Ecophysiology of the filamentous *Alphaproteobacterium Meganema perideroedes* in activated sludge

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**Abstract**

A comprehensive study of the ecophysiology of the filamentous *Meganema perideroedes* affiliated to the *Alphaproteobacteria*, possessing a “Nostocoida limicola Type II” filamentous morphology was conducted. This morphotype often causes serious bulking problems in activated sludge wastewater treatment plants, and hardly anything is known about its physiology. The study was carried out by applying a suite of in situ methods in an industrial activated sludge treatment plant with excessive growth of this species. The experiments revealed a very versatile organism able to take up a large variety of organic substrates under aerobic conditions. It had a remarkably high storage capacity forming polyhydroxyalkanoates from most substrates tested. When nitrate was present as e-acceptor, the number of substrates to be consumed by *M. perideroedes* was more restricted compared to aerobic conditions. With nitrite as e-acceptor, only acetate and glucose among the substrates tested could be assimilated and used for storage and possibly growth. This indicated that *M. perideroedes* might be able to denitrify under certain conditions, which is unusual for filamentous bacteria in activated sludge. No substrate uptake or storage was seen under anaerobic conditions.

*M. perideroedes* was relatively hydrophobic, compared to other filamentous bacteria and microcolonies present in the sludge, indicating the presence of a hydrophobic sheath. Several excreted surface-associated exoenzymes were detected in the sludge, but *M. perideroedes* never showed any activity, except once after a breakdown in the production facility. This confirmed that *M. perideroedes* mainly grows on soluble substrates. Based on the studies of the ecophysiology of *M. perideroedes*, potential control strategies are suggested.

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**Keywords:** Meganema perideroedes; Microautoradiology; Ecophysiology; Activated sludge

**1. Introduction**

Bulking and foaming due to excessive growth of filamentous bacteria cause problems in many activated sludge wastewater treatment plants worldwide. More than 30 different filamentous bacterial types have been described on the basis of morphology [1,2], but the true identity of many of these filamentous microorganisms is still unknown. Recently, filamentous *Alphaproteobacteria* have been detected and found in substantial quantities in several activated sludge plants. Filamentous bacteria belonging to this group of *Proteobacteria* have never been described before. Three species named *Candidatus Alysiosphaera europaea*, *Candidatus Monilibacter batavus* and *Candidatus Alysiomicrobium bavaricum* are described, but do not yet exist in pure culture [3,4]. *Candidatus Alysiosphaera europaea* grows only in coexistence with yeast, while *Candidatus Monilibacter batavus* and *Candidatus Alysiomicrobium bavaricum* could not be cultivated, and only partial 16S rDNA sequences exist [3,4]. So far, only one species has been isolated and characterized in detail [4,5], and a new genus (*Meganema*) and the species name of *Meganema perideroedes* were proposed. Specific oligonucleotide
In order to control filamentous bacteria in activated sludge treatment plants not only the identity, but also knowledge about the physiology and ecology of the bacteria is of importance. Little is known about the physiology of filamentous Alphaproteobacteria and the morphotype “Nostocoida limicola Type II”. A few physiological characteristics of *M. perideroedes* are known from the strains maintained on R2A media [5]. Growth was not well supported on a mineral, salt and vitamin medium supplemented with carbon sources, limiting a detailed physiological characterization of the pure cultures. The bacteria are obligate aerobic, oxidase and catalase-positive [5]. Most isolates stained Gram-negative, although one strain was Gram-variable as has also been observed in the morphotype *Nostocoida limicola Type II*. A few investigations of the ecophysiology of gene probe-defined *M. perideroedes* have been conducted [8]. The bacteria consumed acetate and glucose under aerobic conditions and seemed to have an unusually high substrate uptake capability. Furthermore, they were found to have a high substrate affinity for acetate (an apparent *Kₐ* of 2.4 μM). A high substrate affinity is assumed to be a general characteristic for most filamentous bacteria in activated sludge [1,2,9] and together with relatively low substrate uptake rates and storage abilities, compared to floc-formers, these kinetic properties have been used to explain the outcome of the competition between filamentous and floc-forming bacteria in different configurations of wastewater treatment plants [1,2,9]. However, recent studies of the ecophysiology of filamentous bacteria suggest that it is difficult or impossible to make such general statements. For example, the filamentous *Microthrix parvicella* is adapted to specific substrates (lipids) and is able to take up substrates under anaerobic conditions, properties which are different from most other known bacteria, including the floc-formers [10]. Also *Thiothrix* rely on oxidation of reduced sulfur compounds in addition to heterotrophic growth [11]. Based on this new knowledge, our hypothesis is that many of the troublesome filamentous bacteria in activated sludge have a unique physiology and ecology reflecting specific conditions in various activated sludge systems. Thus, if such knowledge about the ecophysiology of the most important filamentous bacteria is combined with detailed knowledge about the process conditions, efficient control measures may be developed.

