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Abstract

A Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) method based on functional [Fe-Fe]-hydrogenase genes was developed for monitoring the hydrogen (H_2)-producing clostridial population in mixed culture bioprocesses. New non-degenerated primers were designed and then validated on their specific PCR detection of a broad range of clostridial *hydA* genes. The *hydA* based CE-SSCP method gave a specific and discriminating profile for each of the *Clostridium* strains tested. This method was validated using H_2-producing mixed cultures incubated at temperatures ranging from 25°C to 45°C. The *hydA* CE-SSCP profiles clearly differed between temperatures tested. Hence, they varied according to variations of the H_2 production performances. The HydA sequences amplified with the new primer set indicated that diverse *Clostridium* strains impacted the H_2 production yields. The highest performances were related to the dominance of *Clostridium sporogenes*-like *hydA* sequences. This CE-SSCP tool offers highly reliable and throughput analysis of the functional diversity and structure of the *hydA* genes for better understanding of the H_2-producing clostridial population dynamics in H_2 dark fermentation bioreactors.

Keywords

Fingerprint; *hydA* functional genes; *Clostridium*; Dark fermentation; Hydrogen production; Temperature.

1. Introduction

Hydrogen (H_2) is considered as a promising energy carrier, because it is energetically dense, clean and sustainable. Among diverse renewable H_2-producing biotechnologies, dark fermentation techniques using anaerobic microbial communities have gained attention not only because of their high H_2 production rates but, also, for the ability to produce H_2 at low cost from complex and unsterilized substrates, such as organic waste materials [1, 2].

Numerous physiologically and phylogenetically diverse microorganisms can produce H_2 by dark fermentation [3]. Pretreatment methods (*i.e.* heat shock) often used to eliminate H_2 consumers (*e.g.* methanogens) which coexist with H_2-producing bacteria (HPB) in the anaerobic mixed communities of bioreactors [4]. However, heat-shock treatments decrease microbial diversity/richness and enrich the culture in sporulating HPB, such as *Clostridium* species. So far, the highest H_2 production yields have been reported with *Clostridium* pure cultures or mixed cultures where *Clostridium* species predominate [2, 5, 6]. Therefore, monitoring the presence, biodiversity and the dynamics of *Clostridium* species in bioprocesses is of major interest.

The genus *Clostridium* is a large and phenotypically heterogeneous bacterial group which as yet is not phylogenetically well defined. About 200 species have been described and are distributed in 19 clusters [7, 8] of which, the largest and phylogenetically the best-defined is cluster I [7]. It includes *Clostridium* (*C.*) *butyricum* as the type species of the genus and has been considered to contain most of the H_2-producing *Clostridium*. Hung *et al.* [9] developed a specific PCR-DGGE assay targeting 16S rDNA of cluster I *Clostridium* species for monitoring the HPB community in dark fermentation. However, *Clostridium* species belonging to others clusters have been shown to produce H_2 from diverse carbon sources, including *C. cellulolyticum* from cluster III [10] or, *C. bifermentas* from cluster IX [11], *C.*

phytofermentas from cluster XIV [10] and, in thermophilic conditions, *C. thermocellum* from cluster III [12]. Distinguishing HPB among other *Clostridium* species in bioprocesses can be difficult if investigation is based only on their 16S rDNA sequences.

Hence, alternatives approaches targeting functional genes can be useful for the specific monitoring of *Clostridium* HPB. [FeFe]-Hydrogenases ([FeFe]-H_2ases) are key enzymes in H_2 production by *Clostridium* species. Diverse primer sets and probe targeting *hydA* genes, encoding catalytic subunit of [FeFe]-H_2ases, have been developed for monitoring H_2-producing *Clostridium* species, generally *C. butyricum*, during continuous H_2 fermentation [13-18]. Despite the presence of numerous reasonable candidate genes for [FeFe]-H_2ases in genome sequence databases, these primers were designed from few number of *Clostridium* *hydA* sequences and which has decreased their ability to target a broad spectrum of HPB. In contrast, many studies have reported degenerate primer sets for detection of a wide range of *hydA* bacterial genes [19-21]. However, degenerate primers are unsuitable for directly assessing gene diversity using such molecular fingerprinting techniques as denaturing gradient gel electrophoresis (DGGE) [22] or single strand conformation polymorphism (SSCP) [23], where they produce multiple band pattern or undistinguishable peaks from single template DNA [24, 25]. Nevertheless, DGGE or CE-SSCP techniques are useful for: (i) producing molecular fingerprints based on both 16S rRNA- and/or functional genes; and (ii) comparing the shift of bacterial population structure and diversity in relation to the changes in environmental parameters [26, 27]. The use of an automatic capillary electrophoresis (CE), laser induced fluorescence system, such as CE-SSCP, makes it possible to carry out a highly sensitive, reproducible and throughput analysis of diverse bacterial communities [28, 29].

This study aimed at developing a CE-SSCP fingerprinting method for monitoring H_2-producing clostridial population in mixed cultures. Non-degenerate primers were designed and evaluated with PCR detection of a large range of clostridial *hydA* genes. The *hydA* CE-

SSCP method was then validated on pure cultures and further applied in H_2 producing mixed cultures incubated at various mesophilic temperatures.

2. Materials and methods

2.1. Bacterial strains

Four group of bacterial strains (Table 1) were used for the evaluation of the specificity of the newly-designed primer set targeting the *hydA* genes: (i) HPB strains belonging to diverse clusters of the *Clostridium* genus, including reference strains of the main clusters: I, III, IX, XIV and XVIII; (ii) strains belonging to H_2-producing species (*Enterobacter cloacae*) or genera (*Enterococcus* and *Klebsiella*), (iii) the *hydA*-carrying fully-sequenced genome HPB strains belonging to other genera (*Desulfovibrio and Syntrophomonas*), (iv) the strains with a fully-sequenced genome, *Rhodobacter sphaeroides* DSM 158^T and *Xanthobacter autotrophicus* $DSM432^T$, carryring putative genes encoding [NiFe]-H_2ase without [FeFe]-H_2ase [30].

