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# Low-temperature anaerobic digestion is associated with differential methanogenic protein expression

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## ABSTRACT

Anaerobic digestion (AD) is an attractive wastewater treatment technology, leading to the generation of recoverable biofuel (methane). Most industrial AD applications, carry excessive heating costs however, as AD reactors are commonly operated at mesophilic temperatures while handling waste streams discharged at ambient or cold temperatures. Consequently, low-temperature AD represents a cost effective strategy for wastewater treatment. The comparative investigation of key microbial groups underpinning laboratory-scale AD bioreactors operated at 37°C, 15°C and 7°C was carried out. Community structure was monitored using 16S rRNA clone libraries, while abundance of the most prominent methanogens was investigated using qPCR. In addition, metaproteomics was employed to access the microbial functions carried out *in situ*. While *δ-Proteobacteria* were prevalent at 37°C, their abundance decreased dramatically at lower temperatures with inverse trends observed for *Bacteroidetes* and *Firmicutes*. *Methanobacteriales* and *Methanosaeta* were predominant at all temperatures investigated while *Methanomicrobiales* abundance increased at 15°C compared to 37°C and 7°C. Changes in operating temperature resulted in the differential expression of proteins involved in methanogenesis, which was found to occur in all bioreactors, as corroborated by bioreactors' performance. This study demonstrated the value of employing a polyphasic approach to address microbial community dynamics and highlighted the functional redundancy of AD microbiomes.


## INTRODUCTION

Employing anaerobic digestion technology in a low-temperature context holds economic incentives over traditional mesophilic (>20°C) and thermophilic (>45°C) approaches through the reduced operation costs associated with the treatment of dilute wastewaters (Connaughton *et al*., 2006; Enright *et al*., 2007; McKeown *et al*., 2009). Low temperature anaerobic digestion has been successfully applied to treat a vast range of wastewater types such as phenolic (Scully et al 2006), chlorinated aliphatic (Siggins *et al*., 2011a), brewery (Connaughton *et al*., 2006), pharmaceutical (Enright *et al*., 2007) and glucose (Akila and Chandra, 2007) -based wastewaters. Evidence of comparable treatment efficiencies to mesophilic setups has also been recorded (McHugh *et al*., 2004, Collins *et al*., 2005; Trzcinski and Stuckey, 2010), as well as the successful treatment of complex wastewater (Alvarez *et al*., 2008 and Gao *et al*., 2011). Despite successful applications, there is a lack of

fundamental knowledge relating to the mechanisms underpinning AD. The design of bioreactors is generally based on rule of thumb, and bioreactor over-dimensioning; process instability and failures are still common. AD is operated based on relationships between bioreactor performance and empirical operating parameters, but the differences between successful and unsuccessful bioreactors are poorly understood. The future full-scale implementation of AD, and particularly the development of promising new applications, such as low-temperature AD, is severely impaired by this knowledge gap. Methanogenic populations have been the focus of many low-temperature AD studies due to their crucial role in biogas formation and biofilm integrity (Liu *et al.*, 2002). Much of this work has focused on uncovering temporal methanogenic community dynamics under various operating temperatures primarily using nucleic acid based methods (Syutsubo *et al.*, 2008; O'Reilly *et al.*, 2009; McKeown *et al.*, 2009). Although important insights have been gathered (e.g. *Methanocorpusculum* prevalence during AD operation at 15°C; McKeown *et al.*, 2009), minimal information relating methanogenic presence to functional significance (metabolic pathways employed, physiological responses etc.) has been documented (Abram *et al.*, 2011; Siggins *et al.*, 2012). Metaproteomics, which can be defined as the identification of proteins expressed at a given time within an ecosystem, is an essential component of such a function-based approach. Linking microbial community structure (DNA-based) to function (protein-based) could provide a greater level of understanding of the AD process occurring at low-temperature. To this end, we employed quantitative real-time PCR, clone libraries and metaproteomics to investigate microbial community composition and function underpinning granular sludge in bioreactor trials operated at 37°C, 15°C and 7°C.

