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Analysing the *dhaT* gene in Colombian *Clostridium* sp. (Clostridia) 1,3-propanediol-producing strains

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Abstract

Objective: to analyze the *dhaT* gene, one of the genes responsible for the 1,3-propanediol (1,3-PD) production, in two native *Clostridium* strains. **Materials and methods:** The *dhaT* gene was amplified by Polimerase Chain Reaction with specific primers designed from *Clostridium butyricum* VPI1718 operon. Bioinformatics tools like BLASTN, ORF finder, BLASTP and ClustalW were used to determine the identity of the sequence and to assign a function. **Results:** DNA amplification products were obtained from Colombian *Clostridium* sp. native strains (IBUN 13A and IBUN 158B) and the *Clostridium butyricum* DSM 2478 strain, which were sequenced. According to the bioinformatics analysis of the above sequences, a high degree of similarity was found with the *dhaT* gene of different bacterial species. The highest percentage of identity was obtained with the *Clostridium butyricum* VPI 1718 strain. **Conclusion:** knowledge of the physical structure of the 1,3-PD operon in native strains opens the way for developing genetic and metabolic engineering strategies for improving processes productivity.

Key words: 1,3-propanediol, 1,3-propanediol dehydrogenase, *dhaT* gene, 1,3-propanediol operon.

Resumen

Análisis del gen *dhaT* en cepas Colombianas de *Clostridium* sp. (Clostridia) productoras de 1,3-propanodiol. Objetivo: Analizar el gen *dhaT*, uno de los responsables de la producción de 1,3-propanodiol (1,3-PD), en dos cepas nativas de *Clostridium*. **Materiales y métodos:** El gen *dhaT* fue amplificado por Reacción en Cadena de la Polimerasa, por medio de cebadores específicos diseñados a partir del operon de *Clostridium butyricum* VPI1718. Herramientas bioinformáticas como BLASTN, ORF finder, BLASTP y ClustalW se usaron para determinar la identidad de la secuencia y asignarle una función. **Resultados:** Se obtuvieron productos de amplificación de DNA a partir de cepas nativas Colombianas de *Clostridium* sp. (IBUN 13A y IBUN 158B) y de la cepa *Clostridium butyricum* DSM 2478, que posteriormente fueron secuenciados. De acuerdo a los análisis bioinformáticos de las secuencias mencionadas, se encontró un alto grado de similitud con el gen *dhaT* de diferentes especies bacterianas. El más alto porcentaje de identidad se obtuvo con la cepa *Clostridium butyricum* VPI 1718. **Conclusión:** El conocimiento de la estructura física del operón 1,3-PD en cepas nativas, abre las puertas al desarrollo de estrategias genéticas y de ingeniería metabólica para mejorar la productividad del proceso.

Palabras clave: 1,3-propanodiol, 1,3-propanodiol deshidrogenasa, gen *dhaT*, operón 1,3-propanodiol.

Resumo

Análise do gene *dhaT* em cepas colombianas de *Clostridium* sp. (Clostridia), produtoras de 1,3-propanodiol. Objetivo: Analisar o gene *dhaT*, um dos responsáveis pela produção de 1,3-propanodiol (1,3-PD) em duas cepas nativas de *Clostridium*. **Materiais e métodos:** O gene *dhaT* foi amplificado por Reação em Cadeia da Polimerase, usando cevadores específicos sintetizados a partir do operon de

Clostridium butyricum VPI1718. Ferramentas de bioinformática, tais como BLASTN, ORF finder, BLASTP e ClustalW foram utilizadas para determinar a identidade da seqüência e atribuir-lhe uma função. **Resultados:** Obtiveram-se produtos de amplificação do ADN a partir das cepas nativas colombianas de *Clostridium* sp. (IBUN 13A e IBUN 158B) e da cepa *Clostridium butyricum* DSM 2478, que foram posteriormente seqüenciados. Segundo a análise bioinformática das seqüências acima mencionadas, encontrou-se um elevado grau de similaridade com o gene *dhaT* de diferentes espécies bacterianas. A maior porcentagem de identidade foi obtida com a cepa *Clostridium butyricum* VPI 1718. **Conclusão:** O conhecimento da estrutura física do operon 1,3-PD em cepas nativas, abre as portas ao desenvolvimento de estratégias de genética e engenharia metabólica para melhorar a produtividade do processo.

Palavras-chave: 1,3-propanodiol, 1,3-propanodiol desidrogenase, gene *dhaT*, operon 1,3-propanodiol.

Introduction

Interest in 1,3-propanediol (1,3-PD), a bifunctional organic molecule having special properties for many synthetic reactions, has increased during the last few years, particularly as a monomer for polycondensations for producing polyesters, polyethers and polyurethanes. 1,3-PD-based polymers have characteristics such as good stability under light conditions, biodegradability and improved elasticity. It has an emerging application as raw material in carpet and textile fibre manufacturing (1; 2). 1,3-PD is chemically produced by acrolein hydration and ethylene oxide hydroformylation; it can also be produced through a biotechnological route using glycerol as a substrate for bacterial bioconversion (1). Some microorganisms are able to produce 1,3-PD by glycerol fermentation in nature. Bacteria from the genera *Citrobacter*, *Clostridium*, *Enterobacter*, *Klebsiella* and *Lactobacillus* are some of them (3).

