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To: Dr. Gerard Muyzer

MiniReviews Editor

Dear Dr. Muyzer,

I would like to submit to your attention a proposal for a review on a novel topic, never reviewed in the past years: "Ecology and biotechnological potential of the thermophilic fermentative *Coprothermobacter* spp**.".** Co-authors of the review are Dr. Maria Camilla Braguglia, Dr. M.C. Gagliano from Water Research Institute, CNR, Monterotondo (RM), Italy and Prof. Maurizio Petruccioli from Department for Innovation in Biological, Agri-food and Forestry systems (DIBAF), Tuscia University, Viterbo (Italy).

The review deals with the microbial ecology and the biotechnological exploitation of the metabolic properties of *Coprothermobacter* spp., proteolytic thermophiles increasingly detected in several anaerobic systems. Thermophilic bacteria have recently attracted great attention because of their potential application in improving different biochemical processes like anaerobic digestion of various substrates, wastewater treatment or hydrogen production.
*Coprothermobacter* spp. are gram negative anaerobic thermophilic bacteria detected mostly at high temperatures (from 50 to 70°C). Members of this genus were found to have strong intracellular and extracellular protease activity to degrade proteins and peptides. Additionally, they are frequently identified in thermophilic anaerobic system as important hydrogen producers; both the behaviors are enhanced with the establishment of syntrophic associations between *Coprothermobacter* and hydrogenotrophic archaea, that can significantly improve process performances by an increased methane production. In all the systems in which *Coprothermobacter* was identified, the formation and consumption of hydrogen are the key processes that enable complete thermophilic anaerobic digestion to proceed.

The relevance of the review is high: there is a large interest in the subject as shown by specific publications recently published on this topic. The review, as described in detail in the specific attached document (attachment #1), is timely because the available information about *Coprothermobacter spp.* is often fragmented and incomplete and an integrated and coherent data analysis and a clear indication on how to direct the future investigations are still missing. Hence, an update of literature about the species of this genus could be useful to understand its role and ecology. The challenge of this paper is to critically analyze the available information on *Coprothermobacter* spp. and to productively use them for optimizing anaerobic plant design and operation with a rational and scientific approach.
Few reviews about microbial community composition in anaerobic reactors were published in recent years, and studies performed so far were mainly related to methanogens (e.g. *Methanosarcina or Methanosaeta*) and not on microorganisms involved on the fermentative phase of anaerobic digestion. *Coprothermobacter* had never been the subject of a review.

The Authors of the proposed review were centrally involved in nearly all recent studies on *Coprothermobacte*r spp. In particular they gave an important contribution on the optimized

FISH protocol and on the design of specific FISH probes for the *in situ* detection of members of this genus in mixed microbial cultures. Moreover they were actively involved in the all recent studies where the impact of syntrophic association of *Coprothermobacter* and methanogens on anaerobic digestion processes was evaluated.

The review is ready for submission and the full contact details of four experts in the field who are familiar with the topic are given in the following:

1.Claudia Etchebehere
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Expert in microbial community analysis of anaerobic environments

Please find enclosed the outline of the review (attachment # 1) and a list of recent key references showing the contributions to the field made by the Authors (attachment # 2).

Thanking you in advance for your kind cooperation, I am looking forward to hearing from you.

Yours Sincerely,

Simona Rossetti

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# Attachment # 1: Outline of the review

**"Ecology and biotechnological potential of the thermophilic fermentative *Coprothermobacter* spp."**

**Introduction:** This section shortly describes the actual scientific scenario and reasons that lead us to critically analyse this topic. *Coprothermobacter* spp. are gram negative anaerobic thermophilic bacteria detected mostly at high temperatures (from 50 to 70°C). Members of this genus were commonly found in anaerobic environments, and they have a strong intracellular and extracellular protease activity to degrade proteins and peptides at thermophilic temperatures. Proteolityc activity is rarely retrieved at high temperatures (only few proteolytic thermophiles have been characterized so far), so *Coprothermobacter* is a good candidate for a potential application in improving different biochemical processes like anaerobic digestion of various substrates, wastewater treatment or hydrogen production. The available information about *Coprothermobacter spp.* is often fragmented and incomplete; hence, an update of literature about the species of this genus could be useful to understand its role and ecology, and to point-out the development of new strategies to deepen investigate its properties.

**Isolation and phylogenetic classification of *Coprothermobacter*.** The isolation of members of the genus *Coprothermobacter,* the isolation sources and the controversial phylogenetic affiliation of the genus to the phylum *Firmicutes* are here described and discussed. *Coprothermobacter* group showed a multitude of affiliations that are more consistent with their ecology than with small subunit ribosomal DNA-based taxonomy. Overall, available data suggest that *Coprothermobacter* genus is mostly related to *Dictyoglomi* and *Thermotogae* genera.

**Proteolitic activity and biotechnological exploitation of proteolytic properties.** Generally, *Coprothermobacter* is associated with the microbial population of anaerobic thermophilic environments because of its proteolytic properties. The aim of this section is to briefly describe how and why this microorganism is associated with the proteolytic function. In addition, since proteases secreted from thermophilic bacteria are of particular interest and have become increasingly useful in a range of commercial applications, we summarize biotechnological applications of *Coprothermobacter*, either as source of thermostable enzymes or as cells for bioaugmentation of anaerobic processes.

**Syntrophic association with methanogens**. In this section we review experimental evidences of improved metabolic activity of *Coprothermobacter* with the establishment of syntrophic associations with hydrogenotrophic archaea as *Methanothermobacter thermoautotrophicus*. Studies described *in vitro* dynamics of this association and highlighted that the presence of an hydrogenotrophic partner is essential to improve the hydrogen production and proteolytic activity of *Coprothermobacter* in anaerobic natural environments.

**Other metabolic abilities of *Coprothermobacter* spp.** Based on the experimental evidences of many studies, the high metabolic versatility of this microorganism is discussed. This bacterium shows several metabolic abilities as thiosulfate reduction and cyanide detoxification, reduction of iron, fermentative production of pyruvate, formate, buthanol and acetate, syntrophic acetate oxidation, resistance to butanol and isobutanol, involvement in cellulose degradation pathway, etc. All these metabolic abilities are reflected in the wide variety of habitats in which *Coprothermobacter* has been found.

**Distribution and identification of *Coprothermobacter* in biological anaerobic systems.** In this section all the experimental conditions in which *Coprothermobacter* was identified are listed, with the aim to better understand its role in anaerobic processes and how its metabolic abilities are exploited in the context of complex microbial communities. In recent years, *Coprothermobacter* spp. were identified especially in studies focused on the microbial community structure of anaerobic processes, but its presence in non-conventional anaerobic systems (Microbial Fuel Cells, petroleum reservoirs, cellulolytic cultures, etc.) highlights that it can survive under different conditions (also extreme), not only at high temperature.

**Proteolytic activity and substrate availability.** Strictly linked to the content of the previous section, this paragraph describes how *Coprothermobacter* growth seems to be mainly related to proteinaceous substrate availability and its level of hydrolyzation in different kinds of habitats in which it was identified as proteolytic fermentative bacterium. Several studies underlines also that the source of proteinaceous substrate is of particular importance for *Coprothermobacter* growth.

