Investigation of the diversity of homoacetogenic bacteria in mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase gene

P. Ryan, C. Forbes and E. Colleran

ABSTRACT

Homoacetogenic bacteria are strict anaerobes capable of autotrophic growth on H_2/CO_2 or CO, and of heterotrophic growth on a wide range of sugars, alcohols, methoxylated aromatic compounds and one carbon compounds, yielding acetate as their sole metabolic end-product. Batch activity tests on anaerobic granular sludge, using H_2/CO_2 as a substrate and 2-bromoethanesulfonate (BES) as a specific methanogenic inhibitor revealed that H_2/CO_2 conversion and concomitant acetate production commenced only after a lag period of 60–100 h. This finding suggests that the homoacetogenic population of digester sludge could be maintained by heterotrophic growth on sugars or other organic compounds, rather than by autotrophic growth on H_2/CO_2. In the present study, two upflow anaerobic sludge bed (UASB) reactors were operated at 37°C and 55°C for two distinct trial periods, each characterised by the application of influents designed to enrich for homoacetogenic bacteria. Specific primers designed for the amplification of the functional gene encoding formyltetrahydrofolate synthetase (FTHFS), a key enzyme in the acetyl-CoA pathway of acetogenesis, were used as a specific probe for acetogenic bacteria. The diversity of acetogens in the granular sludge cultivated in each reactor was revealed by application of FTHFS targeted PCR. Results show that biomass acetogenic composition was dependent upon the operational temperature of the reactor and the substrate supplied as influent.

Key words | bromoethanesulfonate, formyltetrahydrofolate synthetase, granular sludge, homoacetogens, UASB

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INTRODUCTION

Acetogenesis contributes an estimated 10% to the approximately 10^{13} kg of acetate produced annually in anaerobic environments (Wood & Ljungdahl 1991). Consequently, the organisms carrying out this process are very important participants in global carbon cycling. Acetogens, also known as the homoacetogenic bacteria, are among the most phylogenetically diverse bacterial functional groups. To date, approximately 100 homoacetogenic species have been identified and are phylogenetically classified in 21 different genera (Drake *et al.* 2006). Despite their significance, homoacetogens remain an understudied functional group of bacteria. This is due in part to the inherent difficulties in detecting, identifying, and characterising diversity and distributions of acetogenic bacteria in anaerobic biomass, due to their phylogenetic diversity. Molecular biological approaches have previously been very useful for studies of the ecology of many functional groups of microorganisms (Murrell & Radajewsli 2000). Homoacetogens, however, present some interesting challenges, in particular with reference to their role within the anaerobic digestion process. They are capable of autotrophic growth on H_2/CO_2 and of heterotrophic growth on a wide range of sugars, alcohols,

doi: 10.2166/wst.2008.059

methoxylated aromatic compounds and single carbon compounds, such as methanol and formate (Li *et al.* 1994). H_2-utilising acetogens are known to be present within digester sludges with numbers in the range of 10^5–10^7 cfu/ml or 10^8–10^{11} MPN/ml (Zang & Noike 1994). The internal pressures which allow maintenance of such high numbers of homoacetogens in digesters are poorly understood, since, under the low partial pressures of H_2 prevailing in well-functioning digesters, homoacetogens should, in theory, be out-competed by the hydrogenophilic methanogens which have a much lower Ks value for H_2 (Diekert & Wohlfarth 1994). Thus, the role of homoacetogenic bacteria in anaerobic digestion remains unclear. Despite being phylogenetically diverse homoacetogens have a shared functionality, due to their ability to autotrophically convert CO_2 to acetate *via* the acetyl-CoA pathway. A key enzyme in this CO_2 fixation pathway, formyltetrahydrofolate synthase (FTHFS), which catalyzes the ATP-dependent activation of formate, is conservative in structure and function and can be used to identify homoacetogens. As such, the gene sequence encoding FTHFS has been used as a target in both DNA probe hybridization (Lovell & Hui 1991), and species specific polymerase chain reaction (PCR) (Leaphart & Lovelle 2001) in order to localize and identify homoacetogens in anaerobic biomass. The objective of the present study was to employ previously designed oligonucleotide primers, to amplify the DNA sequence encoding FTHFS (Lephart & Lovell 2001; Leaphart *et al.* 2003) in order to assess the diversity of homoacetogens in anaerobic reactor sludges cultivated under different conditions. Biomass was periodically sampled from anaerobic upflow reactors operated at 37°C (R1) and 55°C (R2) in order to identify the homoacetogenic species present in reactors supplied with a variety of single and more complex carbon compounds as substrates. PCR and cloning of the FTHFS gene was successfully applied to DNA isolated from four different anaerobic reactor sludge samples.