## MATERIALS AND METHODS

**Source of biomass.** Anaerobic granular sludge samples were obtained from three laboratory-scale expanded granular sludge bed (EGSB) bioreactors operated at 37°C (R1), 15°C (R2) and 7°C (R3; Siggins *et al.*, 2011a,b). Each bioreactor had a working volume of 3.5 L. R1 and R2 were operated for 631 days, while R3 was operated for 609 days including 438 days at 7°C after acclimation from 15°C (Siggins *et al.*, 2011a,b). The bioreactors treated a synthetic volatile fatty acid (VFA) wastewater consisting of acetic acid, propionic acid, butyric acid and ethanol with a chemical oxygen demand (COD) ratio of 1:1:1:1 buffered with $NaHCO_3$. At the time of sampling, the bioreactors' influent contained a total of 3 g COD $L^{-1}$ for R1 and R2 and 0.75 g COD $L^{-1}$ for R3. Initially, R3 organic loading rate was of 3 g COD $L^{-1}$ (as that of R1 and R2) but this had to be decreased after 231 days of operation, due

to VFA accumulation in the bioreactor (Siggins *et al.*, 2011b). A hydraulic retention time of 24 h was applied to all bioreactors, which were originally seeded with anaerobic granular sludge from a full-scale (1500$m^3$) internal circulation anaerobic digester, operated at 37°C at the Carbery Milk Products plant (Ballineen, Co. Cork, Ireland). Biomass was sampled for this study at the end of the trial, at day 631 for R1 and R2 and at day 609 for R3. At the time of sampling COD removal was greater than 80% with a biogas production containing >70% $CH_4$ in all bioreactors (Siggins *et al.*, 2011a,b).

**DNA extraction.** Total genomic DNA was extracted from 0.5 g of granular sludge biomass at the conclusion of the trial using an automated nucleic acid extractor (Magtration 12GC, PSS Co., Chiba, Japan). Prior to extraction, granular biomass was finely crushed under liquid nitrogen using a mortar and pestle, and resuspended in sterile double distilled water to a ratio of 1:4 (vol/vol). A 100 µl aliquot of the biomass suspension was loaded per extraction. Each extraction was performed in duplicate and the extracted DNA was eluted in Tris-HCl buffer (pH 8.0) and stored at -20 °C until use.

**Clone library analysis of bacterial 16S rRNA gene.** Partial bacterial 16S rRNA gene sequences were amplified using forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG -3'; Delong, 1992) and reverse primer 1392R (5'ACGGGCGGGGRC -3'; Lane *et al*., 1985). The PCR conditions and component concentrations were identical to those outlined by Siggins *et al.* (2011a). Subsequent construction of clone libraries (TOPO®XL), amplified ribosomal DNA restriction analysis (ARDRA) and plasmid sequencing were performed as described by Collins *et al.* (2003). Any vector contamination was removed by screening sequence data using National Center for Biotechnology Information (NCBI) Vecscreen software. The resulting sequence data were compared to nucleotide databases using basic local alignment NCBI search tool (BLASTn). Sequences were aligned using MacClade 4.0 software (Sinauer Assoc) with nearest relatives from the BLASTn database and selected sequences downloaded from the Ribosomal Database Project (RDP). The resulting partial 16S rRNA gene sequences were deposited in the Genbank database under the accession numbers (R1) HQ655412-HQ655420; (R2) HQ655421-HQ655434; (R3) HQ655435-HQ655457.

**qPCR analysis of archaeal 16S rRNA gene.** Quantitative real-time PCR was performed on technical replicate samples using a LightCycler 480 (Roche, Mannheim, Germany). Four-

methanogenic 16S rRNA gene primer and probe sets were used, specific for two hydrogenotrophic orders, *Methanomicrobiales* (857F: 5'-CGWAGGGAAGCTGTTAAGT-3'; 1059R: 5'TACCGTCGTCCACTCCTT-3'; 929P: -5'AGCACCACAACGCGTGGA-3'; Yu *et al.*, 2005) and *Methanobacteriales* (282F: 5'-ATCGRTACGGGTTGTGGG-3'; 832R :5'-CACCTAACGCRCATHGTTTAC-3'; 749P: 5'-TYCGACAGTGAGGRACGAAAGCTG-3';Yu *et al.*, 2005) and two acetoclastic families, *Methanosaetaceae* (702F: 5'-GAAACCGYGATAAGGGGA-3'; 862R :5'-TAGCGARCATCGTTTACG-3'; 753P: 5'-TTAGCAAGGGCCGGGCAA-3';Yu *et al.*, 2005) and *Methanosarcinaceae* (380F: 5'-TAATCCTYGARGGACCACCA-3'; 828R :5'-CCTACGGCACCRACMAC-3'; 492P: 5'-ACGGCAAGGGACGAAACGTAGG-3';Yu *et al.*, 2005) accounting for most methanogens present in anaerobic digesters (Yu *et al.*, 2005; Lee *et al.*, 2009). Experimental procedures, including reaction mixtures, LightCycler conditions and standard curve preparation was undertaken as previously described (Lee *et al.*, 2009; Siggins *et al.*, 2011a). The volume-based concentrations (gene copies $\mu l^{-1}$) were converted into the biomass-based concentrations (gene copies gVSS $\mu l^{-1}$). VSS was determined gravimetrically according to Standard Methods American Public Health Association.

