

Prabha Iyer · Mary Ann Bruns · Husen Zhang ·
Steve Van Ginkel · Bruce E. Logan

H₂-Producing bacterial communities from a heat-treated soil inoculum

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Abstract Hydrogen gas (~60% H₂) was produced in a continuous flow bioreactor inoculated with heat-treated soil, and fed synthetic wastewater containing glucose (9.5 g l⁻¹). The pH in the bioreactor was maintained at 5.5 to inhibit consumption of H₂ by methanogens. The objective of this study was to characterize bacterial communities in the reactor operated under two different hydraulic retention times (HRTs of 30-h and 10-h) and temperatures (30°C and 37°C). At 30-h HRT, the H₂ production rate was 80 ml h⁻¹ and yield was 0.91 mol H₂/mol glucose. At 10-h HRT, the H₂ production rate was more than 5 times higher at 436 ml h⁻¹, and yield was 1.61 mol H₂/mol glucose. Samples were removed from the reactor under steady-state conditions for PCR-based detection of bacterial populations by ribosomal intergenic spacer analysis (RISA). Populations detected at 30-h HRT were more diverse than at 10-h HRT and included representatives of Bacillaceae, Clostridiaceae, and Enterobacteriaceae. At 10-h HRT, only Clostridiaceae were detected. When the temperature of the 10-h HRT reactor was increased from 30°C to 37°C, the steady-state H₂ production rate increased slightly to 463 ml h⁻¹ and yield was 1.8 mol H₂/mol glucose. Compared to 30°C, RISA

fingerprints at 37°C from the 10-h HRT bioreactor exhibited a clear shift from populations related to *Clostridium acidisoli* (subcluster Ic) to populations related to *Clostridium acetobutylicum* (subcluster Ib).

Introduction

The recent surge in H₂ production research promises to make this clean fuel an important part of the energy balance of the global economy (Benemann 1996; Melis and Happe 2001; Van Ooteghem et al. 2002). The environmental friendliness of this fuel, however, has been undermined by the fact that the vast majority of H₂ currently is produced from non-renewable sources such as natural gas (Chaudhary and Goodman 1999; Suzuki et al. 2000). It is important to move toward more sustainable methods, one of which is H₂ production by microorganisms during fermentation of organic wastes from wastewater or other renewable sources.

Most research on conversion of sugars or organic wastes to H₂ via fermentation has been performed with pure cultures, such as *Bacillus licheniformis* (Kalia et al. 1994), *Enterobacter aerogenes* (Rachman et al. 1998), *Clostridium butyricum* (Karube et al. 1982) and *Clostridium acetobutylicum* (Chin et al. 2003). These studies were performed under aseptic conditions and reported H₂ recoveries between 1.1 mol H₂/mol glucose and 2.0 mol H₂/mol glucose. The maximum yield per mole of glucose is considered to be 4 mol H₂ when acetic acid is produced (Gottschalk 1986). Several studies have also reported H₂ production from wastewater or solid waste by mixed cultures in batch or chemostat reactors (Fang and Liu 2002; Lay et al. 1999; Lin and Lay 2004; Mizuno et al. 2000; Noike and Mizuno 2000; Ueno et al. 1995, 1996). Most of these studies were performed at temperatures between 35°C and 60°C with H₂ yields being as high as 2.7 mol H₂/mol hexose (Yokoi et al. 2002).

Although significant yields of H₂ have been obtained from mixed cultures, very little information is available on the microbial species which produce H₂ under these

P. Iyer · M. A. Bruns (✉)
Department of Crop and Soil Sciences, The Pennsylvania State University,
116 ASI Bldg,
University Park, PA, 16802, USA
e-mail: mvb10@psu.edu
Tel.: +1-814-8630779
Fax: +1-814-8637043

H. Zhang · S. Van Ginkel · B. E. Logan
Department of Civil and Environmental Engineering, The Pennsylvania State University,
116 ASI Bldg,
University Park, PA, 16802, USA