*M. perideroedes* has been responsible for severe bulking in industrial wastewater treatment plants and is present in many other treatment plants. In this study, we wanted to investigate the ecophysiology of this interesting filamentous *Alphaproteobacterium* in detail and learn more about its physiology, eventually to find efficient control measures.

A number of in situ methods have been applied in combination with FISH. Microautoradiography (MAR) was applied to provide information regarding substrate preference and e-acceptor conditions [8,12]. The ability of bacteria to convert carbon sources under defined e-acceptor conditions into storage polymers, e.g. polyhydroxyalkanoates (PHAs), is a potential advantage in a dynamic environment such as activated sludge. Therefore, the PHA content in single cells was quantified using the Nile blue stain, where the fluorescence intensity is proportional to the PHA content of the cells [13,14]. The presence of exoenzymes can provide information about the preferred substrates (dissolved low molecular substrates or macromolecules which require a hydrolytic degradation), or it can indicate unfavourable conditions such as phosphorus limitation [15]. Exoenzymes located on the cell surfaces can be visualized in the sludge by applying Enzyme-Labeled Fluorescence substrates (ELF-97®) [10,16]. Finally, also the cell surface hydrophobicity, which is of major importance in floc formation and substrate adsorption [10], can be determined by Microsphere Adhesion to Cells, MAC [17].

2. Materials and methods

2.1. Activated sludge

The experiments were carried out with activated sludge from an industrial wastewater treatment plant (WTP) in Grindsted, Denmark. The WTP is operated at 15–20 °C with a sludge age of 10–12 days. The load is 4200–5800 kgCOD day⁻¹ with the following concentrations: COD, 5.8–8.0 g l⁻¹; total N, 0.1–0.2 g l⁻¹; total P, 0.02–0.05 g l⁻¹. Most of the COD is dissolved and consists mainly of acetate, ethanol, propanol, glucose, and other organic compounds, but little information is available. Soluble sulfides are present at certain times. The salinity is high (5–7 g l⁻¹) with a conductivity of 15–20 mS cm⁻¹. The treatment plant mainly removes organic matter and receives wastewater from a pharmaceutical plant. Nitrogen is added in the form of ammonium to ensure sufficient nitrogen for growth. However, partial nitrification/denitrification is performed during the summertime. The plant is an aerobic, completely mixed activated sludge system without any selector. The activated sludge was collected and stored at 4 °C the day before the experiments were performed. One
hour before the experiments were conducted, it was acclimatized to room temperature (20–22 °C). The sludge had a content of suspended solids (SS) of 3–4 gSS l⁻¹. The volatile suspended solids (VSS) were 75 ± 2% of SS. Before use, the sludge was diluted to 1 gSS l⁻¹ with filtered nitrate and nitrite free supernatant from the activated sludge.

2.2. Identification

The filamentous bacteria were morphologically identified using the Eikelboom classification system [6]. Furthermore, fluorescence in situ hybridization (FISH) was applied with 16S and 23S rRNA-targeted nucleic acid probes as listed in probeBase [18], targeting the groups of the Alpha-(ALF968), Beta-(BET42a), and Gamma-proteobacteria (GAM42a), and Cytophaga Flavobacterium group of the Bacteroidetes (CF319a). Also, species-specific probes for M. perideroedes (Meg983 mixed with Meg1028) and Thiothrix spp. (TNI) were used [5,19]. Development, optimization and determination of applied formamide concentration for the gene probes Meg983 and Meg1028 was performed by Thomsen et al. (accepted). The sequence of Meg983: 5'-CGG GAT GTC AAA AGG TGG-3' and for probe Meg1028: 5'-CTG TCA CCG AGT CCC TTG C-3'. Hybridization and washing buffer were made according to [20]. Oligonucleotides were labeled with 5(6)-carboxyfluorescein-N-hydroxy-succinimide ester (FLUOS) or with the sulfoindocyanine dyes (Cy3 and Cy5) (Thermohybaid Interactive, Ulm, Germany). A Confocal laser scanning microscope (LSM 510-meta, Carl Zeiss, Oberkochen, Germany) equipped with a UV laser (351 and 364 nm), an Ar ion laser (458 and 488 nm), and two HeNe lasers (543 and 633 nm) were used to record fluorescent signals from the various probes.