**Molecular methods for *Coprothermobacter* identification.** Since in most of the studies cited in the previous sections *Coprothermobacter* spp. were identified only by applying PCR based approaches (using universal primers for the domain Bacteria), this paragraph review the few studies that developed strategies for the specific identification of members of this genus. Two

primer pairs for Real-time PCR and PCR application, and two FISH probes were described and compared each other to evaluate their efficiency vs *Coprothermobacter* sequences available in SILVA database. The design of a specific fluorescent probe that allowed for the first time to estimate the relative abundance of *Coprothermobacter* by using FISH quantification was described as the most promising strategy for a quickly identification in natural environments, considering also possible drawbacks for the contemporary identification of its syntrophic partner *M. thermoautotrophicus.*

**Concluding remarks.** This paragraph outlines the actual metabolism and ecology of *Coprothermobacter* spp. and their importance and potential in industrial and biotechnological processes. Two current projects of genome sequencing of *Coprothermobacter proteoliticus* and *Coprothermobacter platensis* are a good starting point to delineate the most pressing needs and priorities towards the completely understanding of the ecology and microbiology of this genus.

# Attachment # 2: list of recent key references showing the contributions to the field made by the Authors.


1. Gagliano M.C., Braguglia C.M., Gianico A., Mininni G., Nakamura K., Rossetti S. (2014) Thermophilic anaerobic digestion of thermal pretreated sludge: role of microbial community structure and correlation with process performances. Accepted on Water Research. DOI:10.1016/j.watres.2014.10.031

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3. Gagliano M.C. , C.M. Braguglia, A. Gallipoli, A. Gianico, S. Rossetti (2014) Microbial diversity in innovative mesophilic/thermophilic temperature phased anaerobic digestion of sludge. Environmental Science and Pollution Research, (Epub ahead of print) DOI 10.1007/s11356-014-3061-y

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10. Gianico, A, Braguglia, CM, Cesarini, R, Mininni, G (2013) Reduced temperature hydrolysis at 134 C before thermophilic anaerobic digestion of waste activated sludge at increasing organic load, Bioresource technology, 143, 96-103

11. Braguglia, CM, Gianico, A, Mininni, G (2012) Comparison between ozone and ultrasound disintegration on sludge anaerobic digestion, Journal of environmental management, 95, S139-S143

12. Braguglia, CM, Gagliano, MC, Rossetti, S (2012) High frequency ultrasound pretreatment for sludge anaerobic digestion: Effect on floc structure and microbial population, Bioresource technology, 110, 43-49

13. Braguglia, CM, Gagliano, MC, Gallipoli, A, Rossetti,S (2012) Enhanced anaerobic digestion performances: effect of sludge ultrasound pre-treatment and role of the microbial population.J of Environmental Engineering & Management Journal (EEMJ), 11(10), 1803-1810

14. Tomei, MC, Braguglia, CM, Cento, G, Mininni, G (2009) Modeling of anaerobic digestion of sludge, Critical Reviews in Environmental Science and Technology, 39(12), 1003-1

# Phylogeny, ecology and biotechnological potential of the thermophilic fermentative *Coprothermobacter* spp.

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## 1. Why Coprothermobacter?

Thermophilic bacteria have recently attracted great attention because of their potential application in improving different biochemical processes like anaerobic digestion of various substrates, wastewater treatment or hydrogen production. In particular, they possess enzymes with enhanced thermostability and hence may have a potential industrial application (Ollivier et al., 2000).

Indeed, there is considerable demand for a new generation of stable enzymes that are able to withstand severe conditions in industrial processes by replacing or supplementing traditional chemical processes (Elleuche et al., 2014).

Although proteolitic activity seems to be a common characteristic among mesophilic bacteria, only few proteolytic thermophiles have been characterized so far (Kersters et al., 1994; Cai et al., 2011), and the majority of the enzymes that are currently used in the industry are obtained from fungi or mesophilic bacteria (Elleuche et al., 2014).

*Coprothermobacter* spp. are gram negative anaerobic thermophilic bacteria detected mostly at high temperatures (from 50 to 70°C). Member of this genus were found to have strong intracellular and extracellular protease activity to degrade proteins and peptides. Additionally, they are frequently identified in thermophilic anaerobic system as important hydrogen producers; both of these behaviors are improved with the establishment of syntrophic associations between *Coprothermobacter* and hydrogenotrophic archaea, that can significantly improve process performances by an increased methane production. In all the systems in which *Coprothermobacter* was identified, the formation and consumption of hydrogen are the key processes that enable complete thermophilic anaerobic digestion to proceed (Tandishabo et al., 2012 b).

The available information about *Coprothermobacter spp.*, is often fragmented and incomplete; hence, an update of literature about this microorganism could be useful to understand its role and ecology and to point-out the development of new strategies to deepen investigate its properties.

## 2. Isolation and phylogenetic classification of *Coprothermobacter*

The first member of the genus *Coprothermobacter* was originally isolated from a themophilic (55°C) digester fermenting tannery wastes and cattle manure (Ollivier et al., 1985). The isolate was classified as a member of the genus *Bacteroides* and named *Thermobacteroides proteolyticus*, strain BT. In 1993 Rainey and Stackebrandt proposed *Thermobacteroides proteolyticus* as the type species of the new genus *Coprothermobacter,* belonging to a deep-rooting phylum of the domain Bacteria and not

to the Bacteroides-Cytophaga phylum as previously considered. Because of the isolation source, the species described in Ollivier et al. (1985) got the name *Coprothermobacter*.

Kersters et al. (1994) carried out a further characterization of the species, with the isolation of *Coprothermobacter proteolitycus,* strain I8 from biokitchen waste digestate resulting from an anaerobic composting process at 55°C.

A new species, *Coprothermobacter platensis,* was isolated from a methanogenic mesophilic reactor treating a protein-rich wastewater (Etchebehere et al., 1998). Recently, Tandishabo et al. (2007) isolated *Coprothermobacter* strain IT-3 from an enrichment culture containing organosolv lignin, characterized by an higher hydrogen production with respect to the other type strains (Tandishabo et al., 2012 a).

A further study of the same research group (Tandishabo et al., 2012 b) identified and characterized the new strain IT3 as closely resembling the *C. proteolyticus* strain BT by DNA-DNA hybridization, but differentiate these strains by analyzing their growth and hydrogen production rates within the temperature range of 45-70 °C.

In Sasaki et al. (2011) *Coprothermobacter proteolyticus* strain CT-1 was isolated from a thermophilic packed-bed reactor. The authors reported that strain CT-1-related microorganisms were often observed in thermophilic reactors degrading organic solid wastes.

Other isolated strains are listed in Table 1.

*Coprothermobacter* spp. were always identified as rod-shaped cells; however, in Tandishabo et al. (2012 b) was highlighted for the first time the extensive filamentation of strains IT3 and BT at high thermophilic temperatures (60 – 65 °C).

