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Amplicon-guided isolation and cultivation of previously uncultured microbial species from activated sludge

Maarten D. Verhoeven,[1] Per H. Nielsen,[1] Morten K. D. Dueholm[1]

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ABSTRACT Microbes are fundamental for biological wastewater treatment. However, most microbial species found in activated sludge (AS) from wastewater treatment plants (WWTPs) have never been isolated and grown as pure cultures, thus limiting our understanding of the underlying biological processes. To change this, we here introduce an experimental setup where the plating of dispersed AS bacteria is combined with 16S rRNA gene amplicon sequencing of the total plate biomass for rapid identification of growth conditions that allow for the isolation of key microbial species in AS. We show that agarose plates composed of AS fluid supplemented with various carbon sources support the growth of many previously uncultivated AS bacteria. To confirm that the approach can also be used to isolate previously uncultured species, we picked 200 colonies from the plates for growth in liquid medium. This resulted in 185 enriched cultures representing at least 76 strains based on unique 16S rRNA gene V1-V3 amplicon sequence variants (ASVs). Classification of the ASVs with the MiDAS 5.1 database revealed 39 distinct genera, including AAP99, *Ca*. Brachybacter, *Ca*. Proximibacter, Ellin6067, midas_g_12, and midas_29279, which all lack pure culture representatives. Among the ASVs that obtained species-level classification, we observed 26 unique species of which 16 were only classified based on the MiDAS placeholder taxonomy highlighting the potential for culturing many novel taxa. Purification by restreaking and preparation of glycerol stocks resulted in 10 pure cultures of species commonly found in WWTPs globally, including *Rhodoferax* midas_s_1744, *Thauera* midas_s_1356, *Acidovorax* midas_s_1484, *Tessaracoccus* midas_s_1151, and *Sphingopyxis* midas_s_983.

IMPORTANCE Biological wastewater treatment relies on complex microbial communities that assimilate nutrients and break down pollutants in the wastewater. Knowledge about the physiology and metabolism of bacteria in wastewater treatment plants (WWTPs) may therefore be used to improve the efficacy and economy of wastewater treatment. Our current knowledge is largely based on 16S rRNA gene amplicon profiling, fluorescence *in situ* hybridization studies, and predictions based on metagenome-assembled genomes. Bacterial isolates are often required to validate genome-based predictions as they allow researchers to analyze a specific species without interference from other bacteria and with simple bulk measurements. Unfortunately, there are currently very few pure cultures representing the microbes commonly found in WWTPs. To address this, we introduce an isolation strategy that takes advantage of state-of-the-art microbial profiling techniques to uncover suitable growth conditions for key WWTP microbes. We furthermore demonstrate that this information can be used to isolate key organisms representing global WWTPs.

KEYWORDS wastewater, microbiology, culturomics, 16S RNA

Wastewater treatment is a vital technology in urbanized areas, as it protects public health and the environment and enables resource recovery. The most common

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wastewater treatment process worldwide is the conventional activated sludge (AS) process. This process relies on complex microbial communities that grow as suspended aggregates using nutrients from the wastewater as feed and converting it into excess biomass. This biomass is then separated from the cleaned effluent through sedimentation (1). By understanding the physiology and metabolic potential of the microbes common in wastewater treatment plants (WWTPs), we can improve the efficacy and stability of wastewater treatment, reduce the release of greenhouse gases, and recover valuable resources like nitrogen, phosphorus, and biopolymers (1–3).

The emergence of widely accessible DNA sequencing technologies has dramatically improved our knowledge on the distribution, diversity, and phylogeny of microorganisms present in different environments on Earth (4). Two major studies have investigated the global diversity of bacteria in WWTPs, the Global Water Microbiome Consortium (GWMC) project (5), and the Global Microbial Database for Activated Sludge (MiDAS) project (6). The latter features a full-length 16S rRNA gene amplicon sequence variant (ASV)-resolved reference database for all common bacteria and archaea in WWTPs worldwide (MiDAS 4). This database contains a unique seven-rank (domain to species) taxonomy for all reference sequences including reproducible placeholder names for environmental taxa lacking official taxonomic classifications (6, 7). The placeholder taxa are defined based on identity thresholds and may not always align with the phylogenetic evolution of the taxa (7). They can be easily identified by their names, which follow the format "midas_x_y." Here, "x" is a one-letter abbreviation for the taxonomic rank, and "y" refers to the ASV number of the corresponding reference sequence. Using this database, the abundance of all community members in WWTPs, as well as information about their phylogenetic relation to known species, has been described (6). However, our current understanding of their metabolism and community function is still limited, as it is based only on a small subset of species for which isolates, complete genomes, and physiological data are available (8).

Although advances in obtaining metagenome-assembled genomes (MAGs) have shed light on the ecological roles of some uncultured microbial species, as exemplified by the recent recovery of more than a thousand high-quality (HQ) MAGs from Danish WWTPs (9), predicting their physiology becomes increasingly challenging as more novel and distinct taxa are discovered. This is because many of the protein-encoding sequences in these novel microbes show low similarity with those that have been experimentally characterized in previous research. To further advance our understanding of individual bacteria in WWTPs, it is crucial to conduct more wet lab studies that can verify genomics-based predictions. This is especially important for species for which data on their physiology, metabolism, and cell biology is not currently available (4).

While metabolomics, proteomics, transcriptomics, and in-depth physiological studies of mixed or enriched cultures are possible, the results are often challenging to interpret as the community dynamics complicate pinpointing the specific traits of the species of interest. Moreover, it is not possible to stock and reproduce active biomass from environmental samples, which is required to ensure experiments can be reproduced. It is therefore important that we obtain pure cultures from the species of interest in AS (1, 4, 10).

At present, the vast majority of the bacterial species that make up the microbial communities in AS have not been cultured individually, and as such, very few representative pure cultures are available (11). Specifically, we would like to isolate representatives for the core and conditional rare or abundant taxa (CRAT) previously found in WWTPs across the globe (6), as these are assumed to have the highest impact on the treatment efficacy. While there are a few studies available in which global AS core bacterial species (6), such as *Acidovorax caeni* (12), *Microlunatus phosphovorus* (13), *Microthrix parvicella* (14), *Nitrospira defluvii* (15), and *Zoogloea caeni* (16), were isolated, in most cases, available AS isolates represent species that are low abundant *in situ* and may therefore not contribute significantly to the microbial community in WWTPs.

