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1 **Development and application of a functional CE-SSCP fingerprinting**
2 **method based on [Fe-Fe]-hydrogenase genes for monitoring hydrogen-**
3 **producing *Clostridium* in mixed cultures**

4

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19

20 **Abstract**

21 A Capillary Electrophoresis Single Strand Conformation Polymorphism (CE-SSCP) method
22 based on functional [Fe-Fe]-hydrogenase genes was developed for monitoring the hydrogen
23 (H₂)-producing clostridial population in mixed culture bioprocesses. New non-degenerated
24 primers were designed and then validated on their specific PCR detection of a broad range of
25 clostridial *hydA* genes. The *hydA* based CE-SSCP method gave a specific and discriminating
26 profile for each of the *Clostridium* strains tested. This method was validated using H₂-
27 producing mixed cultures incubated at temperatures ranging from 25°C to 45°C. The *hydA*
28 CE-SSCP profiles clearly differed between temperatures tested. Hence, they varied according
29 to variations of the H₂ production performances. The HydA sequences amplified with the new
30 primer set indicated that diverse *Clostridium* strains impacted the H₂ production yields. The
31 highest performances were related to the dominance of *Clostridium sporogenes*-like *hydA*
32 sequences. This CE-SSCP tool offers highly reliable and throughput analysis of the functional
33 diversity and structure of the *hydA* genes for better understanding of the H₂-producing
34 clostridial population dynamics in H₂ dark fermentation bioreactors.

35

36 **Keywords**

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

39 1. Introduction

40

41 Hydrogen (H₂) is considered as a promising energy carrier, because it is energetically dense,
42 clean and sustainable. Among diverse renewable H₂-producing biotechnologies, dark
43 fermentation techniques using anaerobic microbial communities have gained attention not
44 only because of their high H₂ production rates but, also, for the ability to produce H₂ at low
45 cost from complex and unsterilized substrates, such as organic waste materials [1, 2].

46 Numerous physiologically and phylogenetically diverse microorganisms can produce H₂ by
47 dark fermentation [3]. Pretreatment methods (*i.e.* heat shock) often used to eliminate H₂
48 consumers (*e.g.* methanogens) which coexist with H₂-producing bacteria (HPB) in the
49 anaerobic mixed communities of bioreactors [4]. However, heat-shock treatments decrease
50 microbial diversity/richness and enrich the culture in sporulating HPB, such as *Clostridium*
51 species. So far, the highest H₂ production yields have been reported with *Clostridium* pure
52 cultures or mixed cultures where *Clostridium* species predominate [2, 5, 6]. Therefore,
53 monitoring the presence, biodiversity and the dynamics of *Clostridium* species in
54 bioprocesses is of major interest.

55 The genus *Clostridium* is a large and phenotypically heterogeneous bacterial group which as
56 yet is not phylogenetically well defined. About 200 species have been described and are
57 distributed in 19 clusters [7, 8] of which, the largest and phylogenetically the best-defined is
58 cluster I [7]. It includes *Clostridium (C.) butyricum* as the type species of the genus and has
59 been considered to contain most of the H₂-producing *Clostridium*. Hung *et al.* [9] developed a
60 specific PCR-DGGE assay targeting 16S rDNA of cluster I *Clostridium* species for
61 monitoring the HPB community in dark fermentation. However, *Clostridium* species
62 belonging to others clusters have been shown to produce H₂ from diverse carbon sources,
63 including *C. cellulolyticum* from cluster III [10] or, *C. bifermentas* from cluster IX [11], *C.*

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

67 Hence, alternatives approaches targeting functional genes can be useful for the specific
68 monitoring of *Clostridium* HPB. [FeFe]-Hydrogenases ([FeFe]-H₂ases) are key enzymes in
69 H₂ production by *Clostridium* species. Diverse primer sets and probe targeting *hydA* genes,
70 encoding catalytic subunit of [FeFe]-H₂ases, have been developed for monitoring H₂-
71 producing *Clostridium* species, generally *C. butyricum*, during continuous H₂ fermentation
72 [13-18]. Despite the presence of numerous reasonable candidate genes for [FeFe]-H₂ases in
73 genome sequence databases, these primers were designed from few number of *Clostridium*
74 *hydA* sequences and which has decreased their ability to target a broad spectrum of HPB. In
75 contrast, many studies have reported degenerate primer sets for detection of a wide range of
76 *hydA* bacterial genes [19-21]. However, degenerate primers are unsuitable for directly
77 assessing gene diversity using such molecular fingerprinting techniques as denaturing
78 gradient gel electrophoresis (DGGE) [22] or single strand conformation polymorphism
79 (SSCP) [23], where they produce multiple band pattern or undistinguishable peaks from
80 single template DNA [24, 25]. Nevertheless, DGGE or CE-SSCP techniques are useful for: (i)
81 producing molecular fingerprints based on both 16S rRNA- and/or functional genes; and (ii)
82 comparing the shift of bacterial population structure and diversity in relation to the changes in
83 environmental parameters [26, 27]. The use of an automatic capillary electrophoresis (CE),
84 laser induced fluorescence system, such as CE-SSCP, makes it possible to carry out a highly
85 sensitive, reproducible and throughput analysis of diverse bacterial communities [28, 29].