P. Iyer · M. A. Bruns · B. E. Logan
Penn State Institutes of the Environment, The Pennsylvania State University,
116 ASI Bldg,
University Park, PA, 16802, USA

conditions. In some cases, *Clostridium* spp. were presumed to be the H_2 -producers (Chen et al. 2002; Lee et al. 2001; Liu and Fang 2002). Consistent with this assumption, some researchers have attempted to manipulate the microbial community by heat treatment of the inoculum to eliminate non-spore-formers such as methanogens (Lay 2000; Lin and Lay 2004; Logan et al. 2002; Van Ginkel and Sung 2001). The efficacy of this procedure has been variable, and H_2 yields have ranged from 0.92 H_2 /mol glucose (Logan et al. 2002) to 4.8 mol H_2 /mol sucrose (Lin and Lay 2004). The only studies identifying the bacteria present in a mixed H_2 -producing reactor have been by two groups, Fang and coworkers (Fang et al. 2002a,b) and Ueno and coworkers (Ueno et al. 2001). The inoculum used in each case was treated sewage sludge. *Clostridium* spp. were observed in all these studies; other genera detected included *Sporolactobacillus* (Fang et al. 2002b), *Streptococcus* (Fang et al. 2002a) and *Thermoanaerobacterium* (Ueno et al. 2001).

The objective of this study was to characterize bacterial communities in a bioreactor inoculated with heat-treated soil and operated at two different hydraulic retention times (HRT; 30 h and 10 h) and temperatures (30°C and 37°C). We used a soil inoculum that had exhibited highly reproducible steady-state H_2 production rates and yields in previous batch tests (Logan et al. 2002). Biogas samples but not soluble products were measured to determine H_2 production rates and yields from glucose.

Materials and Methods

Source of inoculum

Surface soil from tomato plots on the Penn State University Park campus was sampled on two different dates. Soil was oven-dried without additional moisture at 105°C for 2 h to kill vegetative bacteria as described previously (Logan et al. 2002). Inocula consisted of 10-g samples for independent runs at 30-h HRT and 10-h HRT.

Reactor inoculation and operation

A continuous flow stirred tank reactor (CSTR) with a working volume of 2 l was used for all experiments. The reactor was inoculated and operated in batch mode until H_2 production was detected, after which it was operated in continuous mode. Synthetic wastewater feed consisted of the following components (in g l⁻¹ concentrations): glucose (9.46); NH_4Cl (0.88); KH_2PO_4 (0.40); K_2HPO_4 (0.40); $MgSO_4 \cdot 7H_2O$ (0.48); $FeCl_3$ (0.075); $NiSO_4$ (0.048); $CaCl_2$ (0.075); $Na_2BO_7 \cdot H_2O$ (0.011); $ZnCl_2$ (0.035); $CoCl_2 \cdot 6H_2O$ (0.032); $CuCl_2 \cdot 2H_2O$ (0.015); $MnCl_2 \cdot 4H_2O$ (0.045). The pH was maintained at 5.5 with 0.1 M NaOH to suppress methanogenic activity. Establishment of steady-state conditions at 30-h and 10-h HRTs was assessed by measuring total gas and H_2

production and determining that production rates were constant over a period of three consecutive HRTs.

Chemical assays

Glucose was estimated by the phenol sulfuric acid assay for reducing sugars (Dubois et al. 1956). Biogas production was continuously monitored with a bubble meter calibrated according to the manufacturer's instructions (Challenge Environmental Systems AER-200 respirometer, Fayetteville, Ark.). The composition of biogas in the reactor (H_2 , CO_2 , and CH_4) was measured by gas chromatography (Logan et al. 2002). Reactor biomass was estimated by the Mixed liquor suspended solid (MLSS) method according to Standard Methods 2540 (APHA 1995).

Sample removal and DNA extraction

Samples (2 ml) were withdrawn periodically from the reactor with a needle inserted through a port on the top surface. At 30°C and 30-h HRT, biomass samples were taken at 300, 450, and 600 h following the switch to continuous mode. At 30°C and 10-h HRT, biomass samples were taken at 48, 72, 96, and 120 h, after which the temperature was raised to 37°C. Once the 30-h HRT reactor was switched to 37°C, biomass samples were obtained after 12, 24, and 48 h. All samples were centrifuged immediately after removal from the bioreactor to obtain cell pellets. Supernatant medium (1 ml) was removed from each tube and replaced with 1 ml sterile glycerol. Mixtures were stored at -20°C until analysis.

