Alkalibaculum bacchi gen. nov., sp. nov., a CO-oxidizing, ethanol-producing acetogen isolated from livestock-impacted soil

Toby D. Allen,¹ Matthew E. Caldwell,¹ Paul A. Lawson,¹
Raymond L. Huhnke² and Ralph S. Tanner¹

Correspondence
Ralph S. Tanner
rtanner@ou.edu

¹Department of Botany and Microbiology, University of Oklahoma, Norman, OK 73019, USA

²Department of Biosystems and Agricultural Engineering, Oklahoma State University, Stillwater, OK 74078, USA

Phenotypic and phylogenetic studies were performed on three strains of an acetogenic bacterium isolated from livestock-impacted soil. The bacterium stained Gram-negative and was a non-spore-forming rod that was motile by peritrichous flagella. The novel strains had an optimum pH for growth of 8.0–8.5 and utilized H₂:CO₂, CO:CO₂, glucose, fructose, mannose, turanose, ribose, trimethylamine, pyruvate, methanol, ethanol, *n*-propanol and *n*-butanol as growth substrates. Acetate was produced from glucose. Acetate, CO₂ and ethanol were produced from CO:CO₂. 16S rRNA gene sequence analysis indicated that the novel strains formed a new subline in the family *Eubacteriaceae* (rRNA cluster XV) of the low G+C-containing Gram-positive bacteria of the class *Clostridia*. The DNA G+C base composition was 34 mol%. Cell wall analysis revealed the existence of a novel B-type peptidoglycan similar to the B2 α -type (B4) configuration with a variation containing aspartic acid. Based on phylogenetic and phenotypic evidence, it is proposed that the new isolates represent a novel genus and species, for which the name *Alkalibaculum bacchi* gen. nov., sp. nov. is proposed. The type strain of the type species is CP11^T (=ATCC BAA-1772^T=DSM 22112^T).

Biological ethanol (bioethanol) production has captured worldwide attention as a result of fluctuating oil prices as well as 'green' initiatives to displace petroleum and lower CO₂ emissions. Most bioethanol produced in the USA is derived from the fermentation of corn starch. The result of increasing bioethanol production is thus direct competition with a food commodity and so this source is not viewed as a long-term sustainable option (Pimentel, 2003). Cellulosic ethanol production is a desirable alternative but current technologies have not overcome the obstacles (e.g. pretreatment and waste disposal) associated with the direct fermentation of cellulosic material (Lynd *et al.*, 2008). An alternative to direct fermentation is the indirect fermentation of lignocellulosic biomass to ethanol. Using this method, biomass (e.g. switchgrass, corn stover, etc.) is pyrolysed to produce synthesis gas (CO:CO₂:H₂) (McKendry, 2002; van der Drift *et al.*, 2001). Some acetogenic bacteria can convert the synthesis gas to ethanol through anaerobic fermentation

(Datar *et al.*, 2004). Phylogenetically, acetogens are a diverse group of organisms within the Gram-positive, low G+C *Clostridia*, of which representative micro-organisms are found in eight of 19 *Clostridium* clusters (I, V, VI, IX, XI, XII, XIVa and XV) (Collins *et al.*, 1994; Drake *et al.*, 2006).

However, while indirect fermentation of biomass by acetogens is an attractive option for bioethanol production, relatively few micro-organisms are known that can convert synthesis gas to ethanol (Tanner, 2008). Of the most promising acetogens to date, *Clostridium ljungdahlii*, *Clostridium carboxidivorans* and a micro-organism also isolated in our laboratory, *Clostridium* sp. strain P11 (each in the *Clostridium* rRNA cluster I), have potential for industrial applications for the fermentation of synthesis gas to ethanol. In this report, the phenotypic and phylogenetic characteristics of an anaerobic, alkali-tolerant bacterium that converts CO:CO₂ to acetate, CO₂ and ethanol (strain CP11^T) are described. 16S rRNA gene sequence analysis placed strain CP11^T within cluster XV of the class *Clostridia* (Collins *et al.*, 1994), for which the family name *Eubacteriaceae* within the order *Clostridiales* has been proposed (Ludwig *et al.*, 2009). The family *Eubacteriaceae* contains several acetogenic bacteria including *Acetobacterium woodii*, *Acetobacterium bakii*, *Acetobacterium tundrae*,

Abbreviations: BESA, 2-bromoethanesulfonic acid; FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CP11^T is FJ438469.