2.2. Hydrogen production experiments

The H_2-producing experiments were carried out in 500 ml glass bottles in batch conditions. The inoculum was a digested sludge pretreated by heat/shock (90°C for 10 min). Two milliliters of the pretreated inoculum (final concentration of 225 $mgCOD.l^{-1}$) were inoculated in the following culture medium: 40 mM MES, 10 g sucrose, 1l deionized water. The total working volume was 200 ml. The initial pH was adjusted to 5.5. Each bottle was flushed with

nitrogen for 5 min to maintain anaerobic conditions. The bottles were then capped with a rubber stopper and incubated at 25, 30, 35, 40 or 45°C for 24 days. Each experiment for the different conditions was carried out in triplicates. Four milliliters of mixed culture were collected daily during the first 12 days of the experiments and every 2^{nd}-3^{rd} day during the last 12 days of the experiments, then centrifuged (20,000 g for 10 min). The supernatants and the pellets were then stored at -20°C for further chemical analysis and DNA extraction, respectively.

2.3. Chemical analysis

Biogas production was measured using an acidified water displacement method. Biogas composition (CH_4, CO_2, H_2 and N_2) was analyzed by gas chromatography (GC-14A, Shimadzu) as previously described by Aceves-Lara *et al.* [31]. The biogas produced contained only H_2 and CO_2, without detectable CH_4. Sugar concentration was assessed by High Performance Liquid Chromatography analysis coupled to refractometry detection. The H_2 production performances were evaluated according to the H_2 production yield (mol H_2/mol hexose consumed), potential (ml H_2/l) and rates (mol H_2/day).

2.4. DNA extraction, primer design and PCR amplification

For each experiment, the molecular analysis of bacterial communities were only performed on samples corresponding to maximum H_2 production rate and H_2 potential (this latter corresponding to H_2 yield).

Genomic DNA was extracted and purified from cell pellets using the Wizard Genomic DNA Purification kit (Promega). The quality and amount of DNA were measured by spectrophotometry (Infinite NanoQuant M200, Tecan).

For the PCR amplification of *hydA* genes, two new non-degenerated primers, *hydAClos*F (5' – ACCGGTGGAGTTATGGAAGC – 3', *C. pasteurianum* position F1258) and *hydAClos*R (5' – CATCCACCTGGACATGCCAT – 3', *C. pasteurianum* position R1508), were designed from two consensus sequences retrieved from an alignment of 31 *hydA Clostridium* reference sequences (Table 2). The two consensus sequences were designed using the most conserved nucleotide that occurs at each position of the selected region of the alignment. These primers span an about 200 bp long region with high variability for PCR product differentiation by CE-SSCP analysis. To avoid redundant information, no more than one *hydA* sequence for each *Clostridium* species was included in the alignment. For instance, only the *hydA* sequence of *C. butyricum* str. DSM10702 [EF627973] was selected because those of the strains CGS5 [EF450251], FBR1 [EU689097], and W5 [EU366290] were identical in the region chosen for primer design. The properties of the oligonucleotides (GC content, melting temperature, hairpin formation and self-complementarity) were checked using the Oligonucleotide Properties Calculator [32]. The ability to anneal specific sites was investigated *in silico* by FastPCR software [33]. The *hydA* sequences used for primer design and carried by *Clostridium* strains (Table 2) as well as *hydA* sequences from strains belonging to the 17 following genera were tested: *Alkaliphilus*, *Bacteroides*, *Dehalococcoides*, *Desulfitobacterium*, *Desulfotalea*, *Desulfovibrio*, *Ethanoligenens*, *Eubacterium*, *Megasphaera*, *Pelobacter*, *Rhodopseudomonas*, *Shewanella*, *Symbiobacterium*, *Syntrophomonas*, *Thermoanaerobacter*, *Thermotoga* and *Treponema*. Virtual PCRs were performed according to the following criteria: (i) 4 mismatches allowed in the last 10 bases of

3’ end primer, (ii) minimal complement primer length of 10 bases, and (iii) 75% of local alignment similarity.

The designed primers were tested with a PCR mixture (50 µl) containing 1X *Pfu* Turbo DNA polymerase buffer, 200 µM of each dNTP, 0.5 µM of each primer, 0.5 U of *Pfu* Turbo DNA polymerase (Stratagene) and 10 ng of genomic DNA. For CE-SSCP analysis, one primer was labeled with 5’-fluorescein phosphoramidite. Reactions were performed in a Mastercycler thermal cycler (Eppendorf). The *hydA* genes were amplified as follows: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 10 min. The presence and size of about 200 bp PCR products were determined by ethidium bromide-stained (2%) gel electrophoresis.

The 16S rRNA genes were amplified using *Pfu* Turbo DNA polymerase (Stratagene) and universal primers W49 (5′-ACGGTCCAGACTCCTACGGG-3′, *Escherichia coli* position F331) [34] and 5′-fluorescein phosphoramidite-labeled W104 (5′-TTACCGCGGCTGCTGGCAC-3′, *E. coli* position R533) [34], targeting about 200 bp of the V3 region of the 16S rRNA gene. The PCR reactions were performed as described above, except that 25 cycles and primer hybridization at 61 °C were applied and 130 ng of each primer were used. For HPB mixed cultures, the *Clostridium* genus was specifically analyzed by nested PCR. First, the 16S rRNA genes of the *Clostridium* genus were amplified using AccuPrime *Taq* DNA polymerase (Invitrogen), a bacterial domain forward primer W18 (5′-GAGTTTGATCMTGGCTCAG -3′, *E. coli* position F9) [23] and a *Clostridium*-specific reverse primer W109 (5′- CCCTTTACACCCAGTAA -3′, *E. coli* position R561) [35], as described by Peu *et al.* [36]. Then, the PCR products were diluted 100 times, and 1 µl of each product was used as a template for a universal 16S rDNA PCR, carried out using the primer set W49/W104.

186 **2.5. CE-SSCP electrophoresis and statistical analysis**

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188 One microliter of 5- to 2000-fold diluted PCR product was mixed with 18.8 µl of formamide
189 and 0.2 µl of internal standard GeneScan ROX (Applied Biosystems). Samples were heat-
190 denatured at 95°C for 5 min and immediately cooled in ice. CE-SSCP electrophoresis was
191 performed in an ABI Prism 3130 genetic analyser (Applied Biosystems) with 50 cm-long
192 capillary tubes filled with a non-denaturing 5.6% conformation analysis polymer (Applied
193 Biosystems). Samples were eluted at 12 kV and 32°C for 30 min (for 16S rRNA gene) or 64
194 min (for *hydA* gene). Raw CE-SSCP data were analyzed using GeneScan software (Applied
195 Biosystems).
196 The CE-SSCP profiles were aligned with internal standard ROX. The sum of peak areas were
197 normalized to 1 before statistical analysis. All the transformation and statistical analysis from
198 CE-SSCP profiles were conducted using StatFingerprints sofware [37]. The matrices of
199 similarity were calculated on the basis of Euclidean distances between each pair of CE-SSCP
200 profiles. An analysis of similarity (ANOSIM) was used to investigate the effects of the
201 temperature, considered as a qualitative variable, on the variation of similarity data [38].
202 Comparisons of mean distances were used to calculate the ANOSIM R-statistic (R), where
203 R=1 indicates that the communities are dissimilar, and R=0 indicates that communities are
204 random.

