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ORIGINAL ARTICLE

Genomic insights into members of the candidate phylum Hyd24-12 common in mesophilic anaerobic digesters

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Members of the candidate phylum Hyd24-12 are globally distributed, but no genomic information or knowledge about their morphology, physiology or ecology is available. In this study, members of the Hyd24-12 lineage were shown to be present and abundant in full-scale mesophilic anaerobic digesters at Danish wastewater treatment facilities. In some samples, a member of the Hyd24-12 lineage was one of the most abundant genus-level bacterial taxa, accounting for up to 8% of the bacterial biomass. Three closely related and near-complete genomes were retrieved using metagenome sequencing of full-scale anaerobic digesters. Genome annotation and metabolic reconstruction showed that they are Gram-negative bacteria likely involved in acidogenesis, producing acetate and hydrogen from fermentation of sugars, and may play a role in the cycling of sulphur in the digesters. Fluorescence *in situ* hybridization revealed single rod-shaped cells dispersed within the flocs. The genomic information forms a foundation for a more detailed understanding of their role in anaerobic digestion and provides the first insight into a hitherto undescribed branch in the tree of life.

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Introduction

Production of methane by anaerobic digestion (AD) is widely used to convert organic waste into biogas and forms an important part of the transition from fossil fuel to sustainable energy production. The AD process is divided into four sequential steps that are performed by specialized microbes: hydrolysis, fermentation (acidogenesis), acetogenesis (dehydrogenation) and methanogenesis (acetoclastic or hydrogenotrophic) (Angenent *et al.*, 2004). Hence, the overall function, stability and efficiency of the AD process are dependent on tightly coupled synergistic activities of the complex microbial communities (Schink, 1997; Weiland, 2010). However, the microbial communities in AD are still poorly understood, and relatively little is known about their diversity and function (Chouari *et al.*, 2005; Werner *et al.*, 2011; Sundberg *et al.*, 2013; De Vrieze *et al.*, 2015). In addition, most of the microorganisms have no pure culture representatives, and, given the

synergistic interactions of members of the community, a reductionist approach to understand the ecology of the system is not possible (Kaeberlein *et al.*, 2002; Fuhrman *et al.*, 2015).

The AD environment also harbours extensive diversity of previously uncharacterized bacterial phyla, often known only by their 16S rRNA gene sequence, making it an ideal environment for the study of novel bacterial lineages (Guermazi *et al.*, 2008; Pelletier *et al.*, 2008; Limam *et al.*, 2014; Sekiguchi *et al.*, 2015). New developments in single-cell genomics and metagenomics have in recent years provided a glimpse into the ecology and evolution of many novel candidate phyla (Dinis *et al.*, 2011; Albertsen *et al.*, 2013; Rinke *et al.*, 2013; Brown *et al.*, 2015; Nobu *et al.*, 2015; Sekiguchi *et al.*, 2015). The genomes have enabled construction of metabolic models that attempt to explain the physiology of these organisms in detail. The genome-based models form the basis of more extensive investigations, such as *in situ* single-cell characterization, metatranscriptomics and proteomics (Koch *et al.*, 2014).

In this study, extensive 16S rRNA gene amplicon sequencing was used to screen anaerobic digesters for the presence of members of the Hyd24-12 lineage, which remains one of the few known candidate phyla for which no genomic information is available with nothing known about their morphology, physiology or ecology (Rinke *et al.*, 2013). Selected

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samples were subjected to metagenome sequencing and used for retrieval of three near-complete genomes of Hyd24-12 through differential coverage binning. The genomes were used for detailed metabolic reconstruction and design of oligonucleotide probes for the first *in situ* visualization of these hitherto unrecognized players in AD.

Materials and methods

Sample collection and storage

A total of 306 biomass samples were obtained from 29 anaerobic digesters at 17 Danish wastewater treatment facilities (see Supplementary Table S1). Most digesters were mesophilic (22), whereas 7 were thermophilic. A volume of 50 ml was sampled, homogenized and stored as 2 ml aliquots at -80°C for DNA extraction.

For fluorescence *in situ* hybridization (FISH) analyses, diluted biomass samples (1:4 in $1\times$ phosphate-buffered saline) were fixed with 4% (w/v) paraformaldehyde and stored in 50% (v/v) ethanol/ $1\times$ phosphate-buffered saline solution at -20°C , as previously described by Daims *et al.* (2005).

DNA extraction

DNA was extracted from anaerobic digester sludge using the FastDNA Spin kit for soil (MP Biomedicals, Santa Ana, CA, USA), following the standard protocol except for four times increased bead beating duration and a sludge input volume of 50 μl . These digester-sample-specific modifications to the protocol were found to provide the best trade-off between DNA yield/biomass and DNA integrity (Supplementary Figure S4).

Community profiling with 16S rRNA gene amplicon sequencing

Bacterial community profiling was carried out as recommended by Albertsen *et al.* (2015). The bacterial primers used were 27F (AGAGTTTGAT CCTGGCTCAG) (Lane, 1991) and 534R (ATTACC GCGGCTGCTGG) (Muyzer *et al.*, 1993), which amplify a DNA fragment of ~ 500 bp of the 16S rRNA gene (variable V1–V3 region). PCR amplification was performed using $1\times$ Platinum High fidelity buffer, 400 μM dNTP, 1.5 mM MgSO_4 , 2 mU Platinum Taq DNA Polymerase High Fidelity, 5 μM illumina barcoded V1–V3 adaptor mix (see Supplementary Data 1), and 10 ng template DNA. PCR conditions were 95°C for 2 min, 30 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s, and a final step of elongation at 72°C for 5 min. PCR products were purified using Agencourt AmpureXP (Beckman Coulter, Brea, CA, USA) with a ratio of 0.8 bead solution/PCR solution. Barcoded amplicons were pooled and paired-end sequenced on the Illumina MiSeq platform (v3 chemistry, 2×300 bp). The paired-end reads were trimmed using trimmomatic (v. 0.32) (Bolger *et al.*,

