Genomic and metabolic insights into *Thermoanaerobacterium thermosaccharolyticum* GSU5 solvent production

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April 28, 2020

Abstract

Thermoanaerobacterium thermosaccharolyticum GSU5 was isolated from animal dung collected in a pasture plain in Buenos Aires, Argentina. This thermophilic and anaerobic microorganism was able to produce butanol and ethanol, but not acetone, using sugars such as xylose, arabinose, glucose, galactose, fructose, sucrose and cellobiose. Key metabolic enzymes leading to solvent production were identified in its genome. A detailed analysis of the solvent and organic acid biosynthetic pathway genes of sequenced strains revealed new insights into the unique metabolic features of this species. The large range of fermentable substrates and the ability to produce both ethanol and butanol without acetone makes this species an interesting candidate for second generation biofuel production.

Introduction

Climate change is a threat to the several life forms of our planet. This phenomenon is partially due to the preferential use of fossil fuels to sustain human activities such as heating, agriculture or transportation. The use of biofuels is one of the most promising alternatives, as it would help reduce the effects of climate change, while avoiding the use of non-renewable resources such as petroleum or gas as sources of energy (Hill, Nelson, Tilman, Polasky, & Douglas, 2006). Among the most common biofuels are alcohols that can be obtained from microbial fermentation using different kinds of carbon sources. Ethanol is the best known and most studied alcohol that can be obtained by microbial fermentation using yeast or bacteria, while butanol is a less common but increasingly attractive biofuel due to several characteristics, since it is capable of producing more energy if properly harnessed, and has a lower vapor pressure and is less hygroscopic than ethanol (Dürre, 2007). Many different processes have been developed to obtain alcohols from sugars or starch, but the use of these substrates to produce biofuels would compete with food supplies. To avoid this problem, biofuels should be obtained from non-food substrates, such as lignocellulosic biomass. Several different approaches have been employed to use this abundant substrate, most of which start with the hydrolysis of the biomass to obtain sugars that can be subsequently fermented to the desired compounds. This hydrolysis is normally achieved through energy intensive processes, involving high temperatures and aggressive chemical conditions. Consolidated bioprocessing is an alternative that integrates enzyme production, saccharification and fermentation (Yamada, Hasunuma, & Kondo, 2013) that can be achieved in mild conditions. Consolidated bioprocesses that use anaerobic thermophilic organisms capable of degrading lignocellulosic biomass are expected to meet sustainability standards, as they would enable production of biofuels from renewable resources by means of low energy demanding procedures (Lynd, Weimer, Zyl, & Pretorius, 2002).

The use of cellulolytic thermophilic bacteria, such as *C. thermocellum* , has been extensively studied for the production of ethanol from cellulose (Lamed & Zeikus, 1980; Tian et al., 2016). However, most of these organisms are unable to produce butanol, and/or to ferment pentoses derived from hemicellulose degradation, thereby limiting the efficient use of lignocellulosic biomass (Demain, Newcomb, & Wu, 2005). In recent years, several species of the thermophilic genus *Thermoanaerobacterium* have received increased attention due to

their capability to use different biomass substrates to produce solvents. The genus *Thermoanaerobacterium* comprises 8 validly described species: *T . aciditolerans, T. aotearoense, T. saccharolyticum, T. thermosaccharolyticum, T. thermostercoris* (formerly *T. thermostercus*), *T. thermosulfurigenes, T. xylanolyticum* and *T. butyriciformans* (Onyenwoke & Wiegel, 2015). *T. saccharolyticum* and *T. thermostercoris* can produce ethanol (Romano et al., 2010; Shaw et al., 2008) and several *T. thermosaccharolyticum* isolates have been reported to produce hydrogen (Cao et al., 2009) and/or butanol (Li, Zhang, Yang, & He, 2018) from different biomass sources. These results point out the potential of these microorganisms for the synthesis of diverse bioproducts, and particularly biofuels, from untreated or minimally treated biomass, and spur further research in order to analyze their potential application in consolidated bioprocesses.

We have isolated and characterized a new strain, *T. thermosaccharolyticum* GSU5, an anaerobic thermophilic bacterium that is capable of producing ethanol and butanol from a variety of substrates. In this work we present the genomic sequence of GSU5 and analyze its phenotypic traits, especially those pertaining to solvent production, in comparison to the type strain of the species. Additionally, genes related to solventogenesis of all sequenced *Thermoanaerobacterium* are compared to gain new insights into their unique metabolic properties.

Materials and methods

Bacterial strains and growth conditions

Thermoanaerobacterium thermosaccharolyticum GSU5 was isolated from animal dung collected in a pasture plain in Buenos Aires, Argentina, in 1987. The strain was originally designated *Clostridium thermopapyrolyticum* due to its phenotypic characteristics (Mendez, Pettinari, Ivanier, Ramos, & Siñeriz, 1991). Stock cultures were kept at 4°C in Hungate tubes containing growth medium with a strip of filter paper for several decades. *Thermoanaerobacterium thermosaccharolyticum* DSM 571 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany).

Both strains were grown at 60 ºC in 5 ml screw cap tubes using the Hungate method (Hungate, 1950) in TSC medium (Shaw et al., 2012) containing per liter: 2.0 g sodium citrate tribasic dihydrate, 1.85 g of (NH4)2SO4, 0.1 g FeSO4.7H2O, 2.0 g MgSO4.7H2O, 1.0 g KH2PO4, 0.1 g CaCl2.2H2O, resazurin 2 mg 8.5 g yeast extract, 10 g glucose. The pH was adjusted to 6.7 with NaOH 10 M.

For metabolite production analysis, the strains were grown in TSC medium supplemented with 10 g/L of different carbon sources: monosaccharides xylose, arabinose, glucose, galactose and fructose; and disaccharides sucrose and cellobiose. The pH was adjusted to 6.7 with NaOH 10 M. The strains were grown at 60ºC for 48 hours.

Fermentations were performed in a 2.5 L BiostatA bioreactor with 1.5 L of TSC supplemented with 10g/L of carbon sources (glucose or xylose). After 17 hs 5g/L of the corresponding sugar were added to avoid carbon source depletion. The agitation was kept at 100 rpm. The pH was adjusted initially at 7 and was regulated with NaOH 2M and H2SO4 1M to keep it over 5. TSC pre-cultures were grown in serum bottles containing 50 ml of medium at anaerobic conditions, 150 ml of inoculum was added to the bioreactor (DO= 0,3). The strains were grown at 60ºC for 35 hours.