Glycerol is fermented by *Clostridium butyricum* in a process involving two pathways: the oxidative and the reductive pathways. The oxidative is involved in glycolysis and the reductive leads to 1,3-PD synthesis. The oxidative pathway involves glycerol being dehydrogenated by a glycerol dehydrogenase (GDH) to dihydroxyacetone (DHA) which is then phosphorylated by a dihydroxyacetone kinase (DHAK) and funnelled to glycolysis (4; 5). In strains of *C. acetobutylicum* it has been demonstrated that the oxidation of glycerol proceeds through the glycerol kinase and glycerol-3-phosphate dehydrogenase (6). The reductive pathway involves glycerol being dehydrated by a glycerol dehydratase to form 3-hydroxypropionaldehyde (3-HPA) which is then reduced to 1,3-PD by 1,3-propanediol dehydrogenase, also called 1,3-propanediol oxydoreductase (1,3-PDOR) (4; 5). Both routes are important because by the oxidative pathway it is produced ATP and pyruvate, which is an intermediate producing several products (7; 8). The physiological role of 1,3-PD formation is to regenerate reducing equivalents (such as $NADH+H^+$) which are released when DHA is formed and during glyceraldehydephosphate oxidation (8). **Figure 1** shows the metabolic pathway for glycerol assimilation in *Clostridium butyricum* and the enzymes implicated in it. Genes for the reductive pathway are also shown.

The key enzymes in glycerol assimilation are encoded by the *dha* regulon which is expressed when DHA or glycerol are present in the culture medium (10). This regulon has been investigated and characterised in bacteria such as *Klebsiella pneumoniae, Citrobacter freundii, Clostridium perfringens, Clostridium pasteurianum* and *Clostridium butyricum* (5). **Figure 2** shows the *dha* regulon and related genes from these microorganisms (5). In the case of *C. butyricum* VPI 1718, *dhaB1*, *dhaB2* and *dhaT* genes are grouped in the 1,3-PD operon forming part of the *dha* regulon (11). These genes encode a 1,3-PD dehydrogenase enzyme and a glycerol dehydratase enzyme and its activator protein.

A total of 178 saccharolytic clostridia-related bacterial strains were isolated from Colombian agricultural sources by the Bioprocesses and Bioprospecting group from the Instituto de Biotecnología at the Universidad Nacional de Colombia (IBUN). Thirteen of the strains produced more total solvents from glucose than *Clostridium acetobutylicum* ATCC 824 (12). These strains have been biochemically and molecularly characterised, showing that they are closely related to *C. butyricum* (12, 13, 14, 15, 16, 17). The native strain *Clostridium* sp. IBUN 13A showed 50% greater 1,3-PD volumetric productivity ($Q_{1,3\text{-PD}} = 0.060$ mg mL^{-1} h^{-1}) from glycerol than *Clostridium butyricum* DSM 523 ($Q_{1,3\text{-PD}} = 0.030$ mg mL^{-1} h^{-1}); similar results were obtained with the *Clostridium* sp. IBUN 158B strain (18). The *dhaT* gene necessary for 1,3-PD production was analysed in these two native strains during the course of this work.

Materials and methods

Microorganisms

The strains were obtained from the IBUN gene and strain bank (Von Humboldt accession number 090). The strains used in this study were Colombian *Clostridium* sp. IBUN 13A and IBUN 158B and the *Clostridium butyricum* DSM 2478 reference strain.