The discrepancy between the number of species that can be cultured in the lab compared with the vastly higher number being present in the samples from a natural habitat has been known for decades and is often referred to as "the great plate anomaly" (4, 17). The difficulty of isolating species can be attributed to various factors including (i) the challenges associated with dispersing bacterial aggregates and separating individual cells and (ii) the difficulty in predicting and recreating the specific environmental conditions necessary for the proliferation of targeted microbial community members (18).

In the present study, we address these challenges by introducing a simple isolation strategy where AS bacteria dispersed using sonication, filtered to remove aggregates, and subsequently plated on growth media based on AS fluid (ASF) supplemented with various carbon sources are combined with 16S rRNA gene amplicon sequencing of total plate biomass for rapid identification of growth conditions that allow for the isolation of individual microbial community members. Tailoring the medium composition closely toward the conditions experienced in WWTPs resulted in a higher species isolation yield compared with traditional media, allowing for the isolation of previously uncultured bacteria.

RESULTS AND DISCUSSION

Preparation of single cell suspensions from activated sludge flocs

Most microbes within AS grow in flocs with multiple species. Cultivation of single strains, therefore, requires dispersal of the flocs, preferably without significant impact on the overall viability of bacterial cells present. To disrupt the flocs, AS samples obtained from the Aalborg West (AAW) WWTP were homogenized and sonicated, after which each sample was passed sequentially through 40-µm and 5-µm cell strainers. LIVE/DEAD staining of the dispersed samples showed predominantly single cells, and there was no detectable difference in viability between the single cell suspension compared with the source AS (Fig. 1a). Furthermore, 16S rRNA gene amplicon sequencing of DNA extracted from AS and the derived single-cell suspension showed no loss of species (ASV richness) during the preparation process (see Fig. 1b; Fig. S1a). In fact, the overall diversity was significantly higher in the single-cell preparation. Community composition analysis showed that specific genera, such as *Ca*. Phosphoribacter (previously classified as *Tetrasphaera*), *Nitrospira*, and *Ca*. Microthrix, decreased in relative abundance across all replicates, whereas others, such as *Rhodoferax*, *Ca*. Brachybacter (previously classified as OLB8), midas_g_171, and *Acidovorax* increased (Fig. 1c; Fig. S1b). The reduced relative abundance of highly abundant AS genera explains the increased diversity observed for the single-cell preparations.

AS fluid supports the growth of previously uncultured species

To mimic the environmental conditions found in the WWTP, we extracted ASF directly from the source AS. For this, the supernatant of settled AS from the AAW WWTP was ultra-filtrated to yield a clear solution without particulate matter. Subsequently, the filtrate was concentrated by reverse osmosis and the retentate filter sterilized. The resulting ASF was supplemented to agarose culture plates with a variety of carbon sources in low concentration to mimic the oligotrophic environment encountered by the AS bacteria *in situ* (19). Agarose was used to eliminate any potential effects of impurities present in more regularly used agar (20, 21). Ammonium was added as an additional nitrogen source, except for plates with tryptone or casamino acids. Approximately 1,000 single cells derived from AS were spread on each plate, and these were incubated for 2–3 weeks under either oxic or anoxic conditions at 25°C.

To determine the effect of the different substrates, we performed 16S rRNA gene amplicon sequencing on DNA extracted from the entire biomass scraped off each agarose plate. Taxonomic classification of the resulting ASVs with the MIDAS 5.1 reference database allowed for genus- and species-level classification for most ASVs

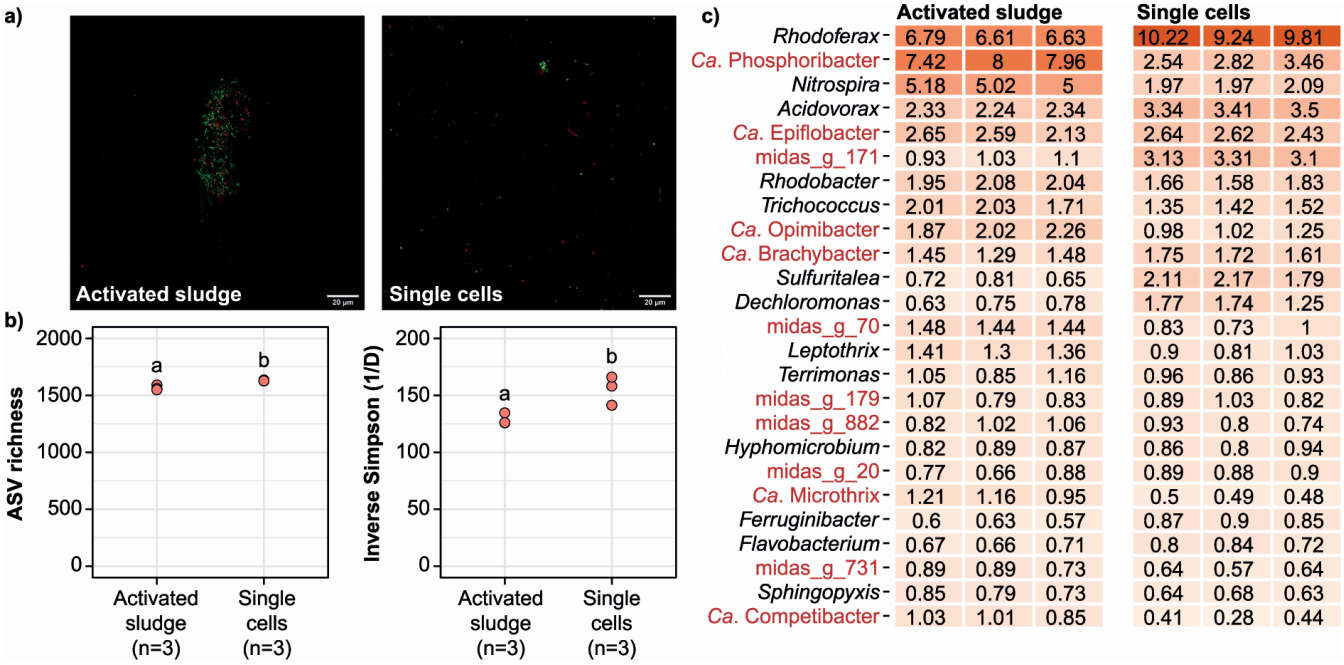

FIG 1 Preparation of single cell suspensions from activated sludge. (a) Microscopy images of LIVE/DEAD-stained untreated activated sludge and single cell suspension. Live cells appear in green and dead in red. (b) Alpha diversity based on 16S rRNA gene V1-V3 amplicon data for technical triplicates. A two-sided *t*-test (Bonferroni correction, α = 0.05) was used for pairwise comparison of individual groups, and the results are shown with compact letter display (groups that do not share letters are significantly different). (c) Heatmap of the 25 most abundant genera in the activated sludge and single cell suspensions based on the V1-V3 amplicon data. Genera marked in red lack pure culture representatives. All figures are based on activated sludge collected the 16th of December 2020 and represent oxic cultivations.