86 This study aimed at developing a CE-SSCP fingerprinting method for monitoring H₂-
87 producing clostridial population in mixed cultures. Non-degenerate primers were designed
88 and evaluated with PCR detection of a large range of clostridial *hydA* genes. The *hydA* CE-

89 SSCP method was then validated on pure cultures and further applied in H₂ producing mixed
90 cultures incubated at various mesophilic temperatures.

91

92

93 **2. Materials and methods**

94

95 **2.1. Bacterial strains**

96

97 Four group of bacterial strains (Table 1) were used for the evaluation of the specificity of the
98 newly-designed primer set targeting the *hydA* genes: (i) HPB strains belonging to diverse
99 clusters of the *Clostridium* genus, including reference strains of the main clusters: I, III, IX,
100 XIV and XVIII; (ii) strains belonging to H₂-producing species (*Enterobacter cloacae*) or
101 genera (*Enterococcus* and *Klebsiella*), (iii) the *hydA*-carrying fully-sequenced genome HPB
102 strains belonging to other genera (*Desulfovibrio* and *Syntrophomonas*), (iv) the strains with a
103 fully-sequenced genome, *Rhodobacter sphaeroides* DSM 158^T and *Xanthobacter*
104 *autotrophicus* DSM432^T, carrying putative genes encoding [NiFe]-H₂ase without [FeFe]-
105 H₂ase [30].

106

107 **2.2. Hydrogen production experiments**

108

109 The H₂-producing experiments were carried out in 500 ml glass bottles in batch conditions.
110 The inoculum was a digested sludge pretreated by heat/shock (90°C for 10 min). Two
111 milliliters of the pretreated inoculum (final concentration of 225 mgCOD.l⁻¹) were inoculated
112 in the following culture medium: 40 mM MES, 10 g sucrose, 1l deionized water. The total
113 working volume was 200 ml. The initial pH was adjusted to 5.5. Each bottle was flushed with

114 nitrogen for 5 min to maintain anaerobic conditions. The bottles were then capped with a
115 rubber stopper and incubated at 25, 30, 35, 40 or 45°C for 24 days. Each experiment for the
116 different conditions was carried out in triplicates. Four milliliters of mixed culture were
117 collected daily during the first 12 days of the experiments and every 2nd-3rd day during the last
118 12 days of the experiments, then centrifuged (20,000 g for 10 min). The supernatants and the
119 pellets were then stored at -20°C for further chemical analysis and DNA extraction,
120 respectively.

121

122 **2.3. Chemical analysis**

123

124 Biogas production was measured using an acidified water displacement method. Biogas
125 composition (CH₄, CO₂, H₂ and N₂) was analyzed by gas chromatography (GC-14A,
126 Shimadzu) as previously described by Aceves-Lara *et al.* [31]. The biogas produced contained
127 only H₂ and CO₂, without detectable CH₄. Sugar concentration was assessed by High
128 Performance Liquid Chromatography analysis coupled to refractometry detection. The H₂
129 production performances were evaluated according to the H₂ production yield (mol H₂/mol
130 hexose consumed), potential (ml H₂/l) and rates (mol H₂/day).

131

132 **2.4. DNA extraction, primer design and PCR amplification**

133

134 For each experiment, the molecular analysis of bacterial communities were only performed on
135 samples corresponding to maximum H₂ production rate and H₂ potential (this latter
136 corresponding to H₂ yield).

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

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

161 3' end primer, (ii) minimal complement primer length of 10 bases, and (iii) 75% of local
162 alignment similarity.

