Classification and Identification of the Bacteria Causing Obligate-Anaerobic Flat Sour Spoilage*1

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Fifty-two strains isolated from spoiled canned coffee and "*Shiruko*" (a sweet bean drink) were examined taxonomically. All the isolates had the same morphological, cultural, and biochemical characteristics except for reduction of nitrate. Therefore, they were divided into two groups (Group A and Group B). The isolates of Group A reduced nitrate to nitrite but those of Group B did not.

Their principal characteristics, which are gram-positive, rod-shaped, obligate-anaerobic, spore-forming, and thermophilic, led us to identify the isolates as members of the genus *Clostridium* or the genus *Desulfotomaculum*. As of April 1983, the genus *Clostridium* has 5 thermophilic species out of 82 approved species and the genus *Desulfotomaculum* has one thermophilic species out of 4 approved species. Comparison of the isolates with the published descriptions (G+C content, sulfate reduction, nutritional requirement, etc.) of the 6 thermophilic species reveals that the isolates are most similar to *Clostridium thermoaceticum*.

A comparison of representative isolates of Groups A and B with a reference strain (*Clostridium thermoaceticum* DSM 521) showed that the morphological, cultural, and biochemical characteristics of Group A are the same as those of *Clostridium thermoaceticum* DSM 521 except for pH requirement and the inability to use xylose, while those of Group B are the same as those of the reference strain except for pH requirement and the inability to use xylose or to reduce nitrate.

Thus, though a few characteristics of the isolates (Group A and Group B) are different from those of *Clostridium thermoaceticum* DSM 521, none of the differences seem sufficiently significant to distinguish the isolates from *Clostridium thermoaceticum*. These facts suggest that the isolates are identical with *Clostridium thermoaceticum*.

Key words: obligate-anaerobic flat sour spoilage; canned coffee; canned "*Shiruko*"; hot vending; classification; identification; genus *Clostridium*; genus *Desulfotomaculum*; *Clostridium thermoaceticum*

Introduction

In previous papers[1)-9)], we showed that, though the appearance and manifestations of the obligate-anaerobic (O. A.) flat sour spoilage in canned drinks kept hot in vending machines in Japan were very similar to those of the well-known so-called flat sour spoilage[10)], the O. A. flat sour spoilage is caused by some obligate anaerobes and is different from the flat sour spoilage caused by facultative anaerobes (*Bacillus stearothermophilus* and *Bacillus coagulans*)[10)]. We also reported that both prior ultraviolet irradiation of the dissolved sugar[5),6)], which is probably the source of the causative bacteria[4)], and addition of some sucrose esters of fatty acids, which have inhibitory effects on the growth of the micro-

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organisms[11,12], are useful as countermeasures to the spoilage[5-9], because the heat resistance of the causative bacteria is so high that the conventional thermal processes are insufficient to sterilize the canned drinks[2-4].

In agreement with our findings[2-4], Matsuda et al.[13] recently reported the isolation of thermophilic, spore-forming, strict anaerobes from spoiled canned "*Shiruko*" (a sweet bean drink) and coffee, and suggested that some of the isolates were identical with *Desulfotomaculum nigrificans* and others were identical with *Clostridium thermoaceticum*. However, their identifications of the isolates were not conclusive.

In this work, all the strains of the causative bacteria isolated from spoiled canned coffee and "*Shiruko*" samples which were incubated at 55°C[2-4] were examined taxonomically. The morphological and physiological natures of selected strains were studied in detail.

Materials and Methods

Microorganisms

The bacteria tested were 52 isolates which have been found to be the causative bacteria of the O. A. flat sour spoilage in previous studies[2-4]. *Escherichia coli* K-12 was used as a standard for deoxyribonucleic acid (DNA) base composition. The strain was obtained from Prof. Isao Shibasaki, Faculty of Engineering, Osaka University. *Clostridium thermoaceticum* DSM 521 was used as a reference strain. The strain was obtained from Deutsche Sammlung von Mikroorganismen.

Morphology of colonies and cells

Colonies and stained cells, which were grown on modified fluid thioglycollate medium (mTGC)[14] plates in a BBL Gas Pak system, were examined by light microscopy.

Flagella were examined with a JEOL JEM-120 transmission electron microscope (JEOL Ltd., Tokyo, Japan). A drop of 10-day-old culture grown in mTGC without agar (mTGC broth) was placed on a collodion-coated copper grid and was shadowed with platinum-palladium (80 : 20).

Biochemical examinations

Gram staining, production of acids from sugar, production of hydrogen sulfide, indole and lecithinase, coagulation of milk, digestion of meat, hydrolysis of gelatin, and reduction of nitrate were examined by the methods described by Harrigan and McCance[15]. Digestion of casein and coagulated egg albumin was examined by the methods described by Sakazaki[16] and Burnett et al.[17], respectively. However, the basal medium used for the test of production of acids from sugar was cystine trypticase agar medium (CTA)[18]; for production of hydrogen sulfide, SIM medium[19]; for indole production, mTGC in which phytone and glucose were omitted; for lecithinase production, mTGC plate; for coagulation of milk, 10% skim milk with iron; for hydrolysis of gelatin, mTGC broth; for nitrate reduction, fluid nitrate medium[20]; for casein digestion, mTGC plate; for digestion of coagulated egg albumin, mTGC.

