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Phylogenetic and physiological studies of four hydrogen-producing thermoanareobes from Icelandic geothermal areas

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ABSTRACT

Hydrogen production capacities of four new thermophilic bacteria (AK_1, AK_{14}, AK_{15} and AK_{17}) isolated from Icelandic geothermal areas were investigated. All isolates were saccharolytic and degrading various mono- and disaccharides. One strain, AK_{17}, degraded cellulose and pectin, at least partially, as well as the amino acids serine and threonine. Strain AK_1 degraded pectin and xylan to some extent. Product formation from glucose fermentation was in all cases acetate, hydrogen and carbon dioxide and one or two of the following: ethanol, butyrate and lactate. Strain AK_1 produced 0.58 to 0.77 mol H_2 per mol of degraded carbohydrates in batch cultures. The values for strains AK_{14}, AK_{15} and AK_{17} ranged from 0.83-1.14, 0.46-1.27 and 0.16-0.70 mol H_2 per mol hexose degraded, respectively. The hydrogen production rates varied from 0.56 mmol H_2 l^{-1} h^{-1} to 4.34 mmol H_2 l^{-1} h^{-1}. Partial hydrogen pressure differentially influenced the amount of hydrogen produced for the four strains. Strain AK_{15} was the most sensitive and strain AK_{17} the least sensitive to hydrogen pressure. Analyses of 16S rRNA revealed that strains AK_1, AK_{14} and AK_{15} belong to various clusters of *Clostridium* whereas strain AK_{17} belongs to the genus *Thermoanaerobacterium*.

Keywords: thermophilic bacteria, hydrogen production, geothermal, phylogenetic, *Clostridium, Thermoanaerobacterium*

YFIRLIT

Vetnisframleiðsla og efnaskipti fjögurra hitakærra, gerjandi baktería (AK_1, AK_{14}, AK_{15}, AK_{17}) sem einangraðar voru úr íslenskum hverum voru rannsakaðar. Allar bakteríurnar reyndust vera sykrusundrandi og brutu niður mismunandi ein- og tvísykrur. Einn stofninn (AK_{17}) reyndist geta að hluta til brotið niður sellulósa og pektín auk amínósýranna seríns og þreoníns. Stofn AK_1 braut niður pektín og xýlan að hluta til. Í öllum tilfellum framleiddu stofnarnir ediksýru, vetni og koltvísýring við niðurbrot á glúkósa. Önnur efni sem framleidd voru, en í mismiklum mæli þó, voru etanól, smjörsýra og mjólkursýra. Við staðlaðar aðstæður í lokuðum ræktum (batch) framleiddi stofn AK_1 frá 0.58 – 0.77 mól af vetni á hvert mól af ein- og tvísykru sem var brotin niður. Fyrir aðra stofna voru þessar tölur 0.83 – 1.14 (stofn AK_{14}), 0.46-1.27 (stofn AK_{15}) og 0.16 – 0.70 (stofn AK_{17}). Vetnisframleiðsluhraðinn var einnig breytilegur eða frá 0.56 mmól H_2 l^{-1} h^{-1} (stofn

AK_{15}) upp í 4.34 mmol H_2 l^{-1} h^{-1} (stofn AK_{17}). Hlutþrýstingur vetnis í ræktunum hafði einnig mismunandi mikil áhrif á vetnisframleiðsluna. Stofn AK_{15} var mest hindraður af vetni en stofn AK_{17} minnst. Raðgreining á 16S rRNA bakteríanna gefur til kynna að stofnar AK_1, AK_{14} og AK_{15} tilheyri mismunandi tengslahópum *Clostridium* baktería en stofn AK_{17} tilheyri ættkvíslinni *Thermoanarobacterium*.

INTRODUCTION

Research on alternative energy sources has recently gained increased interest for several reasons. The excessive use of fossil fuel has lead to the increased concentration of CO_2 in the atmosphere, contributing to a potential climate change. Additionally, the increased demand for energy sources has led to rising oil prices. Biomass is a renewable energy source that may be used as an alternative source of energy. Recently, hydrogen production from biomass has obtained increased attention (Das & Vezioglu 2001, Levin et al. 2004, Rupprecht et al. 2006). Hydrogen can be produced in a number of ways from fossil fuels, e.g. by steam reforming of natural gas or by thermal cracking of natural gas and by coal gasification. However, these processes have several drawbacks, both economically and environmentally. Biologically, hydrogen can be produced by biophotolysis or through photosynthesis (Lichtl et al. 1997, Hansel & Lindblad 1998) and by fermentation (Yokoi et al. 1997, Yokoi et al. 2001). Hydrogen production by fermentative bacteria is generally preferred over photosynthesis since it does not rely on light (Nath & Das 2004).

Among mesophilic hydrogen producing microorganisms are bacteria belonging to the genus *Clostridium*, such as *C. butyricum* (Yokoi et al. 2001), *C. thermolacticum* (Collet et al. 2004) and *C. paraputrificum* (Evvyernie et al. 2001) and facultative anaerobes like, *Citrobacter* (Oh et al. 2003) and *Enterobacter* (Kumar & Das 2000, Nakashimada et al. 2002).