Combinations of MAR-FISH, PHA-FISH, ELF-FISH and MAC-FISH were used for the studies of in situ physiology. All above-mentioned methods were examined by confocal laser scanning microscopy (CLSM) prior to hybridization with gene probes except MAR-FISH. MAR-FISH hybridization with gene probes was performed before applying the photographic emulsion and thus before CLSM examination. Positions of interest were recorded by an automatic stage controller and digital images were recorded. Subsequently, the fresh samples used for ELF and MAC were fixed in 4% paraformaldehyde (PFA) for 3 h on the slide, before the FISH procedure was conducted [20]. The stage control enabled relocation of the recorded microscopic field. The PHA-FISH combination was slightly modified to remove excess Nile blue stain prior to FISH. These slides were pre-treated for 30 min. with 96% ethanol at 4 °C, following the normal FISH procedure. The MAC-FISH and ELF-FISH slides were washed in dH₂O prior to dehydration to remove excess microspheres and precipitates from phosphate-buffered saline medium (PBS), respectively.

2.3. Microautoradiography and MAR-FISH

The microautoradiography experiments were performed using ³H-labeled and ¹⁴C-labeled organic compounds and ¹⁴C-labeled bicarbonate. The procedure, which includes incubation with radiolabeled substrates under defined e-acceptor conditions, fixation, and hybridization with gene probes, addition of a radiosensitive emulsion, exposure, processing, and microscopical investigations, is described in detail elsewhere [11,12,21]. Experiments were repeated on different occasions and incubations were performed on two separate occasions at least. For analysis of MAR-positive M. perideroedes, a minimum of 30 filaments were investigated for each incubation. In brief, two types of studies were conducted. In the first study, various potential substrates were tested for uptake under aerobic conditions to see if M. perideroedes was a specialized or general consumer. For this, a selection of substrates was chosen, representing short and long-chained fatty acids, sugars and amino acids. In the second study, potential use of different e-acceptors was tested by studying uptake of the same organic substrates in the filamentous bacteria under anaerobic conditions with or without nitrate or nitrite present as e-acceptor. In all experiments, 2 ml diluted activated sludge (1 gSS l⁻¹) were transferred to glass serum vials and incubated with a final concentration of substrate of 2 mM, and the labeled fraction was 10 μCi per vial. Incubation time was 3 h (five for bicarbonate). All vials for anaerobic incubations (with or without nitrate or nitrite) were closed with thick, gas-tight rubber stoppers and flushed with ultrapure nitrogen gas prior to incubation. In all anaerobic experiments, a pre-incubation step of 2 h was included with unlabeled organic substrate (2 mM). In this way, only bacteria able to take up large amounts of substrate under these conditions (for storage or growth) would be MAR-positive [22]. After the pre-incubation period, labeled and unlabeled substrate was added to a final substrate concentration of approximately 2 mM. When thiosulfate or nitrate was added, a final concentration of 2 mM was used, while 0.5 mM was used for nitrite. In some experiments, a pre-incubation period of 6 h with unlabeled acetate was applied. As a control for chemohagrophy, sludge was pasteurized at 70 °C for 10 min just before the incubation under defined conditions. In most experiments filamentous bacteria were assessed as MAR-positive or MAR-negative by comparing the number of silver grains on top of the filaments to the background. In some experiments, quantitative MAR (Q-MAR) was used by enumeration of the silver grains [8].
Radiochemicals [3H]acetate, [p-2,3H]glucose, [9,10(\(\alpha\))-3H]oleic acid, [4,5-3H]leucine, [14C]formate, [3H]glycine, [14C]bicarbonate, [3H]mannose and [3H]galactose were purchased from Amersham Biosciences (Amersham Bioscience, Denmark), [1-14C]pyruvate (Na) and [1-\(^{3}\)H]ethanol came from American Radiolabeled Chemical Inc. (Bio Nuclear AB, Sweden) and [1-14C]propionate (Na) and [2,3-3H]butyric acid came from ICN Biochemicals Inc. (Bie & Berntsen, Denmark).

2.4. Storage capabilities

Lipidic storage granules of \(M.\) \(perideroedes\) were investigated by measuring the polyhydroxalkanoates (PHA) content within individual filamentous bacteria by Nile blue staining [23]. The ability of \(M.\) \(perideroedes\) to form PHA from different carbon substrates under different e-acceptor conditions was examined. Samples were incubated under similar conditions as in the MAR experiments without addition of radioactive tracer and with slightly shorter incubation length (2 h). Prior to the experiment, all samples were aerated for 1 h in order to remove easily degradable substrates otherwise present in the sludge. After incubation, samples were spread out onto gelatine-coated glass and stained with Nile blue. All samples were examined with the CLSM. At least 22 filamentous \(M.\) \(perideroedes\) in each sample were investigated, and the average fluorescence intensity of PHA-containing granules was expressed as intensity per filament. The intensity was measured using image analysis software (ImageJ 1.33s, Rasband W, National Institutes of Health, USA, http://rsb.info.nih.gov/ij/). For each filament, a segment of at least 15 \(\mu\)m was analysed by recording maximum intensity in each horizontal line perpendicular to the filament image. An average of these measurements (min 100) was calculated for each analysed image of a given incubation. At least 22 images were examined for each substrate incubation and average fluorescence intensity and standard deviations were calculated. Student’s \(t\) test was used as statistical tool to evaluate significant PHA formation compared to the control.