DNA was extracted from quickly thawed cell pellets after removal of supernatant. Cells were lysed with a 2-min bead-beater treatment at high speed (BioSpec, Bartlesville, Okla.) and DNA was purified with an Ultraclean Soil DNA Kit according to the manufacturer's protocol (Mobio Laboratories, Carlsbad, Calif.). DNA yields were between 500 ng and 2 µg per 2 ml sample. DNA was quantified and its purity verified spectrophotometrically with a Lambda 40 UV/VIS spectrophotometer (Perkin Elmer, Norwalk, Conn.).

Ribosomal intergenic spacer fingerprinting

In previous work by the authors, ribosomal intergenic spacer analysis (RISA) fingerprinting was referred to as ribosomal intergenic transcribed spacer (ITS) analysis (Zhang et al. 2002), and similar methods were used in this study. Purified DNA was PCR-amplified with the bacteria-specific 16S rDNA primer set 926f (16S rRNA) and 115r (23S rRNA) (Lane 1991). PCR buffer (Promega, Madison, Wis.), 2.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promega), 30 pmol each primer, 400 µM each deoxynucleoside triphosphate and 10–40 ng template were used per reaction in a final volume of 50 µl. Amplification

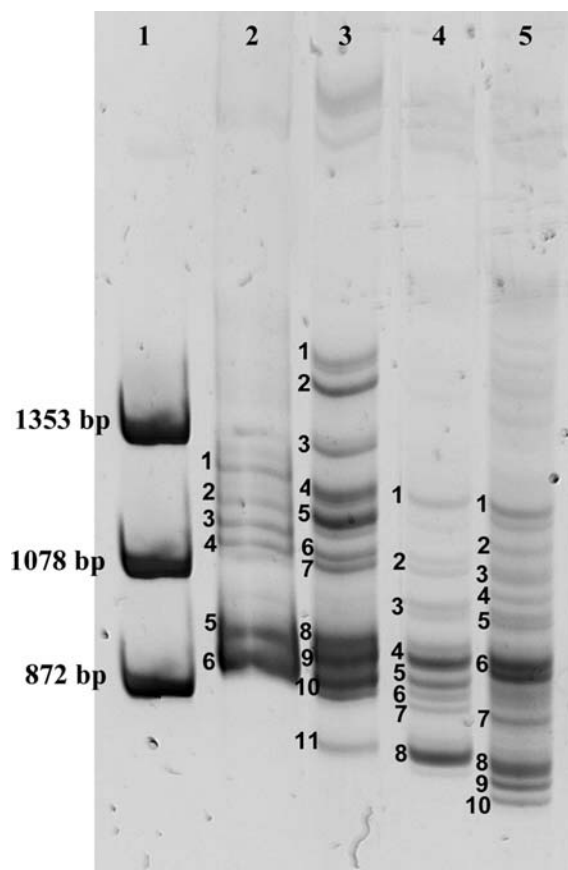
Table 1 Biomass, biogas, and H₂ production rates and yields at 30°C and 30-h hydraulic retention time (HRT), 30°C and 10-h HRT, and 37°C and 10-h HRT

Parameter	Hydraulic retention time		
	30-h	10-h	
Sampling time (h) after conditions established	600	96	48
Temperature (°C)	30	30	37
Biomass (mg/l)	675	946	950
Glucose removed (%)	97	96	96.5
Biogas (ml/h)	147	780	813
H ₂ (ml/h)	80	436	463
Yield (mol H ₂ /mol glucose)	0.9	1.6	1.8

was performed according to the following thermal cycle in a GeneAmp PCR system 9600 (Perkin Elmer): 5 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 54°C and 1 min at 72°C; followed by a 5-min final extension at 72°C.

