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Characterization of the psychrotolerant acetogen strain SyrA5 and the emended description of the species *Acetobacterium carbinolicum*

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Abstract

A psychrotolerant, obligate anaerobic, acetogenic bacterium designated strain SyrA5 was isolated from black anoxic sediment of a brackish fjord. Cells were Gram-positive, non-sporeforming rods. The isolate utilized H_2/CO_2, CO, fructose, glucose, ethanol, ethylene glycol, glycerol, pyruvate, lactate, betaine and the methyl-groups of several methoxylated benzoic derivatives such as syringate, trimethoxybenzoate and vallinate. The optimum temperature for growth was 29 °C, whilst slow growth occurred at 2 °C. The strain grew optimally with NaCl concentrations below 2.7% (w/v), but growth occurred up to 4.3% (w/v) NaCl. Growth was observed in the range from pH 5.9 to 8.5, optimum at pH 8. The G + C content was 44.1 mol%. Based upon 16S rRNA gene sequence analysis and DNA–DNA reassociation studies, the organism was classified in the genus *Acetobacterium*. Strain SyrA5 shared a 16S rRNA sequence similarity with *A. carbinolicum* of 100%, a *fthfs* gene (which codes for the N5,N10 tetrahydrofolate synthetase) sequence identity of 98.5–98.7% (amino acid sequence similarities were 99.4–100%) and a RNA–DNA hybridization homology of 64–68%. Despite a number of phenotypic differences between strain SyrA5 and *A. carbinolicum* we propose including strain SyrA5 as a subspecies of *A. carbinolicum* for which we propose the name *Acetobacterium carbinolicum* subspecies *kysingense*. The type strain is SyrA5 (= DSM 16427^T, ATCC BAA-990).

Introduction

Methoxylated aromatic compounds are monomeric degradation products of lignin, which is one of the major components of wood. The metabolism of these compounds is of interest because of their quantitative significance in nature and their large structural diversity (Drake 1994). Homoacetogenic bacteria play a major role in the anaerobic degradation of the different phenyl methyl ethers and are found in many habitats such as soils, sediments, sewage and the gastrointestinal tracts of several animals, including man (Drake 1994). *Acetobacterium woodii* was the first bacterium reported to be able to cleave methyl phenyl ether bonds in the absence of molecular oxygen and to ferment the methyl residues (analogous to methanol) with CO_2 to acetate (Bache and Pfennig 1981). The aromatic ring structure was not degraded. Similar findings were made later with other homoacetogenic bacteria: *Eubacterium callanderi* (Mountfort et al. 1988), *Sporomusa termitida* (Breznak et al. 1988),

Butribacterium methylotrophicum (Heijthuijsen and Hansen 1990), *Moorrella thermoacetica* (formerly *Clostridium thermoaceticum*) (Fontaine et al. 1942; Drake and Daniel 2004) and *Clostridium methoxybenzovorans* (Mechichi et al. 1999a). These organisms were all able to demethylate aromatic compounds to their corresponding hydroxylated derivatives and gain energy by the conversion of the *O*-methyl group to acetic acid. Most of these strains are Gram-positive bacteria with a low G + C content. Within the genus of *Acetobacterium*, seven out of eight validly described species were able to grow methylotrophically on the *O*-methyl substituent of aromatic compounds (unpublished observations).

The genus *Acetobacterium* was first described by Balch et al. in 1977, and at present it comprises the following species: *A. woodii* (Balch et al. 1977), *Acetobacterium wieringae* (Braun and Gottschalk 1982), *Acetobacterium carbinolicum* (Eichler and Schink 1984), *Acetobacterium malicum* (Tanaka and Pfennig 1988), *Acetobacterium bakii*, *Acetobacterium paludosum*, and *Acetobacterium fimetarium* (Kotsyurbenko et al. 1995), *Acetobacterium psammolithicum* (Krumholz et al. 1999), *Acetobacterium tundrae* (Simankova et al. 2000) and *Acetobacterium dehalogenans* (Traunecker et al. 1991; Engelmann et al. 2001). Three further strains of *Acetobaterium* sp. have been isolated and characterized (Schuppert and Schink 1990; Frings and Schink 1994; Frings et al. 1994) but their taxonomic positions have not yet been confirmed by phylogenetic analysis. In this paper, we report on the isolation and characterization of an acetogenic bacterium. The organism was isolated as part of a study of the degradation of methoxylated aromatic compounds in an anoxic brackish sediment. For the isolate we propose the name *Acetobacterium carbinolicum* subspecies *kysingense*.

Methods

Origin of enrichment cultures and isolate

Strain SyrA5 was isolated from black anoxic sediment of Kysing Fjord, a small embayment of the brackish water estuary Norsminde Fjord connected to Aarhus Bay in Denmark (N 56°01′ 08.3″, E 10°15′26.1″). Sediment samples were collected in March 2002 from the top sediment using 30 cm long and 5 cm wide Plexiglas tubes. The salinity was 10% and the water temperature was 6 °C. The sediment consisted of fine sand and mud.

Isolation and culture conditions

For enrichment, isolation and subsequent cultivation the following brackish water (BW) mineral medium was used (g l^{-1} distilled water): NaCl, 15; $MgCl_2 \cdot 6H_2O$, 2.0; KCl, 0.7; NH_4Cl, 0.2; KH_2PO_4, 0.2; $CaCl_2 \cdot 2H_2O$, 0.2. After autoclaving, the medium was cooled under an atmosphere of N_2:CO_2 (90/10 v/v) and the following solutions were added (ml l^{-1}): $NaHCO_3$, 30 (8.3% w/v); trace metal SL10a (Widdel et al. 1983), 2; Na_2SeO_3 and Na_2WO_4 (10 μM) (Tschech and Pfennig 1984), 2; vitamin mix, 1; vitamin B12, 1; thiamine chloride, 1 (Widdel and Bak. 1992); $Na_2S \cdot 9H_2O$ (0.5 M), 3. pH was adjusted to 7.2–7.4 with HCl (1 M). Cultures were grown without headspace in screw-cap bottles at 30 °C. Approximately 5 ml of sediment from 2.5 cm depth were used to inoculate 50 ml enrichment cultures supplied with 5 mM syringate as carbon and energy source. Isolation of pure cultures was carried out by repeated application of the agar shake dilution method (Pfennig 1978). For subsequent cultivation of the isolate, the addition of 0.05% (w/v) yeast extract was required for reproducible initiation of growth. Growth was monitored by microscopic inspection and optical density measurements at 600 nm (OD_{600}).