A transmission electron micrograph showing cells of strain CP11^T is available with the online version of this paper.

Acetobacterium carbinolicum, *Acetobacterium paludosum*, *Acetobacterium fimetarium*, *Acetobacterium wieringae*, *Acetobacterium malicum*, *Eubacterium limosum* and *Eubacterium aggregans* (Drake *et al.*, 2006). Of these bacteria, *Acetobacterium woodii*, *Acetobacterium bakii*, *Acetobacterium carbinolicum*, *Acetobacterium paludosum*, *Acetobacterium fimetarium*, *Acetobacterium tundræ* and *E. limosum* have been shown to grow autotrophically using CO. In almost all cases, the sole end product of metabolism with H₂:CO₂ or CO is acetate, with the exception of *E. limosum* in which acetate, ethanol, butyrate, butanol and CO₂ are formed (Genthner *et al.*, 1981; Worden *et al.*, 1991), and *E. aggregans* in which acetate and formate are detected (Mechichi *et al.*, 1998). It has been shown that '*Butyribacterium methylotrophicum*' is a strain of *E. limosum* (Jansen & Hansen, 2001; Tanner, 2008). *A. woodii* has been shown to produce ethanol in addition to acetate under phosphate-limiting conditions (Buschhorn *et al.*, 1989). Here we present evidence for a new acetogen, strain CP11^T, within the family *Eubacteriaceae* that forms ethanol as an end product of CO oxidation.

Saturated soil adjacent to a livestock pen in Stillwater, OK, USA, was collected and incubated at 40 °C in anaerobic enrichment medium (pH 8.0) containing (l⁻¹): 10 ml mineral solution (Tanner, 2007), 10 ml vitamin solution (Tanner, 2007), 10 ml trace metal solution (Tanner, 2007), 20 g TAPS (Sigma-Aldrich), 0.05 g yeast extract (Difco) and 5.0 g NaHCO₃. 2-Bromoethanesulfonic acid (BESA) was added from a sterile, anoxic stock solution to a final concentration of 5 mM in order to inhibit methanogens. The medium was reduced with cysteine sulfide (Tanner, 2007) and CO (240 kPa gauge) was added as the substrate. After dilution on agar roll tubes (Hungate, 1969), the resulting isolates (strains CP11^T, CP13 and CP15) were cultivated on the same medium with the following changes: yeast extract was increased to 1 g l⁻¹ and BESA was omitted. Incubations were at 37 °C unless otherwise noted. The strains were characterized biochemically by using a combination of conventional tests as previously described (Tindall *et al.*, 2007) and by API Rapid ID 32A and API ZYM test systems according to the manufacturer's instructions (bioMérieux). Cells growing on glucose with a N₂ headspace were used for pH and temperature experiments. For pH experiments, the following Good's buffers (Sigma-Aldrich) were used (30 g l⁻¹): MES (pH 6 and 6.5); TES (pH 7 and 7.5); TAPS (pH 8 and 8.5); CAPSO (pH 9) and CAPS (pH 10 and 10.5). Cellular morphology was examined using a transmission electron microscope (JEM 2000 FX; JEOL) as previously described (Liou *et al.*, 2005). The Gram reaction was examined using the Hucker method (Beveridge *et al.*, 2007) and a KOH lysis test (Tindall *et al.*, 2007). Nutritional requirements and substrate use were examined as previously described (Liou *et al.*, 2005). Strains were grown anaerobically on methanol before being tested on O-methylated aromatic substrates. Positive results were scored by an increase in optical density and serial passage compared with negative controls. Acids and alcohols were quantified as described previously

