Revised

MS for Environmental Microbiology

Article Type: Research Article

High and stable substrate specificities of microorganisms in enhanced biological phosphorus removal plants

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Running Title: Substrate Specificities of Microorganisms in EBPR Plants

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Summary

Microbial communities are typically characterized by conditions of nutrient limitation so the availability of the resources is likely a key factor in the niche differentiation across all species and in the regulation of the community structure. In this study we have investigated whether four species exhibit any *in situ* short-term changes in substrate uptake pattern when exposed to variations in substrate and growth conditions. Microautoradiography was combined with fluorescence *in situ* hybridization to investigate *in situ* cell-specific substrate uptake profiles of four probe-defined coexisting species in a wastewater treatment plant with enhanced biological phosphorus removal. These were the filamentous "*Candidatus* Microthrix" and *Caldilinea* (type 0803)*,* the polyphosphate-accumulating organism "*Candidatus* Accumulibacter", and the denitrifying *Azoarcus*. The experimental conditions mimicked the conditions potentially encountered in the respective environment (starvation, high/low substrate concentration, induction with specific substrates, and single/multiple substrates). The results showed that each probe-defined species exhibited very distinct and constant substrate uptake profile in time and space, which hardly changed under any of the conditions tested. Such niche partitioning implies that a significant change in substrate composition will be reflected in a changed community structure rather than the substrate uptake response from the different species.

Introduction

The enhanced biological phosphorus removal process (EBPR) is one of the most advanced wastewater treatment configurations which removes carbon, nitrogen, and phosphorus from wastewater. A range of modern molecular methods has revealed that the EBPR process is carried out by microbial communities consisting primarily of uncultured microorganisms; that a low number of core microorganisms is critical to the process, and that the communities in a wide range of EBPR plants seem to be stable at the genus level (Nielsen *et al*., 2010). Many of the important microbes have been identified and their main functions and metabolic potential determined, making the EBPR process one of the best characterized ecosystems in environmental biotechnology to date (Albertsen *et al.,* 2012, Nielsen *et al*., 2012).

Microbial community diversification is influenced by several environmental factors, such as phage predation (Šimek *et al*., 2010) and substrate availability (Jasmin and Kassen, 2007). The EBPR communities typically live under dynamic conditions and nutrient limitation, thus the availability of the resources is likely to be the key factor in the regulation of populations within the community. Presence of specific substrates is important for the community structure in wastewater treatment systems, where very specialized heterotrophs have been found in several functional groups, such as denitrifiers (Thomsen *et al*., 2007; Morgan-Sagastume *et al*., 2008) and many filamentous bacteria (Nielsen *et al*., 2009).

Pure culture studies, however, do not always support the concept that high substrate specificity is a general feature among heterotrophic bacteria. Batch and chemostat experiments with *Escherichia coli* and other pure cultures have shown, that under carbon/energy limited slow growth conditions, they change their substrate uptake profiles from consumption of only a few different substrates to utilization of many carbon sources (Ihssen and Egli, 2005; Liu *et al*., 2005; Egli, 2010). It is hypothesized that expression of such behavior increases the chance of survival because, in natural environments, where the concentration of available carbon and energy sources is low, the expression of multiple transporters and catabolic enzymes gives the cell metabolic flexibility and makes the asset competitive.

The difference between studies carried out under *in situ* conditions and studies performed in pure culture raises the question of whether bacteria present in EBPR ecosystems and other complex ecosystems have a phenotypic plasticity to carry out short-term physiological change to be able to consume other substrates in response to changes in growth conditions such as starvation or variation in type or number of substrates available. Alternatively, such changes can only take place after genetic adaptation, typically after many generations, by adaptive mutations, gene loss, or horizontal gene transfer (Blount *et al*., 2008; Shapiro *et al*., 2009; Philippot *et al*., 2010), allowing them to utilize the new resources and occupy new niches. It is

proposed that genetic microdiversity among closely related strains of a species in a natural environment provides a pool of bacteria able to take over when their fitness is changing (Cohan, 2006, 2011; Ward, 2006). If this change is represented by the availability of another substrate, we should expect this to become visible and of importance only when new clonal offspring become abundant after several generations, unless the individual bacteria are immediately able to adjust to such a new situation and change their substrate uptake patterns.

In this study, we hypothesized that (i) frequently observed functional bacterial groups in EBPR communities (i.e., nitrifiers, denitrifiers, poly-phosphate accumulating bacteria, and filamentous bacteria) are generally highly specialized in their substrate uptake profiles, i.e. have a clear niche partitioning, and that (ii) these profiles are strongly preserved under exposure to short-term variations (few hours, but repeatedly) such as substrate or oxygen concentrations, and type and number of substrates present in the EBPR system. To reveal the short-term physiological changes, we have investigated the *in situ* substrate uptake profiles of probe-defined species, by means of microautoradiography combined with fluorescence *in situ* hybridization (MAR-FISH) under the above-mentioned conditions in a wastewater treatment plant system. Four different probe-defined uncultured populations, here defined as "species", that are typical for EBPR communities, were selected: two filamentous species, the actinobacterial "*Candidatus* Microthrix" (hereafter called *Microthrix*) and a *Caldilinea* (phylum *Chloroflexi*), and two microcolony-forming bacteria, "*Candidatus* Accumulibacter" (hereafter called *Accumulibacter*), a key microorganism involved in the phosphate removal, and *Azoarcus*, a common denitrifier. Previous studies have shown that these bacteria form part of the core community in EBPR plants, they are abundant (each usually comprising >2% of the entire community) and show clear differences in substrate uptake patterns (Nielsen *et al*., 2010; 2012).

