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ORIGINAL ARTICLE

Genome sequencing of a single cell of the widely distributed marine subsurface *Dehalococcoidia*, phylum *Chloroflexi*

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Bacteria of the class *Dehalococcoidia* (DEH), phylum *Chloroflexi*, are widely distributed in the marine subsurface, yet metabolic properties of the many uncultivated lineages are completely unknown. This study therefore analysed genomic content from a single DEH cell designated 'DEH-J10' obtained from the sediments of Aarhus Bay, Denmark. Real-time PCR showed the DEH-J10 phylotype was abundant in upper sediments but was absent below 160 cm below sea floor. A 1.44 Mbp assembly was obtained and was estimated to represent up to 60.8% of the full genome. The predicted genome is much larger than genomes of cultivated DEH and appears to confer metabolic versatility. Numerous genes encoding enzymes of core and auxiliary beta-oxidation pathways were identified, suggesting that this organism is capable of oxidising various fatty acids and/or structurally related substrates. Additional substrate versatility was indicated by genes, which may enable the bacterium to oxidise aromatic compounds. Genes encoding enzymes of the reductive acetyl-CoA pathway were identified, which may also enable the fixation of CO_2 or oxidation of organics completely to CO_2. Genes encoding a putative dimethylsulphoxide reductase were the only evidence for a respiratory terminal reductase. No evidence for reductive dehalogenase genes was found. Genetic evidence also suggests that the organism could synthesise ATP by converting acetyl-CoA to acetate by substrate-level phosphorylation. Other encoded enzymes putatively conferring marine adaptations such as salt tolerance and organo-sulphate sulfohydrolysis were identified. Together, these analyses provide the first insights into the potential metabolic traits that may enable members of the DEH to occupy an ecological niche in marine sediments.

Introduction

Marine subsurface sediments harbour immense quantities of microbial cells with the most recent estimate suggesting that they contain prokaryotic cell numbers equivalent to those estimated for the ocean water column and terrestrial soils, separately (Kallmeyer *et al.,* 2012). These vast numbers suggest that microbes in the marine subsurface are key catalysts in global biogeochemical cycles, especially on geological timescales (D'Hondt *et al.,* 2002; Wellsbury *et al.,* 2002; D'Hondt *et al.,* 2004). To date, numerous investigations have shed light on the phylogenetic composition of microbial life within marine subsurface sediments. From these investigations, it has repeatedly emerged that bacteria affiliated with the phylum *Chloroflexi* are widely distributed and in some cases represent up to 80% of the bacterial 16S rRNA gene sequences in deep sediments (Parkes *et al.,* 2005), and average ∼17% of the bacterial 16S rRNA gene sequences recovered from various sites and depths (Fry *et al.,* 2008). *Chloroflexi* are hence of particular interest in terms of understanding microbial life and biogeochemical cycles within the marine subsurface. In spite of this, essentially nothing is known about the metabolic properties or ecological roles of marine subsurface *Chloroflexi* because these bacteria continue to evade cultivation in the laboratory (D'Hondt *et al.,* 2004; Toffin *et al.,* 2004; Batzke *et al.,* 2007; Webster *et al.,* 2011).

Among the *Chloroflexi*, sequences affiliated with a distinct class-level clade known as the *Dehalococcoidia* (DEH) (previously known as the *Dehalococcoidetes*) (Löffler *et al.,* 2012) are the most widespread and frequently detected in the marine subsurface (Inagaki *et al.,* 2003; Parkes *et al.,* 2005; Inagaki *et al.,* 2006; Webster *et al.,* 2006; Biddle *et al.,* 2008; Nunoura *et al.,* 2009; Blazejak and Schippers 2010; Biddle *et al.,* 2011). To date, our knowledge about the metabolic properties of members of this clade is derived from several closely related cultivated strains, that is, *Dehalococcoides mccartyi* strains (Löffler *et al.,* 2012), *Dehalogenimonas lykanthroporepellens* strains (Moe *et al.,* 2009), *Dehalogenimonas alkenigignens* strains (Bowman *et al.,* 2012) and 'Dehalobium chlorocoercia' strain DF-1 (May *et al.,* 2008). These isolates are unified by their ability to grow via organohalide respiration, that is, they use halogenated organic compounds as terminal electron acceptors while using hydrogen as an electron donor in an anaerobic respiration (Tas *et al.,* 2010). In addition, other relatively closely related DEH, including some marine phylotypes, have been implicated in organohalide respiration by enrichment or stable-isotope probing experiments (Fagervold *et al.,* 2005; Watts *et al.,* 2005; Bedard *et al.,* 2007; Fagervold *et al.,* 2007; Kittelmann and Friedrich 2008a, b). Nevertheless, there are numerous 16S rRNA gene sequences within the whole DEH clade that are considerably divergent from these organohalide-respiring organisms. For these diverse and divergent phylotypes, assumptions about their metabolic properties, such as organohalide respiration on the basis of their 16S rRNA phylogeny, are not possible. It is therefore critical that attempts to understand the metabolic properties of these 'unknown' DEH are made.

In this study, a single-cell genomics approach was used to gain access to the genomic content of a marine subsurface DEH bacterium. Methods for single-cell genomics are now well-established and have been applied to microbial cells from various environments (Marcy *et al.,* 2007; Woyke *et al.,* 2009; Swan *et al.,* 2011; Martinez-Garcia *et al.,* 2012; Lloyd *et al.,* 2013). Such an approach is therefore well suited for the study of uncultivated subsurface DEH because it can provide insights into their metabolic potential without the need for cultivation in the laboratory. To this end, we sequenced a large portion of a genome from an uncultivated member of this clade, which was obtained from sediments of Aarhus Bay, Denmark. This enabled us to predict key metabolic and phenotypic properties of the bacterium. It also serves as a reference for other related and unknown DEH organisms, and as a source to understand evolutionary aspects of DEH, such as the distribution of the genetic potential for organohalide respiration and the evolution of central metabolic pathways.

Materials and methods

Sampling, single-cell sorting, whole-genome amplification and PCR screening

All procedures for sediment sampling, extraction of microbial cells from sediments and separation of cells from sediment particles were performed as previously described (Lloyd *et al.,* 2013) and during the same sampling expedition. Marine sediment was collected with a gravity corer on 22 March 2011, from an area of Aarhus Bay (56°9′35.889N, 10°28′7.893 E) characterised by shallow methane gas accumulations below ∼160 cm below sea floor (cmbsf) (Jensen and Bennike, 2009). The water depth at the sampling site was 16.3 m and the water had an *in situ* temperature of 2.5 °C at the sea floor. Sediment from a depth of 10 cmbsf was used for cell extraction and subsequent single-cell sorting. Procedures for single-cell sorting of fluorescently stained cells, cell lysis, whole-genome amplification and PCR screening of single amplified genomes were previously described (Lloyd *et al.,* 2013). The sample processing described in this report were performed during the same sample processing run as reported previously (Lloyd *et al.,* 2013), at the Bigelow Laboratory Single Cell Genomics Center (SCGC, www.bigelow.org/scgc). To outline the main steps of these procedures, cells were extracted from the sediment by diluting 1:5 in $1 \times$ phosphate-buffered saline to form a slurry. The slurry was treated by sonication on ice for 2×20 s by placing the sonicating probe in the ice outside of the tube. The sonicated slurry was further diluted 1:8 with $1 \times$ phosphate-buffered saline, vortexed briefly, and larger sediment particles were allowed to settle for 10 min. The supernatant was collected and sediment particles were further removed by a density gradient centrifugation step, whereby 0.75 ml of a 60% Nycodenz solution (w/v) was injected below the cell suspension with a fine needle and syringe, and was followed by centrifugation $10500 \times g$ for 60 min at 4 °C. The upper phase was then collected, and $1 \times$ TE and 5% w/v glycerol (final concentration) was added. The collected cells were stored at -80 °C and sent on dry ice for single-cell sorting in April 2011.