2014) and then merged using FLASH (v. 1.2.11) (Magoč and Salzberg, 2011). The reads were screened for potential PhiX contamination using USEARCH (v. 7.0.1090) (Edgar, 2010). The reads were clustered into operational taxonomic units (OTUs, sequence identity $\geq 97\%$) using USEARCH and subsequently classified using the RDP classifier (Wang *et al.*, 2007) with the MIDAS database (v. 1.20) (McIlroy *et al.*, 2015). Further processing was carried out in the R environment (v. 3.1.2) using the ampvis package (Albertsen *et al.*, 2015) (v. 1.24.0), which wraps a number of packages including the phyloseq package (v. 1.8.2) (McMurdie and Holmes, 2013), ggplot2 (v. 1.0.1), reshape2 (v. 1.4.1) (Wickham, 2007), dplyr (v. 0.4.2), vegan (v. 2.3-0), knitr (v. 1.10.5), Biostings (v. 2.36.1), data.table (v. 1.9.4), DESeq2 (v. 1.8.1) (Love *et al.*, 2014), ggdendro (v. 0.1–15) and stringr (v. 1.0.0). The samples were subsampled to an even depth of 10 000 reads per sample, and the fraction of reads classified as Hyd24-12 was obtained. The survey data are available at the SRA with the accession IDs ERS861217-ERS861224.

In silico analysis of Hyd24-12 source locations

The Genbank IDs of the sequences classified as Hyd24-12 in SILVA (v. 121, 1982 sequences in total) (Quast *et al.*, 2013) were used to download the corresponding Genbank files. The fields 'isolation source' and 'PUBMED' were extracted to classify the sequences as originating from either engineered or natural systems.

Metagenome sequencing, assembly and binning

Illumina TruSeq DNA PCR free libraries were prepared for DNA extracts from three of the mesophilic digesters (Supplementary Table S1) according to the manufacturer's protocol and paired-end sequenced on the Illumina HiSeq 2000 platform (2×150 bp) and Illumina MiSeq platform (v3 chemistry, 2×300 bp). The metagenomic assembly and binning process was carried out as described by Albertsen *et al.* (2013) and detailed at 'madsalbertsen.github.io/mmgenome/'. Unmerged reads were quality-trimmed and filtered using default settings in CLC Genomics Workbench (v. 7.5.1; CLC Bio, Aarhus, Denmark). The metagenomic reads were assembled separately for each plant using default settings in CLC Genomics Workbench. Reads were mapped to the assemblies using default settings in CLC Genomics Workbench. The assemblies and mappings were exported as .fasta and .sam files, respectively. The exported files and the mmgenome workflow script 'data.generation.2.1.0.sh' were used to generate the files necessary for the binning process. 16S rRNA gene sequences were extracted from the assemblies using 'rRNA.sh' and classified using the SINA Alignment service (SILVA v 121) (Pruesse *et al.*, 2012); essential genes were called

using Prodigal (Hyatt *et al.*, 2010). Binning was carried out using differential coverage binning in the R environment (v. 3.1.2, R Core Team, 2016) using the R package ‘mmgenome’ (github.com/MadsAlbertsen/mmgenome v. 0.4.1) (Albertsen *et al.*, 2013). The genome bins were checked for completeness, essential single copy genes and coverage distribution using CheckM (v. 0.9.7) (Parks *et al.*, 2015) and the metrics in the mmgenome package. Average nucleotide identity between the genome bins was calculated using JSpecies (Richter and Rosselló-Móra, 2009), and CRISPR arrays were identified with CRT (v. 1.1) (Bland *et al.*, 2007). The genome sequence data have been submitted to DDBJ/EMBL/GenBank databases under accession numbers LKHB00000000, LKHC00000000 and LKHD00000000.

Genome sequence-based phylogenetic analysis

The genomes were placed within the reference genome tree of CheckM (Parks *et al.*, 2015) (v. 0.9.7) and subsequently visualized in ARB (Ludwig *et al.*, 2004).

Phylogeny of the 16S rRNA gene and FISH probe design

Phylogenetic analysis and FISH probe design were performed with the ARB software package (Ludwig *et al.*, 2004). Potential probes were assessed *in silico* with the mathFISH software (Yilmaz *et al.*, 2011) for hybridization efficiencies of target and potentially weak, non-target matches (Yilmaz *et al.*, 2011). Unlabelled helper probes (Fuchs *et al.*, 2000) were designed for calculated inaccessible regions. Unlabelled competitor probes were designed for single-base mismatched non-target sequences (Manz *et al.*, 1992). The Ribosomal Database Project (RDP) PROBE MATCH function (Cole *et al.*, 2009) was used to identify non-target sequences with indels (McIlroy *et al.*, 2011). Probe validation and optimization were based on generated formamide dissociation curves (Daims *et al.*, 2005), where average relative fluorescent intensities, of at least 50 cells calculated with ImageJ software (National Institutes of Health, New York, NY, USA), were measured for varied hybridization buffer formamide concentrations in increments of 5% (v/v) over a range of 5–50% (v/v) (data not shown). Where available, weak base mismatch non-target axenic cultures were used for probe optimization, otherwise full-scale anaerobic digester sludge was used (Table 1).

FISH

FISH was performed essentially as described by Daims *et al.* (2005). Probes were applied, with recommended competitors and helpers, at the stringency conditions given in Table 1 or their original publications. The NON-EUB nonsense probe was used as a negative hybridization control (Wallner *et al.*, 1993). Oligonucleotide probes were