Results

To study the substrate uptake pattern of the probe-defined species, standard incubation conditions were defined as 0.5-2 mM substrate and air saturation (defined as high substrate and high oxygen conditions). These conditions are comparable to other ecophysiological studies carried out earlier and summarized by Nielsen *et al*., (2009, 2010). The uptake patterns of 11 substrates commonly encountered in wastewater influent were evaluated by the MAR-FISH procedure and were in agreement with results from earlier studies conducted under similar conditions over the past 10 years, giving a very consistent substrate uptake pattern for each of the probe-defined species (Fig. 1 and Table S1).

Preferred position of Figure 1

The results show four specialized probe-defined species with different substrate uptake profiles. The majority of the probe-defined filamentous *Microthrix* (> 80%) took up solely oleic acid with high uptake activity (i.e., cells were heavily covered with silver grains) (Fig. 1A). Over 80% of filamentous *Caldilinea* took up glucose with high activity. A fraction consumed oleic acid, mannose, and galactose as well, indicating that *Caldilinea* was able to take up sugar compounds and oleic acid with glucose as the preferred substrate (Fig. 1B). Microcolony-forming *Accumulibacter* and *Azoarcus* had more similar uptake profiles and took up short-chain fatty acids such as acetate, propionate, and pyruvate (Figs. 1C and 1D). The pure culture of *E. coli* MG1655, included as a control organism typically used in laboratory studies, exhibited very dynamic substrate uptake profile. Unlike the uptake profiles of probe-defined EBPR species, the *E. coli* substrate uptake profile changed depending on the conditions applied. *E. coli,* from exponential as well as stationary phase, took up a variety of organic compounds, such as short-chain fatty acids, sugar compounds, amino acids, and glycerol (Figs. 1E and 1F and Fig. S1). The percentage of active cells and the activity level of substrate uptake varied depending on the substrate.

Substrate uptake at low substrate concentrations

In order to investigate substrate uptake at low concentration, which is often encountered at some stages of the wastewater treatment process, low substrate concentration was tested, here defined as 5-20 μM. Under this condition, most *Microthrix* cells took up oleic acid, as in the case of high substrate concentration, but the activity level was considerably lower. It was demonstrated by the lower number of silver grains formed on top of individual cells (Fig. 1A), indicating that the substrate concentration was below the saturation concentrations of the uptake system. *Caldilinea* utilized the same substrates under the low and high substrate concentration conditions, although the number of filaments utilizing mannose and galactose increased at the low substrate concentration (Fig. 1B). No difference was observed for *Accumulibacter* and *Azoarcus* compared to the uptake at high substrate concentrations (Figs. 1C and 1D). Interestingly, the pure culture of *E. coli* showed a weaker uptake capability of several substrates under the low substrate concentration condition. This observation was most pronounced for stationary phase cells (Figs. 1E and 1F), and the change in *E. coli* substrate uptake pattern was much more pronounced than in the case of microorganisms from natural communities.

Effects of starvation

Microorganisms in aeration tanks or clarifiers often experience starvation for 5-10 h, thus, in order to investigate the effect of starvation on the substrate uptake profile, activated sludge (and *E. coli*) samples were first deprived of an external carbon source for 12 h, combined with aeration, before the substrate uptake profiles of the bacteria were investigated at high and low substrate and oxygen concentrations, respectively. The substrate uptake patterns at high substrate and oxygen concentrations after starvation did not change for *Microthrix*, *Accumulibacter* and *Azoarcus*. Minor differences concerning the percentage of the population with a positive substrate uptake were observed for *Caldilinea*. In the case of incubation with galactose at high substrate concentration, the number of positive cells increased for both high and low O_2 conditions while in the case of incubation with the same substrate under low substrate concentration, the

number of positive cells decreased under low O_2 conditions. In the case of incubation with glucose at low substrate concentration for both high and low O_2 conditions, a decrease in glucose uptake activity was observed. For *E. coli* the fraction of positive cells decreased for a few substrates. The differences were most pronounced in the case of stationary phase *E. coli* incubated with high substrate concentration (for the cells in exponential phase changes were observed in case of glycine (high substrate concentration) and acetate and glycine (low substrate concentration); for the cells in stationary phase a decrease in the uptake of 6 out of 9 substrates was observed when compared to standard high substrate concentration conditions, while no changes were observed for low substrate concentration) (Figs. 1 E and F).

In separate series of experiments, the oxygen level was reduced from 20% to 1% (corresponding to approx. 10 and 0.5 mg-oxygen l^{-1}, respectively) during starvation mimicking conditions commonly encountered in activated sludge treatment plants. As some bacteria in activated sludge plants are considered microaerophilic, e.g. *Microthrix* (Rossetti *et al.,* 2005), high oxygen concentration may be toxic and prevent substrate uptake. However, no differences were observed in the uptake pattern of the probe-defined species compared to the experiments carried out at air saturation (results not shown).

Effects of induction

Microorganisms in EBPR plants experience variations in type and concentrations of substrate during the process due to variations in incoming wastewater content, e.g. from specific industries. In order to see whether a prolonged exposure of the specialized bacteria to various specific substrates can induce a change in uptake pattern, a series of experiments with only one substrate present in prolonged time (12 h), here called induction experiments, was carried out. Samples were incubated for 12 h with selected substrates under standard conditions (0.5-2 mM), followed by MAR incubations under the same substrate conditions to observe the possible change in the substrate uptake profile. No changes were observed for *Microthrix*, *Accumulibacter*, and *Azoarcus* (Figs. 1A, 1C, and 1D). For *Caldilinea,* slight changes were noticed. In the

case of glucose, the fraction of positive cells remained the same, although the observed activity level for uptake decreased after induction with all substrates tested. In the case of mannose and galactose, the active fraction of probe-defined bacteria increased with all substrates tested after the induction period (Fig. 1B). For *E. coli* cells changes in the uptake pattern of galactose and both amino acids were observed in both exponential and stationary phases (Figs. 1E and 1F). The uptake of glycine in exponential phase decreased significantly after the leucine induction. Similarly, the active fraction of cells taking up leucine in stationary phase decreased after the leucine induction. In the case of galactose, the uptake activity was higher in both *E. coli* samples after induction with other sugars.