At the SCGC, cells were were diluted $1000 \times$ in DNA-free Sargasso Sea water and filtered through a 40 μm mesh-size cell strainer (BD Biosciences, San Jose, CA, USA). Cells were stained for up to 120 min with SYTO-9 DNA stain (5 μM; Invitrogen, Carlsbad, CA, USA) and sorted by a MoFlo (Beckman Coulter, Carpenteria, CA, USA) flow cytometer using a 488 nm argon laser for excitation, a 70 μm nozzle orifice and a CyClone robotic arm for droplet deposition. Cells were sorted based on nucleic acid fluorescence and side-scatter, and using the 'purify 0.5 drop' mode for maximal purity. Gates with high fluorescence signals were sorted to minimise the chances of sorting autofluorescent sediment particles. Sorted cells were deposited into

384-well plates containing 600 nl $1 \times$ TE buffer per well and stored at $-80\,^{\circ}\mathrm{C}$ until being subjected to lysis. For each of the 384 wells plate, 315 were used for single cells, 66 were dedicated as negative controls (no droplet deposition) and three received 10 cells each (positive controls).

Sorted cells were lysed by an initial freeze-thawing treatment (five cycles) and further lysed and, DNA was denatured by a cold alkaline KOH solution according to Raghunathan *et al.* (2005). Genomic DNA from the lysed cells was amplified using multiple displacement amplification (MDA) in $10\,\mu l$ final volume with Repliphi polymerase (Epicentre, Madison, WI, USA). The MDA reactions were incubated at $30\,^{\circ}\mathrm{C}$ for 12–16 h and inactivated at $65\,^{\circ}\mathrm{C}$ for 15 min. Kinetics of MDA reactions was monitored by measuring the SYTO-9 fluorescence using a FLUOstar Omega microplate fluorescence reader (BMG Labtech, Cary, NC, USA).

Decontamination procedures for workspaces at the SCGC were performed as previously described (Stepanauskas and Sieracki, 2007) and included bleaching of sheath lines and subsequent flushing with DNA-free deionized water. Ultraviolet treatment of MDA reagents was used to remove high-molecular weight DNA contaminants (Woyke *et al.*, 2011). Cell sorting and MDA setup were performed in a high-efficiency particulate air-filtered environment.

The PCR screening of MDA-derived DNA was performed using PCR with the primers 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) that target most bacteria (Lane 1991). Sequencing of products was aided by adding sequencing primers M13F (5′-GTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGCTATGACC-3′) to the general bacterial primers and using these for priming sequencing reactions (Lloyd *et al.*, 2013). From the 630 sorted 'single cells', 71 high-quality 16S rRNA gene sequences were obtained and one well was selected for further analysis. This well contained *Chloroflexi*-related DNA. The full systematic name of the studied single amplified genome is 'DEH bacterium SCGC AB-539-J10', which we abbreviated to 'DEH-J10' throughout this report.

Sequencing of DNA, quality control of sequencing reads and genome assemblies
In order to obtain a sufficient quantity of DNA for shotgun sequencing, the single-cell MDA-derived DNA was reamplified in a second round of MDA, that is, eight replicate $125\,\mu l$ reactions were performed and then pooled together. Barcoded sequencing was performed by GATC Biotech AG, Konstanz, Germany. Pyrosequencing using 454 chemistry was performed using the Genome Sequencer FLX System (Roche, Branford, CT, USA) generating 119 Mbp (340706 reads). Illumina sequencing was performed using the HiSeq2000 system (Illumina Inc., San Diego, CA, USA) in 50-bp single read mode generating 1.71 Gbp (33.5 M reads).

Unassembled Illumina sequence reads were analysed to identify reads coding for 16S rRNA genes of potentially contaminating microorganisms as described by Lloyd *et al.* (2013). Three reads carrying 16S rRNA gene fragments derived from other single-cell genomes that were sequenced in parallel were detected. Raw 454-pyrosequence reads were also checked, yet no foreign 16S rRNA gene fragments were detected in the 454-pyrosequence data. This suggested a post-sequencing mis-assignment of Illumina reads, that is, bioinformatic mis-assignment of barcoded reads into incorrect data sets due to sequencing errors within barcodes, as opposed to contamination of the original DNA, reagents or materials. Identification of mis-assigned Illumina reads, as well as their removal from the DEH-J10 data set, was conducted as previously detailed (Lloyd *et al.*, 2013). In brief, this involved all-against-all BLASTN searches for all contigs from parallel sequenced single-cell assemblies to identify contigs present in multiple assemblies. These contigs were then inspected for read coverage values, which showed that for the few contigs found in multiple single-cell assemblies, read coverage values were always high for each contig in only one assembly and substantially lower in other assemblies. The assemblies which harboured contigs with high-coverage values were therefore considered the original source of each contig. Mis-assigned reads were removed by mapping reads from the single-cell genome assembly to mis-assigned contigs using *Bowtie 2* (Langmead and Salzberg, 2012). After mis-assigned reads were removed, the remaining Illumina reads were assembled using *SPAdes* assembler version 2.3.0 (Bankevich *et al.*, 2012) (parameters: *-k 21,33,45 –sc*). The 454-pyrosequence reads were dereplicated using cd-hit-454 (Niu *et al.*, 2010) with a 98% similarity cutoff and assembled using *GS De Novo Assembler* version 2.6 (*gsAssembler*, Roche) (parameters: -mi 98 -ml 50). The two assemblies were finally combined using *Sequencher* version 5.0.1 (Genecodes) (Lloyd *et al.*, 2013).

Gene annotations
Automatic gene annotations were initially performed using the MicroScope annotation pipeline (http://www.genoscope.cns.fr/agc/microscope/) (Vallenet *et al.*, 2013) and the RAST server (Aziz *et al.*, 2008). All predicted protein sequences were also extracted from the software platforms and were analysed by BLASTP comparisons against protein sequences from previously annotated reference DEH strains, that is, all DEH-J10 proteins were compared against custom databases of total protein sequences from *D. mccartyi* strain CBDB1, strain 195 and *D. lykanthroporepellens* BL-DC-9, separately, using an e-value threshold of 10^{-10}. All DEH-J10 annotations for protein sequences that provided positive hits and revealed the same annotation as the

previously annotated reference protein sequences were kept, whereas discrepancies were manually inspected and edited. All proteins that were not automatically assigned a function by the automatic annotation platforms were also analysed by BLASTP comparisons against the NCBI non-redundant database in order to assign tentative putative functions to proteins. Protein sequences described in this study were also heavily scrutinised with regards to local genomic context and synteny by manual inspections. Gene annotations described in the Results sections are listed in Supplementary Table 2, and gene numbers are prefixed by 'DEHJ-10'. For many short contigs, the automatic open reading frame (ORF) prediction software did not call complete ORFs and therefore these contigs were binned into a 'fragment' data set. This fragment data set was analysed by BLASTX against the NCBI non-redundant database using an e-value threshold of 10^{-5}.

