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Metagenomic evidence of a novel family of anammox bacteria in a subsea environment

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Summary

Bacteria in the order '*Candidatus* Brocadiales' within the phylum *Planctomycetes* (*Planctomycetota*) have the remarkable ability to perform anaerobic ammonium oxidation (anammox). Two families of anammox bacteria with different biogeographical distributions have been reported, marine *Ca*. Scalinduaceae and freshwater *Ca*. Brocadiaceae. Here we report evidence of three new species within a novel genus and family of anammox bacteria, which were discovered in biofilms of a subsea road tunnel under a fjord in Norway. In this particular ecosystem, the nitrogen cycle is likely fuelled by ammonia from organic matter degradation in the fjord sediments and the rock mass above the tunnel, resulting in the growth of biofilms where anammox bacteria can thrive under oxygen limitation. We resolved several metagenome-assembled genomes (MAGs) of anammox bacteria, including three *Ca*. Brocadiales MAGs that could not be classified at the family level. MAGs of this novel family had all the diagnostic genes for a full anaerobic ammonium oxidation pathway in which nitrite was probably reduced by a NirK-like reductase. A survey of published molecular data indicated that this new family of anammox bacteria occurs in many marine sediments, where its members presumably would contribute to nitrogen loss.

Introduction

In 1995, our understanding of the nitrogen cycle changed, when the anaerobic ammonium oxidation (anammox) process was uncovered in an anaerobic pilot plant (Mulder *et al*., 1995). Anammox bacteria were found to couple the oxidation of ammonium to nitrite reduction, resulting in the production of nitrogen gas (van de Graaf *et al*., 1995). Since then, a comprehensive picture of these bacteria and their relevance to the global nitrogen cycle has emerged (Kuypers *et al*., 2018). Microorganisms capable of performing the anammox process were identified as novel members of the phylum *Planctomycetes* (Strous *et al*., 1999), with all known anammox bacteria so far being part of the order '*Candidatus* Brocadiales' (Jetten *et al*., 2010). Anammox bacteria have been shown to contribute to nitrogen turnover in many oxygen-limited natural environments (Kuypers *et al*., 2003), and their unique metabolism has resulted in new biotechnologies for more sustainable biological nitrogen removal in wastewater treatment plants (Jetten *et al*., 1997; van Dongen *et al*., 2001; Lackner *et al*., 2014). Anammox bacteria have quite a versatile metabolism, for example being able to use several organic acids (Kartal *et al*., 2013) and various electron acceptors such as nitrate, nitrite, metal oxides, nitric oxide (NO) and electrodes (Hu *et al*., 2019; Shaw *et al*., 2020).

To this date, anammox bacteria are yet to be isolated. Nonetheless, enrichments and molecular approaches have revealed the existence of two anammox families, *Ca*. Scalinduaceae and *Ca*. Brocadiaceae. These families seem to occupy different ecological niches, with salinity largely influencing their biogeography (Sonthiphand *et al*., 2014). *Ca*. Scalinduaceae is commonly associated with marine environments, with *Ca*. Scalindua as the only described genus. *Ca*. Brocadiaceae are frequently observed in freshwater

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communities and wastewater bioreactors. Four genera of *Ca*. Brocadiaceae are known: *Ca*. Jettenia (Quan *et al*., 2008), *Ca*. Brocadia (Strous *et al*., 1999), *Ca*. Anammoxoglobus (Kartal *et al*., 2007) and the model anammox bacterium *Ca*. Kuenenia (Strous *et al*., 2006).

New, undescribed groups of anammox bacteria might exist, and there are hints of their existence from environmental surveys. For example, in the SILVA 138 database (Quast *et al*., 2013) there is a group of 16S rRNA gene sequences named as GWA2-50-13, and in the GTDB 06-RS202 taxonomy (Parks *et al*., 2018, 2020) there is a family with placeholder name f__2-02-FULL-50-16-A. In phylogenetic trees, these groups cluster within the anammox bacteria. Most anammox bacteria have been described from enrichments, but culture-independent methods such as single-amplified genomes and metagenome-assembled genomes (MAGs) are complementary approaches that can be used to unravel novel microorganisms (Rinke *et al*., 2013; Hug *et al*., 2016).

One of the first MAGs ever resolved was the genome of *Ca*. Kuenenia stuttgartiensis (Strous *et al*., 2006). MAGs of anammox bacteria had been recovered and described from enrichments (Strous *et al*., 2006; Oshiki *et al*., 2015), wastewater treatment plants (Speth *et al*., 2016; Park *et al*., 2017), but also from environmental surveys (Speth *et al*., 2017; Zhao *et al*., 2020). MAGs can be used to predict anammox metabolism based on phylogenetic information and the presence of genes linked to the anammox process. Although, to this date (2022-02-22), there are 122 *Ca*. Brocadiales genomes at the NCBI assembly database, many of them were sampled in engineered environments like wastewater bioreactors. Thus, databases are likely biased towards relatively fast-growing anammox bacteria which are adapted to nitrogen-rich and low-salinity conditions.