(Allen *et al.*, 2008). CO and CO₂ were quantified by GC with thermal conductivity detection (Shimadzu) with a molecular sieve 5A or a Porapak Q column (Supelco), respectively. Fatty acid methyl ester (FAME) analysis was performed on overnight cultures grown on TSB at 37 °C by Microcheck, Inc., USA, according to previously described methods (Miller, 1982; Sasser, 1990). Peptidoglycan analysis was performed by Dr Peter Schumann at the DSMZ according to previously described methods (Schleifer, 1985; Schleifer & Kandler, 1972). Quantitative analysis of cell-wall amino acids was performed by GC according to MacKenzie (1987).

All strains were rods, 1.5–2.2 µm in length and 0.5–0.8 µm wide, and were motile by peritrichous flagella (see Supplementary Fig. S1 available in IJSEM Online). Overnight cultures stained Gram-variable and older cultures (>40 h) stained Gram-negative. Cells also lysed rapidly in 3 % KOH, consistent with the Gram stain results. Spores were not observed in cultures up to 4 weeks old. Growth was observed between 15 °C and 40 °C, with the optimum temperature for all strains being 37 °C. Additionally, strain CP15 grew at 45 °C. All strains grew at pH values ranging from 6.5 to 10.5, with the pH optimum between 8.0 and 8.5. Vitamins were required for growth. The addition of yeast extract (≥0.05 g l⁻¹ for glucose-grown cells and ≥0.5 g l⁻¹ for CO:CO₂-grown cells) greatly stimulated growth. Casamino acids (0.1 g l⁻¹) could partially replace yeast extract for growth on glucose. The following aromatic compounds were not used as substrates: anisic acid, 3-methoxyphenol, 3-methoxycinnamic acid, 4-methoxysalicylic acid, 5-methoxysalicylic acid and syringic acid. However, the ability to O-demethylate methoxylated aromatic compounds has been demonstrated in the acetogens, including the phylogenetic neighbours of strain CP11^T, *E. limosum* and *Acetobacterium woodii* (DeWeerd *et al.*, 1988; Häggblom *et al.*, 1993). The substrates used by all strains are listed in the species description. Strain CP11^T grown on glucose (N₂ headspace) produced acetate as the main product, with trace amounts of ethanol and CO₂ detected. Fermentation of CO:N₂:CO₂ (80:15:5, 200 kPa gauge) by strain CP11^T produced acetate, CO₂ and ethanol. Although the batch fermentations using CO described above produced ethanol as a minor product, continuous fed-batch fermentations using CO produced between 28 and 49 mM ethanol for all strains. Increased production of ethanol by strain CP11^T, including strain/process improvement, is currently under investigation.

Cellular fatty acid profiles of the strains included several dimethyl acetal moieties totalling approximately 29 % of total fatty acids. The detailed fatty acid profiles for all strains are shown in Table 1. The FAME profile of strain CP11^T (as well as those of strains CP13 and CP15) did not correspond to any pattern for recognized bacteria within the databases examined.

The composition of cell-wall peptidoglycan has been shown to be an important taxonomic characteristic,

Table 1. Cellular fatty acid composition of the novel strains

Values are percentages of total identified fatty acids. ND, Not detected or present at <1 %; DMA, dimethyl acetal.

Fatty acid	CP11 ^T	CP13	CP15
C _{12:0}	1.7	2.1	1.9
C _{14:0}	35.8	34.8	38.4
C _{14:0} DMA	2.4	3.0	2.4
C _{16:0}	3.8	4.2	4.1
C _{16:0} aldehyde	4.5	4.3	4.9
C _{16:0} DMA	13.7	13.7	14.9
C _{16:1} ω7c	3.8	4.9	3.9
C _{16:1} ω7c DMA	2.4	1.4	2.0
C _{16:1} ω9c	5.1	7.6	5.5
C _{18:0}	1.4	1.4	1.3
C _{18:0} DMA	1.8	2.8	2.1
C _{18:1} ω7c DMA	5.8	5.1	5.2
C _{18:1} ω9c	ND	1.4	ND
C _{18:1} ω9c DMA	3.1	2.7	2.7