MATERIALS AND METHODS

Reactor design and experimental setup

Two 4.5 l glass laboratory scale anaerobic upflow sludge bed (UASB) reactors; R1 and R2 were seeded with a granular mesophilic anaerobic sludge from a full-scale internal circulation reactor treating milk-processing wastewater from Carbery Milk Products, (Balineen, Co.Cork, Ireland) to a final sludge concentration of $11.5\,g\,l^{-1}$ volatile suspended solid (VSS). Initially, both reactors were operated at 37°C and at a hydraulic retention time (HRT) of 48 hours. The influent supplied to both R1 and R2 during start-up, consisted of sucrose/acetate at a chemical oxygen demand (COD) ratio of 60:40, to a total COD of 10 g COD l^{-1} which was buffered and supplemented with macro and micro nutrients, as recommended by Shelton & Tiedje (1984). On day 128, the temperature of R2 was increased to 55°C to encourage the growth of thermophilic anaerobes. On day 373, the influent to both R1 and R2 was changed to sodium vanillate, a complex methoxylated aromatic compound which is a substrate for the heterotrophic metabolism of homoacetogens. The volumetric loading rate was increased by increasing the influent concentration from 2 g COD $l^{-1}\,d^{-1}$ to a final loading rate of 10 g COD $l^{-1}\,d^{-1}$ by day 464, while the HRT was maintained at 48 hours. Samples of reactor effluent and biogas were routinely analysed by gas chromatography to detect acetate and CH_4 to confirm the activity of acetogens and the presence of an active methanogenic consortium in the biomass. The COD of the influent and effluent were determined on a regular basis to assess the efficiency of the anaerobic process. Reactor sludges were sampled for microbial analysis on days 349 and 419, corresponding to periods of stable reactor operation on each distinct influent.

Batch experiments

A modified version of the specific methanogenic activity (SMA) test (Colleran *et al.* 1992) was used to determine the rate of homoacetogenic conversion of H_2CO_2 to acetate. Test vials were set up in triplicate and contained seed sludge biomass, anaerobic buffer and 50 mM BES to inhibit methanogenic activity (Bouwer & McCarthy 1983). Decreasing pressure in the vials was measured over time using a portable pressure transducer. In-vial acetate and % biogas methane concentrations were determined by gas chromatography, as described previously (Colleran *et al.* 1994).

DNA extraction

Sludge samples were crushed with a pestle and DNA isolated from the crushed sludge, using the MoBio Soil DNA extraction kit (Cambio, Cambridge, UK), according to the manufacturer's instructions. DNA was extracted from each sample in triplicate and pooled prior to further analysis. The isolated DNA was stored at −20°C.

Generation of FTHFS clone libraries, analysis of RFLPs, and phylogenetic classification

Amplification of a portion (1,102bp) of the FTHFS gene using PCR was carried out as previously described, using the primer sequences FTHFS forward; (5'-TTYACWGGH-GAYTTCCATGC-3') and FTHFS reverse; (5'-GTATTGDG-TYTTRGCCATACA-3') designed by Leaphart & Lovelle (2001). Amplicons were ligated into vector PCR 2.1 TOPO® (Invitrogen) and used to transform chemically competent *Escherichia coli* cells. Clone libraries of the sludges from both reactors were constructed on day 349 of the trial, while both reactors were exhibiting stable operation on sucrose/acetate as influent, and on day 419 when the biomass of both reactors was adapted to sodium vanillate as substrate. Distinct FTHFS gene homologs were identified by screening clones with the tetrameric restriction enzyme *Hae III* (McHugh *et al.* 2004). Unique clonal restriction patterns were identified, and representatives of these sequenced. The retrieved sequences were compared to previously identified sequences using the web-based Basic Local Alignment Search Tool, (BLASTn) (www.ncbi.nlm.nih.gov/BLAST) and aligned using CLUSTALX (Thompson *et al.* 1997). Phylogenetic classification was carried out using the software package Paup*4.0.