Thermophilic bacteria capable of hydrogen production have recently gained increased attention. Thermophiles, and especially hyperthermophiles, show superior hydrogen production rates in comparison to mesophiles and fewer fermentation byproducts are produced (van Niel et al. 2002, van Niel et al. 2003). Among well-known thermophiles are *Clostridium* (Krivenko et al. 1990), *Caloramator* (Seyfried et al. 2002), *Thermoanaerobium* (Kondratieva et al. 1989), *Thermoanaerobacter* (Wiegel & Ljungdahl 1981) and *Thermoanaerobacterium* (Liu et al. 1996). The best known extreme thermophiles are *Caldicellulosiruptor saccharolyticus* (van Niel et al. 2002, van Niel et al. 2003), *Thermotoga elfi* (de Vrije et al. 2002, van Niel et al. 2002), *T. neapolitana* (Childers et al. 1992, van Ooteghem et al. 2004), *T. maritima* (Schröder et al. 1994), and various species within *Thermococcus* and *Pyrococcus* (Blamey et al. 1999, Hoaki et al. 1994).

Most hydrogen production by microbes is driven by the metabolism of pyruvate, which is an intermediate formed during the degradation of many substrates. Pyruvate degradation occurs either through pyruvate formate lyase (enteric bacteria) or pyruvate ferredoxin oxidoreductase (strict anaerobes). The overall yield of the reaction is relatively low, usually only one to two moles of hydrogen per molecule of degraded pyruvate. Theoretically, four moles of hydrogen can be produced per mole of glucose, assuming that all pyruvate is converted to acetate (Hawkes et al. 2002). However, in reality this is not the case. A balance of oxidized and reduced end products is usually produced from carbohydrates, which lowers the hydrogen yield (Hawkes et al. 2002). For mesophilic clostridia these end products are often a mixture of acetate and butyrate; for mesophilic facultative bacteria other end products are also produced such as ethanol and lactate. For moderate thermophiles a mixture of volatile fatty acids, as well as ethanol and lactate, are produced (Hawkes et al. 2002, van Groenestijn et al. 2002). However, extreme thermophiles have been shown to produce acetate as a single end product together with hydrogen. In general, the partial pressure of hydrogen that builds up during degradation of carbohydrates influences

the flow of electrons towards more reduced end products (van Niel et al. 2002, van Niel et al. 2003). High temperature favours the kinetics and thermodynamics of hydrogen production. Therefore, thermophilic microorganisms have a higher hydrogen yield than mesophilic organisms and a lower production of reduced end products (van Niel et al. 2002). Thus, in general, mesophilic and moderate thermophilic bacteria produce less than 1.5 mol per mol of glucose degraded, whereas thermophiles and extreme thermophiles produce 1.5-2.5 mol and above per mol, respectively. The highest yield observed is for *Caldicellulosiruptor saccharolyticus* and *Thermotoga maritima*, 3.3 and 4.0 moles of hydrogen per mol of carbohydrate degraded, respectively (van Niel et al. 2002, Schröder et al. 1994).

As hydrogen production of thermophilic bacteria has not been investigated in detail the metabolism of four hydrogen-producing isolates from two different geothermal areas in Iceland is described. Both biochemical and phylogenetic (16S rRNA) characterization were done on all isolates.

MATERIALS AND METHODS

Sampling sites

Samples were collected in May 2004 from the geothermal area at Hengill in Grændalur in SW Iceland and from the Krafla area, NE Iceland. The temperature of the hot spring from where AK_1 was collected was 47.8°C and the pH was 7.6. The temperature and pH values for the other strains were 51°C and pH 7.6 for strain AK_{14}, 60°C and pH 8.6 for strain AK_{15}, and 70°C and pH 6.5 for strain AK_{17}.

Media

The medium (per litre) consisted of: KH_2PO_4 1.5 g, Na_2HPO_4 2.3 g, NH_4Cl 2.2 g, NaCl 3.0 g, $CaCl_2$ 8.8 g, $MgCl_2$ x $6H_2O$ 0.8 g, yeast extract 2.0 g, resarzurine 1 mg, trace element solution 1 ml, vitamin solution 1 ml and $NaHCO_3$ 0.8 g. Carbon and energy sources were 20 mM or in the case of polymers, 3 g l^{-1}. The vitamin solution was according to DSM141. The trace element solution (per litre) consisted of: $FeCl_2$ x 4 H_2O 2,0 g, EDTA 0.5 g, $CuCl_2$ 0.03g, H_3BO_3, $ZnCl_2$, $MnCl_2$ x 4 H_2O, $(NH_4)_6Mo_7O_{24}$ x $4H_2O$, $AlCl_3$, $CoCl_2$ x 6 H_2O, $NiCl_2$, all 0.05 g, Na_2S x $9H_2O$ 0.30 g and 1 ml of concentrated HCl. The medium was prepared by adding the buffer to distilled water, which was then boiled for 5-10 min and cooled while flushing with nitrogen. The mixture was then transferred to cultivation bottles using the Hungate technique (Hungate 1969) and then autoclaved. All other components of the medium were added separately through filter sterilized solutions.