In the present bacterial taxonomic system, the genus *Coprothermobacter* is classified into the phylum *Firmicutes*, class *Clostridia*, and affiliated with family *Thermodesulfobiaceae*, which is differently branched from families including most of aminoacid degrading bacteria in the phylum *Firmicutes* (Sasaki et al., 2011). Despite this, Etchebehere et al. (1998) highlighted for the first time a possible phylogenetic relationship between the genus *Coprothermobacter* and the *Thermotogales*.

Tang et al. (2004) showed by 16S rRNA gene clonal analysis of a mixed microbial community growing in a thermophilic anaerobic municipal solid-waste digester the occurrence of two OTUs phylogenetically assigned to the phylum *Thermotogae* and closely related to *Coprothermobacter proteolyticus*.

Generating a Supertree based on subtree prune-andregraft (SPR) distance, Whidden et al., (2014) highlighted that *Coprothermobacter proteolyticus* showed a particularly interesting affinity, grouping

with *Thermotogae* rather than *Clostridia*. Supertree methods reconcile a set of phylogenetic trees into a single structure that is often interpreted as a branching history of species.

In the work of Kunisawa (2010) regarding the phylogenetic classification of *Thermodesulfovibrio yellowstonii*, *Coprothermobacter proteolyticus* was treated as representing a separate phylum, 'Coprothermobacter'. In this work, orthologous relationships of genes among different genomes were examined, and the comparison between gene arrangements of different bacterial phyla showed a consistent grouping of the major phyla, except for 'Coprothermobacter'.

Placing of *Coprothermobacter proteolyticus* within Clostridia is not supported by either ribosomal protein-based phylogeny (Yutin et al., 2012) or whole-genome analysis (Beiko, 2011; Nishida et al., 2011).

An automated computational pipeline for the identification of r-protein genes, applied to 995 completely sequenced bacterial genome, was developed by Yutin et al. (2012). The phylogenetic tree produced from this alignment showed that the topology of the r-protein tree is generally compatible with the commonly accepted bacterial taxonomy, but several notable deviations existed.

In particular, *Coprothermobacter proteolyticus* DSM 5265 was placed in the *Dictyoglomia-Thermotogae-Aquificae* group, sister to *Dictyoglomia*.

Beiko et al. (2011) by analyzing a set of over 1000 genomes using new tools and techniques for taxonomic classification, highlighted that *Coprothermobacter* group showed a multitude of affiliations that are more consistent with their ecology than with small subunit ribosomal DNA-based taxonomy. With a distance-based approaches (as the neighbor-joining algorithm) they calculated an unusually positioned genome in the set for *C. proteolyticus*, which branches with other major thermophile-containing phyla *Dictyoglomi, Synergistetes,* and *Thermotogae* near the base of the bacterial tree. It is unclear whether this association is driven by legitimate genetic affinities, or is simply a consequence of a lack of affinity of these groups for other phyla in the tree. Additionally, using a model-based phylogenetic analysis of orthologous genes to calculate the intergenomic affinities of species, Beiko and colleagues found that the position of *Coprothermobacter* is connected to the three *Firmicutes* genera *Bacillus*, *Clostridium* and *Thermoanaerobacter*, but is also linked to *Dictyloglomi*, which are in turn linked to all genera within phylum *Thermotogae*.

**Table 1** – Isolated strains belonging to *Coprothermobacter* genus and their isolation source, as described in the NCBI Taxonomy database.

| Isolated strains | Source | Taxonomy ID | Reference |
|---|---|---|---|
| *C. proteolyticus* DSM 5265 | themophilic digestor fermenting tannery wastes and cattle manure | 35786, 309798 | Ollivier et al., 1985 |
| *C. proteolitycus* strain I8 | biokitchen waste digestate | - | Kersters et al. 1994 |
| *C. platensis* DSM 11748 | methanogenic mesophilic reactor treating protein rich wastewater | 108819, 1259795 | Etchebehere et al., 1998 |
| *Coprothermobacter* strain IT-3 | lignin degrading enrichment culture | - | Tandishabo et al., 2007 |
| *Coprothermobacter* sp. BHI60-1 | enrichment cultures from east pacific rice | 1259795 | Cambon-Bonavita et al., 2002 (unpublished) |
| *Coprothermobacter* sp. BHI60-2 | enrichment cultures from east pacific rice | 187080 | Cambon-Bonavita et al., 2002 (unpublished) |
| *Coprothermobacter* sp. Dex80-3 | enrichment cultures from east pacific rice | 187082 | Cambon-Bonavita et al., 2002 (unpublished) |
| *Coprothermobacter* sp. Dex80-4 | enrichment cultures from east pacific rice | 187081 | Cambon-Bonavita et al., 2002 (unpublished) |
| *Coprothermobacter* sp. GK5 | thermophilic anaerobic digester | 700502 | Kanno et al., 2013 |
| *Coprothermobacter* sp. CT-1 | thermophilic packed bed reactor | 1032348 | Sasaki et al., 2011 |
| *Coprothermobacter* sp. P1 | thermophilic digested sludge | 263907 | Nagaya et al., 2004 (unpublished) |
| *Coprothermobacter* sp. PM9-2 | offshore oil reservoir | 1298737 | Urushibata and Morikawa, 2013 (unpublished) |
| *Coprothermobacter* sp. TERIGK51 | thermophilic oil field treatment plant | 467142 | Kaur and Lal, 2007 (unpublished) |

In the work of Nishida et al. (2011), concerning the phylogenetic position of the anaerobic thermophilic bacterial genus *Dictyoglomus*, *C. proteolyticus* was excluded from the large cluster of *Firmicutes.* They demonstrated that *Coprothermobacter* clusters to *Dictyoglomi* and *Thermotogae* and not to *Firmicutes*, indicating that *C. proteolyticus* is not a member of *Firmicutes* but represents another taxonomic group most closely related to *Dictyoglomi*. These results support that *Coprothermobacter*, *Dictyoglomi* and *Thermotogae* diverged from a common ancestor at an early stage of bacterial evolution.

**3. Proteolitic activity**

Generally, *Coprothermobacter* is associated with the microbial population of anaerobic digesters because of its proteolytic properties.

Ollivier et al. (1985) showed that *C. proteolyticus* strain BT grew well on peptides, as demonstrated by $H_2$ production and glucose degradation rate when rumen fluid or trypticase peptone was added to culture media; fermentation of sugars was poor and acetate was the major volatile acid produced. The fermentation products were acetic acid, $H_2$, and $CO_2$, along with smaller quantities of isobutyric, isovaleric, and propionic acids.

In Kersters et al. (1994), *C. proteolyticus* strain I8 fermented a wide variety of proteins (gelatin, bacto-peptone, casein, tryptone and bovine serum albumin) and acetate was a major end-product. Strain I8 ferments sugars as also ascertained for strain BT. By analyzing gelatin degradation by *C. proteolyticus*, Kersters et al. (1993) showed that the gelatin concentration in the medium strongly affected the ammonification: the highest percentage of organic nitrogen conversion to ammonium was observed at a gelatin concentration ranging between 2.7 and 10 g/l. At higher concentrations (10-50 g/l) ammonification was reduced but higher production of acetate was observed. Moreover, *C. proteolyticus* expressed its proteolytic activity also when grew poorly.