**Metaproteomics.** Proteins were extracted from 50 ml granular sludge samples and subsequently separated by 2-dimensional gel electrophoresis (2-DGE) using a sonication protocol as described previously (Abram *et al.*, 2011). Briefly, the first dimension consisted of isoelectric focusing (IEF) using 7 cm IPG strips with linear pH gradients (pH 4 to 7; Amersham). The second dimension polyacrylamide (12% w/v) gels were run in pairs along with molecular weight markers with a range of 10-225 kDa (Broad Range Protein Molecular Markers, Promega). Gel images were captured by scanning with an Epson Perfection V350 photo scanner at a resolution of 800 dpi. Duplicate independent protein extractions and four technical replicates were analyzed for each bioreactor. Gel images were processed using PDQuest-Advanced software, version 8.0.1 (BioRad). Spot counts were obtained using the spot detection wizard enabling the Gaussian model option as recommended by the manufacturer. Protein expression ratios were determined for each bioreactor and only ratios greater than two-fold were considered significant. Proteins of interest were excised from the gels prior to analysis using nanoflow liquid chromatography-electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS) on an ABSciex QStar XL instrument. MS/MS data for +1 to +5 charged precursor ions which exceeded 150 cps was processed using the Paragon™

and Pro group™ search algorithms (Shilov *et al.*, 2007; Xiao *et al.*, 2010) within ProteinPilot 4.0 software (ABSciex, Foster City, CA) against NCBInr database with no species restriction. Only proteins with ≥ 2 peptides identified at > 99% confidence with a competitor error margin (Prot Score) of 2.00 were considered.

## RESULTS AND DISCUSSION

### *Bacteroidetes* and *Methanosaetaceae* are prevalent at low temperature in AD systems.

The bacterial community was found to be dominated by *δ-Proteobacteria* at 37°C, accounting for 61% of the clones identified (Fig. 1). Their relative abundance decreased in low-temperature biomass, representing 19% and 13% of the clone libraries at 15°C and 7°C, respectively. Interestingly, *Bacteroidetes*, only accounting for 10% of the bacterial clones at 37°C increased to 16% and 47% at 15°C and 7°C, respectively. In addition, while the relative abundance of *Chloroflexi* decreased at low temperature, *Firmicutes* were found to be more prevalent at 15°C and 7°C when compared to 37°C (Fig. 1). The importance of *Methanosaetaceae* in AD systems operated at low temperature was highlighted by qPCR (Fig. 2). This correlated with previous observations reporting that *Methanosaeta*-like clones accounted for 29%, 75% and 93% at 37°C, 15°C and 7°C, respectively (Siggins *et al.*, 2011a,b). Strikingly, *Methanomicrobiales* levels were found to increase at 15°C compared to 37°C and 7°C, likely indicating an important role for these hydrogenotrophic methanogens at this temperature (Fig. 2). This was also in agreement with previous work (O'Reilly *et al.*, 2010; Abram *et al.*, 2011; Siggins *et al.*, 2011a,b). Particularly, an increase from 2% to 19% of *Methanomicrobiales*-like clones at 15°C, compared to 37°C was previously observed (Siggins *et al.*, 2011a), while no clones assigned to this taxonomic order were detected at 7°C (Siggins *et al.*, 2011b). *Methanobacteriales* were found to be abundant in all bioreactors and were detected at the highest level at 37°C (Fig. 2), in accordance with previous clone libraries results indicating the presence of 30%, 2% and 7% of *Methanobacteriales*-like clones at 37°C, 15°C and 7°C, respectively (Siggins *et al.*, 2011a,b). Finally, *Methanosarcinaceae* were detected below the quantification limit of the qPCR assay ($10^6$ copies $gVSS^{-1}$) in all bioreactors. Taken together, the bioreactor operating temperature was found to have a profound effect on microbial community composition particularly among bacteria and highlighted an important role for *Bacteroidetes* and *Methanosaetaceae* at low temperature and for *δ-Proteobacteria*, *Methanosaetaceae* and *Methanobacteriales* at 37°C in AD systems.