especially among Gram-positive bacteria (Schleifer & Kandler, 1972). Quantitative analysis of the peptidoglycan amino acids of strain CP11^T showed the following molar ratio: 0.9 Orn:0.8 Ser:0.8 Asp:1.0 Glu:1.2 Ala. In addition, the peptides Orn–Asp, L-Ser–D-Glu and Asp–Orn–D-Ala were observed after partial hydrolysis of the peptidoglycan (Rhuland *et al.*, 1955). N-terminal amino acids were not detected using dinitrophenylation (Schleifer, 1985). From these data, the cell wall of strain CP11^T did not correspond to any peptidoglycan structure published thus far. While the occurrence of the peptide L-Ser–D-Glu and absence of L-Ala–D-Glu suggest a peptidoglycan cross-linked according to B-type, strain CP11^T contained L-serine at position 1 of the subunit whereas most B-type peptidoglycans have glycine in position 1. The B-type peptidoglycan B2α (or B4) contains L-serine at position 1 of the subunit and occurs in strains of the phylogenetic relatives of strain CP11^T, *E. limosum* (formerly '*Butyrivibrio rettgeri*') (Guinand *et al.*, 1969; Schleifer & Kandler, 1972; Tanner *et al.*, 1981) and *Acetobacterium woodii* (Kandler & Schöberth, 1979). The major peptidoglycan cross-linking amino acid in *E. limosum* is D-lysine, with D-ornithine occurring about one-third of the time. As no lysine was found in strain CP11^T, it is reasonable to assume that the strain possesses a peptidoglycan type B2α variation with D-ornithine instead of D-lysine in the interpeptide bridge, similar to *Acetobacterium woodii*, which has D-ornithine as its cross-linking amino acid. However, the peptidoglycans of *E. limosum* and *Acetobacterium woodii* do not contain aspartic acid (Guinand *et al.*, 1969; Kandler & Schöberth, 1979). While the elucidation of the fine structure of the peptidoglycan from strain CP11^T will require further studies, the data clearly indicate the presence of a new variation of a B-type peptidoglycan and further support the establishment of strain CP11^T as representing a novel genus and species.

DNA was isolated using a MO BIO Ultraclean Microbial DNA Isolation kit or by a standard phenol/chloroform extraction. The G+C content of DNA (mol%) was determined by HPLC (Mesbah *et al.*, 1989) using a Prevail C₁₈ reversed-phase column (Alltech) at room temperature with a mobile phase of 25 mM KH₂PO₄:CH₃CN (96:4), pH 2.5. The G+C content of the genomic DNA was 34±0.6 mol%. The universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-GGCTACCTTGTACGACTT) (numbers on primers refer to the 5'-positions relative to *Escherichia coli* 16S rRNA) were used to amplify the 16S rRNA genes as previously described (Rios-Hernandez *et al.*, 2003). The amplified products were treated with ExoSAP-IT (USB Corporation) and directly sequenced with primers directed towards conserved positions of the rRNA gene. Sequencing reactions were performed using the BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystems Inc.) as described by the manufacturer and were analysed using an Applied Biosystems 3730 DNA Analyzer. The closest recognized relatives of the new isolates were determined by performing database searches using the FASTA program (Lipman & Pearson, 1985). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequences using the SEQtools program (Rasmussen, 2002). The resulting multiple sequence alignment was corrected manually using the GeneDoc program (Nicholas *et al.*, 1997), and a phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) with the SEQtools and TreeView programs (Page, 1996). The stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same programs. All major branching nodes were confirmed by maximum-parsimony analysis (data not shown).

