The cells were harvested, washed twice and resuspended in 0.1 M K-PO_4 buffer, pH 7.2, containing 0.075% Na-thioglycollate. The incubations were carried out in Warburg vessels. Each vessel contained cell suspension (8.4 mg dry weight) and additions in a total volume of 2.6 ml. Side arm 1 contained 0.2 ml 1.0 M $KHCO_3$, side arm 2 0.2 ml 0.1 M $NaH^{14}CO_3$ (20 μCi). The Warburg vessels were flushed with H_2 or argon for 15 min, the solutions in the side arms were tipped and the reaction was allowed to proceed for 7 h at 37° C. The reaction was stopped by adding H_2SO_4. The remaining $^{14}CO_2$ was removed by evacuation and by bubbling air through the reaction mixture. The volatile acids were separated from the reaction mixture by steam destillation. 75% of the total radioactivity were present in volatile acids.

Table 3. *Incorporation of $^{14}CO_2$ into volatile acids by strains of* C. formicoaceticum

	Total radioactivity in organic acids (cpm) Cells asuspended in					
	Water		Yeast-malt extract medium [a]			
			− Fructose		+ Fructose [b]	
	H_2	Ar	H_2	Ar	H_2	Ar
5(1)b	4,100	4,100	7,100	6,100	518,000	419,000
12(1)c	3,400	4,600	46,200	49,800	722,800	653,400
12(3)b	4,900	6,600	42,600	29,100	219,300	394,700
26(1)a	11,500	8,400	59,300	51,400	274,200	211,400
29(2)b	6,700	1,200	108,200	106,400	325,600	497,300
32(1)b	1,900	2,100	59,500	49,500	182,400	150,400
34(1)a	3,400	3,100	2,800	7,200	371,000	466,300

[a] The yeast-malt extract medium contained vitamins, trace metals, 0.2% yeast, 0.3% malt extract.

[b] The fructose concentration was 1%. The assays were carried out as described under Table 2.

stituted only 2% of the amount incorporated during the fermentation of fructose. Furthermore, hydrogen did not stimulate $^{14}CO_2$ fixation under any condition. The data given in Table 3 show that the remaining seven strains behaved similarly. 52 to 74% of the radioactivity fixed in the presence of fructose was present in volatile acids. The remaining radioactivity was predominantly located in amino acids as was shown by chromatography on Dowex-50 (H^+) and paper chromatography. The radioactivity in acetate constituted approximately 50% of the total radioactivity of volatile acids. This was demonstrated by converting acetate enzymatically into citrate and separating formate and citrate on Dowex-1 formate.

Optimum Growth Temperature

Since the isolated strains differ from *C. aceticum* in their incapability to use gaseous hydrogen as reductant in the synthesis of acetate and formate from CO_2 it seemed desirable to determine the optimum growth temperature in order to judge the relationship of these strains to *C. thermoaceticum*. In Fig.2 the growth curves of strains *A 1* and *29(2)b* obtained at different temperatures are summarized. The optimum growth temperature of these strains lies around 37° C. All strains failed to grow at 52° C. Therefore, these strains like El Ghazzawi's strain are clearly distinguished from *C. thermoaceticum*.