Effects of multiple substrates

As the presence of certain substrates is necessary for uptake of other substrates under *in situ* conditions (Kong *et al*., 2004; Thomsen *et al*., 2007), a number of experiments were carried out to reveal such potential dependencies. The substrate uptake pattern for all probe-defined species remained virtually unchanged after co-incubations with chosen substrates, except for two important changes (Fig 1). Firstly, *Microthrix* was able to utilize glycerol only when oleic acid was present as a co-substrate (Figs. 1A and 2A-2F). Secondly, *Accumulibacter* and *Azoarcus* were only capable of utilizing leucine when acetate was present as a co-substrate (Figs. 1C and 1D and 2G-2I). Interestingly, *E. coli* exhibited changes in the uptake pattern for a significant fraction of the substrates tested. These included changes in both the percentage of positive cells and the activity level of substrate uptake. For exponential *E. coli* cells a decrease in the fraction of positive cells could be observed in the case of 6 substrates tested. The response of stationary *E. coli* cells was more diverse as both decrease as well as increase in the fraction of metabolically active cells could be observed for different substrates. Such a heterogeneous behavior could be noticed in the case of glycerol as the presence of different co-substrates induced different metabolic responses.

Preferred position of Figure 2

Discussion

This study shows that microorganisms growing under dynamic conditions in EBPR plants were strongly specialized in their choice of substrate and that any changes in their *in situ* substrate uptake profiles were very limited when exposed to short-term variations in substrate and growth conditions, typical for the environment investigated. Different types of substrates, concentrations, starvation as well as induction with selected substrates did not induce the uptake of other substrates. Only in the presence of multiple substrates three cases were observed where substrates not consumed under standard conditions were co-utilized in the presence of a second substrate. Our observations suggest that the phenotypic plasticity of the core species in the EBPR plant was low as originally hypothesised, and thus a possible adaptation could only occur through an evolutionary response and the acquisition of mutation that allow access to a novel substrate. These results were distinctly different from the patterns exhibited by *E. coli,* where the ability to utilize individual substrates depended on the environmental conditions. These pure-culture observations are consistent with other literature reports on the state of preparedness of bacteria grown in pure cultures to utilize multiple substrates simultaneously for low (Ihssen and Egli, 2005; Liu *et al*., 2005) as well as high (Egli, 1995) substrate concentration conditions.

The four probe-defined species had distinctly different substrate uptake profiles, showing the existence of different stable ecological niches in the wastewater system investigated. This is not only the case for the four species investigated here, but also for most of the approx. 30 core species present in the EBPR system, where the substrate profiles have been stable for several years in the specific probe-defined populations (Nielsen *et al*., 2010; 2012). As an example, the substrate uptake profile of *Microthrix* was investigated regularly over the past 15 years in many different plants, always with the same result (Andreasen and Nielsen, 1997; 2000, Nielsen *et al*., 2002, and this study). These results show that the species present in the EBPR plants are very stable in substrate uptake profile. Although genetic adaptation potentially will occur

over many generations in the presence of a multitude of related niches and perfect conditions for acquiring adaptive genes, such offspring did not seem to be competitive, nor able to establish themselves. This is important for past and future *in situ* investigations of substrate uptake profiles in EBPR plants, in other engineered systems, and potentially in other natural ecosystems.

Little is known about the microdiversity among activated sludge bacteria, but recently, significant microdiversity was shown in *Accumulibacter* from full-scale treatment plants (Albertsen *et al*., 2012). The differences between the genome of the activated sludge *Accumulibacter* and the only existing reference genome (Garcia Martin *et al*., 2006) are primarily related to the genes involved in the production of extracellular polymers and different virus-related genes and are not linked to the central metabolic pathways. Presently, no other genomes exist with a sufficient similarity to the core species in the EBPR plants to perform more detailed comparisons (Albertsen *et al*., 2012). Wastewater treatment plants certainly expose microbial populations to conditions that could favor periodic selections or "sweeps" (Cohan and Koeppel, 2008; Fraser *et al*., 2009), but no new strains with changed substrate uptake pattern seemed to evolve.

In the case of *Accumulibacter*, *Azoarcus,* and *Microthrix*, the presence of a second substrate induced the uptake of substrates that were not taken up when present as sole substrates. *Accumulibacter* and *Azoarcus* took up leucine only when acetate was present, as reported previously (Kong *et al*., 2004; Thomsen *et al*., 2007). It was hypothesized that leucine could potentially be used as a nitrogen source, but the detailed mechanism for its uptake and use is not clear. A notable fraction of *Microthrix* was able to consume glycerol but only when oleic acid was present. As *Microthrix* produce extracellular lipases that degrade triglycerides into long chain fatty acids and glycerol (Nielsen *et al*., 2002), it seems rational that they can consume both types of substrate, but the exact mechanism is not known, and no genomic information is available.

Although it is not known how common the simultaneous uptake of several substrates by probe-defined species in activated sludge is, this physiological trait presumably gives a competitive advantage, particularly in the dynamic complex communities, in which several organic compounds might be available in the local environment. However, the results show that the exact substrate uptake profile for a certain species cannot always be based on single-substrate experiments only.