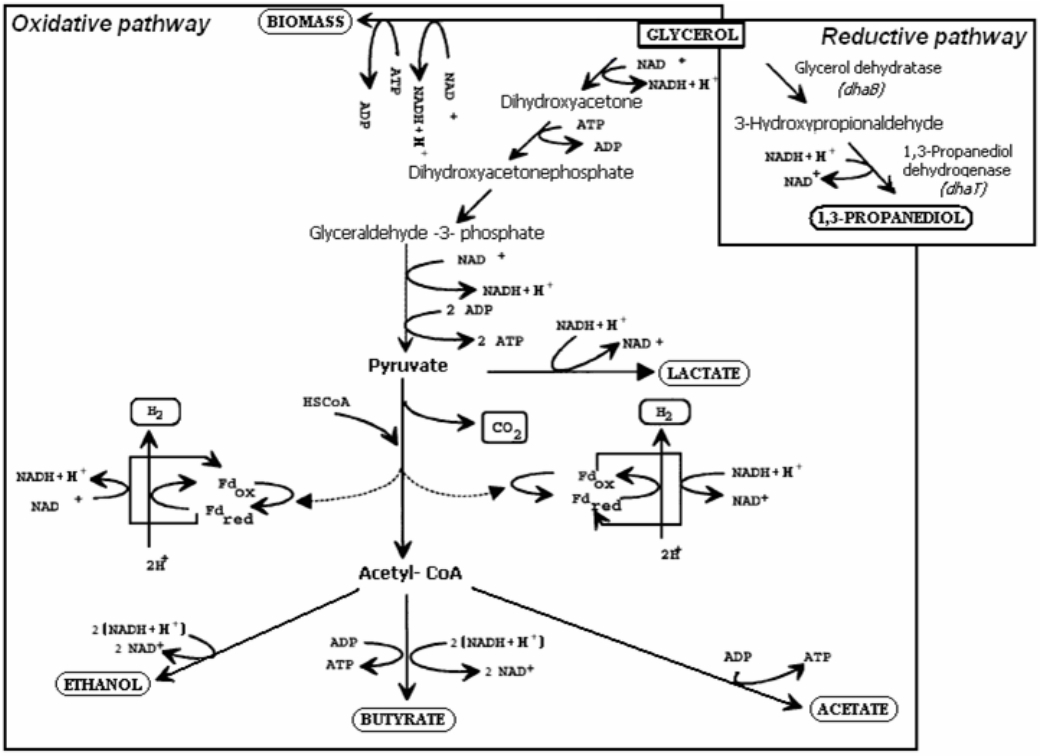

Figure 1. Metabolic pathways for glycerol fermentation by ***Clostridium butyricum*** (adapted from 9). Glycerol dehydratase is encoded by *dhaB*1 and 1,3-propanediol dehydrogenase by *dhaT*.

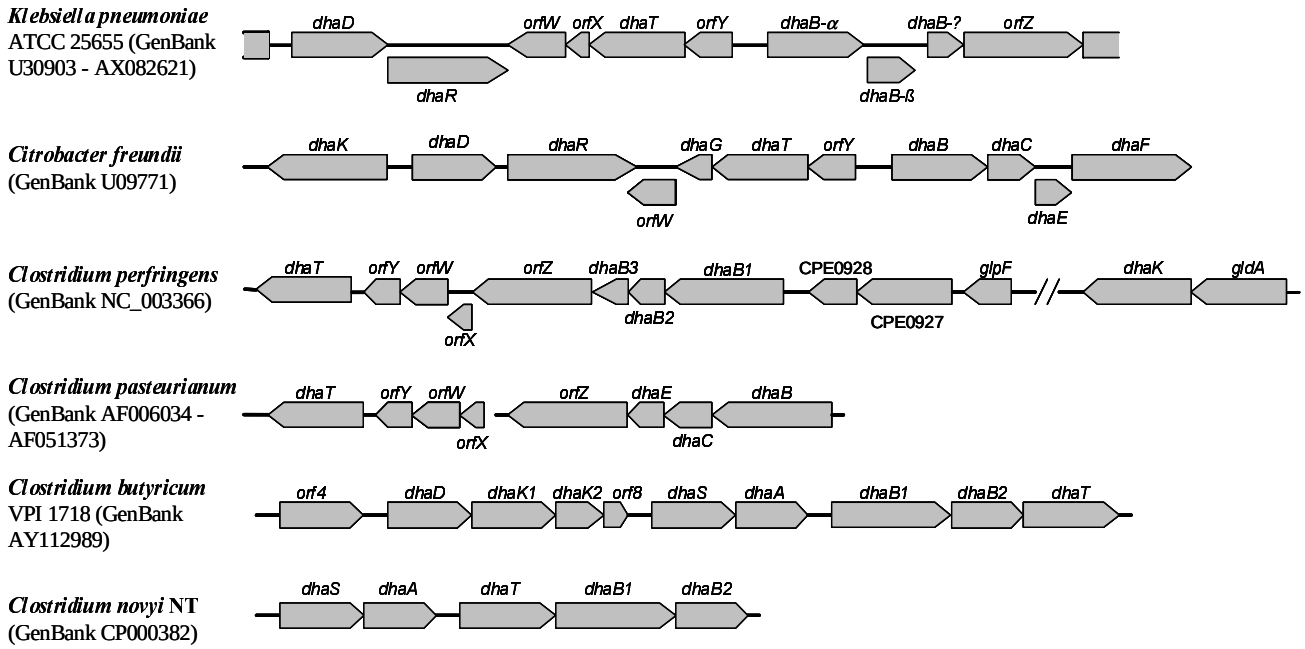

Figure 2. *Dha* regulon and related genes in different microorganisms (modified from 5).