205

206 **2.6. Assignation of the partial *hydA* sequences in mixed cultures**

207

208 The PCR products obtained from the HPB mixed cultures were purified using a PCR
209 Purification Kit (Qiagen). The *hydA* clone libraries were constructed using the TA Cloning kit
210 (Invitrogen). The PCR-CE-SSCP profile of each PCR product, amplified using plasmid-

targeted primers T7 and P13, was compared with the HPB culture profiles for peak assignation. The cloned inserts were sent for sequencing with primers T7 (MilleGen company, Toulouse, France). The KB Basecaller software (Applied Biosystems) was used for base calling of the sequences. The confidence in base-calls was higher than 99.9%. The protein sequences, deduced from the nucleotide *hydA* sequences, were aligned with reference sequences retrieved from the Genbank database using the CLUSTALW program [39], and further refined manually using the BioEdit program [40]. Distances were estimated by the Kimura method [41], and the neighbor-joining method was used for inferring the tree topologies [42]. Tree topology confidence was determined by bootstrap analysis on 1000 replicates [43]. All phylogenetic programs were implemented with SeaView software [44]. The *hydA* gene sequences were deposited in the Genbank database under the accession numbers HM210801 to HM210803.

3. Results and discussion

3.1. Design and evaluation of *hydA* PCR primer set

The newly designed primer set, *hydAClos*F/*hydAClos*R, showed high *in silico* specificity and selectivity in binding two conserved regions of the active site domain (H-cluster) of the Fe-H_2ases: the first corresponded to an upstream region (amino acids 420 to 426, *C. pasteurianum* HydA numbering) consisting of the consensus motif TGGVMEA, and the second was a downstream region (amino acids 497 to 503) consisting of the consensus motif MACPGGC in the first part of the L3Fe signature [30]. On the one hand, the forward primer *hydAClos*F was able to anneal the *hydA* sequences of 18 genera (*i.e., Alkaliphilus, Bacteroides, Clostridium, Dehalococcoides, Desulfitobacterium, Desulfotalea, Desulfovibrio,*

Ethanoligenens, Eubacterium, Megasphaera, Pelobacter, Rhodopseudomonas, Shewanella, Symbiobacterium, Syntrophomonas, Thermoanaerobacter, Thermotoga and *Treponema*). The *hydA* sequences of *Alkaliphilus, Dehalococcoides, Syntrophomonas* and *Thermoanaerobacter* species had only one or two mismatches with the forward primer sequence (with 90-95 % identity). The forward primer sequences showed 85.6% identity (on average) with the *Clostridium hydA* gene sequence, corresponding to 2.9 mismatches in average. A maximum of seven mismatches were found between this primer sequence and the *hydA* sequence of *C. thermocellum* (Table 2). On the other hand, the reverse primer *hydAClos*R was highly specific to the *Clostridium hydA* sequences. Among the 31 *Clostridium hydA* gene sequences tested, 26 were targeted by this reverse primer. *hydAClos*R showed 84% identity (on average) with *Clostridium hydA* gene sequences and a maximum of seven mismatches with the *hydA* sequence of *C. methylpentosum* (Table 2).

Specific *hydA* PCR products of expected size (about 200 bp) were obtained using the *hydAClos*F/*hydAClos*R primer set from all the nine tested strains representing six distinct clusters of *Clostridium* genus (based on 16S rRNA similarities) (Table 1). Although primer-template mismatches could have decreased the efficiency of PCR amplification [45-47], the use of *Pfu* DNA polymerase, which presents proofreading activity, favored mismatched nucleotide removal at the 3′ ends of the primers, and therefore improved the elongation and amplification steps [48, 49]. No PCR product was detected from the strains belonging to other distantly-related genera (*e.g., Enterobacter* or *Klebsiella*) (Table 1). This result showed the high specificity and selectivity of the newly designed primer set for the detection of the *hydA* genes from a broad range of *Clostridium* strains.

3.2. Validation of the *hydA* CE-SSCP method on pure *Clostridium* strains

The *hydA* PCR products obtained from the *Clostridium* strains using either a 5'FAM-labeled forward primer or a 5'-FAM-labeled reverse primer were analyzed by CE-SSCP in order to evaluate the discriminative capability of this functional fingerprinting method. The CE-SSCP profiles obtained from independent PCR runs on the same DNA templates, using the same primers, showed peaks with an identical migration position, confirming the reliability of the CE-SSCP analysis [29].

A specific CE-SSCP profile consisting of one major peak was obtained for each of the nine strains, whichever the strand labeled (Figures 1A and 1B). The major peak was usually preceded by two minor peaks, using the reverse strand (Figure 1A). The presence of multiple peaks has been shown previously with 16S rDNA based CE-SSCP profiles [29]. The occurrence of artifactual peaks can be due to 3'-recessed end fragments produced by the exonuclease activity of the proofreading DNA polymerase [50].

Different migration behaviors were observed with forward or reverse strands for each *Clostridium* strain (Figures 1A and 1B). The largest range of CE-SSCP electrophoretic mobility was obtained when the reverse strand was labeled (Figure 1A). Contrasting results were found in the literature from 16S rDNA strands of diverse bacterial strains [29, 51]. Consequently, a CE-SSCP analysis with forward or reverse labeling should be tested prior to the application of a method based on CE-SSCP technique, because divergent results can be found depending on genes or strains tested. The analysis of reverse strands was carried out only for the experiments performed on mixed cultures because a better discrimination of the *Clostridium* species was obtained.