**Changes in operating temperature lead to the differential expression of enzymes from methanogenesis.** Sixty protein spots were excised from 2-DGE gels (267 ± 26.4 spots detected; n=24) and analyzed by nLC-ESI-MS/MS, which led to the identification of 41 distinct proteins (Table 1) present in each sample. Overall, 36 proteins (88%) were assigned to methanogenic archaea, with 29 proteins from *Methanosaeataceae*, 1 from *Methanosarcinaceae*, 4 from *Methanobacteriales* and 2 from *Methanomicrobiales*. Amongst these, 16 proteins were involved in methanogenesis (from $CO_2$ and acetate) including 7 proteins displaying differential expression in a temperature dependent manner (Table 1). Remarkably, a protein involved in methanogenesis from $CO_2$, (tetrahydromethanopterin S-methyltransferase subunit H; Table 1), which was found to be expressed at higher level at 37°C, was assigned to *Methanoseata concilii*. Until recently, *Methanoseata* were considered to exclusively produce methane from acetate. Rotaru *et al*., (2014), however, demonstrated that these methanogens were able to reduce $CO_2$ to methane using metatranscriptomics and radiotracer experiments. The detection of this protein (tetrahydromethanopterin S-methyltransferase) in the three bioreactors investigated in the present study indicates that *M. concilii* was actively producing methane from both $CO_2$ and acetate (see corresponding proteins in Table 1) at the time of sampling. Interestingly, a subunit of methyl-coenzyme M reductase, catalyzing the last step of methanogenesis, assigned to *Methanoculleus marisnigri* (*Methanomicrobiales*) showed reduced levels of expression as the operating temperature decreased with an expression ratio of 18.6 fold decrease at 7°C compared to 37°C and 11.7 fold decrease between 15°C and 7°C (Table 1). The same enzyme assigned to *Methanospirullum hungatei* was found to have an increased expression at 15°C (Table 1), somewhat correlating with the increased level of *Methanomicrobiales* detected by qPCR at this temperature (Fig.2). Another subunit of methyl-coenzyme M reductase assigned to *Methanothermobacter* was found to show a reduced level of expression at 15°C, when compared to 37°C and 7°C (Table 1). This was also supported by the decrease in abundance of *Methanobacteriales* reported at 15°C by qPCR (Fig. 2). Strikingly, 2 homologues of acetyl-coA synthetase, converting acetate to acetyl-CoA, assigned to *Methanoseata concilii* were found to be differentially regulated at different temperatures. While one homologue was expressed at higher level at low temperatures compared to 37°C (expression ratios of -2.6 for 37/15 and -4.5 for 37/7), the other was expressed in an opposite fashion displaying a higher level of expression at 37°C when compared to 15°C and 7°C (expression ratios of 15.8 for 37/15 and 8.5 for 37/7; Table 1). In addition, these two enzymes were expressed at similar level at 15°C and 7°C (Table 1), suggesting a differential regulation at 37°C. Ten proteins