Isolations and enrichments

Samples were collected using an extended pole equipped with grip arms placed at the end. Anaerobic bottles (120 ml) were fixed at the end, opened and completely filled with geothermal liquid/mud samples and closed with butyl rubber and aluminium caps. A 5 ml aliquot from each sample was inoculated into 45 ml serum bottles containing 2 g l^{-1} yeast extract (YE) and 20 mM glucose and incubated at temperatures slightly below the experimental site temperatures. In most cases, because of the dense inoculum it was not possible to follow growth with increased absorbance by using a spectrophotometer. Therefore, after five days, an aliquot of 5 ml of each enrichment culture was transferred into a new fresh glucose-containing medium. Positive samples were diluted (tenfold dilutions) and inoculated in the same medium with 20 g l^{-1} of Gelrite® in Hungate roll tubes. Visible colonies were picked up with sterile Pasteur pipettes and inoculated into fresh media. Seventeen isolates were obtained and analysed for partial 16S rRNA sequence analyses. Full 16S rRNA analyses were done on four of the isolates.

Growth conditions

Strains AK_1 and AK_{14} were incubated at 50°C at pH 7.0 in all experiments. Strain AK_{15} was incubated at 65° at pH 7.0 and strain AK_{17} at 60° at pH 6.0.

Hydrogen production capacity

Screening of hydrogen production capacity

was performed in small culture serum bottles with 10 ml of medium and 13 ml of gas phase in batch cultures. Various carbon sources were tested and added into the media after autoclaving from filter sterilized stock solutions. The following carbon sources were tested: fructose, galactose, glucose, mannose, arabinose, xylose, ribose, lactose, sucrose, cellulose, pectin, xylan, lactate, succinate, pyruvate, malate, oxalate, inisitol, lysine, alanine, threonine, serine, cysteine, histidine. Control bottles contained yeast extract (2 g l^{-1}) as the sole carbon source. Strains were inoculated from stock cultures kept frozen in glycerol. Before inoculation, samples were thawed and the cultures were grown overnight. From these culture bottles, 0.1 ml was inoculated in the experimental bottles. Hydrogen was measured after the growth had stopped. In one set of experiments, hydrogen production capacity was investigated with different ratios of liquid and gas phase on glucose (20 mM). The liquid phase varied from 2 ml up to 90 ml in bottles containing a total volume of 120 ml. Thus, the liquid–gas ratios varied from 0.016 to 0.75. In another set of experiments hydrogen production rates were measured. This was done in 120 ml serum bottles containing 50 ml of medium. In all growth experiments, two sample parallels were used. The controls were media without inoculation and serum bottles without carbon sources. Determination of growth was performed on a CARY spectrophotometer at 600 nm. Log-phase growth rates (μ) were derived from the absorbance (OD) data using the standard equation $\ln(X/X_0) = (\mu)(t)$, where X is the measured culture OD, X_0 is the initial culture OD, and t is the elapsed time.

Analytical methods

Hydrogen was analysed with a Perkin Elmer gas chromatograph equipped with a thermoconductivity detector. Argon was used as the carrier gas at a rate of 3 ml min^{-1}, with another 17 ml min^{-1} as make-up gas in the detectors. The separation was performed on a Supelco 1010 Carboxen GC Plot Capillary Column. The oven temperature was 65°C and the injector and detector temperatures were kept at 200°C. Samples for volatile fatty acids (VFA), lactate, glucose and ethanol were centrifuged (1ml) for 20 min at 6000g. The supernatants were acidified with 25% formic acid, and n-propanol and crotonate were used as internal standards. The analysis of VFA's and ethanol was performed by gas chromatograph (HP 5890II) using an FID detector with a 30 m DB-FFAP capillary column (Agilent Industries Inc, Palo Alto, CA, US). Glucose and lactate were analysed by diluting samples (2.5 times) using isovaleric acid as the internal standard. Analyses were carried out with an HPLC (n-1000 Refractive Index Detector, WGE DR, Bures GmbH & CoKG, Waters 510 HPLC pump, and Marathong autosampler [Spark Holland BV]). The column was SUGAR SH1011 (8.0 mm ID x 300 mm L) lot number 050620.

Phylogenetic analysis

DNA was extracted from the strains and used as templates in 16S rRNA PCR reactions. 16S rRNA genes were ampliÞed with primers F9 and R1544, which are speciÞc for bacterial genes (Skirnisdottir et al. 2000). The PCR products were sequenced with the universal 16S rRNA primers F9, F515, F1392, R357, R805, R1195, and R1544 (Skirnisdottir et al. 2000) by using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Subsequently, the DNA was analysed with a 3730 DNA analyser from Applied Biosystems. The nucleotide sequence was displayed and analysed with Sequencher (Gene Code Corporation).

The species used for the analysis (with gene accession numbers in parentheses) were as follows: *Anaerobacter polyendosporus* (Y18189), *Clostridium fallax* (M59088), *Clostridium intestinale* (AY781385), *Clostridium putrifaciens* (Y18177), *Clostridium thermoamylolyticum* (X76743), *Clostridium uzonii* (Y18182), *Thermoanaerobacterium thermosulfurigenes (X58351), Thermoanaerobacterium saccharolyticum* (L09169), *Thermoanaerobacterium aotearoense* (X93359), *Thermoanaerobacter-*

ium bryantii (AY140670), *Thermoanaerobacterium xylanolyticum* (L09172), *Ruminococcus callidus* (X85100), *Anaerotruncus colihominis* HKU19 (DQ002932), *Ruminococcus flavefaciens* JM1 (AY349157), *Ruminococcus albus* (X85098). *Archaeoglobus profundus* (AF322392), *Morella thermoacetica* (M59121), and *Thermoanaerobacterium thermosaccharolyticum* (AB059411).