PCR products generated with bacterial primers were separated by electrophoresis in an Adjustable Slab Gel Kit, model ASG-250 (CBS Scientific, Del Mar, Calif.) and 1 mm-thick (22 cm × 16 cm glass plates) polyacrylamide gels with the polyacrylamide concentration at 5%. The gels were prepared and run in 1× TBE buffer (pH 8). Samples were electrophoresed for 20 h at 95 V. Polyacrylamide gels were stained with SYBR Green (Applied Biosystems, Foster City, Calif.) for 1 h. The UV gel image was captured with an Epi Chemi II Darkroom unit (UVP Laboratory Products, Upland, Calif.). DNA bands were excised and eluted from the gel by the crush and soak procedure (Sambrook et al. 1989). Eluted DNA fragments were ligated with the pCR4-TOPO vector and transformed into TOP 10 competent cells (TOPO TA cloning kit, Invitrogen, Carlsbad, Calif.). Plasmids were isolated from monoclonal cultures and the insert sequenced in both directions with an ABI Hitachi Genetic Analyzer and M13 universal and M13 reverse primers. Two clones were sequenced per band observed in each RISA fingerprint. Sequences were checked for chimeric PCR products with the CHIMERA CHECK (Maidak et al. 1999) and Bellerophon (Hugenholtz and Huber 2003) programs.

Unique operational taxonomic units (OTUs) were identified only from the 16S rRNA gene portions of the RISA-PCR products (i.e., spacer regions were not used in the analysis). Closest affiliations for 16S rRNA gene sequences were identified by database searches with BLAST (Altschul et al. 1990). CLUSTALX (Thompson et al. 1997) was used to align sequences obtained from RISA bands with sequences from previously described bacteria. Phylogenetic trees were constructed based on approximately 600 nucleotide positions (*Escherichia coli* positions 910–1,510) and maximum likelihood analysis with the SEQBOOT, DNAML, DNADIST, CONSENSE and FITCH programs in PHYLIP (Felsenstein 1993). GenBank accession numbers for unique OTUs are AY591771–AY591783.

**Fig. 1** Inverted image of SYBR green-stained polyacrylamide gels showing DNA bands in ribosomal intergenic spacer analysis (RISA) fingerprints. Lanes: 1 DNA marker, 2–5 samples from bioreactors operated as follows: 2 30-h HRT/30°C after 600 h, 3 10-h HRT/30°C reactor after 72 h, 4 10-h HRT/37°C after 12 h (earlier), 5 10-h HRT/37°C after 48 h (later)

Results

Hydraulic retention time

Both biogas and H₂ production rates increased approximately 5-fold when HRT was decreased 3-fold from 30 h to 10 h (Table 1). H₂ yield also increased by 77% from 0.91 mol H₂/mol glucose to 1.61 mol H₂/mol glucose. Biomass at 10-h HRT (946 mg l⁻¹) was only slightly higher than biomass at 30-h HRT (675 mg l⁻¹). Greater than 96% of the glucose was consumed at either HRT (Table 1). Further, the proportion of H₂ and CO₂ in the gas evolved remained constant at approximately 57% and 43%, respectively. No CH₄ was detected in any sample.

Temperature

Changing the temperature of the 10-h HRT bioreactor from 30°C to 37°C had only a slight impact on gas production under the conditions studied. Gas and H₂ production were slightly higher at 37°C than at 30°C, but the increases were less than 10% (Table 1). H₂ yield at

Table 2 Bacterial 16S rDNA sequences detected under varying bioreactor conditions. OTU Operational taxonomic unit