Purity was checked microscopically and by growth tests in BW medium containing syringate (0, 1, and 5 mM, respectively), yeast extract (0.5% w/v) and trypticase peptone (0.5% w/v). Contamination with facultative anaerobic bacteria was tested by streaking out a drop of a SyrA5 culture on a nutrient broth agar plate. Incubations were carried out at 30 °C.

Morphology

Cell morphology was examined with a Zeiss Bright Field microscope. Phase contrast micrographs were obtained with a Photometics Imagepoint cooled CCD video camera connected to an Axiovert 100 Zeiss microscope.

For transmission electron microscopy (TEM) exponentially growing cells were placed on a

carbon-coated, glow-discharged copper grid (mesh 400), washed with PBS (phosphate buffered saline) and subsequently stained with 1% (w/v) ammonium molybdate (pH 7) (Klemm et al. 1994). Micrographs of the cells were obtained with a JEOL 1010 transmission electron microscope.

The fine structure was studied using a JEOL 1200 EX electron microscope. Whole cells were fixed with 2.5% (v/v) glutaraldehyde overnight at 4 °C and then treated with 1% (w/v) OsO_4 for 2 h at room temperature. To obtain thin sections, cells were embedded in Epon-812. The sections were stained with uranyl acetate and lead citrate.

Physiological tests

All physiological tests were carried out in 16 ml Hungate tubes filled with 10 ml of culture under a N_2:CO_2 (90/10 v/v) atmosphere.

For the growth experiments, BW medium supplied with 5 mM syringate and 0.05% (w/v) yeast extract was employed. Growth curves were obtained by measuring the change in optical density at 600 nm (OD_{600}) directly in Hungate tubes using a Novaspec spectrophotometer. Prior to OD measurements, the tubes were thoroughly shaken on a vortex mixer to avoid clumping of the cells. Additionally, growth was checked by microscopy. All tests were performed in duplicate.

The temperature range for growth was determined by incubating cultures from 2 to 55 °C at intervals of 2–5 °C in a temperature gradient block. The activation energy (E_a) was estimated from the slope of an Arrhenius plot and Q_{10} was calculated by the following equation: $Q_{10} = \exp[(E_a * 10)/RT * (T + 10)]$. R is the gas constant (8.31 J K^{-1} mol^{-1}) and T is the absolute temperature (K).

To estimate the pH dependence of growth, the pH of the medium was adjusted to values ranging from 5.2 to 8.5 by addition of NaOH or H_2SO_4 from sterile stock solution (1 M). Minor pH adjustments were carried out after inoculation. Growth at pH values above 8.5 was not determinable by OD measurements due to precipitation of the carbonate buffer. The NaCl requirement for growth was examined by growing the isolate at 21 different NaCl concentrations ranging from 0 to 5% (w/v).

Substrate tests were carried out in the presence of 0.05% (w/v) yeast extract. Cultures supplemented with 0.05% yeast extract alone served as controls. Substrates were added from sterile solutions to give the concentrations desired. H_2 and CO were tested by injecting 3 ml of the gases into the mixture of N_2:CO_2 (90/10 v/v). Utilization of H_2 and CO was followed by gas chromatography. Culture growth was evaluated by OD_{600} measurements and microscopic inspection. Growing cultures were sub-cultured three times in the same medium with the respective substrate combination, before growth was considered positive.

Analytical procedures

The aromatic acids, syringate and gallate, were analyzed by reversed phase HPLC using a MicroSphere C18 column (Varian). A solution of H_2SO_4 (5 mM) and methanol (40% v/v) were used as eluent. The flow rate was 0.5 ml min^{-1}, the oven temperature was 30 °C and the injected sample volume was 60 μl. The compounds were measured with a UV detector at 228 nm. Samples were centrifuged and filter-sterilized prior to analyses.

For determination of short-chained volatile fatty acids (acetate, propionate, butyrate and valerate), samples were centrifuged and sterilized through a filter of 0.22 μm. The volatile fatty acids were analysed according to Soerensen et al. (1991) on an HP 5890 Series II gas chromatograph (Hewlett Packard) equipped with a Nucol capillary column (15 m, 0.53 mm ID, Supelco) and a glass column (6 ft, 2 mm ID) packed with 60/80 Carbopack C, 0.3% Carbowax 20 M, 0.1% H_3PO_4, respectively. The chromatograph was connected to a flame ionization detector (FID), the carrier gas was nitrogen and the oven temperature was 103 °C.

CO and H_2 were analysed on a ML GC 82-22 gas chromatograph (Mikrolab, Aarhus, Denmark) equipped with a thermo conductivity detector (TCD). The compounds were separated on a stainless steel column (1.5 m × 1.8 in.) packed with Molecular sieve 5A. The oven temperature was 100°C and helium was used as a carrier gas for CO analysis. Measuring H_2, the oven temperature was 40 °C and nitrogen was the carrier gas. The flow rate was 25 ml min^{-1} for both gases.

Biochemical tests

Gram staining was performed using the standard method described by Seeley et al. (1991). Standard

catalase test was carried out according to Smibert and Krieg (1994). For testing of cytochrome oxidase triplicate test-strips from Merck were employed according to the manufacturer's instructions.

Antibiotic sensitivity was determined by measuring OD_{600} in cultures of SyrA5 supplemented with antibiotics at concentrations of 10 and 100 μg ml^{-1}. The isolate was grown in BW medium with syringate (5 mM) and yeast extract (0.05% w/v) in addition of one of the following antibiotics: streptomycin, erythromycin, tetracyclin, penicillin G, chloramphenicol. Cultures showing growth in the presence of antibiotics were transferred four times to the same medium and antibiotic concentration.

Requirement of vitamins and yeast extract for growth was tested by growing the isolate in BW medium with syringate (5 mM) in the absence of these components. Growing cultures were subcultured three times.

Extraction of respiratory lipoquinones and polar lipids

Respiratory lipoquinones and polar lipids were extracted from 100 mg of freeze-dried cell material using the two stage method described by Tindall (1990a, 1990b). Respiratory quinones were extracted using methanol:hexane (Tindall 1990a, 1990b) and the polar lipids were extracted by adjusting the remaining methanol:0.3% aqueous NaCl phase (containing the cell debris) to give a choroform:methanol:0.3% aqueous NaCl mixture (1:2:0.8, v/v/v). The extraction solvent was stirred overnight and the cell debris pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform: methanol:0.3% aqueous NaCl mixture to a ratio of 1:1:0.9 (v/v/v).