DNA preparation

A preinoculum was prepared by adding 3.0 ml of previously activated culture to 40 ml TGY medium in anaerobic conditions and incubated for 14 h at 37°C (12; 16). 7.5 ml of the preinoculum were transferred to 100 ml TGY and incubated at 37°C until reaching 0.3 to 0.5 optical density at 680 nm. The cells were placed on ice for 30 to 45 minutes and harvested by centrifugation at 6,000 rpm for 10 min at 4°C (the same conditions were used for all centrifugation steps). The cells were then washed twice with 10 ml SSE (0,9% NaCl and 1 mM EDTA) and suspended in 567 µl TE buffer (10 mM Tris-HCL and 1 mM EDTA). SDS and proteinase K were added to 0,5% and 100 µg/ml final concentration, respectively, and the mixture was incubated at 37°C for 1 h. 100 µl NaCl 5 M and 80 µl CTAB/NaCl were then added and incubation proceeded at 65°C for 10 min. An equal volume of chloroform was added and mixed until the two phases emulsified and the mixture was centrifuged. The aqueous phase was transferred to a 2-ml Eppendorf tube and an equal volume of phenol/chloroform/isoamyl alcohol solution (25:24:1) was added and mixed by inversion. This mixture was centrifuged again and the aqueous phase was transferred to a new Eppendorf tube. The procedure was repeated twice more after adding CTAB and NaCl to 700 µl final volume. The aqueous phase was transferred to a 2 ml tube and incubated at 37°C for 20 min. Five µl RNase (20 mg/ml) were added and incubation proceeded at 37°C for 30 min. Two volumes of isopropanol were added, mixed and incubated for 30 min at -20ºC. The sample was spun for 20 min and the supernatant was decanted. Successive washings were done with 500 µl absolute ethanol and 500 µl 70% ethanol. Supernatant was decanted and the DNA pellet was dried at 37°C until the ethanol was completely eliminated. The pellet was finally suspended with 50 µl TE buffer and incubated at 4ºC for 12 h before storing at -20°C.

Partial *dhaT* gene amplification

The partial gene was amplified in a Hybaid Omn-E thermocycler using the PATB and PDH4 primers described by Raynaud *et al*. (11; **Table 1**). These primers were modified by replacing ambiguous nucleotides for those reported for the corresponding positions in *C. butyricum dha* regulon (GenBank AY112989). The reaction mixture was made up to a total 25 µl volume containing 50 ng total DNA, 0.5 mM of each primer, 250 mM dNTPs, 2.0 mM $MgCl_2$, 1.0 U DNA polymerase BIOLASE (Bioline) and 1X corresponding PCR buffer. The PCR programme was as follows: 94°C for 2 min (1 cycle); 94°C for 10 s, 60°C for 30 s and 72°C for 1 min (30 cycles).

Complete *dhaT* gene amplification

The F1, H1 and I1 primers were designed according to the 1,3-PD operon region of *C. butyricum* VPI 1718 (Table 1), using free primer design software, such as Primer3 (http://primer3.sourceforge.net/), OligoPerfect (http://www.invitrogen.com/content.cfm?pageid=9716) and Primer Quest (http://www.idtdna.com/Scitools/Applications/Primerquest/). The strategy was based on the taxonomic closeness and operon 1,3-PD sequence similarity between *C. butyricum* VPI 1718 and the Colombian *Clostridium* native strains. Two different amplifications were carried out in a Hybaid Omn-E thermocycler using a reaction mixture consisting of 50 ng total DNA, 0.25 mM of each primer, 250 mM DNTPs, 2.0 mM $MgCl_2$, 2.0 U DNA polymerase BIOLASE (Bioline) and 1X corresponding PCR buffer. The F1-H1 primer pair was used in a reaction with the following PCR programme: 94°C for 2 min (1 cycle); 94°C for 15 s, 52°C for 30 s and 72°C for 1 min 30 s (25 cycles); 94°C for 15 s, 60°C for 30 s and 72°C for 7 min (1 cycle). The PATB-I1 primer pair was used in a second reaction with the following programme: 94°C for 2 min (1 cycle); 94°C for 15 s, 52°C for 30 s and 72°C for 1

Table 1. Primers used for amplifying the *dhaT* gene in *Clostridium* sp. strains

Primer	Sense	Sequence 5´- 3´	Position *
PATB	Forward	GGAATTGCTGCAACACATGAAGG	6382-6404
PDH4	Reverse	GAATCCTTTAAATAGTATTAATTAATAAGC	7233- 7204
F1	Forward	TTGCAGGATATAGTGCACAG	4971- 4990
H1	Reverse	AGCTACTATTGGTGGCAATG	6465- 6446
I1	Reverse	CTTGTTCTTTGTTGTTAGCC	7301- 7282

* Position of primers in the *C. butyricum dha* regulon sequence (GenBank AY112989).

min 30 s (25 cycles); 94°C for 15 s, 60°C for 30 s and 72°C for 7 min.

Evaluation of amplified products

PCR products were analysed by electrophoresis on 1.5% (for amplifications with F1-H1 and PATB-I1) and 2% (for amplifications with PATB-PDH4) agarose gels with 0.5X TBE. HyperLadder II (Bioline, 50-2000 bp) was used as the molecular weight marker. Gels were stained with ethidium bromide (5mg/ml) and digitalised with a Bio-Rad transilluminator. PCR products were purified and sequenced by Macrogen (http://www.macrogen.com).