The discrimination efficiency of the *hydA* CE-SSCP method was compared to the 16S rDNA CE-SSCP analysis of the same *Clostridium* pure strains (Figure 1C). As expected, the different species displayed different 16S rDNA CE-SSCP patterns. However, the very closely related *C. acetobutylicum* and *C. butyricum* species (100% identity regarding the V3 region of

16S rDNA) exhibited identical 16S rDNA CE-SSCP fingerprints, as expected, while these strains were clearly separated by the *hydA* CE-SSCP analysis. Indeed, the use of the 16S rDNA genetic marker was not always sufficient for distinguishing between bacterial species of the same genus [52]. The difficulty in distinguishing between *Clostridium* cluster I species in H_2 dark fermentation systems using universal bacterial primers has also been reported by Hung *et al.* [9]. Here, it was shown that the *hydA* gene could be used as an alternative functional marker as a complement to the 16S rDNA phylogenetic marker for identifying closely-related species within the *Clostridium* genus.

hydA primers previously used for investigating a broad HPB diversity spanned 500 to 600 bp regions [19, 21]. These regions contained two or three signature motifs of the catalytic H-cluster domain of [FeFe]-H_2ases, named FeFe-P1, -P2 and -P3 [30]. Our results showed that the genetic variation within the short *hydA* region studied, located between the FeFe-P2 and -P3 motifs, was sufficient for a successful discrimination between the H_2-producing *Clostridium* species and was therefore suitable for assessing the diversity of HPB mixed populations using CE-SSCP.

3.3. Application of the *hydA* CE-SSCP method on mixed cultures

The *hydA* CE-SSCP method was used for comparing specifically the H_2-producing clostridial populations exhibiting significantly different H_2 production potentials and yields depending on the increasing temperature (Figure 2).

With regard to the maximum H_2 production rates (Figure 2C), the H_2 production yields (Figure 2A) and potentials (Figure 2B) were significantly affected by the temperature ($p<0.05$). An increase of H_2 production yields and potentials was observed with the increasing temperatures from 30 °C to 40 °C; however, these performances decreased with a further

311 increase in temperature from 40 °C to 45 °C. The highest H_2 production potential (75.56 ±
312 3.33 ml H_2/l) and yield (1.52 ± 0.06 H_2 mol/mol hexose) were observed at 40°C. Many
313 studies have reported similar H_2 production trends, *i.e.* temperature optima occurring between
314 35°C and 40°C and maximum yields ranging from 1.5 to 2 H_2 mol/mol hexose, using heat-
315 shocked anaerobic seed sludge microflora in the mesophilic range from 30°C to 45°C [53],
316 33°C to 41°C [54] and 20°C to 55°C [5]. Interestingly, higher H_2 production potentials, close
317 to those observed at 37°C, were obtained at 25°C (Figure 2B), suggesting the occurrence of a
318 second optimum of H_2 dark fermentation below 25°C. The occurrence of two peaks of
319 fermentation temperatures for H_2 production associated to the dominance of two different
320 bacterial species has already been observed at 60°C and 70°C within a temperature range
321 from 37 to 85°C [55]. Nevertheless, the features of microbial H_2 production below 25°C have
322 received little attention so far. Some pure *Clostridium* strains belonging to cluster I, such as *C.*
323 *gasigenes*, have been reported to produce H_2 at low temperatures in the dark fermentation of
324 organic substrates [56].
325 The *hydA* CE-SSCP profiles corresponding to the H_2 production potentials were significantly
326 influenced by the temperature (ANOSIM R=0.75, $p<0.001$) (Figure 3). Different *hydA* CE-
327 SSCP patterns, consisting of one or two major peaks, were observed according to the
328 temperature (Figure 3). The CE-SSCP profiles were very similar at 30°C, 35°C and 40°C
329 (consisting of one identical major peak), whereas a divergence was observed at 25°C or 45°C,
330 with the lowest H_2 production yields (Figure 2A).
331 Whatever the temperature, highly similar universal bacterial- and *Clostridium*-specific 16S
332 rDNA CE-SSCP fingerprinting patterns were obtained from all the H_2 producing cultures
333 (data not shown). This result indicated that the *Clostridium* genus was largely dominant and
334 played a major role in our H_2-producing experiments. This finding can be explained by the

335 use of a heat-shock pretreated sludge as inoculum, which was likely to have contained a large
336 majority of HPB from *Clostridium* genus [2, 5].
337 Three different peaks dominated the *hydA* CE-SSCP profiles and a shift occurred in
338 accordance with the temperature gradient (Figure 3). Peak 1 was present in both 25°C and
339 30°C profiles but was undetectable at higher temperatures. The HydA sequence of clone that
340 match with peak 1 showed 86.2% identity with that of *C. acetobutylicum*, an efficient H_2
341 producer in pure culture [57]. It was similarly dominant at 25°C, but its intensity decreased at
342 30°C (Figure 3), when H_2 production yield increased (Figure 2A). Peak 2 was found in all
343 CE-SSCP patterns whatever the temperature, showing a wider ecological niche compared to
344 the other species of the seed inoculum. Interestingly, peak 2 dominated the CE-SSCP profiles
345 when the H_2 production yield increased with the rise in temperature from 30 °C to 40 °C. In
346 contrast, its intensity decreased when the H_2 production yield decreased with an increase of
347 temperature from 40°C to 45°C. The HydA sequence of clone that match with peak 2 was
348 affiliated to the HydA sequence of *C. sporogenes* (70% identity), Consequently, the increase
349 of H_2 production potential and yield in our experiments (Figures 2A and 2B) may be
350 associated to the growth of a *C. sporogenes hydA* like gene-carrying strain whose optimal
351 activity was at 40°C. At this temperature, Chang *et al.* [13] also obtained a sequence (KL3-2)
352 affiliated to *C. sporogenes* (71.2% identity) (Figure 4) from fermentative H_2 producing batch
353 cultures using heat-pretreated compost as the inoculum. This observation supports the key
354 role of this species in H_2 production in our experiments. The maximum H_2 production rates
355 for all temperatures (Figure 2C) were associated with similar *hydA* CE-SSCP profiles
356 dominated by peak 2 (data not shown). This indicated that *C. sporogenes* also seemed to be
357 involved in maximal H_2 production rates. Finally, peak 3 was only detected in cultures at the
358 highest temperature (45°C). The HydA sequence of clone that match with peak 3 was closely
359 related to that of *C. perfringens* ATCC 13124 (91.2% identity). In a recent study, *C.*

perfringens was found to be the most dominant H_2 producer in a bioreactor operated at 37°C and using a heat-pretreated cattle dung compost as the inoculum [17].