Caldilinea species remained specific towards four substrates throughout all the conditions applied, although slight changes in sugar uptake activity were observed at low substrate concentration, starvation, and induction conditions. No K_m values are reported in the literature for *Caldilinea*; thus it is not clear if substrate affinity or other factors are responsible for the observed changes in the uptake of monosaccharides. In some cases, the probe-defined *Caldilinea* showed increased galactose and mannose uptake while the activity of glucose uptake was lowered. However, the ability to modify individual substrate uptake was highly dependent on the type of conditions applied. It seems possible that the *Caldilinea* population was able to respond to unfavorable conditions (starvation, diminished substrate availability) as well as to induction with all three monosaccharides tested. It was not an uptake of new substrates, but a difference in the relative uptake rate of the substrates.

The specificity of FISH probes applied in this study was high, typically covering a single or few species in a genus as characterized by 16S rRNA gene phylogeny. Three out of four probe-defined species showed very consistent substrate profiles for all bacteria targeted by the probes, indicating little diversity in terms of substrate specificity. Only the *Caldilinea* group exhibited a slightly different substrate uptake pattern for a subpopulation presumably due to the presence of more than one phenotype or due to clonal heterogeneity (Elowitz *et al.* 2002).

Trying to extrapolate potential substrate capabilities of the uncultured probe-defined *Azoarcus* and *Caldilinea* based on their isolates is clearly not possible. *Azoarcus* spp. can grow on glucose and ethanol in pure cultures (Zhou *et al*., 1995), but was not able to consume these substrates under *in situ* conditions. Furthermore, pure culture of filamentous *Caldilinea* sp. isolated from wastewater treatment plants could utilize acetate and pyruvate (Yoon *et al*., 2010), in contrast to *in situ* studies. Also other closely related species isolated from plant roots or rivers showed very different substrate uptake capabilities (Misko and Germida, 2002; Freese *et al*., 2010). The conclusion from our results indicates that, although great diversity may evolve around each species present in the EBPR ecosystems, the strong competition in the community only allows a single or very few strains of this species with conserved substrate uptake profile to multiply and become abundant. It is well known from pure culture studies that just a few hundred generations may be enough for new subpopulations to evolve and adapt to new distinct substrates (Jasmin and Kassen, 2007; Blount *et al*., 2008; Lee *et al*., 2009), but under *in situ* conditions in EBPR plants, they appear not to have the enhanced fitness to become dominant. However, they may be present in very low abundance not observed by the FISH method applied, that could potentially be seen as a range of closely related strains or species with different metabolic potentials for substrate utilization. This underlines the need for obtaining reference genomes or isolates from the actual dominant members of EBPR communities, and not from other closely related strains, for predicting their function in the ecosystem by metabolic reconstruction.

Our results suggest limitations concerning the use of leucine as an indicator of protein synthesis (heterotrophic production), bacterial growth, or activity in mixed ecosystems (Kirchman *et al*., 1985; Alonso-Sáez *et al*., 2010). We showed that leucine was not incorporated by any of the four probe-defined bacteria under the conditions tested, with two exceptions (uptake of leucine by *Accumulibacter* and *Azoarcus* when acetate was present). Leucine uptake in *E. coli* was strongly dependent on the conditions applied. This is in agreement with investigations of an estuarine community, where the incorporation of

leucine was not suitable to determine/estimate the cell activity (Mayali *et al*., 2011). These results show that leucine cannot be used as a general measure for microbial activity in natural ecosystems without careful controls.

The study clearly demonstrates that bacteria present in the EBPR ecosystem, forming a stable core community across many plants, were specialized in uptake of specific substrates, although with some substrate overlap. It is, however, interesting that we have not been able to find a broad generalist able to consume the majority of the substrates tested in this or previous studies (summarized by Nielsen *et al*., 2010; 2012). The niche overlap observed for most substrates stresses the fact that other physiological factors are also important for defining the niches for various species under natural dynamic conditions, such as substrate affinity, activity under different electron acceptor conditions, and the ability to store substrates. The implication of such niche partitioning is also that a significant change in the incoming substrate composition will immediately be reflected in a changed community structure and not in alterations in the substrate uptake response from the individual species. This is in agreement with the observations of community shift in full-scale plants with external carbon addition for improved denitrification (Hagman *et al*., 2008). This observation further underlines that neutral models that do not take such niches into account when describing factors shaping communities (Ofiteru *et al*., 2010) cannot reliably describe all microbial ecosystems.

Interestingly, the results indicate that, with some precautions, it is possible to make community-wide predictions of microbial substrate networks in the EBPR system and other engineered microbial ecosystems, if relevant reference genomes are known and the system is calibrated by proper measurements of the substrate uptake capabilities under the actual *in situ* conditions. Such predictions would be difficult if extensive, short-term, physiological adaptations took place as illustrated by the studies of *E. coli*. If *E. coli* substrate uptake should be predicted under different environmental conditions, based on its genomic

potential only, our results show that this would be difficult, unless coupled with analyses of expressed genes, proteins or other data showing the actual *in situ* activity. To what extent this is also the case for bacteria in more oligotrophic natural environments remains to be investigated.

Experimental procedures

Activated sludge samples

Activated sludge was collected from the Aalborg West full-scale WWTP. The plant is designed for a population equivalent of 300,000 and performs biological nitrogen and phosphorus removal by the alternating BioDenipho process (Seviour *et al.*, 2010). The WWTP has previously been described in detail (Nielsen *et al*., 2002). All samples were collected from the aeration tanks on the same day as the experiments were conducted. Before the experiments, activated sludge was gently homogenized and aerated for 1-2 h to reduce possible background substrate in the samples.