Analysis of similarities and gene prediction

Sequences were assembled and compared to the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) using the BLASTN 2.2.17 application with the default conditions (http://www.ncbi.nlm.nih.gov/blast/) and the algorithm Megablast to find similarities. The query coverage percentages were between 90 and 100%. The F1-H1 sequences were assembled with PATB-I1 sequences and then aligned with the *C. butyricum dhaT* gene sequence (GenBank AY112989); the region aligned with the gene was taken as the analysis sequence. Open reading frames (ORFs) in the sequenced PCR products were identified using ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), using the bacterial code. Every ORF having more than 300 nucleotides was translated into amino acids and the resulting sequences were compared to the UniProt protein database using BLASTP 2.2.17 from NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the algorithm blastp, with the default parameters.

Multiple alignments were carried out using CLUSTALW software (http://align.genome.jp/) with default conditions and the matrix BLOSUM62 to observe the taxonomic relatedness of sequenced *dhaT* genes to already reported sequences. Sequences for the *dhaT* gene from *Clostridium* strains were obtained from the GenBank database; these were aligned with the sequences obtained in this work whilst *Klebsiella* and *Citrobacter* sequences were taken as outgroups. A dendrogram was then drawn up using MEGA 4 software (http://www.megasoftware.net/mega.html) using Neighbour Joining as grouping method with 500 bootstrap repetitions.

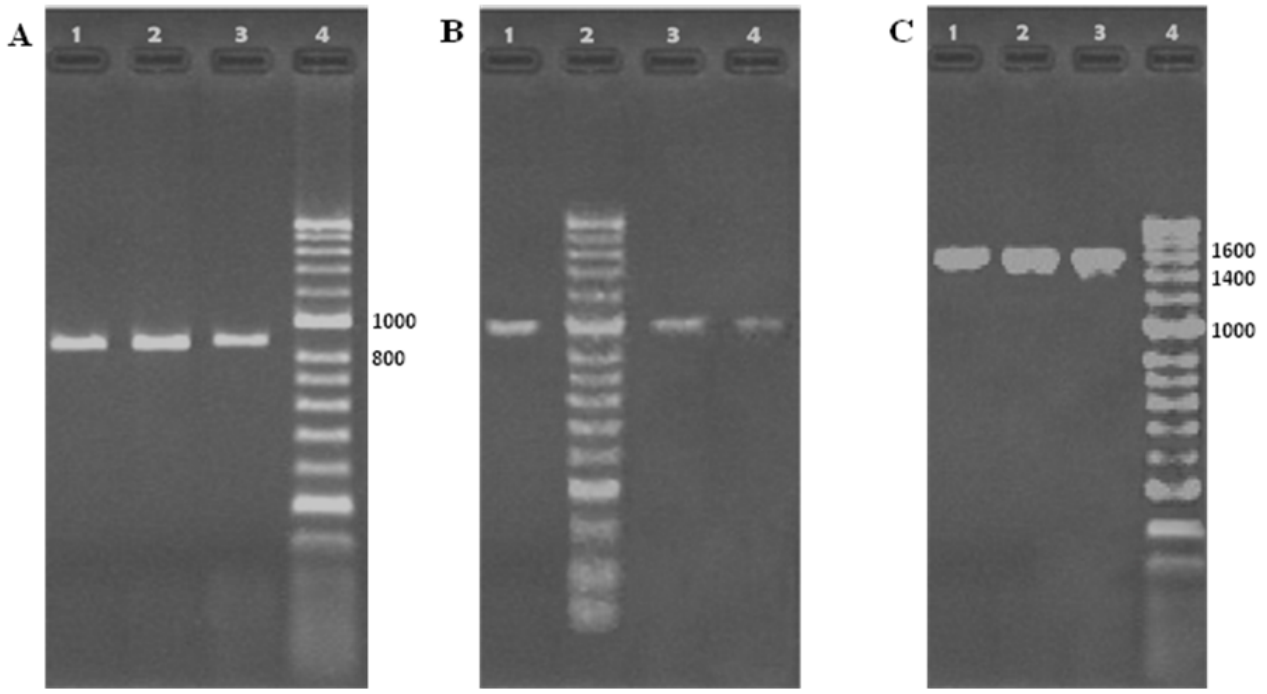

Figure 3. Amplification of the *dhaT* gene. (A) PATB and PDH4 primers. Lane 1, *Clostridium butyricum* DSM 2478; 2, *Clostridium* sp. IBUN 13A; 3, *Clostridium* sp. IBUN 158B; 4, Hyper Ladder II (BIOLINE). (B) PATB and I1 primers. Lane 1, *Clostridium butyricum* DSM 2478; 2, Hyper Ladder II (BIOLINE); 3, *Clostridium* sp. IBUN 13A; 4, *Clostridium* sp. IBUN 158B. (C) F1 and H1 primers. Lane 1, *Clostridium butyricum* DSM 2478; 2, *Clostridium* sp. IBUN 13A; 3, *Clostridium* sp. IBUN 158B; 4, Hyper Ladder II (BIOLINE).