RESULTS AND DISCUSSION

Bacterial activity tests

Initial studies focused on the use of batch activity tests to investigate homoacetogenic physiology, using H_2/CO_2 as substrate in the presence and absence of BES, as a specific methanogenic inhibitor. Figure 1a illustrates the pattern of H_2/CO_2 conversion by the seed sludge in the presence and absence of 50 mM BES. Stoichiometric conversion of

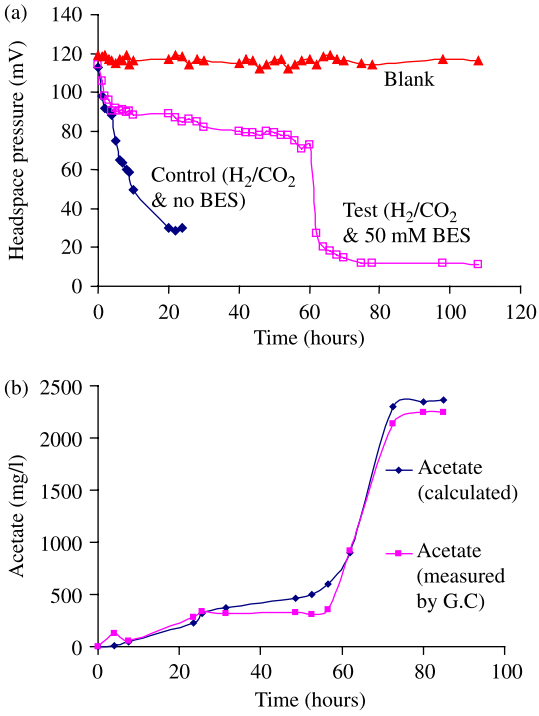

Figure 1 | (a) Headspace pressure decrease in batch activity test vials containing H_2/CO_2, in the presence and absence of 50 mM BES. (b) Measured and calculated (based on H_2/CO_2 utilisation) acetate in-vial concentrations.

H_2/CO_2 to methane was obtained in the absence of BES within 20 hours. In contrast, a lag phase of approximately 60 hours prior to H_2/CO_2 depletion was noted in test vials containing BES. This delayed H_2/CO_2 conversion was associated with acetate accumulation (Figure 1b), indicating the involvement of the homoacetogenic species. As the tests were carried out under non-growth conditions, these findings suggest that the delayed onset of acetate accumulation in the presence of BES was due to bacterial adaptation rather than growth. Similar patterns were noted during tests on biomass sampled on days 349 and 419 (data not shown). Molecular based techniques were subsequently applied to identify key organisms and assess the diversity of homoacetogenic bacteria in the biomass.

Homoacetogenic population dynamics within R1 and R2

The phylogenetic placement of the sequences retrieved from sludges sampled on day 349 are presented in Figure 2. Seven unique sequences out of a total 124 screened were

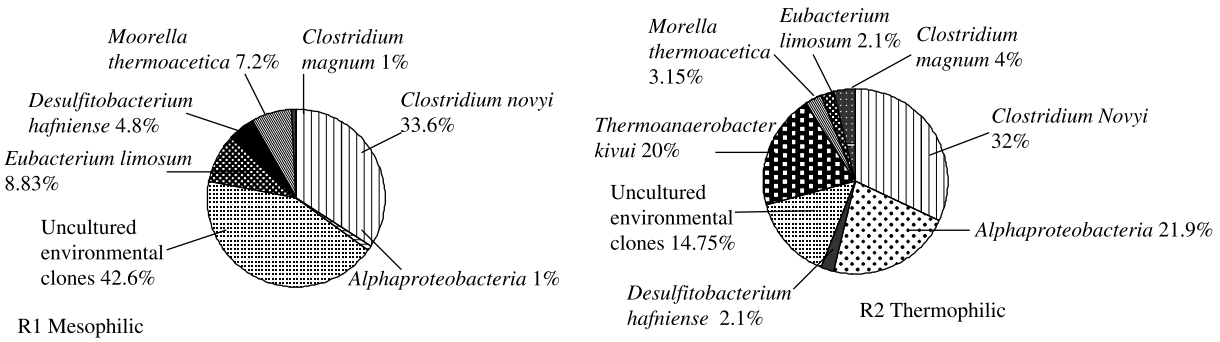

Figure 2 | Affiliation and distribution of FTHFS sequences analysed from the mesophilic (R1) and thermophilic (R2) reactor sludges sampled on day 349.