Only *hydA* sequences affiliated with those of *Clostridium* cluster I species were retrieved from our bioreactors (Figure 4). This confirmed the predominant role of this cluster in H_2-producing mixed cultures [9, 17]. However, *Clostridium* cluster III-like *hydA* sequences have been detected using newly-described primers in others experiments on other substrates (data not shown), proving that other *Clostridium* clusters could also be targeted as demonstrated on pure strains (*e.g. C. cellulolyticum*). The topology of the HydA tree also highlighted the inconsistencies occurring between HydA- and 16S rDNA-based phylogenies [20]. For instance, the HydA sequences of *C. saccharoperbutylacetonicum* and *C. beijerinkii* (Cluster I based on 16S rDNA similarity) was affiliated with the HydA sequences of *Clostridium* species belonging to others clusters : *C. phytofermentas* of cluster XIV or *C. thermocellum* of cluster III (Figure 4), supporting the likelihood of the new primers as able to target different *Clostridium* clusters.

In summary, our results supported a correlation between the changes in *hydA* population structure and H_2 production performance. These results were consistent with previous studies showing the correlation between H_2 percentages or H_2 production rates and the succession of dominant bacteria in continuous reactors [17-19]. The correlation between *hydA* gene expression and/or abundance of *C. butyricum* and H_2 production fluctuations in batch or continuous reactors has already been well established [14, 15, 58, 59]. Consequently, the analysis of the functional *hydA* structure, diversity and/or abundance proved to be a powerful molecular tool for monitoring H_2 performances in dark fermentation bioreactors.

4. Conclusions

This work demonstrated the potential of using the *hydA*-based CE-SSCP method for monitoring the structure and the dynamics of H_2-producing clostridial populations which play a key role in H_2 production in bioreactors. New non-degenerate primers were designed and successfully validated for the specific PCR detection of a broad range of *hydA* genes from diverse *Clostridium* strains belonging to different 16S rDNA-based clusters. The functional CE-SSCP method obtained successful discrimination between *Clostridium* strains and offered reliable and high throughput analysis of *hydA* genes in mixed-culture bioprocesses. This is the first study reporting the features of functional gene-based CE-SSCP profiles from pure strains, which are useful for studying the diversity of functional populations. This valuable molecular tool could be used for better understanding of the links between the dynamics of H_2-producing functional populations and the H_2 cycle in diverse environments.

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Captions to figures

Figure 1. CE-SSCP profiles of DNA fragments amplified using primer sets: 5'-FAM-labeled *hydAClos*F/*hydAClos*R (A), *hydAClos*F/5'-FAM-labeled *hydAClos*R (B), w49/w104 (C). The numbers represent the CE-SSCP profiles of the different *Clostridium* strains tested. Numbers: (1) *C. pasteurianum* DSM525^T; (2) *C. spiroforme* DSM 1552^T; (3) *C. saccharoperbutylacetonicum* DSM14923^T; (4) *Clostridium leptum* DSM753^T; (5) *C. cellulolyticum* DSM5812^T; (6) *C. phytofermentans* DSM 18823^T; (7) *C. acetobutylicum* DSM792^T; (8) *C. butyricum* DSM10702^T; (9) *C. hiranonis* DSM13275^T. For comparisons between samples, the different CE-SSCP profiles were aligned on the basis of the common ROX internal standard, and normalized. The x and y axis of each CE-SSCP profiles represent relative migration distance and relative intensity, respectively.

Figure 2. Effect of the temperature in mixed cultures on H_2 production: (A) yields (B) potentials and (C) rates. Values are mean (n=3) ± standard deviations (error bars).

Figure 3. CE-SSCP profiles of *hydA* gene fragments retrieved from H_2-producing experiments conducted at 25-45°C, at maximum H_2 production yields. For comparisons between samples, the different CE-SSCP profiles were aligned on the basis of the common ROX internal standard, and normalized. The x and y axis of each CE-SSCP profiles represent relative migration distance and relative intensity, respectively. A representative CE-SSCP profile from the three replicates is presented for each temperature. The prominent peaks of each CE-SSCP profile was marked with a number. The phylogenetic affiliation of sequenced clones that match a numbered peak was given in Figure 4.

Figure 4. Neighbor-joining phylogenetic tree of the HydA sequences (134 amino acids). The Genbank accession numbers (in brackets) were obtained from the protein sequence databases. The sequences detected by PCR using newly-developed *hydA* primer sets are indicated in bold. The sequences retrieved from mixed cultures are in bold and highlighted. The sequences used for primer design are marked by an asterisk. Circles at the branch nodes represent bootstrap confidence level percentage*s* obtained from 1000 replicates: large black circles = 95-100%; small black circles = 75-95%; small white circles = 50-75%. The scale bar indicates 10% sequence divergence. The *Desulfovibrio* HydA sequences (AAS96246, AAA23373 and CAA72423) were used as outgroups. The clusters of *Clostridium* species (*Clos.* cluster) at the right-hand side of the tree were based on 16S rRNA gene similarities.

Table

Table 1. Bacterial strains tested in this study

Organism (source)	***Clostridium* cluster**	**Class**	**Hydrogen production**	**PCR detection with new primers**
Clostridium acetobutylicum ATCC 824 (DSM792^T)	Ia	*Clostridia*	+	+
Clostridium butyricum (DSM10702^T)	Ia	*Clostridia*	+	+
Clostridium pasteurianum (DSM525^T)	Ii	*Clostridia*	+	+
Clostridium saccharoperbutylacetonicum N1-4 (DSM14923^T)	I	*Clostridia*	+	+
Clostridium cellulolyticum H10 (DSM5812^T)	III	*Clostridia*	+	+
Clostridium leptum (DSM753^T)	IV	*Clostridia*	+	+
Clostridium hiranonis TO-931 (DSM13275^T)	XI	*Clostridia*	+	+
Clostridium phytofermentans ISDg (DSM 18823^T)	XIVa	*Clostridia*	+	+
Clostridium spiroforme (DSM 1552^T)	XVIII	*Clostridia*	+	+
Desulfomicrobium baculatum (DSM1743)	-	*Deltaproteobacteria*	n.d.	-
Desulfovibrio gigas (DSM496)	-	*Deltaproteobacteria*	n.d.	-
Desulfovibrio vulgaris subsp. *vulgaris* str. Hildenborough (DSM644 ^{T})	-	*Deltaproteobacteria*	+	-
Enterobacter cloacae La 1313 (NEU1027)	-	*Gammaproteobacteria*	n.d.	-
Enterococcus casseliflavus Bet 1 (NEU110)	-	*Bacilli*	n.d.	-
Enterococcus faecium (DSM2918)	-	*Bacilli*	n.d.	-
Klebsiella pneumoniae subsp. *pneumoniae* (DSM30104^T)	-	*Gammaproteobacteria*	n.d.	-
Raoultella terrigena (DSM2687^T)	-	*Gammaproteobacteria*	n.d.	-
Rhodobacter sphaeroides 2.4.1 (DSM158^T)	-	*Alphaproteobacteria*	+	-
Syntrophomonas wolfei subsp. *wolfei* str. Göttingen (DSM2245B)	VIII	*Clostridia*	+	-
Xanthobacter autotrophicus Py2 (DSM432^T)	-	*Alphaproteobacteria*	n.d.	-

Table

Table 2. *hydA* sequence homology between the designed primers and *hydA* gene sequences of different clostridial species. The strains used for primer testing are indicated in bold.