Acetylmethylcarbinol production, blood hemolysis, catalase production, esculin hydrolysis, and urease production were examined by the methods described by Holdeman et al.[21] However, the basal medium used for the test of acetylmethylcarbinol production was fluid VP medium[22]; for catalase production, mTGC plate; for esculin hydrolysis, CTA medium.

All the liquid media were sealed with sterile liquid paraffin and all the agar plates were placed in a BBL Gas Pak anaerobic system immediately after inoculation. All these media were incubated at 55°C for 10 days.

DNA base composition

Deoxyribonucleic acid (DNA) was isolated by the method of Marmur[23]. However, the DNA of causative bacteria of the O. A. flat sour spoilage was extracted from the cells grown in mTGC broth at 55°C for 2 days. The cells were partially digested with lysozyme at 50°C for 20 min and then lysed with sodium dodecyl sulfate (SDS) at 60°C for 20 min. The DNA of *Escherichia coli* K-12 was extracted from cells grown in a Bacto nutrient broth (Difco Laboratories, Michigan, USA) at 35°C for 1 day on a rotary shaker. The cells were lysed with SDS at 60°C for 10 min as described by Marmur[23].

The guanine plus cytosine (G+C) content of the DNA was determined by thermal de-

naturation[24)-26)]. Determinations of thermal melting point (Tm) were made with a Hitachi 124 spectrophotometer (Hitachi Ltd., Tokyo, Japan) equipped with a Komatsu KPC-3 temperature program controller (Komatsu Electronics Co., Ltd., Kanagawa, Japan), a Komatsu SPR DC power controller, and a Hitachi SPD electrically heated cell block. The temperature of the DNA solution was measured in the cuvette with a thermocouple (Cu-Con, 0.2 mmϕ). The DNA solution was diluted to give an absorbance of approximately 0.5 at 260 nm in 0.1×standard saline citrate (SSC: 0.15 *M* sodium chloride solution plus 0.015 *M* trisodium citrate solution, pH 7.0). The temperature of the DNA solution was raised at a rate of 0.5°C/min and the absorbance changes at 260 nm were recorded on a Hitachi 056 recorder with two pens.

The Tm was determined as the temperature at the midpoint of the absorbance rise. The equation used to relate Tm to mol percent G+C content in 0.1×SSC was that used by Owen and Hill[27)]. The mol% G+C of the unknown DNA can be expressed relative to the reference DNA (the DNA of *Escherichia coli* K-12) by the following equation:

mol%G+C=51.2+2.08 (Tm unknown+Tm *E. coli* K-12)

Fermentation end-product analysis

To detect acids as end products of fermentation, a culture (mTGC broth) incubated at 55°C for 10 days was passed through a 0.22 μm Millipore filter (Millipore Corporation, Massachusetts, USA), by pressure filtration to avoid losses. A 50 ml aliquot of the filtrate was passed through a Dowex 50W-X8 (H^+) column (1.5×29 cm) (Dow Chemical Co., Michigan, USA) and the effluent was passed through an Amberlite IR-45 (OH^-) column (1.5×29 cm) (Rohm & Haas Co., Pennsylvania, USA). Acids adsorbed on the Amberlite IR-45 column were eluted with 2*N* ammonia water. The eluted solution was concentrated in a rotary vacuum evaporator under reduced pressure below 45°C. The concentrated solution (approximately 50 ml) was passed through a Dowex 50W-X8 (H^+) column (1.5×14 cm). The effluent was neutralized with 0.1*N* sodium hydroxide solution and evaporated to 50 ml. A 1 ml aliquot of the solution was taken as a sample for high-performance liquid chromatography and the remaining 49 ml was evaporated to dryness. For gas chromatography, the residue was esterified with butanol and the esters were extracted with *n*-hexane by the method described by Yamashita et al.[28)]

As a control, the medium without inoculation was treated in the same way.

High-performance liquid chromatography

A Shimadzu LC-3A liquid chromatograph (Shimadzu Seisakusho Ltd., Kyoto, Japan) equipped with an analytical Shimadzugel SCR-101H column (300×7.9 mm) was used for the separation and identification of acids in the eluates. The acids were detected with a Shimadzu SPD-2A spectrophotometric detector and determined by the absolute calibration curve method with a Shimadzu Chromatopac C-R1A (a recorder and data processor). The conditions of operation were as follows: mobile phase, deionized water (pH 2.1 by perchloric acid); flow rate, 0.50 ml/min; column temperature, room temperature; wavelength of detection, 210 nm; sample volume for injection, 0.5 μl.