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

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

185

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

187

188 One microliter of 5- to 2000-fold diluted PCR product was mixed with 18.8 μ l of formamide
189 and 0.2 μ l 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 software [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. Assignment 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-

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

223

224 **3. Results and discussion**

225

226 **3.1. Design and evaluation of *hydA* PCR primer set**

227

228 The newly designed primer set, *hydAClosF/hydAClosR*, showed high *in silico* specificity and
229 selectivity in binding two conserved regions of the active site domain (H-cluster) of the Fe-
230 H₂ases: the first corresponded to an upstream region (amino acids 420 to 426, *C.*
231 *pasteurianum* HydA numbering) consisting of the consensus motif TGGVMEA, and the
232 second was a downstream region (amino acids 497 to 503) consisting of the consensus motif
233 MACPGGC in the first part of the L3Fe signature [30]. On the one hand, the forward primer
234 *hydAClosF* was able to anneal the *hydA* sequences of 18 genera (*i.e.*, *Alkaliphilus*,
235 *Bacteroides*, *Clostridium*, *Dehalococcoides*, *Desulfitobacterium*, *Desulfotalea*, *Desulfovibrio*,

236 *Ethanoligenens*, *Eubacterium*, *Megasphaera*, *Pelobacter*, *Rhodopseudomonas*, *Shewanella*,
237 *Symbiobacterium*, *Syntrophomonas*, *Thermoanaerobacter*, *Thermotoga* and *Treponema*). The
238 *hydA* sequences of *Alkaliphilus*, *Dehalococcoides*, *Syntrophomonas* and *Thermoanaerobacter*
239 species had only one or two mismatches with the forward primer sequence (with 90-95 %
240 identity). The forward primer sequences showed 85.6% identity (on average) with the
241 *Clostridium hydA* gene sequence, corresponding to 2.9 mismatches in average. A maximum
242 of seven mismatches were found between this primer sequence and the *hydA* sequence of *C.*
243 *thermocellum* (Table 2). On the other hand, the reverse primer *hydAClosR* was highly specific
244 to the *Clostridium hydA* sequences. Among the 31 *Clostridium hydA* gene sequences tested,
245 26 were targeted by this reverse primer. *hydAClosR* showed 84% identity (on average) with
246 *Clostridium hydA* gene sequences and a maximum of seven mismatches with the *hydA*
247 sequence of *C. methylpentosum* (Table 2).

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

258

259 **3.2. Validation of the *hydA* CE-SSCP method on pure *Clostridium* strains**

260

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

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

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

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

286 16S rDNA) exhibited identical 16S rDNA CE-SSCP fingerprints, as expected, while these
287 strains were clearly separated by the *hydA* CE-SSCP analysis. Indeed, the use of the 16S
288 rDNA genetic marker was not always sufficient for distinguishing between bacterial species
289 of the same genus [52]. The difficulty in distinguishing between *Clostridium* cluster I species
290 in H₂ dark fermentation systems using universal bacterial primers has also been reported by
291 Hung *et al.* [9]. Here, it was shown that the *hydA* gene could be used as an alternative
292 functional marker as a complement to the 16S rDNA phylogenetic marker for identifying
293 closely-related species within the *Clostridium* genus.

294 *hydA* primers previously used for investigating a broad HPB diversity spanned 500 to 600 bp
295 regions [19, 21]. These regions contained two or three signature motifs of the catalytic H-
296 cluster domain of [FeFe]-H₂ases, named FeFe-P1, -P2 and -P3 [30]. Our results showed that
297 the genetic variation within the short *hydA* region studied, located between the FeFe-P2 and -
298 P3 motifs, was sufficient for a successful discrimination between the H₂-producing
299 *Clostridium* species and was therefore suitable for assessing the diversity of HPB mixed
300 populations using CE-SSCP.

301

302 **3.3. Application of the *hydA* CE-SSCP method on mixed cultures**

303

304 The *hydA* CE-SSCP method was used for comparing specifically the H₂-producing clostridial
305 populations exhibiting significantly different H₂ production potentials and yields depending
306 on the increasing temperature (Figure 2).

307 With regard to the maximum H₂ production rates (Figure 2C), the H₂ production yields
308 (Figure 2A) and potentials (Figure 2B) were significantly affected by the temperature
309 ($p < 0.05$). An increase of H₂ production yields and potentials was observed with the increasing
310 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 H₂ production potential (75.56 ±
312 3.33 ml H₂/l) and yield (1.52 ± 0.06 H₂ mol/mol hexose) were observed at 40°C. Many
313 studies have reported similar H₂ production trends, *i.e.* temperature optima occurring between
314 35°C and 40°C and maximum yields ranging from 1.5 to 2 H₂ 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 H₂ 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 H₂ dark fermentation below 25°C. The occurrence of two peaks of
319 fermentation temperatures for H₂ 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 H₂ 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 H₂ at low temperatures in the dark fermentation of
324 organic substrates [56].