Additional DNA contamination controls
PCR and sequencing of rRNA genes were also used to check the MDA-derived DNA for contamination by exogenous DNA. MDA-derived DNA from the single cell was diluted 1:50 and screened with primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTGAGTTT-3') targeting 16S rRNA genes of most *Bacteria* (Lane, 1991), primers ARC-8F (5'-TCCGGTTGATCCTGCC-3') and ARC-1492R (5'-GGCTACCTTGTTACGACTT-3') targeting 16S rRNA genes of most *Archaea* (Teske *et al.,* 2002) and primers A (5'-GAAACTGC GAATGGCTCATT-3') and B (5'-CCTTCTGCAGGTT CACCTAC-3') targeting 18S rRNA genes of *Eukarya* (Medlin *et al.,* 1988). PCR products from positive reactions were cloned into pGEM-T Easy Vector System (Promega, Mannheim, Germany) and sequenced via the Sanger method.

In order to assess the genomic origin of assembled contigs, all contigs which had at least one complete ORF predicted (that is, all non 'fragment' data) were examined for the relatedness of their genetic content to genetic content derived from known DEH and other *Chloroflexi* genomes. All predicted proteins and ribosomal genes predicted by the annotation software (see above) were examined by BLASTP or BLASTN analyses (using an e-value threshold of 10^{-5}), respectively, against the whole NCBI non-redundant database. Contigs were classified as 'DEH-affiliated' if hits were identified in the top five hits to the genera *Dehalococcoides, Dehalogenimonas, Caldilinea, Anaerolinea, Ktedonobacter, Chloroflexus, Rosei-flexus, Oscillochloris, Sphaerobacter, Thermo-baculum* or *Thermomicrobium*. If contigs did not harbour genetic content that gave a top five hits to any DEH or *Chloroflexi*, they were classified as 'non-affiliated'.

Estimation of genome recovery and genome size
The total genome size was estimated based on conserved single copy gene and tRNA analyses (Woyke *et al.,* 2009). To identify relevant conserved single copy genes for the DEH-J10 genome, the genomes of *D. mccartyi* strains CBDB1, 195, BAV1, GT and VS, and *D. lykanthroporepellens* BL-DC-9, that were available in January 2012 on the Joint Genome Institute's Integrated Microbial Genomes website (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) (Markowitz *et al.,* 2009), were included in the analysis. In total, we identified 462 conserved single copy gene in the reference genomes. The number of corresponding conserved single copy genes present in the DEH-J10 genomic content was then used to estimate the genome size. A second estimation was done by comparing tRNA gene numbers of DEH-J10 with the numbers of tRNA genes in the above described reference genomes.

Real-time PCR for quantification of DEH-J10 phylotype in Aarhus Bay sediments
DNA used in the real-time PCR assays was extracted from marine sediments in triplicate using a Fas-tDNA Spin for Soil Kit (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions with the following exceptions: 0.8 g of sediment was added to the initial tube containing the beads, and 780 μl of sodium phosphate buffer was added. After the DNA-binding step, the silica matrix and bound DNA was allowed to settle for 30 min. Each sample was eluted in 50 μl of supplied DNA elution solution water and combined.

Real-time PCR assays were performed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Quantification of total *Bacteria* was conducted using primers 341f (5'-C CTACGGGAGGCAGCAG-3') and 534r (5'-ATTAC CGCGGCTGCTGGCA-3'), which complement highly conserved regions in a highly diverse range of bacterial 16S rRNA genes (Wang and Qian 2009). The primers J10-16S-F (5'-GAGAGTGTAGGCGG CTCCCT-3') and J10-16S-R (5'-GGTCGATACCTCCTA TATCT-3'), which were designed in this study to specifically target the 16S rRNA gene of DEH-J10 and closely related phylotypes, were used for the quantification of DEH-J10. PCR reactions (total volume of 20 μl) contained 10 μl of 2 × SensiMix SYBR Kit PCR Master Mix (Bioline, Luckenwalde, Germany), 1 and 5 μM of each primer for bacterial and DEH-J10 assays, respectively, 1.0 μl of DNA template and deionized water up to 20 μl. PCR cycling conditions included an initial 'enzyme activation' step at 95 °C for 10 min, a short touchdown programme over five cycles consisting of 95 °C for 30 s, 64 °C (− 1.0 °C per cycle until a final temperature of 59 °C was reached) for 30 s and 72 °C for 30 s, and this was followed by an additional 35 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. Acquisition of fluorescence signal was performed during the 72 °C extension step of

each cycle. Melt-curve analyses were performed after each run and PCR products were also checked by standard agarose gel electrophoresis. The DNA standards used in real-time PCR assays consisted of a serial dilution of purified PCR product derived from a cloned DEH-J10 16S rRNA gene, and a cloned DEH 16S rRNA previously retrieved in our laboratory from sediments of the Chilean margin were used for the DEH-10 and 'total *Bacteria*' assays, respectively. These gene sequences were PCR amplified directly from colonies using M13 vector-specific primers, checked using standard agarose gel electrophoresis, extracted and gel purified using a Wizard SV Gel and PCR Clean-Up Kit (Promega) according to the manufacturer's instructions. DNA concentrations were determined using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE, USA) in triplicate. Measured concentrations of purified PCR product were then converted to copies per microliter, and the concentration was adjusted to 1×10^{11} copies μl^{-1} before performing 10-fold serial dilutions. A standard curve (1×10^{6} to 1.0×10^{2} copies per reaction) was generated and included in each run in triplicate. The detection limit was therefore also set at 1.0×10^{2} copies per reaction for the DEH-J10 assay. Data and copy numbers were analysed using the real-time PCR systems accompanying software (STEPONE version 2.0, Applied Biosystems) following the manufacturers guidelines. The specificity of the assay using primers J10-16S-F and J10-16S-R was evaluated by comparing the primers ability with amplify DNA derived from other phylogenetically distinct DEH single cells, and by cloning and sequencing (12 clones) of the amplification products from the primers. A distance matrix of aligned sequences obtained was produced by *Mega5* version 5.02 (Tamura *et al.*, 2011) and revealed the maximum amount of sequence divergence among the obtained sequences was 1%. This suggests that the quantitative PCR assay is highly specific for 16S rRNA genes with ⩾99% sequence identity.