224 involved in energy and metabolism were found to be expressed in all bioreactors, amongst
225 which 2 proteins were differentially expressed as a function of operating temperature (Table
226 1). Active pathways included glycolysis/gluconeogenesis possibly using acetate from the
227 bioreactors' influent as a precursor. Enzymes involved in the pentose phosphate pathway,
228 TCA cycle and amino acid biosynthesis, such as leucine, which can be derived from pyruvate
229 metabolism (a glycolytic intermediate) were also detected (Table 1). Of particular note was
230 the differential expression of the bifunctional enzyme Fae/Hps, which abundance increased as
231 the operating temperature decreased (expression ratios of -3.5 for 37/15 and -2.9 for 15/7;
232 Table 1). This enzyme contains 2 domains: i) one homologous to formaldehyde-activating
233 enzyme (Fae) and ii) the other to 3-hexulose-6-phosphate synthase (Hps; Grochowski *et al.*,
234 2005), also separately detected in all bioreactors at similar levels (Table 1). Interestingly, the
235 two reactions catalyzed by Fae/Hps involve formaldehyde as a substrate. The first enzyme
236 converts formaldehyde and tetrahydromethanopterin to 5,10-methylene-
237 tetrahydromethanopterin, an intermediate of methanogenesis from $CO_2$, while the second
238 enzyme combines formaldehyde with D-ribulose-5-phosphate (an intermediate from the
239 pentose phosphate pathway) to produce hexulose-6-phosphate, which can be further
240 converted to D-fructose-6-phosphate, an glycolytic intermediate. The production of
241 formaldehyde could result from the anaerobic oxidation of $CH_4$ to methanol (Caldwell *et al.*,
242 2008). An increased expression of Fae/Hps is likely to be related to an increase in
243 formaldehyde production, which could possibly result from an increase in anaerobic $CH_4$
244 oxidation. This, in turn, is supported by the increased solubility of $CH_4$ with decreasing
245 temperatures (Servio and Englezos, 2002), which would facilitate microbial access for
246 methane oxidation. This could potentially limit the success of anaerobic digestion at
247 temperature as low as 7°C, where $CH_4$ production could be partially diverted towards
248 carbohydrate metabolism *via* the process of anaerobic $CH_4$ oxidation. Finally 8 proteins
249 detected in all bioreactors were found to be involved in cell maintenance processes including
250 4 displaying chaperone activities indicating an important role for these enzymes in AD
251 systems. Of particular note was the differential expression of GroEL assigned to *δ-*
252 *Proteobacteria*, detected at higher levels at 37°C compared to 15°C and 7°C (expression
253 ratios of 2.5 for 37/15 and 3.9 for 37/7; Table 1), which correlated with the marked increased
254 abundance of *δ-Proteobacteria*-like clones observed at this temperature (Fig. 1). Similarly a
255 protein of unknown function assigned to this taxonomic class was found to be expressed at
256 higher level at 37°C with expression ratios of 2.5 for 37/15 and 2.2 for 37/7. Taken together

the phylogenetic analyses (using qPCR and clone libraries) are largely supporting metaproteomic assignment.

Overall this study has highlighted the importance of *Methanosaetaceae* to the AD process with the assignment to this methanogenic group of 29 proteins out of the 41 identified. Importantly, while methanogenesis was found to occur at all temperatures investigated, differential expression of methanogenic enzymes was observed at 37°C, 15°C and 7°C. Interestingly, this did not indicate the regulation of specific steps of the methanogenesis pathway but seemed to correlate with changes in microbial community composition. For example, proteins involved in the last step of methanogenesis that were assigned to *Methanomicrobiales*, were found to be expressed at higher levels at 15°C when compared to 37°C, while those assigned to *Methanobacteriales* followed the opposite trend. This was supported by phylogenetic analyses, which correspondingly reported similar observations using both qPCR and clone librairies (Siggins *et al.*, 2011a,b). Taken together this study has highlighted a functional redundancy within the AD microbiome where different microbial groups carry out the same functions under different operating conditions.

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**Table 1:** Identification and putative function of proteins excised from 2-DGE gels from anaerobic granular biomass of reactors operated at 37°C, 15°C and 7°C.