325 The *hydA* CE-SSCP profiles corresponding to the H₂ 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 H₂ 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 H₂ producing cultures
333 (data not shown). This result indicated that the *Clostridium* genus was largely dominant and
334 played a major role in our H₂-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 H₂
341 producer in pure culture [57]. It was similarly dominant at 25°C, but its intensity decreased at
342 30°C (Figure 3), when H₂ 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 H₂ production yield increased with the rise in temperature from 30 °C to 40 °C. In
346 contrast, its intensity decreased when the H₂ 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 H₂ 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 H₂ producing batch
353 cultures using heat-pretreated compost as the inoculum. This observation supports the key
354 role of this species in H₂ production in our experiments. The maximum H₂ 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 H₂ 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.*

360 *perfringens* was found to be the most dominant H₂ producer in a bioreactor operated at 37°C
361 and using a heat-pretreated cattle dung compost as the inoculum [17].
362 Only *hydA* sequences affiliated with those of *Clostridium* cluster I species were retrieved
363 from our bioreactors (Figure 4). This confirmed the predominant role of this cluster in H₂-
364 producing mixed cultures [9, 17]. However, *Clostridium* cluster III-like *hydA* sequences have
365 been detected using newly-described primers in others experiments on other substrates (data
366 not shown), proving that other *Clostridium* clusters could also be targeted as demonstrated on
367 pure strains (*e.g.* *C. cellulolyticum*). The topology of the HydA tree also highlighted the
368 inconsistencies occurring between HydA- and 16S rDNA-based phylogenies [20]. For
369 instance, the HydA sequences of *C. saccharoperbutylacetonicum* and *C. beijerinckii* (Cluster I
370 based on 16S rDNA similarity) was affiliated with the HydA sequences of *Clostridium*
371 species belonging to others clusters : *C. phytofermentas* of cluster XIV or *C. thermocellum* of
372 cluster III (Figure 4), supporting the likelihood of the new primers as able to target different
373 *Clostridium* clusters.

374 In summary, our results supported a correlation between the changes in *hydA* population
375 structure and H₂ production performance. These results were consistent with previous studies
376 showing the correlation between H₂ percentages or H₂ production rates and the succession of
377 dominant bacteria in continuous reactors [17-19]. The correlation between *hydA* gene
378 expression and/or abundance of *C. butyricum* and H₂ production fluctuations in batch or
379 continuous reactors has already been well established [14, 15, 58, 59]. Consequently, the
380 analysis of the functional *hydA* structure, diversity and/or abundance proved to be a powerful
381 molecular tool for monitoring H₂ performances in dark fermentation bioreactors.

382

383

384 **4. Conclusions**

385

386 This work demonstrated the potential of using the *hydA*-based CE-SSCP method for
387 monitoring the structure and the dynamics of H₂-producing clostridial populations which play
388 a key role in H₂ production in bioreactors. New non-degenerate primers were designed and
389 successfully validated for the specific PCR detection of a broad range of *hydA* genes from
390 diverse *Clostridium* strains belonging to different 16S rDNA-based clusters. The functional
391 CE-SSCP method obtained successful discrimination between *Clostridium* strains and offered
392 reliable and high throughput analysis of *hydA* genes in mixed-culture bioprocesses. This is the
393 first study reporting the features of functional gene-based CE-SSCP profiles from pure
394 strains, which are useful for studying the diversity of functional populations. This valuable
395 molecular tool could be used for better understanding of the links between the dynamics of
396 H₂-producing functional populations and the H₂ cycle in diverse environments.

397

398

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400

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413

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- 592

593 **Captions to figures**

594

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

605

606 **Figure 2.** Effect of the temperature in mixed cultures on H₂ production: (A) yields (B)
607 potentials and (C) rates. Values are mean (n=3) ± standard deviations (error bars).

608

609 **Figure 3.** CE-SSCP profiles of *hydA* gene fragments retrieved from H₂-producing
610 experiments conducted at 25-45°C, at maximum H₂ production yields. For comparisons
611 between samples, the different CE-SSCP profiles were aligned on the basis of the common
612 ROX internal standard, and normalized. The x and y axis of each CE-SSCP profiles represent
613 relative migration distance and relative intensity, respectively. A representative CE-SSCP
614 profile from the three replicates is presented for each temperature. The prominent peaks of
615 each CE-SSCP profile was marked with a number. The phylogenetic affiliation of sequenced
616 clones that match a numbered peak was given in Figure 4.