PCR assays for the detection of reductive dehalogenase genes
MDA-derived DNA was examined for the presence of genes encoding reductive dehalogenases by PCR assays with established primers and PCR conditions (Hölscher *et al.*, 2004; Chow *et al.*, 2010). MDA-derived DNA used as template in PCR assays was used undiluted or diluted 1:20 and 1:50.

Sequence accession
The obtained 16S rRNA gene was deposited in the GenBank database under the accession number KC880080 and the genomic data (contigs > 200 bp) are present as BioProject PRJNA196991 in the GenBank database.

Results and discussion

*Isolation of the single cell 'DEH-J10' and quantification in Aarhus Bay sediment*
Sediments from Aarhus Bay, Denmark, were sampled from a depth of 10 cmbsf and used to obtain single cells by flow cytometric sorting of fluorescently stained cells. After cell lysis, MDA of the DNA and sequencing of 16S rRNA gene amplicons from sorted cells, a single cell designated 'DEH-J10' was selected for genome sequencing based on its high degree of divergence from cultivated strains and its unique phylogenetic position within the DEH (Figure 1). The closest cultivated strain was *D. lykanthroporepellens* strain BL-DC-9, which had only 86% sequence identity with the 16S rRNA gene of DEH-J10. The 16S rRNA gene of DEH-J10 phylogenetically affiliates with the previously termed 'subphylum II' clade of the *Chloroflexi* (Inagaki *et al.*, 2006).

Quantitative real-time PCR analysis of the DEH-J10 16S rRNA gene phylotype in the Aarhus Bay core showed that it was detectable at around 10^{5} copies per gram at 10 cmbsf and numbers were slightly increased at 40 cmbsf (Figure 2). The numbers then gradually decreased with sediment depth in an almost linear fashion and the phylotype was not detectable below 160 cmbsf, whereas copy numbers of 'total' *Bacteria* were still above 10^{8} copies g^{-1} at this depth. The DEH-J10 phylotype should therefore be regarded as a relatively 'shallow' subsurface phylotype that may represent DEH inhabiting the shallow subsurface, but not the deep subsurface.

General description of the genomic data obtained from the single cell DEH-J10
A combination of 454-pyrosequence and Illumina reads were assembled into 1.44 Mbp distributed across 629 contigs (Table 1). This assembled data were separated into two data sets: (i) the first 'primary' data set included contigs that had at least one complete ORF called by the ORF prediction software; and (ii) a second 'fragment' data set that encompassed contigs for which no full ORFs were called by the ORF prediction software and was therefore examined separately by BLASTX. Only encoded proteins from the fragment data set that were predicted to perform functions fitting into the context of metabolic pathways identified from primary data set are discussed and specified in the text with respect to their origins.

Because DNA contamination from exogenous sources was a major concern, various procedures to assess the degree of genomic purity were performed. The 16S rRNA gene sequence obtained by PCR when screening MDA products with broad-range 16S rRNA gene targeting primers before genome sequencing was identical to the sequence identified in the genome assembly. Further, PCR
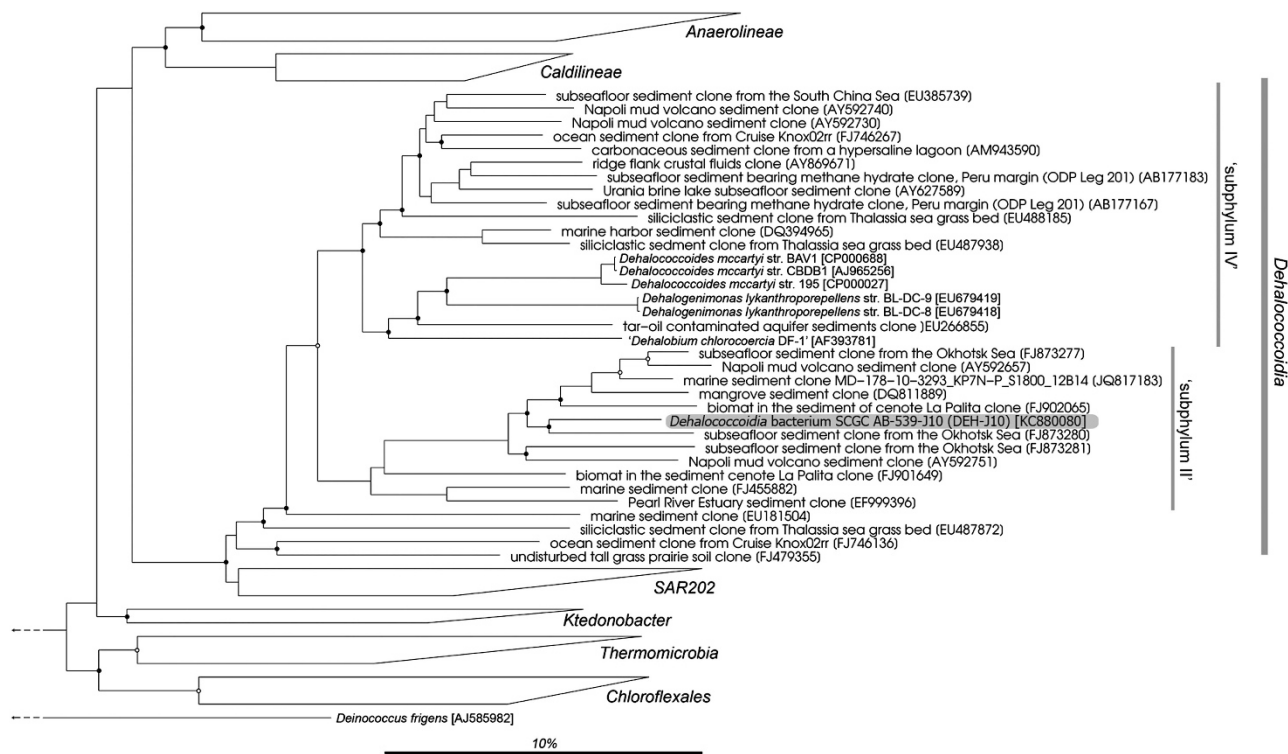
Figure 1 Phylogenetic tree based on 16S rRNA genes to examine the phylogeny of the single cell DEH-J10 (highlighted) compared with cultivated and uncultivated members of the *Dehalococcoidia* and other major groups of the *Chloroflexi*. The tree is based on the neighbor-joining algorithm with bootstrap resampling (1000 times). Nodes with bootstrap values $\geqslant 50\%$ are indicated by filled circles (●) and nodes with bootstrap values of $\geqslant 90\%$ are indicated by open circles (○). *Deinococcus frigens* (GenBank no. AJ585982) was used as an outgroup to root the tree. The scale bar represents 10% sequence divergence. Previously defined *Chloroflexi* subphylums 'II' and 'IV' (Inagaki *et al*, 2006) are also indicated for comparative purposes.
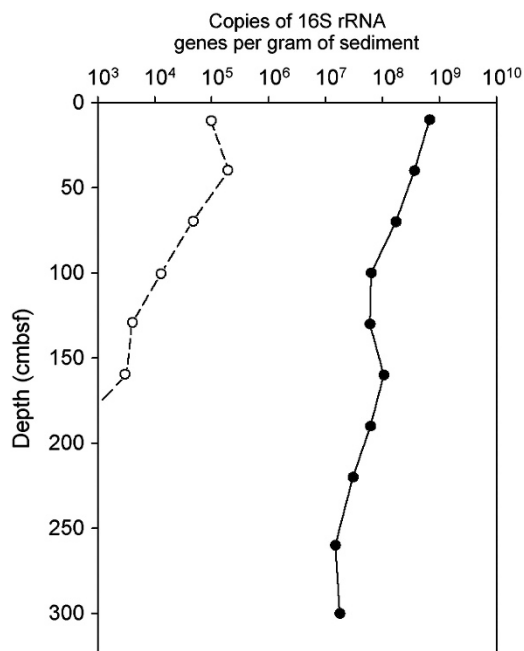
Figure 2 Quantification of 'total' bacterial 16S rRNA genes and genes amplified by specific primers designed for the 16S rRNA gene of the single cell DEH-J10 through depths of the Aarhus Bay sediment core. 'Total' *Bacteria* are represented by filled circles (●), DEH-J10 by open circles (○).