Table 1 FISH probes designed and optimized in this study

Probe	<i>Escherichia coli</i> pos.	Target group	Coverage ^a	Non-target hits	Sequence (5'–3')	Validated against	Required ^b [FA]%
Hyd24-12_468	468–488	Hyd24-12 clade B-1AC	5/6	0	GTA ACG TCA GGC AAT GGA CAT	Damhusaaen full-scale AD	NA
Hyd24-12_468_h1	450–467	Helper for Hyd24-12_468	NA	NA	ATT GCT CCC ACT GCT GTT C	Damhusaaen full-scale AD	Yes
Hyd24-12_468_h2	489–506	Helper for Hyd24-12_468	NA	NA	CGG GAG CTT TCT CTG GGG	Damhusaaen full-scale AD	Yes
Hyd24-12_659	659–675	Hyd24-12 clade B-1AC	6/6	2	TCC GCA CAC CTC TCC CG	Damhusaaen full-scale AD	NA
Hyd24-12_659_c1	659–675	Competitor for Hyd24-12_659	NA	NA	TCC AGA CAC CTC TCC CG	<i>Phenyllobacterium falsum</i> DSM18556	Yes
Hyd24-12_659_c2	659–675	Competitor for Hyd24-12_659	NA	NA	TCC GTA CAC CTC TCC CG	ND	NA
Hyd24-12_659_c3	659–675	Competitor for Hyd24-12_659	NA	NA	TCC GCT CAC CTC TCC CG	<i>Moricaulis maris</i> DSM4734	Yes
Hyd24-12_659_c4	659–675	Competitor for Hyd24-12_659	NA	NA	TCC GGA CTC CTC TCC CG	ND	ND
Hyd24-12_731	731–748	Hyd24-12 clade zEL51	15/17 ^d	0	ACT TGG CCA GTA TGC CGG	NA	ND
Hyd24-12_842	842–858	Hyd24-12 clades zEL51, Hyd-32 & B9, b18	17/31 ^d	0	CGG CTC TGA AGG GGT TGA	NA	ND
Hyd24-12_842_c1	842–858	Competitor for Hyd24-12_842	NA	NA	CGG CAC TGA AGG GGT TGA	NA	ND
Hyd24-12_842_h1	820–841	Helper for Hyd24-12_842	NA	NA	GAC CYC CAA AAC CYA GTR CCC A	NA	ND
Hyd24-12_842_h2	860–881	Helper for Hyd24-12_842	NA	NA	CGG GGY ACT TAA YGC GTT ARC T	NA	ND

Abbreviations: NA, not applicable; ND, not determined.

^aCoverage of groups as defined in MIDAS Database, Release 1.21 (McIlroy *et al.*, 2015) (see Figure 2). Values given as group hits/ group totals.

^bRequirement of accessory probes: competitor probes assessed on ability of the mismatch alone to prevent non-target binding at the recommended formamide concentration (note: it is recommended that un-validated competitor probes are included); helper probes assessed on ability to improve the fluorescence signal of the associated labelled probe.

^cDetermined optimal formamide concentration for use in FISH hybridizations.

^dTogether, these probes cover 26/31 sequences of the Hyd24-12 clades zEL51, Hyd-32 and B9, b18.

labelled on both the 3' and 5' ends with either 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) or with the sulphoindocyanine dyes (Cy3 and Cy5) (DOPE-FISH) (Stoecker *et al.*, 2010). Microscopic analysis was performed with an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Genome analysis

Genome annotation was performed in the 'MicroScope' annotation pipeline (Vallenet *et al.*, 2009, 2013). Automatic annotations were manually curated for all genes described using the integrated bioinformatics tools and the proposed annotation rules, which include an amino acid identity of at least 40% to classify homologues and an identity of at least 25% with the support of conserved domains to determine putative homologues (Vallenet *et al.*, 2009, 2013). The set of bioinformatics tools includes BlastP (Altschul *et al.*, 1990) homology searches against the full non-redundant protein sequence databank UniProt (Uniprot Consortium, 2014) and against the well-annotated model organisms *Escherichia coli* K-12 and *Bacillus subtilis* 168 (Vallenet *et al.*, 2013), enzymatic classifications based on COG (Tatusov *et al.*, 2003), InterPro (Mitchell *et al.*, 2015), FIGFam (Meyer *et al.*, 2009) and PRIAM (Claudel-Renard *et al.*, 2003) profiles, and prediction of protein localization using the TMHMM (Sonnhammer *et al.*, 1998), SignalP (Bendtsen *et al.*, 2004) and PSORTb (Gardy *et al.*, 2005) tools. Synteny maps (i.e. conservation of local gene order) were used to validate the annotation of genes located within conserved operons (Vallenet *et al.*, 2009). Metabolic pathways were subsequently identified

with the assistance of the integrated MicroCyc (Vallenet *et al.*, 2009) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa *et al.*, 2014).

Results and discussion

Survey of 16S rRNA genes of Hyd24-12 in anaerobic digesters

The survey of 22 full-scale mesophilic and 7 thermophilic anaerobic digesters from 17 Danish wastewater treatment plants over 3 years revealed that members of the Hyd24-12 lineage were stably present in most mesophilic but no thermophilic anaerobic digesters (Figure 1). In most mesophilic digesters, they were among the five most abundant bacterial OTUs and constituted around 1–3% and, in some cases, up to 8.2% of all sequenced bacterial reads (see Supplementary Figure S1). No 16S rRNA gene sequences from Hyd24-12 were detected in the incoming surplus sludge from the activated sludge treatment plants, which demonstrates that these bacteria were actively growing in the digesters. The other abundant bacterial phyla in the mesophilic digesters were Actinobacteria, Firmicutes, Chloroflexi, Synergistetes and Bacteroidetes (Figure 1). The best (LCA) classification is shown in Figure 1, but the lack of closely related organisms in the databases and a curated taxonomy hampers taxonomic classification for a number of the most abundant OTUs. In general, the abundance stability of these top genera was high, and that may be due to relatively similar growth conditions for all digesters: feed was primary sludge and surplus activated sludge, temperature in the interval 34–37 °C, pH 7.1–8.2 and total ammonium 0.57–1.1 g N/l (see Supplementary Table S1).