Etchebehere et al. (1998) described that cells of *C. platensis* were proteolytic, because growth was observed with different proteinaceous substrates. They also highlighted an extracellular protease activity, which increased during growth.

In a metaproteomic study on cellulose methanization process (Lü et al., 2014 b), *C. proteolyticus* was described as scavenger and/or predator performing proteolysis and fermentation. Among the numerous identified proteins, 22 putatively involved in ammonia assimilation and amino acid biosynthesis and in peptidase activities were affiliated to *C. proteolyticus*, highlighting its intensive proteolytic activity during the process. The latter suggested a role in degrading several distinct

proteins sources during cellulose degradation, like EPS, the abundant extracellular enzymes or other secreted proteins and dead cell material generated during the incubation by the counter-selection of poorly competitive strains. Moreover, Lü and colleagues further supported this proteolytic activity by other retrieved protein functions. In particular, seven ABC transporter clusters were attributed to *C. proteolyticus* including three related to peptide transport, suggesting an important peptide import/export activity.

Proteases secreted from thermophilic bacteria are of particular interest and have become increasingly useful in a range of commercial applications (do Nascimento and Martins, 2004). Thus, proteolytic properties make *Coprothermobacter* a possible "source" of enzymes for different applications in biotechnological processes at high temperatures in an industrial perspective, as discussed in the next section.

### *3.1 Biotechnological exploitation of proteolytic properties*

Enzymes from extremophiles, so called extremozymes, offer the opportunity to expand the reaction conditions compatible with biocatalytic conversions (Toplak et al., 2013). They are capable of catalysing their respective reactions in non-aqueous environments, water/solvent mixtures, at extremely high pressures, acidic and alkaline pH, at temperatures up to 140°C, or near the freezing point of water (Elleuche et al., 2014). Actually, only few extremozymes have found their way in the market, as the application of Taq polymerase from *Thermus acquaticus* in Polymerase Chain Reaction (Holland et al., 1991). Proteolytic enzymes, especially thermostable proteases, are extensively used in the detergent, food, leather, pharmaceutical, and textile industry because higher processing temperatures resulting in faster reaction rates and in increasing solubility of nongaseous reactants and products (do Nascimento and Martins, 2004; Elleuche et al., 2014). Heat-active enzymes with optimal proteolytic activity at high temperatures are required for use under rough process conditions (Toplak et al., 2013).

*C. proteolyticus* showed high levels of intracellular and extracellular protease activity (Majeed et al., 2013; Lü et al., 2014 b). For these proteolitic properties, *Coprothermobacter* may be a good candidate for facilitating treatment and processing of protein-rich wastewater at high temperature.

A comprehensive survey of the protease complement (or degradome) in the genome of *C. proteolyticus* was recently reported (Cai et al., 2011); it possesses a core degradome structure that may be common in the thermophilic bacteria, as shown by the comparison with *Moorella thermoacetica* and *Thermoanaerobacter tengcongensis*, the most closely related sequenced species

in the family *Thermoanaerobacteriaceae*. Functional characterization of these enzymes in this bacterium may provide a better understanding of the mechanisms of physiological adaptation to hot temperature and a better assessment of its potential application to wastewater processing.

Antranikian and Klingeberg (1994) were the first to isolate and patent a proteolytic enzyme extracted from *C. proteolyticus*. They discovered a serine-protease, showing an extraordinary thermostability as well as thermoactivity, with an optimum temperature in the range of 75 to 95° and a pH optimum in the range of 6.5-10. Protease was also resistant to inhibition from different chemicals (Ethylenediaminetetraacetate, iodoacetate and phenylmethylsulphonylfluoride) (Klingeberg et al., 1991). Due to the unique properties of the protease, it was added to a detergent composition comprising one or more surfactants or additionally enzymes.

Another possible application of protease from *C. proteolyticus* was described in Majeed et al. (2013), which is the first report of the biochemical characterization of a recombinant protease from *C. proteolyticus*. The enzyme is a serine protease with an alkaline pH optimum and functions at elevated temperature. The protease has also the desirable property of retaining high activity in the presence of a wide variety of surfactants, thus indicating potential utility of this enzyme in detergent applications. The production, purification and biochemical characterization of a *C. proteolyticus* protease in *E. coli*, namely proteolysin, was recently described by Toplak et al. (2013). Proteolysin can hydrolize proteins at temperatures as high as 90 °C, and showed an high tolerance towards organic solvents. Because of its thermostability, relaxed specificity, the broad pH range, and resistance to routinely used protein denaturants and DTT, proteolysin is a candidate for proteomics studies when protein digestion under extreme conditions is required.

Besides the use of enzymes, Palatsi et al. (2011) suggested to enrich the anaerobic digestion process with species of peptide-fermentative microorganisms like *Coprothermobacter* in order to treat successfully substrates as slaughterhouse and residual waters. Bioaugmentation may be indeed a cost-effective strategy to exploit the proteolitic activity of *Coprothermobacter* and improve degradation processes at high temperatures. Bioaugmentation of thermophilic, anaerobic sludge with *Coprothermobacter* was successfully applied by Lü et al. (2014 a). In general, the proteins degradation rate and efficiency were found to slightly increase with the increase of *C. proteolyticus* inoculation density. However, *C. proteolyticus* itself did not improve the final methane yield. On the contrary, when *C. proteolyticus* was added together with a granular anaerobic sludge, the methane yield was significantly enhanced. This could be explained by the fact that *C. proteolyticus* can produce $H_2$ from proteinaceous materials and, consequently, promote syntrophic cooperation with hydrogenotrophic methanogens, as described in the next section.

## 4. Syntrophic association with methanogens

Growth of *Coprothermobacter* spp. on protein substrates is closely associated with hydrogen production and hydrogen is one of the most important carriers in interspecies electron transfer between *Coprothermobacter* spp. and methanogens (Tandishabo et al., 2012). *Coprothermobacter* spp. should therefore benefit from the activities of the hydrogenotrophic methanogens in anaerobic digesters. In particular, *Coprothermobacter* can accomplish protein degradation in syntrophic association with hydrogenotrophic methanogenic archaea (Sasaki et al., 2011).

In fact, *Coprothermobacter* activity is improved by the establishment of a syntrophy with hydrogenotrophic methanogens like *Methanothermobacter thermoautotrophicus,* commonly found as component of methanogenic population in many thermophilic anaerobic systems. Hydrogen is the primary energy source of this methanogen, even when in situ hydrogen concentrations are very low (Kato et al., 2008).

Sasaki et al. (2011), comparing a monoculture of *Coprothermobacter proteolyticus* to a co-culture with *Methanothermobacter thermoautotrophicus*, reported that in co-colture *Coprothermobacter* growth rate increased 4-fold in presence of *Methanothermobacter*, with respect to the pure culture.