Condition	Total bands	Number of bands with OTU	Band numbers in Fig. 1	OTU (accession no.)	Family	Closest relative in GenBank (accession no.)	Similarity (%)
30-h HRT, 30°C	6	3	2,3,6	30H-30-1 (AY591771)	Bacillaceae	<i>Bacillus myxolacticus</i> (D16274)	99
		1	5	30H-30-2 (AY591772)	Clostridiaceae (Ic)	<i>Clostridium acidisoli</i> (AJ237756)	98
		1	1	30H-30-3 (AY601654)	Bacillaceae	<i>Bacillus racemilacticus</i> (D16278)	95
		1	1	30H-30-4 (AY591781)	Enterobacteriaceae	<i>Klebsiella ornithinolytica</i> (AF251467)	98
		1	4	30H-30-5	n.a. (chimera)	n.a	n.a
10-h HRT, 30°C	11	10	1–10	10H-30-1 (AY601655)	Clostridiaceae (Ic)	<i>C. acidisoli</i> (AJ237756)	96–98
		1	10	10H-30-2 (AY591773)	Clostridiaceae (Ib)	<i>Clostridium</i> sp. 44a-T5zd (AY082483)	97
		1	11	10H-30-3 (AY591774)	Clostridiaceae (Ib)	<i>Clostridium acetobutylicum</i> (U16166)	97
10-h HRT, 37°C earlier ^a	8	5	1,4,5,7,8	10H-37-1 (AY591775)	Clostridiaceae (Ib)	<i>C. acetobutylicum</i> (U16166)	96–97
		6	1,3,5,6,7,8	10H-37-2 (AY591776)	Clostridiaceae (Ib)	<i>Clostridium</i> sp. 44a-T5zd (AY082483)	98
		1	2	10H-37-3 (AY591777)	Clostridiaceae (Ic)	<i>C. acidisoli</i> (AJ237756)	97
10-h HRT, 37°C later ^b	10	7	2-9	10H-37-4 (AY591778)	Clostridiaceae (Ib)	<i>C. acetobutylicum</i> (U16166)	96–97
		4	4,6,7,8	10H-37-5 (AY591779)	Clostridiaceae (Ib)	<i>Clostridium</i> sp. 44a-T5zd (AY082483)	98–99
		1	10	10H-37-6 (AY591780)	Clostridiaceae	Uncultured <i>Clostridium</i> (AF191232)	92
		1	2	10H-37-7 (AY591782)	Enterobacteriaceae	<i>Citrobacter braakii</i> (AF025368)	99
		1	1	10H-37-8 (AY591783)	Enterobacteriaceae	<i>Enterobacter cloacae</i> (AF511434)	99

^a12 h after raising temperature from 30°C to 37°C^b48 h after raising temperature to 37°C

37°C was also slightly higher at 1.8 mol H₂/mol glucose, compared to 1.6 mol H₂/mol glucose at 30°C. The increase in temperature appeared to have little effect on biomass. The proportion of H₂ in biogas was similar (56–57%) at both temperatures.

RISA fingerprints

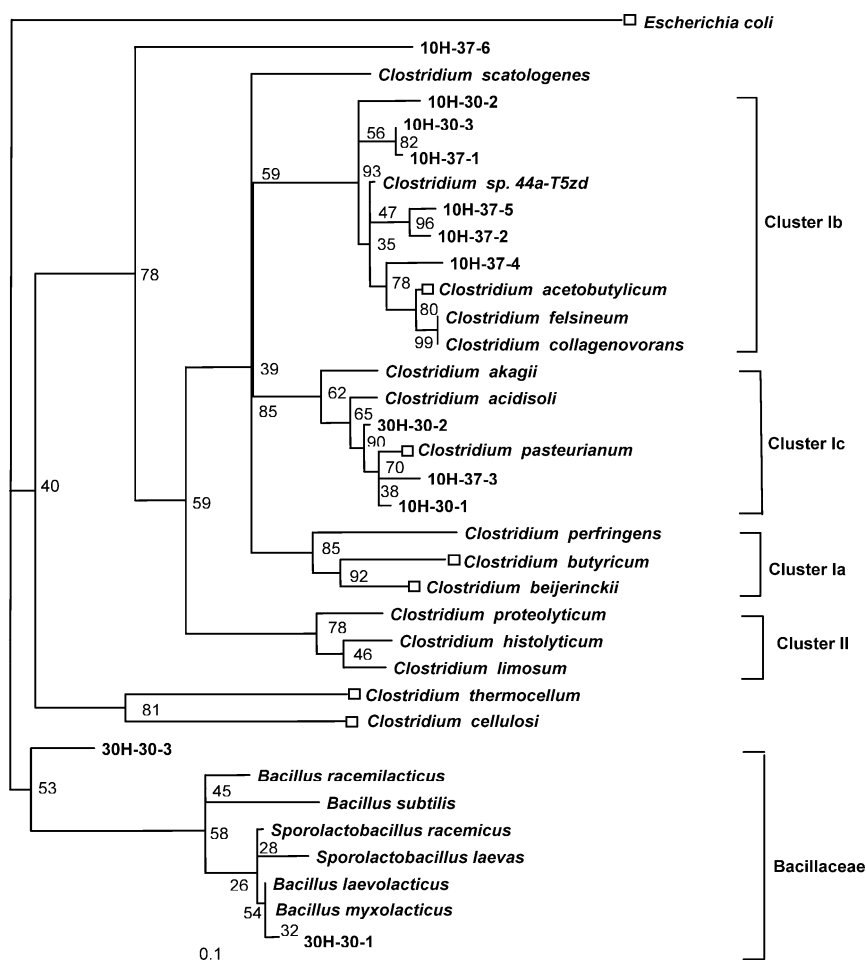
Bioreactor communities at 30-h HRT and 30°C produced RISA fingerprints containing the same six DNA bands after 300, 450, and 600 h; the latter fingerprint is shown in Fig. 1, lane 2. When the bioreactor was operated in a separate run at 10-h HRT and 30°C, samples taken after 48, 72, 96, and 120 h all contained the same 11 bands. The RISA fingerprint produced at 72 h is shown in Fig. 1, lane 3.