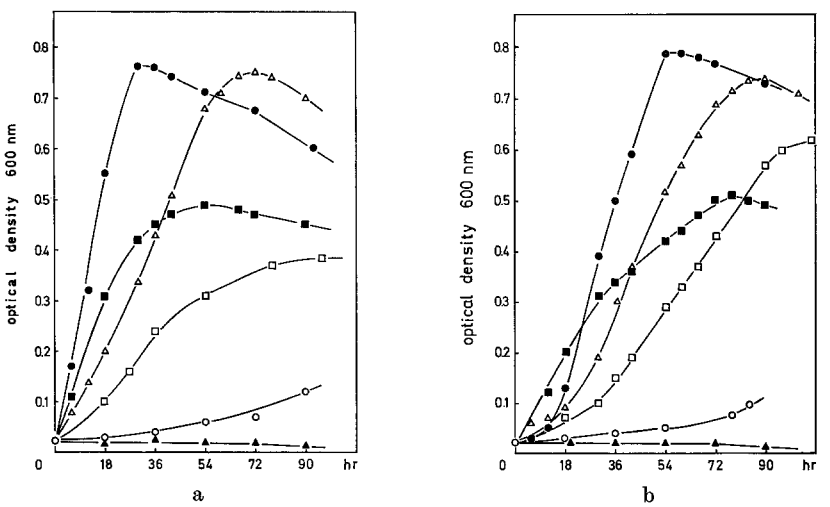

Fig. 2a and b. Effect of temperature on growth of *C. formicoaceticum* strains. a) strain *A 1*; b) strain *29(2)b*; o——o 22° C; □——□ 28° C; △——△ 32° C; ●——● 37° C; ■——■ 44° C; ▲——▲ 52° C

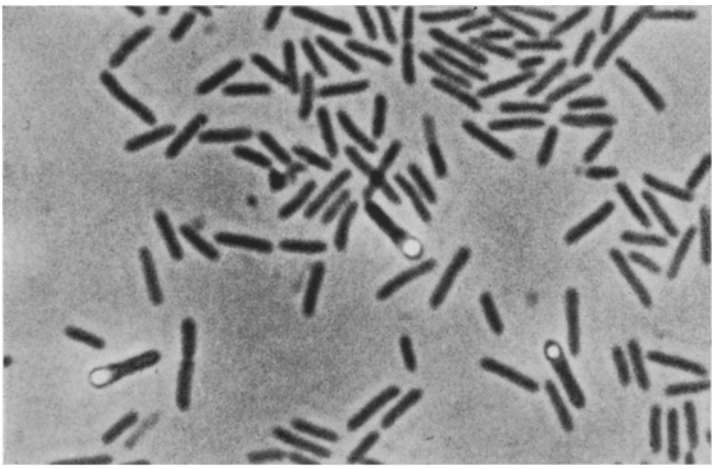

Fig. 3. *Clostridium formicoaceticum* strain *A 1*. Phase contrast microscopy of a culture grown on agar slant. Magnification: 750×

Fig. 4. Peritrichous flagellation of *C. formicoaceticum A 1*. Shadow cast preparation photographed at a magnification of 7000×

Since the isolated strains differ from *C. aceticum* and *C. thermoaceticum* with respect to important properties it is proposed to create a new species *C. formicoaceticum*. A description of the morphological and physiological properties of the type strain *A 1* is given below.

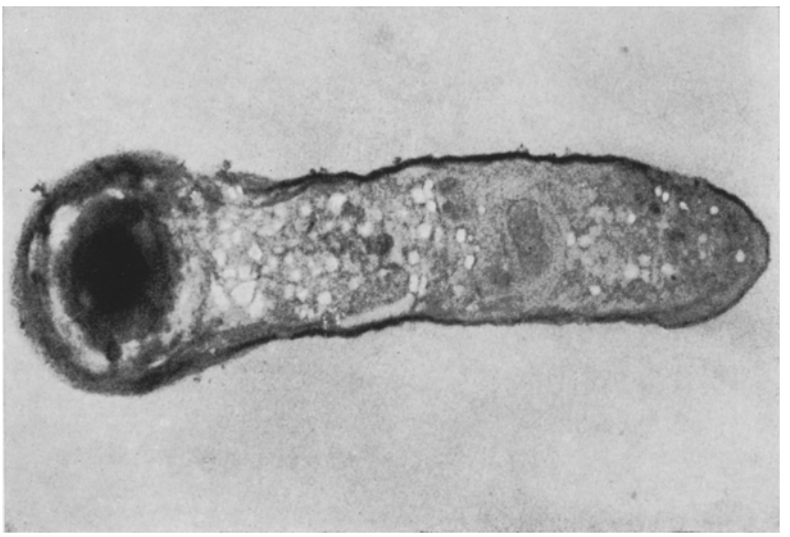

Fig. 5. Thin section through *C. formicoaceticum A 1*. Fixation with OsO_4—$K_2Cr_2O_4$, magnification: 15,000 ×

Morphology

In actively growing cultures *C. formicoaceticum* strain *A 1* is unicellular (Fig. 3). The straight or slightly curved rods are 1.2 to 2.0 μm wide and 5 to 12 μm long. They are motile by means of peritrichous flagella (Fig. 4). The terminal spore is spherical (Fig. 5) and measures 2.8 to 3.6 μm in diameter. Strain *A 1* and the other isolated strains are gram-negative.