Anammox bacteria are obligate anaerobes, and thus they can occur in habitats with limited oxygen supply such as thick biofilms (Suarez *et al*., 2019) and marine oxygen minimum zones (Woebken *et al*., 2008). Other largely unexplored anoxic environments could harbour novel anammox bacteria. For example, in marine sediments, oxygen is rapidly used as terminal electron acceptor, with anoxic conditions just a few millimetres below the sediment surface (Glud, 2008). Deep subseafloor sediments are poorly surveyed environments and despite their nutrient limitation, rich and distinct microbial communities, including anammox bacteria, can thrive in these ecosystems (Parkes *et al*., 1994; Kallmeyer *et al*., 2012; Inagaki *et al*., 2015; Hoshino and Inagaki, 2019; Zhao *et al*., 2020). Sampling of subseafloor communities can be challenging as underwater drilling is required. In subsea road tunnels, however, microbial subseafloor communities are accessible as water seepage from surrounding bedrock occurs (Grønhaug, 1978; Hagelia, 2011). One such example is the Oslofjord tunnel in Norway, a subsea road tunnel with a maximum depth of 134 m below sea level, where saline water seepage from cracks in the bedrocks results in growth of microbial biofilms on the rough sprayed concrete surfaces used for rock support of the tunnel. The biofilms are rich in manganese oxide and iron hydroxide biominerals. Redox reactions within the biofilms involve the sulfur, carbon, nitrogen, iron and manganese biogeochemical cycles, sometimes leading to acidification (Hagelia, 2007, 2011; Karačić *et al*., 2018).

Surveys of the Oslofjord site using 16S rRNA gene sequencing showed a microbial community where nitrogen-transforming microorganisms are abundant (Karačić *et al*., 2018). Both ammonium-oxidizing bacteria and ammonium-oxidizing archaea are present and presumably oxidize ammonium to nitrite. Amplicon sequence variants classified as the marine anammox *Ca*. Scalindua were present in these datasets. In addition, a group of unclassified *Ca*. Brocadiales were the second most abundant taxa in some samples (Karačić *et al*., 2018). It is unclear if these unclassified sequences belonged to anammox bacteria, as their potential function was only inferred from 16S rRNA gene taxonomy. To explore the *Ca*. Brocadiales community in the Oslofjord site, and describe their taxonomy and potential functions, we used shotgun metagenomic sequencing with the aim of resolving MAGs of these unclassified potentially new anammox bacteria.

Results and discussion

Three MAGs represent a novel family of anammox

Analysis of MAGs from two sites in the tunnel yielded seven Oslofjord Tunnel MAGs (OFTMs) that, with GTDB-Tk, were classified within the order *Ca*. Brocadiales: OFTM134, OFTM214, OFTM301, OFTM1, OFTM180, OFTM351 and OFTM256. They were detected in five out of eight samples, with the sum of their relative abundance ranging between 0.05% and 5.37%. OFTM134, OFTM214 and OFTM301 could not be classified at family level and were 80%–95% complete (Table 1), while the other four MAGs were affiliated to the genus *Ca*. Scalindua. These seven MAGs were placed in a phylogenetic tree of 92 concatenated core bacterial genes (Fig. 1), which revealed that the three unclassified MAGs (OFTM134, OFTM214 and OFTM301) may represent novel anammox taxa.

In order to investigate the level of taxonomic novelty, average amino acid identity (AAI) was computed for a set of genomes including the three novel MAGs (OFTM134, OFTM214 and OFTM301), representatives of the four known anammox genera, and two non-anammox

Table 1. Summary of genomic information of three anammox MAGs (completeness and contamination was determined by checkM; rRNA molecules and number of scaffolds by DRAM).

MAG	Scaffolds	Completeness (%)	Contamination (%)	5S rRNA	16S rRNA scaffold (begin-end bp)	23S rRNA scaffold (begin-end bp)	tRNA count
OFTM134	114	95.1	3.4	None	OFTM134_10 (2548–3239)	OFTM134_10, (2–2148)	48
OFTM214	142	93.9	5.7	None	OFTM214_0 (6–843)	None	45
OFTM301	769	82.8	9.7	None	none	None	32

Planctomycetes genomes (Fig. 2). AAI values among the three OFTMs ranged from 60% to 63%. Between the three OFTMs and genomes representative of the four known anammox genera, AAI ranged from 48% to 50%. Interestingly, AAI between non-anammox and anammox genomes was 38%–39%. Taking into consideration previously reported AAI thresholds of 60%–80% AAI for genus level and >85% AAI at the species level (Luo *et al*., 2014), these results indicate that OFTM134, OFTM214 and OFTM301 may represent a novel family of anammox bacteria, and that each genome may represent a distinct species within this new genus.

The novel MAGs have genomic potential for full anammox metabolism

Genomic potential for the full anaerobic ammonium oxidation pathway as well as components of electron transport chain were identified in OFTM134, OFTM214 and OFTM301 (Fig. 3). Genes encoding nitrate reductase/nitrite oxidoreductase (*nxrABC*) were identified in the three MAGs. This protein complex may catalyse nitrite oxidation to nitrate (van de Graaf *et al*., 1996; Chicano *et al*., 2021). Nitrite could be reduced to NO via a NirK-type nitrite reductase identified in OFTM134 and OFTM301. Copper-containing NirK proteins have been reported in *Ca*. Jettenia (Hira *et al*., 2012; Mardanov *et al*., 2019) and *Ca*. Brocadia caroliniensis (Park *et al*., 2017), while *Ca*. Kuenenia stuttgartiensis, and *Ca*. Scalindua harbour a heme-iron NirS-type nitrite reductase (Strous *et al*., 2006; van de Vossenberg *et al*., 2013; Oshiki *et al*., 2017) (Fig. S3). Both NirK and NirS were absent in OFTM214, as also reported for several *Ca*. Brocadia (Oshiki *et al*., 2015; Narita *et al*., 2017; Okubo *et al*., 2021) (Fig. S3). A multiheme hydroxylamine oxidoreductase (HAO)-encoding gene, present in this MAG, might catalyse this reaction (Ferousi *et al*., 2021). Alternatively, the MAG, which is ∼94% complete, might be missing a nitrite reductase-encoding gene.