Analysis of respiratory lipoquinones

Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by thin layer chromatography on silica gel (Macherey-Nagel Art. No. 805 023), using hexane:*tert*-butylmethylether (9:1 v/v) as solvent. UV absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 mm $\times$ 125 mm, 3 μm, RP18) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm.

Analysis of polar lipids

Polar lipids were separated by two dimensional silica gel thin layer chromatography (Macherey-Nagel Art. No. 818 135). The first direction was developed in chloroform:methanol:water (65:25:4, v/v/v), and the second in chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (a-glycols), Dragendorff (quaternary nitrogen), and anisaldehyde-sulphuric acid (glycolipids).

Fatty acid analysis

Fatty acids were analysed as the methyl ester derivatives prepared from 10 mg of dry cell material. Cells were subjected to differential hydrolysis in order to detect ester linked and non-ester linked (amide bound) fatty acids (Tindall, unpublished). Fatty acid methyl esters were analysed by gas chromatography using a 0.2 μm $\times$ 25 m non-polar capillary column (Hewlett Packard Ultra 2 [crosslinked 5% phenylmethylsiloxane], film thickness 0.33 μm, internal column diameter 0.2 mm) and flame ionisation detection. The run conditions were: injection and detector port temperature 300 °C, carrier gas hydrogen at an inlet pressure of 60 kPa, split ratio 50:1, injection volume 1 μl, with a temperature program from 130 (hold for 2 min) to 310 °C at a rate of 4 °C min^{-1}.

DNA extraction, PCR amplification, and sequencing

DNA was extracted from a freshly grown 50 ml culture of strain SyrA5 using previously described

methods (Henckel et al. 1999; Lueders et al. 2004) involving lysis of cells by bead-beating, phenol–chloroform–isoamyl alcohol extraction and polyethylene glycol precipitation of DNA. Nearly full length 16S rRNA gene fragments were amplified from genomic DNA of pure cultures using primers 27F/1492R (Lane 1991) as described previously (Friedrich 2002). 16S rRNA gene PCR products were sequenced with ABI BigDye terminator cycle sequencing chemistry on an ABI Prism 377 sequencer (Applied Biosystems, Weiterstadt, Germany). A nearly complete 16S rRNA gene sequence of 1433 nucleotides was obtained after sequencing both strands with the following primers: 27F and 1492R (the numbers referring to positions in *Escherichia coli* (Brosius et al. 1981)). The *fthfs* gene was partially amplified following the protocol of Leaphart et al. (2001) with modifications. The PCR thermal profile encompassed an initial denaturant step (94 °C, 2 min) followed by 35 cycles of denaturing (94 °C, 30 s), annealing (55 °C, 45 s), and extension (72 °C, 60 s), and a final extension step (72 °C, 6 min). PCR products were purified and cloned into *E. coli* using the pGem-T vector system (Promega, Mannheim, Germany). Clones were randomly selected, checked for correct insert size, and sequenced using vector targeting primers.

The strain SyrA5 16S rRNA gene and the *fthfs* gene sequences have been deposited in GenBank (Accession nos. AY744449 and DQ152900-DQ1 52908, respectively).

Phylogenetic analysis

Small subunit rRNA gene sequences were used for phylogenetic analysis. The closest relatives of strain SyrA5 were found by comparing the 16S rRNA gene sequence to those available in the Ribosomal Database Project (RDP-II) and GenBank using the Sequence Match version 2.7 and MegaBLAST algorithm (National Center for Biotechnology), respectively (Maidak et al. 2001; Wheeler et al. 2002). The sequences were aligned using RDP-II Sequence Aligner version 1.7. The multiple alignments were corrected manually employing the sequence alignment editor BioEdit version 5.0.9 (Hall 1999). Only unambiguously aligned sequence positions were retained for phylogenetic analyses conducted in the MEGA program version 2.1 (Kumar et al. 2001). A total of 1343 nucleotides were included in the final data matrix. Phylogenetic trees were constructed by use of two treeing algorithms, distance matrix (neighbor joining) (Saitou and Nei 1987) and maximum parsimony (Fitch 1971; Eck and Dayhoff 1980). Distances in the neighbor joining algorithm were generated under assumption of models of Jukes and Cantor (1969) and Kimura (1980). Robustness of individual branches was estimated by bootstrap analysis with 1000 replications for both algorithms (Felsenstein 1985). Partial *fthfs* gene sequences were analyzed using the ARB software package (Ludwig et al. 2004).

DNA–DNA hybridization

DNA reassociation was performed to determine the genomic relatedness between strain SyrA5 and *A. carbinolicum* DMS 2925. DNA was isolated as described by Cashion et al. (1977) and Escara and Hutton (1980). DNA–DNA reassociation, performed under optimal conditions (2× SSC at 66 °C) (Huss et al. 1983; Jahnke 1992), was recorded with a Gilford 2600 spectrophotometer. The analysis was carried out at the identification service laboratories of the DSMZ (Braunschweig, Germany).

G + C content of the DNA

Biomass of strain SyrA5 for determination of the genomic G + C content was extracted from 1 L of culture and the analysis was carried out at the identification service laboratories of the DSMZ (Braunschweig, Germany). The DNA was isolated according to Visuvanathan et al. (1989) and purified as described by Cashion et al. (1977). The G + C content was determined by HPLC analysis (Tamaoka and Komagata 1984; Mesbah et al. 1989).

Tests carried out with Acetobacterium carbinolicum

A. carbinolicum (DSM 2925^T) was tested for the following properties. Growth at different NaCl concentrations, utilization of CO, fumarate, succinate, 3,4-dimethoxybenzoate, vanillate, isovanillate, syringate, ferulate, and guaiacol.

Results

Enrichment and isolation

Bacterial growth with concomitant gas production (presumably CH_4) was observed within 1–2 weeks of incubation. In subcultures, gas production ceased after four consecutive transfers. The enrichment culture was dominated by large rod-shaped cells and was routinely transferred every second week. Pure cultures were obtained by several passages through deep agar dilutions. The colonies were whitish and lens-shaped. Well-separated colonies were withdrawn with sterile Pasteur pipettes and transferred to fresh medium supplemented with syringate (5 mM). Growth was visible as increasing medium turbidity within 3–4 days. One of the isolates designated strain SyrA5 was used in a detailed study.