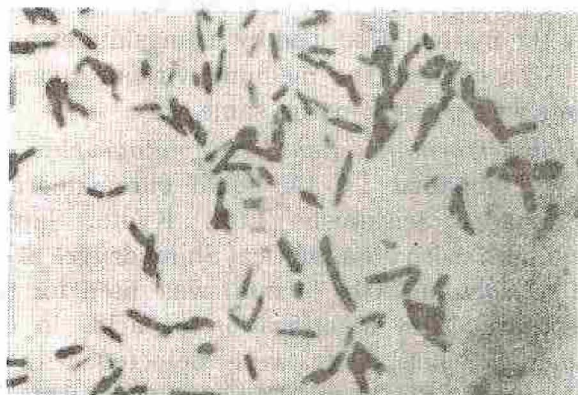
Gas chromatography

A Yanaco gas chromatograph, model G80 (Yanagimoto Mfg. Co., Ltd., Kyoto, Japan), equipped with a flame ionization detector and a Yanaco YR-201 recorder was used. A glass column, 2.25 m by 3 mm (inside diameter), was packed with 15% Polyester FF on Neosorb NS 60-80 mesh (Nishio Industry Co., Ltd., Tokyo, Japan). The column was operated isothermally for 3 min at 80°C, programmed at the rate of 12°C/min to a temperature of 200°C, and then held isothermally for 15 min at 200°C. The injection port was operated at 250°C. Helium was used as the carrier gas at the rate of 25 ml/min. *n*-Dodecane was used as an internal standard. About 2 μl of the *n*-hexane solution was injected into the gas chromatograph.

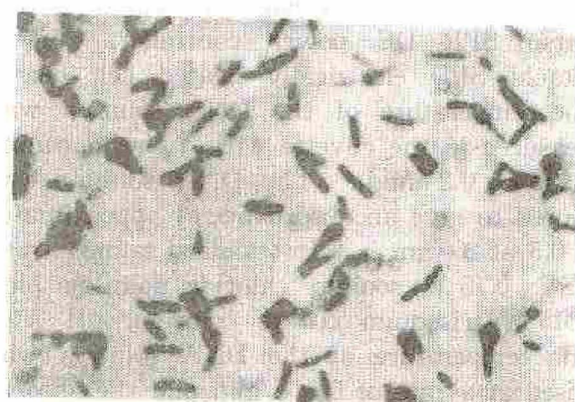
Growth temperature and pH requirement

Growth in mTGC broth was measured in terms of optical density at 660 nm (OD_{660}) with a Shimadzu Bausch & Lomb Spectronic 70. mTGC broth in a test tube (18×180 mm), including a small Teflon-covered magnet bar

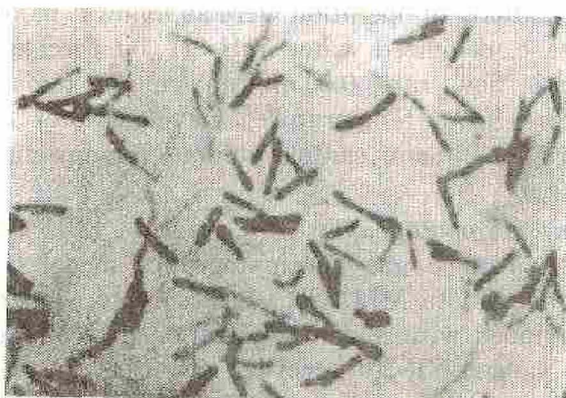
(4.5 × 15 mm) for stirring to make a uniform suspension of cells before measurement of OD_{660}, was sealed with sterile liquid paraffin immediately after autoclaving. The medium was inoculated with one-twentieth volume of a 48-hr-old culture grown in mTGC broth. The incubation temperatures of water baths were adjusted from 40°C to 70°C in 5°C increments. The OD_{660} of the media was measured at 6-hr intervals for 7 days.



Strain No. 24-1

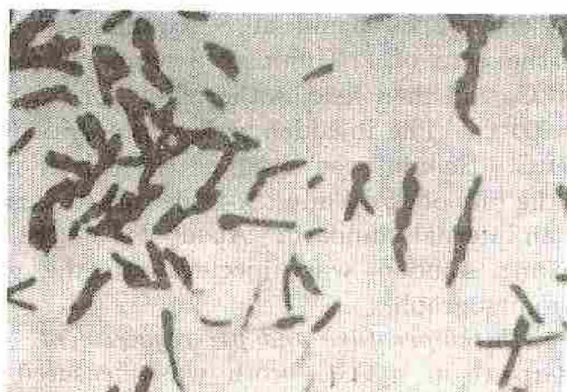


Strain No. 13-1



Strain No. 26-11

Strain No. 27-8



Strain No. 28-3

Strain No. 28-4

Fig. 1. Light micrographs of Gram-stained representative isolates ×1700.

The pH requirement study was performed by a procedure similar to that employed in the study on growth temperature. However, the pH values of mTGC broth were adjusted from 4.5 to 8.5 in increments of approximately 0.5 pH unit. The media were inoculated with one-hundredth volume of a 7-day-old culture grown in mTGC broth. The media were incubated at 55°C and the OD_{660} of the media was measured at 24-hr intervals for 7 days.