The almost complete 16S rRNA gene sequences (>1500 nt) of the three strains were determined. Pairwise analysis showed that strain CP11^T had 100 % 16S rRNA gene sequence similarity to strains CP13 and CP15. Sequence database searches confirmed that the unidentified organism did not correspond to any recognized bacterium and represented a previously unknown taxon. The results of the neighbour-joining analysis are shown in Fig. 1 and confirmed the association of the unknown bacterium (as exemplified by strain CP11^T) as a member of the family Eubacteriaceae of the order Clostridiales (Ludwig *et al.*, 2009). Specifically, the novel bacterium shared a branching node with *Alkalibacter saccharofermentans* Z-79820^T (92.6 % sequence similarity) and formed a loose association with organisms present within cluster XV in the low G+C containing Gram-positive bacteria of the class Clostridia (Collins *et al.*, 1994). These organisms include: *Anaerofustis stercorihominis* (90.9 % sequence similarity), members of the genus *Acetobacterium* spp. (89.3–89.8 %), members of the genus *Eubacterium* (89.1–90.5 %), *Garciella nitratireducens* DSM 15102^T (89.2 %) and *Pseudoramibacter alactolyticus* ATCC 23263 (88.1 %). There is no

Fig. 1. Unrooted phylogenetic tree showing the relationships of strain CP11^T in the family *Eubacteriaceae* (rRNA cluster XV) and some close relatives. The tree was constructed using the neighbour-joining method and was based on a comparison of approximately 1300 nt. Bootstrap values expressed as a percentage of 1000 replications are given at the branching points; only values >90 % are shown. Bar, 1 % sequence divergence.

precise correlation between the 16S rRNA gene sequence divergence and species delineation, but it is generally recognized that divergence values of 1.3 % or more are significant (Stackebrandt & Ebers, 2006). In addition to tree topology considerations and sequence divergence values of 7 % or greater with the aforementioned taxa, the separateness of the novel bacterium was supported by phenotypic, biochemical and chemotaxonomic findings, namely the unique variation of the B-type peptidoglycan and other characteristics listed in Table 2. Therefore, the unidentified bacterium is only distantly related to other taxa and merits classification at a similar taxonomic rank (i.e. genus). The name *Alkalibaculum bacchi* gen. nov., sp. nov. is proposed for the novel isolates. Characteristics which are useful in distinguishing *Alkalibaculum bacchi* gen. nov., sp. nov. from the aforementioned taxa are shown in Table 2.

Description of *Alkalibaculum* gen. nov.

Alkalibaculum (Al.ka.li.ba'cu.lum. Arabic n. *al-qaliy* the ashes of saltwort; N.L. n. *alkali* alkali; L. neut. n. *baculum* stick; N.L. neut. n. *Alkalibaculum* alkali stick).

Cells are non-sporing, strictly anaerobic rods that are motile by means of peritrichous flagella. Cells stain Gram-negative and are positive for KOH lysis. The pH optimum for growth is 8.0–8.5. Some fatty acids contain dimethyl acetal moieties. Cell wall analysis yields a peptidoglycan

amino acid molar ratio of 0.9 Orn:0.8 Ser:0.8 Asp:1.0 Glu:1.2 Ala and indicates a novel B-type configuration, a variation similar to a B2 α -type configuration containing aspartic acid. The G+C content of the DNA is 34 mol%. The type species is *Alkalibaculum bacchi*.

Description of *Alkalibaculum bacchi* sp. nov.

Alkalibaculum bacchi (bac'chi. L. gen. n. *bacchi* of Bacchus, Roman god of wine, referring to the production of ethanol by this organism).

Cells are 1.5–2.2 µm in length and 0.5–0.8 µm wide. Growth occurs between 15 °C and 40 °C; some strains grow at 45 °C (optimum temperature is 37 °C). No growth occurs at 4 °C or at 50 °C. All strains grow at pH 6.5–10.5; optimum is 8.0–8.5. Starch, aesculin, gelatin and casein are not hydrolysed. Sulfate or nitrate is not reduced. Cellulose and agar are not degraded. The end products of glucose fermentation are acetate with trace amounts of ethanol and CO₂. Vitamins are required for growth; yeast extract greatly stimulates growth. H₂:CO₂, CO:CO₂, glucose, fructose, mannose, turanose, ribose, trimethylamine, pyruvate, methanol, ethanol, *n*-propanol and *n*-butanol are used as growth substrates. Arabinose, galactose, trehalose, raffinose, melibiose, sucrose, maltose, lactose, melezitose, fucose, xylose, cellobiose, glycerol, dulcitol, mannitol, malonic acid, glycolic acid, salicin, amygdalin, choline, betaine, methylamine, dimethylamine, DMSO, adonitol,