Morphology and cellular properties

Cells of strain SyrA5 were mostly straight rods with slightly pointed ends varying in length from 1.5 to 2.3 μm and in width from 0.6 to 0.9 μm (Figure 1a). Cells mainly occurred in pairs, sometimes also in chains of 3–15 cells. The cellular morphology varied somewhat with culture conditions. Without yeast extract in the medium, cells appeared swollen and were arranged in clusters (Figure 1b). Very elongated cells were observed at high NaCl concentrations (2.7–4.3% w/v) (Figure 1c).

Cells stained Gram-positive. They were motile by means of a single subterminal flagellum (Figure 2a), but motility usually was absent in ageing cultures. Electron microscopic examination of ultra-thin sections of the isolate showed typical Gram-positive cell wall structures as well as electron dense areas, which could resemble chromosomal structures (Figure 2b, c). The isolate did not form endospores. No growth occurred in heat-sterilized cultures (85 °C, 1 h) with syringate (5 mM) and yeast extract (0.2% w/v) even after 8 weeks of incubation. Strain SyrA5 was negative for both catalase and cytochrome *c* oxidase.

Physiological properties

The isolate was an obligate anaerobe and halotolerant. It grew well in mineral medium

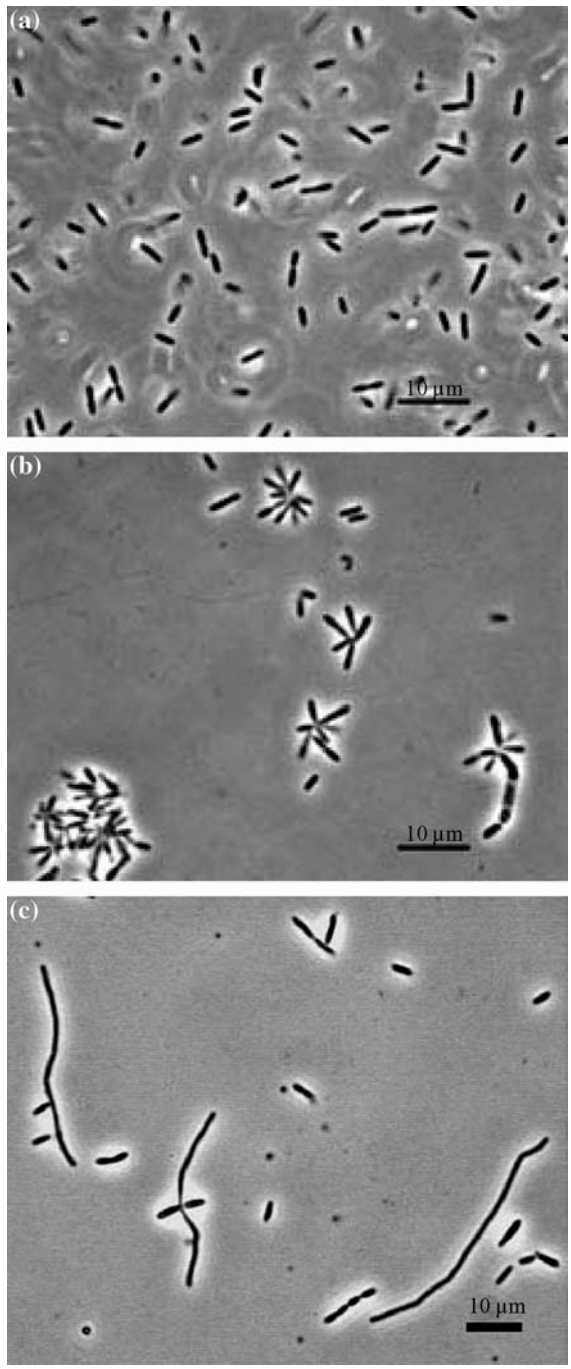

Figure 1. Cell morphology of strain SyrA5 grown on BW medium with 5 mM syringate and 0.05% (w/v) yeast extract (a). In (b) the same medium and substrate was applied but without yeast extract. The NaCl concentration in (c) was 2.7% (w/v). Incubations at 30 °C.