Physiology

Reduction of nitrate and formation of H_2S, acetoin and indole are not observed. Pectine supports growth, but starch, gelatin and casein are not hydrolyzed. The nutritional properties of strain *A 1* are summarized in Table 4. Fructose, gluconate, glucuronate, galacturonate and 2-keto-3-deoxygluconate are fermented vigorously. Ribose and a number of organic acids also serve as substrates. The growth curves with a number of substrates are given in Fig. 6. The compound most readily fermented by *C. formicoaceticum* is fructose, and the experiments reported in the subsequent sections of this publication have been done with fructose as energy source.

It has already been shown that the addition of bicarbonate to the medium is necessary for growth of *C. formicoaceticum*. As is evident from Fig. 7 formate can replace bicarbonate as hydrogen acceptor. Since

Table 4. *Utilization of various substrates by* C. formicoaceticum A 1

D-sucrose	–	5-ketogluconate	–
D-fructose	+++	D-2-deoxyglucose	–
D-glucose	–	D-glucosamine	–
D-galactose	–	D-xylose	–
D-mannose	–	D-ribose	++
D-gluconate	+++	L-arabinose	–
D-mannonate	++	L-glutamate	++
D-galactonate	++	fumarate	+
D-glucuronate	+++	succinate	–
D-galacturonate	+++	malate	++
pectine	+	glycine	–
		glycerol	++
alginic acid	–	D-glycerate	++
2-keto-3-deoxygluconate	+++	lactate	++
		pyruvate	+

Media containing the substrates indicated were inoculated with a washed cell suspension. Lactate and pyruvate were added to give a final concentration of 0.5%, in all other experiments the substrate concentration was 1%.

+++ = very good; ++ = good; + = moderate; – = no growth.

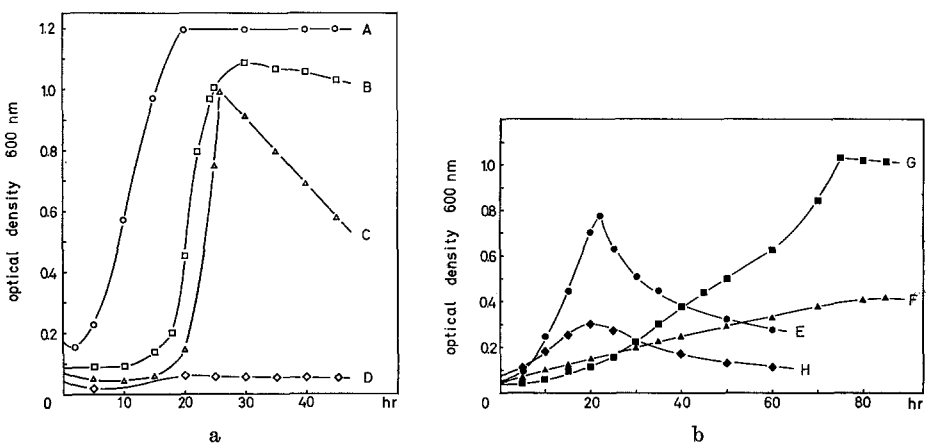

Fig. 6a and b. Growth of *C. formicoaceticum A 1* with various organic substrates. a) *A* Fructose; *B* Na-gluconate; *C* Na-glutamate; *D* no substrate; b) *E* Na-malate; *F* Na-fumarate; *G* Na-lactate; *H* Na-Pyruvate

the growth medium of *C. formicoaceticum* in addition to fructose contains 0.5% peptone or 0.2% yeast extract it was tested whether the latter substances influence the utilization of formate as hydrogen acceptor. From Fig. 8, this seems unlikely because growth of *C. formicoaceticum* depends on peptone in a similar manner, regardless whether carbon dioxide or formate is present.