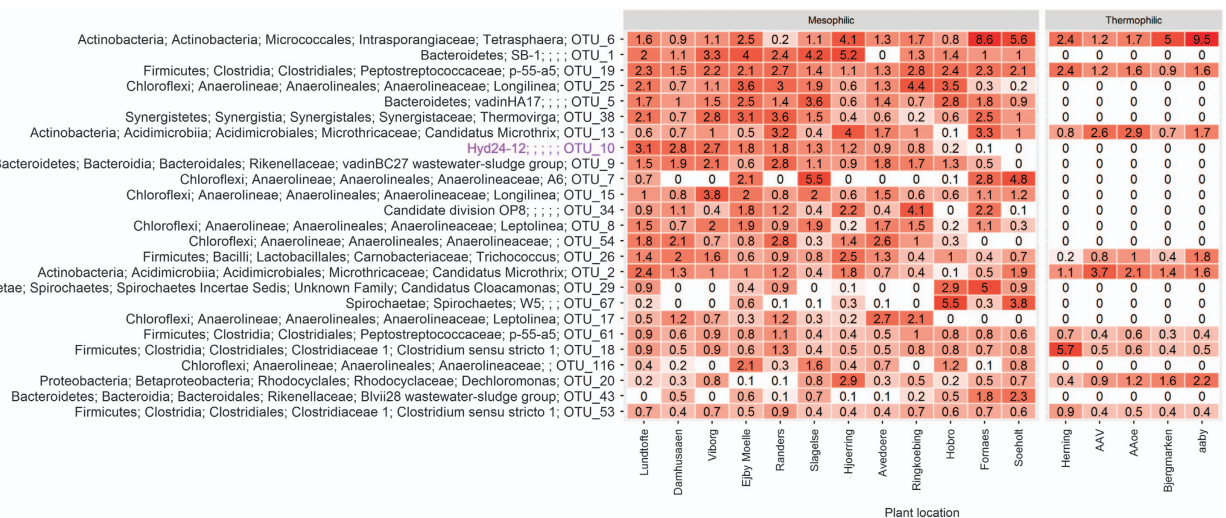


Figure 1 Heatmap of the 25 most abundant bacterial OTUs in mesophilic digesters at wastewater treatment plants along with their abundance in thermophilic digesters at 17 wastewater treatment plants. The OTU classified as belonging to the Hyd24-12 candidate phylum (purple) was detected exclusively in mesophilic reactors. Classification levels presented are phylum, class, order, family and genus and are separated by a semicolon. The field is empty where no classification at a given level could be provided. The abundance profiles show mean abundances for plants with more reactors (1–4 reactors at each WWTP) and 2–97 samples for each plant over 4 years (Supplementary Table S1). The OTUs are sorted on the basis of the mean abundance across the mesophilic samples.

In silico analysis of 16S rRNA gene sequences within Hyd24-12 of SILVA (Quast *et al.*, 2013) from other surveys confirmed that members of Hyd24-12 are widespread in anaerobic environments. The sequences originate from 48 separate studies, with engineered systems such as anaerobic bioreactors accounting for 10 studies, and natural systems such as marine sediments, microbial mats in hydrogen, methane-rich waters and mud volcanoes accounting for 38 studies (see Supplementary References). Furthermore, the 48 studies show that members of Hyd24-12 are globally dispersed (Supplementary Figure S3 and Supplementary Table S2) and are potentially important in many microbial ecosystems besides ADs (Mills *et al.*, 2005; Harris *et al.*, 2012). Some of the surveys of full-scale anaerobic digesters detected some Hyd24-12 sequences (e.g., De Vrieze *et al.*, 2015), while others did not (Sundberg *et al.*, 2013). This was likely because they used the RDP database, where Hyd24-12 sequences are classified as ‘unclassified bacteria’.

Recovering genomic information from Hyd24-12

Three full-scale anaerobic digesters were sampled for metagenomic analyses. To ensure differential abundance of microorganisms needed to bin genomes based on coverage profiles (Albertsen *et al.*, 2013), biomass samples were either taken from the sludge and foam layer of reactors or from the same reactor weeks apart. More than 50 gigabases of metagenomic data were generated, and population genomes were recovered by differential coverage binning (Albertsen *et al.*, 2013) from each of the three plants (Table 2). The three population genomes were ~2.2 Mbp with a GC content of ~64%, and the completeness of the genomes were estimated by CheckM (Parks *et al.*, 2015) to be between 86% and 91% with less than 2.2% estimated contamination (Table 2). However, the level of completeness may be underestimated, given that members of the Hyd24-12 are distantly related to other characterized

organisms, and the genes used in the marker sets might be too divergent or simply not present (Rinke *et al.*, 2013; Brown *et al.*, 2015; Sekiguchi *et al.*, 2015). The three genomes each contained a single rRNA operon and shared identical 16S rRNA gene sequences, which suggests that they belong to the same species (Yarza *et al.*, 2014). The JSpecies program determined that these three genomes shared between 99.8% and 99.9% average nucleotide identity (ANIb), supporting the close taxonomic relationship observed from the 16S rRNA gene analysis (Kim *et al.*, 2014). In order to further evaluate the similarity between the strains, the raw metagenome reads from each digester were mapped to the assembled Hyd24-12 genomes obtained from the other two digesters. Complete coverage of all genomes with the metagenome reads from the other digester revealed that the Hyd24-12 genomes were almost identical. This also indicates, along with the high ANIb, that the genomes are more complete than estimated in Table 2 by CheckM. Indeed, the data suggested that the three strains might actually be variants of the same strain with single-nucleotide polymorphisms only. This is very interesting as the digesters were from different parts of Denmark without any exchange of sludge or feed. This could indicate that they are highly adapted to the specific AD environment in this type of mesophilic digesters.

Hyd24-12 phylogeny, FISH probe design and morphology

The 16S rRNA genes obtained have a sequence identity of 86% with the original clone Hyd24-12 sequence (AJ535232) (Knittel *et al.*, 2003) and classify to the Hyd24-12-lineage (Figure 2a). Additional phylogenetic analyses, based on the genome sequence, placed the Hyd24-12 genomes within the Fibrobacteres-Chlorobi-Bacteroidetes superphylum (Figure 3). The Hyd24-12 genomes are distantly related to all currently available genomes, supporting its status as a novel phylum.

Table 2 Genome statistics for the three Hyd24-12 genomes

Genome bin identifier	Dam_1	Ran_1	Vib_1
Source digester	Damhusaaen	Randers	Viborg
Closest env. 16S clone	6E6_cons (EF688250)	6E6_cons (EF688250)	6E6_cons (EF688250)
No. of contigs	247	168	224
Total length	2 182 231	2 013 453	2 188 467
Longest contig	90 522	171 008	113 480
N50 (contigs)	27 448	38 589	27 030
GC (%)	63.8	64.2	63.7
Genome completeness (%) ^a	86.1	90.7	90.7
Genome contamination (%) ^a	2.17	1.09	2.17
No. of tRNA genes	44	41	48
rRNA genes found in genome	5S, 16S, 23S	5S, 16S, 23S	5S, 16S
No. of CDS	2336	1995	2349
No. of CRISPR array	2 (33 repeats in total)	0	0
Coding density (%)	91.8	93.4	92.8

^aEstimates provided by CheckM.