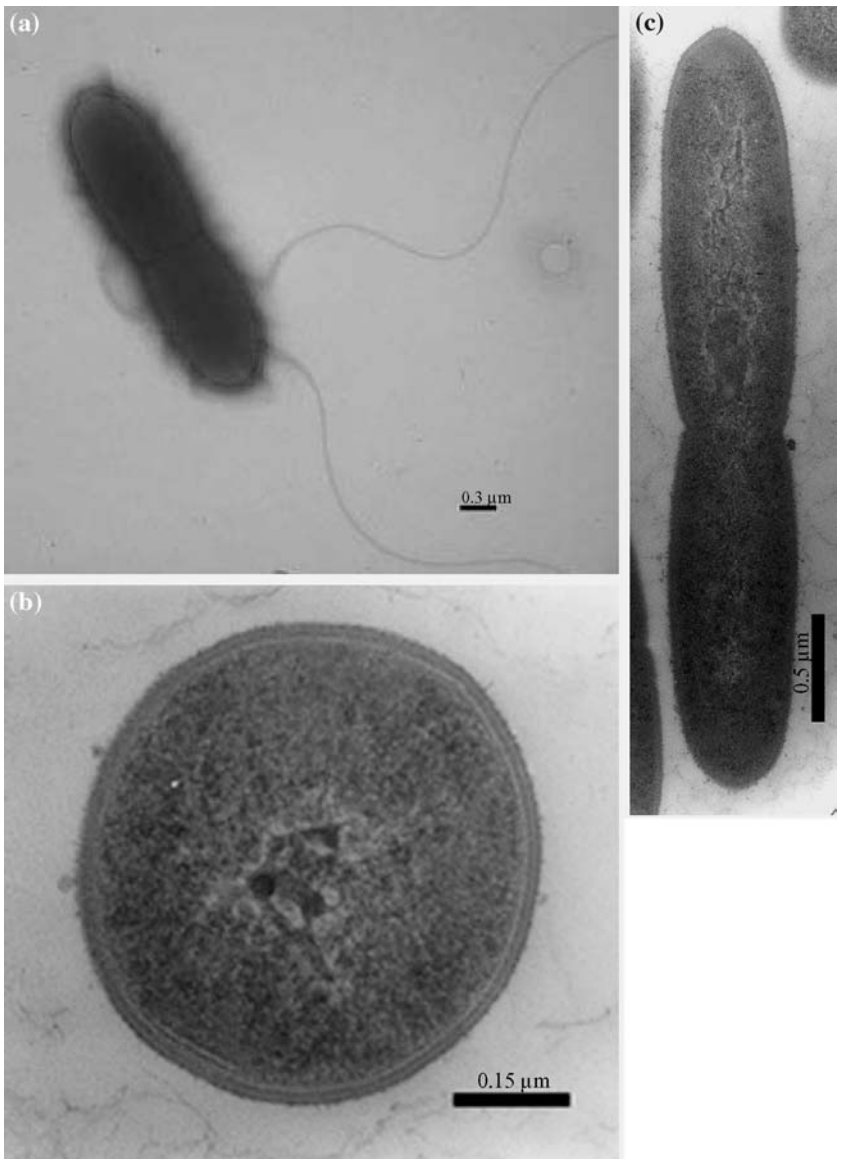

Figure 2. Transmission electron micrographs of strain SyrA5. A pair of dividing cells is shown in (a) with one subterminal flagella evident on each. Thin sections elucidate Gram-positive cell wall structure (b), and electron dense areas that resemble chromosomal structures (c).

containing up to 2.7% (w/v) NaCl with growth rates (μ) varying between 0.029 and 0.057 h^{-1} ($t_d = 26.6–16.3$ h). Slow growth was observed with NaCl concentrations in the range of 2.8–4.3% (w/v) ($\mu < 0.016$ h^{-1}; $t_d > 46$ h). Growth occurred between pH 5.9 and 8.5 with an optimum at pH 8.0. The optimal growth temperature of strain SyrA5 was 29 °C ($\mu = 0.042$ h^{-1}; $t_d = 16.5$ h) with growth occurring from 2 °C (the lowest temperature in the test, $\mu = 0.0006$ h^{-1}; $t_d = 1155$ h) to 36 °C ($\mu = 0.005$ h^{-1}; $t_d = 143$ h). Growth was not observed at 39 °C (Figure 3). In the temperature interval of 10–25 °C the activation energy (E_a) of the reaction of syringate demethylation was 70.7 kJ mol^{-1} and the Q_{10} was 3.

Originally, the isolate was grown without yeast extract. After 12 transfers of the enrichment culture, however, the addition of 0.05% (w/v) yeast extract became necessary in order to assure reproducible growth initiation. Cells grew faster

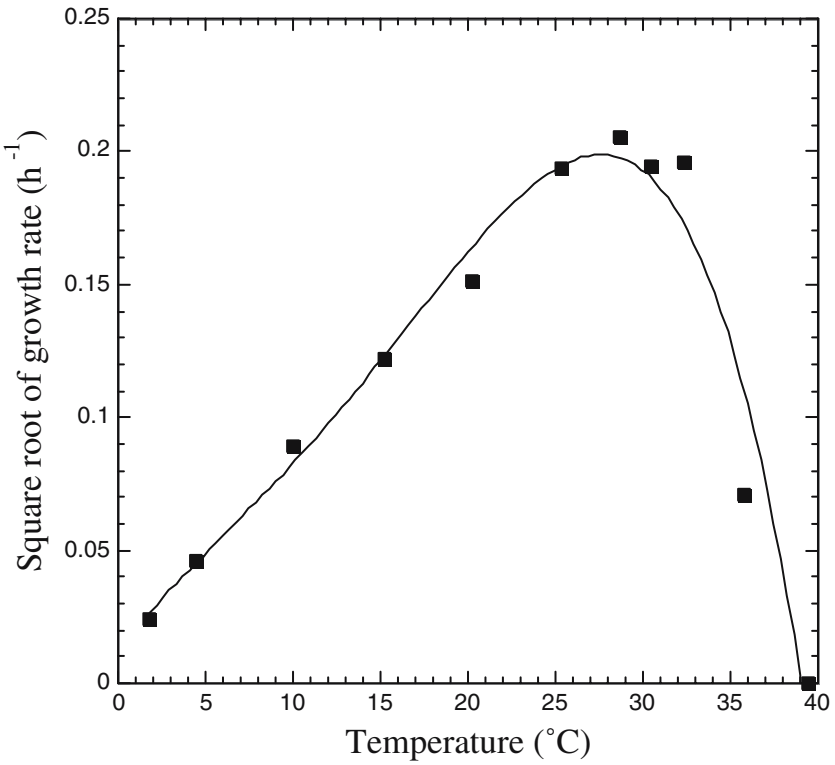

Figure 3. Growth of strain SyrA5 at different incubation temperatures. The growth rates are presented as the square root of the growth rate as a function of the incubation temperature. The non-transformed growth rates at the lowest temperature tested, the optimal temperature and the maximum temperature are given in the text.

and to higher cell densities with increasing concentrations of yeast extract (0–0.2%) w/v (OD_{600} in stationary phase = 0. 2 and 0.3 respectively).

Fructose (10 mM), glucose (10 mM), ethanol (10 mM), ethylene glycol (5 mM), glycerol (5 mM), pyruvate (10 mM), lactate (10 mM), and betaine (5 mM) were used as substrates. The highest cell density was obtained with fructose. SyrA5 did not metabolize propanol, butanol and pentanol. Growth was observed with H_2/CO_2 and CO but not with formate. Several methoxylated derivatives of benzoic acids were used as sole energy sources. The results of all substrate tests are summarized in Table 1. Syringic acid was demethylated to the corresponding hydroxy derivative, gallate, plus acetate indicating that both *O*-methyl groups were utilized. The aromatic ring was not degraded. Short chain volatile fatty acids other than acetate were not produced. Quantitatively 6.6 mM acetate was produced from 4.8 mM syringic acid, resulting in an acetate to *O*-methyl group ratio of 0.69. This stoichiometry is in good agreement with the following theoretical equation:

$$2C_9H_{10}O_5 + 2CO_2 + 2H_2O \rightarrow 2C_7H_6O_5 + 3CH_3COOH$$

Mixotrophic growth was observed when strain SyrA5 concomitantly consumed H_2/CO_2 and syringate. However, the growth rate and cell density were similar to those measured when cultures grew heterotrophically on syringate in the presence of N_2/CO_2.