Sequence analysis

The sequence was obtained using a whole-genome shotgun strategy with a Roche 454 GS FLX Titanium pyrosequencer at INDEAR, Argentina, achieving ˜37-fold coverage. Assembly was done using Newbler version 2.6 and generated 73 contigs, the largest of which has 81,110 bases. The annotation was performed using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) and Rapid Annotations using Subsystems Technology (RAST) (Overbeek et al., 2014). The genome of GSU5 was deposited in Genbank with the accession number MINB01000001-MINB01000073.

Genome to genome distance calculations were performed using the GGDC program (https://ggdc.dsmz.de/), that provides an in silico estimation of DNA-DNA hybridization values (Auch, Klenk, & Göker, 2010)

and Average Nucleotide Identity based on blast (ANIb) http://jspecies.ribohost.com/ jspeciesws (Richter, Rosselló-Móra, Oliver Glöckner, & Peplies, 2016).

Analytical determinations

Cultures were centrifuged for 5 minutes at 6000 rpm, the supernatants were filtered through 0.22 μm nylon membranes (MSI, USA) and stored at -20 ºC for analytical determinations.

Organic acids were determined by high-performance liquid chromatography (HPLC) using an LC-20AT Prominance equipment (Shimadzu Corp., Kyoto, Japan) with an HPX-87-H Aminex column (Cat no. 125-170 0140; Bio Rad Laboratories Inc., Hercules, CA) at 50ºC. The mobile phased consisted of 5mM sulfuric acid at a flow rate 0f 0,6 ml/min. The metabolites were quantified with a SPD-20AV UV detector (Shimadzu 171 Corp.).

Ethanol and butanol were measured by Manual Head Space GC-FID using an Agilent 7820A GC–FID with manual head space injection. The separation was conducted on a HP-INNO- WAX capillary column (30 m, 0.25 μm film thickness and 0.25 mm ID). The supernatants were diluted $\frac{1}{2}$ in K2CO3 1g/ml, and 5 μl of isobutanol 5g/L was added as an internal standard. The samples were heated at 60°C for 1h and 1ml of the gas phase was injected manually in a GC-FID. The GC oven was initially heated at 35 °C for 2 min, then to 45 °C at 30 °C/min and held for 1 min, finally, was heated to 120ºC at 50ºC/ml and maintained for 1 min. The injector and FID temperatures were set at 150 and 300°C, respectively. Nitrogen was used as the carrier gas at a column flow of 2 ml/min with a 5:1 split ratio.

Results

Genomic relatedness of *T. thermosaccharolyticum* GSU5

The draft genome sequence of *T. thermosaccharolyticum* GSU5 was obtained in 73 contigs with a total length of 2.7 kbases, with a mean G+C content of 33.9 %. Analysis of the genome using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) revealed 2668 genes, comprising 2501 predicted coding regions, 102 pseudogenes and 65 RNA genes, including one 16S rRNA gene. The RAST annotation covered 339 subsystems, including 44% of the coding sequences, and classified 897 open reading frames (ORFs) as hypothetical proteins.

Comparison of the 16S rRNA gene of GSU5 against sequences deposited in the Ribosomal Database Project revealed that GSU5 clusters together with other strains of *Thermoanaerobacterium thermosaccharolyticum* and is closest to strain TG57 (Figure 1).

The overall genomic phylogenetic relatedness of strain GSU5 with other strains of the genus *Thermoanaerobacterium* was analyzed through*in silico* genome comparisons. Values obtained by means of*in silico* determination of genome to genome distances between GSU5 and all other available *Thermoanaerobacterium* genomes corroborate that it belongs to the *T. thermosaccharolyticum*species. Values of 87.30%, 76.40%, 75.40 % and 72.10% were obtained when GSU5 was compared with *T. thermosaccharolyticum* TG57, T.*thermosaccharolyticum* DSM 571, T. *thermosaccharolyticum*M5, and *T. thermosaccharolyticum* M0795 respectively, while the values obtained with all other strains ranged between 26.40 and 24.20% (Table 1). When Average Nucleotide Identity based on BLAST (ANIb) was calculated, the values obtained (96.28 - 98.04%) when GSU5 was compared with other *T. thermosaccharolyticum* strains provided further evidence that it belongs to this species (Table 1).

Among the 11 *Thermoanaerobacterium* genomes available, the best represented species is *T. thermosaccharolyticum,* with five sequenced strains, followed by *T. saccharolyticum* , with two strains, and a single representative each for *T. xylanolyticum*and *T. aotearoense* . The other two genomes correspond to strains RBIITD and PSU2. When the genome of *Thermoanaerobacterium sp. RBIITD* was compared against all strains of the genus*Thermoanaerobacterium* DDH values ranged between 21.3 and 24.70 % and ANI values ranged between 75.92 and 79.93 % (Table S1). Similar results were obtained for *Thermoanaerobacterium* sp. PSU2, with DDH values ranging from 21.30 and 58.30 % and ANI values between 76.68 and 94.20 %

(Table S2). These results indicate that these strains do not belong to any of the species with sequenced representatives: *T. thermosaccharolyticum, T. saccharolyticum, T. xylanolyticum* or *T. aotearoense* .

Solvent synthesis pathways

The key enzymes involved in the synthesis of butanol, ethanol and acetone were searched in the genome of GSU5 and other sequenced *Thermoanaerobacterium* strains using the RAST annotation Server, BLAST and the Biocyc database collection in order to analyze the corresponding pathways.

As shown in Table 2, all genes coding for key enzymes of butanol synthesis were detected in GSU5 and all sequenced *T. thermosaccharolyticum* strains (locus tags in Table S3). Genes coding for the crotonase (*crt*), butyryl-CoA dehydrogenase (*bcd*), electron transfer flavoprotein (*etfAB*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*), and acetyl-CoA C-acetyltransferase (*thl*), are located in a cluster, followed by *but* , that codes for a butyryl-CoA: acetate-CoA transferase, and *rex* , corresponding to a redox dependent transcriptional regulator (Figure 2). In the well-known butanol producer *Clostridium acetobutylicum* seven of the genes are also in a genomic cluster, the *bcs* operon, albeit with a different organization: in this organism *thl* is located in a different region, *rex* is situated upstream from *crt* , and *but* is absent.

