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Characterization by length heterogeneity (LH)-PCR of a hydrogen-producing community obtained in dark fermentation using coastal lake sediment as an inoculum

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Abstract

Background: Sustainable biohydrogen production can be achieved by dark fermentation of organic wastes, and studies were carried out using a range of substrates and inocula. The bacterial populations involved were mainly identified as facultative anaerobes (*Enterobacteriaceae*) and strictly anaerobes (*Clostridiales*), and their dynamics in relation to H_2 and metabolite production depends on the physical and environmental conditions of the bioreactor. This study has evaluated the use of length heterogeneity (LH)-PCR fingerprinting to detect changes in the microbial community during continuous hydrogen production under dark fermentation.

Methods: A mesophilic continuous dark fermentation was established using coastal lake sediment as an inoculum in a synthetic medium with glucose as a substrate. The LH-PCR profiling associated to the sequencing of 16S rRNA genes was used for the characterization of the bacterial community and identification of species during a continuous production of H_2.

Results: The resulting dominant units of the communities present at both 24 h (batch) and 210 h (continuous culture) were affiliated to species of the genus *Clostridium*. Fluctuations based on their relative abundance over time were observed. At 24 h, a higher ratio was detected for the group *Clostridium butyricum - Clostridium tertium* followed by *Clostridium bifermentans* and *Clostridium perfringens*, while at 210 h, the group *Clostridium aurantibutyricum - Clostridium acetobutylicum* was the most abundant.

Conclusions: The LH-PCR profiling has proven to be a sensitive and rapid method for the evaluation of the dynamics of a functional consortium formed by species of the genus *Clostridium* and has a potential for studies aimed at the optimization of biohydrogen production.

Keywords: Biohydrogen, Dark anaerobic fermentation, *Clostridium*, 16S rRNA genes, Length heterogeneity-PCR fingerprinting

Background

Biohydrogen can be obtained by dark fermentation of carbohydrates under both thermophilic and mesophilic conditions, and the exploitation of this process for the production of an environmentally friendly energy source could also reduce the dependence on fossil fuels [1].

Mesophilic fermentation has shown an interesting H_2 production rate and seems to be promising for its economic feasibility [2], but the application of this process still needs improvement. Studies for the enhancement of the yield and the production rate have evaluated the effect of pH, temperature, bioreactor design, and substrates [3-5], as well as the use of inocula made of pure cultures or artificial consortia [6,7]. In order to increase the sustainability of the process, experiments have been conducted using wastewater or organic wastes as

substrates [8,9]. Slurry and other environmental matrices have also been selected and used as inocula since they are rich in H_2 producers [3,4,7-9]. The members of bacterial communities during H_2 production were mainly identified as facultative anaerobes, belonging to the *Enterobacteriaceae* and several species of the strict anaerobe *Clostridium* [1]. The structure and dynamics of communities using different substrates in relation to H_2 and metabolite production were investigated using denaturant gradient gel electrophoresis (DGGE) of the 16S rRNA gene [10,11], and more recently, the capillary electrophoresis single-strand conformation of the 16S rRNA and Fe hydrogenase (*hydA*) genes of *Clostridiales* has been proposed [12].

The objective of this study was to develop a sensitive method for the evaluation of microbial community dynamics during continuous H_2 production in a model dark fermentation. The data presented are part of a large experiment aimed at the selection and enrichment of a microbial consortium pool for biohydrogen production using a coastal lake sediment as an inoculum and testing different organic loads and retention times. The evaluation of metabolites during the process is not reported in this paper.