Growth was completely inhibited by erythromycin, tetracycline, penicillin G, and chloramphenicol. Resistance to streptomycin was observed.

Chemotaxonomy

Acetobacterium carbinolicum DSM 2925 and strain SyrA5 did not contain any known respiratory lipoquinones. The polar lipid composition was

Table 1. Phenotypic (physiological and biochemical) properties that differentiate strain SyrA5 from its closest relatives of the genus *Acetobacterium*.

Characteristics	Strain SyrA5	*A. carbinolicim*[a,d]	*A. woodii*[b,d]
Origin	Anoxic mud (brackish water)	Anoxic mud (fresh water)	Black marine sediment
Cell shape	Rods	Rods	Oval rods
Length (μm)	1.5–2.3	1.5–2.5	1.0–2.0
With (μm)	0.6–0.9	0.8–1.0	0.8–1.2
Motility	+	+	+
Flagella	Subterminal	n.d.	Subterminal
Slime production	–	n.d.	n.d.
Endospores	–	–	–
Gram staining	+	+	+
DNA G+C content (mol%)	44.1	38.5 ± 1.0	39
16S rDNA similarity to strain SyrA5 (%)	–	100	99.4
Temp. optimum/range (°C)	29/2–36	27/15–40	30/n.d.
NaCl optimum/range (% w/v)	< 2.7/ < 4.3	n.d./ < 1.5[e]	n.d.
pH optimum/range	8.0/5.9–8.5	7/6–8	Survive at 5
Growth requirements	Yeast extract	–	Pantothenate
Catalase	–	n.d.	–
Cytochromes	n.d.	–	n.d.
Substrate degraded			
H_2/CO_2	+	+	+
CO	+	+[e]	n.d
Formate	–	+	+
Fructose	+	+	+
Glucose	+	+	–
Methanol	–	+	+
Ethanol	+	+	–
Propanol	–	+	–
Butanol	–	+	–
Pentanol	–	+	–
Ethyleneglycol	+	+	+
Glycerol	+	+	+
2-Methoxyethanol	–	–	n.d.
Pyruvate	+	+	+
Fumerate	–	–[e]	–
Malate	–	–	–
Succinate	–	–[e]	–
Lactate	+	+	+w
Betaine	+	+	+[d]
Choline	–	–[d]	–
Gallate	–	n.d.	n.d
3,4-Dimethoxybenzoate	+	+[e]	n.d
TMB	+	+	+
Vanillate	+	+[e]	+
Isovanillate	+	+[e]	n.d
Syringate	+	+[e]	+
Ferulate	–	±[e]	+
Guaiacol	+	+[e]	n.d
Glycolate	–	–[d]	–
Methoxy acetate	–	–[d]	–
Trimethylamine	–	–[d]	–[d]

Symbols: +: positive; –: negative; n.d.: no data available. The following substrates were not utilized by strain SyrA5: benzoate, 2-methylbenzoate, 2,6-dimethylbenzoate, 2,4-dihydroxybenzoate, 4-methoxybenzoate (anisic acid), 2-hydroxy-5-methoxybenzoate, phenol, pyrocatechol, resorcinol, pyrogallol, phloroglucinol, yeast extract.[a]Eichler and Schink (1984).[b]Balch et al. (1977), Bache and Pfennig (1981).[c]Krumholz et al. (1999).[d]Tanaka and Pfennig (1988).[e] Results of the present study (Paarup et al.).

rather complex, with a number of glycolipids and unidentified components being present. Most striking was the absence of significant amounts of phospholipids. The two strains had almost identical polar lipid compositions.

The fatty acid patterns of the two strains were virtually identical, which is consistent with the fact that the two strains are probably members of the same species (Table 2). The fatty acid composition comprised largely straight chain saturated and straight chain unsaturated, as well as branched, unsaturated fatty acids.

Phylogenetic analysis

The G+C mol% of the DNA of SyrA5 was 44.1 mol%. According to the phylogenetic analyses, the isolate affiliated with the genus of *Acetobacterium* (Figure 4). The 16S rRNA gene sequence of strain SyrA5 was 100% identical to the sequence of *A. carbinolicum* (DSM 2925^T). Sequence identities to other members of the genus *Acetobacterium* ranged between 97.2 and 99.4%. More distantly related to strain SyrA5 were species of the genera *Eubacterium* (~94% similarity), *Pseudoramibacter* (~90% similarity) and *Clostridium* (~85% similarity). Since branching patterns were identical in all trees created with the distance matrix and the maximum parsimony approaches, only the distance matrix tree is shown (Figure 4). Bootstrapping supported the branching in trees based on the distance matrix approach with values above 68. *A. carbinolicum* (DSM 2925^T) was also the closest relative of strain SyrA5 using the *fthfs* gene sequence, which codes for the N5,N10 tetrahydrofolate synthetase enzyme, as an additional phylogenetic marker. Replicate cloned sequences from each strain were highly similar to each other (>99.6–00%; $n=5$, each). In contrast to the 16S rRNA gene, the *fthfs* gene sequences of these strains were not identical but had differences in 14 nucleic acid positions (out of 1060 positions), which corresponds to 98.7% sequence identity. Translated amino acid sequences of Fthfs of these strains were highly similar (99.4–100%) indicating the frequent occurrence of silent mutations. More distantly related to SyrA5 were *A. psammolithicum* and *A. woodii* (90 and 89% amino acid sequence identity, respectively).

Chromosomal DNA–DNA reassociation was performed to determine the relatedness between strain SyrA5 and *A. carbinolicum* (DSM 2925^T). The strains showed 64–68% relatedness at the genomic level. These values are close to the boundary value of 70% used for species delineation (Wayne et al. 1987).

Table 2. Relative amounts of cellular fatty acids (%) of strain SyrA5 and *Acetobacterium carbinolicum* (DSM 2925).