Table 1 Summary of genome assembly properties

Assembly statistic		Total assembly	Primary data set	Fragment data set
Assembly size (Mbp)		1.44	1.29	0.15
Average GC% content		47.3	47.5	45.6
No. of contigs		629	203	426
Mean contig length (kbp)		2.3	6.3	0.4
Longest contig length (kbp)		79.1	79.1	1.5
Shortest contig length (bp)		115	201	115
Predicted CDS		1557	1557	n.a.
Estimation of genome	(tRNA)	59.57–60.86%	n.a.	n.a.
recovered (%)	(CSCG)	50.65–50.87%	n.a.	n.a.
Genome size estimation (Mbp)		2.36–2.84	n.a.	n.a.

Abbreviations: CDS, coding sequence; CSCG, conserved single copy gene; n.a., not applicable.

using broad-range bacterial 16S rRNA gene primers and MDA-derived DNA as template, cloning and sequencing of the amplicons, revealed that all 83 clones with good quality sequence reads were almost identical to the 16S rRNA gene identified in the assembly, with few single base pair differences in some sequences likely due to PCR or sequencing errors. PCR using broad-range *Eukarya*-specific 18S rRNA or *Archaea*-specific 16S rRNA gene primers did not give amplification products. As an additional bioinformatic means to assess the genomic purity, BLASTP and BLASTN analyses

were also used to examine all contigs from the primary assembly for evidence of genetic content related to known DEH or other *Chloroflexi*. This analysis showed that the vast majority of contigs (harbouring 89.75% of the primary data set) could be directly linked to genetic content related to known DEH or other *Chloroflexi*. Together, these analyses strongly indicate that the genetic material described in this study was derived from the genome of a single DEH cell.

The analysis of the numbers of conserved single copy and tRNA genes in comparison with known DEH suggested that the 1.44 Mbp assembly represented 50.65–60.86% of the whole DEH-J10 genome (Table 1). On the basis of this information, an estimated genome size of 2.36–2.84 Mbp was deduced. When comparing all encoded proteins from the primary data set with the NCBI non-redundant database by BLASTP, 19.1% of the proteins had best hits to proteins from cultivated DEH and 3.6% to other *Chloroflexi* (Supplementary Table 1). Many best BLASTP hits were to proteins derived from other anaerobic groups such as *Deltaproteobacteria* (6.5%), *Firmicutes* (4.2%), methanogenic *Archaea* (4.2%) and syntrophic bacteria (1.5%). BLASTP analyses against protein sequences from known DEH genomes using an e-value threshold of 10^{-10} revealed overall protein

sequence identities of 44.1–45.7% for positive hits. The genomes of sequenced *D. mccartyi* strains contain two high-plasticity regions around the origin of replication that harbour the vast majority of putative terminal reductases required for organohalide respiration (Kube *et al.,* 2005; McMurdie *et al.,* 2009). BLAST comparisons of DEH-J10 proteins with several *D. mccartyi* genomes revealed that genes from DEH-J10 are highly underrepresented in these regions and hits were generally weaker than hits to other regions of the genomes (Supplementary Figure 1). Together, the data strongly suggest that although the DEH-J10 genome shares most similarity with genomes of previously sequenced DEH of the genera *Dehalococcoides* and *Dehalogenimonas*, it harbours a larger genome and appears considerably different in terms of overall gene content and arrangement.

Central carbon metabolism

Central metabolic pathways predicted from the genome annotations are depicted in Figure 3. Possible carbon assimilation paths include the uptake of organic compounds, carbon dioxide fixation via the reductive acetyl-CoA (Wood-Ljungdahl) pathway and carboxylation reactions. Three subunits of the carboxylating pyruvate:ferredoxin oxidoreductase
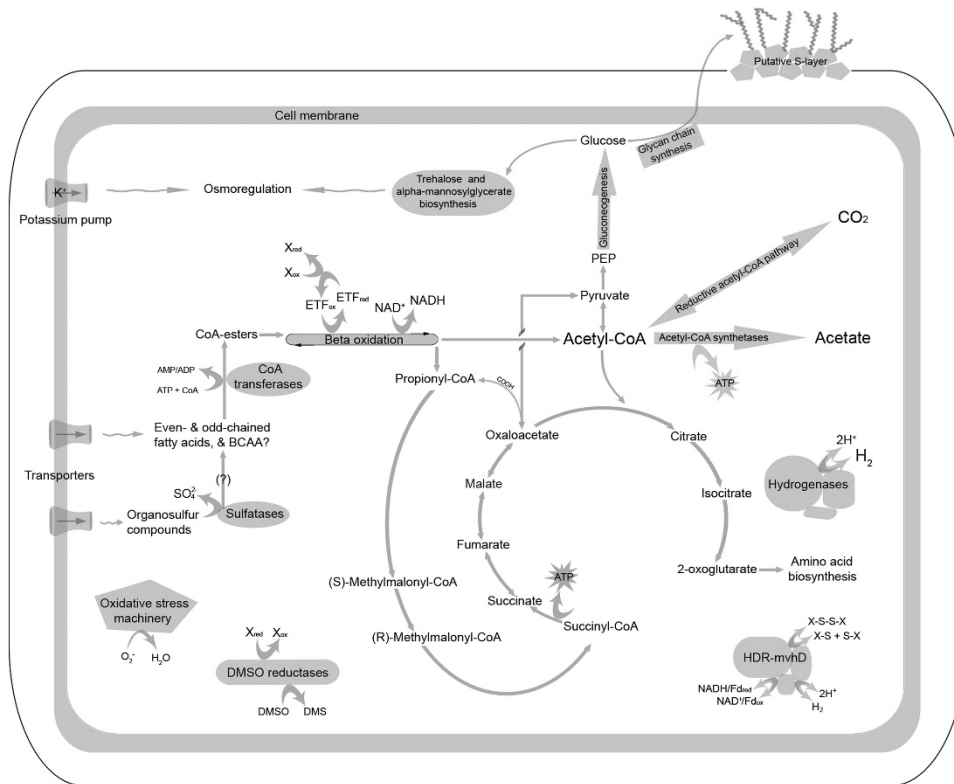
Figure 3 Schematic depiction of the overall metabolic and phenotypic features of single cell DEH-J10, as predicted from single-cell genome sequencing and gene annotations. BCAA, branched chain amino acids; ETF, electron transfer protein complex; HDR, heterodisulfide reductase-like proteins; mvhD, methyl-viologen-reducing hydrogenase delta subunit; DMSO, dimethyl sulfoxide; DMS, dimethyl sulphide. X–unknown electron carrier.