Because all RISA fingerprints contained several bands of different spacer lengths but nearly identical sequences

in the 16S rRNA gene regions, the 16S rRNA gene sequences with >99% identity from each bioreactor community were grouped into OTUs (Table 2). At 30°C, rRNA gene sequence diversity at 30-h HRT was more diverse than at 10-h HRT. Bacillaceae, Clostridiaceae, and Enterobacteriaceae were detected at 30-h HRT, while only Clostridiaceae were detected at 10-h HRT. Of the 11 bands, 10 in the 10-h HRT fingerprint yielded DNA sequences affiliated with Cluster Ic clostridia, while 2 bands gave rise to sequences from Cluster Ib clostridia (Table 2). Relationships among OTUs related to low G+C Gram-positive bacteria and Enterobacteriaceae are shown in phylogenetic trees in Figs. 2 and 3, respectively.

After 120 h operation of the 10-h HRT reactor, the temperature was raised from 30°C to 37°C. The first DNA sample, taken 12 h after the temperature increase, produced a RISA fingerprint containing eight DNA bands (Fig. 1, lane 4), seven of which yielded sequences affiliated with Cluster Ib clostridia (Table 2). As quickly as

Fig. 2 Maximum likelihood tree showing the phylogenetic relationships of 11 clones from the H₂ bioreactor aligned with reference strains from the Clostridiaceae and Bacillaceae families based on 16S rDNA sequences. Bootstrap values are based on analysis of 1,000 replicates. Bar 0.1 substitutions per nucleotide position. Known H₂-producing bacterial species. GenBank accession numbers for the sequences used in phylogenetic analyses are: *Escherichia coli* V00348, *Clostridium scatologenes* AJ427628, *Clostridium* sp. 44a-T5zd AY082483, *C. acetobutylicum* NC 003030, *C. felsineum* AF270502, *C. collagenovorans* X73439, *C. akagii* AJ 237755, *C. acidisoli* AJ237756, *C. pasteurianum* M23930, *C. perfringens* NC 003366, *C. butyricum* AJ289706, *C. beijerinckii* X68719, *C. proteolyticum* X73448, *C. histolyticum* M59094, *C. limosum* M59096, *C. thermocellum* L09173, *C. cellulosi* L09177, *Bacillus laevolacticus* D16270, *B. myxolacticus* D16274, *B. racemilacticus* D16279, *B. subtilis* Z99104, *Sporolactobacillus racemicus* D16291, *S. laevas* D16288



12 h after the temperature was raised to 37°C, therefore, 87% of cloned sequences were affiliated with Cluster Ib clostridia (10H-37-1 and 10H-37-2), whereas only two cloned sequences (13%) grouped with Cluster Ic (10H-37-3) (Table 2). After 48 h, the RISA fingerprint contained 10 bands (Fig. 1, lane 5), but no representatives of Cluster Ic clostridia were detected among the 20 cloned sequences. Close relatives of *C. acetobutylicum* in Cluster Ib clostridia (10H-37-4, 10H-37-5) constituted 80% of the clones examined. Interestingly, populations outside this cluster were detected in the RISA fingerprint at 48 h. These were represented by one clone (10H-37-6) associated with a *Clostridium* sequence which did not group with Cluster I (Fig. 2) and two clones (10H-37-7, 10H-37-8) associated with Enterobacteriaceae (Fig. 3).