In the next step, NO and ammonium can then serve as substrates for a hydrazine synthase (*hzsABC*). Genes encoding HzsABC were identified in OFTM134, OFTM214 and OFTM301. Hydrazine can finally be converted to dinitrogen gas by a hydrazine dehydrogenase (HDH). Interestingly, each MAG had several genes encoding proteins with multiheme motifs annotated as HDH or HAO (DOI: 10.5281/zenodo.5524859). Of six genes annotated as HAO/HDH in OFTM134, one had a best BLASTP hit to kustc0694 HDH, four to kustc1061 hydroxylamine oxidase (HOX), and one to HAO kuste4574, part of the Rieske complex R/b-3. In OFTM214, one gene had a best BLASTP hit to HDH and one to HOX, while in OFTM301, one gene had a best BLASTP hit to HDH and two to HOX. Moreover, the three MAGs had a hydroxylamine reductase (EC: 1.7.99.1, K05601) gene (*hcp*), encoding a protein hypothesized to be used for ammonification – the disproportionation of hydroxylamine in the absence of nitrite into dinitrogen gas and ammonium (van der Star *et al*., 2008).

Almost complete electron transport chains were identified in OFTM134, OFTM214 and OFTM301 (DOI: 10.5281/zenodo.5524859). All genes encoding subunits of the complex I, NADH dehydrogenase (*nuoA-N*), were present, except *nuoG*. Two genes were annotated as *nuoF* in OFTM134 and OFTM301, and *nuoE* was absent in OFTM301. A series of genes were concomitantly annotated as subunits of both the sodium-pumping NADH:quinone oxidoreductase (NQR) and the RNF complex, and scattered genes in the genomes had significant BLASTP hits to NQR subunits, but no MAGs had all genes. Given the high homology between the two complexes and the previous miss-annotation of *RnfEA* as *NqrDE* (de Almeida *et al*., 2016), and that *nqrA* was missing in the three MAGs, but all subunits of the RNF complex were present in all MAGs, we assume that these six genes are *rnfABCDEG* in each of the three MAGs. That would mean that NQR is absent in these organisms. However, as genomic evidence is inconclusive, physiological investigation would be required to elucidate the potential presence of NQR. Succinate dehydrogenase genes *sdhABC* (complex II) were present in OFTM134 and OFTM214, while OFTM301 only harboured *sdhC*.

There are three Rieske/cyt$_b$ complexes (complex III) in *K*. *stuttgartiensis*: R/*b*-1, R/*b*-2 and R/*b*-3 (Kartal *et al*., 2011, 2013; de Almeida *et al*., 2016). The Rieske complex R/*b*-1 (identified via BLASTP analyses using