were encoded and may provide a link between the reductive acetyl-CoA pathway and other anabolic pathways. In addition, a gene encoding a membrane subunit of a sodium-translocating oxaloacetate carboxylase was present, possibly involved in Na^+-dependent pyruvate carboxylation to oxalo-acetate. In addition, this enzyme could operate in the opposite direction as a bifunctional priopionyl-CoA/oxaloacetate transcarboxylase, which could transfer the carboxyl group from oxaloacetate to propionyl-CoA and thereby perform a necessary step of the methylmalonyl pathway (described below) (Kosaka *et al.*, 2006).

The genome of DEH-J10 encoded several key gluconeogenesis functions, that is, phosphoenol-pyruvate synthase, phosphoglucerate mutase, a 'type V' bifunctional fructose-1,6-bisphosphate aldolase/fructose-1,6-bisphosphatase enzyme, an additional 'type II' fructose-1,6-bisphosphatase and glucose-6-P isomerase (Supplementary Table 2). The bifunctional fructose-1,6-bisphosphate aldolase/fructose-1,6-bisphosphatase is typical of strict anae-robes and may confer unidirectionality to gluconeo-genesis (Say and Fuchs, 2010). Its presence indicates that DEH-J10 is not able to catalyse glycolysis and therefore is not able to grow on sugars, similarly as described for cultivated DEH. Genes encoding enzymes of the tricarboxylic acid cycle were detected and are likely used for anabolic purposes, for example, for amino-acids biosynthesis.

Enzymes required for cobalamin salvage were encoded (*cobS*, *cobT*, *cobU* and *cobC*) and may enable the organism to remodel cobinamids to a functional cobalamin (Yi *et al.*, 2012). Functional cobalamins would therefore be available to act as cofactors for methyltransferases required for the reductive acetyl-CoA pathway, as well as methyl-malonyl-CoA mutases and reductive dehalogenases, all of which are discussed below.

Acetyl-CoA synthetases

Several genes encoding enzymes with highest sequence similarities to ADP-forming acetyl-CoA synthetases and succinyl-CoA synthetases were identified (Supplementary Table 2). Most of the top BLASTP hits for these predicted proteins were to proteins from the archaeal genera *Pyrococcus* and *Thermococcus*, which are known to catalyse the one-step formation of acetate from acetyl-CoA with the concomitant phosphorylation of ADP to ATP (Bräsen *et al.*, 2008). The predicted beta-subunit of at least one of the putative acetyl-CoA synthetases contained a conserved histidine residue typical of well characterised acetyl-CoA synthetases that specifically form acetate and differentiate them from other structurally related succinyl-CoA synthetases (Bräsen *et al.*, 2008). DEH-J10 may therefore gain ATP by substrate-level phosphorylation during the conversion of acetyl-CoA to acetate (McInerney *et al.*, 2007; Bräsen *et al.*, 2008).

Reductive acetyl-CoA (Wood-Ljungdahl) pathway

The DEH-J10 bacterium harboured almost all genes required for the reductive acetyl-CoA pathway (Wood-Ljungdahl pathway), which is present only in strictly anaerobic prokaryotes (Figure 3 and Supplementary Table 2). This is in contrast to *D. mccartyi* strains, which lack genes encoding methylene-tetrahydrofolate reductase and the beta-subunit of the carbon monoxide dehydro-genase/acetyl-CoA decarbonylase-synthase complex (CODH/ACDS) (Seshadri *et al.*, 2005), but is similar to *D. lykanthroporepellens* strain BL-DC-9 which also contains these genes. The only gene missing in DEH-J10 is the gene for 10-formyl tetrahydrofolate synthase. This gene is present in the genomes of all known DEH, suggesting that it was missed owing to the incomplete genome recovery. The reductive acetyl-CoA pathway may enable the bacterium to assimilate CO_2 and other C1-compounds (Berg, 2011; Fuchs, 2011). Because this pathway runs close to thermodynamic equilibrium, it can also operate in the opposite direction for the complete oxidation of organics via acetyl CoA to CO_2 (Berg, 2011; Fuchs, 2011). It has also been shown that the pathway can function in both directions within a single organism using the same enzymatic machinery, depending on the physiological conditions (Schauder *et al.*, 1986; Hattori *et al.*, 2005). Such a metabolic feature would enable DEH-J10 to switch metabolic strategy if environmental conditions necessitate.

Intriguingly, the genetic information for the carbonyl-branch of the reductive acetyl-CoA pathway appears to be of archaeal origin, which is in contrast to corresponding genes in known DEH genomes. Two separate gene clusters (referred to as 'A' and 'B') contained genes for two different enzyme complexes constituting the bifunctional CODH/ACDS complex. CODH/ACDS gene cluster A contained genes for all five subunits of the complex, whereas cluster B did not contain genes for gamma and delta subunits. The contig harbouring cluster B was, however, truncated in the vicinity of these genes and therefore genes for the gamma and delta subunits may be present in the missing genomic content. Both CODH/ACDS gene clusters contained genes encoding epsilon subunits that are characteristic of archaeal CODH/ACDS complexes (Lindahl and Chang, 2001), and phylogenetic analysis of the catalytic alpha-subunit protein sequences affiliated both with archaeal-derived proteins (Supplementary Figure 2). These facts suggest that both gene clusters may have been horizontally transferred from an archaeon, which has also been previously described for the subsur-face bacterium *Desulforudis audaxviator* MP104C (Chivian *et al.*, 2008). In addition, genes encoding subunits of a formylmethanofuran dehydrogenase (subunits B, D and G) were associated with CODH/ACDS gene cluster B. These are suggestive that an archaeal-like reductive acetyl-CoA pathway may operate, in which most of the typical methanogenic

pathway could be used for conversions of acetyl-CoA to and from CO_2 (Klenk *et al.*, 1997; Anderson *et al.*, 2011; Berg, 2011). No genes for a methyl-CoM reductase or other coenzymes and prosthetic groups required for CO_2 reduction to methane by methanogens (Kaster *et al.*, 2011a) were detected. The absence of genes for such features suggests that the reductive acetyl-CoA pathway might only be used for the fixation of CO_2 or oxidation of acetyl-CoA.