Discussion

When the HRT of the 30°C bioreactor was shortened from 30 h to 10 h, biogas and H₂ production increased and the community composition shifted from more phylogenetically diverse sequences (Bacillaceae, Clostridiaceae, and Enterobacteriaceae) to sequences grouping solely within *Clostridium* spp. (Table 2). The detection of sequences belonging to *Bacillus* and *Clostridium* spp. at 30-h HRT

was not surprising, since spore-forming bacteria were likely to have survived the soil heat treatment. Sequences from two DNA bands at 30-h HRT and 30°C did not group with either Bacillaceae or Clostridiaceae (Table 2). One of these sequences (30H-30-5) was a chimera (Hugenholz and Huber 2003), while the second (30H-30-4) was most closely related to *Klebsiella ornithinolytica* (98%). *Klebsiella* spp. are known to include fermentative H₂-producers (Brosseau and Zajic 1982; Kumar and Vatsala 1989; Kumar and Das 2001; Solomon et al. 1995; Yokoi et al. 1997). The detection of sequences from non-spore-forming bacteria had not been expected and indicated that the dry-heat soil treatment had not destroyed all vegetative cells prior to bioreactor inoculation.

Sequences belonging to *Bacillus* spp. at 30-h HRT and 30°C were most closely related to *Bacillus racemilacticus* and *Bacillus myxolacticus*, both homofermentative lactic acid producers (Holzapfel and Botha 1988; Nakayama and Yanoshi 1967). The presence of homoacetogens could have contributed to the lower H₂ production observed at 30-h HRT (Table 1), because, at the shorter HRT, these populations could have been washed out of the reactor. Our observations are consistent with a previous study by Ueno and coworkers who reported higher H₂ production at 12-h HRT than at longer HRTs. Their study proposed that homoacetogens predominated at the longer HRTs, while

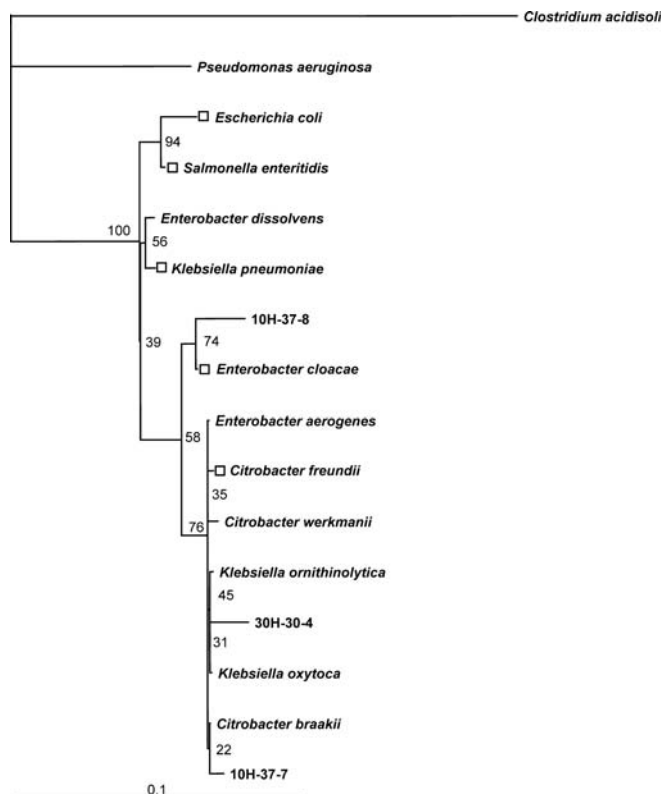


Fig. 3 Maximum likelihood tree showing the phylogenetic relationships of four clones from the H_2 bioreactor aligned with reference strains from the Enterobacteriaceae family based on 16S rDNA sequences. Bootstrap values are based on analysis of 1,000 replicates. Bar 0.1 substitutions per nucleotide position. Known H_2 -producing bacterial species. GenBank accession numbers for the sequences used in phylogenetic analyses are: *Clostridium acidisoli* AJ237756, *E. coli* V00348, *Pseudomonas aeruginosa* AE004949, *Salmonella enteritidis* U90318, *Enterobacter dissolvens* Z96079, *E. cloacae* AF511434, *E. aerogenes* AB004750, *Citrobacter freundii* AJ233408, *C. werkmanii* AF025373, *C. braakii* AF025368, *Klebsiella oxytoca* U78183, *K. ornithinolytica* AJ251467, *K. pneumoniae* AJ233420

acidogens took over at the shorter HRT, although microbiological studies were not undertaken to verify this (Ueno et al. 1996).