617

618 **Figure 4.** Neighbor-joining phylogenetic tree of the HydA sequences (134 amino acids). The
619 Genbank accession numbers (in brackets) were obtained from the protein sequence databases.
620 The sequences detected by PCR using newly-developed *hydA* primer sets are indicated in
621 bold. The sequences retrieved from mixed cultures are in bold and highlighted. The sequences
622 used for primer design are marked by an asterisk. Circles at the branch nodes represent
623 bootstrap confidence level percentages obtained from 1000 replicates: large black circles =
624 95-100%; small black circles = 75-95%; small white circles = 50-75%. The scale bar indicates
625 10% sequence divergence. The *Desulfovibrio* HydA sequences (AAS96246, AAA23373 and
626 CAA72423) were used as outgroups. The clusters of *Clostridium* species (*Clos.* cluster) at the
627 right-hand side of the tree were based on 16S rRNA gene similarities.

Table 1. Bacterial strains tested in this study

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

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	
	<i>hydAClosF</i>	<i>hydAClosR</i>
	5' -ACCGGTGGAGTTATGGAAGC-3' 3' -TGGCCACCTCAATACCTTCG-5'	5' -CATCCACCTGGACATGCCAT-3' 3' -GTAGGTGGACCTGTACGGTA-5'
Targeted consensus motif		
Clostridium cluster I ^c		
Clostridium acetobutylicum [NC_003030]G..G..C.....	..C..C..G..C..G.....
<i>Clostridium beijerinckii</i> [NC_009617]	..C..T.....T.G.....A..T.....
<i>Clostridium botulinum</i> [NC_010674]	..T.....A..T.GA.....	..C..A.....C..T.....
Clostridium butyricum [EF627973]	..T.....C...T...
<i>Clostridium carboxidivorans</i> [NZ_ACVI01000015]	..A.....A..T..A.....A..T.....C.....
<i>Clostridium kluyveri</i> [NC_009706]T..C..T..C..C.....
<i>Clostridium novyi</i> [NC_008593]	A.A.....CCGT.....A.....
<i>Clostridium paraputrificum</i> [AB159510]	..A.....T.....A...
Clostridium pasteurianum [M81737]	..A..G.....A..TA...T.....
<i>Clostridium perfringens</i> [NC_008261]	..A.....T.....
<i>Clostridium saccharobutylicum</i> [U09760]	..T.....C.....C..T.....
Clostridium saccharoperbutylacetonicum [AY827554]	..A..T..A...GA.....
<i>Clostridium sporogenes</i> [NZ_ABKW02000004]A.....	..C..ACT.....G.....
<i>Clostridium tyrobutyricum</i> [FJ226584]G..A.....G..C.....
Clostridium cluster III ^c		
Clostridium cellulolyticum [NC_011898]C.....	..T..C..G.....T.....
<i>Clostridium papyrosolvans</i> [NZ_ACXX01000005]T.....AG...TG...
<i>Clostridium thermocellum</i> [NC_009012]	A...G..A..CCGA.....C..C.....
Clostridium cluster IV ^c		
Clostridium leptum [NZ_ABCB02000021]G..G.....C..C..G..C..CGT...
<i>Clostridium methylpentosum</i> [NZ_ACEC01000002]G..G.....C..	..C..C..C..C..CGT...
Clostridium cluster XI ^c		
<i>Clostridium bartlettii</i> [NZ_ABEZ02000007]	..A.....A...G.A..C..T.....T.....
<i>Clostridium difficile</i> [NC_009089]	.CA.....A.T.....T.....
Clostridium hiranonis [NZ_ABWP01000030]	..T.....A.....T.....T.....
Clostridium cluster XIV ^c		
<i>Clostridium asparagiforme</i> [NZ_ACCJ01000406]G..G..C.....	..C..C..G.....GG....
<i>Clostridium bolteae</i> [NZ_ABCC02000011]	..C..G..G..T.....	..C..C.....C..G....
<i>Clostridium hathewayi</i> [NZ_ACIO01000001]	..T..G..G.....	..C..C..G..C..G....
<i>Clostridium hylemonae</i> [NZ_ABYI02000019]G..A.....C..C..G.....G....
<i>Clostridium nexile</i> [NZ_ABWO01000184]A.....	..C..A..G.....G....
Clostridium phytofermentans [NC_010001]	..A.....GT.....	..T.....T..C.....
<i>Clostridium scindens</i> [NZ_ABFY02000067]	..A..G.....C..G..C..G....
Clostridium cluster XVIII ^c		
Clostridium spiroforme [NZ_ABIK02000014]	.C.....A..T.....	..T.....TG....
<i>Clostridium ramosum</i> [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.