Electron donating and processing reactions

Beta-oxidation of hydrocarbons. Numerous genes encoding enzymes of the beta-oxidation pathway are present in the genome of DEH-J10 (Figure 3 and Supplementary Table 2). Beta-oxidation pathways typically enable the oxidation of fatty acids and structurally related compounds such as alkanes or alkenes of varying chain lengths (after activation), aromatics (after dearomatising) or branched-chain amino acids. A variety of genes encoding putative enzymes with CoA-transferase activities were identified, indicating that various organic substrates could be activated for beta-oxidation (Supplementary Table 2). Most CoA-transferases were related to 'family III' type enzymes, which are typically highly substrate specific (Heider 2001). Genes encoding enzymes of the methylmalonyl-CoA pathway were detected and included at least eight copies of methylmalonyl-CoA mutases in the primary data set and a propionyl-CoA carboxylase in the fragment data set (Supplementary Table 2). The methylmalonyl-CoA pathway can be used for the oxidation of activated odd-chain fatty acids and propionyl-CoA. One gene cluster contained a gene encoding an alpha-methylacyl-CoA racemase, in association with various genes for typical beta-oxidation enzymes, as well as methylmalonyl-CoA mutase subunits. This particular enzyme may suggest that the organism could use modified fatty acids such as methyl-branched fatty acids (Sakai *et al.*, 2004).

Genes predicted to encode both alpha and beta subunits of an electron transfer flavoprotein complex were present. These may serve as an electron acceptor for acyl-CoA dehydrogenases (Beckmann and Frerman, 1985; Husain and Steenkamp, 1985; Zhang *et al.,* 2004), such as those present in the beta-oxidation pathways. The reducing equivalents could then be transferred to an electron carrier before being transferred to an electron transport chain and could therefore provide a means for linking the oxidation of organics by beta-oxidation to energy conserving mechanisms.

Many of the genes for beta-oxidation enzymes were located in the vicinity of gene clusters encoding 'ABC' or 'branched-chain amino acid' transporters. This suggests functional associations, that is, uptake of defined molecules by specific transporters, followed by the activation through ligation with CoA and subsequent beta-oxidation.

Such genomic linkages have been previously observed, for example, in the short-chain fatty acid utilising bacterium *Syntrophobacter acidotrophicus* (McInerney *et al.,* 2007). All together, the genetic information related to beta-oxidation constitute a notable portion of the genomic content that differentiates DEH-J10 from known DEH and may suggest that beta-oxidation pathways represent an important metabolic route to obtain carbon and reducing equivalents for DEH-J10.

Catabolism of aromatics. Genes predicted to encode subunits of a class I benzoyl-CoA reductase were identified (Supplementary Table 2). Benzoyl-CoA reductases are key enzymes in the central metabolism of aromatic compounds (Löffler *et al.,* 2011). Two subunits (gamma and beta) were present on one contig, whereas genes for possible alpha and delta subunits were present on a separate contig. The alpha and delta subunits contain ATP-binding sites of the acetate kinase/sugar kinase/Hsp70 actin family domains and therefore an ATP-dependent reduction of an aromatic ring typical of facultative anaerobes could be hypothesised (Selesi *et al.,* 2010). The only other gene predicted to encode an enzyme involved in the oxidation of aromatic compounds to acetyl-CoA was a gene annotated as a subunit of succinyl-CoA:benzylsuccinate CoA-transferase. The presence of such genes suggests that the DEH-J10 may also have the capacity to oxidise substituted aromatics.

Hydrogenases and associated proteins. Multiple genes and operons encoding hydrogenases and accessory proteins, for example, a hydrogenase assembly chaperon and a cofactor insertion complex, were identified (Supplementary Table 2). All these genes had high sequence identities to genes from known DEH strains. The hydrogenase encoding genes included genes for cytoplasmic HymABC subunits and so-called 'periplasmic' NiFe 'hup' hydrogenases found in known DEH. The hup hydrogenase has been previously discussed to be a good candidate for shuttling electrons into the electron transport chain in cultivated *Dehalococcoides* strains (Seshadri *et al.,* 2005). However, despite having high overall amino acid similarity to hup hydrogenases from known DEH, the hup small subunit in DEH-J10 does not contain a twin-arginine translocation export signal peptide or a transmembrane helix, as it appears to be truncated at the N terminus in comparison with these subunits in known DEH. Further, a gene encoding a putatively membrane-bound iron-sulfur-cluster binding domain-containing protein, which is typically directly adjacent to genes for subunits of the hup hydrogenases in known DEH, is absent in DEH-J10. Together, this data suggest that this protein complex is not membrane associated and is therefore not a key respiratory enzyme complex for electron input.

Alternatively, this hydrogenase may be involved in proton/hydrogen processing within the cytoplasm.

Heterodisulfide reductase and associated proteins. A cluster of genes encoding two heterodisulfide reductase-like 'alpha' subunits, four putative methyl viologen-reducing hydrogenase delta subunits and two formate dehydrogenase-like 'beta' subunits, was identified (Supplementary Figure 3 and Supplementary Table 2). This cluster was one of the longest and most obvious stretches of ORFs identified (encoding at least 20 ORFs) in our data set, whereby no homologues in known DEH could be identified by BLASTP analyses, yet was downstream of known DEH genes. We hypothesise that the heterodisulfide reductase-like enzymes have important roles in cytoplasmic electron transfer and energy conserving mechanisms, like in other anaerobes such as sulphate reducers, acetogens and methanogens (Stojanowic *et al.*, 2003; Strittmatter *et al.*, 2009; Kaster *et al.*, 2011b; Callaghan *et al.*, 2012). These complexes might be especially important for transferring reducing equivalents released during beta-oxidation and/or conversions of succinate to acetyl-CoA (via the methylmalonyl-CoA pathway), to and from ferredoxins or NADH, possibly by electron bifurcating/confurcating mechanisms that may be linked to other metabolic steps (Buckel and Thauer, 2012; Grein *et al.*, 2012). The truncation of the contig containing these genes precluded linking the predicted proteins directly to other related mechanisms by genomic associations, and the exact mechanisms of such complexes are not understood in many other microorganisms. Nevertheless, its presence indicates the capacity for energy processing mechanisms unique to DEH-J10 in comparison with known DEH.

Electron accepting reactions

Potential terminal reductases. Genes predicted to encode terminal reductases included four genes encoding subunits of complex iron-sulfur molybdoenzyme family proteins (Supplementary Table 2). Phylogenetic analysis of the three different catalytic subunit A protein sequences placed them in a distinct branch containing dimethyl sulfoxide (DMSO) reductases, which also often have activity towards trimethylamine N-oxide (Supplementary Figure 4). In addition to the genes for subunit A proteins, a gene encoding one copy of a 'four-cluster protein' subunit (subunit B) was identified, whereas no genes were identified for putative homologues of complex iron-sulfur molybdoenzyme subunit C proteins, which typically act as membrane anchors for many multimeric complex iron-sulfur molybdoenzyme complexes (Rothery *et al.*, 2008). Further, twin-arginine translocation translocation signal peptides were not identified for the predicted A or B subunits, and together, suggest that this complex may be cytoplasmic or interacting with other membrane-bound respiratory proteins by unknown

mechanisms. It is important to note that some complex iron-sulfur molybdoenzyme terminal reductases are known to lack the membrane-bound C subunits while retaining activity (McEwan *et al.*, 2002). DMSO is widely distributed in pelagic marine environments and may be deposited to sediments in association with sinking particulate matter (Hatton 2002) and can then be utilised as an electron acceptor in reduced marine sediments (Kiene and Capone, 1988; López and Duarte, 2004). It could therefore represent an effective electron acceptor for bacteria living in the shallow subsurface, as the redox potential of DMSO ($+160\,mV$) (Wood, 1981) is between the redox potentials of other favourable anaerobic electron acceptors typically used in the shallow subsurface, such as Mn(IV) and Fe(III). Compounds such as DMSO and trimethylamine N-oxide may be especially useful to test as a terminal electron acceptor for further enrichment attempts.