Sequences from 16S rRNA analyses were submitted to the NCBI database using the nucleotide-nucleotide BLAST (BLASTn; Cole et al. 2006). Phylogenetic trees were made by using the program BioEdit (Hall 1999) and final alignments (multiple alignments) were carried out with the program ClustalX (Thompson et al. 1997). Finally, the trees were viewed (displayed) with the program TreeView.

RESULTS

Hydrogen production and growth rates from glucose

Figure 1 shows hydrogen production over time as well as biomass production for the four strains. The doubling time for strain AK_1 was 2.56 h and the hydrogen production rate 0.96 mmol $l^{-1}h^{-1}$ (Figure 1A). Strain AK_{14} produced hydrogen at the fastest rate of the four strains investigated (4.34 mmol $l^{-1}h^{-1}$) with a doubling time of 1.31 h (Figure 1B). Strain AK_{15} had the lowest growth rate (13.6 h) and the lowest hydrogen production rate (0.56 mmol $l^{-1}h^{-1}$; Figure 1C). Strain AK_{17} was the fastest growing strain (doubling time = 1.24 h) but at a much lower hydrogen production rate (1.91 mmol $l^{-1}h^{-1}$) than strain AK_{14} (Figure 1D).

End product formation from glucose

Degradation of glucose to various end products was investigated for the four strains (Table 1). All strains degraded glucose completely during the experimental time (24 h). The moderate thermophilic strains (AK_1 and AK_{14}) produced small

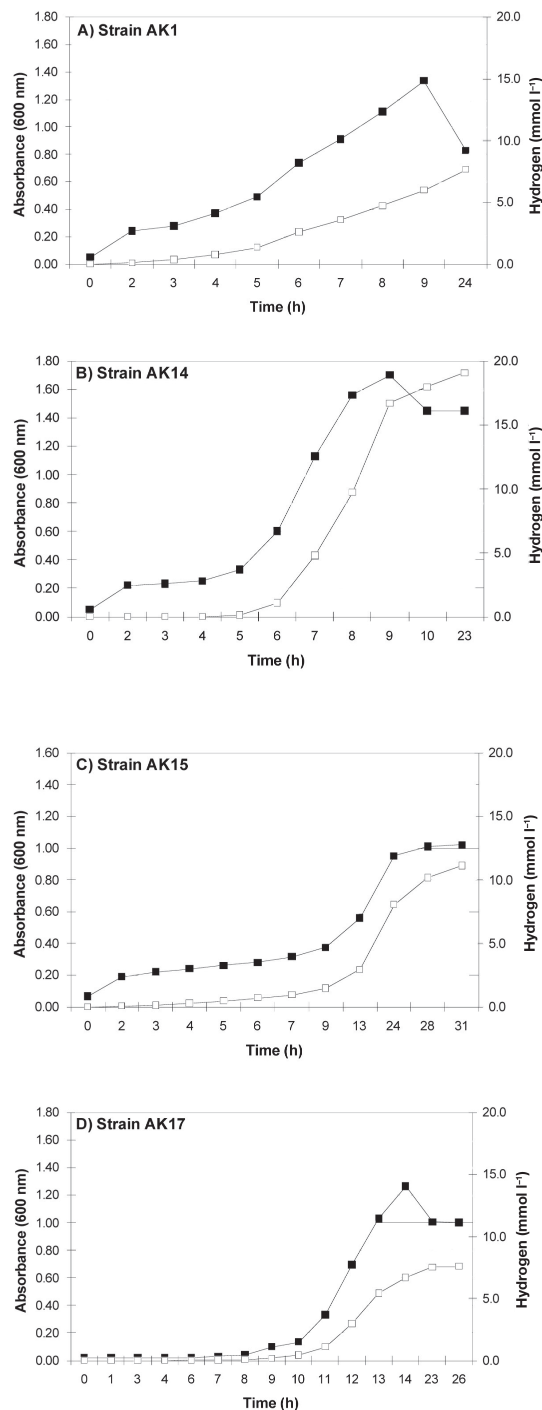

Figure 1. Increase in growth (absorbance) and hydrogen production over time for strains AK_1 (A), AK_{14} (B), AK_{15} (C) and AK_{17} (D) grown on glucose. Optical density (■), Hydrogen (□).

Table 1. End product formation (in mM) from glucose (20 mM) for the four thermophilic bacteria strains.

Strain	Ethanol	Acetate	Butyrate	Lactate	H_2	CO_2
AK_1	29.7	10.5	0.0	4.0	7.7	40.3
AK_{14}	0.0	8.3	12.0	3.3	19.1	32.3
AK_{15}	16.6	8.3	0.0	3,5	11.1	24.9
AK_{17}	31.1	11.4	0.0	0.0	7.6	42.6

amounts of acetate and lactate as well as the gaseous end products (hydrogen and carbon dioxide). The main fermentation end product for strain AK_1 was, however, ethanol and butyrate for strain AK_{14}. The thermophilic strains (AK_{15} and AK_{17}) both produced acetate and ethanol as the main fermentation products and in the case of strain AK_{15} small amounts of lactate were produced.