When the 10-h HRT reactor temperature was raised from 30°C to 37°C, gas production and H_2 yield per mole of glucose increased slightly. The effect of temperature change on RISA band patterns was also subtle, revealing a shift in sequences within Cluster I clostridia (Stackebrandt and Hippe 2001). Sequences detected at 30°C consisted of *C. acidisoli* relatives (subcluster 1c), whereas at 37°C sequences were related to *C. acetobutylicum* (subcluster 1b) following an intermediate stage where both types were detected. This shift is consistent with temperature optima reported in the literature for *C. acidisoli* (25–30°C), an acid-tolerant isolate from forest litter (Kuhner et al. 2000) and the solventogenic *C. acetobutylicum* (37°C) (McCoy and Fred 1941).

The *Clostridium* genus comprises many species displaying diverse metabolic and morphological properties (Mitchell 2001). Several *Clostridium* spp. are known to

produce H_2 (Nandi and Sengupta 1998). In fact the majority of studies of fermentative H_2 production have been performed with *Clostridium* spp. (Breure et al. 1986; Brosseau and Zajic 1982; Chin et al. 2003; Evvyermie et al. 2001; Karube et al. 1976; Wang et al. 2003). All but one of the clostridial species (Fig. 1) detected in our study belonged to *Clostridium* Cluster 1 (Collins et al. 1994; Stackebrandt and Hippe 2001). Cluster I clostridia have been consistently detected in H_2 -producing reactors (Fang et al. 2002a,b), although cluster III (Ueno et al. 2001) and cluster IV (Fang et al. 2002b; Ueno et al. 2001) have also been observed in some studies.

The RISA technique used in this study is the third type of approach reported for molecular characterization of bacterial populations in H_2 bioreactors. Earlier studies have used 16S rDNA clone libraries (Fang et al. 2002b) and denaturing gradient gel electrophoresis (DGGE) (Fang et al. 2002a; Ueno et al. 2001). In contrast to clone libraries, DNA fingerprinting techniques such as RISA and DGGE offer more convenient and comprehensive means for temporal and spatial tracking of microbial populations in bioreactors. Although Fang and Liu (2002) used DGGE analysis to study the effect of pH on reactor microbial diversity and reported greater diversity at higher pH values, they did not identify the organisms present under different pH conditions. Our study thus appears to be the first to follow bacterial population shifts in a H_2 -producing reactor under different conditions.

RISA is not a quantitative community analysis approach because it is based on standard PCR, which is associated with potential amplification bias. Thus, PCR primers developed from extant genetic databases may not amplify DNA from unrecognized populations, and DNA bands in RISA fingerprints may not appear if populations comprise less than 1% of total community DNA (Borneman and Triplett 1997). Furthermore, RISA band intensities cannot be interpreted to indicate relative population abundance because bacterial genomes may contain more than one rRNA operon; for example, the recently sequenced genome of *Clostridium acetobutylicum* ATCC 824 contains 11 rRNA operons with identical 16S rRNA genes (one of the 11 is reversed) and spacer lengths of 178 bp or 179 bp (Nolling et al. 2001). If 16S-23S rRNA spacer lengths vary among rRNA operons within a genome, DNA from an individual population may give rise to more than one RISA band. In our study it was not possible to determine whether bands having different lengths but nearly identical 16S rRNA gene regions were from the same population (Fig. 1, Table 2).

The advantage of RISA is that it offers easy access to qualitative DNA sequence data from key microbial populations undergoing changes over time. The use of community fingerprinting approaches that do not involve sequencing, such as T-RFLPs (Liu et al. 1997), may not have detected the heterogeneity within Cluster I clostridia or the population shifts that occurred with temperature increase in our study. DNA sequences obtained through RISA aid in the design of appropriate primers, probes, or restriction analyses for faster and more quantitative

community diagnosis in routine screening or monitoring. Detection of populations that reduce or interfere with H₂ production may be an important diagnostic tool for maintaining optimal bioreactor performance.

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