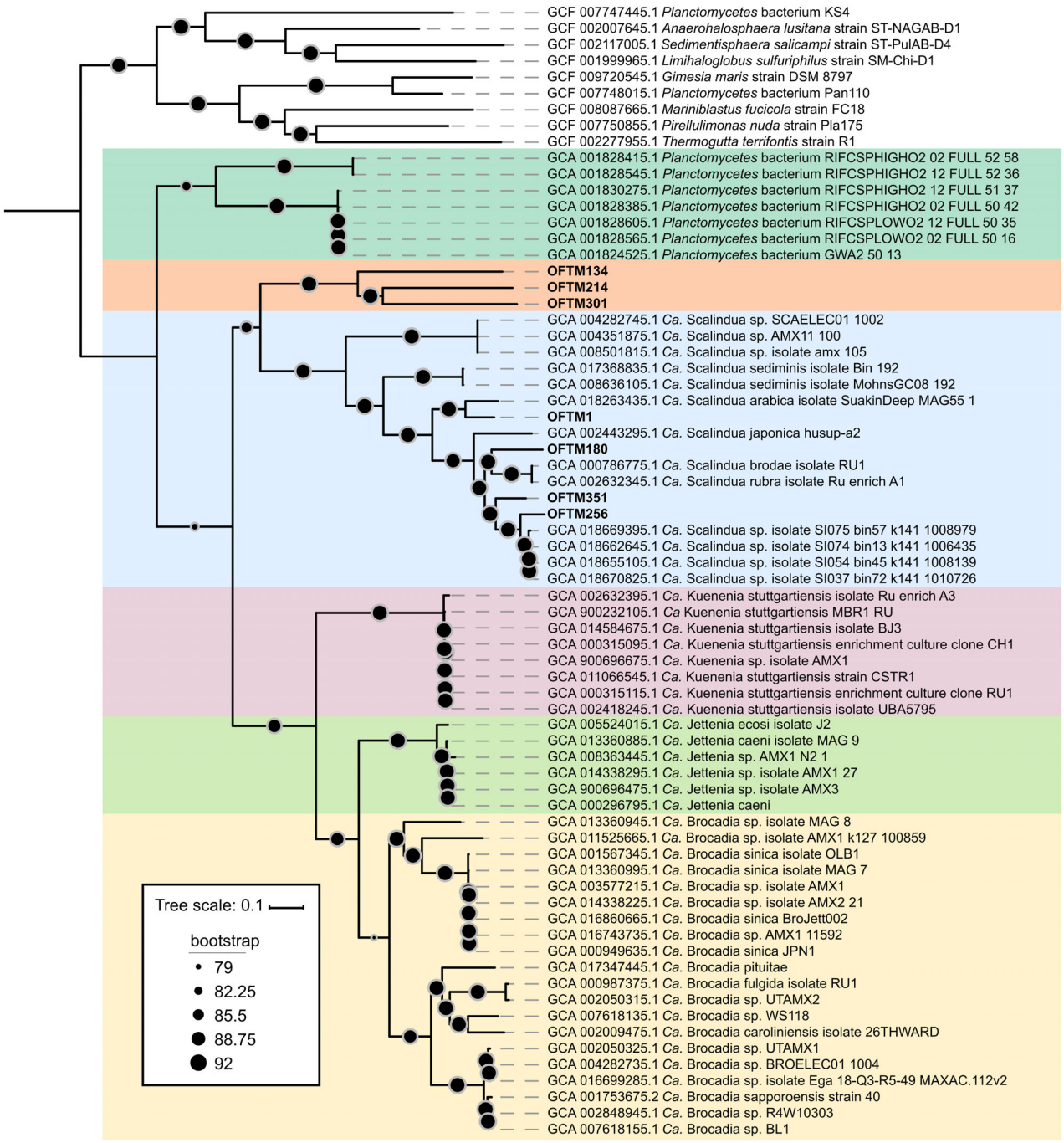

Fig. 1. Phylogenetic tree built with the UBCG pipeline using 92 concatenated core bacterial genes retrieved from 70 genomes. Non-anammox *Planctomycetes* genomes form a clade at the top of the tree. The GTDB "f__2-02-FULL-50-16-A" clade is highlighted in darker green. Novel anammox (this study) are highlighted in orange, while known genera *Ca*. Scalindua, *Ca*. Kuenenia, *Ca*. Jettenia and *Ca*. Brocadia are highlighted in blue, pink, light green and yellow respectively. Reference genomes start with GCA or GCF reference numbers, and MAGs from this study start with OFTM.

kuste3096-7) was identified in OFTM134, OFTM214 and OFTM301: three copies in OFTM134, three copies in OFTM214 and five copies in OFTM301. A *c*-type cytochrome-encoding gene was frequently present upstream or downstream of the two R/*b*-1 subunits. In several instances, this gene was a homologue of cytochrome c_{554}, which accepts electrons from HAO in the ammonium oxidizer *Nitrosomonas europaea* (Yamanaka and Shinra, 1974). OFTM134 and OFTM214 had five out of six genes encoding subunits of R/*b*-2 (kustd1480-85);

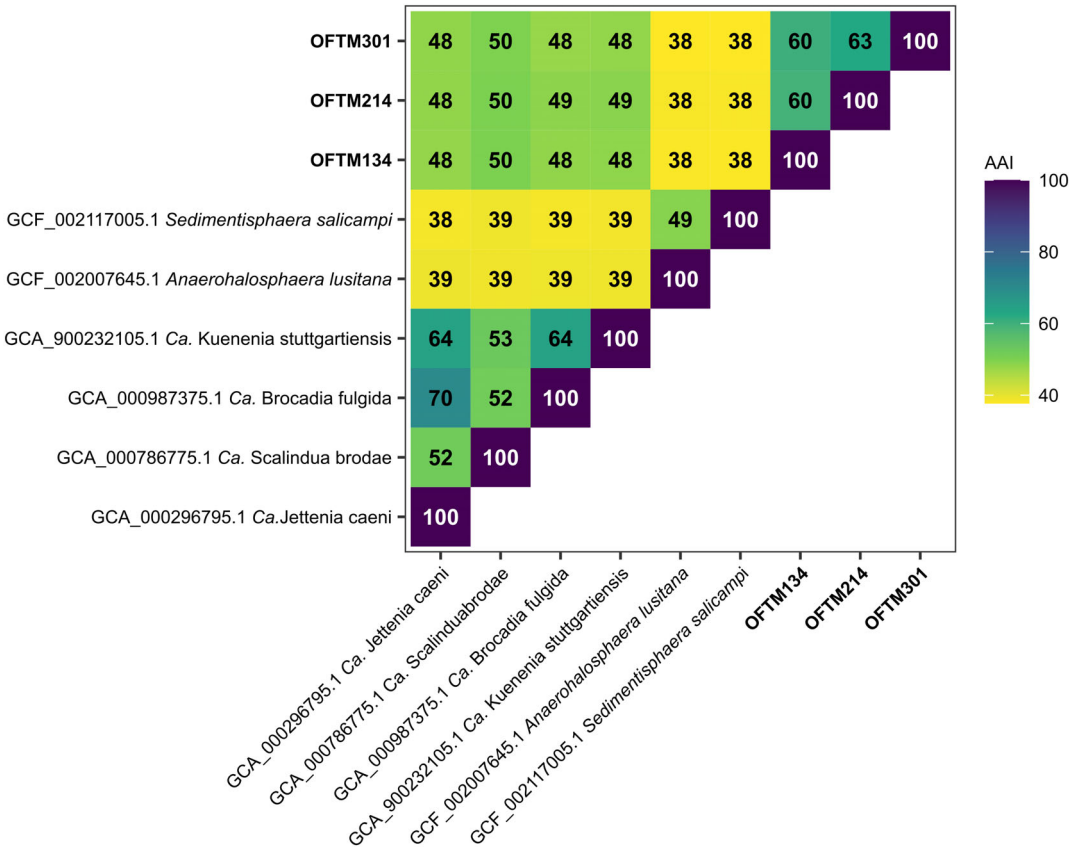

Fig. 2. Average AAI matrix. Numbers express percent of AAI similarity between genome pairs.