Glucose determination

To compare the production of acetic acid with the consumption of glucose in the medium, glucose, both in a control medium without inoculation and in a cultured medium after incubation, was determined by the method described by Somogyi[29),30)]

Results

Morphology

The morphological characteristics of colonies of the isolates grown on mTGC plates in the BBL Gas Pak anaerobic system at 55°C for 7 to 10 days were as follows: shape, circular; size, 1 to 2 mm in diameter; color, slightly milk-white or yellow-white; opacity, opaque; elevation, slightly convex; surface, smooth; edge, entire.

The isolates consisted of straight rods, 0.6 to 0.8 μm by 2.0 to 6.0 μm, with terminal spores 1.1 to 1.2 μm in diameter. The 6 representative isolates described in *Biochemical examinations* (below) were peritrichous. Fig. 1 shows vegetative and sporulated cells of the 6 representative isolates and Fig. 2 shows electron micrographs depicting flagella of two isolates among the 6 isolates.

Biochemical examinations

In previous papers[1)-5)], were reported that all the isolates were gram-negative and that Strain No. 13-1 hydrolyzed gelatin. However, the detailed reexaminations in this study on the staining and hydrolysis of gelatin showed that all the isolates were gram-positive and that Strain No. 13-1 did not hydrolyze gelatin.

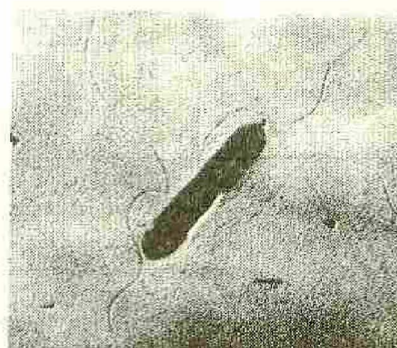
Therefore, all of these isolates were gram-positive, and produced acids from glucose but not from lactose, sucrose or salicin[2)-4)]. These isolates did not produce indole, but did produce hydrogen sulfide[2),4)]. The tests for coagulation of milk, digestion of coagulated egg albumin and meat, and hydrolysis of gelatin all gave negative results[2)-4)]. These isolates did not reduce sulfate, but did reduce sulfite[2),4)]. However, the isolates could be divided into two groups as regards the ability to reduce nitrate as described previously[3),4)].

Strain Nos. 24-1, 13-1, 26-11, 27-8, 28-3, and 28-4 were selected and studied in detail. Strain Nos. 24-1, 13-1, 26-11, and 27-8 were isolated from canned coffee[2),3)]. Strain Nos. 28-3 and 28-4 were isolated from canned "*Shiruko*"[4)]. Group A (Strain Nos. 26-11 and 28-3) reduced nitrate but Group B (Strain Nos. 24-1, 13-1, 27-8, and 28-4) did not[2),4)].

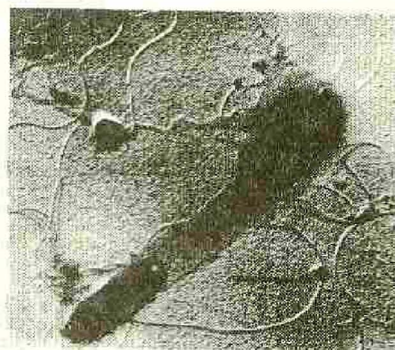
Table 1 lists other biochemical characteristics of the 6 isolates. The isolates all gave the same results in the tests of all the biochemical characteristics shown in Table 1.

DNA base composition

The mol% G+C contents of the isolates are shown in Table 2. The values ranged



Strain No. 24-1



Strain No. 26-11

Fig. 2. Electron micrographs depicting flagella of Strain No. 24-1 and Strain No. 26-11 Bar = 1 μm.

Table 1. Other Biochemical Characteristics of the 6 Representative Strains

Assays giving negative results:		
Acetylmethylcarbinol production		Blood hemolysis
Casein hydrolysis		Catalase production
Lecithinase production		Urease production
Acid from		
Fructose		
No acid from		
Adonitol	Amygdalin	Arabinose
Cellobiose	Cellulose	Dulcitol
Esculin	Erythritol	Galactose
Glycerol	Glycogen	Inositol
Inulin	Maltose	Mannitol
Mannose	Melezitose	Melibiose
Raffinose	Rhamnose	Ribose
Sorbitol	Sorbose	Starch
Trehalose	Xylose	

Table 2. Base Compositions of DNAs from the 6 Representative Strains

Strain No.	G+C content of DNA (mol %)
24-1	49.6
13-1	50.3
26-11	50.4
27-8	51.0
28-3	52.8
28-4	51.7

Table 3. Determined Amounts of Acetic Acid in mTGC Broths

Strain No.	By the HPLC method (mg%)	By the GC method (mg%)
24-1	180.0	199.7
13-1	181.6	195.0
26-11	34.1	55.6
27-8	157.2	197.9
28-3	29.3	54.3
28-4	11.3	27.6

Each of the strains was incubated at 55°C for 10 days.

from 49.6 to 52.8 mol%.