observed. By supplementing conventional culture media [Reasoner's 2A (R2A) and Tryptic Soy Broth (TSB)], which have been previously utilized for cultivating and isolating bacteria from AS (10, 22), with ASF, we observed an increase in the ASV richness by 1.8–4.8-fold and a rise in the inverse Simpson's diversity by 1.5–4.0-fold. (Fig. 2a). The effect could also be observed directly on the agarose plates based on the diversity of colony morphologies (Fig. S2). Compared with the conventional bacterial culture media, the ASV diversity that emerged on ASF plates supplemented with single carbon sources was significantly higher (Fig. 2a).

To further investigate the effect of media composition on the growth of the AS bacteria, we performed beta diversity analyzes (Fig. 2b). Principal coordinates analysis (PCoA) plots of Bray-Curtis and Jaccard distances for ASVs revealed that each growth condition promoted the growth of a specific subset of the AS bacteria and that similar carbon sources such as tryptone and casamino acids, or glucose and mannose, resembled each other more closely when it comes to ASV diversity and abundance. This suggests that specific growth conditions should be considered for targeted isolation of specific species. The sample clustering was more pronounced under anoxic compared with oxic conditions according to PERMANOVA, which suggest that fermentative bacteria in general were more substrate specific.

To pinpoint which media promote the growth of abundant AS bacteria, we compared the relative abundance of the most abundant genera in the dispersed AS with those obtained from the agarose plates (Fig. 3). Because we only spread approx. 1,000 bacterial cells on each agarose plate and the harvested microbial biomass was visible by eye (containing billions of cells), we assume that any detectable reads in the amplicon data relate to the actual growth of the associated species. With this in mind, we were able to detect growth of 68 (oxic) and 72 (anoxic) of the top 100 most abundant genera in the dispersed AS using medium with the ASF, whereas media without ASF only allowed growth of 23 (oxic) and 4 (anoxic) genera. This clearly demonstrates the importance of

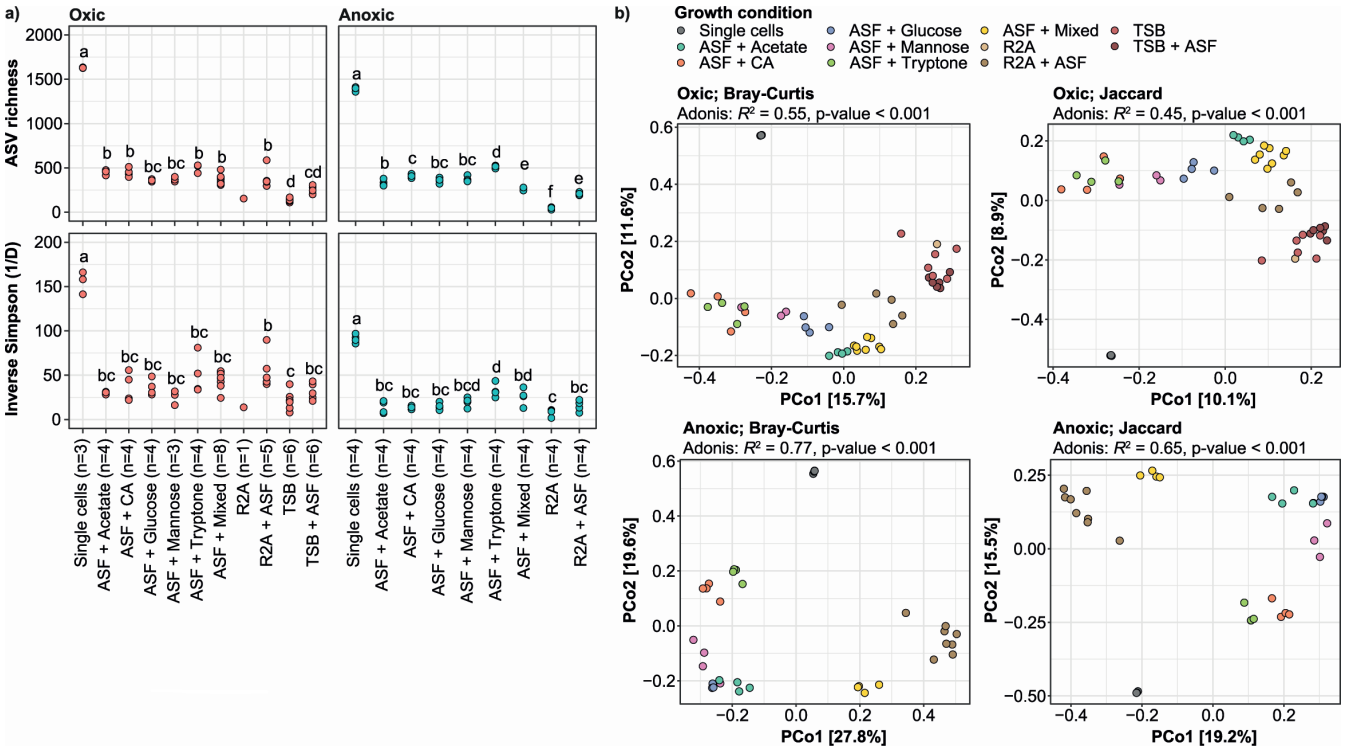

FIG 2 Microbial diversity of activated sludge single cell suspensions grown on various agarose media under oxic or anoxic conditions. (a) Alpha diversity based on 16S rRNA gene V1-V3 amplicon data. A two-sided *t*-test (Bonferroni correction, α = 0.05) was used for pairwise comparison of individual groups, and the results are shown with compact letter display (groups that do not share letters are significantly different). (b) Beta diversity based on 16S rRNA gene V1-V3 amplicon data. Bray-Curtis and Jaccard diversity were calculated at the ASV level. The fraction of variation in the microbial community explained by the growth condition was determined by permutational multivariate analysis of variance (PERMANOVA) (Adonis R^2 values). Exact *P-values* < 0.001 could not be confidently determined due to the permutational nature of the test. ASF, activated sludge fluid; CA, casamino acids.

mimicking the source environment when trying to isolate new bacteria from a specific ecosystem.