Simultaneously, the number of cells of *Methanothermobacter* decreased without affecting methane production rate. In addition, in co-culture the soluble protein content decreased more than in monoculture. This means that the presence of an hydrogenotrophic partner was essential to improve the proteolytic activity of *Coprothermobacter.*

In the work of Lü et al. (2014 b) on cellulose methanisation process, *C. proteolyticus* was proposed as important $H_2$ producer in the system and might have established efficient syntrophy with *Methanothermobacter*.

Moreover, in a thermophilic digester at 70°C the shift in bacterial population to *Coprothermobacter* dominance corresponded to the predominance of *Methanothermobacter* among Archaea (Ge et al., 2012).

Proteolysis in a thermophilic model bioelectrochemical co-culture (*C .proteolyticus* and hydrogenotrophic *Methanothermobacter thermoautotrophicum* strain ΔH) and single-culture (*C. proteolyticus*) was examined by using a bioelectrochemical system to control electron flow in culture medium (Sasaki et al., 2012). In non-bioelectrochemical environments, hydrogen-consuming methanogens help in scavenging hydrogen and increasing proteolytic activity. This study showed that the growth of *C.proteolyticus* and proteolysis were accelerated as the result of increased hydrogen

consumption, which occurred because of the better growth of strain ΔH in the thermophilic bioelectrochemical culture system. These observations highlighted the role of *Methanothermobacter* to optimize $H_2$ consumption avoiding its accumulation and the consequent inhibition of fermentative bacteria.

In the work of Ho (2014) analyzing the microbial community in a thermophilic anaerobic reactor treating sewage sludge, *Coprothermobacter* appeared to be the key microorganism along the process, while methanogenic community shifted from *Methanosarcina* to *Methanothermobacter* as the temperature increased from 55°C to 65°C; a possible syntrophic association at lower temperature with *Methanosarcina* was speculated, but it have to be further investigated.

*Coprothermobacter* and *Methanothermobacter* have been detected in the same thermophilic anaerobic systems in several studies (Tang et al., 2008; Tatara et al., 2008; Tang et al., 2011; Ritari et al., 2012; Luo et al., 2013; Lü et al., 2014 b; Guo et al., 2014; Gagliano et al., 2014 a; Gagliano et al., 2014 b) with different substrates as waste activated sludge, municipal solid waste, or food wastes.

## 5. Other metabolic abilities of *Coprothermobacter* spp.

Besides the proteolytic attitude of *Coprothermobacter spp*. described in the previous paragraph, other metabolic activities are reported in literature.

The ability of *C. proteolyticus* and *C. platensis* to use thiosulfate as electron acceptor was reported by Etchebehere et al. (1998). Thiosulfate reduction, alanine production and hydrogen inhibition of glucose utilization were studied in pure cultures of members of the genus *Coprothermobacter* by Etchebehere and Muxì (2000). They found that thiosulfate enhanced the growth of the *C. platensis* strain on carbohydrates and proteinaceous substrates. Moreover, the addition of the only thiosulfate to the culture media without any substrate did not result in an increase in growth, confirming the inability of the bacterium to use thiosulfate as energy source. Hydrogen addition to growing cultures of *C.proteolyticus* and *C.platensis* strains immediately caused growth to cease. It was also shown that hydrogen accumulation caused inhibition of glucose utilization thereby explaining the preferential utilization of proteins my member of this genus. Furthermore, both members of the genus *Coprothermobacter* were shown to produce alanine during glucose and pyruvate fermentation.

During the growth of *Coprothermobacter*, thiosulfate is reduced to hydrogen sulfide, which mainly reacts with the $Fe^{+2}$ in the medium to form black iron sulfide precipitates. In the work of Tandishabo et al. (2012 b) the increased opacity caused by an increase in the number of cells and the precipitation

of iron sulfides was used as an indicator of growth, to compare the growth of several strains of *Coprothermobacter* on thiosulfate.

In the work of Tandishabo et al. (2007) the strain IT3 was showed to be involved in cyanide detoxification by means of an extracellular rhodanese activity, and this activity was found also in *C. proteolitycus* and to a lesser extent in *C. platensis*. This ability strongly correlated with thiosulfate reduction in many anaerobic bacteria, and could play an important role in the treatment of wastes from cassava processing, a plant containing cianogenic glucosides, commonly used as source of calories in developing tropical countries.

In a study on the negative effects of iron oxides on methanogenesis in a microbial community obtained from a thermophilic anaerobic digester, Yamada et al. (2014) showed the presence of *Coprothermobacter* as the dominant bacteria by applying T-RFLP. *Coprothermobater* relative abundance increased with the increasing concentrations of ferrihydrite, suggesting the ability of *Coprothermobacter* to acquire energy with the reduction of iron, together with the ability to tolerate high concentration of ferrihydrite.

A metaproteomic approach revealed that *Coprothermobacter* may be also involved in different pathways during anaerobic degradation of organic matter (Lü et al., 2014 b). They included fermentative production of pyruvate, formate, buthanol and acetate, syntrophic acetate oxidation (SAO) with consequent production of hydrogen, ammonia assimilation, aminoacids biosyntesis and general protein turnover. In addition, a possible role in microcin (a bacterial toxin) synthesis was discovered; several proteins from *C. proteolyticus* related to oxidative stress response or to virulence factor were also identified, but their role remains unclear.

In the works of Ho et al., (2014) and Ho (2014) a possible involvement of *Coprothermobacter* in SAO was suggested, during thermophilic anaerobic digestion of sludge at thermophilic temperatures with high ammonia and acetate concentration. In particular, monitoring the utilization of $^{13}C$- labelled acetate together with nanoscale secondary ion mass spectrometry (nanoSIMS) and RNA-based stable isotope probing (RNA-SIP), *Coprothermobacter* was identified as the possible primary acetate degrader.

*Coprothermobacter* was also found to be the dominant bacteria during carbon monoxide biomethanation integrated to anaerobic digestion of sewage sludge, suggesting a possible role in the conversion chain of CO into methane (Luo et al., 2013).

Recently, Kanno et al. (2013) identified a new strain of *Coprothermobacter*, GK5, in an enrichment culture under anaerobic conditions in the presence of butanol and isobutanol, highlighting its resistance to these solvents. In particular, the maximum concentrations of butanol and isobutanol

towards which *Coprothermobacter* showed tolerance was 2.0% (v/v). Moreover, *Coprothermobacter* GK5 was the only thermophilic strain isolated under these conditions.
All these metabolic abilities are reflected in the wide variety of habitats in which *Coprothermobacter* was identified, as discussed in the next section.