Putative binding sites for the transcriptional regulator Rex (ROP: Rex operator site) were observed upstream from *thl* (Figure 2) and *adhE* in the genome of GSU5, suggesting that Rex could regulate the expression of these genes in *Thermoanaerobacterium* . Butanol synthesis genes were also found in *Thermoanaerobacterium sp. RBIITD* but with a genetic organization different from the one present in *T. thermosaccharolyticum,* since *thl* is located in the *bcs* operon but *rex* is located upstream from *crt* (Figure 2). In contrast, these genes were absent in *T. xylanolyticum LX-11, T. saccharolyticum JW/SL-YS485* , *T. saccharolyticum NTOU1, T. aotearoense SCUT27* and *Thermoanaerobacterium sp. PSU-2* , which suggests that they are not capable of producing butanol (Table 2).

There are 5 genes encoding alcohol dehydrogenases distributed throughout the genome of GSU5 and one encoding an aldehyde dehydrogenase (*ald*). This last gene was present in about half of the analyzed genomes, including three out of five sequenced *T. thermosaccharolyticum* (Table 2). All *Thermoanaerobacterium* strains carry *adhE* , coding for the bifunctional alcohol/aldehyde dehydrogenase, in a monocistronic operon with a similar location in all genomes analyzed, as well as *bhd* and two other alcohol dehydrogenases. Interestingly, one of the dehydrogenases, *adh3* , was located upstream from genes coding for a NADH-dependent reduced ferredoxin: NADP oxidoreductase (*nfnAB*) in all genomes.

The well characterized *C. acetobutylicum* produces butanol and acetone through the ABE (Acetone-Butanol-Ethanol) pathway, so the possibility that *T. thermosaccharolyticum* produced this compound was studied. The genes that code for acetone synthesis enzymes were searched in the genomes of *T. thermosaccharolyticum* GSU5 and in all available *Thermoanaerobacterium* genomes using the genes from *C. acetobutylicum* as queries. None of the genomes analyzed carried *ctfAB* or *adc* , the genes that code for the butyrate-acetoacetate CoA-transferase (subunits A and B), and the acetoacetate decarboxylase respectively (Table 2).

Genes corresponding to enzymes involved in the synthesis of acetate and butyrate (Table 2) were also identified. *T. thermosaccharolyticum* GSU5 presents the gene coding for butyryl-CoA: acetate CoA- transferase (But) which catalyzes the conversion of butyryl-CoA to butyrate, located downstream from the butanol synthesis genes. This gene was also found in all other strains of this species, and in *Thermoanaerobacterium* sp. RBIITD, albeit in this last case *but* is located in a different part of the genome (Figure 2). On the other hand *T. xylanolyticum* LX-11*, T. saccharolyticum* JW/SL-YS485, *T. saccharolyticum* NTOU1, *T. aotearoense* SCUT27 and *T. sp.*PSU-2 carry the genes coding for Ptb (that catalyzes the synthesis of butyryl-CoA to butyryl-P), and Buk (that catalyzes the conversion of butyryl-P to butyrate).

Production of metabolites from different carbon sources

The synthesis of solvents and acids by *T. thermosaccharolyticum* GSU5 was analyzed in cultures grown using different substrates in 5 ml tubes, and compared with the type strain of the species, DSM 571. Both strains grew well on several monosaccharides: glucose, xylose, arabinose and galactose, and also on the disaccharides

sucrose and cellobiose. They grew slightly more when using fructose that on pentoses and other hexoses, and also showed a modest preference for cellobiose compared to sucrose (despite their specific epithet). In all cases they produced alcohols and acids, but no acetone. Ethanol was the most abundant alcohol, and butanol was also observed in all cultures in lower amounts. Among the acids, butyrate was the most abundant, followed by acetate, with lower amounts of lactate (Figure 3).

The relative production of the metabolites depended on the substrate used, and some differences were observed between the strains. The main product of GSU5 was butyrate in all conditions tested, ranging from 0.93 to 2.07 g/L, while DSM 571 synthesized butyrate as a main product (0.99 to 1.35 g/L) in all cultures except those using glucose and xylose, in which similar amounts of ethanol and butyrate were observed (Figure 3). Both strains produced much more ethanol than butanol. GSU5 produced between 0.12 and 0.73 g/L ethanol and 0.005 to 0.048 g/L butanol, with the highest amount of butanol produced in sucrose. DSM 571 produced between 0.25 and 1.25 g/L ethanol and 0.014 to 0.034 g/L butanol with the highest amount of butanol obtained in glucose.

Strain GSU5 produced 3.2 times more butanol than DSM 571 in sucrose (0.048 vs 0.015 g/L; $p<0.05$), and also accumulated more butanol than DSM571 in arabinose, while the opposite was observed in fructose and cellobiose, in which strain DSM571 produced more butanol than GSU5. When ethanol production was compared, strain DSM 571 produced a greater concentration of ethanol than GSU5 in all hexoses and in xylose, in which the highest amount was observed (0.98 g/L), while GSU5 accumulated more ethanol in arabinose and with the disaccharides sucrose and cellobiose, with the maximum production in this last carbon source (0.73 g/L).

Bioreactor cultures

Strain GSU5 was grown in bioreactor batch cultures using glucose or xylose as carbon sources in order to analyze solvent production in a larger scale. Several tests were performed to determine the best pH conditions for growth and solvent production, since previous studies had indicated that solvent production was enhanced by low pH in other *T. thermosaccharolyticum* strains. Preliminary tests in glucose cultures with initial starting pH of 6.7 or 7.5 did not allow adequate growth, so all cultures were performed with an initial pH of 7, and pH was controlled to prevent values lower than 5. Sugars were added at an initial concentration of 10 g/L, and replenished after 17 h by adding half the initial amount to avoid sugar depletion.

Growth was more vigorous in glucose cultures, achieving 52% more biomass than when using xylose (1.9 vs 1.0 g/L). Solvent production was also much higher in glucose, while xylose cultures produced more acids than solvents (Figure 4). Butyric acid remained the main product in xylose cultures, but glucose cultures produced slightly more ethanol than butyrate. The final butanol concentration obtained was 0.33 ± 0.01 g/L in glucose and 0.26 ± 0.02 g/L in xylose, while the final ethanol concentration was 4.34 ± 0.06 g/L in glucose and 0.78 ± 0.49 g/L in xylose (Table 3).