Methods
Experiment
A dark fermentation was established in a continuously stirred tank reactor of 2.5 l with 1 l of working volume and a synthetic basal fermentation medium [13], pH 6.7, buffer 0.1 M with 10 g l^{-1} of glucose as a sole carbon source, and inoculated with 5 g of sediment. The sediment was manually collected from the coastal lake Fogliano, near Mar Tirreno (Central Italy), at a depth of about 1 m and stored at –20°C for 9 months until use. The sampling area is characterized by anoxic conditions even a few millimeters below the water surface [14]. The tank reactor with the medium was sterilized before use, and all the operations were conducted under sterile conditions in order to evaluate the sediment community only. The reactor was sealed and sparged with N_2 to establish anaerobic conditions. A constant temperature of 37°C and continuous mixing were obtained in a heating plate equipped with an aluminum jacket. The reactor operated for 24 h in batch mode to avoid the washing out of the microbial pool. Then, a continuous feeding was established by tubes connected to peristaltic pumps. The hydraulic retention time was set to 24 h during the first 3 days of operation and was then changed to 12 h. The reactor was connected to a water displacement system in order to measure the biogas production, and the H_2 content was evaluated by a gas chromatograph (Focus GC, Thermo Scientific, West Palm Beach, FL, USA) equipped with a thermal conductivity detector and a 3-m stainless steel column packed with Hayesep Q (800/100 mesh; Valco Instruments Co. Inc., Houston, TX, USA). Metabolite production and physical parameters as well as the amount of gas produced and its composition were evaluated several times daily. The reactor was operated continuously, and the cell viability was periodically determined using the Live/Dead® Bacterial Viability Kit, purchased from Molecular Probes Inc., Eugene, OR, USA, with five replications [15].

Community characterization
DNA was extracted from frozen sediment and pellets of the bioreactor cultures using the Fast DNA Spin Kit for Soil (QBiogene, Vista, CA, USA) and a modified protocol. Samples were mixed to 978 μl of sodium phosphate buffer and 122 μl of MT buffer, shaken with a vortex for 30 s, incubated for 5 min at 65°C, and centrifuged for 1 min at 10,000×*g*. The supernatant was processed according to the manufacturer, and the DNA samples were used for LH-PCR of the 16S rRNA V1 + V2 domains with the primer pair 27 F-6-FAM (fluorescently labelled) and 355R, as has been reported earlier [16]. The fragments were separated using the ABI Prism® 3730 genetic analyzer (PE Biosystems, Foster City, CA, USA) under the setting Microsatellite, and the results were obtained by means of the software ABI Prism GeneMapper® V 4.0 that provided the evaluation of the size (bp) and the intensity for each amplicon of a given electropherogram, with respect to the internal ROX standard, using a minimum noise threshold of 50 fluorescent units. The analysis was conducted on replicate DNA extractions for each sample with two replicate LH-PCR profiles each. Binning and normalization were performed according to Mills et al. [16] in order to eliminate errors related to the data collection and analysis software. Thus, for each group of replicates, only fragments detected in three fourth of the profiles and relative ratios greater than 1% were considered reproducible and included in the analysis. The relative ratio for each amplicon was evaluated by dividing the peak height by the total intensity of the electropherogram, and the mean relative ratios were evaluated. Cloning and sequencing of the amplified 16S rRNA genes were performed according to [16], and the sequences were identified with respect to the NCBI GenBank database, using the MegaBLAST search option. LH-PCR was performed on the DNA from the clones, and the amplicon size was recorded as previously described. In addition, the cloned sequences were aligned with ClustalX [17] to the primers used for LH-PCR, and the virtual size of the corresponding fragments was evaluated (*in silico* analysis). The nucleotide sequences generated in this study were deposited in NCBI GenBank with the accession numbers: [GenBank:JX012104-JX012128].