| Protein[a] | Accession No | % Cov[b] | Protein Assignment | Phylogenetic classification | Ratio[c] 37/15 | 37/7 | 15/7 | Predicted Function |
|---|---|---|---|---|---|---|---|---|
| **Cellular information processing** | | | | | | | | |
| PIN domain-containing protein | gi\|330507600 | 9.1 | *Methanosaeta concilii* | *Methanosaetaceae* | **-2.6** | 1.3 | **3.1** | Ribonuclease activity |
| **Chaperone & cell maintenance** | | | | | | | | |
| Chaperonin GroEL | gi\|253698887 | 7.3 | *Geobacter sp.* | *δ- proteobacteria* | **2.5** | **3.9** | 1.6 | Protein folding |
| | gi\|197116649 | 7.3 | *Geobacter bemidjiensis* | | | | | |
| | gi\|189425979 | 7.3 | *Geobacter lovleyi* | | | | | |
| Chaperone DnaK | gi\|330507176 | 4.2 | *Methanosaeta concilii* | *Methanosaetaceae* | **4.5** | **2.4** | -3.4 | Protein folding |
| LPXTG-motif cell wall anchor domain protein | gi\|395612836 | 13.9 | *Streptococcus pneumoniae* | *Firmicutes* | 1.9 | -1.3 | -1.5 | Cell wall maintenance |
| | gi\|395574460 | 13.9 | *Streptococcus pneumoniae* | | | | | |
| Proteasome α-subunit | gi\|73669981 | 12.6 | *Methanosarcina barkeri* | *Methanosarcinaceae* | **-2.5** | -1.2 | **-2.1** | Cell regulation |
| | gi\|52550031 | 5.7 | Uncultured archaeon | | | | | |
| Proteasome α-subunit | gi\|386002841 | 13.2 | *Methanosaeta harundinacea* | *Methanosaetaceae* | 1.8 | 1.6 | -1.2 | Cell regulation |
| | gi\|116754662 | 10 | *Methanosaeta thermophila* | | | | | |
| S-layer duplication domain containing protein | gi\|330507267 | 22.8 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.2 | -1.2 | -1.6 | Cell envelope maintenance |
| Thermosome α-subunit | gi\|330506447 | 52.4 | *Methanosaeta concilii* | *Methanosaetaceae* | **-3.3** | **-5.0** | -1.2 | Molecular chaperone |
| Thermosome δ-subunit | gi\|330507490 | 37.1 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.0 | 1.5 | 1.6 | Molecular chaperone |
| **Energy & metabolism** | | | | | | | | |
| 3-hexulose-6-phosphate synthase | gi\|330506676 | 15.7 | *Methanosaeta concilii* | *Methanosaetaceae* | -1.6 | 1.2 | -1.5 | Pentose phosphate pathway |
| 3-isopropylmalate dehydratase large subunit | gi\|330507399 | 7.7 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.2 | 1.8 | 1.4 | Leucine biosynthesis |
| Aspartate-semialdehyde dehydrogenase | gi\|330507317 | 7.9 | *Methanosaeta concilii* | *Methanosaetaceae* | **2.3** | **2.1** | 1.6 | Amino acid biosynthesis |
| S-adenosylmethionine synthetase | gi\|330508591 | 14.9 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.1 | 1.2 | 1.1 | Methionine metabolism |
| Bifunctional enzyme Fae/Hps | gi\|330507450 | 14.7 | *Methanosaeta concilii* | *Methanosaetaceae* | **-3.5** | **-2.0** | **-2.9** | Pentose phosphate pathway/methanogenesis |
| Enolase | gi\|33050645 | 40.1 | *Methanosaeta concilii* | *Methanosaetaceae* | -1.9 | -1.3 | 1.8 | Glycolysis |
| Manganese-dependent inorganic pyrophosphatase | gi\|330507876 | 42.4 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.2 | 1.9 | 1.8 | Cellular energy |
| Succinyl-CoA synthetase β-subunit | gi\|116749138 | 2.8 | *Syntrophobacter fumaroxidans* | *δ- proteobacteria* | 1.1 | 1.7 | 1.6 | TCA cycle |
| V-type ATP synthase α-subunit | gi\|330508339 | 24.8 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.8 | -1.5 | -1.5 | ATP synthesis |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| V-type ATP synthase β -subunit | gi\|330508338 | 45.8 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.7 | 1.7 | 1.2 | ATP synthesis |
| **Methanogenesis** | | | | | | | | |