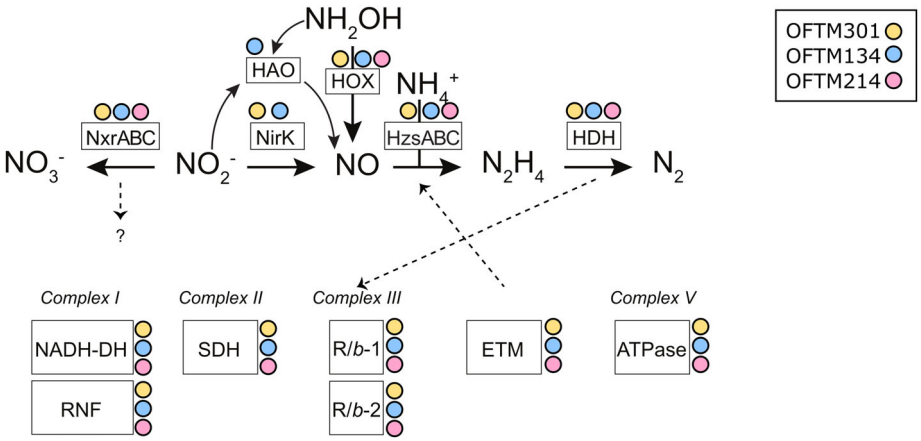

Fig. 3. Summary of metabolic potential identified in the three MAGs. The upper section highlights proteins identified in the novel MAGs involved in the pathway for anaerobic ammonium oxidation, and the bottom section displays proteins involved in the electron transport chain. Solid arrows indicate the flux of substrates, and dashed arrows indicate the flow of electrons. NxrABC, nitrate reductase/nitrite oxidoreductase (EC:1.7.5.1 1.7.99.-); NirK, NO-forming nitrite reductase (EC:1.7.2.1); HzsABC, hydrazine synthase (EC:1.7.2.7); HDH, hydrazine dehydrogenase (EC:1.7.2.8); HAO, hydroxylamine oxidoreductase (EC:1.7.2.6); HOX, hydroxylamine oxidase (homologue to kustc1061); NADH-DH, NADH dehydrogenase *nuoA-N* (EC:7.1.1.2); RNF, Rnf complex *rnf*ABCDEG (EC:7.2.1.2); SDH, succinate dehydrogenase (EC:1.3.5.1), R/*b*-1-2: Rieske-heme b complexes 1 and 2; ETM, electron transfer module.

the only gene missing was a homologue of kustd1482, a gene of unknown function. OFTM301 had four out of six genes encoding R/*b*-2; kustd1480 and kustd1482 were missing. Finally, genes encoding subunits of R/*b*-3 (homologues to kuste4569-74) were not detected. Out of the six R/*b*-3 genes, significant BLASTP hits to gene

kuste4570 were identified in the three MAGs, and OFTM134 also had a hit to kuste4574, the subunit with high similarity to HAO.

Both subunits of the electron transfer module (ETM; kuste2855-6) were identified in OFTM134, OFTM214 and OFTM301 (two copies in OFTM301). In each MAG, a gene encoding a cytochrome c_{554} was present upstream or downstream of the two ETM subunits. Interestingly, genes encoding the cytochrome *bd* complex subunits I and II (*cyd*AB) were present: *cyd*A was present in two to three copies in each MAG, and *cyd*B was present in one copy in OFTM134 and OFTM301 (in OFTM214 it was missing). CydAB might be used to counteract oxidative and nitrosative stress (Giuffrè *et al*., 2014). The gene *cydA* has been previously identified in anammox bacteria (de Almeida *et al*., 2016); however, given that *cydX* was missing in all three MAGs, it is unlike that these organisms would have cytochrome *bd* oxidase activity (VanOrsdel *et al*., 2013). Finally, a complete F-type ATPase was present in OFTM134 and OFTM214, while in OFTM301, six of the eight subunits were present. MAG OFTM134 also had a complete V-/A-type ATPase. Complete MAG annotation, BLASTP results, and gene nucleotide and amino sequences are available at DOI: 10.5281/zenodo.5524859.

The presence of the most important diagnostic genes associated to the anammox process and the phylogenetic affiliation of OFTM134, OFTM301 and OFTM214 within *Ca*. Brocadiales strongly suggest that these are new bacteria, able to perform the anammox process.

Potential metabolic versatility was identified in the novel MAGs

Besides genes encoding proteins involved in anammox metabolism, the MAGs had potential for sulfur, hydrogen and an alternative nitrogen cycle pathway. OFTM134, OFTM214 and OFTM301 had a nickel-dependent hydrogenase and corresponding maturation protein-encoding genes, as well as a sulfhydrogenase-encoding gene (*hydB*) involved in polysulfide reduction to sulfide (Ma *et al*., 1993), which seems to be absent in some *Ca*. Brocadia (Fig. S3). OFTM134 also had a sulfide:quinone oxidoreductase-encoding gene (*sqr*) for sulfide oxidation to elemental sulfur (Cherney *et al*., 2010) and a thiosulfate reductase/polysulfide reductase chain A-encoding gene (*phsA*) for thiosulfate reduction to sulfide (Hinsley and Berks, 2002). The *sqr* gene is lacking in most anammox bacteria, with the exception of *Ca*. Brocadia sinica, while *phsA* is only observed in *Ca*. Scalindua sediminis and *Ca*. Kuenenia (Fig. S3). OFTM134 and OFTM214 had a *sorA* gene encoding a cytochrome-dependent sulfite dehydrogenase for sulfite oxidation to sulfate (Kappler *et al*., 2000).