Interestingly, ASF was able to stimulate the growth of several candidate genera and genera only described based on the MiDAS placeholder taxonomy (Fig. 3). These include genera of key importance for the wastewater treatment process, such as the polyphosphate accumulating organisms (PAOs) *Ca*. Accumulibacter and *Ca*. Phosphoribacter (23, 24). Because these genera have been key targets for isolation for many years, we investigated these genera in depth at the ASV level (Fig. S3). The most common *Ca*. Accumulibacter species, *Ca*. *Accumulibacter phosphatis*, was unable to grow under any of the applied conditions, even though it was present in the dispersed AS. It is, therefore, expected that this species lacks a vital nutrient in the media or a syntrophic partner. However, other *Ca*. Accumulibacter species exhibited growth potential, particularly under anoxic conditions. Growth potential was also observed for several strains, defined by ASVs, belonging to *Ca*. Phosphoribacter, which is the most abundant PAO worldwide (24). Once again, growth was most pronounced under anoxic conditions. Accordingly, it should be possible to isolate members of this genus.

The discrepancy between high abundance in AS and low relative abundance of *Ca*. Phosphoribacter on culture plates could be due to several factors. While 16S rRNA gene amplicon sequencing of the single cell material confirmed the presence of *Ca*. Phosphoribacter, it cannot be ruled out that the sonication treatment of the AS has disproportionately impacted the viability of this genus (25). Detailed investigation on the impact of sonication on taxa-specific viability could provide insight into how to obtain live single cells from the bacteria of interest.

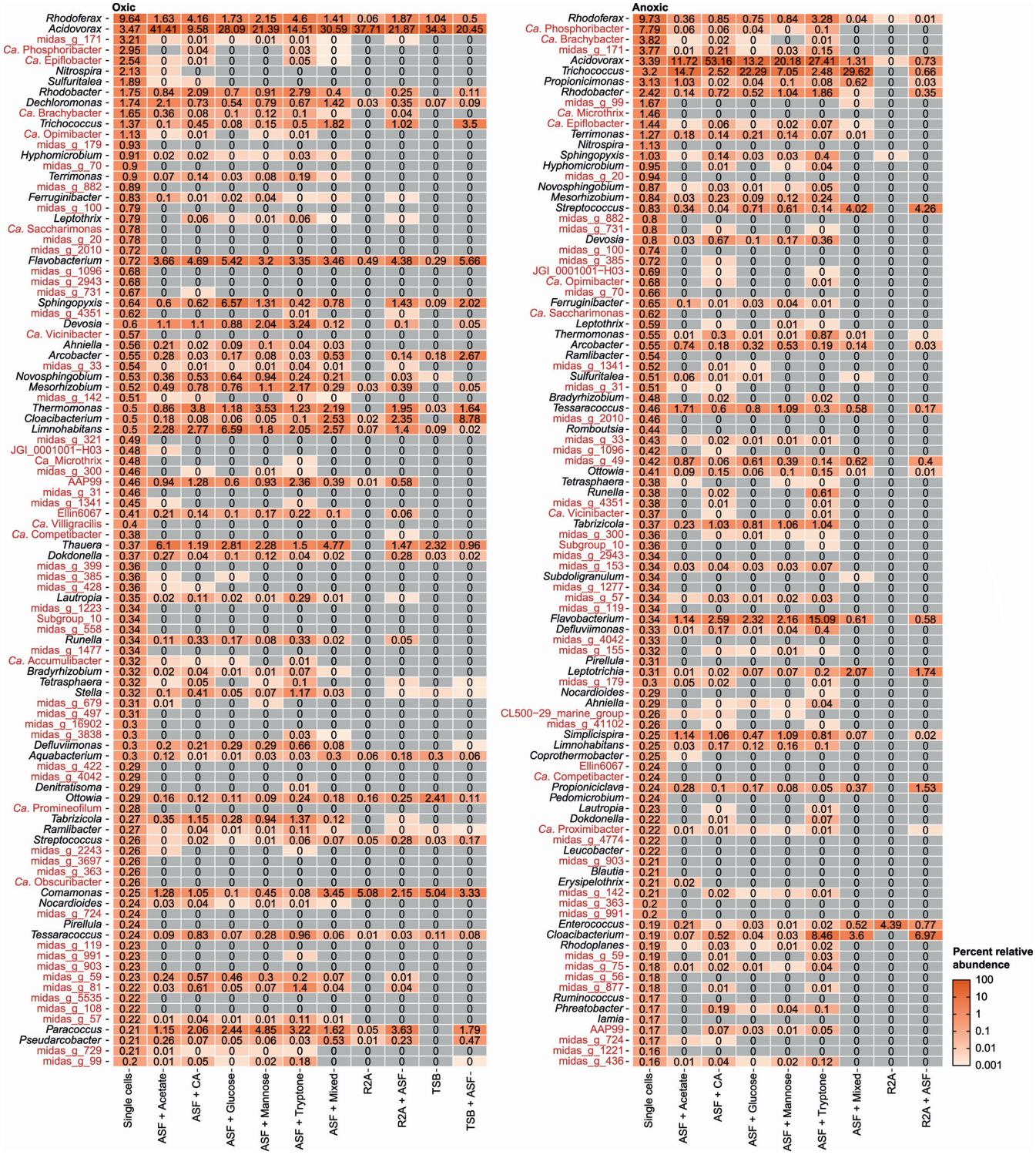

FIG 3 Heat map showing the top 100 genera found in the AS and the corresponding abundance values found on each plate incubation under oxic and anoxic conditions. Values are the average abundance from four separately processed agarose plates. Genera colored in red have no pure culture representatives. ASF, activated sludge fluid; CA, casamino acids.

Visual inspection of the culture plates prior to scraping colonies showed substantial variation in colony size (Fig. S2). Since the 16S rRNA gene amplicon data solely provide the relative abundance for growth on plates, it underrepresents slow growing organisms forming small colonies. Long-term survey data from Danish WWTPs show that *Ca.*

Phosphoribacter is highly enriched (up to 30%) within some plants while only present at low levels in sewage influent (0.05%) (11). Nonetheless, 16S rRNA gene amplicon sequencing data (Fig. 3) imply that *Ca*. Phosphoribacter is likely forming colonies, albeit likely of small size.