Conserved domains were identified in proteins having complete *dhaT* gene sequences. Translations into amino acids from the obtained ORFs were compared to the NCBI Conserved Domains Database (CDD) (http://130.14. 29.110/Structure/cdd/wrpsb.cgi). The corresponding 1,3-PD dehydrogenase sequences for *Clostridium* sp. IBUN 13A and IBUN 158B and *C. butyricum* DSM 2478 were automatically registered in TrEMBL under accession codes Q0G9F0, Q0G9E9 and Q0G9F1 respectively. TrEMBL accessions were simultaneously included in the protein family and Pfam and InterPro domain databases.

Nucleotide sequence accession numbers

The three sequences obtained for the complete *dhaT* gene were submitted to the GenBank database; the corresponding accession numbers were: DQ901407 for *C. butyricum* DSM 2478, DQ901408 for *Clostridium* sp. IBUN 13A and DQ901409 for *Clostridium* sp. IBUN 158B.

Results

Predicting the partial *dhaT* gene

Fragments of around 851 bp were amplified with PATB and PDH4 primers with DNA from *Clostridium* sp. IBUN 13A and IBUN 158B and *C. butyricum* DSM 2478 strains (**Figure 3A**). The size of the assembled sequence was 769 bp with DNA from *Clostridium* sp. IBUN 13A, 781 bp for IBUN 158B and 766 bp for *C. butyricum* DSM 2478. The three nucleotide sequences presented closely related identity with part of the *C. butyricum dhaT* gene sequence (GenBank AY112989). **Table 2** summarises the ORFs found for each sequence; the highest level of homology was found with *C. butyricum* VPI 1718 1,3-PD dehydrogenase (a protein having 385 residues).

Predicting the complete *dhaT* gene

Fragments of around 919 bp were amplified with primers PATB and I1 using total DNA from the strains *Clostridium*

Table 2. ORFs identified in *C. butyricum* DSM 2478, *Clostridium* sp. IBUN 13A and IBUN 158B sequences analysed with BLASTP

Sequence	ORF[a]	Position	Length[b]	Homology (BLAST)[c]	E value (identity)[d]
DSM2478 partial *dhaT*	+1	19 - 765	747 (248)	*gb|AAM54730.1| 1,3-propanediol dehydrogenase [Clostridium butyricum]*	3e-122 (99%)
IBUN 13A partial *dhaT*	+1	1 - 768	768 (256)	*gb|AAM54730.1| 1,3-propanediol dehydrogenase [Clostridium butyricum]*	3e-136 (98%)
IBUN 158B partial *dhaT*	+1	13 - 780	768 (256)	*gb|AAM54730.1| 1,3-propanediol dehydrogenase [Clostridium butyricum]*	4e-132 (96%)
DSM2478 complete *dhaT*	+2	14 - 1170	1158 (385)	*gb|AAM54730.1| 1,3-propanediol dehydrogenase [Clostridium butyricum]*	0.0 (99%)
IBUN 13A complete *dhaT*	+2	14 - 1170	1158 (385)	*gb|AAM54730.1| 1,3-propanediol dehydrogenase [Clostridium butyricum]*	0.0 (99%)
IBUN 158B complete *dhaT*	+2	14 – 1170	1158 (385)	*gb|AAM54730.1| 1,3-propanediol dehydrogenase [Clostridium butyricum]*	0.0 (99%)

[a] +: ORF on 5' – 3' positive chain, [b] Nucleotides (amino acids), [c] Matched with the highest score obtained after searching in GenBank with BLASTN (accession number is displayed), [d] E value of the alignment made by BLAST (identity percentage).

sp. IBUN 13A, IBUN 158B and *C. butyricum* DSM 2478, whilst the F1 and H1 primers led to fragments of around 1,494 bp long using the same DNA samples (**Figures 3B** and **3C**). Whole *dhaT* assembled from sequences obtained for F1-H1 and PATB-I1 fragments for every strain analysed were 1.171 bp, being equal to the size of the *C. butyricum dhaT* sequence (GenBank AY112989). High similarity was also found with the *dhaT* gene from different bacterial species, such as *C. butyricum, C. pasteurianum, C. novyi, C. perfringens, K. pneumoniae* and *C. freundii* (**Figure 4**).

The highest level of homology was obtained with *C. butyricum* VPI 1718 1,3-PD dehydrogenase when comparing the amino acid sequences translated from every predicted coding sequence against the UniProt protein database (**Table 2**). High similarity with the Fe-ADH functional domain (Pfam: 00465), corresponding to iron-containing alcohol dehydrogenase, was always obtained after looking for conserved domains. TrEMBL accessions were classified under Pfam domain Fe-ADH and InterPro entry IPR001670 (both corresponding to iron-containing alcohol dehydrogenases). Some representative enzymes from this domain include propanediol oxidoreductase from *E. coli,* butanol dehydrogenases from *C. acetobutylicum* and 1,3-PD dehydrogenases from *K. pneumoniae* and *C. freundii*.