As expected, fermentor cultures corresponding to both sugars had a much higher biomass and solvent production that 5 ml tube cultures. Although the main products were the same observed in tube cultures, the relative amounts of acids and alcohols varied. A higher relative amount of butanol was observed with both substrates when all major metabolites were considered, showing a slight increase in carbon fluxes towards butanol in bioreactor cultures (Figure 5). When cultures grown using glucose were compared to tube cultures, solvent production was observed to increase more than biomass, with the most important increase observed in butanol production. While an 8 fold change was observed in biomass (1.78 *vs* 0.22 g/L), final concentrations of ethanol and butanol increased 10 and 14 times respectively, so that the amount of alcohols produced in the fermentor was similar to that of acids (Figure 5). Results obtained with xylose were different, as a 6 fold increase in biomass (1.04 *vs* 0.20 g/L) was accompanied by a similar increase in butanol concentration, while ethanol concentration only increased 1.5 fold when fermentor cultures were compared with 5 ml cultures (Figure 5).

Discussion

In this work we describe a novel strain of *Thermoanaerobacterium thermosaccharolyticum* (strain GSU5) through genome analysis and metabolic characterization. The phylogenetic analysis revealed that strain GSU5 belongs to the *T. thermosaccharolyticum* species and is closest to strain TG57. A genomic analysis of all sequenced strains of *Thermoanaerobacterium* was also performed, including two strains that have not yet been assigned to any species, PSU2 and RBIITD. When strain PSU2 was first described, it was classified as a strain of*T. thermosaccharolyticum* (O-Thong, Prasertsan, Karakashev, & Angelidaki, 2008), and when its genome was published, the authors reported that according to in silico studies, it did not belong to the*T. xylanolyticum* species (O-Thong et al., 2017). The results presented in this work indicate that neither PSU2 nor RBIITD belong to*T. saccharolyticum, T . thermosaccharolyticum, T. xylanolyticum* or *T. aotearoense* . It remains to be studied whether these strains belong to species with no sequenced representatives, or to previously undescribed*Thermoanaerobacterium* species.

T. thermosaccharolyticum synthesizes several products of biotechnological importance, among which ethanol and butanol are the most relevant, along with hydrogen (Bhandiwad, Guseva, & Lynd, 2013). Strains GSU5 and DSM 571 produced both alcohols from all carbon sources tested in this study, including glucose, fructose, galactose, xylose, arabinose, cellobiose and sucrose. The organization of genes involved in butanol synthesis is the same in both strains, and different from the organization of the well-known *bcs* operon found in solventogenic*Clostridium* (Berezina, Brandt, Yarotsky, Schwarz, & Zverlov, 2009) in which the *thl* gene is not part of the *bcs* operon, but constitutes a monocistronic operon in another part of the genome (Wietzke & Bahl, 2012).

It is known that in *C. acetobutylicum* the transcriptional regulator Rex plays a fundamental role in the regulation of solvent synthesis (Panitz et al., 2014) and binds to specific sequences identified upstream from *thl, crt*(the first gene of the *bcs* operon), and *adhE* (Wietzke & Bahl, 2012). A gene coding for Rex was identified downstream from the*bsc* operon in *T. thermosaccharolyticum* GSU5 and DSM571, and putative Rex binding sequences (ROP) were found both upstream from*thl* (the first gene of the *bsc* operon) and of *adhE* , suggesting that Rex could regulate the expression of these genes, as described in *Clostridium* . Butanol synthesis has been reported previously not only in several strains of *T. thermosaccharolyticum* but also in *Thermoanaerobacterium* sp. RBIITD (Biswas et al., 2018). In this last organism, the structure of the *bcs* operon is the same as in *T. thermosaccharolyticum,*except that *rex* is upstream from the *bcs* operon, as in*C. acetobutylicum* .

All genomes analyzed carry *adhE* , coding for the bifunctional alcohol/aldehyde dehydrogenase, while *ald* , corresponding to an aldehyde dehydrogenase, was only found in GSU5 and in some butanol producing and non-butanol producing strains. *T. thermosaccharolyticum* DSM 571 and M5 do not have genes coding for this enzyme, but produce both butanol and ethanol (Bhandiwad et al., 2013; Jiang et al., 2018; Li et al., 2018). These results suggest that*ald* is not essential for alcohol synthesis in*Thermoanaerobacterium* , in agreement with previous reports that indicated that AdhE was responsible for the synthesis of n-butanol from butyryl-coA and ethanol from acetyl-coA (Bhandiwad et al., 2014). All strains also carried genes coding for other dehydrogenases, including Bhd, so a possible role of these enzymes in the synthesis of alcohols in *Thermoanaerobacterium* cannot be ruled out.

When *T. thermosaccharolyticum* GSU5 or DSM 571 were grown in different carbon sources, several of the typical metabolites associated to butanol producers were detected, such as butyrate, acetate, lactate, ethanol and butanol, but no acetone could be found in any of the cultures. In addition, it has been reported that butanol producing strains RBIITD and TG57 do not produce acetone during fermentation (Biswas et al., 2018; Li et al., 2018). A search of the genome of GSU5 for the presence of genes related to acetone synthesis revealed that it lacks *adc* , coding for an acetate decarboxylase, and *ctfAB* , which codes for both units of a butyrate-acetoacetate CoA-transferase (Figure 6).

In recent years analysis of the genome of several strains of *T. thermosaccharolyticum* revealed important differences in the biosynthesis of butanol and butyrate when compared to the pathway known in the well characterized *C. acetobutylicum* (Jones & Woods, 1986). Previous reports indicated that *adc* was absent in*T. thermosaccharolyticum* M5 (Jiang et al., 2018), and that both*adc* and *ctf* were absent in *T. thermo-*

*saccharolyticum*TG57 (Li et al., 2018). The analysis performed in this work revealed a general absence of these genes in all the genomes, indicating that none of the strains analyzed would be able to produce acetone during fermentation due to the absence of *adc* and *ctf* . Since the analysis involved all available *Thermoanaerobacterium* genomes representing half of the known species, these results suggest that this is a common trait in this genus.

Butyrate production has been reported for several *T. thermosaccharolyticum* strains (Biswas et al., 2018; Freier-Schröder, Wiegel, & Gottschalk, 1989; Li et al., 2018), and is the main metabolite produced from most carbon sources by both strains tested in this work. Among the known butyrate producing pathways, the most common is the acetyl-CoA pathway, that has two variants: i) the two step conversion catalyzed by the phosphotransbutyrylase (Ptb) and butyrate kinase (Buk) with a phosphorylated intermediate that allows the formation of ATP, commonly found in bacteria that have the ABE pathway, and ii) the one step conversion of Butyryl-CoA to Butyrate catalyzed by a butyryl-CoA:acetate-CoA transferase (But) (Vital, Howe, & Tiedje, 2014). Only the one step pathway seems to be performed by strain GSU5 and all other *T. thermosaccharolyticum* strains, as they carry*but* (denominated *ach* in *T. thermosaccharolyticum*TG57), and not *ptb* or *buk* (Bhandiwad et al., 2013; Li et al., 2018) (Figure 6). The two step pathway has been proposed to exist in strain M5 (Jiang et al., 2018) but its genome does not carry genes coding for Ptb or Buk.