## 6. Distribution and identification of *Coprothermobacter* in biological anaerobic systems

In recent years, *Coprothermobacter* spp. were identified in several studies focused on the microbial community structure of anaerobic processes, especially under thermophilic conditions.
In Tandishabo et al. (2012 a) the molecular enumeration of *Coprothermobacter* spp. was performed on samples from seven anaerobic digesters fed with garbage, dairy cattle manure and food processing waste, using specific primers targeting the 16S rDNA of the genus. The largest number of *Coprothermobacter* spp. cells was found in a thermophilic anaerobic digester treating dairy cow manure.
Lee et al. (2009) detected *Coprothermobacter* spp as the key microbe in the hyperthermophilic acidogenesis step (70 °C) and in the methanogenic step (55°C) of an hyperthermophilic two-phased digestion system treating kitchen garbage and waste activated sludge; 23% of the 16S rDNA clones during acidogenesis step were affiliated to this genus.
Kobayashi et al. (2008) identified *Coprothermobacter* during thermophilic anaerobic digestion of waste activated sludge; 73% of clones were affiliated to this genus, and real-time PCR highlighted that *Coprothermobacter* spp. were the main bacteria in the digester. The same result was evidenced in a following study (Kobayashi et al., 2009) on temperature-phased anaerobic digestion where 70% and 15.3% of total clones were affiliated to *Coprothermobacter*, during thermophilic and mesophilic stage, respectively.
In the study of Cheon et al. (2008) *Coprothermobacter* was detected in a full scale thermophilic digester (55°C) with the random cloning method.
The presence of *Coprothermobacter* was evidenced in coexistence with *Lutispora thermophila* in a thermophilic anaerobic reactor of a two-phased AD treating activated sludge (Pervin et al, 2013).
Ho et al., (2014) analysed the microbial community structure during two-phased anaerobic digestion of sludge by pyrosequencing and identified several *Coprothermobacter* related OTUs during the thermophilic phase. Their abundance increased with the increase of temperature from 55 to 65°C, dominating in the latter condition.

In Gagliano et al. (2014 a), *Coprothermobacter* was identified and quantified by FISH, during the thermophilic stage of an innovative two- stage (mesophilic-thermophilic) anaerobic digestion of activated sludge. Its relative abundance decreased along time, inversely with respect to the total VFA (Volatile Fatty Acids) concentration in the system.

In a study on long term performance of a municipal sludge anaerobic digestion process with a transition from mesophilic to thermophilic conditions (from 36°C to 60°C), Tezel et al., (2014) found that *Coprothermobacter* 16S rRNA gene copies number, measured by quantitative PCR analysis, decreased significantly during time with the increase of temperature and VFA concentration.

Lienen et al. (2014) detected *Coprothermobacter* by DGGE in laboratory-scale digesters fed with digested sludge and operating at the temperature range of 37-56 °C.

In Palatsi et al., (2011) during the anaerobic digestion of slaughterhouse waste mixture, with a metabolism related to long-chain fatty acid degradation, proteolityc activity of *Coprothermobacter* was retrieved together with *Anaerobaculum mobile*.

*Coprothermobacter* was also associated to carbohydrate fermentation at 55°C during phased anaerobic digestion fed with a mixture of primary and secondary sludge (Zamanzadeh et al., 2013).

Zhang et al. (2009) analyzed the effect of a Focused-Pulsed (FP) sludge pretreatment on the bacterial diversity of a mesophilic full-scale anaerobic digester treating a mixture of primary and waste activated sludge, and showed that *Coprothermobacter* population decreased from 10% to 1% after the pretreatment, in line with the observed dramatic shift from $H_2$-utilizing to acetoclastic methanogens after FP pretreatment.

*Coprothermobacter* was the predominat phylotype detected in a thermophilic digester treating municipal solid waste by RNA-Based Stable Isotope Probing (Hatamoto et al., 2008). Moreover, *Coprothermobacter* was identified in a CSTR fed with a mixture of biowaste and sewage sludge (Ritari et al., 2012) and in a bioelectrochemical reactor for methanogenic fermentation of thickened sewage sludge (Sasaki et al., 2013).

*Coprothermobacter* spp. were also found in non-conventional anaerobic systems.

For instance, 16S rRNA gene sequences closely related to *C. proteolyticus* were found in an anaerobic packed-bed reactor with carbon fiber textiles (CFT) as the supporting material treating an artificial garbage slurry (Sasaki et al., 2007).

In Tatara et al. (2008) 17% of total clones in the liquid and biofilm fractions of thermophilic down-flow anaerobic packed-bed reactor treating propionate were represented by *Coprothermobacter*. In a packed bed reactor treating volatile fatty acids (Ueno et al., 2008) *Coprothermobacter* was the predominant clone in the class of *Thermoanaerobacteriaceae*.

*Coprothermobacter* was selected during thermophilic dry anaerobic digestion of model garbage with ammonia stripping (Yabu et al., 2011) and found in thermophilic dry methanogenic systems fed with garbage stillage (Tang et al., 2011).

Kawagoshi et al. (2005) identified *Coprothermobacter* by DGGE in an hydrogen producing reactor inoculated with six different sources and treating a synthetic medium, while Nissilä et al. (2010) detected *Coprothermobacter* in a batch enrichment of hydrogen producing, cellulolytic cultures. *Coprothermobacter* was found at the biocathode during hydrogen production in a two-chambered microbial electrolysis cells, representing 19.8% of total clones (Fu et al., 2013 c).

In the work of Köllmeier (2013), *Coprothermobacter* was detected by clonal analysis in a cellulolytic enrichment culture (generated from an agricultural biogas plant) fed with filter paper and growing at 60°C.

The detection of *Coprothermobacter* in a cellulolityc habitat was also described in Tang et al. (2008), working on a glucose-degrading methanogenic consortium at 65°C and in Motte et al., (2014) during the dry phase of thermophilic dark fermentation of wheat straw, in which was the dominant member of the class *Clostridia*.

*Coprothermobacter* was also detected in enrichment cultures generated from high temperature petroleum reservoirs (Nazina et al., 2006; Lan et al., 2011). Mbadinga et al. (2012) detected *Coprothermobacter* in an alkane-dependent methanogenic community growing at 55°C, and its presence was related to thiosulfate reduction.

Sulfate reduction ability of this microorganism was also reported in Sarti et al. (2009). *Coprothermobacter* was detected at high sulfate concentration (3 g $SO_4^-$ /l) in an anaerobic sequencing batch reactor treating sulfate rich wastewater. *C. proteolitycus* was also found by Amplified Ribosomal DNA Restriction Analysis (ARDRA) in an anaerobic thermophilic oleate-degrading enrichment culture (Menes and Muxì, 2001).

Moreover, *Coprothermobacter* species were identified in several studies on thermophilic microbial fuel cells (MFCs) (Jong et al., 2006; Wrighton et al., 2008; Ha et al., 2013; Fu et al., 2013 a and b; Hussain et al., 2011). In all of these studies, its presence was supposed to be independent by anodic electron transfer, but related to its presence in the startup sludge inoculum and its ability to do extracellular electron transfer.

Additionally, there are some studies in which 16S rRNA gene sequences belonging to *Coprothermobacter* spp. were retrieved during thermophilic anaerobic digestion, but were not explicitly affiliated to this genus (Hori et al., 2006; Sasaki et al., 2006; Hatamoto et al., 2007; Rivere et al., 2009; Krakat et al., 2010).

Overall, the metabolic versatility described in previous sections is supported by the identification of *Coprothermobacter* in several habitats and/or in presence of different kind of substrates.

## 7. Proteolytic activity and substrate availability

Besides the different kinds of habitat in which *Coprothermobacter* was identified as a proteolytic fermentative bacteria, its growth seems to be mainly related to proteinaceous substrate availability and its level of hydrolyzation.