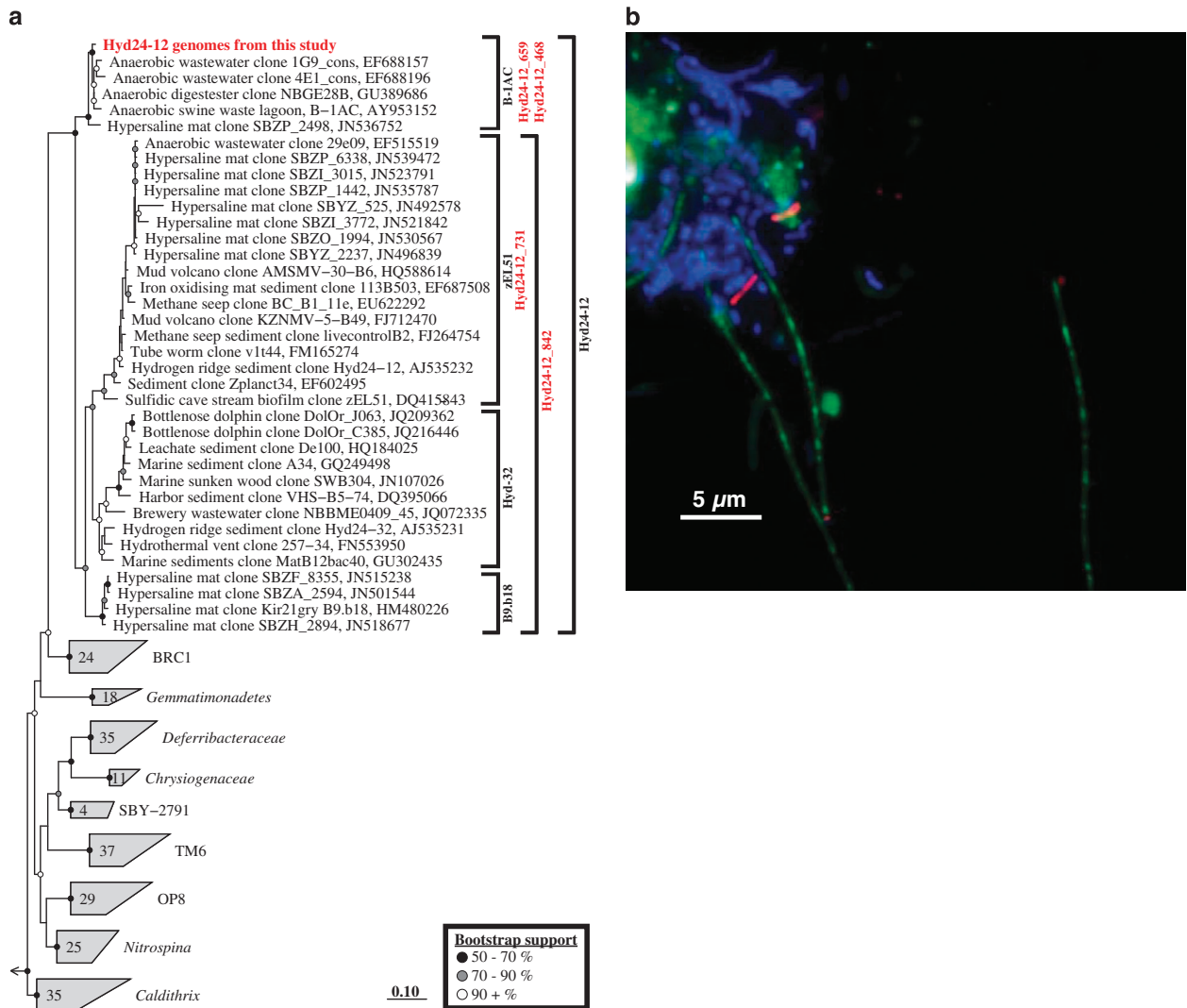


Figure 2 Phylogenetic analysis and design of FISH probes. **(a)** Maximum-likelihood (PhyML) 16S rRNA gene phylogenetic tree of target groups and selected related sequences (all > 1200 bp). Phylogenetic classification is taken from the MiDAS database (Release 1.21), which is a version of the SILVA database (Release 119 NR99) (Quast *et al.*, 2013) curated for activated sludge sequences (McIlroy *et al.*, 2015). Clades of the Hyd24-12 lineage are shown in red. Phylogenetic classification is taken from the MiDAS database (Release 1.21), which is a version of the SILVA database (Release 119 NR99) (Quast *et al.*, 2013) curated for activated sludge sequences (McIlroy *et al.*, 2015). Clades of the Hyd24-12 lineage are shown in red. Probes covering clades are shown in red. The scale bar represents substitutions per nucleotide base. Bootstrap values from 100 re-samplings are indicated for branches when > 50%. **(b)** Composite FISH micrographs of the B-1AC clade members in the Randers anaerobic digester sludge. B-1AC cells appear red (Hyd24-12_659, Cy3), other bacterial cells appear blue (EUBmix probe set (Amann *et al.*, 1990; Daims *et al.*, 1999), Cy5) and archaeal cells appear green (ARCH915 (Stahl and Amann, 1991), FLUOS).

Several FISH probes were designed to target different clades within the phylum. In the MiDAS taxonomy (v. 1.21) (McIlroy *et al.*, 2015), a version of the SILVA taxonomy (Quast *et al.*, 2013) that is curated for activated sludge-related organisms, the Hyd24-12 lineage is delineated into four clades, designated B-1AC, zEL51, Hyd-32 and B9.18. The Hyd24-12_468 and Hyd24-12_659 probes were designed to cover the B-1AC clade, which includes the Hyd24-12 genome sequences obtained in this study (Figure 2). The former probe covers almost all the B-1AC sequences, with the closest non-target sequence match having three internal base mismatches. The Hyd24-12_659 probe is less specific, having one perfectly matched non-target sequence and several with mismatches not covered by the

competitor probes. Overlap in the coverage of these two probes, labelled with different fluorochromes, allows greater confidence in their specificity. A suitable probe to cover the entire Hyd24-12 lineage was not found. However, the Hyd24-12_731 and Hyd24-12_842 probes provide good coverage of the other sequences in the phylum (see Table 1). As sequences covered by these additional probes were not detected in the full-scale anaerobic digesters studied here, optimization and assessment of these probes were not pursued.