| $H_4$MPT S-methyltransferase subunit H | gi\|330507199 | 29.9 | *Methanosaeta concilii* | *Methanosaetaceae* | **2.6** | **2.9** | 1.3 | Methanogenesis from $CO_2$ |
| 5,10-methylene-$H_4$MPT reductase | gi\|333988092 | 26.8 | *Methanobacterium sp.* | *Methanobacteriales* | -1.1 | -1.4 | 1.2 | Methanogenesis from $CO_2$ |
| Acetyl-CoA synthetase | gi\|330506786 | 20.1 | *Methanosaeta concilii* | *Methanosaetaceae* | **-2.6** | **-4.5** | 1.7 | Methanogenesis from acetate |
| Acetyl-CoA synthetase | gi\|330506787 | 8.2 | *Methanosaeta concilii* | *Methanosaetaceae* | **15.8** | **8.5** | 1.2 | Methanogenesis from acetate |
| CO dehydrogenase /acetyl-CoA synthase α-subunit | gi\|330507409 | 13.8 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.5 | 1.8 | 1.9 | Methanogenesis from acetate |
| CO dehydrogenase /acetyl-CoA synthase β-subunit | gi\|330507407 | 32.9 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.2 | 1.3 | -1.7 | Methanogenesis from acetate |
| CO dehydrogenase /acetyl-CoA synthase δ-subunit | gi\|330507405 | 17.1 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.3 | **2.7** | **2.6** | Methanogenesis from acetate |
| Methyl-coenzyme M reductase I α-subunit | gi\|333988259 | 16.0 | *Methanobacterium* sp. | *Methanobacteriales* | 1.6 | 1.2 | 1.4 | Methanogenesis |
| Methyl-coenzyme M reductase I α-subunit | gi\|330506955 | 33.6 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.3 | 1.7 | 1.3 | Methanogenesis |
| Methyl-coenzyme M reductase I β-subunit | gi\|126178567 | 4.1 | *Methanoculleus marisnigri* | *Methanomicrobiales* | -1.4 | **18.6** | **11.7** | Methanogenesis |
| Methyl-coenzyme M reductase I β-subunit | gi\|517427 | 5.9 | *Methanothermobacter thermautotrophicus* | *Methanobacteriales* | 1.9 | -1.2 | -1.5 | Methanogenesis |
| | gi\|3891379 | 5.8 | *Methanothermobacter marburgensis* | | | | | |
| Methyl-coenzyme M reductase I β-subunit | gi\|88603391 | 6.1 | *Methanospirillum hungatei* | *Methanomicrobiales* | **-3.1** | **-2.4** | **2.3** | Methanogenesis |
| Methyl-coenzyme M reductase I β-subunit | gi\|330506958 | 56.7 | *Methanosaeta concilii* | *Methanosaetaceae* | -1.1 | 1.1 | 1.5 | Methanogenesis |
| Methyl-coenzyme M reductase I γ-subunit | gi\|330506956 | 41.2 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.2 | 1.1 | 1.4 | Methanogenesis |
| Methyl-coenzyme M reductase I γ-subunit | gi\|304315294 | 9.2 | *Methanothermobacter marburgensis* | *Methanobacteriales* | **13.9** | **6.5** | **-2.7** | Methanogenesis |
| | gi\|3334251 | 9.1 | *Methanothermobacter thermautotrophicus* | | | | | |
| Putative methanogenesis marker protein 15 | gi\|330506647 | 21.3 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.4 | 1.8 | 1.1 | Methanogenesis |
| **Transport** | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Family 5 extracellular solute-binding protein | gi\|330508239 | 16.0 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.1 | -1.9 | 1.3 | ABC transporter |
| ABC transporter substrate-binding protein | gi\|502902166 | 3.8 | *Segniliparus rotundus* | *Actinobacteria* | 1.1 | **3.9** | **4.8** | ABC transporter |
| **Unknown function** | | | | | | | | |
| Hypothetical protein | gi\|330508095 | 13.8 | *Methanosaeta concilii* | *Methanosaetaceae* | **-2.0** | -1.4 | -1.4 | Unknown |
| Hypothetical protein | gi\|310823886 | 6.1 | *Stigmatella aurantiaca* | *δ- proteobacteria* | **2.5** | **2.2** | 1.6 | Unknown |
| Hypothetical protein | gi\|330509044 | 41.8 | *Methanosaeta concilii* | *Methanosaetaceae* | **-3.3** | **2.0** | 1.8 | Unknown |
| Hypothetical protein | gi\|330507300 | 10.3 | *Methanosaeta concilii* | *Methanosaetaceae* | 1.5 | 1.3 | 1.4 | Unknown |