***Clostridium* strains [Genbank Accession number]**[a]	**Primer sequence**[b]	
	*hydAClos*F 5′-ACCGGTGGAGTTATGGAAGC-3′	*hydAClos*R 5′-CATCCACCTGGACATGCCAT-3′
Targeted consensus motif	3′-TGGCCACCTCAATACCTTCG-5′	3′-GTAGGTGGACCTGTACGGTA-5′
Clostridium cluster I[c]		
Clostridium acetobutylicum [NC_003030]	.....G..G..C.....	..C..C..G..C..G.....
Clostridium beijerinckii [NC_009617]	..C..T.....T.G.....	.....A..T.....
Clostridium botulinum [NC_010674]	..T.....A..T.GA.....	..C..A.....C..T.....
Clostridium butyricum [EF627973]	..T.....	.....C....T...
Clostridium carboxidivorans [NZ_ACVI01000015]	..A.....A..T..A.....	.....A..T.....C.....
Clostridium kluyveri [NC_009706]	.....	..T..C..T..C..C.....
Clostridium novyi [NC_008593]	A.A........CCGT.....	.........A.....
Clostridium paraputrificum [AB159510]	..A........T.....	.....A...
Clostridium pasteurianum [M81737]	..A..G.....	.....A..TA....T.....
Clostridium perfringens [NC_008261]	..A.....	.....T.....
Clostridium saccharobutylicum [U09760]	..T.....	.....C.....C..T.....
Clostridium saccharoperbutylacetonicum [AY827554]	..A..T..A....GA.....	.....
Clostridium sporogenes [NZ_ABKW02000004]	.....A.....	..C..ACT......G.....
Clostridium tyrobutyricum [FJ226584]	.....G..A.....	.....G..C.....
Clostridium cluster III[c]		
Clostridium cellulolyticum [NC_011898]	.....C.....	..T..C..G.....T.....
Clostridium *papyrosolvens* [NZ_ACXX01000005]	.....T.....	.....AG...TG....
Clostridium thermocellum [NC_009012]	A....G..A..CCGA.....	.....C..C.....
Clostridium cluster IV[c]		
Clostridium leptum [NZ_ABCB02000021]	.....G..G.....C..	.....C..G..C..CGT...
Clostridium methylpentosum [NZ_ACEC01000002]	.....G..G.....C..	..C..C..C..C..CGT...
Clostridium cluster XI[c]		
Clostridium bartlettii [NZ_ABEZ02000007]	..A.....A...G.A..C..	.....T.....T.....
Clostridium difficile [NC_009089]	.CA.....A.T.....	.....T.....
Clostridium hiranonis [NZ_ABWP01000030]	..T.....A.....	.....T.....T.....
Clostridium cluster XIV[c]		
Clostridium asparagiforme [NZ_ACCJ01000406]	.....G..G..C.....	..C..C..G.....GG....
Clostridium bolteae [NZ_ABCC02000011]	..C..G..G..T.....	..C..C.....C...G....
Clostridium hathewayi [NZ_ACIO01000001]	..T..G..G.....	..C..C..G..C...G....
Clostridium hylemonae [NZ_ABYI02000019]	.....G..A.....C..	.....C..G.....G....
Clostridium nexile [NZ_ABWO01000184]	.....A.....	..C..A..G.....G....
Clostridium phytofermentans [NC_010001]	..A.....GT.....	..T.....T..C.....
Clostridium scindens [NZ_ABFY02000067]	..A..G.....	.....C..G..C...G....
Clostridium cluster XVIII[c]		
Clostridium spiroforme [NZ_ABIK02000014]	.C.....A..T.....	..T.....TG....
Clostridium ramosum [NZ_ABFX02000004]	.CA.....A..T.....	.....C..T.....G....

[a] Genbank accession numbers of *hydA* gene sequences.
[b] The points indicate identical nucleotides in a consensus table.
[c] Clusters of *Clostridium* species based on 16S rRNA gene similarities.