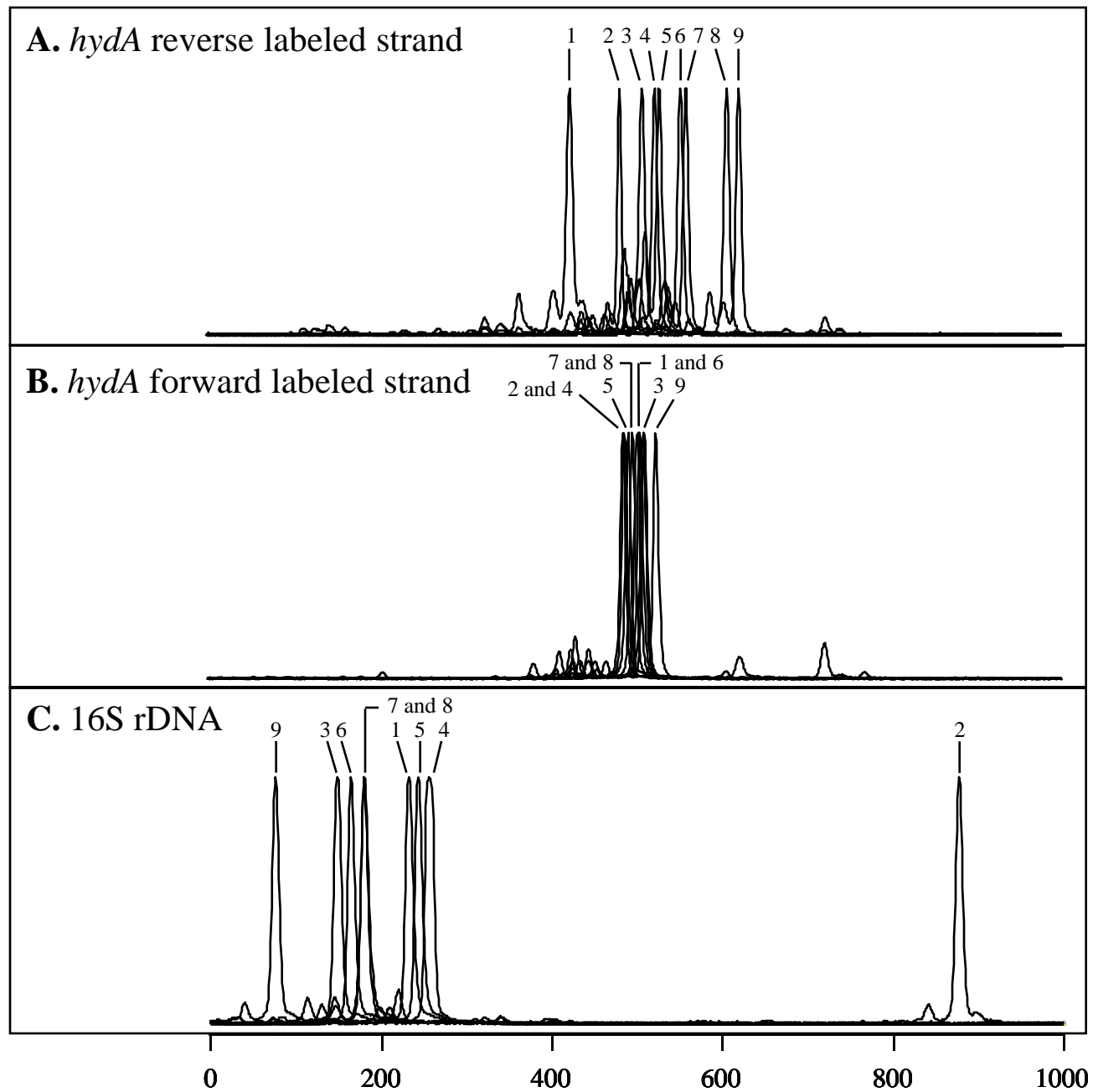


Figure 1