The one step conversion of butyryl-CoA to butyrate has been extensively studied in *C. kluyveri* , that seems to compensate the lack of the ATP producing step by means of an electron bifurcating mechanism involving the crotonyl-CoA reductase, that couples the reduction of crotonyl-CoA to the reduction of ferredoxin using NADH as the electron donor for both reactions. The reduction of crotonyl-CoA to butyryl-CoA is catalyzed by the cytoplasmic butyryl-CoA dehydrogenase complex, coded by *bcd* and *etfAB* , while the reduction of the ferredoxin is catalyzed by NfnAB, a NADH-dependent reduced ferredoxin:NADP oxidoreductase (Wang, Huang, Moll, & Thauer, 2010). These genes are present in all the butanol and butyrate producing strains: *T. thermosaccharolyticum* and *Thermoanaerobacterium* sp. RBIITD (table 2). Furthermore, the ferredoxin dependent activity of the butyryl-CoA dehydrogenase was experimentally demonstrated in *T. thermoanaerobacterium* DSM 571 (Bhandiwad et al., 2014). Based on this information, it can be hypothesized that all butanol producing*Thermoanaerobacterium* are able to obtain energy during the one step butyrate synthesis through the ferredoxin mediated electron bifurcation mechanism (Figure 6). In contrast, genes coding for Ptb and Buk were identified in *T. xylanolyticum* , *T. saccharolyticum* , *T. aotearoense* and *Thermoanaerobacterium*sp. PSU-2. These strains lack genes needed for butanol synthesis (*bcs* operon), and are also devoid of genes coding for the cytoplasmic butyryl-CoA dehydrogenase complex (*bcd* and*etfAB*), suggesting that they are unable to synthesize either butanol or butyrate. In these microorganisms *pbt* and *buk*are clustered together with a gene that codes for a leucine/valine/phenylalanine dehydrogenase. This genetic organization has been previously observed in *Bacillus megaterium* . In this organism Ptb expression was induced in the presence of valine and isoleucine, and the enzyme could use butyryl-CoA and 2-methyl-propionyl CoA as substrates (Vazquez, Pettinari, & Méndez, 2001). In *B. subtilis* these genes are part of the *bkd* operon, involved in the degradation of branched chain amino-acids (Debarbouille, Gardan, Arnaud, & Rapoport, 1999). It is possible that in *T. xylanolyticum* ,*T. saccharolyticum* , *Thermoanaerobacterium* sp. PSU-2 and*T. aotearoense* Ptb and Buk could be involved in branched chain amino-acid degradation as in *Bacillus* .

Strain GSU5 was able to simultaneously produce both organic acids and alcohols in different carbon sources in a similar manner as the control strain DSM 571. The main acid produced was butyrate, the most abundant metabolic product in most conditions. Both strains were able to synthesize ethanol and smaller amounts of butanol from all carbon sources tested. These results are similar to those obtained in previous studies performed using *T. thermosaccharolyticum* DSM 571 grown on cellobiose (Bhandiwad et al., 2013), and also using strain M5 grown on xylan (Jiang et al., 2018). In contrast, Li et al report that *T. thermosaccharolyticum* TG57 produce butyrate, acetate and butanol, but no ethanol, when grown using glucose, cellobiose, cellulose or xylan, in spite of the fact that it has all enzymes required for the synthesis of both alcohols (Li et al., 2018).

When GSU5 was grown in bioreactors, it achieved a higher biomass, and some differences were observed in the relative amounts of acids and alcohols produced. For example, while butyrate was the most abundant metabolite produced in 5 ml tube cultures using all substrates tested, including glucose and xylose, fermentor cultures with those substrates had a greater proportion of alcohols. Furthermore, ethanol was the most abundant metabolite in glucose cultures. This could suggest that in higher density cultures, carbon flow towards the synthesis of alcohols increases contributing to the reoxidation of excess NADH or NADPH, thus favoring the production of alcohols over acids. Solventogenic anaerobes like the broadly studied model bacterium *C. acetobutylicum* have a fermentation behavior characterized by two distinct phases: formation of acids during the first phase is followed by a solventogenic phase in which growth slows down, and solvents are produced (Amador-Noguez, Brasg, Feng, Roquet, & Rabinowitz, 2011). When the dynamics of acids and alcohols production was analyzed in *T. thermosaccharolyticum*GSU5, metabolite synthesis was observed to accompany growth, and no clear solventogenic phase could be distinguished. This had been reported for *T. thermosaccharolyticum* M5 (Jiang et al., 2018) and careful observation of metabolite curves displayed in studies carried out with different solventogenic *Thermoanaerobacterium* strains show that in all cases alcohols and acids are synthesized throughout growth. This can be clearly perceived in an early work performed using strain DSM 571 (Freier-Schröder et al., 1989) and in the metabolite curves shown in studies that analyze the production of hydrogen in different strains of*T. thermosaccharolyticum* (Cao et al., 2009; Cao, Zhao, Wang, Wang, & Ren, 2014; Khamtib & Reungsang, 2012), using both sugars or lignocellulosic biomass for growth. These results suggest that solventogenesis in *Thermoanaerobacterium* is not subject to the same regulatory mechanisms described for the ABE metabolism in

Clostridium.

Several species of *Thermoanaerobacterium* have been studied for the production of different bioproducts. Among them, *T. thermosaccharolyticum* seems to be the most promising, and different strains have been analyzed as candidates for the production of hydrogen (Cao et al., 2009, 2014), ethanol (Bhandiwad et al., 2013, 2014; Jiang, Guo, et al., 2018; Li et al., 2018) and 1,2 propanediol (Altaras, Etzel, & Cameron, 2001; Cameron & Cooney, 1986) from several carbon sources, including lignocellulosic biomass. Knowledge on their physiology and unique metabolic properties will enable the use of these microorganisms to obtain biotechnologically relevant bioproducts in sustainable and environmentally friendly processes.

Acknowledgements

We wish to thank Dr. Beatriz Méndez for her expert advice and Dr. Laura Raiger Iustman for allowing us to use the bioreactor. M.J.P. and J.R. are career investigators from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). R.D.P., D.E.E., and D.S.A. hold doctoral fellowships from CONICET.

Conflicts of interest .

Authors declare no conflict of interest.