Table 2. Characteristics useful for distinguishing between strain CP11^T and members of related genera

Taxa: 1, CP11^T; 2, *Alkalibacter saccharofermentans* DSM 14828^T (data from Garnova *et al.*, 2004); 3, *Eubacterium limosum* DSM 20543^T (Wade, 2009); 4, *Anaerofustis stercorihominis* ATCC BAA-858^T (Finegold *et al.*, 2004); 5, *Acetobacterium woodii* DSM 1030^T (Balch *et al.*, 1977; Buschhorn *et al.*, 1989); 6, *Garciella nitratreducens* DSM 15102^T (Miranda-Tello *et al.*, 2003); 7, *Pseudoramibacter alactolyticus* ATCC 23263^T (Willems & Collins, 1996). +, Positive; –, negative; +/-, positive for some strains; ND, no data available; A, acetate; B, butyrate; C, caproate; E, ethanol; F, formate; L, lactate.

Characteristic	1	2	3	4	5	6	7
Motility	+	–	–	ND	+	+	–
pH							
Range for growth	6.5–10.5	7.2–10.2	ND	ND	ND	5.5–9.0	ND
Optimum	8.0–8.5	9.0	ND	ND	ND	7.5	ND
Utilization of:							
Ethanol	+	–	–	ND	+	–	ND
H ₂ :CO ₂	+	–	+	ND	+	–*	–
Mannitol	–	+	+	+	–	+	+/-
Methanol	+	–	+	ND	–	–	ND
Peptone	–	+	+	+	ND	–	ND
Pyruvate	+	–	ND	ND	–	+	+
Trimethylamine	+	–	ND	ND	ND	ND	ND
Tryptone	–	+	ND	ND	ND	–	ND
Xylose	–	+	+/-	+	–	+	–
Major products from glucose	A	A, E, F, H ₂ , CO ₂	A, B, L, H ₂ , CO ₂	A, B	A	L	A, B, C, F, H ₂
DNA G + C content (mol%)	34	42	47	70	39	31	61

*Negative result for H₂ utilization reported.

inositol, aesculin, gum arabic, formate, acetate, propionate, butyrate, lactate, fumarate, 2-butanol, 2-propanol, hexanol, anisic acid, 3-methoxyphenol, 3-methoxycinnamic acid, 4-methoxysalicylic acid, 5-methoxysalicylic acid, syringic acid, peptone, tryptone, Casamino acids and yeast extract are not utilized. Using the API Rapid ID 32A kit, a weak reaction is observed only for glutamic acid decarboxylase. No activity is detected for urease, arginine dihydrolase, α -galactosidase, β -galactosidase, β -galactosidase 6-phosphate, α -glucosidase, β -glucosidase, α -arabinosidase, *N*-acetyl- β -glucosaminidase, α -fucosidase, alkaline phosphatase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, pyroglutamic acid arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase or serine arylamidase. Additionally from the API Rapid ID 32A, mannose and raffinose are not fermented, indole is not produced and nitrate is not reduced. Using the API ZYM kit, enzyme activity is detected only for esterase (C4) (weak reaction), with some strains indicating very weak activity for naphthol-AS-BI-phosphohydrolase (CP11^T and CP13) and *N*-acetyl- β -glucosaminidase (CP13 and CP15). Enzyme activity is not detected for alkaline phosphatase, esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, α -mannosidase or α -fucosidase. Approximately 29 % of the total fatty

acids contain dimethyl acetal moieties. The G + C content of the genomic DNA is 34 mol%.

The type strain, CP11^T (=ATCC BAA-1772^T=DSM 22112^T), was isolated from livestock-impacted soil.

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