2.5. Enzyme-Labeled Fluorescence (ELF), ELF-FISH

The presence of exoenzyme activity was determined using Enzyme-Labeled Fluorescence (ELF-97\(^{\circ}\), Molecular Probes, Eugene, OR, USA), where substrates upon enzymatic cleavage form a fluorescent precipitate (excitation 345 nm/emission 530 nm) on the surfaces of bacteria or microcolonies within flocs [10,16]. All commercially available ELF\(^{\circ}\) 97 enzymes were evaluated for activity: ELF\(^{\circ}\) 97 esterase substrate (ELF\(^{\circ}\) 97 acetate), ELF\(^{\circ}\) 97 lipase substrate (ELF\(^{\circ}\) 97 palmitate), ELF\(^{\circ}\) 97 \(\beta\)-d-galactosidase substrate (ELF\(^{\circ}\) 97 \(\beta\)-d-galactopyranoside), ELF\(^{\circ}\) 97 \(\beta\)-d-glucuronidase substrate (ELF\(^{\circ}\) 97 \(\beta\)-d-glucuronidase, ELF\(^{\circ}\) 97 chitinase/N-acetylglucosaminidase substrate (ELF\(^{\circ}\) 97 N-acetylglucosaminidase; ELF\(^{\circ}\) 97 NAG), and ELF\(^{\circ}\) 97 Endogenous Phosphatase detection kit. Stock solutions of enzymes were dissolved in methanol or DMSO (chitinase) according to the guidelines of the manufacture. Hundred \(\mu\)l sludge was incubated with approx. 0.2 mM of either substrate and placed in the dark for 2–3 h. Sub-samples were spread out onto gelatine-coated glass and examined with the CLSM prior to FISH analysis. The enzyme samples were PFA fixed and stored in \(1 \times PBS\) at 4 °C and could be stored without significant loss in fluorescence intensity for at least 6 months.

2.6. Microsphere Adhesion to Cells, MAC-FISH

Surface properties were investigated using microsphere adhesion to cells where sulfate-modified microspheres rendering hydrophobic characteristics and a diameter of 0.1 \(\mu\)m were applied [17,24]. Yellow-green fluorescent polystyrene microspheres with excitation and emission properties (505 nm/515 nm) were used (Molecular Probes, Eugene, OR, USA). The microspheres were diluted in dH\(_2\)O to a concentration of 0.02% (w/v) and sonicated for 100 min at 60 W. Five \(\mu\)l of microspheres and 10 \(\mu\)l sludge were mixed with 100 \(\mu\)l of dH\(_2\)O and vortexed for two min. Sub-samples were spread out onto gelatine-coated cover glass and examined immediately before drying under CLSM prior to FISH.

3. Results

3.1. Identification and description

The industrial wastewater treatment plant investigated has suffered from filamentous bulking for several years with a sludge volume index (SVI) above 200 ml g\(^{-1}\) and often much higher. Three types of filamentous bacteria have been dominating over a period of several years as determined by FISH, and for the period of investigation, the numbers of filamentous bacteria were: \(M.\) \(perideroedes\) constituted 60–95%, \(Thiothrix\) spp. (5–35%), and bacteria affiliating to the \(Cytophaga Flavobacterium\) group of the \(Bacteroidetes\) (5–10%) (Fig. 1A and B). \(M.\) \(perideroedes\) grew as slightly coiled filaments with a cell diameter of approx. 1.5 \(\mu\)m, disc-shaped cells and a length of often up to 0.5 mm. \(M.\) \(perideroedes\) filaments were always Gram-negative, even though some cells within some filaments occasionally stained Gram-positive. Some cells in the filaments contained Neisser-positive granules, indicating polyphosphate inclusions, but most cells were Neisser-negative.
The cells contained PHA granules as determined by Nile blue staining. Two phenotypes of *M. perideroedes* were observed in the sludge based on MAR experiments (see later). The applied gene probes Meg983 mixed with Meg1028 targeted both phenotypes in the sludge, so the difference was mainly based on the differences in substrate uptake pattern, where one of the morphotypes was less versatile than the other. However, it was present in small amount and could not be distinguished systematically by morphology, so it was not investigated in detail.