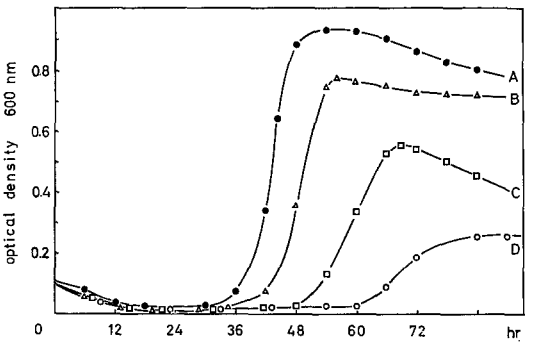

Fig. 7. The dependence of growth of *C. formicoaceticum A 1* on the concentration of formate. The inoculum was grown in a medium containing fructose and 2% $NaHCO_3$. After washing with sterile K-PO_4 buffer, it was added to the test media containing the formate concentrations indicated. *A* 60 mM; *B* 20 mM; *C* 6 mM; *D* no formate

Since acetate and formate are the end products of the fermentation of fructose by *C. formicoaceticum* it is of interest whether or not acetate and formate are produced in a constant proportion during growth. As shown in Fig. 9, actively growing cells of *C. formicoaceticum* produce only acetate. Formate does not appear among the fermentation products until the stationary growth phase is reached. The amounts of acids produced and of fructose consumed during the fermentation are given in Fig. 10 on a molar basis. During the exponential growth phase *C. formicoaceticum* performs a homoacetate fermentation. The production of formate in the stationary growth phase is accompanied by a decrease of acetate formation to 2 moles per mole of fructose fermented. In the experiment depicted in Fig. 9, the pH of the medium decreases from 7.8 to 7.2. That this decrease of the pH is not responsible for the halt of growth was shown in a separate experiment in which the pH of the growth medium was adjusted to 7.2 prior to inoculation. The course of fermentation was similar to that shown in Fig. 9.

Growth Yield and Path of Fructose Degradation

The determination of the growth yield was complicated by the fact that peptone or yeast extract are required for growth of *C. formicoaceticum*. Although the bacteria did not grow with peptone or yeast extract alone it is not excluded that these substances are utilized for energy production along with fructose. In the experiment shown in Fig. 11 fructose was fermented by *C. formicoaceticum* in the presence of 0.1% yeast extract. Aliquots were withdrawn and the dry weight and the