[a] Proteins identified with at least 2 peptides > 99% confidence through Paragon™ and Progroup™ search algorithms.
[b] Protein sequence coverage estimated by the percentage of matching amino acids from the identified peptides with a confidence level of ≥ 95%.
[c] Ratios relate to differential abundance of protein for the two biomass samples at 37°C, 15°C and 7°C. Proteins present at reduced levels are expressed as a negative reciprocal. Significantly expressed proteins are in bold.
Fae/Hps, formaldehyde activating enzyme/ hexulose-6-phosphate synthase; $H_4$MPT, Tetrahydromethanopterin; CoA, coenzyme.

## FIGURE LEGENDS

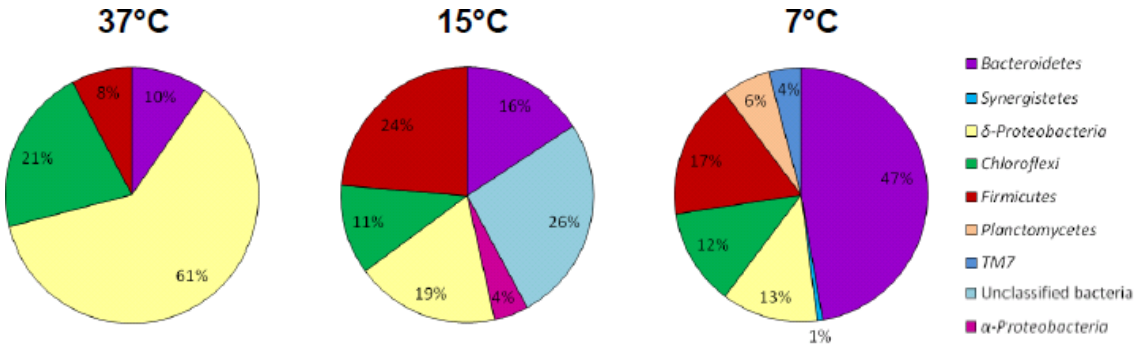
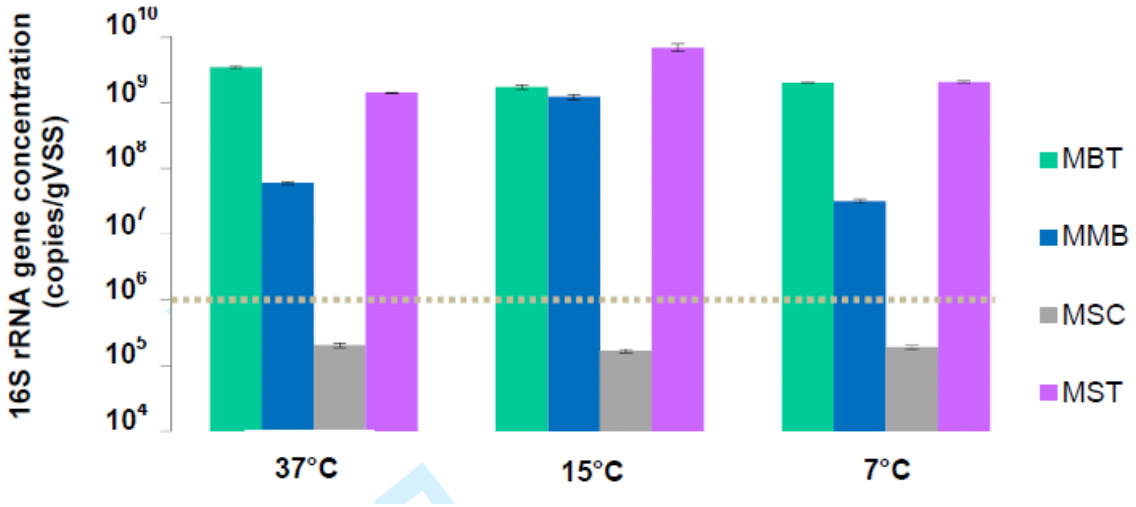



**Figure 1:** Phylogenetic affiliation of bacterial 16S rRNA gene sequences obtained from biomass samples from bioreactors operated at 37°C, 15°C and 7°C. Pie charts were constructed using the frequency of 16S rRNA sequences assigned to the relevant taxonomic groups (n=155, 114 and 116 at 37°C, 15°C and 7°C, respectively).

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376 **Figure 2:** Quantification of 16S rRNA gene concentrations for methanogens: MBT
377 *Methanobacteriales*; MMB *Methanomicrobiales*; MSc *Methanosarcinaceae*; MSt
378 *Methanosaetaceae* present in 37°C, 15°C and 7°C bioreactor biomass samples. Error bars
379 indicate the standard deviation and are the result of two technical replicates. Dashed line
380 relates to the detection limit of the assay ($10^6$ gene copies per gram of VSS).

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