Substrate spectrum and hydrogen production

Hydrogen production was tested on various carbon substrates (sugars, organic acids, amino acids). None of the four strains produced hydrogen above the amounts produced from controls (YE) from lactate, succinate, malate, oxalate, inisitol, lysine, alanine, cysteine and histidine. Strain AK_1 degraded all the mono- and disaccharides tested, except arabinose. The highest hydrogen production was observed from lactose, glucose and mannose and the lowest from sucrose (Figure 2A). In addition, small amounts of hydrogen were produced from ribose, pyruvate, pectin and xylan (not shown in Figure 2 for polymers). Strain AK_{14} degraded six out of eleven of the carbohydrates tested; five monosaccharides were degraded as well as the disaccharide sucrose. The highest hydrogen production was from xylose and the lowest from mannose (Figure 2B). No hydrogen was produced from xylan, pectin and cellulose by this strain. Small amounts of hydrogen were produced from pyruvate although no biomass was produced. Strain AK_{15} degraded the same carbohydrates as AK_{14} except for also degrading ribose, at least partially. In general less hydrogen was produced by strain AK_{15} as compared to the other strains (Figure 2C). Strain AK_{17} degraded all mono- and disaccharides tested. Most hydrogen produced was from mannose and the lowest from arabinose (Figure 2D). Additionally, elevated hydrogen production as well as an increase in absorbance was found in bottles emended with pyruvate and the amino acids serine and threonine as well as from cellulose and pectin (not shown in Figure 2D).

The influence of partial pressure on the hydrogen production

To investigate the influence of the partial pressure of hydrogen (pH_2) on hydrogen production simple batch experiments were performed with different liquid and gas phase ratios on 20 mM of glucose (Figure 3). Strain AK_1 produced 34% of the theoretical maximum yield with the lowest liquid/gas phase ratio, dropping to 18-20% with the highest liquid/gas phase ratio. Strain AK_{15} was most dramatically influenced by pH_2, dropping from 52 to 11% of the theoretical yield from the lowest to highest liquid/gas phase ratios. Strains AK_{14} and AK_{17} , however, showed much less sensitivity towards a higher pH_2. Strain AK_{14} produced 43% of the theoretical yield at the lowest liquid/gas phase ratio, which dropped only to 36% at the highest liquid/gas phase ratio. Strain AK_{17}, was only slightly affected by the partial pressure of hydrogen. This can be seen further in Figure 4,which shows that the production of hydrogen leveled off dramatically for strain AK_{15} as the gas phase decreased from 90 ml to 60 ml (ratio change: 0.25 to 0.50). This was less dramatic for strains AK_1 and AK_{17}. The threshold for

strain AK_{14} was between a liquid to gas phase ratio of 0.5 and 0.75 but this strain produced the most hydrogen of the strains tested.

16S rRNA analyses of the four bacterial strains

All four strains fall into various clusters or sub-clusters of the phylum *Clostridium* (Figure 5). A full 16S rRNA analysis placed strain AK_1 into Cluster IV with the closest phylogenetic relationship with various *Ruminococcus* species and *Anaerotruncus colihomins* (89.3%). Strain AK_{14} is placed into Cluster I with the closest phylogenetic relationship with *Anaerobacter polyendosporus* (95.1%). Strain AK_{15} was closely related with *Clostridium uzonii* (98.8%) and strain AK_{17} was closely related to the genus *Thermoanaerobacterium,* and closest to *T. aotoense* (97.8%).

DISCUSSION

Hydrogen-producing microorganisms were successfully enriched from two geothermal areas in Iceland. In total, seventeen carbohydrate degrading isolates were obtained, of which four were further investigated in detail, both physiologically and phylogenetically.

The original purpose of this study was to isolate thermophilic hydrogen- (and ethanol-) producing bacteria because of the increased interest in hydrogen as an energy carrier. High temperatures favour the kinetics and thermodynamics of hydrogen production (van Groenestijn et al. 2002). The highest values of H_2 produced per mol of glucose degraded have indeed been reported from the hyperthermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga mari-*