No genes encoding homologues of reductive dehalogenase enzymes, associated membrane-bound anchor proteins or transcriptional regulators, which are required for respiration with organohalide compounds, were detected in the genomic data. Further, genes for reductive dehalogenases were not detected with PCR using primers targeting these genes using MDA-derived DNA as template. Considering there is speculation that some marine subsurface DEH-affiliated bacteria could perform reductive dehalogenation (Adrian, 2009; Futagami *et al.,* 2009; Valentine, 2010; Durbin and Teske, 2011; Wagner *et al.,* 2012), the apparent absence of genes encoding reductive dehalogenases is worthy to note because this is the first genomic data from relatives of known organohalide-respiring DEH. Even if genes for reductive dehalogenases were in the missing genomic content, the DEH-J10 bacterium is considerably different to cultivated DEH because it does not appear to harbour a high proportion of genetic material dedicated to organohalide respiration. This can be assumed because if DEH-J10 harboured high copy numbers of genes for reductive dehalogenase homologues like in analogy to cultivated DEH (for example, up to 36 copies in *D. mccartyi* strains) (Kube *et al.,* 2005; Seshadri *et al.,* 2005; McMurdie *et al.,* 2009; Siddaramappa *et al.,* 2012), the chances of detecting these genes would be high even if a partial genome was recovered. Together with the indications for other energy conserving mechanisms described above, this strongly suggests that the DEH-J10 bacterium does not depend on organohalide respiration as a means of energy conservation like cultivated DEH. It also provides a first indication that it is not a conserved trait within the class DEH to harbour high proportions of genomic content dedicated to organohalide respiration.

Environmental adaptations

Osmoprotection. A gene cluster was identified that encodes enzymes possibly involved in the synthesis of

osmoprotectants such as trehalose and alpha-mannosylglycerate (Styrvold and Strom, 1991; Empadinhas *et al.,* 2004), and in the regulation of cellular osmolarity (Supplementary Table 2). The genetic potential for trehalose synthesising enzymes appears unique to DEH-J10 in comparison with known DEH, whereas genes for alpha-mannosylglycerate synthesising enzymes are present in terrestrial DEH (Styrvold and Strom, 1991; Empadinhas *et al.,* 2004) and may possibly represent an evolutionary remnant of a marine DEH strain, or even an adaptation to osmotic fluctuations in terrestrial environments. Downstream were genes encoding a potassium uptake transporter that gave best BLASTP hits to proteins from marine methanogens and halotolerant microorganisms. This transporter might also be involved in the regulation of cytoplasmic osmotic strength by regulation of potassium ion concentrations (Roberts, 2004).

Oxygen protection. The presence of genes for enzymes related to oxygen and/or reactive oxygen species protection such as superoxide reductase/desulfoferrodoxin, superoxide dismutase and catalase (Supplementary Table 2), which are all absent in cultivated DEH, might represent adaptations to growth in shallow marine sediments, where organisms in sediments subject to bioturbation may be periodically exposed to oxygen.

Sulfatases (sulfohydrolases). Several genes predicted to encode enzymes with sulfatase activity were detected on two separate contigs (Supplementary Table 2). In addition, a gene encoding a sulfatase-maturating enzyme was identified, which is critical for post-translational modification and functionality of sulfatases (Benjdia *et al.,* 2011). Sulfatases catalyse the removal of sulphate groups from organic molecules and thereby enable further catabolism of the carbon backbones of various organic compounds (Kertesz, 2000; Glöckner *et al.,* 2003; Woebken *et al.,* 2007). Organosulphur compounds can be particularly abundant in the pelagic marine environment (Glöckner *et al.,* 2003) and may further arise from sulphurisation of organic compounds through diagenetic reactions during burial in marine sediments (Schmidt *et al.,* 2009). Genes encoding sulfatases are well represented in marine sediment metagenomes (Quaiser *et al.,* 2011) and are highly represented in the genomes of marine versus freshwater *Planctomycetes* (Woebken *et al.,* 2007). Sulfatases may therefore be a particular adaptation of DEH-J10 to organosulphur compounds found in marine sediment environments. Interestingly, most genes encoding sulfatases in DEH-J10 were most related to genes derived from pelagic bacteria, suggesting a degree of genetic continuity exists between pelagic and subsurface microorganisms.

Cell wall formation. No indications for peptidoglycan formation were found in the genomic data, suggesting that the analysed cell did not contain a rigid cell wall. This is in line with known DEH that also do not encode the enzymatic machinery for peptidoglycan cell wall biosynthesis, yet are known to contain proteinaceous surface layers (S-layers) (Maymó-Gatell *et al.,* 1997; Adrian *et al.,* 2000). It has also been suggested that the monoderm nature of the *Chloroflexi* is evolutionary conserved throughout the whole phylum (Sutcliffe, 2011). In contrast to known DEH, however, the capacity to glycosylate S-layer proteins was suggested by a gene cluster encoding various enzymes putatively involved in the synthesis of glycan chains (Supplementary Table 2). These genes mostly had high similarity to genes from other organisms with S-layers. Glycosylated S-layers could have ecological implications such as providing protection against proteolytic enzymes, improvement of cell wall integrity or alter surface charges and thereby influence interactions with other microorganisms or sediment particles.

Concluding remarks. This study provides the first insights into the genome of a bacterium belonging to the marine DEH-affiliated *Chloroflexi*, as revealed by partial sequencing of a single-cell genome. Although to date such single-cell genome approaches are limited in that complete genomes are rarely retrieved, the considerable portion of the genome obtained in this study provides invaluable information about the metabolic potential of an organism for which nothing was previously known. The data indicate that the DEH-J10 genome likely confers metabolic versatility to the organism, much more than previously found for organohalide-respiring DEH. It appears that DEH-J10 could employ the beta-oxidation pathway to use various organics as a source for carbon and reducing equivalents. The organism could use the reductive acetyl-CoA pathway to completely oxidise the organics processed via beta-oxidation pathways, or it could use this same pathway to obtain carbon by autotrophy. In contrast to known DEH, the DEH-J10 bacterium likely does not rely on organohalide respiration for energy conservation and might instead use DMSO as an electron acceptor. The organism may also generate ATP in a non-respiratory manner via conversions of acetyl-CoA to acetate. The observation that populations of the DEH-J10 phylotype are restricted to relatively shallow subsurface sediments together with the genomic data suggests that the bacterium is linked to the degradation of organic matter in the upper sediments of the Aarhus Bay site. Because of the pronounced diversity within the class DEH, further studies will be required to unravel the properties of other DEH genotypes from the many other divergent phylogenetic clusters of the DEH.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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