Bacterial strain and growth conditions

Wild-type *E. coli* K-12 MG1655 was used as a control in all experiments. Complex medium used for *E. coli* cultivation contained 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 12.8 g l^{-1} $Na_2HPO_4{\cdot}2H_2O$, 3 g l^{-1} KH_2PO_4, 1.77 g l^{-1} $(NH_4)_2SO_4$, 130 mg l^{-1} $MgCl_2{\cdot}6H_2O$, 80 mg l^{-1} $CaCO_3$, 77 mg l^{-1} $FeCl_3{\cdot}6H_2O$, 11 mg l^{-1} $MnCl_2{\cdot}4H_2O$, 1.5 mg l^{-1} $CuSO_4{\cdot}5H_2O$, 1.3 mg l^{-1} $CoCl_2{\cdot}6H_2O$, 4 mg l^{-1} ZnO, 1.2 mg l^{-1} H_3BO_3, 8.5 mg l^{-1} $NaMoO_4$, 790 mg l^{-1} EDTA $Na_4{\cdot}2H_2O$ as described by Ihssen and Egli (2004, 2005). All salts were added as concentrated solutions to the medium. Bacteria were grown in Erlenmeyer flasks at 37°C on rotary shaker (at 150 rpm) and collected during the exponential (2.5 h, $OD_{546nm} = 1.3 \pm 0.4$) and stationary (24 h, $OD_{546nm} = 7.8 \pm 0.5$) phases. Each sample was washed 3 times with the mineral medium to remove carbon source from the medium and resuspended in the mineral medium preheated to 37°C. The mineral medium was prepared in the same way as the complex medium above, but without tryptone and yeast extract.

Incubation procedures

Multiple experiments were conducted to study the effect of high or low substrate concentration, starvation, induction with other substrates, and multiple substrates, on the substrate uptake specificity of investigated bacterial species. Starvation experiments were performed both under high oxygen concentration (20%, corresponding to normal air saturation, 10 mg l^{-1}) and low oxygen concentration (1%, 0.5 mg l^{-1}). Low oxygen concentration in the headspace was achieved by injecting appropriate volume of air into the serum bottles after repeated (three times) evacuation of the headspace with high purity N_2 (99.999%). Each starvation condition tested was followed by incubation with both high and low substrate concentrations. Inductions and multiple substrate incubations were followed by incubation under the high oxygen and high substrate condition. An overview of experimental procedures is shown in Table S2 and S3.

Organic substrates labeled with radioisotopes used for MAR incubations are described in SI Methods. All unlabeled organic compounds were prepared as concentrated stock solutions (20 or 80 mM) with the pH adjusted to 7.5. In all the incubations, the ratio between labeled and unlabeled substrates as well as the ratio between the substrate and biomass concentration (specific activity, 5 μCi $mgSS^{-1}$) were identical to allow the substrate uptake to be compared between different experiments. The conditions were adjusted in order to ensure that the substrates were not depleted during the incubations. All incubations were conducted for 2 h, aerobically in the dark at 20°C (activated sludge) or 37°C (*E. coli*). The serum bottles were sealed with gas-tight rubber stoppers and gently shaken at 150 rpm during the incubation. Biological replicates were performed. For each biological replicate two technical replicates were prepared. After MAR incubation, all samples were fixed for 3 h (activated sludge) or 1 h (*E. coli*) either with 4% [w/v] paraformaldehyde (Gram-negative bacteria, i.e., *Caldilinea*, *Accumulibacter*, *Azoarcus*, and *E. coli*) or 50% [v/v] ethanol (Gram-positive bacteria, i.e., *Microthrix*).

High and low substrate concentration conditions. Activated sludge was diluted with filtered effluent water from the same treatment plant to a final SS (dry matter) concentration of 1 g-SS l^{-1} for experiments at the high substrate concentration condition (0.5-2 mM). At low concentrations (5-20 μM) the sludge was diluted to 0.01 g-SS l^{-1} to ensure a low overall substrate removal rate and that substrates were not depleted. *E. coli* was diluted with mineral media to a final dry matter concentration of 0.1 g-SS l^{-1} and 0.001 g-SS l^{-1} for high and low substrate concentration conditions, respectively. All samples were incubated with individual radioactively labeled and unlabeled organic substrates. The concentrations of all substrates used are listed in Table S2. For the high substrate concentration condition, 2 ml of diluted sample was transferred to 10-ml serum bottle. For the low concentration condition, 20 ml of diluted sample was transferred to 110-ml serum bottle.

Starvation conditions. Undiluted activated sludge (30 ml, 3.5 g-SS l^{-1}) and *E. coli* were transferred to 300-ml serum bottles and starved for 12 h under aerobic conditions. Serum bottles were incubated on rotary shaker (150 rpm) at 20°C (activated sludge) and 37°C (*E. coli*). After starvation, the sludge and *E. coli* were incubated under both high and low substrate and oxygen conditions, according to the procedure described above.

Induction conditions. Four induction experiments were performed (Table S2). 50 ml of diluted-sludge (0.2 g-SS l^{-1}) and *E. coli* (0.1 g-SS l^{-1}) were transferred to 300-ml serum bottles. Organic substrates (3 mM) were added, and cells were induced for 12 h under aerobic conditions. Serum bottles were incubated on a rotary shaker (150 rpm) at either 20°C (activated sludge) or 37°C (*E. coli*). After induction, the sludge and *E. coli* were washed three times with filtered tap water and incubated with chosen labeled substrates (Table S2) under the high substrate and oxygen concentration conditions, according to the procedure described above.

Multiple substrate conditions. Diluted-sludge (2 ml, 1 g-SS l^{-1}) and *E. coli* (0.1 g-SS l^{-1}) were transferred to 10-ml serum bottles and incubated with one labeled substrate and one unlabeled organic substrate (Table S3).