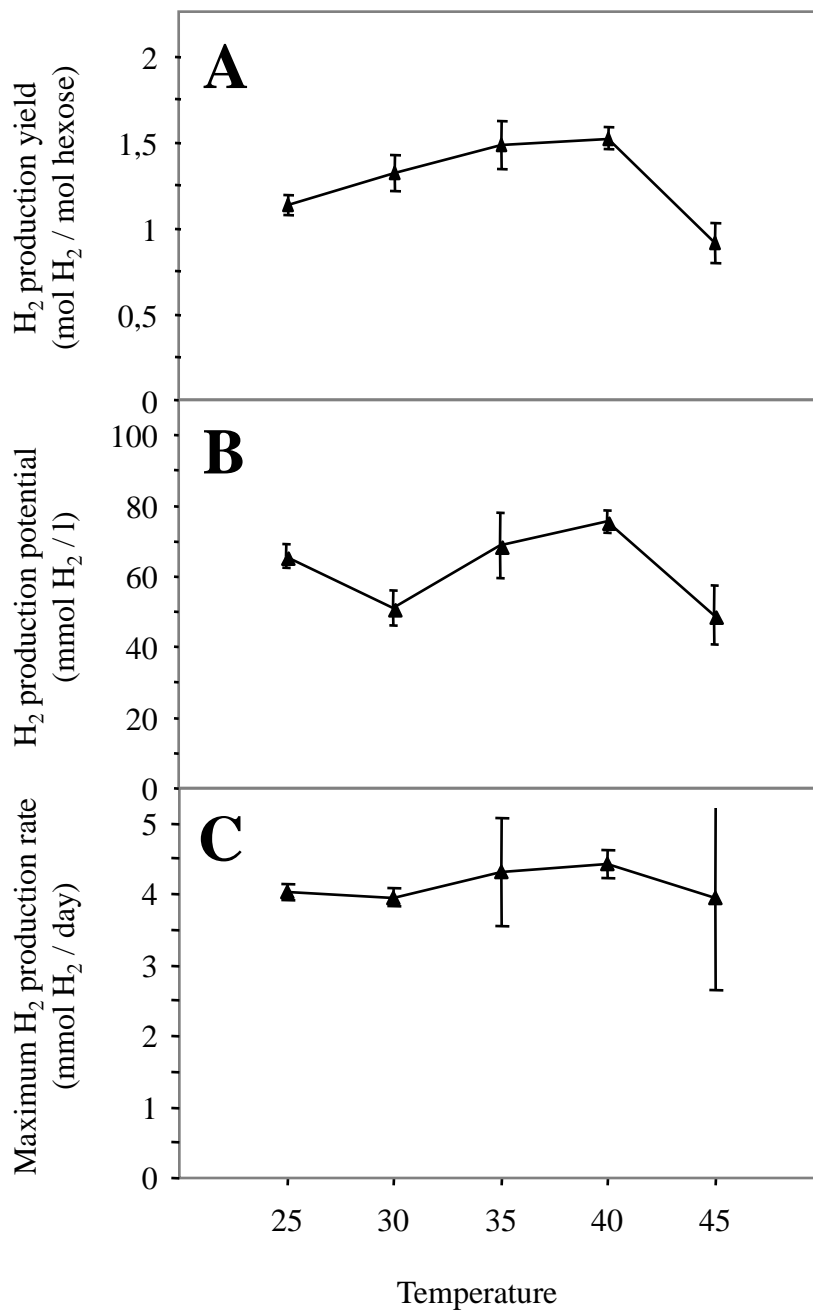


Figure 2

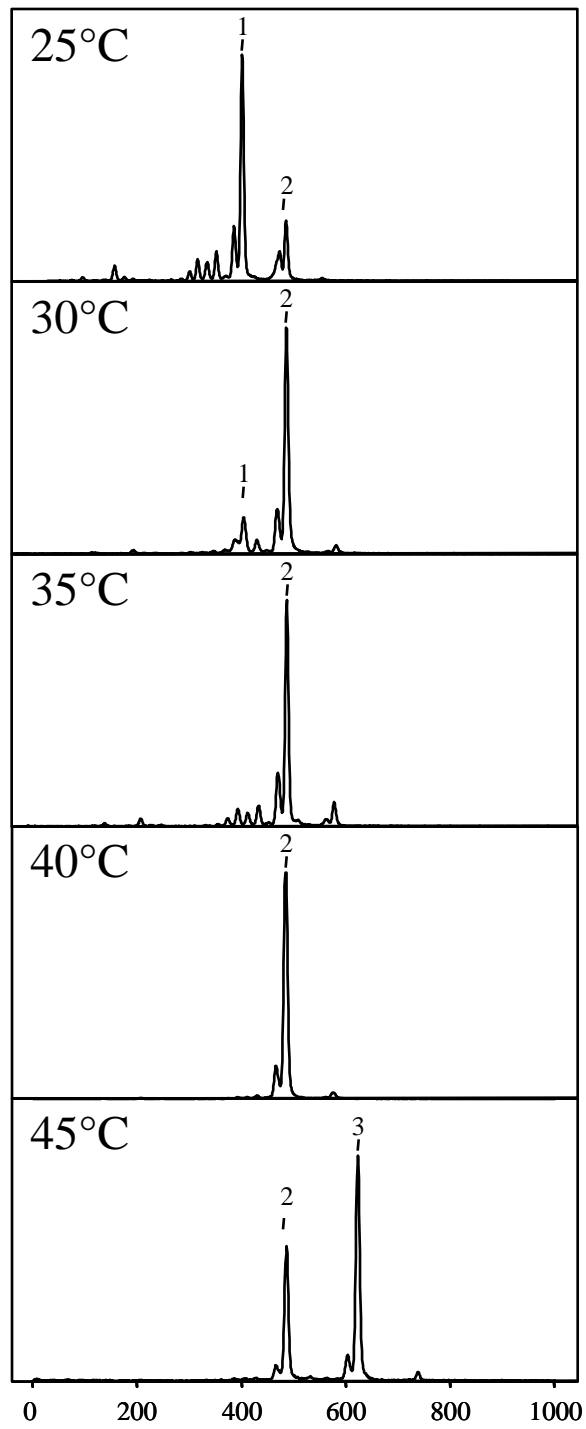