Discussion

Colombian *Clostridium* strains IBUN 13A and IBUN 158B have been shown to be good 1,3-PD producers from glycerol (18). Glycerol assimilation involves two metabolic pathways for energy production and recovery of the necessary oxidative cofactors ($NADH+H^+$). Essential genes for these two routes have been found to be associated in the *dha* regulon (5; 7; 10). Genes for the *C. butyricum* VPI 1718 reductive pathway are located together in the 1,3-PD operon (11) and since Colombian strains are closely related to this species (13; 17) it is presumed that they should bear the same genes integrated in a similar fashion. It was thus decided to analyse these genes. The *dhaT* gene was initially analysed to ascertain 1,3-PD operon structure; it was selected because it is the most conserved gene from this operon within the 1,3-PD-producing bacterial group (5). It was also decided to analyse the reference strain *C. butyricum* DSM 2478, since it has been shown to be a 1,3-PD-producer (18; 19) and no records of genes related to

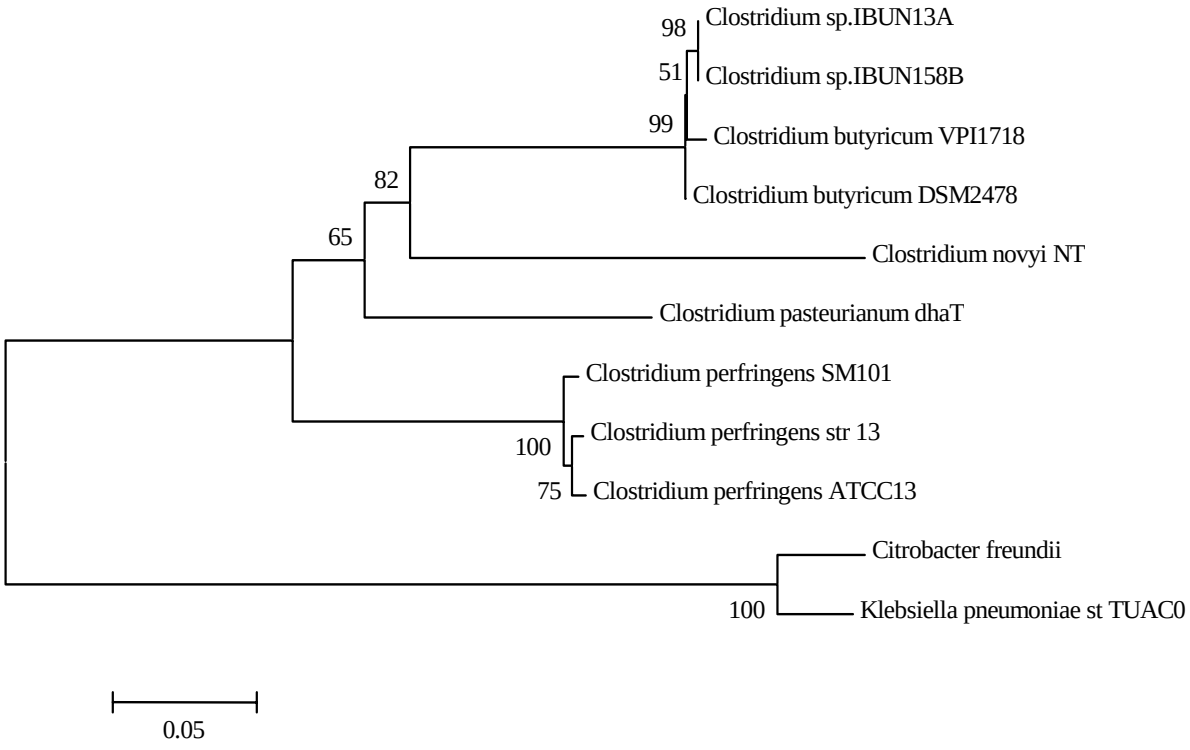

Figure 4. Dendrogram obtained using MEGA software and Neighbour Joining with 500 bootstrap repeats. It can be seen that *Clostridium* sp. IBUN 13A and 158B are closely related to the strain *Clostridium butyricum* DSM 2478.

1,3-PD production from this strain have been registered in GenBank to date (http://www.ncbi.nlm.nih.gov/Genbank).

Fragments of 851 bp, obtained when the partial gene was amplified with the primers PATB and PDH4, were in agreement with the expected size, according to bioinformatics analyses carried out on the *C. butyricum* 1,3-PD operon sequence (GenBank AY112989). A high similarity was found when comparing the predicted partial gene sequence from every native strain with that of the *C. butyricum dhaT* gene (GenBank AY112989), meaning that native strains could have a very similar gene.