Fluorescence *in situ* hybridization and DAPI staining

Fixed activated sludge and *E. coli* cells were washed three times with filtered tap water and then resuspended in a 1:1 volume ratio of phosphate-buffered saline (PBS) and 99.9% ethanol. Fixed sludge was spread onto gelatin-coated glass slides, air-dried, and dehydrated consecutively in 50%, 80%, and 99.9% [v/v] ethanol. FISH was carried out according to Amann (1995). The following oligonucleotide probes were used for FISH: EUBmix [equimolar concentrations of EUB338 (Amann *et al.*, 1990), EUB338II and EUB338III (Daims *et al.*, 1999)] targeting most of the *Bacteria*, MPA60 (Erhart *et al.*, 1997) targeting *Microthrix* (class *Actinobacteria*, phylum *Actinobacteria*), T0803-0654 (Kragelund *et al.*, 2011) targeting "type 0803" within the *Caldilinea* genus (class *Caldilineae*, phylum *Chloroflexi*), Acc-I-444 (Flowers *et al.*, 2009) targeting *Accumulibacter* clade I (class *Betaproteobacteria*), and Azo644 (Hess *et al.*, 1997) targeting *Azoarcus (*order *Rhodocyclales,* class *Betaproteobacteria)*, EUBmix probe was labeled with 5,(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS), all remaining probes were labeled with sulfoindocyanine dye (Cy3). Fixed *E. coli* cells were spread onto gelatin-coated glass slides, airdried and stained with DAPI solution for 5 min in the dark (Porter and Feig, 1980) after the microautoradiographic (MAR) procedure.

Microautoradiography

The combination of MAR and FISH was carried out as described by Nielsen and Nielsen (2005). Slides with activated sludge (after FISH procedure) and *E. coli* cells were coated with LM-1 emulsion (GE Healthcare UK Ltd., Little Chalfont, United Kingdom), exposed in the dark for 3 and 10 days and then developed with Kodak D-19 developer, as described by Nielsen and Nielsen (2005). In this report, the

results after 10 days exposure are discussed (Fig. 1). However, it must be noted that the substrate uptake patterns observed after 3 and 10 days exposure were similar (data not shown).

Microscopy

An epifluorescence microscope (Axioskop 2 Plus, Zeiss, Oberkochen, Germany) equipped with a charge-couple device (CCD) camera (CoolSNAP HQ, Photometrics, Oberkochen, Germany) was used to examine all the slides. Silver grain formation on probe-defined or DAPI-stained bacteria was observed with light microscopy. MAR-positive and MAR-negative cells were assessed visually by comparison of the number of silver grains developed on top of the cells to the background level.

Acknowledgements

This research was partially supported by the JSPS Institutional Program for Young Researcher Overseas Visits (T. Kindaichi), by research foundation from Public Association for Construction Service Chugoku region (T. Kindaichi), by FøSu (M. Nierychlo and P.H. Nielsen), and by Aalborg University.

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Figure legends

Figure 1

Summary of the substrate-uptake specificity for four probe-defined bacterial functional groups (*Microthrix*, *Caldilinea*, *Accumulibacter*, *Azoarcus*) from Aalborg West activated sludge (A-D) and pure culture *E. coli* MG 1655 from exponential and stationary phase (E and F, respectively), based on MAR experiments with 11 different labeled substrates performed under 15 different aerobic conditions. High [S] and low [S] in the header mean high or low concentration of substrate, 0.5-2 mM and 5-20 μM, respectively. Standard conditions: 0.5-2 mM substrate and air saturation. High oxygen: air saturation. Low oxygen: 1% in headspace, 0.5 mg-oxygen l^{-1}. Starvation: 12 h pre-incubation without any substrates. Induction: 12 h pre-incubation with organic substrate (3 mM). Multiple substrates: incubation with two different (one labeled and one unlabeled) substrates. The colors indicate fractions of active population and uptake activity. Green: no substrate uptake, most cells negative (> 80% of total cells); yellow: some positive cells (20-80% of total cells) exhibiting substrate uptake; red: most of the cells positive (> 80% of total cells) and highly covered with silver grains (i.e., high activity); orange shows a decrease in uptake activity compared to red, most of the cells positive (> 80% of total cells) with less silver grains per cell; purple shows an increase in uptake activity compared to red, most of the cells positive (> 80% of total cells) with more silver grains per cell. Gray: not determined.

Figure 2

MAR-FISH micrographs of Aalborg West WWTP activated sludge sample incubated with labeled glycerol under the multiple substrates (oleic acid + glycerol) condition (A-C), with labeled glycerol under the high substrate concentration (standard) condition (D-F), with labeled leucine under the multiple substrates (acetate + leucine) condition (G-I), and with labeled leucine under the high substrate concentration condition (J-L). *In situ* hybridizations were performed with a combination of the FLUOS-labeled mixed EUB338 probes (green), Cy3-labeled probes MPA60 (A and D), and Cy3-labeled probes

Acc-I-444 (G and J). *Microthrix* cells (A and D) and *Accumulibacter* cells (G and J) appear yellow, other bacteria appear green. Panel B, E, H, and K are bright-field MAR images, corresponding to the FISH images (A, D, G, and J, respectively). Panel C, F, I, and L are overlay images showing uptake of glycerol in presence of oleic acid by *Microthrix* cells (C), lack of glycerol uptake by *Microthrix* cells when present as a single substrate (F), uptake of leucine in presence of acetate by *Accumulibacter* cells (I), lack of leucine uptake by *Accumulibacter* cells when present as a single substrate (L). The bars represent 10 μm. Arrows indicate MAR-positive or MAR-negative cells.