OFTM134, OFTM214 and OFTM301 had one or two copies of a histidine ammonia-lyase-encoding gene (*hutH*) for amino acid utilization and ammonia production (Schwede *et al*., 1999), which could potentially enter the ammonium oxidation pathway. This gene is also present in *Ca*. Scalinduaceae and in some *Ca*. Brocadia (Fig. S3). OFTM214 had a D-lactate dehydrogenase-encoding gene (*dld*), potentially involved in D-lactate utilization (Pinchuk *et al*., 2009). Finally, OFTM214 and OFTM301 had periplasmic nitrate reductase-encoding genes (*napAB*), for potential nitrate reduction to nitrite (Jepson *et al*., 2007). Reflecting the potential metabolic versatility of these organisms, heterodisulfide reductase-encoding genes (*hdr*) and Methyl-viologen-reducing hydrogenase-encoding genes (*mvh*) were identified: *hdrABC* in OFTM134, *hdrD* and *mvhD* in OFTM214, and *hdrABCD* and *mvhD* in OFTM301. Both *hdrABC* and *mvhD* are also observed among *Ca*. Scalinduaceae, but not among *Ca*. Brocadiaceae (Fig. S3).

For some anammox bacteria, the use of organic acids such as acetate has been reported (Kartal *et al*., 2008; van de Vossenberg *et al*., 2013). Bacteria incorporate acetate into acetyl-CoA via two main pathways: one uses an AMP-forming acetyl-CoA synthetase (ACS), and another requires both acetate kinase (AckA) and phosphate acetyltransferase (Pta), although this pathway can also be used for acetate formation (Wolfe, 2005). Genes with homology to kustc1128 ACS were found in OFTM134, OFTM214 and OFTM301. The AckA + Pta pathway was present in both OFTM301 and OFTM134. OFTM301 also had a putative acetate permease (ActP). However, at least for *K. stuttgartiensis*, the ability to incorporate acetate is still under discussion (Russ *et al*., 2012; Lawson *et al*., 2021). Additionally, OFTM214 had an ADP-forming acetyl-CoA synthetase, which has been linked to acetate production from acetyl-CoA in archaea and some bacteria (Schäfer *et al*., 1993; Schmidt and Schönheit, 2013). Both *AckA* and *Pta* seem to be absent in *Ca*. Kuenenia and *Ca*. Jettenia, but they are observed in *Ca*. Scalindua and *Ca*. Brocadia; *ActP* is also observed in some *Ca*. Scalindua (Fig. S3).

The novel MAGs have a limited geographical distribution

We did a BLASTP search with ribosomal protein S3 sequences to resolve if similar novel anammox were present in published metagenomes. The highest hits had between 66% and 73% identity to the novel anammox protein sequences. When comparing 16S rRNA gene sequences, one hit with 95% identity to OFTM134 and 93% to OFTM214 was retrieved. This corresponds to the NCBI accession number KM019039.1, which was sampled at brine–seawater interface of the Kebrit Deep brine pool at the Red Sea (Guan *et al*., 2015). This is an

environment characterized by its anoxic and hypersaline conditions. Additional *Ca*. Brocadiales 16S rRNA genes were recovered with a targeted assembly of the 16S rRNA gene from the metagenome (Gruber-Vodicka *et al*., 2020); although some of them grouped with *Ca*. Scalinduaceae, others were part of the OFTM134, OFTM214 and KM019039.1 groups (Fig. S4). The latter were part of a new clade, which was different to all *Ca*. Brocadiales families in the SILVA 138 database (Fig. S4). Thus, based on 16S rRNA gene phylogeny, there is evidence of four major clades among *Ca*. Brocadiales: *Ca*. Scalinduaceae, *Ca*. Brocadiaceae, GWA2-50-13 and the novel family from this study.

The near absence of this family in databases suggests that the novel MAGs could be rare, have a limited geographical distribution or thrive in poorly sampled ecosystems. Standard 16S rRNA V3–V4 and V4 primers can detect these anammox bacteria (see Supporting Information). However, a search against the 16S rRNA amplicon datasets at the NCBI resulted in similar sequences only being observed in 49 SRA samples. Of them, 25 corresponded to 'marine sediment metagenome' samples, 11 to 'sediment metagenome' and eight to 'marine metagenome' samples.

Other phylogenetic markers for anammox bacteria exist. One of them is HDH, a gene where PCR primers had been used for environmental surveys (Yang *et al*., 2020). For OFTM134, two sequences with around 99% similarity were found. These and two other related sequences are from surveys in Jiaozhou Bay sediments, suggesting that these might be similar species (Fig. 4). These HDH sequences seem to correspond to the anammox novel clade II reported in Dang *et al*. (2010). A lower similarity was observed for OFTM301 and OFTM214, but they seem to be part of the same clade (Fig. 4). The poor representation of these sequences in HDH clone libraries could be in part due to multiple mismatches in commonly used HDH primers (See Supporting Information).

In the absence of adequate primers, metagenomics improves the identification of HDH in environmental samples. One approach that could be used is protein-level assembly (Steinegger *et al*., 2019). We used this to successfully recover several HDHs that clustered either with *Ca*. Scalinduaceae or with the novel anammox clade (Fig. 4). HDH recovered from MAGs in other surveys suggests that other undescribed anammox clades may exist (Fig. 4), as also suggested by 16S rRNA and concatenated protein trees (Fig. S4, Fig. 1). Primer mismatches in HDH amplicon studies and misclassification in 16S rRNA amplicon studies could lead to an underestimation of anammox bacteria in marine sediments, which could affect estimations of total nitrogen turnover.

Since the novel anammox family appears to be present in marine sediments, this would indicate adaptations to salinity. Indeed, OpuC, encoding a transporter for glycine betaine and proline, was present in all three MAGs. OMTF214 harboured a gene encoding a BCCT transporter, which is involved in the uptake of several compatible solutes (Ziegler *et al*., 2010). OpuC appears to be absent in Ca. *Brocadia* and BCCT transporters are only observed in *Ca*. Kuenenia and *Ca*. Scalindua arabica (Fig. S3). Instead of using compatible solutes, another adaptation to salinity could be to maintain high intracellular salt concentrations, as it has been suggested for *Ca*. Scalindua rubra (Speth *et al*., 2017).