Competition from fast-growing bacteria

Although many AS bacteria were able to grow on the ASF-based agarose plates, their relative abundance on the plates was in general low. To learn more about the competing bacteria, we investigated the genera with the highest relative abundance on the agarose plates (Fig. S4). These were *Acidovorax*, *Pseudomonas*, *Diaphorobacter*, and *Flavobacterium* under oxic conditions and *Lactococcus* (mainly on R2A), *Acidovorax*, *Uliginosibacterium*, and *Trichococcus* under anoxic conditions.

Overall, it seems that the cultivation conditions on plates with ASF stimulated growth of many organisms that are more abundant in sewage compared with AS (26, 27). This indicates that the growth conditions on plates more closely resemble a nutrient-rich sewer system environment than the oligotrophic environment found in the WWTPs, where the latter inherently selects for microbes that manifest a kinetics-based strategy for proliferation (k-strategists) (19). Accordingly, culturing on plates is more suited for isolating rate-based strategists (r-strategists), since, with readily available substrate and virtually no competition from other cells, colonies can grow unhindered (4). *Acidovorax* has been commonly observed in WWTP that manifests a short sludge retention time (28). Moreover, it is one of the most abundant genera in the influent wastewater at Danish WWTPs (26, 27). Yet, in most Danish WWTPs, it seems that *Acidovorax* is not proliferating in the AS system and their presence is likely largely due to the high influx of these species from the sewage system (29). *Trichoccocus* was among the most abundant genera observed of the tested anoxic culture conditions, and cultured representatives from this genus are facultative anaerobes that can grow at low temperatures and manifest fermentative growth (30, 31). Similar to *Acidovorax*, multiple studies have identified *Trichococcus* as one of the most prevalent genera in sewage systems (32, 33).

Process-important bacterial species can be isolated

An important goal of this study was to demonstrate that pure cultures of previously uncultured AS species can be isolated based on our optimized growth conditions. To do this, colonies were randomly picked from plates incubated under oxic conditions containing ASF supplemented with acetate, tryptone, or casamino acids taking care to select both large and small colonies. The colonies were transferred to liquid isolation medium in 96-deep-well plates and incubated for 2 weeks at 25°C under oxic conditions. Hereafter, 16S rRNA gene amplicon sequencing was performed on DNA extracted from 185 growing cultures. Sequencing revealed the enrichment of 76 strains based on unique ASVs. The average read abundance of these ASVs was 55% with 176 out of 185 below 95%, indicating that subsequent single colony isolation was required to obtain pure cultures. Classification of the ASVs using the MiDAS 5.1 database revealed enrichment of 39 genera and 26 species (many ASVs were not classified at the species level) (Supplementary Data S1). The enriched genera included previously uncultivated genera, such as *Ca*. Brachybacter (previously OLB8) (34), AAP99, Ellin6067, and *Ca*. Propionivibrio (23), which are all abundant in WWTPs, and most of the species (16 out of 26) were only classified based on the MiDAS placeholder taxonomy, highlighting that several previously uncultivated taxa can be isolated using the ASF medium.

Performing high-throughput restreaking, colony picking, and microbial profiling without laboratory automation can be resource intensive. Therefore, a selection of 20 cultures was made based on their significance in wastewater treatment and average abundance in WWTPs in Denmark (6). After restreaking and growing these cultures for two weeks on agarose plates with isolation medium, four colonies of each culture were picked and grown in liquid isolation medium. 16S rRNA gene amplicon sequencing on DNA extracted from the liquid cultures showed the successful isolation for three of the

TABLE 1 Relative abundance of targeted species throughout the isolation process[a]

Isolate	ASV	MIDAS 5.1 taxonomy	Best hit in LTP	AS	Plate	Colony	Restreak	Glycerol stock
098_3	ASV29,42,58,65,56,60	*Acinetobacter* sp.	97.6%–99.2%	0.02%	0.25%	59.5%	39.4%	99.9%
121_4	ASV2	*Paracoccus* sp.	99.6%	0.05%	0.78%	64.2%	99.8%	100.0%
076_1	ASV17	*Rhodoferax* midas_s_1744	95.6%	0.03%	0.80%	56.8%	99.3%	100.0%
060_1	ASV4	*Acidovorax* sp.	100.0%	0.94%	22.67%	96.4%	96.0%	100.0%
175_2	ASV13	*Sphingopyxis bauzanensis*	100.0%	0.38%	0.36%	63.3%	37.3%	99.8%
150_1	ASV6	*Thauera* midas_s_1356	98.8%	0.02%	2.67%	31.6%	86.3%	99.9%
070_2	ASV12	*Acidovorax* midas_s_1484	98.8%	1.88%	1.01%	92.8%	57.5%	100.0%
105_4	ASV59	*Tessaracoccus* midas_s_1151	94.8%	0.04%	0.02%	32.1%	19.5%	98.4%
185_4	ASV9	*Thermomonas carbonis*	99.6%	0.08%	0.45%	99.1%	34.0%	100.0%
172_3	ASV18	*Sphingopyxis* midas_s_983	100.0%	0.26%	1.49%	11.9%	37.3%	99.5%
163_2	ASV34	*Zoogloea* midas_s_1080	99.2%	0.03%	0.31%	27.3%	8.4%	0.0%
100_4	ASV22	Ellin6067 midas_s_284	89.2%	0.02%	0.03%	62.8%	60.5%	0.0%
138_5	ASV74	*Rhodobacter* sp.	97.6%	0.33%	2.03%	38.6%	43.2%	0.0%
181_2	ASV19	*Rhodoplanes* midas_s_187	98.4%	0.16%	0.02%	39.4%	48.9%	0.0%
179_3	ASV48	AAP99 sp.	97.2%	0.27%	1.01%	84.0%	3.0%	0.0%
083_1	ASV23	*Hyphomicrobium* sp.	95.2%	0.09%	0.01%	14.2%	55.5%	0.0%
142_1	ASV8	*Ca.* Proximibacter danicus	98.8%	0.02%	0.03%	96.1%	94.3%	0.0%

[a]AS, ASV abundance in the source-activated sludge. Plate, ASV abundance after AS single cells were plated and incubated under oxic conditions. Colony and Restreak, ASV abundance of picked colony from the ASF plate and the subsequent restreak after being incubated in isolation medium for 2 weeks. Glycerol stock, ASV abundance of colonies after restreaking the final glycerol stock. Best hit in LTP indicated the highest percentage identity of the ASVs when mapped against the living tree project database version LTP_05_2023.