Fermentation end-product analysis

The only acid which could be identified by the high-performance liquid chromatography (HPLC) method was acetic acid. However, there were also 1 or 2 unidentified peaks which were not found in the control.

In gas chromatography (GC) only one peak was detected and identified as acetic acid, and no peak was found in the control.

Table 3 shows the amounts of acetic acid produced by the 6 isolates in the mTGC broths as determined by the HPLC method and the GC method. The amounts of acetic acid produced by the 6 isolates ranged from approximately 10 to 180 mg% (HPLC method) or from approximately 30 to 200 mg% (GC method). The recoveries in the HPLC method and the GC method were 85.5% and 86.4%, respectively.

Growth temperature and pH requirement

Growth curves of the 6 isolates at various temperatures are shown in Fig. 3. The figure shows that the growth rates and cell yields vary considerably.

Fig. 4 shows the growth rate and cell yield at each temperature as percentages of the maximum growth rate and cell yield, respectively. None of the isolates grew below 40C° or over 70°C. For Strain Nos. 26-11, 28-3, and 28-4 the temperatures of maximum growth rate and maximum cell yield were almost the same. For Strain Nos. 24-1, 13-1, and 27-8 the temperatures of the maximum growth rate were higher than those of the maximum cell yield by 5°C to 10°C. In all cases, the optimum growth temperatures of the isolates

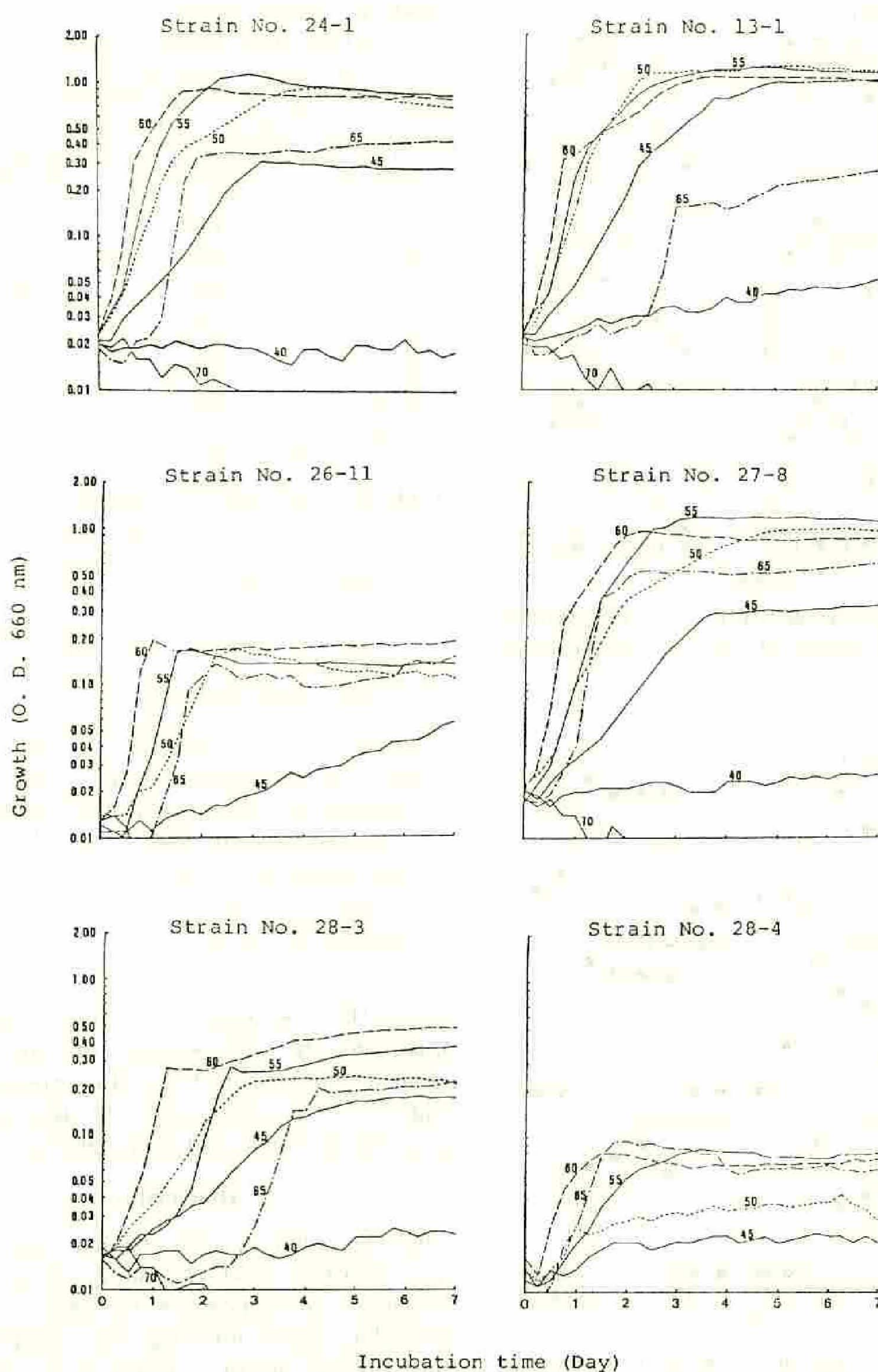


Fig. 3. Growth curves of the 6 representative strains at various temperatures
Numerals on the plots are temperatures (°C).

were between 55°C and 65°C.