As expected, complete gene sequences revealed great similarity with *dhaT* gene sequences from different bacteria and, just like that obtained with partial sequences, the greatest similarity was obtained with the *C. butyricum* VPI 1718 *dhaT* sequence. It was also observed that the gene was not truncated, since start and stop codons were localised in the same positions as those belonging to the *C. butyricum* VPI 1718 *dhaT* gene for every strain analysed; such start and stop codons had not been previously found in partial gene sequences. A high similarity with the Fe-ADH functional domain, corresponding to iron-containing alcohol dehydrogenases was also found in all complete gene sequences.

C. butyricum dhaB1 and *dhaB2* gene (GenBank DQ901408) similarity was also found with sequences obtained in the same Colombian strains (unpublished results); this fact led to the conclusion that 1,3-PD operon genetic organisation in the native strains and in *C. butyricum* DSM 2478 was similar to that of *C. butyricum* VPI 1718. The former results were particularly relevant as *C. butyricum* VPI 1718 glycerol dehydratase, encoded in the 1,3-PD operon, has been the only B_{12} coenzyme-independent enzyme of its kind reported so far. B_{12} coenzyme-dependence is one of the key limitations in the biotechnological process for obtaining 1,3-PD from renewable sources such as glucose, given that it is a very expensive reagent which must be added to the culture media. B_{12} coenzyme-independent glycerol dehydratase from *C. butyricum* and similar enzymes could thus significantly influence the cost of producing 1,3-PD from renewable sources (11).

On the other hand, it should be noted that the *C. butyricum dha* regulon is considerably different to that of other microorganisms (5). Glycerol dehydratase is encoded by a single gene in *C. butyricum* (*dhaB1*), as well as its activator protein, encoded by *dhaB2*. These enzymes are usually encoded by three and two genes respectively, leading to the corresponding amount of subunits (5). A second difference lies in the 1,3-PD regulator of *C. butyricum*, a two-component signal transduction system encoded by two genes named *dhaS* and *dhaA*, instead of the *dhaR* gene encoding a single regulator protein in other bacteria (5). It should also be stressed that all genes belonging to the reductive pathway in 1,3-PD-producing *Clostridium* species are transcribed in the same direction, unlike 1,3-PD-producing Gram(-) enterobacteria *K. pneumoniae* and *C freundii* (5).

Identifying the *dhaT* gene in the native strains has contributed to knowledge regarding the genetics of 1,3-PD production in these strains, thereby laying the foundations for new processes for improving their production rate. Ongoing work is focused on sequencing other genes related to 1,3-PD production from glycerol in the Colombian native strains, namely those neighbouring *dhaT* in the 1,3-PD operon which encode the remaining reductive pathway enzymes. An attempt is also being made to characterise enzymes involved in 1,3-PD production to improve their activity. Improvement through recombinant DNA or metabolic engineering strategies could open the way forward for using high glycerol content industrial effluents as fermentation substrate, for instance those arising from high-scale biodiesel production, in such a way that the effluent can be used as raw material and process costs can be lessened (20).

The high percentage of identity (more than 70% in nucleotide sequences and more than 25% in amino acid sequences) found after comparing sequences from the strains *Clostridium* sp. IBUN 13A, *Clostridium* sp. IBUN 158B and *C. butyricum* DSM 2478 with the GenBank database indicated that these strains bore a similar *dhaT* gene to that of *C. butyricum*, *C. pasteurianum* and *C. perfringens*. The fact that complete encoding sequences (including start and stop codons) were identified suggested native Colombian strains ability to encode a protein homologous to reported 1,3-PD dehydrogenase from these typical 1,3-PD-producing microorganisms (i.e. having similar biochemical functions and being descended from a common ancestor).

Conclusions

According to the results, the arrangement of the genes for reductive glycerol metabolism in the native *Clostridium* sp. IBUN 13A and IBUN 158B and *C. butyricum* DSM 2478 strains was likely to be similar to the *C. butyricum* VPI 1718 1,3-PD operon which contained a B_{12}-independent glycerol dehydratase. Colombian *Clostridium* strains could encode an equivalent B_{12}-independent enzyme, therefore becoming ideal candidates for developing cost-effective 1,3-PD

production without B_{12} coenzyme-dependence. This work contributes towards enriching available genetic information regarding Colombian *Clostridium* strains as well as the reference strain *C. butyricum* DSM 2478. It was also the first step in gaining knowledge about the genes involved in 1,3-PD production in native strains, in turn leading to the search for the remaining genes contained in the *C. butyricum* 1,3-PD operon (*dhaB1* and *dhaB2* genes encoding glycerol dehydratase and activator protein). A broader investigation aimed at characterising the complete operon (including its promoter and operon-regulating factors) will open the way forward for genetic manipulation or enzymatic activity modulation in native strains, aimed at their future biotechnological exploitation.

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Conflict of interests

The authors state that results presented in this article do not involve conflict of interests.

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