When applied to several full-scale anaerobic digester sludge samples, the Hyd24-12_468 and Hyd24-12_659 probes hybridized to small rods, approx. $2 \times 0.4 \mu\text{m}$ in size, dispersed through the flocs (see Figure 2b). Good overlap was observed for

these probes, supporting their specificity. Of the two probes, a much higher signal was observed for the Hyd24-12_659 probe. There was no observed overlap between the signal of two Hyd24-12 probes and the universal bacterial EUBmix probe set (see Figure 2b), which is supported by the absence of the target site for the probes of the latter in the Hyd24-12 sequences. Quantitative FISH was very difficult to carry out in the digesters due to high levels of background fluorescence. Instead, abundance estimates were carried out for the domains Bacteria, Archaea, Eukarya, and the Hyd24-12 lineage, based on read mapping from the PCR free metagenomes to the 16S rRNA genes of the MiDAS database. It showed that Archaea constituted 4–9% of the reads in sludge samples and 7–13% in foam samples. Reads from the Hyd24-12 lineage constituted 0.4–3.5% in the different samples (Supplementary Table S3).

Morphology and motility

The rod shape morphology of B-1AC clade organisms observed by FISH is supported by *mreBCD* and *mrdAB* operons in the Hyd24-12 genomes (see Supplementary Data 2). These operons encode proteins involved in the formation of membrane-bound actin filaments, which are essential for the biogenesis of rod-shape stabilizing peptidoglycans

along the lateral cell wall of rod-shaped bacteria (Kruse *et al.*, 2003, 2005; Osborn and Rothfield, 2007; Bendezú and de Boer, 2008).

The cell envelope characteristics of genome-sequenced bacteria can be determined based on PFAM protein families that are substantially enriched or depleted in archetypical monoderm lineages relative to archetypical diderm lineages (Albertsen *et al.*, 2013). A search for such protein families in the Hyd24-12 genomes revealed an archetypical diderm cell envelope with lipopolysaccharides (see Supplementary Figure S2).

None of the Hyd24-12 genomes encode any flagella-related proteins, suggesting limited motility. However, genes associated with type IV pili were identified using the PilFind algorithm (see Supplementary Data 2) (Imam *et al.*, 2011). These pili enable the bacteria to generate surface-associated twitching motility. This allows them to move effectively through environments that contain shear-thinning viscoelastic fluids, such as the extracellular polymeric substances of biofilms (Conrad *et al.*, 2011; Jin *et al.*, 2011). In addition to motility, type IV pili play a role in the attachment to living and non-living surfaces, including those of other bacteria (Giltner *et al.*, 2012).

No genes associated with spore formation were detected in the Hyd24-12 genomes. This suggests that the Hyd24-12 genomes investigated represent non-sporulating bacteria.

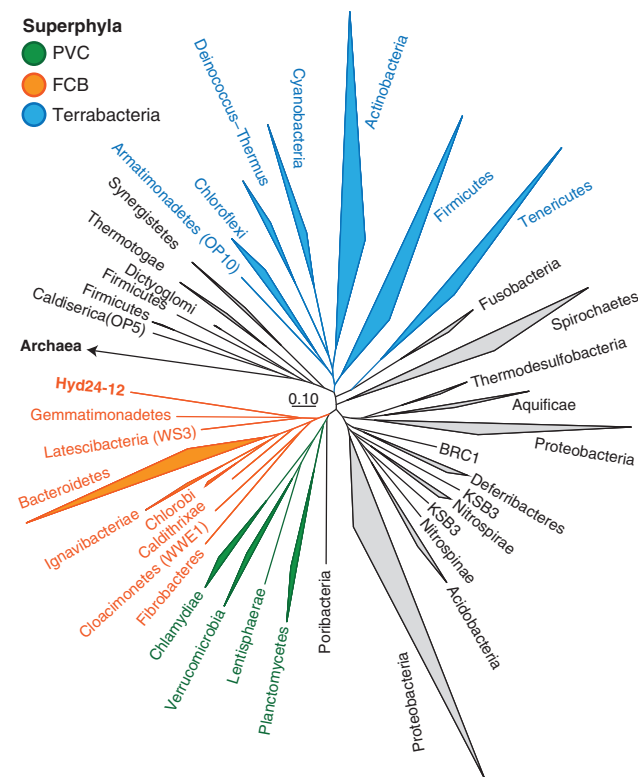


Figure 3 Phylogenetic position of the Hyd24-12 genomes in the reference genome tree generated by CheckM. The CheckM tree is inferred from the concatenation of 43 conserved marker genes and incorporates 2052 finished and 3604 draft genomes from the IMG database (Parks *et al.*, 2015).

Energy metabolism

The three genomes do not contain any genes for respiration with oxygen, nitrate/nitrite or Fe(III) and seem primarily to have a fermentative metabolism. However, the genomes indicate that the organisms may be able to use elemental sulphur as an electron acceptor, see below. The Hyd24-12 genomes encode a complete glycolysis pathway, along with the non-oxidative branch of the pentose phosphate pathway (Figure 4 and see Supplementary Data 2). This allows Hyd24-12 to potentially catabolize a wide range of hexoses and pentoses to pyruvate, thereby providing the cell with energy in the form of ATP and reducing equivalents in the form of NADH (Stincone *et al.*, 2014). The sugars are probably obtained from the environment through a major facilitator superfamily transporter at the expense of the proton motive force (Madej, 2014; Wisedchaisri *et al.*, 2014). The transporter does not share similarity (>30%) with any experimentally validated transporters, and it is therefore impossible to infer a specific substrate preference. It is known that primary sludge and activated sludge fed into the digesters contain many different polysaccharides (Raunkjaer *et al.*, 1994; Frølund *et al.*, 1996). No genes encoding for extracellular glycosylases were identified, which might indicate that Hyd24-12 is reliant on the hydrolytic action of other organisms present within the anaerobic digesters.