Fig. 5 shows the growth rate at various pH values as a percentage of the maximum growth rate. The 6 isolates did not grow at pH below 5.0 to 5.5 or over 8.0 to 8.5. The optimum pH values for growth of the isolates were approximately 7.5 except for Strain No. 28-4 (optimum growth pH, 6.5).

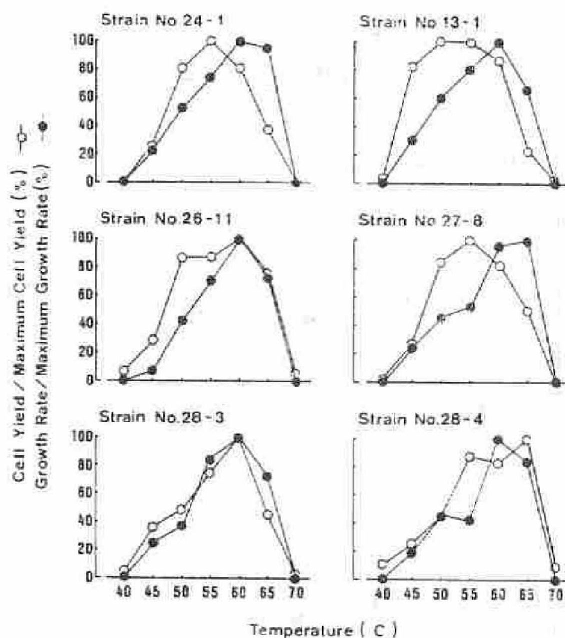


Fig. 4. Effect of temperature on the growth rates and cell yields of the 6 representative strains

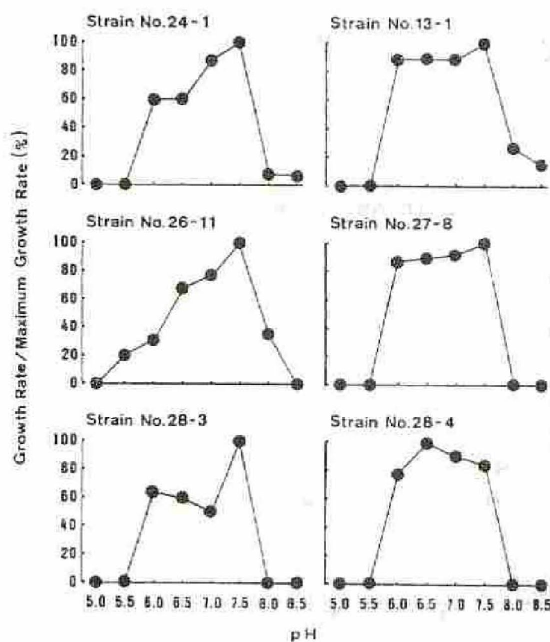


Fig. 5. Effect of pH on the growth rates of the 6 representative strains

Glucose determination

Table 4 shows the concentrations of glucose in the mTGC broth media, which were subjected to fermentation end-product analysis, both without inoculation and with inoculation of the 6 isolates. The amounts of glucose consumed by the isolates, are also shown. From a comparison with the growth curves (Fig. 3), it is clear that the isolates which grew better consumed more glucose.

Table 4. Concentrations of Glucose in the mTGC Broth Media Used for Fermentation End-Product Analysis

Strain No.	Glucose in the medium after incubation (mg %)	Glucose consumed (mg 100 ml of the medium)
Control*	616	0
24-1	354	262
13-1	321	295
26-11	542	74
27-8	337	279
28-3	540	76
28-4	575	41

* Without inoculation.

Table 5. Approved Thermophilic Species in the Genus *Clostridium* and the Genus *Desulfotomaculum* and their DNA Base Compositions

Species	G+C content of DNA (mol%)
Clostridium thermoaceticum	54*1
Clostridium thermoautotrophicum	53−55*2
Clostridium thermocellum	38−39*1
Clostridium thermohydrosulfuricum	29.5−32.0*1
Clostridium thermosaccharolyticum	29−32*1
Desulfotomaculum nigrificans	44.7−46.6*3

*1 Gottschalk, G., et al.[34]
*2 Wiegel, J., et al.[35]
*3 Pfennig, N., et al.[30]

Discussion

All the isolates from spoiled canned coffee and "*Shiruko*" had the same principal characteristics: gram-positive, rod-shaped, obligate-anaerobic, spore-forming, non-gas-producing, thermophilic, sulfite-reducing, and non-sulfate-reducing. On the basis of the identification scheme in Bergey's Manual of Determinative Bacteriology (Eighth Edition)[31),32)], the three characteristics, spore-forming, rod-shaped, and obligate-anaerobic, indicated that the isolates could be identified as members of the genus *Clostridium* or the genus *Desulfotomaculum*.