3.2. Ecophysiology

3.2.1. Uptake of organic substrates under aerobic conditions

In order to determine whether *M. perideroedes* was a specialized or versatile consumer of organic matter, the
uptake of a number of potential substrates under aerobic conditions was tested by MAR (Table 1). An example is shown in Fig. 1C, where the identity and uptake of labeled substrate (MAR-FISH) can be seen. Almost all substrates offered were consumed by *M. perideroedes* under aerobic conditions. Especially acetate, propionate, butyrate, oleic acid and glucose were clearly taken up, and a certain uptake of other sugars and amino acids also took place. The bacteria did not fix labeled bicarbonate in the presence of thiosulphate and thus did not have any mixotrophic/autotrophic activity similar to that of *Thiothrix* spp. present in the same treatment plant. Filamentous bacteria affiliating to the *Bacteroides* were never observed to consume any of the offered substrates under all e-acceptor conditions tested. This is in accordance with previous observations (Nielsen, unpublished results). Non-filamentous bacteria within the activated sludge flocs consumed all substrates tested, thus acting as positive controls.

### 3.2.2. Substrate uptake under anaerobic conditions

The uptake of the organic substrates by *M. perideroedes* was investigated under conditions where nitrate or nitrite served as potential e-acceptors and under anaerobic conditions where no external e-acceptor was added (Table 2). A 2 h pre-incubation period with non-labeled substrates was included in all incubations in order to show that MAR-positive filaments either had a very large storage capacity or that growth took place on the substrate.

When nitrate was present as a potential e-acceptor, *M. perideroedes* took up the same substrates as under aerobic conditions with the exception of butyrate, oleic acid, mannose and the amino acids, which were not assimilated (Table 2). Using nitrite as e-acceptor, *M. perideroedes* uptake was limited to only glucose and acetate. Assimilation of these substrates with both nitrite and nitrate as potential e-acceptors indicated a potential capacity for denitrification by using these substrates. This was further investigated by experiments with a pre-incubation time of 6 h with unlabeled acetate and glucose, using nitrate and nitrite as e-acceptor. An uptake of labeled substrate was still observed after 6 h in both cases (results not shown), suggesting that *M. perideroedes* was able to use nitrate and nitrite for energy production.

In order to see whether the substrate uptake rate was different with oxygen and nitrate as e-acceptors, quantitative MAR was used. Based on an enumeration of silver grains on top of the filamentous bacteria, it was evident that the uptake rate of labeled acetate with nitrate as e-acceptor was reduced to approx. 80% of the uptake rate with oxygen as e-acceptor (Fig. 2). It was also noticed that the uptake rate decreased by 25–35%.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Uptake of substrates by filamentous <em>M. perideroedes</em> under aerobic conditions as investigated by MAR</th>
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<tbody>
<tr>
<td></td>
<td><em>M. perideroedes</em></td>
</tr>
<tr>
<td>Formate</td>
<td>–</td>
</tr>
<tr>
<td>Acetate</td>
<td>++</td>
</tr>
<tr>
<td>Propionate</td>
<td>++</td>
</tr>
<tr>
<td>Butyrate</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>–</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>++</td>
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<tr>
<td>Glucose</td>
<td>++</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
</tr>
<tr>
<td>Glycine</td>
<td>+</td>
</tr>
<tr>
<td>Leucine</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>–</td>
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<tr>
<td>Bicarbonate + thiosulfate</td>
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<table>
<thead>
<tr>
<th>Table 2</th>
<th>Uptake of organic substrates by <em>M. perideroedes</em> under conditions where nitrate or nitrite serve as electron acceptor and anaerobic conditions as investigated by MAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate</td>
<td>Nitrite</td>
</tr>
<tr>
<td></td>
<td><em>M. perideroedes</em></td>
</tr>
<tr>
<td>Formate</td>
<td>–</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
</tr>
<tr>
<td>Propionate</td>
<td>+</td>
</tr>
<tr>
<td>Butyrate</td>
<td>–</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>–</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>–</td>
</tr>
<tr>
<td>Glycine</td>
<td>–</td>
</tr>
<tr>
<td>Leucine</td>
<td>–</td>
</tr>
</tbody>
</table>

–: No silver grains (no substrate uptake).  
+: Few silver grains, but clearly positive.  
++: Positive, many silver grains.  
¹ Microcolonies in sludge flocs.
when a 2 h pre-incubation step with unlabeled substrate was included (Fig. 2). This indicated that the substrate uptake rate was very high in the beginning of the incubation period, probably showing the difference between the substrate uptake rate when both storage and growth took place, and when only growth took place. When pre-incubation was included for nitrate, the same trend was observed, although not statistically significant. The uptake rate of acetate with nitrate as e-acceptor was still approximately 80% of the rate with oxygen as e-acceptor (Fig. 2).