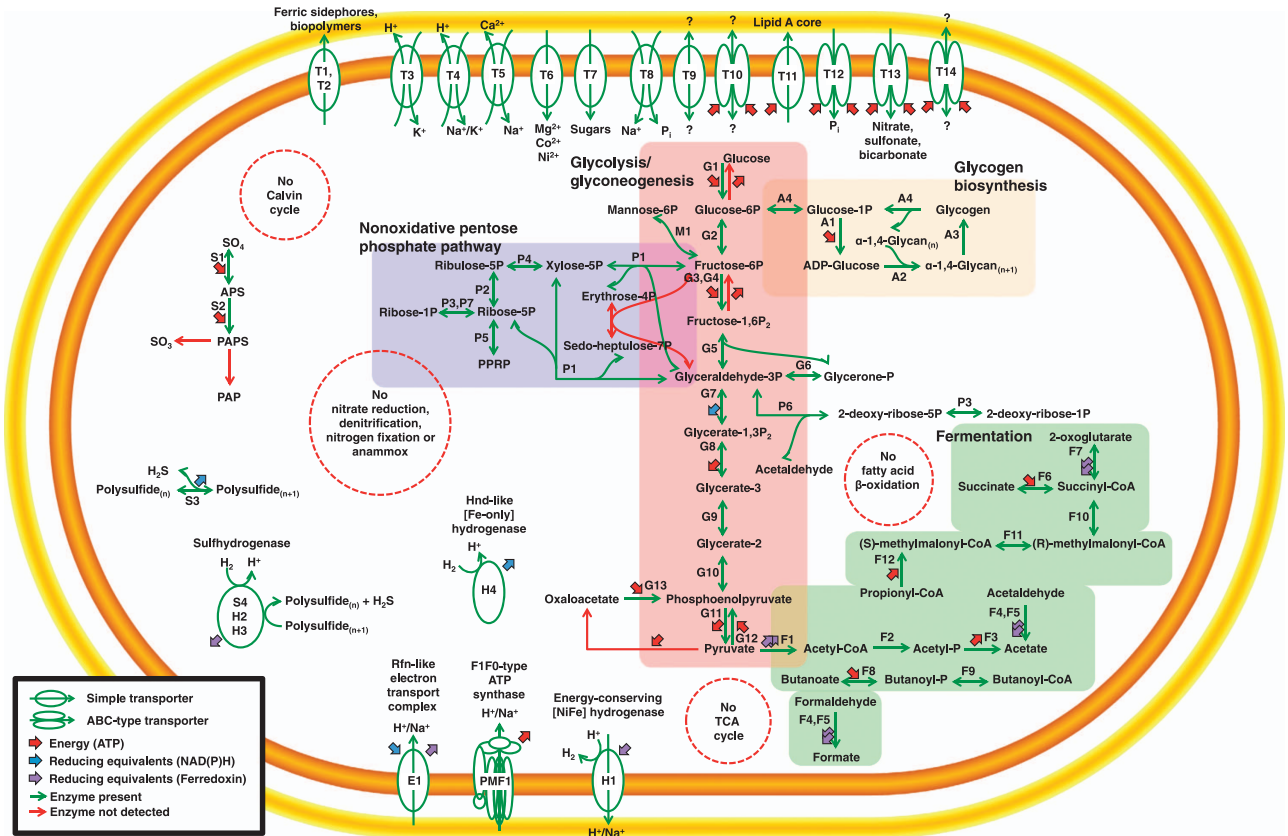


Figure 4 Metabolic model of Hyd24-12 species in mesophilic anaerobic digesters, based on the annotated genome sequences. Selected metabolic pathways important for the bacteria in the anaerobic digestion process are highlighted. Numbers correspond to annotated genes in Supplementary Data 1.

Hyd24-12 encodes for the complete pathway for glycogen biosynthesis and catabolism (Figure 4 and see Supplementary Data 2) (Preiss *et al.*, 1983; Wilson *et al.*, 2010). Hence, glycogen may serve as a carbon and energy storage which can be utilized to mitigate fluctuations in substrate availability. The Hyd24-12 genomes did not encode for pathways for other storage compounds such as trehalose or polyhydroxyalkanoates.

There are limited catabolic options for the pyruvate formed, for example, by glycolysis. The tricarboxylic acid cycle of Hyd24-12 is incomplete (8 of 10 key enzymes are missing) and probably non-functional. However, pyruvate can be converted into acetyl-CoA by a pyruvate ferredoxin oxidoreductase, providing additional reducing equivalents in the form of reduced ferredoxin (Figure 4 and see Supplementary Data 2) (Menon and Ragsdale, 1997). Acetyl-CoA can then be converted into acetate by the action of phosphate acetyltransferase and acetate kinase, thus providing the bacterium with additional ATP (Latimer and Ferry, 1993; Mai and Adams, 1996).

All three Hyd24-12 genomes also encode for two aldehyde ferredoxin oxidoreductases (Figure 4 and see Supplementary Data 2). These may be used to oxidize formaldehyde and acetaldehyde to formate and acetate, respectively, providing the cell with

energy in the form of additional reduced ferredoxin (Mukund and Adams, 1991). However, the enzyme may also be used in the reverse reaction to regenerate oxidized ferredoxin. The presence of a membrane-embedded, energy-conserving hydrogenase allows the cell to establish a proton motive force, based on the energy-rich reduced ferredoxin, which reduces H^+ to H_2 in the process (Strittmatter *et al.*, 2009). The energy stored in the proton motive force may then be harvested through an ATP synthase to yield ATP.