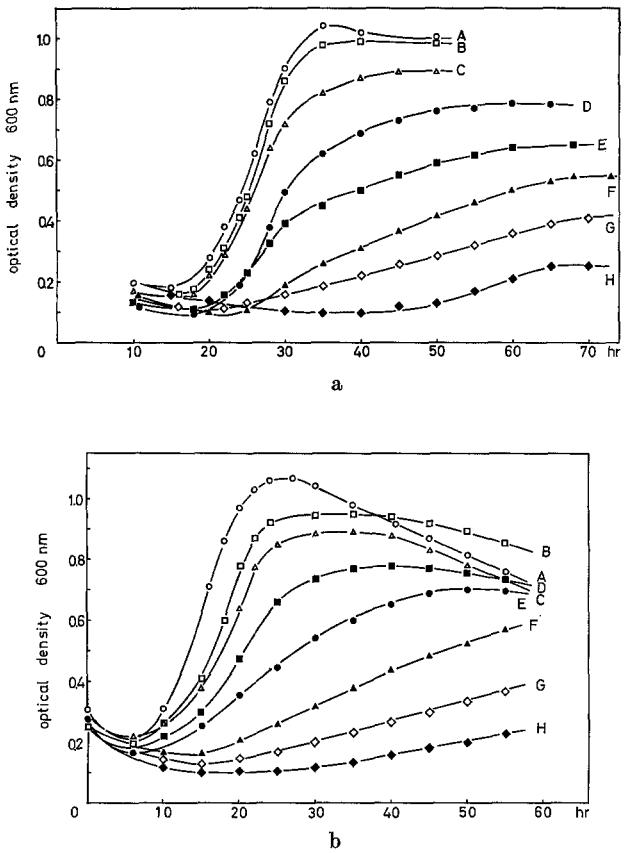

Fig. 8 a and b. The effect of peptone on the growth of *C. formicoaceticum A 1* in the presence of Na-bicarbonate and Na-formate, respectively. The inoculum was grown in a fructose medium containing 60 mM Na-formate. After washing twice with sterile 0.05 M K-PO_4 buffer, pH 7.2, the cells were transferred to the test medium. a) The medium contained 1% fructose, 60 mM $NaHCO_3$ and the following concentrations of peptone: *A* 0.5%; *B* 0.4%; *C* 0.3%; *D* 0.2%; *E* 0.1%; *F* 0.05%; *G* 0.01%; *H* no peptone. b) medium contained 1% fructose, 60 mM Na-formate and peptone concentrations as under a)

fructose concentration were determined. It can be seen that the Y_m value was about 30 g of dry weight per mole of fructose. On the basis of the Y_{ATP} values published (Bauchop and Elsden, 1960; Moustafa and Collins, 1969; Hempfling *et al.*, 1969) this amounts to the synthesis of 2 to 3 moles of ATP per mole of fructose fermented.

Tracer experiments on the fermentation of fructose by *C. formicoaceticum* have shown that carbon atoms 1 and 6 of fructose are incorporated

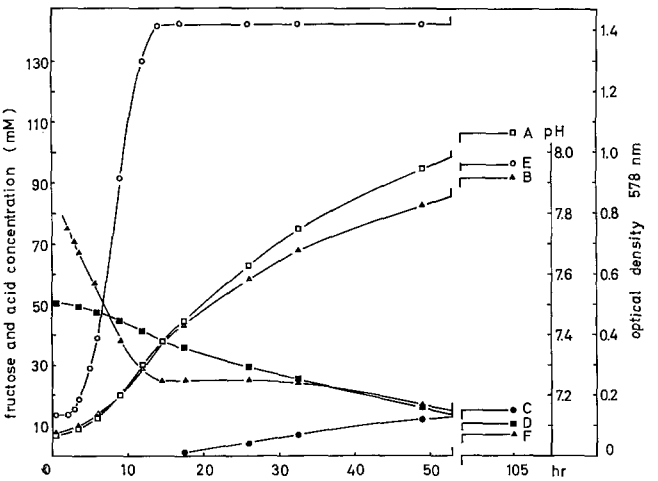

Fig. 9. Course of acid production during the fermentation of fructose by *C. formicoaceticum A 1.* The fermentation was carried out in a 1-l volumetric flask with a device to take samples under anaerobic and sterile conditions. The determinations were performed as described under Methods. *A* total acid; *B* acetic acid; *C* formic acid; *D* fructose; *E* optical density; *F* pH value

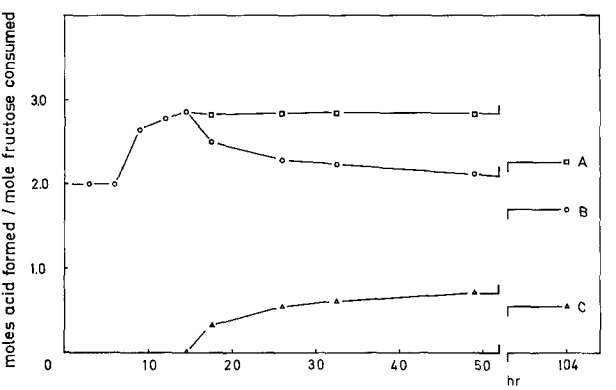

Fig. 10. Ratio of acids produced to fructose consumed during the growth of *C. formicoaceticum A 1.* The ratios were calculated from the differences in acid and fructose concentrations of adjacent samples. The data were taken from Fig. 9

into volatile acids (Linke, 1969; Andreesen and Gottschalk, 1969). This excludes the Entner-Doudoroff pathway and the degradation via pentose phosphates as main routes of fructose catabolism in *C. formicoaceticum.* Additionally, glucose-6-phosphate dehydrogenase and 6-phosphogluco-