targeted taxa based on high relative abundance of the ASV of interest (>95%) and strong enrichment of an additional seven (>50% relative abundance). These liquid cultures, along with seven additional cultures that showed enrichment of intriguing strains, were restreaked for isolation. Subsequently, they were used to prepare glycerol stocks for long-term storage. The stocks were eventually plated, and the resulting colonies were evaluated by amplicon sequencing. This confirmed the successful isolation of 10 species (Table 1; Supplemental Data S1). These species include some species only identified by MiDAS placeholder names, including *Rhodoferax* midas_s_1744, *Thauera* midas_s_1356, *Acidovorax* midas_s_1484, *Tessaracoccus* midas_s_1151, and *Sphingopyxis* midas_s_983. To gauge the novelty of the isolates further, we compared their ASVs with the most recent version of the Living Tree Project (LTP) database (LTP_05_2023) (Table 1). Using the species-level threshold for 16S rRNA genes (<98.7% identity) as suggested by Yarza et al. (35), we determined that two of our isolates likely represent new species. Additionally, two more are on the borderline with a 98.8% identity to their best match. Future experiments will shed light on the physiology and metabolic potential of these taxa.

When evaluating the amplicon abundance data for each step of the isolation process, several aspects seem to stand out: (i) Most species present in high abundance (>50% relative abundance) in cultures derived directly from initial culture plates were successfully isolated after several restreaks. Conversely, most species with low abundance for colony-forming units did not achieve successful species isolation. (ii) Several abundant species on the plates were not able to grow in liquid culture. This could be due to differences in growth conditions since physicochemical conditions might not accommodate proliferation for the species of interest. Additionally, some species might manifest loss of viability when frozen as glycerol stocks at −80°C during the final step of single-colony isolation (36). (iii) While it is plausible that intermediate liquid cultures for some species could have been contaminated by outcompeting bacterial species, sequencing data, for example, from the restreaks of *Zoogloea* midas_s_1080, consistently showed the presence of both the species of interest and an *Enterobacter* species (Supplemental Data S1). This suggests that *Zoogloea* midas_s_1080 may not be able to grow in isolation, indicating a potential obligate syntrophic relationship between the two species (37).

Most isolates are dependent on ASF for growth

The notable increase in microbial diversity associated with ASF supplementation suggests that most microbial species in AS are dependent on ASF for growth. To determine which components in the ASF promote growth, we plated the ten isolates on agarose plates with acetate, NH_4Cl, and either no ASF, fresh ASF, autoclaved ASF, or ashed ASF (Fig. 4). Autoclaving destroys heat-sensitive organic molecules such as signal peptides and certain vitamins in the ASF, whereas ashing removes all organic components, leaving only inorganic compounds, such as minerals and trace elements. We found that none of the isolates were able to grow on acetate and NH_4Cl alone. However, the addition of fresh ASF promoted the growth of all isolates (Fig. 4). Autoclaved ASF was able to recover the growth of seven isolates, whereas no growth was observed with the addition of ashed ASF, indicating an auxotrophy for certain organic metabolites for all the isolates.

To determine if growth inhibition was caused by auxotrophy toward common nutrients, we investigated if addition of buffer and nutrient found in R2A growth medium (yeast extract and sodium pyruvate) as well as additional amino acid source (tryptone and casamino acids) could recover the growth of the isolates that were unable to grow on plates lacking ASF or were supplemented with autoclaved or ashed ASF. Interestingly, no growth was observed unless some form of ASF was supplemented here as well. This indicates that the ASF provides components other than the common nutrients found in R2A media that are essential for the growth of all ten species. The addition of the nutrient mix recovered the growth of several isolates that were unable to grow on either autoclaved or ashed ASF. This suggests that most species are also dependent on one or several inorganic compounds that were exclusively present in ASF and could not be reconstituted using the tested nutrient supplement.

To gain more insight into the elemental composition of ASF, ashed ASF, and the nutrient supplement, we analyzed media containing these using inductively coupled plasma-optical emission spectrometer (ICP-OES) (Table 2). The measured concentrations were found to be in the same order of magnitude for most measured elements. One exception was cobalt, which was roughly four times lower in the nutrient supplement when compared with the ASF. The addition of trace elements is known to stimulate microbial conversions, and specifically, cobalt has been shown to affect overall AS

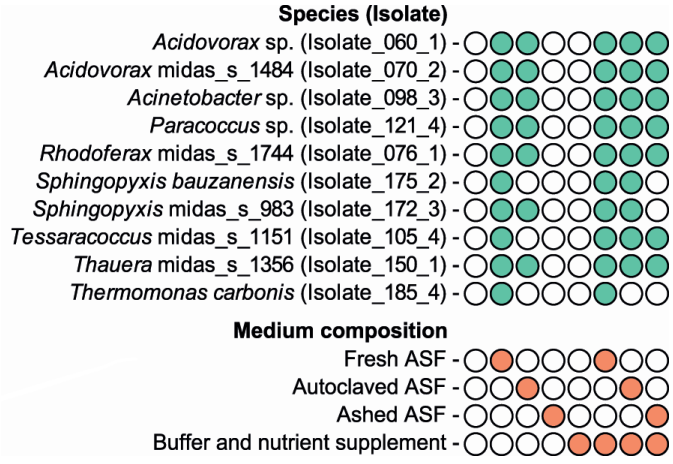

FIG 4 Effect of media composition on the growth of the ten pure culture isolates. Growth as determined using agarose plates containing 250 mg/L acetate and 50 mg/L NH_4Cl supplemented with no ASF, fresh ASF, autoclaved ASF, or ashed ASF. Buffer and nutrient supplement (500 mg/L K_2HPO_4, 50 mg/L $MgSO_4*7H_2O$, 50 mg/L yeast extract, 300 mg/L sodium pyruvate, 100 mg/L casamino acids, and 100 mg/L tryptone) was used to evaluate auxotrophy toward common nutrients. Closed green circles indicate observed growth after 2 weeks of incubation at 25°C. Closed orange circles indicated addition of the specific medium component.

TABLE 2 Concentration of elements measured for the culture medium containing ASF, ashed ASF, and medium supplement using ICP-OES

Element	ASF	Ashed ASF	Nutrient supplement
Al	72 µM	102 µM	61 µM
Ca	2371 µM	1155 µM	408 µM
Co	0.28 µM	0.33 µM	0.07 µM
Cr	27 µM	59 µM	25 µM
Cu	0.4 µM	0.3 µM	0.1 µM
Fe	70 µM	179 µM	55 µM
Mg	458 µM	201 µM	198 µM
Mn	3.4 µM	3.4 µM	0.9 µM
Na	5063 µM	5535 µM	4142 µM
Ni	15 µM	16 µM	5 µM
P	30 µM	1 µM	2445 µM
S	8098 µM	584 µM	304 µM
Zn	2 µM	1 µM	1 µM

growth (38). It would therefore be interesting to examine the specific effect of cobalt in a future study.