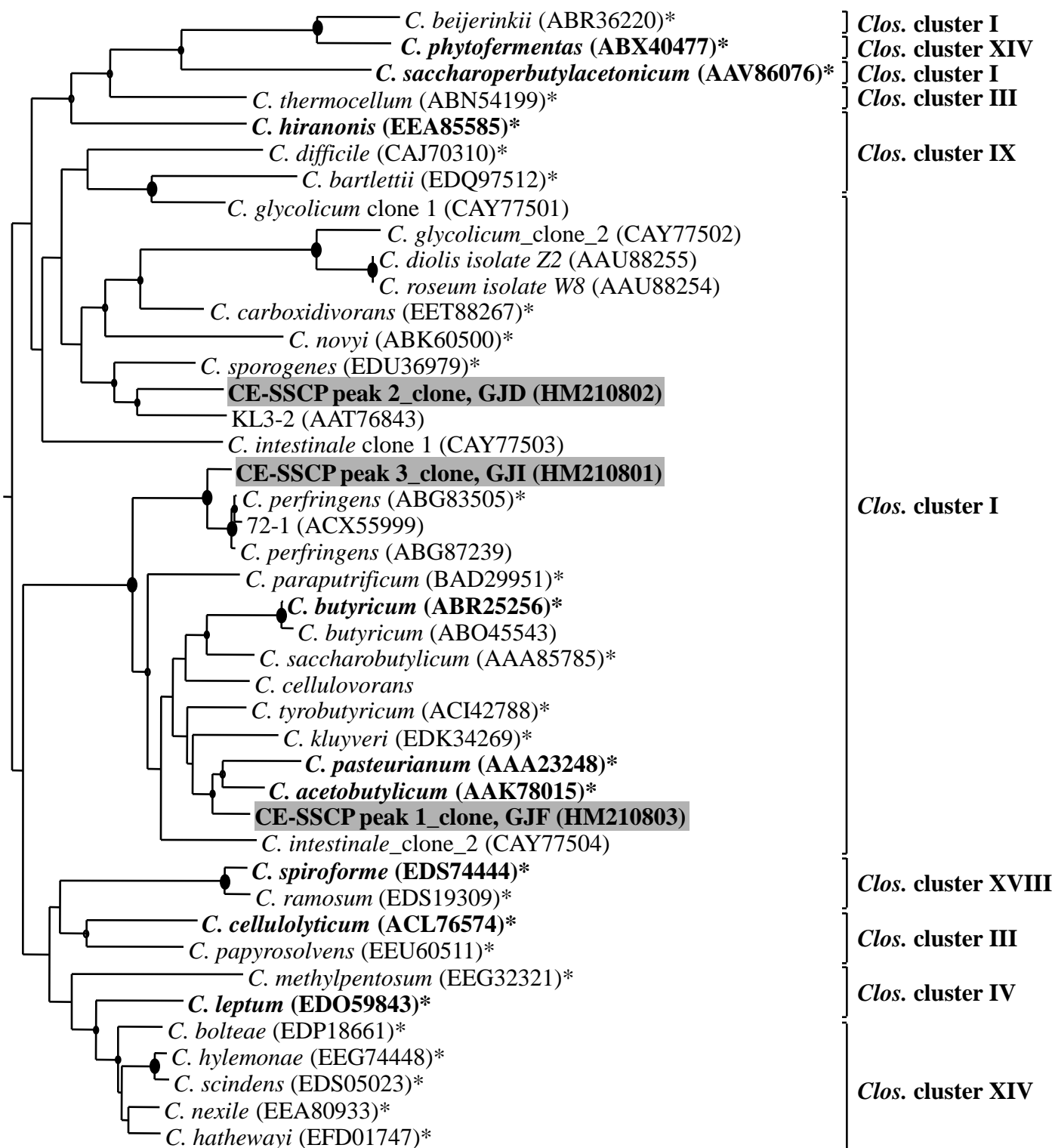


Figure 3



0.1

Figure 4