References

Altaras, N. E., Etzel, M. R., & Cameron, D. C. (2001). Conversion of sugars to 1,2-propanediol by *Thermoanaerobacterium thermosaccharolyticum* HG-8. *Biotechnology Progress* ,*17* (1), 52–56. https://doi.org/10.1021/bp000130b

Amador-Noguez, D., Brasg, I. A., Feng, X. J., Roquet, N., & Rabinowitz, J. D. (2011). Metabolome remodeling during the acidogenic-solventogenic transition in *Clostridium acetobutylicum* . *Applied and Environmental Microbiology* , *77* (22), 7984–7997. https://doi.org/10.1128/AEM.05374-11

Auch, A. F., Klenk, H.-P., & Göker, M. (2010). Standard operating procedure for calculating genome-to-genome distances based on high-scoring segment pairs. *Standards in Genomic Sciences* ,*2* (1), 142–148. https://doi.org/10.4056/sigs.541628

Berezina, O. V., Brandt, A., Yarotsky, S., Schwarz, W. H., & Zverlov, V. V. (2009). Isolation of a new butanol-producing *Clostridium*strain: High level of hemicellulosic activity and structure of solventogenesis

genes of a new *Clostridium saccharobutylicum*isolate. *Systematic and Applied Microbiology* , *32* (7), 449–459. https://doi.org/10.1016/j.syapm.2009.07.005

Bhandiwad, A., Guseva, A., & Lynd, L. R. (2013). Metabolic Engineering of *Thermoanaerobacterium thermosaccharolyticum* for Increased n- Butanol Production for n -butanol production. *Advances in Microbiology* , *2013* (August 2015), 46–51. https://doi.org/10.1016/j.ymben.2013.10.012

Bhandiwad, A., Shaw, A. J., Guss, A., Guseva, A., Bahl, H., & Lynd, L. R. (2014). Metabolic engineering of *Thermoanaerobacterium saccharolyticum* for n -butanol production. *Metabolic Engineering* , *21* , 17–25. https://doi.org/10.1016/j.ymben.2013.10.012

Biswas, R., Huntemann, M., Clum, A., Pillay, M., Palaniappan, K., Varghese, N., ... Gussc, A. M. (2018). Complete Genome Sequence of*Thermoanaerobacterium* sp. Strain RBIITD, a Butyrate- and Butanol-Producing Thermophile Ranjita. *Genome Announcements* ,*6* (2), e01411-17.

Cameron, D. C., & Cooney, C. L. (1986). A novel ferementation: The production of R(-)-1,2-propanediol and acetol by *Clostridium Thermosaccharolyticum* . *Nature Biotechnology* , *4* (August), 719–725.

Cao, G., Ren, N., Wang, A., Lee, D. J., Guo, W., Liu, B., ... Zhao, Q. (2009). Acid hydrolysis of corn stover for biohydrogen production using *Thermoanaerobacterium thermosaccharolyticum* W16.*International Journal of Hydrogen Energy* , *34* (17), 7182–7188. https://doi.org/10.1016/j.ijhydene.2009.07.009

Cao, G., Zhao, L., Wang, A. J., Wang, Z. Y., & Ren, N. Q. (2014). Single-step bioconversion of lignocellulose to hydrogen using novel moderately thermophilic bacteria. *Biotechnology for Biofuels* ,*7* (1), 1–13. https://doi.org/10.1186/1754-6834-7-82

Debarbouille, M., Gardan, R., Arnaud, M., & Rapoport, G. (1999). Role of bkdR, a transcriptional activator of the SigL-dependent isoleucine and valine degradation pathway in *Bacillus subtilis* .*Journal of Bacteriology* , *181* (7), 2059–2066.

Demain, A. L., Newcomb, M., & Wu, J. H. D. (2005). Cellulase, Clostridia, and Ethanol. *Thermophysics and Aeromechanics* ,*69* (1), 124–154. https://doi.org/10.1128/MMBR.69.1.124-154.2005

Dürre, P. (2007). Biobutanol: An attractive biofuel. *Biotechnology Journal* , *2* (12), 1525–1534. https://doi.org/10.1002/biot.20

Freier-Schröder, D., Wiegel, J. irge., & Gottschalk, G. (1989). Butanol formation by *Clostridium thermosaccharolyticum* at neutral pH Doris, *11* (11), 831–836.

Hill, J., Nelson, E., Tilman, D., Polasky, S., & Douglas, T. (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proceedings of the National Academy of Sciences* , *103* (30), 11206–11210. https://doi.org/10.5506/APhysPolB.44.1379

Hungate, R. E. (1950). The Anaerobic Mesophilic Cellulolytic Bacteria.*Bacteriological Reviews* , *14* , 1–49.

Jiang, Y., Guo, D., Lu, J., Dürre, P., Dong, W., Yan, W., ... Xin, F. (2018). Consolidated bioprocessing of butanol production from xylan by a thermophilic and butanologenic *Thermoanaerobacterium* sp. M5.*Biotechnology for Biofuels* , *11* (1), 1–14. https://doi.org/10.1186/s13068-018-1092-1

Jones, D. T., & Woods, D. R. (1986). Acetone-Butanol Fermentation Revisited. *Microbiological Reviews* , *50* (4), 484–524.

Khamtib, S., & Reungsang, A. (2012). Biohydrogen production from xylose by Thermoanaerobacterium thermosaccharolyticum KKU19 isolated from hot spring sediment. *International Journal of Hydrogen Energy* ,*37* (17), 12219–12228. https://doi.org/10.1016/j.ijhydene.2012.06.038

Lamed, R., & Zeikus, J. G. (1980). Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and*Thermoanaerobium brockii* . *Journal of Bacteriology* ,*144* (2), 569–578. https://doi.org/10.1038/ng1296-461

Li, T., Zhang, C., Yang, K. L., & He, J. (2018). Unique genetic cassettes in a *Thermoanaerobacterium* contribute to simultaneous conversion of cellulose and monosugars into butanol. *Science Advances* , *4* (3). https://doi.org/10.1126/sciadv.1701475

Lynd, L. R., Weimer, P. J., Zyl, W. H. van, & Pretorius, I. S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology.*Microbiology and Molecular Biology Reviews* , *63* (3), 506–577. https://doi.org/10.1128/MMBR.66.