Anaerobic conditions without nitrate or nitrite present showed no uptake of any of the substrates tested, indicating that *M. perideroedes* did not possess an anaerobic storage capability or a fermentative metabolism.

A minor subpopulation of *M. perideroedes* showed less ability to take up substrates with other e-acceptors than oxygen (results not shown). No substrates could be assimilated with nitrite as e-acceptor, and only propionate could be consumed with nitrate as e-acceptor.

### 3.2.3. Storage capabilities

In fresh activated sludge samples, all *M. perideroedes* filaments had small PHA-containing granules as visualized by Nile blue and FISH (Fig. 1E and F). The fluorescence intensity among the individual cells varied, indicating some variation in the PHA content of the individual cells within a filament. The initial intensity was low compared to filaments after substrate uptake (Fig. 3A and B), indicating low levels of PHA in most cells. A number of substrates were tested for potential conversion and PHA formation under different e-acceptor conditions by measuring the increase in fluorescence intensity during 2 h incubation with the substrates. For some substrates, a substantial uptake was observed, where the individual cells in the filaments were almost completely filled with PHA granules. There was some variation between filaments, but with rather similar PHA content in cells along a certain filament. The PHA content of the filaments usually showed a normal distribution after uptake of substrate, as exemplified by uptake of acetate with nitrite as e-acceptor (Fig. 3B). For some substrates it is likely that the standard deviation of the PHA content was ascribed to more than one type of *M. perideroedes*, but it was not possible to systematically assign it to a particular morphotype, since discrimination between these was very difficult.

The distribution of the PHA content in the filaments was recorded for all tested substrates and e-acceptor conditions. The results are shown in Fig. 4A–C. Aerobic conversion of substrates into PHA took place for acetate, propionate, oleic acid, galactose and mannose. Glucose and leucine were not converted to PHA. With nitrate as e-acceptor, oleic acid and galactose showed a very big increase in the PHA content, and to a lesser extent, but still significant, also acetate, propionate, glucose, mannose and leucine formed PHA. When nitrite was present as e-acceptor, only acetate and glucose formed enlarged PHA granules. PHA formation under anaerobic conditions without nitrate or nitrite was not observed with any of the carbon sources tested. The
difference in fluorescence intensity between control incubations and incubations that led to PHA formation was at least a factor of 3. Using Student’s t test, all substrates being positive shown in Fig. 4 had a significantly higher content of PHA in the cells than the control incubations. For most substrates, except for glucose and leucine, the formation of PHA was very similar with oxygen and nitrate present as e-acceptors. For some substrates, M. perideroedes filaments formed PHA only under certain e-acceptor conditions as observed for glucose and leucine with oxygen and nitrate as e-acceptors. These observations indicate that the filaments used the uptake of substrate for either direct growth or for another type of storage compounds not detected by PHA stain, e.g. glycogen (Fig. 4A–C). Also, the amount of PHA that was formed depended on e-acceptor conditions, illustrated with oleic acid and galactose with oxygen and nitrate e-acceptor (Fig. 4A–C), where much more PHA was formed with nitrate.

When the granule sizes of PHA were measured for M. perideroedes and Thiothrix sp., it was evident that M. perideroedes had a much higher PHA content per cell than Thiothrix sp. The fluorescence intensity of M. perideroedes PHA granules (from acetate accumulation) was approx. 3 times higher than the intensity from the PHA granules of Thiothrix sp. (data not shown), possibly due to the greater cell diameter. M. perideroedes had 2–5 granules in the individual cells, while Thiothrix sp. only had one (data not shown).

3.2.4. Exoenzyme activities

Presence and location of extracellular enzyme activity in sludge flocs and on the surface of filamentous bacteria were investigated by enzyme-labeled fluorescence assays (Table 3). While microcolonies within the flocs exhibited enzyme activity in five of the six enzymes tested, M. perideroedes hardly ever showed any enzyme activity during the study period. On one occasion, after a shutdown of the industrial production, approximately 15% of the M. perideroedes filaments exhibited lipase activity. Some Thiothrix sp. filaments tested positive in phosphatase and esterase, while filamentous bacteria affiliating to the Bacteroidetes always showed esterase activity.