Results and discussion

Fingerprints of the bacterial communities were obtained at 24 and 210 h that respectively represented the beginning of the continuous phase and the time corresponding to a well-established continuous H_2 production that reached an average of 50% of the total gas produced. At two times of sampling, the average yields were 240 and 238 ml H_2 g^{-1}, and the culture densities were 6.73×10^8 and 1.99×10^9 viable cells/ml, respectively. The community profiles are illustrated in Figure 1A and presented five distinct LH amplicons for both times of sampling indicated with letters corresponding to the sizes (bp): *a* (330 to 331), *b* (334), *c* (336 to 337), *d* (343 to 344), and *e* (346 to 348). The average relative ratio of each amplicon was given in the ordinate and indicated not only the presence of the main community members at both times of sampling but also the fluctuations of their relative abundance. In fact, at 24 h, amplicon *d* was dominant followed by *a* and *e* with relative ratios of 41.13%, 22.9%, and 20.3%, respectively, while at 210 h, amplicon *c* was dominant (38.22%) followed by *e* and *d* (27.96% and 12.46%, respectively). The dominant units during the fermentation were also detected in the fingerprinting of the frozen sediment used as an inoculum (Figure 1B) with a different ratio. Two units present in the sediment profiles were not detected in the fermentation communities as expected when considering the selective stringent conditions of the process. All the cloned sequences were affiliated to species of the genus *Clostridium*. Table 1 shows the list of GenBank accessions presenting the higher identities. The LH-PCR performed on cloned sequences produced a single amplicon for each clone that matched one of the *a* to *e* community units. The size of LH amplicon of clones was confirmed by the virtual *in silico* analysis and allowed the identification of the community units as reported in Table 1. Therefore, clones affiliated to *Clostridium butyricum* and *Clostridium tertium* were associated to amplicon *d* dominant at 24 h, while clones with identity to *Clostridium aurantibutyricum* and
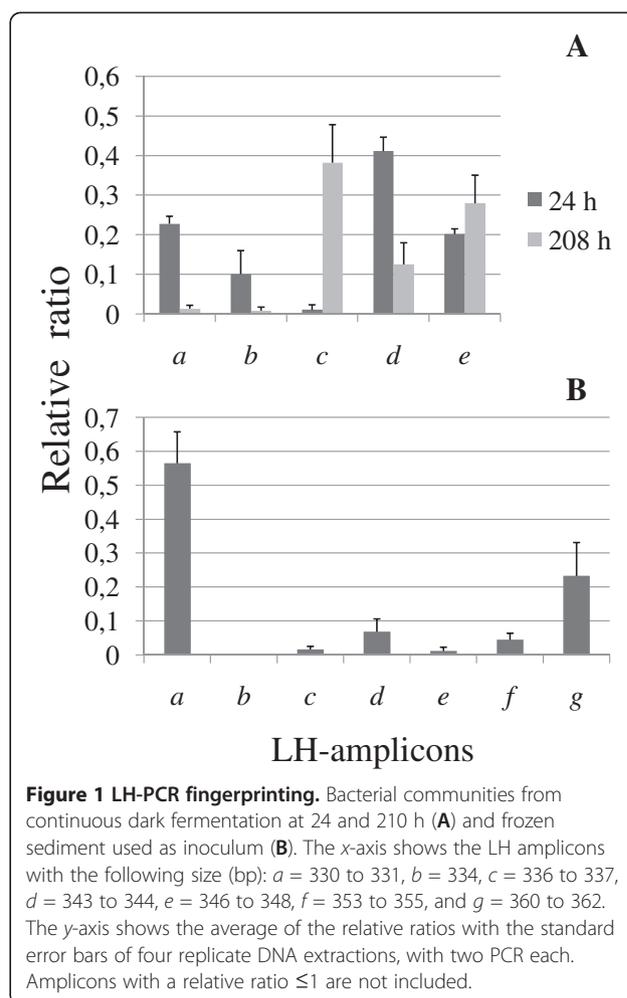

Figure 1 LH-PCR fingerprinting. Bacterial communities from continuous dark fermentation at 24 and 210 h (**A**) and frozen sediment used as inoculum (**B**). The *x*-axis shows the LH amplicons with the following size (bp): *a* = 330 to 331, *b* = 334, *c* = 336 to 337, *d* = 343 to 344, *e* = 346 to 348, *f* = 353 to 355, and *g* = 360 to 362. The *y*-axis shows the average of the relative ratios with the standard error bars of four replicate DNA extractions, with two PCR each. Amplicons with a relative ratio ≤1 are not included.