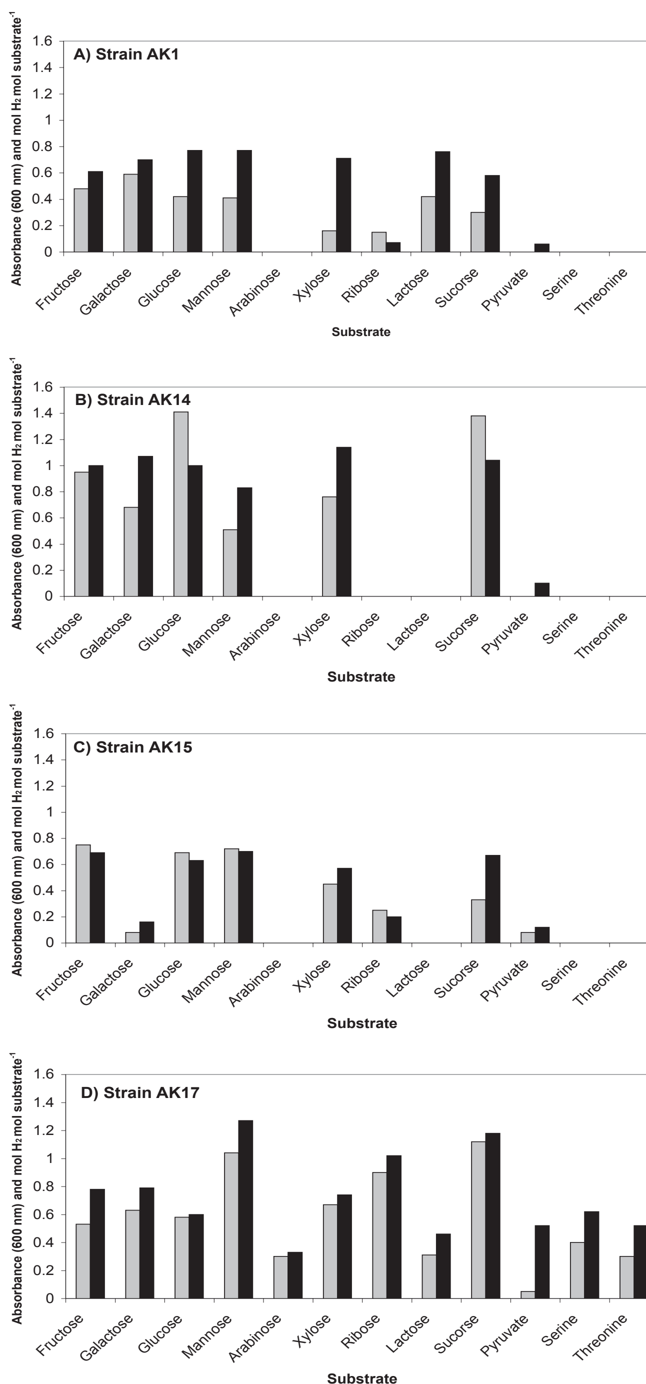

Figure 2. Growth (absorbance) and hydrogen production on various carbohydrates and serine and threonine by four strains of thermophilic bacteria AK_1 (A), AK_{14} (B), AK_{15} (C) and AK_{17} (D). Grey columns are growth (absorbance); the dark-coloured columns are hydrogen given as mol hydrogen per mol substrate consumed.

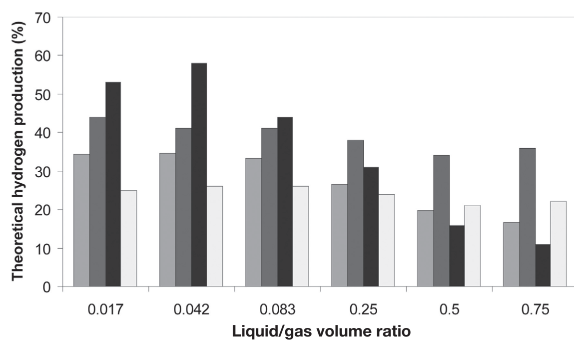

Figure 3. Percentage of theoretical hydrogen production on glucose during various liquid to gas volume ratios. From left to right: Strains AK_1, AK_{14}, AK_{15} and AK_{17}.

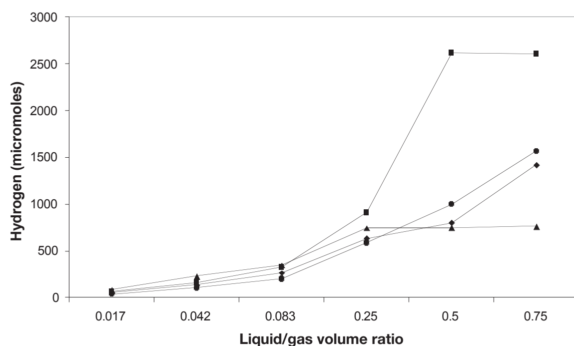

Figure 4. Hydrogen produced during fermentation of glucose (20 mM) with different gas– liquid ratios. Strain AK_1 (◆), strain AK_{14} (■), strain AK_{15} (▲), strain AK_{17} (●).

time, being 3.3 and 4.0 mol H_2 per mol carbohydrate degraded, respectively (Schröder et al. 1994, van Niel et al. 2002).

Most mesophilic organisms, both strict anaerobes like *Clostridium* and facultative anaerobes like *Citrobacter* and *Enterobacter*, have a lower H_2 yield as compared to the thermophilic and hyperthermophilic bacteria (Oh et al. 2003, Kumar & Das 2000). The main reason for this is the production of more reduced end products containing the electrons that would otherwise be rendered as hydrogen gas (Hawkes et al. 2002). The hydrogen gas production capacity of some anaerobic thermophilic organisms belonging to the genus *Thermoanaerobacterium* has also been investigated (Liu et al. 1996). Shin reported that *T. thermosaccharolyticum* and *Desulfotomaculum geothermicum* strains produced hydrogen in a thermophilic acidogenic culture (Shin et al. 2004). A hyperthermophilic archeon, *Thermococcus kodakaraensis* KOD1 with optimum growth temperature of 85°C, was isolated from a geothermal spring in Japan and identified as a hydrogen-producing bacterium (Kanai et al. 2005). *Clostridium thermolacticum* has been reported to produce hydrogen from lactose at 58°C (Collet et al. 2004).