3.2.5. Surface properties

Distribution of slightly or strongly hydrophobic surfaces in the sludge was investigated by MAC. The dominating M. perideroedes was always relatively hydrophobic in comparison with the other filaments present in the sludge as indicated by a high degree of attached hydrophobic microspheres relative to Thiothrix sp. (Fig. 1G–I). The Bacteroidetes filaments showed attachments of microspheres, however, in much smaller amounts than M. perideroedes. Several parts of the sludge flocs were shown to be relatively hydrophobic, as many spheres attached to the floc structure, but variations among microcolonies within single flocs were detected.

4. Discussion

This study presents the first detailed study of the ecophysiology of a filamentous bacterium affiliating to the Alphaproteobacteria, namely M. perideroedes, which was recently isolated and characterized [5]. However, very little is known about its physiology, so presently no specific control measures exist in full-scale treatment plants.
Table 3
Excretion of exoenzymes by bacteria present in the sludge as investigated by Enzyme-Labeled Fluorescence, ELF

<table>
<thead>
<tr>
<th></th>
<th>Esterase</th>
<th>Lipase</th>
<th>Chitinase</th>
<th>Phosphatase</th>
<th>Glucuronidase</th>
<th>Galactosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. perideroedes</td>
<td>–</td>
<td>(+)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Thiothrix sp.</td>
<td>(+++)</td>
<td>–</td>
<td>–</td>
<td>(++)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytophaga-Flavobacterium-group of the Bacteroidetes</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>Flocs*</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>+++</td>
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</tbody>
</table>

#: No enzyme activity (not active).
+: Little enzyme activity, but clearly positive.
(++): Occasionally a fraction positive.
++: Clearly positive, enzyme activity.
+++: Very active, high enzyme activity.
*: Microcolonies in sludge flocs.

4.1. Substrate assimilation profile and storage capacity

The probe-defined M. perideroedes consumed almost all organic substrates tested under aerobic conditions, thus indicating that this bacterium was a very versatile heterotrophic organism able to consume a range of short and long-chain fatty acids, sugars and amino acids. This versatility is greater than that observed for other filamentous bacteria in activated sludge systems studied so far [10,11,21,25]. The substrate uptake pattern was generally in agreement with growth patterns observed by an isolate studied by Levantesi et al. [4]. Whether it is the same species as that investigated in this study is unclear, as gonidia and rosette formation was observed with their isolate, but never observed in situ or with the isolates from Grindsted industrial WWT [5]. Using nitrate as e-acceptor, the number of substrates utilized by M. perideroedes had diminished compared to aerobic conditions, but the main groups of substrates were still assimilated. When nitrate was present, only acetate and glucose were assimilated, indicating that storage and/or growth could take place under these conditions. Under strict anaerobic conditions, no substrate uptake took place, which is also the case for other filamentous bacteria in activated sludge, except M. parvicella [10]. This bacterium is known to take up substrate under anaerobic conditions as a storage compound for later use with oxygen or nitrate as e-acceptor [10,26].

M. perideroedes had a very great capacity to form a storage compound, most probably PHA, with both oxygen, nitrate or nitrite present as e-acceptors. It formed at least 3 times more PHA within the cells than did Thiothrix cells in the same sludge as quantified by the Nile blue staining. Many bacteria are able to accumulate polymeric material which can serve as a reserve of carbon and/or energy during periods of unbalanced growth due to unfavorable conditions [27]. PHA granules have also been found in several filamentous morphotypes from activated sludge [1]. PHA formation has, however, only in one case been reported in extensive amounts in filamentous organisms — this was found in an unidenti-
to grow on this substrate with nitrate as e-acceptor. The results emphasize, however, that although *M. perideroedes* was physiologically active with both oxygen, nitrate, and nitrite as e-acceptor, the substrate uptake pattern and biochemical pathways change significantly upon change in e-acceptor conditions. Different substrates resulted in different quantities of formed PHA under different e-acceptor conditions as illustrated with glucose, leucine, oleic acid, and galactose (with oxygen and nitrate). Interestingly, also the amount of PHA formed from a specific substrate also depended on e-acceptor condition, indicating change in metabolic pathways. In relation to possible control strategies, it is particularly interesting that the number of substrates became more restricted when nitrate, and particularly nitrite, were e-acceptors. This may explain why *M. perideroedes* seems not to be common in treatment plants with denitrification and suggests that introduction of denitrifying conditions may be a way to limit its growth. A restriction of certain substrates with nitrate as e-acceptor is also known from isolated denitrifiers belonging to the genus *Thauera*, which are present in activated sludge. *T. lincollorentis* and *T. terpenica* grow on sugar compounds under aerobic conditions, but not under denitrifying conditions [30].