Aside from the elements measured in our study, it has been shown in several studies that other rare earth metals can be crucial for growth of certain species, including some isolated from extreme habitats (39, 40). While WWTP influent seems unlikely to be a significant source for these elements (41), further research would be needed to identify any rare earth metal dependencies.

The prior attempt to isolate uncultured bacterial species from bovine rumen proved successful by devising and utilizing a culture medium that faithfully mirrored the mineral composition inherent in the rumen itself (42). Similarly, the growth-promoting effects of ashed ASF could be explained by its mineral composition, which more closely mirrors that of the AS, compared with previously used media like R2A.

Perspectives

Among bacteria that were isolated, successfully cultured, and stocked, several novel species are of particular interest for wastewater treatment systems. For instance, species that belong to the *Rhodoferax*, *Tessaracoccus*, and *Thauera* genera have previously been implicated to play a major role in denitrification, phosphate accumulation, and floc formation (9). However, isolation of species of some highly desired genera that are present in the AS core community, such as *Ca*. Phosphoribacter (24), *Ca*. Brachybacter (34), and *Ca*. Accumulibacter (23), was unsuccessful. With species abundance data implying that plate culture conditions more closely mimic sewage rather than AS, adjustments could be made by, for example, decreasing the concentration of certain substrates further or utilizing plates in which substrates are slowly released (43). Beyond plating-based isolation, the workflow proposed in this study could be combined with alternative cultivation and selection techniques such as dilution to extinction, parallel small scale membrane reactors, or cell sorting (4).

Pure cultures of microbes also play a significant role in the transition toward a bio-based economy as, in industry, microorganisms are employed on a massive scale for the production of organic compounds and conversion of resources and as such can provide a plethora of applications in biotechnology (17). However, only several dozen species are commonly used in industry. As with microbial characterization studies, the main obstacle for tapping into the remaining biological resources is our current inability to grow, culture, and isolate these species outside of their natural habitat (4).

Incorporating recent developments in lab automation equipment, including pipetting, DNA extraction, plating, and colony picking robots into the workflow we present in this study, would allow for screening and isolation of bacterial species at

substantially higher volumes. As such, the increase in throughput can be used to optimize medium composition specifically for species of particular interest. Combined with PCR-based screening methods, this would allow for targeted selection of species that are low in abundance or grow slowly in the selected cultivation conditions.

Conclusion

This study represents a first exploration of how 16S rRNA gene amplicon sequencing can be used to guide the isolation of novel microbial species directly from AS. By applying 16S rRNA gene amplicon sequencing to the entire biomass grown on agarose plates with different medium compositions after inoculation with AS bacteria and then classifying the resulting ASVs using the MiDAS 5.1 reference database, we obtained invaluable information on suitable cultivation conditions for core species in the AS. We found that ASF was required for growth and isolation of several bacterial species known for their high abundance in WWTPs. While the initial growth experiments presented here appear to suggest that the ASF substitutes both vital organic growth factors and the unique minerals found in AS, additional research into the underlying mechanisms would offer valuable insights into the determinants of an effective cultivation medium for AS-associated microorganisms.

MATERIALS AND METHODS

Medium preparation

ASF was prepared as follows: 20 L of activated sludge from aeration tanks at AAW WWTP was gathered on 14 October 2020 and 26 January 2021 and ultra-filtrated using Alfa Laval-GR61PP (Alfa Laval, Lund, Sweden) flat disk membranes according to manufacturer specifications (maximum pressure of 7 bar, cross flow 15 L/min). Subsequently, the permeate was concentrated 2× by reverse osmosis using Alva Laval-R099 (Alfa Laval, Lund, Sweden) flat disk filters using the same operating conditions, and the retentate was filter sterilized using Nalgene 0.1-µm bottle-top sterile filter units (Thermo Fisher Scientific, Waltham, MA). For cultivations that required ashed ASF, 500 mL of the concentrate was heated to above 100°C to evaporate 95% of the liquid after which the remainder was subjected to 350°C for 2 hours and subsequently reconstituted to 500 mL with demineralized water. Autoclaved ASF was obtained by autoclaving the concentrate at 121°C for 20 min.

ASF medium was prepared by supplementing 500 mL of 2× ASF the specified carbon and nitrogen sources to a final concentration of 50 mg/L casamino acids, 100 mg/L mannose, 100 mg/L glucose, 100 mg/L tryptone, 125 mg/L acetate, and 53 mg/L NH_3Cl using 100× filter-sterilized stock solutions and adding sterile filter double distilled water (liquid medium) or autoclaved molten 2% DNA pure agarose (VWR, Radnor, PA) (agarose plates) to 1 L. Agarose was used instead of agar and autoclaved alone to reduce the formation of reactive oxygen species which could otherwise inhibit the growth of species sensitive to oxidative stress (20). TSB (Sigma Aldrich #22092, Burlington, MA) was prepared according to the manufacturer's instructions. R2A medium was prepared by combining medium components in a suitable quantity of demineralized water to accommodate a final concentration of 500 mg/L yeast extract, 300 mg/L sodium pyruvate, 500 mg/L proteose peptone, 500 mg/L glucose, 500 mg/L K_2HPO_4, 50 mg/L $MgSO_4$ * $7H_2O$, and 250 mg/L acetate; then, agarose and/or ASF were added after autoclaving at 120°C for 20 min. TSB and R2A agarose plates were solidified using 15 g/L agarose. Single-colony isolates were grown and stocked in an isolation medium composed of R2A supplements, except for glucose and proteose peptone, with ASF, 50 mg/L casamino acids, 100 mg/L tryptone, 125 mg/L acetate, and 53 mg/L NH_4Cl. Growth characterization studies were performed with agarose plate containing a range of compounds with the concentrations as described for the isolation medium.