Distribution of anammox MAGs in the Oslofjord biofilms

Anammox bacteria often coexist with nitrifiers, and such was also the case for the microbial community in the Oslofjord biofilms. MAGs classified as the nitrifiers *Nitrosomonas*, *Nitrosopumilus*, *Nitrospirales* and *Nitrospirota* were recovered (data not shown). This confirms the results of Karačić *et al*. (2018), where nitrifiers were also observed in the amplicon dataset.

Both *Ca*. Scalindua and the novel anammox MAGs coexisted in the Oslofjord biofilm (Fig. 5). In this study, two separate locations were sampled in four different years, but it seems as if OFTM134, OFTM214 and OFTM301 were not represented in all samples (Fig. 5). It is unclear what factors affected the abundance and distribution of anammox bacteria in the Oslofjord tunnel. Local variations in the microbial communities between biofilms and within the biofilms could lead to distinct nitrogen-cycling processes, and thus nitrogen loss by the anammox process could be localized to certain spots.

Oslofjord tunnel biofilms

Biofilm growth has been observed in the Oslofjord subsea tunnel and is clearly linked to concrete biodeterioration (Hagelia, 2011; Karačić *et al*., 2018). The communities sampled represent biofilms associated with water seeps on the tunnel walls, the mode of occurrence being similar to observations of biofilm growth on exposed rock walls in underground mines (Bond *et al*., 2000; Sjöberg *et al*., 2020). Given that anammox bacteria have been identified in sediments (Dang *et al*., 2010; Prokopenko *et al*., 2013; Devol, 2015; Zhao *et al*., 2020), we hypothesize that the origin of anammox bacteria in the tunnel is seawater, seeping through cracks in the bedrock, from overlying marine sediments into the tunnel.

Results from a previous study at the Oslofjord tunnel suggest that nitrogen transformation processes occur in

Fig. 4. Phylogenetic tree of hydrazine dehydrogenase (HDH) amino acid sequences. Circles shown Ultrafast bootstrap support higher than 95%. The HOX kustc1061 of *Ca*. Kuenenia stuttgartiensis was used as outgroup.

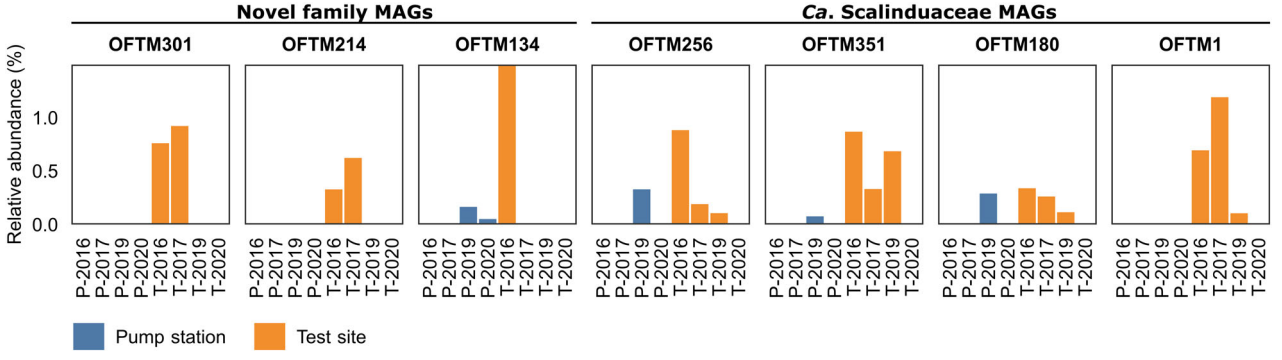

Fig. 5. Relative abundances of *Ca*. Brocadiales MAGs at the two sites in the Oslofjord tunnel.

the tunnel biofilms (Hagelia, 2011). Ammonium loss from water coming from bedrock, passing through biofilms, and falling into ditches below was inferred for some locations (Fig. S1) (Hagelia, 2011). For example, in water collected from rock joints at location V5, the ammonium concentration was 1.7 mg N/L, but in the ditch below it

was 0.003 mg N/L (Hagelia, 2011), potentially due to nitrification and/or anammox activity.

The main source of ammonium in the tunnel could have been organic matter degradation in the marine sediments above the tunnel. In sediments, oxygen is rapidly used as electron acceptor, while ammonium accumulates with increasing depth as organic matter is degraded (Devol, 2015). Data from Hagelia (2011) show that in the water column above the tunnel, at 60 m sea depth, the ammonium concentration was below detection limit at <0.005 mg N/L. However, in the deeper zone of the tunnel, water collected from rock joints typically had about 1–1.5 mg N/L ammonium (Fig. S1). Ammonium concentrations in the tunnel were positively correlated with local sediment thickness (Fig. S2). This supports that the chemical composition of the water changed when passing through sediments, before arriving to the tunnel.

In the absence of oxygen, nitrate and nitrite in the sediments above the tunnel would have been used by denitrifiers and anammox bacteria. Hagelia (2011) observed that for rock joins in the tunnel, nitrate + nitrite concentrations were around 0.006 mg N/L, while the concentration in the Oslofjord water column was 0.15 mg N/L (Fig. S1). Despite the incoming water to the tunnel being poor in nitrate + nitrite, in the tunnel, the oxic environment would favour aerobic ammonia oxidation and further nitrite oxidation. The newly produced nitrite could sustain other nitrogen-converting processes such as anammox.