identified from the R1 mesophilic clone library. The phylum *Firmicutes* dominated the R1 FTHFS library representing 55.31% of total clones. The mesophilic biomass was composed of large numbers of acetogens closely related to *Clostridia novyi* (33.6%). Of the 100 known acetogenic species isolated to date, *Clostridium magnum*, *Morella thermoacetica* and *Eubacterium limosum* related sequences were recovered from R1 biomass, and accounted for 17% of the total clones analysed. These acetogenic isolates are all capable of heterotrophic metabolism converting glucose to acetate, and it is likely that this was their ecophysiological role within the R1 biomass at this time, given the sucrose rich influent. The thermophilic biomass was equally as diverse as R1 sludge, and was composed of 8 unique FTHFS gene homologs. The thermophilic R2 biomass was co-dominated by two distinct Phyla; the *Firmicutes* and the *α-proteobacteria.* Uncultured environmental bacteria were highly represented in the biomass (42.6%). The relative number of clones associated with *Firmicutes,* and in particular *Clostridia,* was higher in the R2 biomass on day 349, which had been operated at 55°C (64.4%) than in the mesophilic (R1) biomass (55.31%). Of 95 clones analysed from R2, 20% were closely related to *Thermoanaerobacter kivui* and 4% to *Clostridium magnum*. These *Firmicutes* are classed as authentic acetogens, and the FTHFS primers used in this study were designed using their sequences, among others (Lovell & Leaphart 2005).

Representative FTHFS sequences retrieved from the biomass sampled on day 349 proved to be less diverse than that of day 419 during which time the reactors were supplied with a sodium vanillate influent from day 373 (Figure 3). Sodium vanillate, a complex methoxylated aromatic compound was employed to enrich for and promote homoacetogenic population diversity and dominance within both reactors. Biomass was removed from R1 and R2 while both reactors exhibited stable operation and COD removal efficiencies were above 75%. The thermophilic sludge was less phylogenetically diverse than its mesophilic counterpart. Of the 102 clones analysed from each library, 8 distinct restriction patterns were noted from the R1 biomass and 6 from the R2 biomass. The higher diversity of sequence homologs evident in the mesophilic reactor biomass libraries in comparison to the thermophilic library indicates greater acetogenic diversity at the lower temperature, despite comparable reactor performance. This is unsurprising, given that most acetogenic isolates are mesophilic (Drake *et al.* 2006). By day 419, the *Firmicutes* remained the most highly represented phylum in both the mesophilic and thermophilic sludges, with the most highly represented species, *Clostridium formicoaceticum* accounting for 53.5% of the mesophilic biomass (Figure 3). The range of substrates for *C. formicoacetium,* originally isolated from sewage sludge, is very similar to those of the first acetogen species to be isolated, *Clostridium aceticum*. Both utilise methoxylated aromatic compounds, such as vanillate, as growth supportive substrates.

Thus it can be postulated that the vanillate influent supplied to the reactors sustained the metabolism of *C. formicoacetium*. Of total clones analysed, 32.35% of FTHFS sequences from R1 and 57% from R2 were highly similar to the gene sequence from *Acetobacterium carbolinicum.* During a study conducted on a subspecies of this bacterium, isolated from anoxic sediment it was noted that this psychrotolerant, obligate anaerobe utilises vanillate and