Fatty acids	DMS 2925	SyrA5
12:0	0.1	0.2
14:1ω5c	0.3	0.3
14:0	2.1	2.0
iso-15:1	1.0	1.0
15:1ω8c	0.2	0.2
15:1ω6c	0.1	0.2
16:1ω9c	1.0	2.3
16:1ω7c	22.3	19.3
16:1ω5c	8.5	8.5
16:0	16.5	16.1
iso-17:1 or anteiso-17:1	25.3	25.2
17:1ω8c	0.6	0.5
17:1ω6c	1.5	1.4
17:0	0.4	0.4
18:1ω9c	1.0	1.3
18:1ω7c	10.4	11.9
18:1ω5c	0.5	0.7
18:0	2.6	2.4
10-methyl 18:0	1.3	0.6
iso-19:1	2.0	2.3

Discussion

In the present study, the isolation and characterization of a strictly anaerobic bacterium designated strain SyrA5 is described. This strain demethylated syringic acid to the hydroxy derivative, gallic acid, and produced acetate. Other methoxylated aromatic compounds such as 3,4,5-trimethoxybenzoic acid, vanillic acid, 3,4-dimethoxybenzoic acid and guaiacol were used as sole organic substrates as well. The isolate showed an acetogenic metabolism with growth on H_2/CO_2 and CO. SyrA5 grew heterotrophically on sugars, organic acids, ethyleneglycol and glycerol (Table 1). Mixotrophic growth on H_2/CO_2 and syringate also occurred. The pathway for carbon flow during syringic acid fermentation by SyrA5 is presumed to involve the oxidation of methyl groups to CO_2 concomitant with methyl group

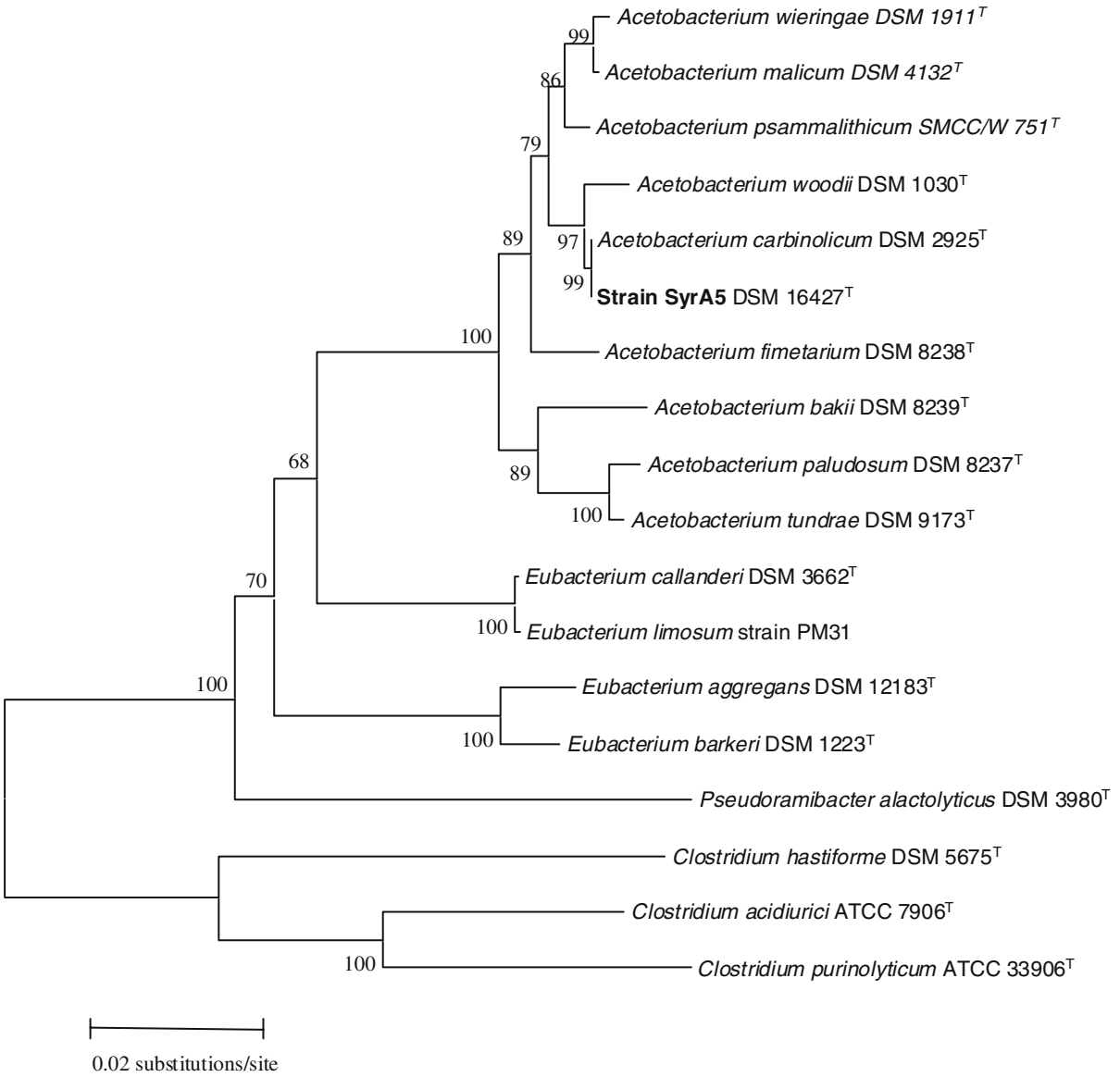

Figure 4. Phylogenetic tree based on 16S rDNA sequences (1343 nt) showing the relationship of strain SyrA5 and its closest relatives as well as other reference organisms of the *Eubacterium*, *Clostridium* and *Pseudoramibacter* genera. The tree was constructed in BioEdit 5.0.9 using the neighbor-joining algorithm (distance matrix) with default settings. Numbers represent percentages of 1000 replicate bootstrap samplings.

conversion to the methyl carbon of acetate (Zeikus 1983). The aromatic ring structure of the hydroxylated product, gallic acid, is not further degraded. The concurrent carbon flow of the methyl substituent has been observed in several acetogens (Zeikus et al. 1980; Kerby et al. 1983). Bache and Pfennig (1981) showed with *A. woodii* that the molar ratio of acetate formed over methoxyl groups consumed is 0.75 when syringate is the substrate of growth. The ratio of 0.69 obtained in our study (6.6 mol acetate were produced from 9.6 mol methoxyl groups during syringic acid fermentation) is in good agreement with the theoretical value. The difference between the measured and the theoretical value is probably caused by the simultaneous assimilation of the acetate into

biomass. Our observation that yeast extract is required for stable growth is in agreement with observation by Krumholz et al. (1999) indicating acetogenic bacteria generally may have complex growths requirements. However, the growth factors in yeast extract required by strain SyrA5 were not determined.