As previously reported, the partial press-ure of hydrogen has a great influence on the hydrogen yield (van Niel et al. 2002, van Niel et al. 2003, Bothun et al. 2004). This was clearly observed for strain AK_{15} in the present study where the theoretical yield of hydrogen increased from 10% to 58% by changing the liquid/gas phase from a ratio of 0.75 to 0.042 (Figure 3). Generally, the hydrogen yield for the four strains investigated were comparable to many mesophilic bacteria. This is perhaps not surprising for strains AK_1 and AK_{14}, which are on the verge of mesophile/thermophile boundaries. In this investigation, simple batch experiments were performed with a fixed liquid/gas-ratio (10/13) on all substrates to compare the hydrogen yield between substrates. Figure 2 shows hydrogen production from mono- and disaccharides as well as from the amino acids serine and threonine). Strain AK_1 and strain AK_{14} produced 0.77 mol and 1.00 mol of hydrogen per mole glucose, respectively. This is 20-25% of the theoretical yield but the yield could be enhanced by lowering the partial pressure of hydrogen to 34% (strain AK_1) and to 43% (strain AK_{14}). Generally, strain AK_{14} was the best hydrogen producer of the four isolates. The hydrogen yield was slightly higher (29%), however, from other carbon sources such as xylose and mannose and could be enhanced by lowering the partial pressure of hydrogen to 34% (strain AK_1) and to 43% (strain AK_{14}). Strains AK_{15} and AK_{17} had a temperature optimum at 60°C but showed lower yields of hydrogen per mole

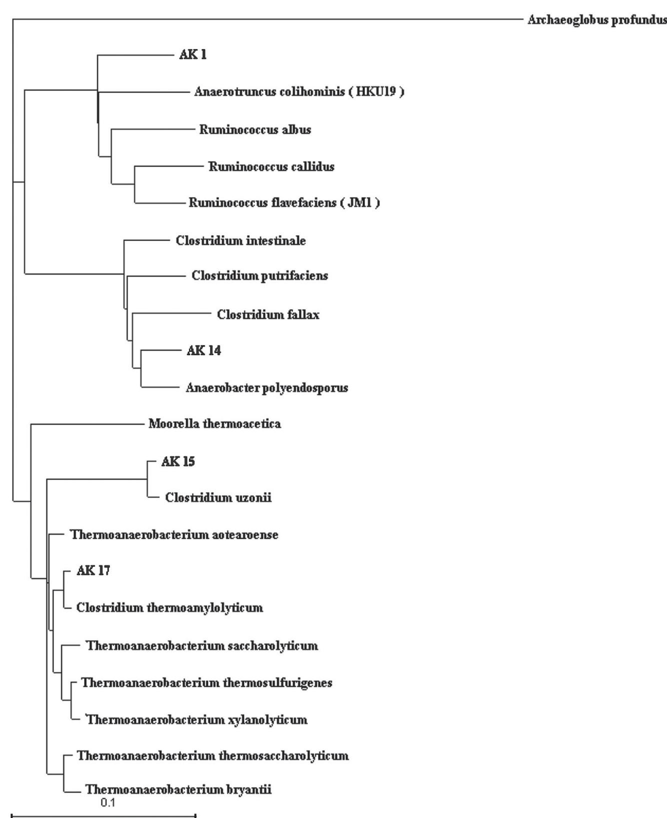

Figure 5. Phylogenetic position of strains AK_1, AK_{14}, AK_{15} and AK_{17} among members of various clusters within Clostridia. Bar represents 10% sequence divergence.

glucose with 13-16% of the theoretical yield. Strain AK_{15} was slightly better than AK_{17} in terms of the mol to mol ratio with glucose as the carbon source. However, strain AK_{17} showed up to 30% of the theoretical yield when mannose was the carbon source. Again, the theoretical hydrogen yield increased by lowering the partial pressure of hydrogen. Other factors such as gradual decreases in pH inhibited hydrogen production since pH affects the activity of the iron-containing enzyme hydrogenase (Dabrock et al. 1992). Therefore, control of pH at the optimum level is required. Initial pH also influences the extent of lag phase in batch hydrogen production. Media composition and temperature are also important parameters affecting the duration of lag phase. Some studies have reported that a low initial pH of 4.0–4.5 causes longer lag periods such as 20 h (Khanal et al. 2004). High initial pH levels decrease lag time but on the other hand lower the yield of hydrogen production (Zhang et al. 2003). The main reason for a low pH during glucose fermentation is from the production of acids into the medium. Thus, by using high glucose (or other carbon sources) concentrations in batch cultures will clearly influence both the hydrogen production yield and rate of formation. This has been shown for *Citrobacter* species where 1 g l^{-1} (5.54 mM), 5 g l^{-1} (27.7 mM) and 20 g l^{-1} (110.8 mM) of glucose yielded 2.5, 1.2 and 0.8 mol of H_2 per mol glucose respectively (Oh et al. 2004).