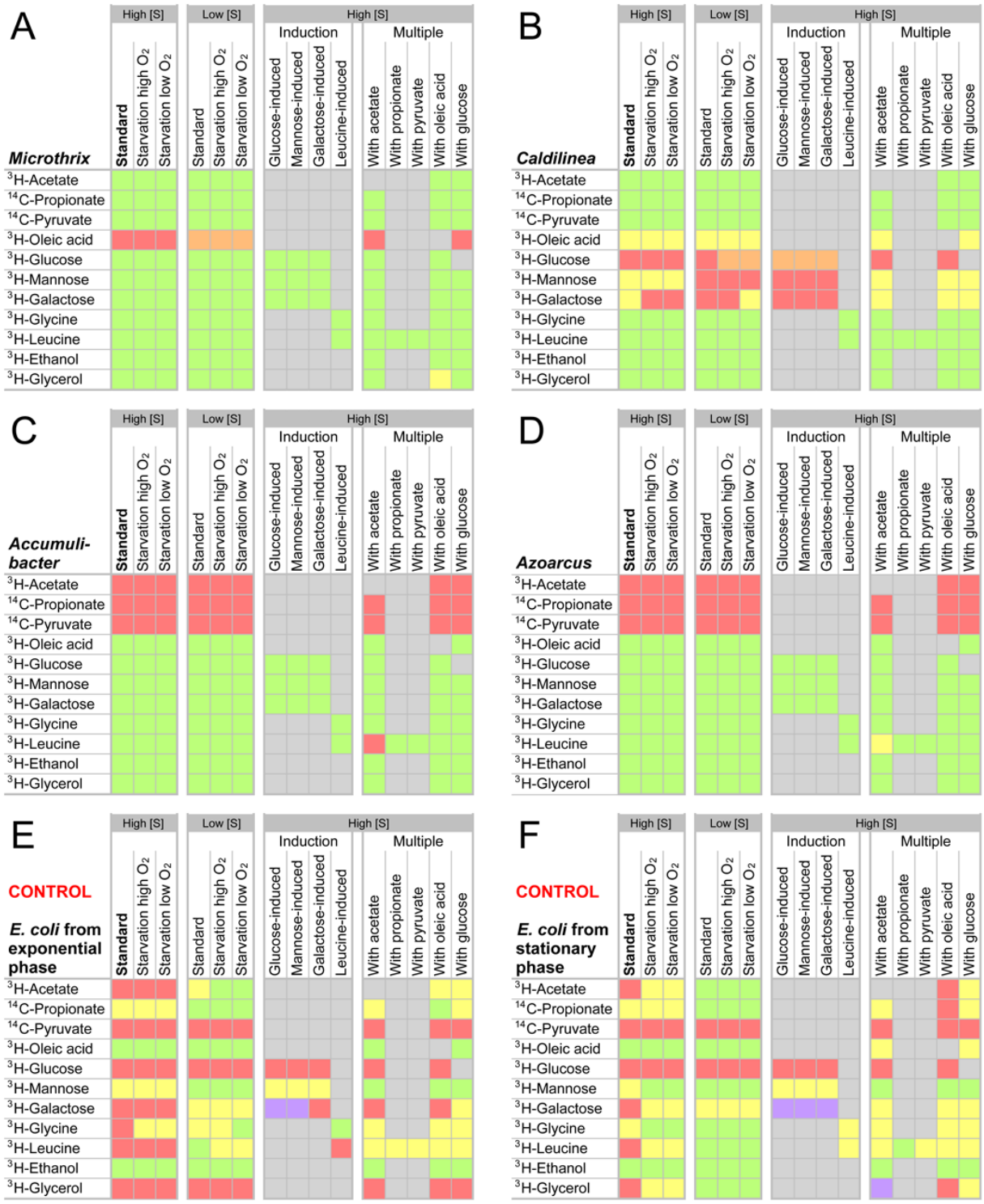
A
Microthrix
B
Caldilinea
C
Accumuli-bacter
D
Azoarcus
E
CONTROL
E. coli from exponential phase
F
CONTROL
E. coli from stationary phase
High [S]
Low [S]
Induction
Multiple
Standard
Starvation high O2
Starvation low O2
Glucose-induced
Mannose-induced
Galactose-induced
Leucine-induced
With acetate
With propionate
With pyruvate
With oleic acid
With glucose
3H-Acetate
14C-Propionate
14C-Pyruvate
3H-Oleic acid
3H-Glucose
3H-Mannose
3H-Galactose
3H-Glycine
3H-Leucine
3H-Ethanol
3H-Glycerol