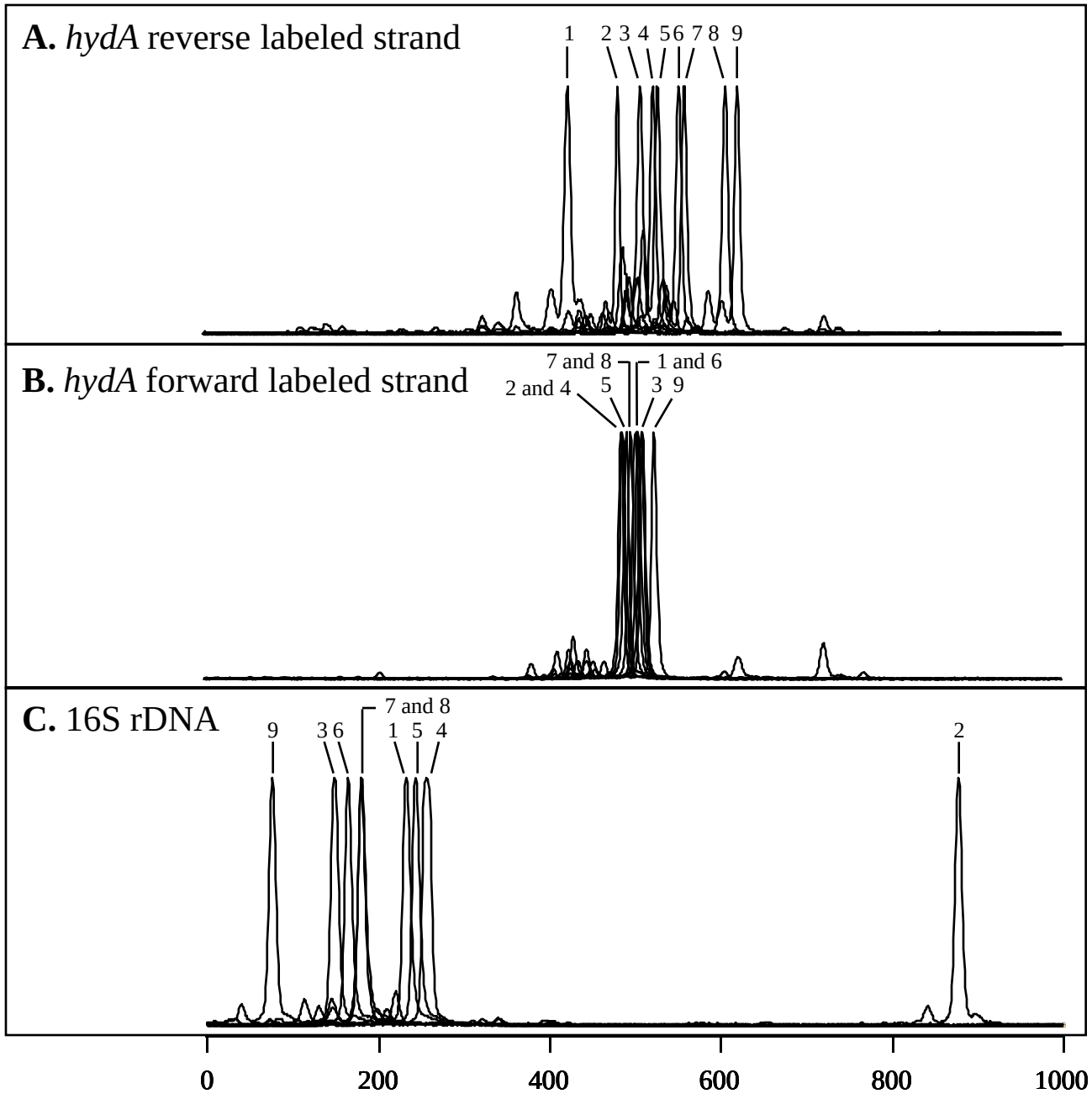
A. hydA reverse labeled strand
1
2 3 4 5 6 7 8 9
B. hydA forward labeled strand
7 and 8
1 and 6
2 and 4
5
3 9
C. 16S rDNA
9
3 6
7 and 8
1 5 4
2
0
200
400
600
800
1000