Mendez, B. S., Pettinari, M. J., Ivanier, S. E., Ramos, C. A., & Siñeriz, F. (1991). *Clostridium thermopapyrolyticum* sp. nov., a cellulolytic thermophile. *International Journal of Systematic Bacteriology* , 281–283. https://doi.org/10.1099/00207713-41-2-281

O-Thong, S., Khongkliang, P., Mamimin, C., Singkhala, A., Prasertsan, P., & Birkeland, N. K. (2017). Draft genome sequence of*Thermoanaerobacterium* sp. strain PSU-2 isolated from thermophilic hydrogen producing reactor. *Genomics Data* , *12* , 49–51. https://doi.org/10.1016/j.gdata.2017.02.012

O-Thong, S., Prasertsan, P., Karakashev, D., & Angelidaki, I. (2008). Thermophilic fermentative hydrogen production by the newly isolated*Thermoanaerobacterium thermosaccharolyticum* PSU-2.*International Journal of Hydrogen Energy* , *33* (4), 1204–1214. https://doi.org/10.1016/j.ijhydene.2007.12.015

Onyenwoke, R. U., & Wiegel, J. (2015). *Thermoanaerobacterium* .*Bergey's Manual of Systematics of Archaea and Bacteria* , 1–18. https://doi.org/10.1002/9781118960608.gbm00755

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., ... Stevens, R. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Research* , *42* (D1), 206–214. https://doi.org/10.1093/nar/gkt1226

Panitz, J. C., Zverlov, V. V., Pham, V. T. T., Stürzl, S., Schieder, D., & Schwarz, W. H. (2014). Isolation of a solventogenic*Clostridium* sp. strain: Fermentation of glycerol to n-butanol, analysis of the bcs operon region and its potential regulatory elements.*Systematic and Applied Microbiology* , *37* (1), 1–9. https://doi.org/10.1016/j.syapm.2013.10.004

Richter, M., Rosselló-Móra, R., Oliver Glöckner, F., & Peplies, J. (2016). JSpeciesWS: A web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* ,*32* (6), 929–931. https://doi.org/10.1093/bioinformatics/btv681

Romano, I., Dipasquale, L., Orlando, P., Lama, L., d'Ippolito, G., Pascual, J., & Gambacorta, A. (2010). *Thermoanaerobacterium thermostercus* sp. nov., a new anaerobic thermophilic hydrogen-producing bacterium from buffalo-dung. *Extremophiles* , *14* (2), 233–240. https://doi.org/10.1007/s00792-010-0303-x

Shaw, A. J., Podkaminer, K. K., Desai, S. G., Bardsley, J. S., Rogers, S. R., Thorne, P. G., ... Lynd, L. R. (2008). Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield.*Proceedings of the National Academy of Sciences* , *105* (37), 13769–13774. https://doi.org/10.1073/pnas.0801266105

Shaw, J. A., Covalla, S. F., Miller, B. B., Firliet, B. T., Hogsett, D. A., & Herring, C. D. (2012). Urease expression in a*Thermoanaerobacterium saccharolyticum* ethanologen allows high titer ethanol production. *Metabolic Engineering* , *14* (5), 528–532. https://doi.org/10.1016/j.ymben.2012.06.004

Tian, L., Papanek, B., Olson, D. G., Rydzak, T., Holwerda, E. K., Zheng, T., ... Lynd, L. R. (2016). Simultaneous achievement of high ethanol yield and titer in *Clostridium thermocellum* .*Biotechnology for Biofuels* , *9* (1), 1–11. https://doi.org/10.1186/s13068-016-0528-8

Vazquez, G. J., Pettinari, M. J., & Méndez, B. S. (2001). Phosphotransbutyrylase expression in *Bacillus megaterium* .*Current Microbiology* , *42* (5), 345–349. https://doi.org/10.1007/s002840010227

Vital, M., Howe, A. C., & Tiedje, J. M. (2014). Revealing the bacterial butyrate synthesis pathways by analyzing (meta)genomic data.*MBio* , *5* (2), 1–11. https://doi.org/10.1128/mBio.00889-14

Wang, S., Huang, H., Moll, J., & Thauer, R. K. (2010). NADP+ reduction with reduced ferredoxin and NADP+ reduction with NADH are coupled via an electron-bifurcating enzyme complex in *Clostridium*

kluyveri .*Journal of Bacteriology* , *192* (19), 5115–5123. https://doi.org/10.1128/JB.00612-10

Wietzke, M., & Bahl, H. (2012). The redox-sensing protein Rex, a transcriptional regulator of solventogenesis in *Clostridium acetobutylicum* . *Applied Microbiology and Biotechnology* ,*96* (3), 749–761. https://doi.org/10.1007/s00253-012-4112-2

Yamada, R., Hasunuma, T., & Kondo, A. (2013). Endowing non-cellulolytic microorganisms with cellulolytic activity aiming for consolidated bioprocessing. *Biotechnology Advances* , *31* (6), 754–763. https://doi.org/10.1016/j.biotechadv.2013.02.007

Table 1: Genome to genome comparisons of GSU5 with other *Thermoanaerobacterium* .

Strain	Acccession	DDH estimate	ANIb
T. thermosaccharolyticum GSU5	MINB01000001-MINB01000073	-	
T. thermosaccharolyticum TG57	CP016893.1	87.30±2.50%	98.04%
T. thermosaccharolyticum DSM 571	CP002171	76.40 ± 2.88%	96.48%
T.thermosaccharolyticum M5	NKHD01000001-NKHD01000077	75.40±3.00%	96.93%
T. thermosaccharolyticum M0795	NC_019970	72.10 ± 2.92%	96.28%
T. saccharolyticum JW/SL-YS485	CP003184.1	24.30±2.39%	81.63%
T. saccharolyticum NTOU1	BBKT01000001-BBKT01000101	26.40±2.50%	83.52%
T. xylanolyticum LX-11	CP002739.1	25.00±2.40%	82.12%
T. aotearoense SCUT27	AYSN01000000	24.20±2.50%	81.76%
Thermoanaerobacterium sp. PSU-2	MSQD00000000.1	24.40±2.50%	81.82%
Thermoanaerobacterium sp. RBIITD	LT906662.1	24.30±2.50%	79.52%

DNA-DNA hybridization (DDH) values were estimated using the Genome-to-Genome Distance Calculator 2.1 (Species cutoff value: 70%). ANIb: Average Nucleotide Identity based on BLAST (Species cutoff value =95%).