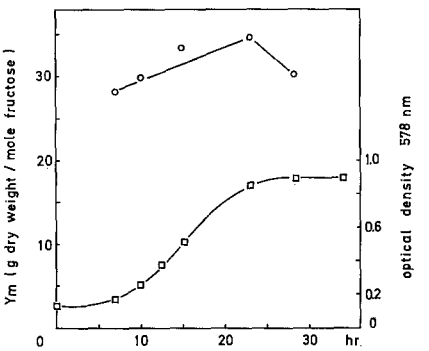

Fig. 11. Determination of the molar growth yield with fructose as energy source. *C. formicoaceticum A 1* was grown in a fructose medium supplement with 0.1% yeast extract. Aliquots of 100 ml cell suspension were withdrawn, the fructose concentration and the dry weight were determined as described under Methods. o——o Y_m value; □——□ optical density

Table 5. *Specific activities of glycolytic enzymes in cell-free extracts of* C. formicoaceticum A 1

E.C. No.	Enzyme	Specific activity (U/g)
2.7.1.1	Hexokinase	18
5.3.1.9	Phosphoglucose isomerase	17
2.7.1.11	Phosphofructokinase	23
4.1.2.7	Fructose-1,6-diphosphate aldolase	34
5.3.1.1	Triosephosphate isomerase	450
1.2.1.12	Glyceraldehyde-3-phosphate dehydrogenase	568
2.7.2.3	Phosphoglycerate kinase	1,050
2.7.5.3	Phosphoglycerate mutase	96
4.2.1.11	Enolase	757
2.7.1.40	Pyruvate kinase	563
1.1.1.27	Lactate dehydrogenase	2

The cells were grown on fructose. Cell-free extracts were prepared and assayed for as described under Methods.

nate dehydrogenase could not be detected in cell-free extracts of *C. formicoaceticum*. As shown in Table 5 all enzymes for the degradation of fructose via the Embden-Meyerhof pathway are present in cell-free extracts. Since the fermentation of fructose to acetate, CO_2 and reducing equivalents is associated with the formation of 4 moles of ATP per mole of fructose, 1 or 2 moles of ATP flow into the synthesis of acetate from CO_2 and reducing equivalents.

Table 6. *^{14}C-distribution in acetic and formic acid after incubation of washed cells of* C. formicoaceticum A 1 *with fructose and $^{14}CO_2$*

Initial CO_2	(μmole/ml)	71.8
Initial CO_2	(cpm/μmole)	885
Final CO_2	(μmole/ml)	68.2
Final CO_2	(cpm/μmole)	810
Final formate	(μmole/ml)	1.0
Final formate	(cpm/μmole)	350
Final acetate	(μmole/ml)	0.76
Final acetate	(cpm/μmole)	171
Final acetate	(cpm/μmole in CH_3—)	82
Final acetate	(cpm/μmole in —COOH)	84
Fructose consumed	(μmole/ml)	0.35

Gas phase, 100% N_2. Reaction mixture (volume 1 l): 360 mg (dry weight) washed cells in 0.066 M K-PO_4 buffer, pH 7.3; $KHCO_3$, 70 mM; Na-thioglycollate, 6.5 mM; fructose, 8 mM; Na_2 $^{14}CO_3$ (50 μCi). After 4 h of incubation 500 ml of the reaction mixture were removed and analyzed as described under Methods.