Figure 1

Figure

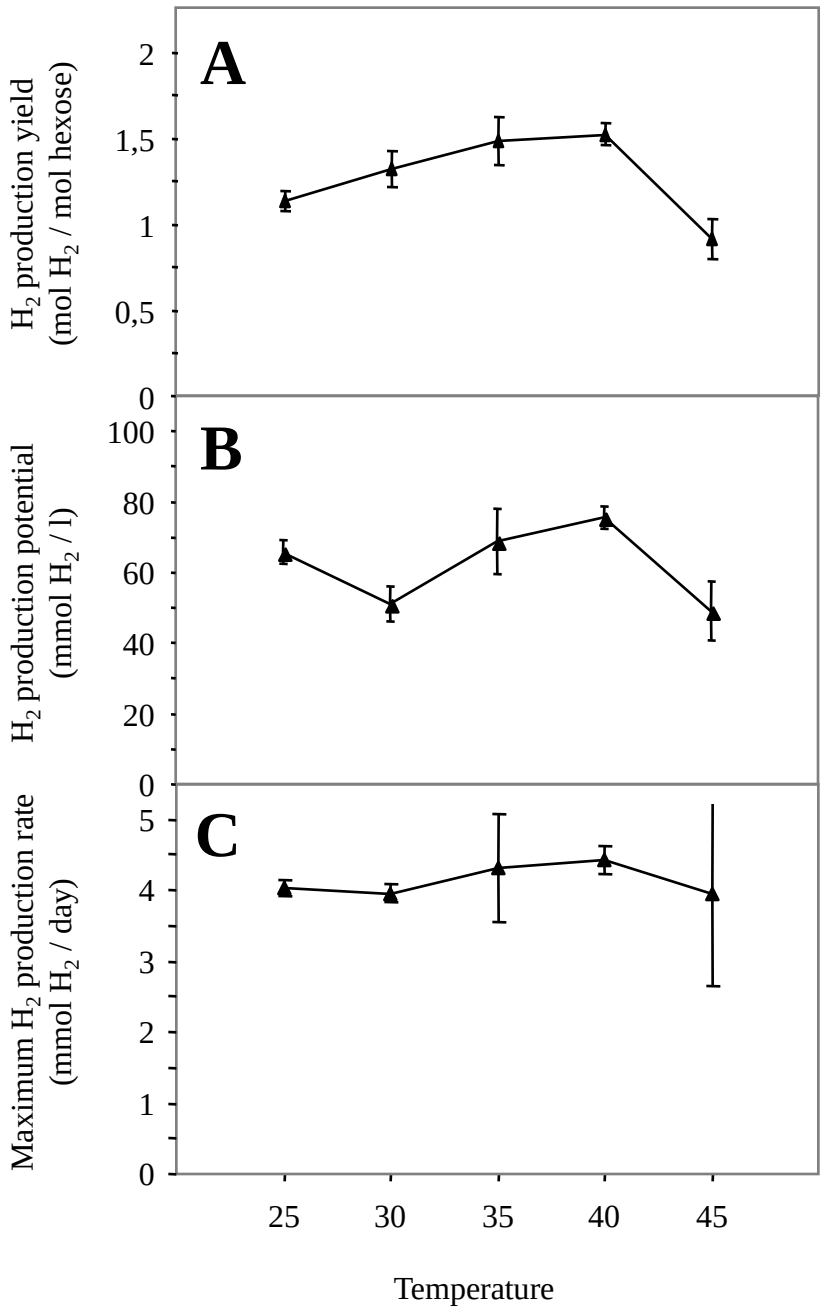

Figure 2

Figure

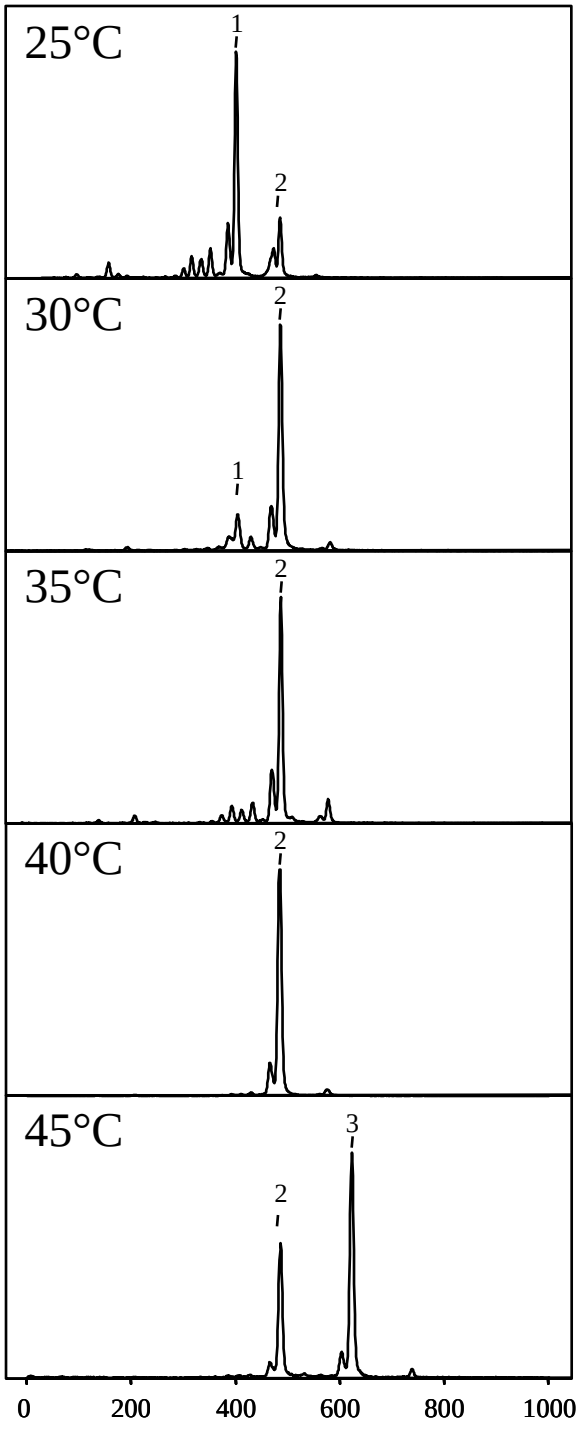

Figure 3

Figure

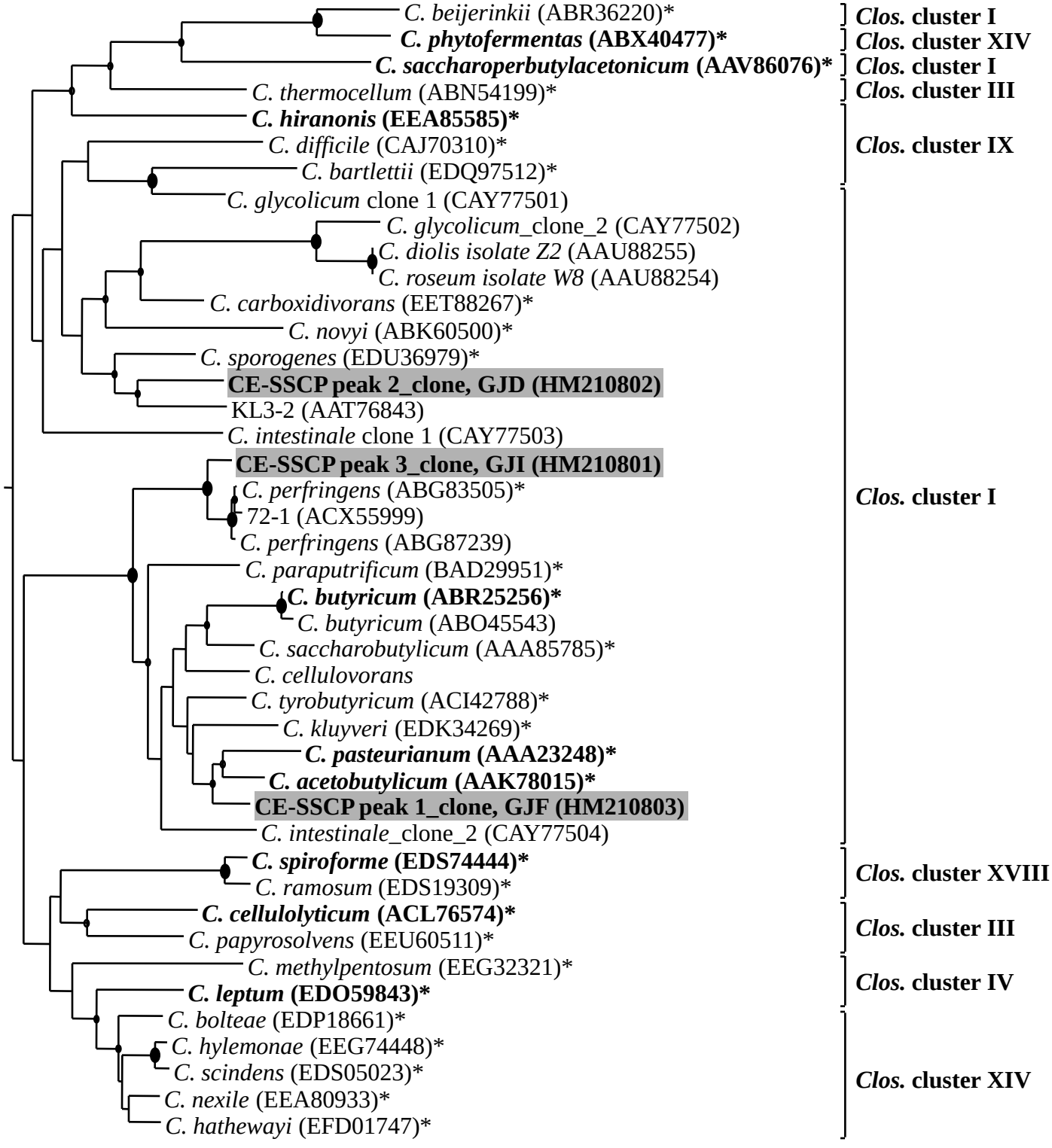

Figure 4