Preparation of activated sludge single cells

Activated sludge (30 mL) from aeration tanks was obtained at the AAW WWTP on 16 December 2020 and 03 March 2021 and immediately homogenized in the lab using the RZR 2020 Benchtop Stirrer (Heidolph, Schwabach, Germany) with a glass/Teflon tissue grinder attached (1 min, 2nd gear). Five milliliters was subsequently sonicated using a Bandelin Sonopuls HD2200 with an MS73 probe (Berlin, Germany) set at 60% amplitude with 6 × 10 s pulses with a 10-s interval. Single cells were separated by centrifugation through a cell strainer with pore sizes of 40 µm (VWR) for 5 min at 3,000 × *g*, followed by centrifugation through a cell strainer with pore sizes of 5 µm (pluriSelect Life Science, Leipzig, Germany) for 2 min at 8,600 × *g*. Cells present in the permeate were counted in a Bürker-Türk counting chamber and subsequently diluted to approx. 10,000 cells/mL.

LIVE/DEAD staining and microscopy

LIVE/DEAD staining was performed using the LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific) following the manufacturer-recommended protocol. Stained cells were analyzed on a white light laser confocal microscope (TCS SP8 X; Leica, Germany).

Bacterial plating and growth

Agarose plate cultivation was performed at 25°C for at least two weeks. Anoxic plate cultivation was done in 3.5 L anaerobic jars with Oxoid AnaeroGen gas generation sachets (Thermo Fisher Scientific) to remove oxygen to yield levels < 1%. When adequate colony formation was observed, the total combined biomass for each plate was harvested using cell scrapers (VWR) and suspended in 300 µL DNase-free water. Single-colony cultures were obtained by picking colonies with 1 µL sterile inoculation loops and transferring them to 800 µL of liquid isolation medium in 2-mL 96-deep-well plates (VWR). These were then incubated for at least two weeks on a benchtop shaker (RT, 200 rpm). Restreaking of the liquid cultures was performed to achieve pure culture isolates. Intermediate cultures were concentrated by centrifugation to yield 200 µL of which 160 µL was used as input for DNA extraction and 40 µL was mixed with 100 µL of 40% glycerol and stored at −80°C. Final isolated species were grown in liquid isolation medium and stocked by supplementing with glycerol to a final concentration of 30% and stored at −80°C.

Elemental composition analysis

Elemental analysis of various culture media was conducted using ICP-OES on an iCAP 6000 Series (Thermo Scientific, Waltham, MA), as previously described (44).

DNA extraction

DNA was extracted from 160 µL AS, single cell suspension, suspended biomass from scraped culture plates, or liquid cultures using the FastDNA Spin Kit for soil (MP Biomedicals) according to the MiDAS protocol for plate extraction (aau_wwtp_dna_v_8.0) available at https://www.midasfieldguide.org/guide/protocols. DNA concentration and integrity were assessed using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and an Agilent 2200 Tapestation (Agilent Technologies, CA, USA), respectively.

16S rRNA gene amplicon sequencing

V1-V3 amplicons were made using the 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) (45) and 534R (5′-ATTACCGCGGCTGCTGG-3′) (46) primers with barcodes and Illumina adaptors (Integrated DNA Technologies) (47). 25 µL PCR reactions in duplicate were run for each sample using 1× PCRBIO Ultra Mix (PCR Biosystems), 400 nM of both forward and reverse

primers, and 10 ng template DNA. PCR conditions were 95°C, for 2 min followed by 20 cycles of 95°C for 20 s, 56°C for 30 s, and 72°C for 60 s, followed by a final elongation at 72°C for 5 min. PCR products were purified using 0.8× CleanNGS beads and eluted in 25 µL nuclease-free water. The 16S rRNA gene V1-V3 amplicon libraries were pooled in equimolar concentrations and paired-end sequenced (2 × 300 bp) on a Illumina MiSeq using v3 chemistry (Illumina, USA). 10% to 20% PhiX control library was added to mitigate low-diversity library effects.

16S rRNA genes V1-V3 were processed using usearch v.11.0.667. Amplicon data were processed differently for plate scrapes and colonies. For plates, forward and reverse reads were merged using the -fastq_mergepairs command, filtered to remove phiX sequences using usearch -filter_phix, and quality filtered using usearch -fastq_filter with -fastq_maxee 1.0. For individual colonies, only forward reads were sequenced, and these were filtered to remove phiX sequences using usearch -filter_phix, trimmed to 250 bp using -fastx_truncate -trunclen 250, and quality filtered using usearch -fastq_filter with -fastq_maxee 1.0. All subsequent steps were the same. Dereplication was performed using -fastx_uniques with -sizeout, and ASVs were resolved using the usearch -unoise3 command. An ASV-table was created by mapping the quality-filtered reads to the ASVs using the usearch -otutab command with the -zotus and -strand plus options. Taxonomy was assigned to ASVs with the MiDAS 5.1 database (48) using the usearch -sintax command with -strand both and -sintax_cutoff 0.8 options.

Amplicon data analyses

Amplicon data were analyzed with R v.4.0.5 (49) through RStudio IDE v.2022.02.3 with the tidyverse v.1.3.1 (https://www.tidyverse.org/), vegan v.2.5 (50), ggplot2 v. 3.3.6 (51), and Ampvis2 v.2.7.9 (52) packages. For all analyses, samples were rarefied to 10,000 reads to obtain the same sensitivity toward low abundant taxa. Alpha diversity (observed ASVs and inverse Simpsons) was calculated using Ampvis2. A multiple two-sided Student's *t*-test was used to determine statistically significant differences in alpha diversity between samples grouped by growth conditions, with a Bonferroni correction applied using an initial $\alpha = 0.05$. Beta diversity distances based on Bray-Curtis (abundance based) and Jaccard (presence/absence based) were calculated at the ASV level using the vegdist function in the vegan R package and visualized by PCoA plots with Ampvis2. A PERMANOVA test was performed on the beta diversity matrices using the adonis function in the vegan package with 999 permutations to determine how much of the variance could be explained by the growth conditions. Raw data for heatmaps were prepared using Ampvis2 and visualized using ggplot2. Figures were assembled and polished in Adobe Illustrator v.26.3.1.

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AUTHOR CONTRIBUTIONS

Maarten D. Verhoeven, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Per H. Nielsen, Conceptualization, Resources, Writing – review and editing, Supervision, Validation | Morten K. D. Dueholm, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Supervision, Validation, Visualization, Writing – review and editing

DATA AVAILABILITY

The raw sequencing data generated in this study have been deposited in the NCBI SRA database under accession code PRJNA981068.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental file 1 (AEM01151-23-s0001.pdf). Fig. S1 to S4.
Supplemental file 2 (AEM01151-23-s0002.xlsx). Data S1.

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