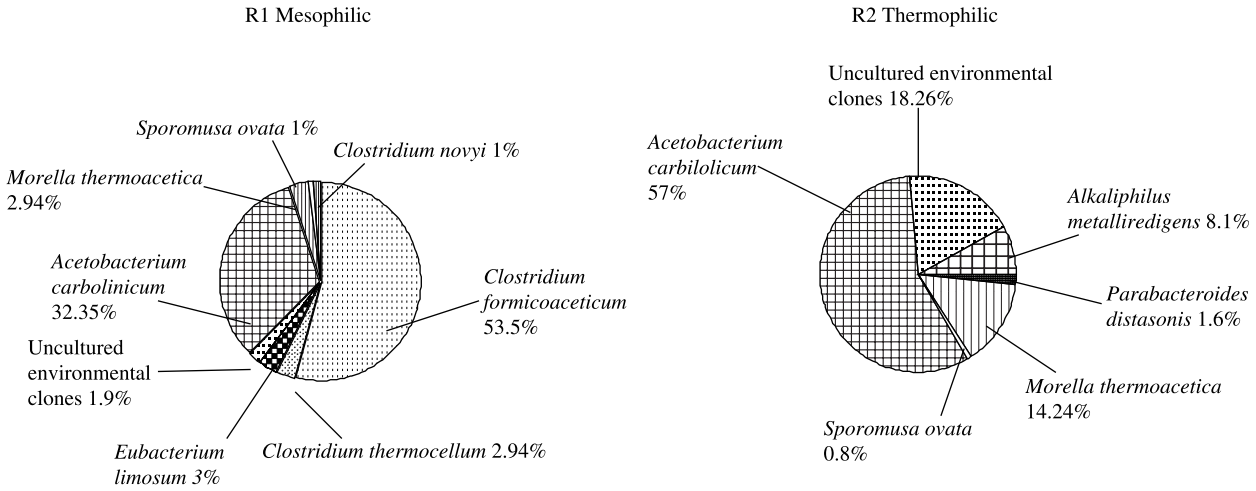

Figure 3 | Affiliation and distribution of FTHFS sequences analysed from the mesophilic (R1) and thermophilic (R2) reactor sludges sampled on day 419.

other methoxylated aromatic compounds such as syringate (Paarup *et al.* 2006). The thermophilic biomass contained a large proportion of thermophilic clostridia species with *M. thermoacetica,* a known acetogen (Fontaine *et al.* 1942) accounting for approximately 14.2% of the total clones. *M. thermoacetica* has an optimum growth temperature of 55–60°C and has been shown to be metabolically robust. Originally isolated as a heterotroph, it was later determined that the carboxyl groups of aromatic compounds can serve as CO_2 precursors in the acetyl-CoA pathway of this organism (Hsu *et al.* 1990).

CONCLUSION

To date, 'known' acetogenic species are predominately from two genera; the *Acetobacteria* and *Clostridium* (Drake *et al.* 2006). Although the results from the current study would largely support this trend, unclassified acetogens were also well represented in the sludges, particularly those sampled on day 349 of the trial. It was evident during comparison of the FTHFS genes in the sludges sampled throughout the trial, that the most diverse acetogenic consortia was cultivated in the mesophilic reactor when the methoxylated aromatic substrate, vanillate was supplied as substrate. The acetogenic libraries constructed from both R1 and R2 biomass, enriched using vanillate as substrate contained abundant sequences which were highly homologous to previously isolated acetogens that are capable of utilizing this substrate. However, the populations in R1 and R2 on day 419 were phylogenetically distinct from those present in the reactors on day 349. Particularly notable was the decrease in abundance of *Clostridium novyi* in the sludge from both reactors between day 349 and 419. From this it can be postulated that this organism has a greater capacity for production of acetate from sugars, as it was not well represented in the sludges cultivated on vanillate. The dominance of uncultured and unclassified sequences diminished between days 349 and 419, which suggests that a large number of as yet unclassified and uncultured acetogens are capable of producing acetate from sugars. The influents supplied throughout the trial were designed to be composed of suitable substrates for the heterotrophic growth of acetogens, and it was expected that acetogenic species would utilise each substrate comparably. However, based on FTHFS analysis, distinct acetogenic populations were cultivated upon each influent from which it can be concluded that as well as being highly phylogenetically diverse, these organisms possess varying metabolic capabilities.

Theoretically, acetogens should be out competed in diverse habitats, such as anaerobic sludge. The specialised metabolic capacities of homoacetogens allow them to utilise aromatic compounds e.g., vanillate, and this characteristic can be exploited for the specific enrichment of these organisms. This was achieved in the present study, and it was evident that the application of this homoacetogenic

specific substrate increased the diversity of 'known' homoacetogens within the biomass, specifically within the mesophilic temperature range.

The use of FTHFS specific PCR primers to recover FTHFS sequences from environmental habitats has the potential to broaden research capabilities into studies of acetogen diversity and distributions. However, for a more thorough understanding of species diversity within anaerobic granular sludge, analysis of *Bacterial* and *Archaeal* consortia should also be carried out.