Table 2. Genes involved in ethanol, butanol and butyrate synthesis in sequenced strains

Strain	*crt*	*bcd*	*etf B*	*etf A*	*hbd*	*thl*	*but*	*ptb*	*buk*	*ald*	*adh1*	*adh2*	*ad*
T. thermosaccharolyticum GSU5	100	100	100	100	100	100	100			100	100	100	10
T. thermosaccharolyticum TG57	100	100	99	100	100	100	100			58	100	99	99
T. thermosaccharolyticum DSM 571	91	98	99	99	100	100	100				99	99	10
T. thermosaccharolyticum M5	99	99	99	99	100	100	100				99	100	99
T. thermosaccharolyticum M0795	99	99	98	95	98	100	100			58		99	99
T. saccharolyticum JW/SL-YS485								0	0	93		92	90
T. saccharolyticum NTOU1								0	0		98	92	92
T. xylanolyticum LX-11								0	0	92		92	92
T.aotearoense SCUT27								0	0	93		92	90
Thermoanaerobacterium sp. PSU-2								0	0			91	91
Thermoanaerobacterium sp. RBIITD	77	96	91	96	88	90	82	0	0	58	67	89	89
Clostridium acetobutylicum ATCC 824	58	72	66	58	69	71		0	0				

Numbers indicate percentage of protein sequence identity with the corresponding protein of strain GSU5. Red cells indicate that the gene is present in the corresponding strain and absent in GSU5. Blank cells indicate absence of the gene in the corresponding strain. Genes code for the proteins indicated as follows, *crt:* crotonase;*bcd* : butyryl-CoA dehydrogenase; *etfAB:* electron transfer flavoprotein; *hbd* : 3-hydroxybutyryl-CoA dehydrogenase;*thl* : acetyl-CoA C-acetyltransferase; *but* : butyryl-CoA: acetate-CoA transferase; *ptb* : phosphotrans butyrylase;*buk* : butyrate kinase; *ald* : aldehyde dehydrogenase;*adh* : alcohol dehydrogenase; *bdh* : butanol dehydrogenase;*adhE* : bifunctional aldehyde/ alcohol dehydrogenase; *pta* : phosphotrans

acetylase; *ak* : acetate kinase; *ctfAB* : acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase;*adc:* acetoacetate decarboxylase. Accession numbers for each genome are indicated in table 1.

Table 3. Fermentation products of *Thermoanaerobacterium thermosaccharolyticum* GSU5

	Product concentration g/L Glu	Product concentration g/L Xyl	Yield a g/g Glu	Yield a g/g Xyl	Yieldb mol /mol Glu	Yieldb mol /mol Xyl	Volumetric productivityc g/(L.h) Glu	Volumetric productivityc g/(L.h) Xyl
Butanol	0.33±0.10	0.26±0.15	0.034±0.008	0.022±0.007	0.09±0.02	0.05±0.01	0.015±0.004	0.013±0
Ethanol	4.35±0.06	0.8±0.5	0.46±0.07	0.059±0.04	1.8±0.1	0.23±0.17	0.19±0.01	0.03±0.
Butyrate	3±1	6±3	0.39±0.24	0.33±0.19	0.8±0.3	0.6±0.3	0.12±0.08	0.28±0.
Acetate	2.3±0.9	0.96±0.2	0.13±0.08	0.10±0.07	0.5±0.2	0.15±0.07	0.11±0.04	0.05±0.
Lactate	1.1±0.3	1.2±0.8	0.15±0.07	0.09±0.07	0.22±0.06	0.2±0.1	0.05±0.01	0.06±0.
Biomass	1.9±0.7	1.0±0.5	0.19±0.09	0.09±0.05	-	-	-	-

Cultures were grown in TSC medium supplemented with glucose or xylose at 10/L in a bioreactor under anaerobic conditions for 36 h. Results represent means ± the standard deviations corresponding to three independent cultures. Glu, Glucose; Xyl, xylose.

a Yield: g of product/ g of substrate

b yield: : mol of product/mol of substrate

c: g of product/ (time . volume)

Figure Legends

Figure 1: Relatedness of GSU5 with other bacteria.

The 16s rRNA gene of GSU5 was compared against the sequences deposited in the Ribosomal Database Project. A phylogenetic tree was obtained using the Mega 6.0 program. At least one representative of each species of *Thermoanaerobacterium* , and all strains with sequenced genomes were included, along with the two species of *Caldanaerobium* , formerly classified within the *Thermoanaerobacterium* genus.*Clostridium acetobutylicum* was used as an outgroup.

*Strains with sequenced genomes

Figure 2: Organization of the *bcs* operon in*Thermoanaerobacterium thermosaccharolyticum* ,*Thermoanaerobacterium sp.* RBIITD and *Clostridium acetobutylicum* . Putative Rex operator sites (ROP) are indicated as red circles.

Figure 3: Production of metabolites in T. *thermosaccharolyticum*GSU5 (A) and T. *thermosaccharolyticum* DSM 571 (B). Strains were grown in TCS supplemented with different carbon sources in 5 ml Hungate tubes under anaerobic conditions for 48 h. Results represent the mean value ± standard deviation of three independent replicates.

Figure 4: Production of metabolites in *T. thermosaccharolyticum*GSU5 grown in TCS supplemented with glucose (A) or xylose (B) at 10 g/L in a bioreactor under anaerobic conditions for 36 h.

Data from a representative fermentation of three independent replicates is presented.

Figure 5: Relative distribution (%) of metabolites produced by *T. thermosaccharolyticum* GSU5 in Hungate tubes with glucose (A), in a bioreactor with glucose (B), in Hungate tubes with xylose (C), and in a bioreactor with xylose (D).

Figure 6: Proposed main metabolic pathways in T. *thermosaccharolyticum.*

Purple solid arrows indicate reactions catalyzed by genes present in the genome. Red dotted arrows indicate reactions catalyzed by genes absent in the genome. Enzymes separated by a slash and/or with asterisks indicate reactions that could be potentially catalyzed by more than one enzyme. Enzyme names are: Crt: crotonase; Bcd: butyryl-CoA dehydrogenase; EtfAB: electron transfer flavoprotein; Hbd: 3-hydroxybutyryl-CoA dehydrogenase; Thl: acetyl-CoA C-acetyltransferase; But: butyryl-CoA: acetate-CoA transferase; Ptb: phosphotrans butyrylase; Buk: butyrate kinase; Ald: aldehyde dehydrogenase; Bdh: butanol dehydrogenase; AdhE: bifunctional aldehyde/ alcohol dehydrogenase; Pta: phosphotrans acetylase; Ak: acetate kinase; CtfAB: acetoacetyl coenzyme A:acetate/butyrate:coenzyme A transferase; Adc: acetoacetate decarboxylase; Fd red: reduced ferredoxin; Fd ox: oxidized ferredoxin.