Table 6. Morphological and Cultural Characteristics of the Representative Isolates and *Clostridium thermoaceticum* DSM 521

Character	Isolates		*Clostridium thermoaceticum* DSM 521	
	Group A*1	Group B*2	This study	Fontaine et al.[37]
Gram stain	positive	positive	positive	positive
Colony				
shape	circular	circular	circular	circular
size*3	1 mm	1–2 mm	1 mm	—
color	slightly milk-white or yellow-white	slightly milk-white or yellow-white	slightly milk-white or yellow-white	—
opacity	opaque	opaque	opaque	opaque
elevation	slightly convex	slightly convex	slightly convex	—
surface	smooth	smooth	smooth	smooth
edge	entire	entire	entire	—
Cell				
shape	straight rod	straight rod	straight rod	rod
size*4	0.6×2–6	0.6–0.8×2–6	0.6×2–5	0.4×2.8
flagella	peritrichous	peritrichous	—	peritrichous
Spore				
location	terminal	terminal	terminal	terminal
shape	nearly round	nearly round	nearly round	very nearly round

*1 Strain Nos. 26-11 and 28-3.
*2 Strain Nos. 24-1, 13-1, 27-8, and 28-4.
*3 In diameter.
*4 μm.

The Approved Lists of Bacterial Names[33] were used to exclude comparison of the isolates with species whose names have no standing in nomenclature. Volumes 30, 31, 32, and Volume 33 (1) and (2) of the International Journal of Systematic Bacteriology were used to include comparison of the isolates with species whose names had been approved during and after 1980.

There are 82 species in the genus *Clostridium* and 4 species in the genus *Desulfotomaculum* as of April 1983. The only 6 thermophilic species out of these 86 species are shown in Table 5. The isolates had G+C contents (49.6–52.8) (Table 2) that were too large to be consistent with identification as *Clostridium thermocellum*, *Clostridium thermohydrosulfuricum*, or *Clostridium thermosaccharolyticum*. None of the isolates reduced sulfate to sulfite, in contrast to the genus *Desulfotomaculum*[33] and the G+C content of *Desulfotomaculum nigrificans* was smaller than those of the isolates by approximately 5 mol%. The other two species, *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* seemed to be similar to the isolates, but the latter grew chemolithotrophically with hydrogen plus carbon dioxide[35]. Accordingly, comparison of the isolates with the published descriptions of the thermophilic species led us to conclude that the isolates were most similar to *Clostridium thermoaceticum*.

A comparison of the representative isolates (Group A and Group B) with a reference strain (*Clostridium thermoaceticum* DSM 521) is shown in Tables 6 and 7. As shown in Table 6, the morphological and cultural characteristics of both Group A and Group B were the same as those of *Clostridium thermoaceticum* DSM 521. Table 7 shows that both Group A and Group B had the same biochemical characteristics except for reduction of nitrate. Therefore, the biochemical characteristics of Group A were the same as those

Table 7. Biochemical Characteristics of the Representative Isolates and *Clostridium thermoaceticum* DSM 521

Reaction	Isolates		*Clostridium thermoaceticum* DSM 521	
	Group A*1	Group B*1	This study	Fontaine et al.[37]
Blood hemolysis	−	−	−	
Coagulation of milk	−	−	−	−
Digestion of				
coagulated egg albumin	−	−	−	−
meat	−	−	−	
Hydrolysis of				
casein	−	−	−	
esculin	−	−	−	
gelatin	−	−	−	−
Nitrite from nitrate	+	−	+	+
Production of				
acetylmethylcarbinol	−	−	−	
catalase	−	−	−	
H_2S	+	+	+	
indole	−	−	−	
lecithinase	−	−	−	
urease	−	−	−	
Acid from				
adonitol	−	−	−	
amygdalin	−	−	−	−
arabinose	−	−	−	−
cellobiose	−	−	−	−
dulcitol	−	−	−	−
esculin	−	−	−	
erythritol	−	−	−	−
fructose	+	+	+	+
galactose	−	−	−	+w*2
glucose	+	+	+	+
glycerol	−	−	−	−
glycogen	−	−	−	−
inositol	−	−	−	−
inulin	−	−	−	−
lactose	−	−	−	−
maltose	−	−	−	−
mannitol	−	−	−	−
mannose	−	−	−	+w*2
melezitose	−	−	−	−
melibiose	−	−	−	
raffinose	−	−	−	−
rhamnose	−	−	−	−
ribose	−	−	−	
salicin	−	−	−	−
sorbitol	−	−	−	
sorbose	−	−	−	
starch	−	−	−	−
sucrose	−	−	−	−
trehalose	−	−	−	−
xylose	−	−	+	+

*1 See Table 6.
*2 Weak.