REFERENCES

Bouwer, E. J. & McCarty, P. L. 1983 Effects of 2-bromoethanesulfonic acid and 2-chloroethanesulfonic acid on acetate utilization in a continuous-flow methanogenic fixed-film column. *Appl. Environ. Microbiol.* **45**, 1408–1410.

Colleran, E., Concannon, F., Golden, T., Geoghegan, F., Crumlish, B., Killilea, E., Henry, M. & Coates, J. 1992 Use of methanogenic activity tests to characterize anaerobic sludges, screen for anaerobic biodegradability and determine toxicity thresholds against individual anaerobic trophic groups and species. *Water Sci. Technol.* **25**(7), 31–40.

Colleran, E., Finnegan, S. & O'Keefe, R. B. 1994 Anaerobic digestion of high-sulphate-content wastewater from the industrial production of citric acid. *Water Sci. Technol.* **30**(12), 263–273.

Diekert, G. & Wohlfarth, G. 1994 Metabolism of homoacetogens. *Antonie van Leeuwenhoek*. ***66***, 209–221.

Drake, H. L., Küsel, L. & Matthies, C. 2006 *Acetogenic Prokaryotes*. The Prokaryotes, Springer, New York, pp. 354–420.

Fontaine, F. E., Peterson, W. H., McCoy, E., Johnson, M. J. & Ritter, G. J. 1942 A new type of glucose fermentationby *Clostridium thermoaceticum* n. sp. *J. Bacteriol.* **43**, 701–715.

Hsu, T., Daniel, S. L., Lux, M. F. & Drake, H. L. 1990 Biotransformations of carboxylated aromatic compounds by the acetogen clostridium thermoaceticum: Generation of growth-supportive CO2 equivalents under CO2- limited conditions. *J. Bacteriol.* **172**, 212–217.

Leaphart, A. & Lovell, C. H. 2001 Recovery and analysis of formyltetrahydrofolate synthetase gene sequences from natural populations of acetogenic bacteria. *Appl. Environ. Microbiol.* **67**, 1392–1395.

Leaphart, A. B., Friez, M. J. & Lovell, C. R. 2003 Formyltetrahydrofolate synthetase sequences from salt marsh plant roots reveal a diversity of acetogenic bacteria and other bacterial functional groups. *Appl. Environ. Microbiol.* **69**, 693–696.

Li, Y., Fang, H. P. & Noike, T. 1994 Recent developments in microbiology and biochemistry of homoacetogens and syntrophic acetogens. *Paper Presented at the Proceedings of 7th International Symposium on Anaerobic Digestion: Oral Paper Preprints*. pp. 54–57 Cape Town, South Africa.

Lovell, C. R. & Hui, Y. 1991 Design and testing of a functional group-specific DNA probe for the study of natural populations of acetogenic bacteria. *Appl. Environ. Microbiol.* **57**, 2602–2609.

Lovell, C. R. & Leaphart, A. B. 2005 Community-level analysis: key genes of CO_2-reductive acetogenesis. *Method. Enzymol.* **397**, 454–469.

McHugh, S., Carton, M. W., Collins, G. & O'Flaherty, V. 2004 Reactor performance and microbial community dynamics during anaerobic biological treatment of wastewaters at 16-37°C. *FEMS Microbiol Ecol.* **48**, 369–378.

Murrell, C. J. & Radajewsli, S. 2000 Cultivation-independent techniques for studying methanotroph ecology. *Res. Microbiol.* **151**, 807–814.

Paarup, M., Friedrich, M. W., Tindall, B. J. & Finster, K. 2006 Characterization of the psychrotolerant acetogen strain SyrA5 and the emended description of the species Acetobacterium carbinolicum. *Antoine Van Leeuwenhoek*. **89**, 55–69.

Shelton, D. R. & Tiedje, J. M. 1984 General method for determining anaerobic biodegradation potential. *Appl. Environ. Microbiol.* **47**, 850–857.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997 The CLISTAL_X windows interface: Flexible strategies for multiple-sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876–4882.

Wood, H. G. & Ljungdahl, L. G. 1991 Autotrophic character of the acetogenic bacteria. In: Barton, L. L. & Shively, J. (eds) *Variations in Autotrophic Life*. Academic Press, San Diego, Calif pp. 201–250.

Zhang, T. C. & Noike, T. 1994 Influence of retention time on reactor performance and bacterial trophic populations in anaerobic digestion processes. *Water Res.* **28**, 27–36.