High concentrations of H_2 inhibit glycolysis and acidogenesis due to thermodynamic considerations (Huang *et al.*, 2015). Hyd24-12 therefore needs a way to remove excess H_2 . This can be achieved by syntrophic association with other microorganisms, or internally by the action of a cytosolic hydrogenase, which couples the oxidation of H_2 with the reduction of NAD^+ (Figure 4 and see Supplementary Data 2). Alternatively, Hyd24-12 may employ a sulfhydrogenase to couple the oxidation of H_2 to H^+ with the reduction of elemental sulphur (S^0) or polysulphide to hydrogen sulphide (H_2S) as is seen for *Pyrococcus furiosus* (Mukund and Adams, 1991). The genomes do not indicate a potential for sulphate reduction. Elemental sulphur is continuously produced in the digesters because activated sludge fed into the digesters contains oxidized iron (Fe(III)), which in the presence of sulphide produces S^0 and

black iron sulphide (FeS) (Rasmussen and Nielsen, 1996; Nielsen *et al.*, 2005; Omri *et al.*, 2011). Sulphide is a normal compound in digesters and is produced from amino acids and reduction of sulphate. Notably, other studies have also detected members of the Hyd24-12 phylum in sulphur-rich environments such as hydrothermal vents, sulphur-rich springs and sediments (Elshahed *et al.*, 2003; Schauer *et al.*, 2011; Pjevac *et al.*, 2014). Thus, Hyd24-12 related organisms potentially play a role in sulphur transformations in digesters and other environments. Such a role requires further investigation.

The Hyd24-12 genomes do not contain the genes required for fatty acid β -oxidation or for the catabolism of amino acids. Sugars are therefore considered the primary energy source of the Hyd24-12 in anaerobic digesters.

Whereas Hyd24-12 is able to take up carbon in the form of amino acids, carbohydrates, etc., it is unable to carry out fixation of CO₂ as such genes are missing.

Amino acid and nitrogen metabolism

Based on the genome annotations, Hyd24-12 is only predicted to be able to synthesize few amino acids (glycine, serine, cysteine, threonine, asparagine, aspartate, glutamate and glutamine). Accordingly, Hyd24-12 might rely on amino acids present within the environment. As most amino acids are found as proteins, which cannot be taken up by the bacterium, Hyd24-12 needs a way to degrade these polymers, and this is achieved by the action of multiple extracellular proteases encoded in the genome, which are likely secreted in a Sec- or Tat-dependent mechanism (Natale *et al.*, 2008) (see Supplementary Data 2). The cells may subsequently import the amino acids using ABC-transporters encoded in the genome. Owing to the lack of experimentally validated homologues from closely related species, it is not possible to predict the substrate specificity of these transporters. A reduced capacity of microorganisms for synthesizing amino acids is known from strict symbionts and, recently, also from a number of candidate phyla with very small genomes (<1 Mbp) (Brown *et al.*, 2015). However, the relatively large size of the Hyd24-12 genomes (~2.2 Mbp) and their dispersed growth in the anaerobic sludge suggest that they are not strict symbionts.

Hyd24-12 does not have the necessary pathways for fixation of nitrogen. The nitrogen metabolism of Hyd24-12 is generally limited. Amino acids may also represent a source of nitrogen. However, nitrogen can also be obtained from ammonium assimilation via the glutamine synthetase/glutamate synthase pathways (Bravo and Mora, 1988).

Oxidative stress protection

The three Hyd24-12 genomes each contains a gene cluster encoding for a superoxide reductase, nitric oxide reductase and ferroxidase. These genes are

probably involved in resistance against oxidative stress, and may allow the bacteria to survive in the presence of oxygen. However, 16S rRNA gene sequences from Hyd24-12 have only been observed in oxygen-depleted environments.

Ecological significance and concluding remarks

This study applied metagenomic sequencing to obtain genomes from the candidate phylum Hyd24-12 and provides the first morphological and physiological information for the lineage. Members of the phylum were shown to be very abundant and stably present in mesophilic anaerobic digesters, occasionally accounting for the most abundant OTU in the samples, but absent in thermophilic reactors. This indicates that they are likely to play a substantial role in the ecology of mesophilic AD systems at wastewater treatment plants fed with primary sludge and surplus activated sludge. Metabolic reconstruction based on the genomic information showed that members of Hyd24-12 are likely to be fermenters relying on simple sugars. In addition, they may also use elemental sulphur as an electron acceptor, thus forming part of the microbial cycling of sulphur in anaerobic systems and partly responsible for production of hydrogen sulphide. Sulphide is unwanted in the biogas due to toxicity and corrosion (Syed *et al.*, 2006), but will also provide more elemental sulphur by reacting with incoming Fe(III). In that case, members of Hyd24-12 may compete with the methanogens for organics. An *in silico* investigation of environmental 16S rRNA gene surveys suggests that members of the phylum are present in anaerobic environments, often associated with sulphurous compounds and methane production, such as sediment mats and anaerobic bioreactors. The fact that the genomes are auxotrophic for several amino acids and lacking putative secreted glycoside hydrolases also indicates a strict reliance on other organisms for nutrients. The genomes generated in this study provide the foundation for future detailed analyses of members of the phylum, such as metatranscriptomics and metaproteomics. The design of FISH probes for the phylum also revealed their morphology and spatial arrangement in anaerobic digesters and will also facilitate future *in situ* investigations of the phylum in digesters and other environments.

Phylogenetic and genomic analyses of the three Hyd24-12 genomes classified them as a single species within a novel phylum located within the Fibrobacteres-Chlorobi-Bacteroidetes superphylum.

We propose the following taxonomic names for the novel genus and species of Hyd24-12:

- '*Candidatus Fermentibacter*' gen. nov.
- '*Candidatus Fermentibacter daniensis*' gen. et sp. nov.

Based on this, we propose the following names for the phylum, class, order, and family:

- ‘*Candidatus Fermentibacteria*’ phyl. nov.
- ‘*Candidatus Fermentibacteria*’ classis nov.
- ‘*Candidatus Fermentibacterales*’ ord. nov.
- ‘*Candidatus Fermentibacteraceae*’ fam. nov.

Etymology. *Fermentibacter* (Fer.men.ti.bac'ter. M.L. n. ferment -um to ferment, Gr. dim. n. bakterion a small rod, M.L. neut. n. Fermentibacter a small fermenting rod-shaped bacterium). *Fermentibacter daniensis* (da.ni. ensis. M.L. fem. adj. daniensis, pertaining to Dania, the Medieval Latin name for the country of Denmark, where the species was first discovered).

Conflict of Interest

The authors declare no conflict of interest.

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