Clostridium acetobutylicum matched amplicon *c* dominant at 210 h. Clones affiliated to *Clostridium bifermentans*, *Clostridium ghonii*, and *Clostridium perfringens* presented the fragment sizes corresponding to amplicons *a*, *b*, and *e*, respectively. Most community studies on biohydrogen production have used the denaturing gradient gel electrophoresis (DGGE) profiling and evaluated the diversity as

Table 1 Affiliation of the 16S rRNA cloned sequences to accessions reported in the GenBank

Number of clones	Time (h)	GenBank affiliation [accession no.]	Identity (%)	LH amplicon
3, 2	0, 24	*C. bifermentans* [GenBank:AB618787]	98 to 100	*a*
1	24	*C. bifermentans* [GenBank:AB538434]	99	*a*
2	24	*C. ghonii* [GenBank:AB542933]	98	*b*
2, 5	24, 210	*C. aurantibutyricum* [GenBank:FJ358641]	96	*c*
2	24	*C. acetobutylicum* [GenBank:AB595131]	96	*c*
3	24	*C. butyricum* [GenBank:AB595129]	99 to 100	*d*
1	24	*C. tertium* [GenBank:AB618789]	99	*d*
1	24	*C. perfringens* [GenBank:AB627081]	97	*e*
1	24	*C. perfringens* [GenBank:EU728702]	98	*e*

The sequences were obtained from frozen sediment (time 0) and mesophilic continuous dark fermentation cultures after 24 and 210 h. The association to the community profiling (amplicons *a* to *e*) obtained by LH-PCR of clones is given.

the presence/absence of bands on gels or the detection of the intensity using densitometric methods that still require visual evaluation [18]. The faint bands on DGGE gels are of difficult evaluation and often ignored [3,10,18,19], while sequencing of the intense bands has sometimes revealed the presence of more than one species [10]. In this study, the ABI collection software allowed the distinction of amplicons differing in size of only a few base pairs and the accurate detection of the relative ratio for each of them, including the less abundant amplicons. The LH-PCR profiling is reported as a fast, reliable, and sensitive method for community studies in environmental samples, biological processes, and bioreactors, where diversity is present on the class/phylum level [16,20-22]. In this study, the taxonomic identification of community members as species of *Clostridium* is based on a limited number of clones that may not represent the whole community. However, all the amplicons during the process are associated to one or two of the clones that could represent the dominant units. Several studies have described communities mainly formed by Clostridia in dark fermentations started by pre-heated inoculum rich in spore-forming hydrogen producers [11,12,18]. In our work, the sediment was frozen until use since we had evidence from previous experiments that such treatment did not significantly affect the survival of H_2-producing populations, allowing the storage of inoculum for serial experiments. Thus, the freezing and the strict control of anaerobiosis may have selected the *Clostridium* species. Although the data presented cannot distinguish the H_2 producers from the other community members, the literature reported H_2 production for strains of *C. butyricum*, *C. bifermentans*, and *C. acetobutylicum* in pure cultures [23-25] and *C. perfringens* in complex communities in bioreactors [11].

Conclusions

The inoculum allowed for establishing a mesophilic dark fermentation, and a microbial pool able to produce H_2 was obtained. The method LH-PCR was reported for the first time in this type of process and proved to be effective for profiling a community formed by Clostridia. Moreover, the ABI collection software allowed the evaluation of community fluctuation based not only on the presence or the absence but also on the relative abundance of each unit, indicating the feasibility of the method for the evaluation of subtle changes in studies aimed at the biohydrogen production using the selected microbial pool.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RDB carried out the molecular studies and drafted the manuscript. AM, GM CP, SR, AS, and CrV carried out the anaerobic fermentations, the evaluation of metabolites, and the cell count. CoV helped in the analysis of molecular data and sequencing. GI coordinated the study and its design. All the authors helped in the revision of the manuscript and approved the final version.

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