Based on 16S rRNA gene sequence comparison strain SyrA5 was affiliated to the genus *Acetobacterium* with *Acetobacterium carbinolicum* as closest relative. The polar lipid and fatty acid composition of *A. carbinolicum* (DSM 2925^T) and strain SyrA5 were virtually identical, which is also reflected in the high DNA–DNA hybridization values, which indicate that the strains are closely related and may be regarded as members of the same species. No data is available on the polar lipid composition of any of the described *Acetobacterium* species, nor of any of the closest 16S rRNA gene sequence based 'relatives', namely *Pseudooramibacter alactolyticus*, *Eubacterium barkeri*, *Eubacterium limosum*, and *Eubacterium callanderi* (i.e. the genus *Eubacterium senso stricto*). In the case of the two strains examined here, the predominance of glycolipids is an unusual feature.

The fatty acid composition of *A. carbinolicum* and strain SyrA5 can be compared with the fatty acid composition of psychrophilic members of the genus *Acetobacterium* (Kotsyurbenko et al. 1995; Simankooa et al. 2000). The published data of Simankova et al. (2000) indicate that the species *A. paludosum*, *A. tundrae*, and *A. bakii* all produce both fatty acids and dimethylacetals, which are indicators of the presence of plasmalogens in the polar lipids. Due to the relatively small degrees of difference between the 16S rRNA gene sequences of the known species in the genus *Acetobacterium* it may not be possible to easily detect reliable groupings within the genus. However, the fatty acid composition of *A. fimetarium* (Simankova et al. 2000) is distinctive, in that dimethylacetals (i.e. plasmalogens) were not detected, a feature also shared by the two strains examined in this study. However, the data presented by Simankova et al. (2000) for *A. fimetarium* contradicts the results of Kotsyurbenko et al. (1995). Initial mass spectroscopic studies were not able to confirm the identity of the fatty acid identified herein as an unsaturated iso- or anteiso-branched 17:1 derivative (Table 2), and further work is needed. However, the results presented here, and those published previously, indicate that further studies on the chemical composition of members of the genus *Acetobacterium* may be fruitful, since there are indications of sub-divisions within this group.

Overall, members of the genus *Acetobacterium* form a tight phylogenetic cluster within the *Clostridium* subphylum of the Gram-positive bacteria. All species of the genus show high 16S rRNA gene identities (96.8–100%) (Kotsyurbenko et al. 1995; Simankov et al. 2000) and thus minor 16S rRNA gene sequence divergences support differentiation among *Acetobacterium* species. For example, *A. carbinolicum* and *A. woodii* show high similarities with respect to 16S rRNA gene sequence (99.4% identity), genomic DNA (69% homology) and G + C contents (both ~39%). They share almost similar cellular and physiological properties and differ mainly in the ability or inability to metabolize primary aliphatic alcohols. In comparison, the 16S rRNA gene sequences of strain SyrA5 and *A. carbinolicum* are 100% identical and their *fthfs* gene sequence are highly similar (98.7% identity); their DNA–DNA homology value is 64–68%; and the G + C mol% of their DNA is 44.1 and 38.5 ± 1.0, respectively. Phenotypically strain SyrA5 differs from all other *Acetobacterium* species by its inability to grow on formate. The pH, temperature and NaCl tolerance ranges of strain SyrA5 were broader than the ranges of most other *Acetobacterium* species, including *A. carbinolicum*. Strain SyrA5 is not able to use four out of five primary aliphatic alcohols, which is characteristic for the described strains of *A. carbinolicum* (Eichler and Schink, 1984). However, despite the phenotypic difference between strain SyrA5 and *A. carbinolicum* and a 5% difference in G + C mol% of the DNA between the strains, which according to Rossello-Mora and Amann (2001) would support species delineation, we propose, based on the molecular data (identical 16S rRNA gene sequences, high *fthfs* gene similarity and the high DNA–DNA hybridization value) and chemotaxonomic data (lipoquinones, polar lipids and fatty acids) to include our isolate as a subspecies into the species *A. carbinolicum*. We propose the name *A. carbinolicum* subspecies *kysingense*, which relates to the habitat strain SyrA5 was isolated from. As a consequence we propose to emend the species description of *Acetobacterium carbinolicum*.

Emended description of Acetobacterium carbinolicum *(Eichler and Schink 1984)*

The characteristics of this species are as described by Eichler and Schink (1984) with the following exception. Primary aliphatic alcohols (C_3–C_5) may be used by some strains. Some strains grow at NaCl concentration of up to 4.3%. Some strains may grow at 2 °C. Some strains including the type strain use a variety of methoxylated aromatic compounds. Yeast extract stimulates growth in some strains. The G + C content is 38.5–44.1 mol%; the type strain has a G + C content of 38.5 ± 1 mol%. The type strain is DSM 2925^T.

Description of Acetobacterium carbinolicum *subspecies* kysingense

Acetobacterium carbinolicum subspecies *kysingense* (ky.sing.en'se. N.L. adj. *kysingense*, from Kysing a small fjord at the east coast of Jutland, Denmark, where the strain was isolated from). Cells are rod-shaped (1.5–2.3 μm long and 0.6–0.9 μm wide) with slightly pointed ends, mostly in pairs. Motile by a single subpolar flagellum. Whitish, lens-shaped colonies in agar shake tubes. Gram-positive, no spore formation. Catalase and oxidase negative, strictly anaerobic. Psychrotolerant, growth at 2 °C, growth occurs up to 36 °C, temperature optimum at 29 °C. pH-range for growth is 5.5–8.5, optimum at 8.0. Halotolerant growth with NaCl concentrations < 4.3%, optimum at 0–2.7% NaCl. Growth on CO and H_2/CO_2. Chemoorganotroph, utilizing fructose, glucose, ethanol, ethyleneglycol, glycerol, pyruvate, lactate, betaine as well as methyl groups of methoxylated aromatic compounds for growth during fermentation. Syringic acid fermented to gallic acid and acetate. Requires 0.05% (w/v) yeast extract for growth. Mixotrophic growth on H_2/CO_2 and syringate. G + C content of DNA is 44.1%. Isolated from sulphide rich anoxic sediment of a brackish fjord at the east coast of Jutland (Denmark). The type strain is SyrA5 (DSM 16427 = ATCC BAA-990). The GenBank accession No. for the 16S rRNA gene is AY744449, and for the *fthfs* genes are DQ152900-DQ152908.

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