Tandishabo et al. (2012 a) showed that *Coprothermobacter* population size in several anaerobic digesters is not only controlled by the thermodynamics of hydrogen production but also by the type of substrates in the respective wastes. In particular, in the study the presence of *Coprothermobacter spp.* cells in a thermophilic anaerobic digester treating dairy cow manure was related to its proteolytic properties, since dairy cow manure can contain up to 16% of crude protein. Despite this, they additionally observed that *Coprothermobacte*r cells seemed to show a severely decreased ability to proliferate in digesters fed with dairy cow manure compared to the digesters fed with food processing wastes, which had an higher content in crude proteins (20-35%).

Kobayashi et al. (2008) correlated the dominance of *Coprothermobacter* during thermophilic anaerobic digestion of waste activated sludge to the high protein content (around 15 g/L) of the feed.

In a thermophilic anaerobic reactor of a two-phased AD treating activated sludge, a temperature shift from 50°C to 65°C caused a change in bacterial population from *Thermotogae* sp. to *Coprothermobacter* and *Lutispora thermophila*, and their presence was related to an increased hydrolysis, since both microorganisms carry out amino-acid fermentation (Pervin et al., 2013); the increased protein hydrolysis was connected to better digestion performances. A similar finding was reported by Ge et al. (2012), due to a temperature shift from 55°C to 70°C in a thermophilic anaerobic digester fed with WAS. At 55°C the dominant organism were affiliated to *Clostridia*, *Thermotogae* and *Sphingobacteria*, while at 70°C *Coprothermobacter* outcompetes and dominated in the thermophilic reactor.

Cheon et al. (2007) compared microbial population dynamics in thermophilic methane digesters fed with garbage, sewage sludge and feedstock waste, and *Coprothermobacter* was detected only in the first two digesters, with higher and increasing relative abundance in the reactor fed with sewage sludge, in which the organic loading rate (OLR) was significantly lower with respect to the other reactors.

A decrease in relative abundance of *Coprothermobacter* population along time was observed in Gagliano et al., (2014 a), during the thermophilic stage of an innovative two- stage (mesophilic-thermophilic) anaerobic digestion of activated sludge caused by the decreased availability of highly degraded proteins and the increase of VFA concentration.

The negative effect of an increase of VFA concentration on *Coprothermobacter* population was also shown by Tezel et al. (2014). However, the overall proteolityc activity was not markedly affected, as evidenced by the total ammonia concentration (from 980 to 2.184 mg/L).

Lee et al. (2009) connected the presence of *Coprothermobacter* in an hyperthermophilic acidogenic reactor treating kitchen garbage and WAS at 70 °C with to high protein solubilization efficiency in waste activated sludge at this temperature. Indeed, comparing their results on protein degradation with a reactor fed with only kitchen garbage, they highlighted an increase of the protein solubilization efficiency from sludge with respect to kitchen garbage.

*Coprothermobacter* was detected in a full scale thermophilic digester (55°C), when the substrate changed from garbage to sewage sludge (Cheon et al., 2008).

In Sasaki et al. (2007) *Coprothermobacter* was found to be responsible for degradation of low-molecule organic matters in packed-bed reactor degrading organic solid waste.

Guo et al. (2014) compared microbial characteristics of the thermophilic and mesophilic anaerobic digesters exposed to elevated food waste loadings. *Coprothermobacter* was detected in thermophilic digester and its abundance increased with OLR (the OLR of the reactor was gradually elevated with a gradient of 0.5 g VS $L^{-1}$ $d^{-1}$ from the initial level of 1.0 g VS $L^{-1}$ $d^{-1}$).

In the study of Wagner et al. (2013) nine complex organic substrates from three classes (protein-, lipid-, and cellulose-rich) were investigated in batch experiments in order to evaluate their potential for utilization as substrates for biogas production. *Coprothermobacter* clones were detected during batch digestions fed with dosing feed (obtained from an anaerobic digester), lawn-clippings, carboxymethylcellulose and tree-cut (wood and branches); thus, sludge seems to be a good substrate for *Coprothermobacter* growth, but it was also involved in cellulose degradation.

This feature underlines that the source of proteinaceous substrate is of particular importance for *Coprothermobacter* growth.

## 8. Molecular methods for *Coprothermobacter* identification

In most of the previously cited studies, *Coprothermobacter spp.* were identified only by applying PCR based approaches, mainly clonal analysis and DGGE, using universal primers for the domain

Bacteria. Very few studies developed strategies for the specific identification of members of this genus. Specific primer set for Real-time PCR and PCR were recently designed by Kobayashi et al. (2008) and Tandishabo et al. (2012 a) (Table 2) for the detection of *Coprothermobacter* species involved in the anaerobic digestion process.

Regarding the specificities of the two primer pairs with respect to the available *Coprothermobacter* 16S RNA gene sequences collected into the SILVA database, both show a good coverage of the matchable sequences (Table 3 a)..

The primer pair COP1029F and COP1179R was successfully applied also in the work of Tezel et al. (2014).

Lü et al. (2014 b) designed an oligonucleotide probe for Fluorescence In Situ Hybridization (FISH) application (Copro929 probe in Table 2), but the specificity was closely related to 16S rDNA sequences retrieved by clonal analysis in that specific study, and no quantification was carried out using this probe.

**Table 2 -** Oligonucleotide primers and probes targeting the 16S rRNA gene of *Coprothermobacter* genus

| Name | Sequence (5'-3') | Lenght | Application | Reference |
|---|---|---|---|---|
| **COP1029f** | ACA GGT GTT GCA TGG CTG TC | 20 | Real-time PCR | Kobayashi et al., (2008) |
| **COP1179r** | CTC CCC TTC CTC TGG CTC TT | 20 | | |
| **A584F** | GTG GTT GTC TGT GTC TCA | 18 | PCR | Tandishabo et al., (2012 a) |
| **B1138R** | CTA TTG TGC TCT GAC CCT | 18 | | |
| **Copro929** | CUC CGC CGC UUG UGC GGA | 18 | FISH | Lü et al. (2014 b) |
| **CTH485** | TCC CTT TCT ACT GGG GTA | 18 | FISH | Gagliano et al., (2014 b) |
| **hCTH439** | CCG TCC TTC CTC GTC CCC CAG T | 22 | FISH | |

The design of a specific fluorescent probe and an oligonucleotide helper targeting *Coprothermobacter* was extensively described in Gagliano et al. (2014 b) (Table 2). The study also described possible drawbacks in in-situ identification of this microorganism and pointed-out a strategy for the contemporary identification of its syntrophic partner *Methanothermobacter thermoautotrophicus*, for which a specific FISH protocol was previously described in Nakamura et al. (2006). The probe

CTH485 and helper CTH439 were successfully applied on samples taken from thermophilic reactors treating waste activated sludge (Gagliano et al., 2014 a; Gagliano et al., 2014 b). This study allowed for the first time to estimate the relative abundance of *Coprothermobacter* by using FISH quantification. The identification of *Coprothermobacter* in pure culture (a) and in a thermophilic anaerobic sludge (b) by applying CTH485 probe together with helper CTH439 is shown in Figure 1.