Table 7. *^{14}C-distribution in acetic acid and carbon dioxide after growth of* C. formicoaceticum A 1 *with fructose in the presence of $Na^{14}COOH$*

Initial formate	(μmole/ml)	49.5
Initial formate	(cpm/μmole)	7,050
Initial acetate	(μmole/ml)	8.3
Initial CO_2	(μmole/ml)	3.1
Initial CO_2	(cpm/μmole)	104
Final formate	(μmole/ml)	0
Final CO_2	(μmole/ml)	25.9
Final CO_2	(cpm/μmole)	3,130
Final acetate	(μmole/ml)	53.5
Final acetate	(cpm/μmole)	4,775
Final acetate	(cpm/μmole in CH_3—)	2,280
Final acetate	(cpm/μmole in —COOH)	2,060
Ratio, $^{14}CH_3$:^{14}COOH		1.11
Fructose consumed	(μmole/ml)	10.0

Cells grown in the presence of Na-formate were inoculated into a growth medium containing 1% fructose and formate instead of bicarbonate. $Na^{14}COOH$ (300 μCi) was added aseptically to a 1 l culture. The gas phase was 100% N_2. For analytical procedures see Methods.

^{14}C-Distribution in Acetate

It has been demonstrated that growth of *C. formicoaceticum* requires the presence of bicarbonate or formate in the culture medium and that $^{14}CO_2$ is incorporated into acetate and formate. In an additional experiment the ^{14}C-distribution in radioactive acetate isolated from the culture medium was determined. Table 6 shows that $^{14}CO_2$ is incorporated into

both carbon atoms of acetate which confirms the results of Linke (1969) obtained with El Ghazzawi's strain. A similar experiment has been carried out using ^{14}C-formate instead of $^{14}CO_2$. The radioactivity of the isolated acetate resided also in both carbon atoms (Table 7).

Discussion

The experiments presented in this publication show very clearly that the isolated clostridial strains perform a homoacetate fermentation. This is demonstrated by the following findings: 1. during active growth up to 2.86 moles of acetate are produced from one mole of fructose; 2. hydrogen is not produced during the fermentation of fructose; 3. growth of these strains is abundant only in the presence of carbon dioxide; and 4. carbon dioxide is incorporated into both carbon atoms of acetate. Thus, as far as the mode of action on carbohydrates is concerned, the isolates very closely resemble *C. aceticum* (Wieringa, 1940) and *C. thermoaceticum* (Fontaine *et al.*, 1942; Barker and Kamen, 1945). On the other hand, important differences between the latter two species and the new isolates were noticed. We think that these differences justify the combination of the new isolates with El Ghazzawi's strain in order to establish the new species *C. formicoaceticum*.

At the end of the logarithmic growth phase and in the stationary phase *C. formicoaceticum* produces formate as additional fermentation product. This has not been reported for *C. aceticum*. *C. thermoaceticum* forms little or no formate (Wood, 1952) and has been shown to be able to utilize formate as precursor in the synthesis of acetate (Lentz and Wood, 1955). With respect to a differentiation of *C. formicoaceticum* from *C. aceticum* the effect of hydrogen on growth and on the synthesis of acetate from carbon dioxide is most remarkable. Wieringa (1940) showed that *C. aceticum* could grow at the expense of hydrogen gas plus carbon dioxide as energy and carbon sources and Karlsson *et al.* (1948) reported that glucose-grown cells of *C. aceticum* were still able to produce acetate with H_2 as hydrogen donor. Although in the present study the original enrichment cultures were set up under an atmosphere of H_2 and CO_2 the isolated strains of *C. formicoaceticum* were neither able to grow with H_2 and CO_2 nor did hydrogen under various conditions stimulate acetate synthesis from CO_2. We believe, however, that the ability of Wieringa's *C. aceticum* to grow with H_2 and CO_2 is such an important property of this microorganism that strains lacking this ability cannot be considered as *C. aceticum*.

C. thermoaceticum has its optimum growth temperature between 55 and 60° C. All strains of *C. formicoaceticum* grow best at about 37° C, none of the strains is able to grow at a temperature of 52° C. Clearly,

C. formicoaceticum is a true mesophilic microorganism. In addition, both species differ considerably in their average size: *C. thermoaceticum* measures 0.4 μm × 2.8 μm (Fontaine *et al.*, 1942) and *C. formicoaceticum* 1.4 μm × 8 μm. Also the behavior of the two microorganisms in the gram reaction, in the reduction of nitrate and the utilization of some substrates shows differences. The clostridial species *C. acidi-urici* (Barker *et al.*, 1940), *C. cylindrosporum* (Barker and Elsden, 1947) and *C. sticklandii* (Stadtman and White, 1954) are also able to synthesize acetate from CO_2. However, with respect to their nutritional properties and their physiology these species have so little in common with the isolated strains that the latter cannot be considered as belonging to one of them.