Hydrogen production rates are of great importance when considering the efficiency of hydrogen-producing bacteria. From the data presented in this investigation it can be concluded that the four tested strains show similar results as *C. thermolacticum* (2.58 mmol $l^{-1}h^{-1}$; Collet et al. 2004) but much lower in comparison with *E. aerogenes* (17.4 mmol $l^{-1}h^{-1}$; Tanisho 1996). Strain AK_{14} was in fact considerably better than *C. thermolacticum* with 4.34 mmol $l^{-1}h^{-1}$, followed by strain AK_{17} with 1.91 mmol $l^{-1}h^{-1}$. These yields may change when optimization experiments have been done testing the influence of pH_2, pH and the glucose concentration. Ongoing research has indeed shown how these factors can affect hydrogen production. Mixed culture experiments of strains AK_{15} and AK_{17} have been performed in a continuously stirred tank reactor (CSTR). Preliminary results show that about 6.5 mmol $l^{-1}h^{-1}$ were produced and that strain AK_{17} became dominant in the culture (90%) after 15 – 20 days (results not shown).

The four isolates characterized in this study fall within different clusters of clostridia (Figure 5). This does not necessarily mean that they are members of the genus *Clostridium*. If bacteria are to be classified as *Clostridium* they have to have the following phenotypic characteristics: anaerobic and rod shaped, spore forming and unable to use sulphate as an electron acceptor. Strains AK_1, AK_{14} and AK_{15} could not use any sulphuric compound and are rod shaped and anaerobic (results not shown). No spores have been detected in strains AK_1 and AK_{14} but it was possible to detect spores in strain AK_{15} (and AK_{17}). Strain AK_{17} could use thiosulphate and produce sulphur (results not shown). From this information it can be concluded that strain AK_{15} belongs to the genus *Clostridium* and strain AK_{17} belongs to the genus *Thermoanaerobaterium*. Strains AK_1 and AK_{14} are, however, possible members of new genera since their phylogenetic distances are far away from any known species and genera.

Results from partial 16S rRNA analysis of strain AK_1 indicated the closest phylogenetic relation to *Ruminococcus* (92%) or other related species. A full 16S rRNA confirmed these results but the closest phylogenetic relation changed to an 88.9% relation to *Anaerotruncus colihomins* and 88.5% to *Ruminococcus albus.* This relation is in phylogenetic context considerably removed from the 97% limit for a new species and also from the 94% limit of a new genus (Stackebrandt et al. 1999). Thus it can at least be concluded that strain AK_1 is a new species and should be placed within the *Clostridium* cluster VI until thermoanaerobic bacteria have been reclassified. Initial results from a partial 16S rRNA analysis of strain AK_{14} gave the closest phylogenetic relation to *Sarcina maxima* (94%). A full 16S rRNA indicated the closest phylogenetic relationship (94.8%) with *Anaerobacter polyendosporus.* Thus, strain AK_{14} is likely a new species within *Clostridium* cluster I (Collins et al. 1994). Most strains within this cluster produce butyric acid (Collins et al. 1994). *Clostridium thermobutyricum* is also in this cluster but is only 89% related to strain AK_{14}. Butyrate production among thermoanaerobes is rare and has only been reported for *Thermoanarerobacterium thermosaccharolyticum* and *Clostridium palmarum* besides *C. thermobutyricum* (McBee 1950, Freier et al. 1981, Wiegel et al. 1989, Canganella et al. 2002). Strain AK_{14} produces 0.55 moles of butyrate per mole of glucose degraded whereas *T. thermosaccharolyticum* and *C. palmarum* produces only 0.1 – 0.4 moles. However, *C. thermobutyricum* produced up to 0.85 moles of butyrate per mole of glucose degraded. Strain AK_{15} is closely related to *Clostridium uzonii* (98.8%) isolated from a hot spring in Kamchatka Peninsula (Krivenko et al. 1990). C. *uzonii* is a deeply branching member of cluster V, comprising members of T*hermoanaerobacter*. From a phylogenetic perspective strain AK_{15} is most likely *C. uzonii.* Comparing the main physiological differences between strain AK_{15} and *C. uzonii* reveals that the only comparable difference is in the ability of *C. uzonii* to degrade arabinose. Analysis of 16S rRNA placed strain AK_{17} in *Thermoanaerobacterium*, with the closest relationship with *T. aotearoense* (97.7%). Members of *Thermoanaerobacter* and *Thermoanaerobacterium* have been isolated from unique areas such as deep surface oil wells (Cayol et al. 1995), geothermal water outlets (Cook et al. 1996, hot springs (Cann et al. 2001) and from the leachate of a waste pile from a canning factory (Lee et al. 1993). The products from glucose fermentation by *Thermoanaerobacterium* have been reported to be acetate, ethanol and CO_2 as well as lower yields of hydrogen, formic acid and lactate. Strain AK_{17} produced large amounts of ethanol but less of acetate and hydrogen. From the phylogenetic data it is clear that DNA:DNA hybridization is needed for *T. aotoense* and *T. amylolyticum* to see whether strain AK_{17} is a new species within the genus *Thermoanaerobacterium*. Interestingly, strain AK_{17} degraded serine and threonine but the ability of saccharolytic thermoanaerobes to degrade amino acids is not common. *Thermoanaerobacter brockii* has been shown to degrade several amino acids (Fardeau et al. 1997).

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