So far, no filamentous bacteria in activated sludge have been reported to perform full denitrification to gaseous dinitrogen, while several filamentous bacteria are reported to reduce nitrate to nitrite. Examples are *Microthrix parvicella* [31], *Thiothrix* sp., and Type 021N [32]. It is not known whether *M. perideroedes* performs a full denitrification, but our results suggest that it may be the case with acetate and glucose as substrates, because they were able to assimilate labeled acetate after 6 h pre-incubation with unlabeled acetate with nitrate OR nitRate as e-acceptor, indicating growth or an extreme storage capacity. Furthermore, with nitrate, the uptake rate of acetate was reduced to approximately 80% of the rate with oxygen, which is typical for denitrifying bacteria due to the lower energy yield [33]. However, as several substrates were not consumed with nitrite as e-acceptor, e.g. propionate and oleic acid, these may only favour a reduction of nitrate to nitrite.

The less abundant *M. perideroedes* phenotype or genotype observed in the activated sludge had a different physiology, compared to the dominant type. It also had a versatile substrate uptake under aerobic conditions, but was not active under any other e-acceptor conditions than oxygen. This was evident in both MAR and PHA experiments. This type could hardly be distinguished from the predominant type by morphology and not by the applied gene probes. As it was present only in small numbers, it was not studied in detail, but shows that there are still several filamentous *Alphaproteobacteria* that remain to be properly identified and characterized.

4.2. Exoenzymatic activity and surface properties

*Meganema perideroedes* did not generally exhibit any exoenzyme activity as assessed from the ELF assays. Only in a situation after the factory shutdown, some lipase activity was observed, indicating that starvation and atypical conditions could induce excretion of certain exoenzymes. Phosphate starvation has been described to promote enhanced phosphatase expression in a biofilm [15]. In general, we found other types of bacteria expressing various enzymes within the flocs. It has been hypothesized that different groups of bacteria express different enzymes, and, for example, phosphatase in activated sludge flocs was associated mainly with bacteria affiliating with the *Cytophaga flavobacterium* group of the *Bacteroidetes* [34]. Also *M. particella*, which is a specialized filamentous lipid degrader, excretes lipases on the cell surface [10]. The lack of exoenzyme production by *M. perideroedes* suggests that this bacterium mainly consumes soluble short-chain substrates without the need of hydrolysis before uptake. This is in agreement with its great ability to assimilate a range of substrates and form large PHA granules. The observed potential for excretion of lipase could be an action for utilizing otherwise inaccessible fat (triglycerides) under starvation conditions.

Cell surface hydrophobicity of bacteria in activated sludge influences the location in the sludge floc and the accessibility of certain substrates. If the bacteria are very hydrophobic, they will mainly be located within sludge flocs or they may form foam or scum. A hydrophobic sheath can also attract hydrophobic substrates such as lipids [10]. *M. perideroedes* form very hydrophobic surfaces in both pure culture [5] and under in situ conditions as assessed by MAC. It is, however, not clear what the benefit of the hydrophobic sheath is for *M. perideroedes* in the particular plant, as it apparently grows mainly on water-soluble substrates where it is an advantage to be present in the bulk water phase and not inside the floc. In other cases, however, we have found this species forming foam in industrial treatment plants, supporting a general hydrophobic nature of the sheath.

4.3. Possible control measures

*Meganema perideroedes* can be characterized as a very versatile consumer of soluble substrates with both oxygen and nitrate as e-acceptor, it has a high substrate affinity, and it has a very large capacity for substrate uptake and formation of the storage polymer PHA. This is to some extent different from the general description of filamentous bacteria in activated sludge [1], stressing that each species has its own specific characteristics promoting growth in specific treatment plants. Many industrial wastewater treatment plants treat wastewater with
a high content of soluble organic compounds, and it is in this type of plant that bulking problems caused by the morphotypes represented by the Alphaproteobacteria are reported. Usually, the most efficient control strategy against many filamentous bacteria is the use of selectors, where most soluble substrates are consumed by flocc-forming bacteria in special selector tanks due to the flocc-formers’ higher substrate uptake and growth rate [9]. However, M. perideroedes has an extremely high substrate uptake rate and storage capacity, which is probably comparable to or higher than those of most flocc-forming bacteria, so a selector will probably only work if the e-acceptor conditions are selective for the flocc-formers. Our study showed that nitrite reduced the number of organic substrates to be consumed by M. perideroedes significantly, compared to conditions with oxygen or nitrate. Thus, a possible control strategy could be to add nitrite in a selector so that a larger fraction of the organic substrates will go to other denitrifying bacteria, thereby outcompeting M. perideroedes.

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References