Table 8. Ratio of Acetic Acid Produced and Glucose Consumed

Group	Strain No.	Acetic acid produced (mM/100 ml)	Glucose consumed (mM/100 ml)	Ratio: acetic/glucose	Mean ratio
A	26–11	1.07	0.411	2.60	2.55
	28– 3	1.05	0.442	2.49	
B	24– 1	3.85	1.45	2.66	2.44
	13– 1	3.76	1.64	2.29	
	27– 8	3.82	1.55	2.46	
	28– 4	0.531	0.228	2.33	
Clostridium thermoaceticum DSM 521		2.96	1.17	2.53	—

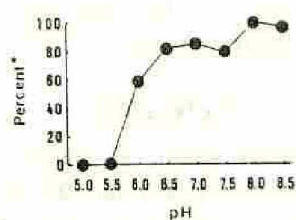


Fig. 6. Effect of pH on the growth rate of *Clostridium thermoaceticum* DSM 521
* Growth rate/maximum growth rate (%).

of *Clostridium thermoaceticum* DSM 521 except for lack of the ability to utilize xylose. The biochemical characteristics of Group B were the same as those of *Clostridium thermoaceticum* DSM 521 except for inability to utilize xylose or to reduce nitrate.

When *Clostridium thermoaceticum* DSM 521 was inoculated into thioglycollate fluid medium (TF)[2], thioglycollate sulfite fluid medium (TSiF)[2], and thioglycollate sulfate fluid medium (TSaF)[2] by the method described in previous papers[2)–4)], it grew well in all the media but blackened only TSiF, like all the isolates described in previous papers[2)–4)]. Thus, it is also sulfite-reducing but not sulfate-reducing.

Table 8 shows the molar ratios of acetic acid formed to glucose consumed as determined by the GC method. The molar ratios of the representative 6 isolates ranged from 2.30 to 2.65. The average values of Group A and Group B were 2.55 and 2.44, respectively. *Clostridium thermoaceticum* DSM 521 was found to produce 2.53 molecules of acetic acid per molecule of glucose by the same method. On the other hand, Fontaine et al.[37] reported that *Clostridium thermoaceticum* fermented glucose at a molar ratio of acetic acid to glucose of 2.55. Therefore, it was concluded that the isolates fermented glucose at almost the same ratio of acetic acid to glucose as *Clostridium thermoaceticum*.

None of the isolates grew at a temperature below 40°C or over 70°C (Fig. 4), and the optimum growth temperatures of the isolates were between 55°C and 65°C. Fontaine et al.[37] reported that the optimum growth temperature of *Clostridium thermoaceticum* ranged from 55°C to 65°C, while the maximum was about 65°C, and the minimum was about 45°C. All the isolates had almost the same growth temperature requirement as *Clostridium thermoaceticum*.

Fig. 6 shows the effect of pH on the growth of *Clostridium thermoaceticum* DSM 521 determined by the same method as used for the isolates. In Fig. 5, the growth rate at each pH value is given as a percentage of the maximum growth rate. Comparison of Fig. 6 with Fig. 5 shows that *Clostridium thermoaceticum* DSM 521 can grow at higher pH than the isolates.

Though a few characteristics of both Group A and Group B of the isolates are different from those of *Clostridium thermoaceticum*, none of these characteristics seem to be major differences which would distinguish the isolates from *Clostridium thermoaceticum*. These results suggest that the isolates are identical with *Clostridium thermoaceticum*.

The isolation of *Clostridium thermoaceticum* from spoiled canned foods as a causative bacteria is very rare. There are no descriptions of this bacteria in most textbooks on food microbiology and food processing, and

there are very few published data on the relation between the bacteria and canned foods. We know of only one paper, by Beerens and Des Rosiers[34] (cited by Walker[31]). They reported that 5 strains of thermophilic *Clostridium*, which were isolated from canned vegetables and were identical with *Clostridium thermoaceticum*, seemed to have caused a phenomenon similar to the O. A. flat sour spoilage and showed characteristics similar to those of the present causative bacteria of the O.A. flat sour spoilage. However, all the strains produced hydrogen sulfide but did not reduce sulfite, and some of the strains fermented xylose but others did not.

As we had described previously[1) 4)], Matsuda et al.[13] recently reported that thermophilic, spore-forming, strict anaerobes were isolated from spoiled canned "*Shiruko*" and coffee, and that some of the isolates were identical with *Desulfotomaculum nigrificans* and others with *Clostridium thermoaceticum*. However, the G+C contents of the isolates were not determined and the results of the tests of acid formation from glucose, mannose, and mannitol varied from strain to strain among the isolates identified as *Clostridium thermoaceticum*. Further, their reactions in the test of nitrate reduction were weak. Thus, though there were several unknown and different characteristics among them, they might be similar to the isolates of Group A in the present study.

These results suggest that there are several small differences among the characteristics of the strains which can be identified as *Clostridium thermoaceticum*.

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