Conclusions

Here we described three novel species within a novel genus and family of anammox bacteria, which were found in metagenomes from a subseafloor biofilm. We suggest the name *Ca*. Anammoxibacter for this new genus and *Ca*. Anammoxibacteraceae for the family, referring to their predicted potential for the anammox process. This study adds to our understanding of the largely unexplored biodiversity of subseafloor environments. Future exploration of other anaerobic communities where ammonium is present could lead to discovery of more anammox bacteria, with implications to the global nitrogen cycle and potential novel biotechnological applications.

Experimental procedures

The Oslofjord subsea road tunnel is located near Drøbak, Norway (59.66472 N, 10.61306 E). In locations where water seepage occurred, biofilms with orange or black colour were observed. The locations with biofilm are shielded from tunnel traffic exhaust fumes by a continuous inner lining of cast concrete elements. The sea depths above the investigated part of the tunnel vary from about 30 to 45 m, with sediment thicknesses of 10–55 m and rock covers ranging from 35 to 75 m. The sediments consist of mud and clays in upper parts and are frequently dominated by fluvio-glacial deposits (sand and gravel) in the deeper parts (Backer and Blindheim, 1999; Haverkamp *et al*., 2014). The bedrock here consists mainly of granitic gneiss and granite pegmatite, being variably affected by deep weathering along rock joint systems with associated clay minerals (illite, kaolinite, montmorillonite), calcite, chlorite and subordinate sulfide (pyrite) (NPRA, unpublished data).

Two separate places in the tunnel, referred to as Pump-station and Test-site, were sampled on four different occasions over a period of 5 years. Sampling and DNA extraction were conducted as previously described (Karačić *et al*., 2018). Shotgun metagenomic sequencing was done in an Illumina NovaSeq6000 platform, generating 150 bp paired-end reads. Reads were normalized to 100× coverage using BBNorm in the BBTools package 38.61b (https://sourceforge.net/projects/bbmap). Co-assembly of normalized reads was performed with Megahit 1.2.9 (Li *et al*., 2015), followed by mapping the reads to the assembly with Bowtie v2.3.5.1 (Langmead and Salzberg, 2012). Metagenomic binning was conducted with both MetaBAT2 v2.15 (Kang *et al*., 2019) and BinSanity v0.5.3 (Graham *et al*., 2017). MAGs obtained with both methods were dereplicated with DASTool v1.1.2 (Sieber *et al*., 2018), resulting in 401 MAGs with less than 10% contamination and completeness higher than 50% as determined with CheckM (Parks *et al*., 2015). Using GTDB-Tk v1.5.0 (Chaumeil *et al*., 2020) with the GTDB 06-RS202 taxonomy (Parks *et al*., 2018, 2020), seven MAGs were classified as *Ca*. Brocadiales. The relative abundance of MAGs was estimated with coverM v0.6.1 (https://github.com/wwood/CoverM) with the *relative_abundance* parameter in *genome* mode using BWA-MEM (Li, 2013).

MAGs were annotated with DRAM v1.0 (Shaffer *et al*., 2020) with default options, except -min_contig_size 1000, and genes of interest were searched in annotation files. While the gene encoding hydrazine synthase subunit A (*hzsA*) was annotated via the PFAM hit to PF18582, subunits B and C, present downstream, were identified via BLASTP analyses and manual inspection of CxxCH motifs. Genes encoding proteins involved in the anammox electron transport chain were searched both via annotation files and via BLASTP using previously identified reference sequences from *Ca*. Kuenenia stuttgartiensis (de Almeida *et al*., 2016; Kartal and Keltjens, 2016).

A UBCG v3.0 (Na *et al*., 2018) phylogenetic tree with 92 genes was constructed to place the MAGs. For that, reference genomes were downloaded from NCBI, and after checkM v1.1.3 inspection, 56 high quality (>90% complete and <5% contaminated) reference genomes were selected. Average AAI between selected genomes was calculated using the Kostas Lab tool (http://enve-omics.ce.gatech.edu/g-matrix/index). For this, genomes were gene-called with Prodigal v2.6.3 (Hyatt *et al*., 2010), and amino acid fasta files were used as input.

The identification of organisms related to the MAGs in this study in other databases deposited on NCBI was attempted via BLASTP using as query sequences the ribosomal protein S3 (RpsC) and hydroxylamine dehydrogenase (HDH) in this study's MAGs, and via BLASTN using as query sequences fragments of the 16S rRNA gene extracted with SSU-align v0.1.1 (Nawrocki, 2009). For HDH, identified and reference amino acid sequences were aligned with MAFFT v7.487 using the L-INS-i option (Katoh and Standley, 2013). This was used to construct a maximum likelihood tree with IQ-TREE v2.1.4 (Minh *et al*., 2020), using 1000 replicates for bootstrap support with ultrafast bootstrap (Hoang *et al*., 2018), and a WAG + R3 model chosen by ModelFinder (Kalyaanamoorthy *et al*., 2017).

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Data Availability

MAGs from this study have been deposited into NCBI (accession numbers: JAIQZO000000000, JAIQZQ000000000, JAIQZP000000000). Metagenome reads and other MAGs from the Oslofjord tunnel biofilms can be found in the BioProject PRJNA755678. Annotated gene and protein files, as well as the DRAM annotation spreadsheet and other DRAM output files, have been deposited in Zenodo (DOI:10.5281/zenodo.5524859), available at https://zenodo.org/record/5524859#.Yg5RqJTMJaQ.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information.