The name *C. formicoaceticum* for the new species was considered by El Ghazzawi (1967) but not proposed according to the rules of the International Code of Nomenclature of Bacteria. Since the name was merely mentioned by El Ghazzawi (1967) it was not validly published [Rule 12c(3), International Code of Nomenclature of Bacteria].

It has been mentioned that formate is produced mainly in the stationary growth phase. The metabolic changes which cause the cells to excrete formate are not known. During active growth formate is able to replace CO_2 as hydrogen acceptor in acetate synthesis—a function of formate which has also been demonstrated for *C. thermoaceticum* (Lentz and Wood, 1955) and *Butyribacterium rettgeri* (Pine and Barker, 1954).

The data obtained from growth yield experiments cannot be interpreted unequivocally. Determinations of enzyme activities of the glycolytic pathway and tracer experiments (Andreesen and Gottschalk, 1969) provided evidence that fructose is metabolized mainly if not exclusively via the Embden-Meyerhof pathway. Therefore, the degradation of fructose gives rise to the formation of two moles of acetate and CO_2 and of four moles of ATP and reducing equivalents (8 H). With the assumption that two moles of ATP are necessary for acetate synthesis from CO_2 two moles of ATP remain for the synthesis of cellular material. Our growth yield data then would give an Y_{ATP} of 14.2 to 17.4. This value is higher than that of Bauchop and Elsden (1960), however, it corresponds to data published recently (Moustafa and Collins, 1969).

Description

Clostridium formicoaceticum nov spec. Andreesen, Gottschalk and Schlegel, 1970.

for.mi.co.a.ce'ti.cum L. noun formica an ant, ML. adj. formicus pertaining to ants, to formic acid L. noun acetum wine-vinegar, ML. adj. aceticus pertaining to vinegar, to acetic acid. ML. adj. formicoaceticus pertaining to formic and acetic acids.

Morphology. Straight or slightly curved rods, 1.2 to 2.0 μm wide (average size: 1.4 μm), 5 to 12 μm long (average size: 8 μm) sporulating cells are 1.9 to 2.7 μm wide and slightly longer (average size: 10 μm). Motile by means of peritrichous flagella. Spores spherical (2.8 to 3.6 μm), in terminal position. Gram negative.

Physiology. Strict anaerobe, does not contain catalase. Mesophilic, optimum temperature about 37° C.

Organotrophic, growth in the presence of either bicarbonate or formate as hydrogen acceptor in addition to one of the following substrates: Fructose, gluconate, glucuronate, galacturonate, 2-keto-3-deoxygluconate, mannonate, galactonate, D-ribose, L-glutamate, malate, glycerol, D-glycerate, lactate, pyruvate, fumarate, pectine. All enzymes of the Embden-Meyerhof pathway present. Gluconate is degraded to pyruvate- and glyceraldehyde-3-phosphate via 2-keto-3-deoxygluconate and 2-keto-3-deoxy-6-phosphogluconate. Homoacetate fermentation during the exponential growth phase, in the stationary growth phase formate is produced in addition. Not utilized as substrates: sucrose, glucose, galactose, mannose, 5-ketogluconate, D-2-deoxyglucose, D-glucosamine, D-xylose, L-arabinose, succinate, glycine. Starch, gelatine, casein, alginic acid are not hydrolyzed.

Vitamin B_6 and casamino acids required for growth.

No reduction of nitrate, no formation of H_2S, acetoin and indole. No formation of hydrogen.

Habitat. Mud of ditches, sewage.

Type. Strain *A 1* (isolated from a sewage plant in Göttingen), deposited with Sammlung für Mikroorganismen Göttingen, Germany (SMG 92).

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