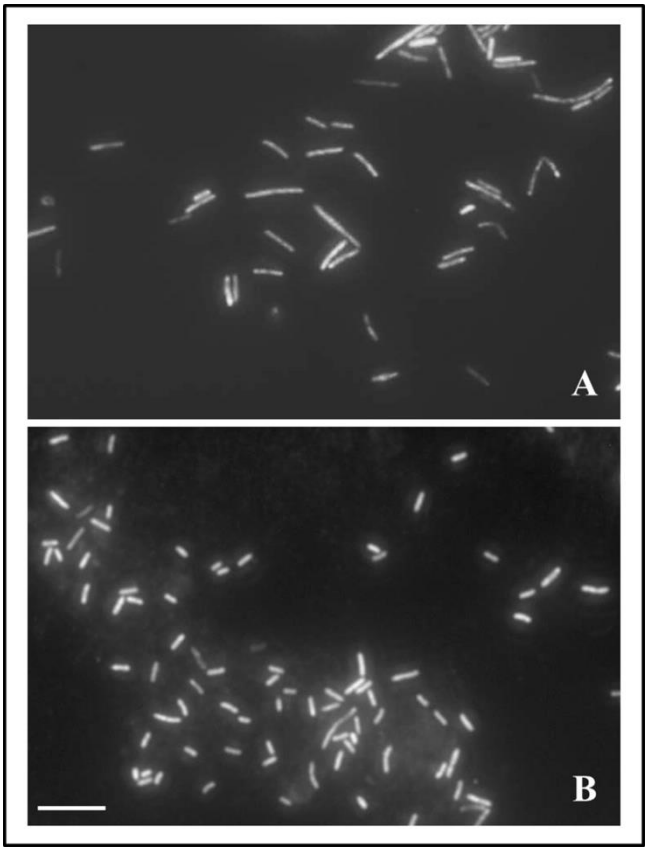


**Fig.1** - FISH analysis of *Coprothermobacter proteolyticus* (DSMZ 5265) (a), and of a thermophilic anaerobic sludge (b) using CTH485 probe together with helper CTH439. Bar is 5 µm.

The current specificities of Copro929 and CTH485 probes are reported in Table 3. CTH485 probe has a lower genus coverage with respect to Copro929 (74.2 vs 90.8 respectively); however the specificity of this probe is higher being the number of outgroup hits markedly lower of those detected by Copro929 probe.

Ho (2014) describe the need of a FISH probe specific for *Coprothermobacter*: it could be used in conjunction with other specific probes to quantitatively determine the syntrophic associations between these bacteria and partner methanogens and their spatial arrangement.

In addition, in situ detection targeting *Coprothermobacter* is an indispensable molecular tool to refine and confirm the data collected from clonal analysis, identifying the metabolically active cells and their variation in relation to operational parameters during different processes; additionally, FISH allow highlight possible changes in cell morphology compared with pure cultured cells, as clearly seen in Figure 1.

**Concluding remarks**

The thermophilic fermentative bacteria *Coprothermobacter* show high metabolic versatility and among the different abilities, the proteolytic activity is the one better described and reported in literature, in particular as regards its role during the fermentative phase of the thermophilic anaerobic digestion process. In perspective, the improvement of thermophilic anaerobic degradation could be obtained with the establishment of *Coprothermobacter* in the microbial community, especially in syntrophic association with methanogenic archaea. This syntrophic association increases the hydrogen production rate of *Coprothermobacter*; therefore, if this ability is exploited in hydrogen production systems, methanogenesis should be inhibited to avoid hydrogen consumption. The ability of *Coprothermobacter* in syntrophic electron transfer is also recently exploited in microbial fuel cells. Recent studies showed that it seems to be also involved in the syntrophic acetate oxidation (SAO), another important step in those thermophilic anaerobic processes in which acetotrophic methanogenesis is lacking.

The identification of *Coprothermobacter* in several different habitats showed that it can survive under extreme conditions, not only at high temperature.

Indeed, its identification in high temperature petroleum reservoirs, its resistance to high concentration of butanol, or its metabolic attitude in iron ad thiosulphate reduction demonstrated the wide versatility of this microorganism. In addition, proteolytic enzymes purified from *Coprothermobacter* showed activity under very extreme conditions, as high temperature and pH. These findings highlighted the ecological importance of members of this genus, also from a biotechnological point of view, with the possible application of *Coprothermobacter* cells or its purified enzymes in industrial processes or chemical formulations.

The study of the ecological role of *Coprothermobacter* has certainly gained more attention in recent years, but it still requires deepen investigation to be fully understood.

To this purpose, the complete genome sequence of *Coprothermobacter proteolyticus* DSM 5265 was recently published (Alexiev et al., 2014); this microorganism was selected in 2002 as part of a

National Science Foundation-funded “Assembling the Tree of Life” project at the Institute for Genomic Research (TIGR) to sequence the genomes of representatives of the seven phyla of bacteria that at the time had cultured representatives but no available genome sequence Additionally, the complete genome sequence of *Coprothermobacter platensis* DSM11748 is currently under investigation inside the project “Continuation of the Genomic Encyclopedia of Bacteria and Archaea pilot project” by the Joint Genome Institute (United States) (NCBI ref. NZ_ARJK00000000.1). A following step investigating about functions of *Coprothermobacter* spp. genes and enzymes its certainly required to set its role and prominency in thermophilic anaerobic processes. In this perspective, the approaches described in the studies of Lü et al. (2014 b) and Ho (2014) (metatranscriptomic and RNA-SIP) are certainly the most promising: the analysis of expression patterns of target genes allows to identify environmental factors that significantly impact the metabolic functions, leading to more rational approaches to optimisation of processes in which *Coprothermobacter* could be involved.

**Table 3 –** Current specificities of primers (a) and probes (b) targeting *Coprothermobacter* group. The primer and probe match was carried out using the TestPrime and TestProbe tools on the SILVA website (www.arb.silva.de). The results are based on the comprehensive rRNA database SILVA (release 117). To obtain the genus coverage value only the matchable sequences were considered (and not the sequences without any data in the probe region).

**(a)**

| Primers | Size of target group | Number of probe hits in target group | Mismatched sequences in the target group | Sequences without any data in the probe region | Genus Coverage (%) | Outgroup hits | Reference |
|---|---|---|---|---|---|---|---|
| **COP1029f COP1179r** | 745 | 345 | 95 | 340 | 79.5 | 6 | Kobayashi et al., (2008) |
| **A584F B1138R** | | 317 | 85 | 378 | 80.1 | 0 | Tandishabo et al., (2012) |

**(b)**

| Probes | Size of target group | Number of probe hits in target group | Mismatched sequences in the target group | Sequences without any data in the probe region | Genus Coverage (%) | Outgroup hits | Reference |
|---|---|---|---|---|---|---|---|
| **Copro929** | 745 | 375 | 38 | 332 | 90.8 | 196199 | Lü et al.,(2014 b) |
| **CTH485** | 745 | 509 | 177 | 59 | 74.2 | 20 | Gagliano et al.,(2014 b) |

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