Figure 1

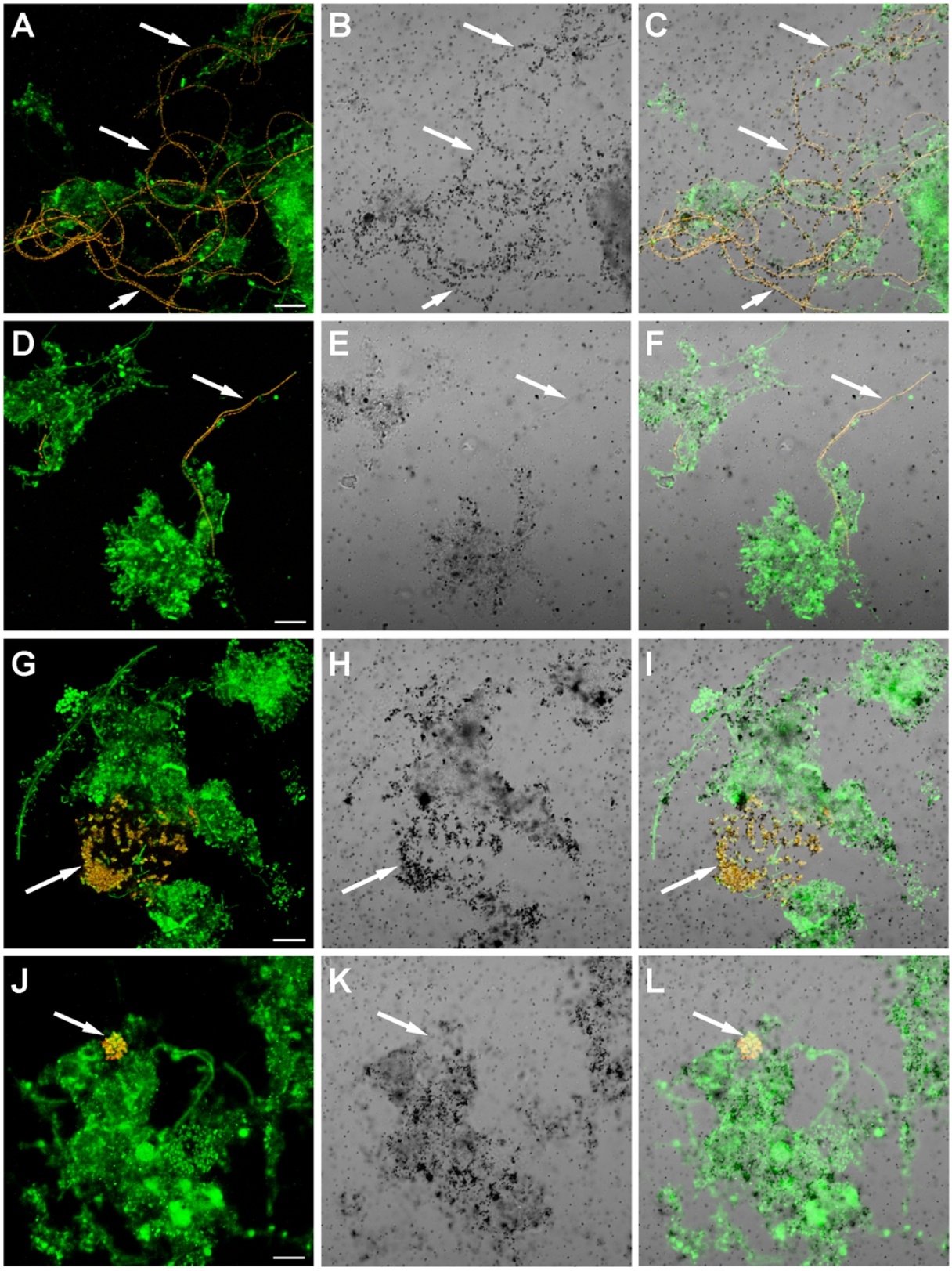

Figure 2

Supporting Information

High and stable substrate specificities of microorganisms in enhanced biological phosphorus removal plants

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Content:

Experimental Procedure

Labeled organic substrates

The labeled substrates under in this study were sodium [^{3}H]acetate (NET003; specific activity: 8000 mCi mmol^{-1}), sodium [2-^{14}C]propionate (ARC0281A; specific activity: 55 mCi mmol^{-1}), sodium [2-^{14}C]pyruvate (NEC256; specific activity: 16 mCi mmol^{-1}), [2-^{3}H]glycerol (ART0188A; specific activity: 20000 mCi mmol^{-1}), [9,10(N)-^{3}H]oleic acid (TRK140; specific activity: 7000 mCi mmol^{-1}), D-[6-^{3}H(N)]glucose (NET100; specific activity: 32300 mCi mmol^{-1}), D-[2-^{3}H(N)]mannose (NET570A; specific activity: 21600 mCi mmol^{-1}), D-[1-^{3}H]galactose (TRK233; specific activity: 7000 mCi mmol^{-1}), [2-^{3}H]glycine (TRK71; specific activity: 23000 mCi mmol^{-1}), L-[4,5-^{3}H]leucine (TRK754; specific activity: 64000 mCi mmol^{-1}), and [1-^{3}H]ethanol (ART0335; specific activity: 5000 mCi mmol^{-1}). Labeled substrates were purchased from PerkinElmer, Inc. (Waltham, MA), American Radiolabeled Chemicals, Inc. (Saint Louis, MO), or Amersham Biosciences (Little Chalfont, United Kingdom).

Figure S1

Micrographs of *E. coli* MG1655 (stationary phase) incubated with [^{3}H]glycerol under the multiple substrates (acetate + glycerol) condition (A-C), with [^{3}H]glycerol under the multiple substrates (oleic acid + glycerol) condition (D-F), with [^{3}H]glycerol under the high substrate concentration after starvation under low O_2 concentration condition (G-I), with [^{3}H]glycerol under the low substrate concentration after starvation under low O_2 concentration condition (J-L), with [^{3}H]glucose under the multiple substrates (acetate + glucose) condition (M-O), with [^{3}H]glucose under the multiple substrates (oleic acid + glucose) condition (P-R), with [^{3}H]glucose under the high substrate concentration after starvation under low O_2 concentration condition (S-U), and with [^{3}H]glucose under the low substrate concentration after starvation under low O_2 concentration condition (V-X). Panel A, D, G, J, M, P, S, and V show DAPI images. Panel B, E, H, K, N, Q, T, and W are bright-filed MAR images, corresponding to the DAPI images (A, D, G, J, M, P, S, and V, respectively). Panel C, F, I, L, O, R, U, and X are overlay images showing strongly high positive (C, O, R, U, and X), positive (F), some positive (I), and negative (L). The bars represent 10 μm.

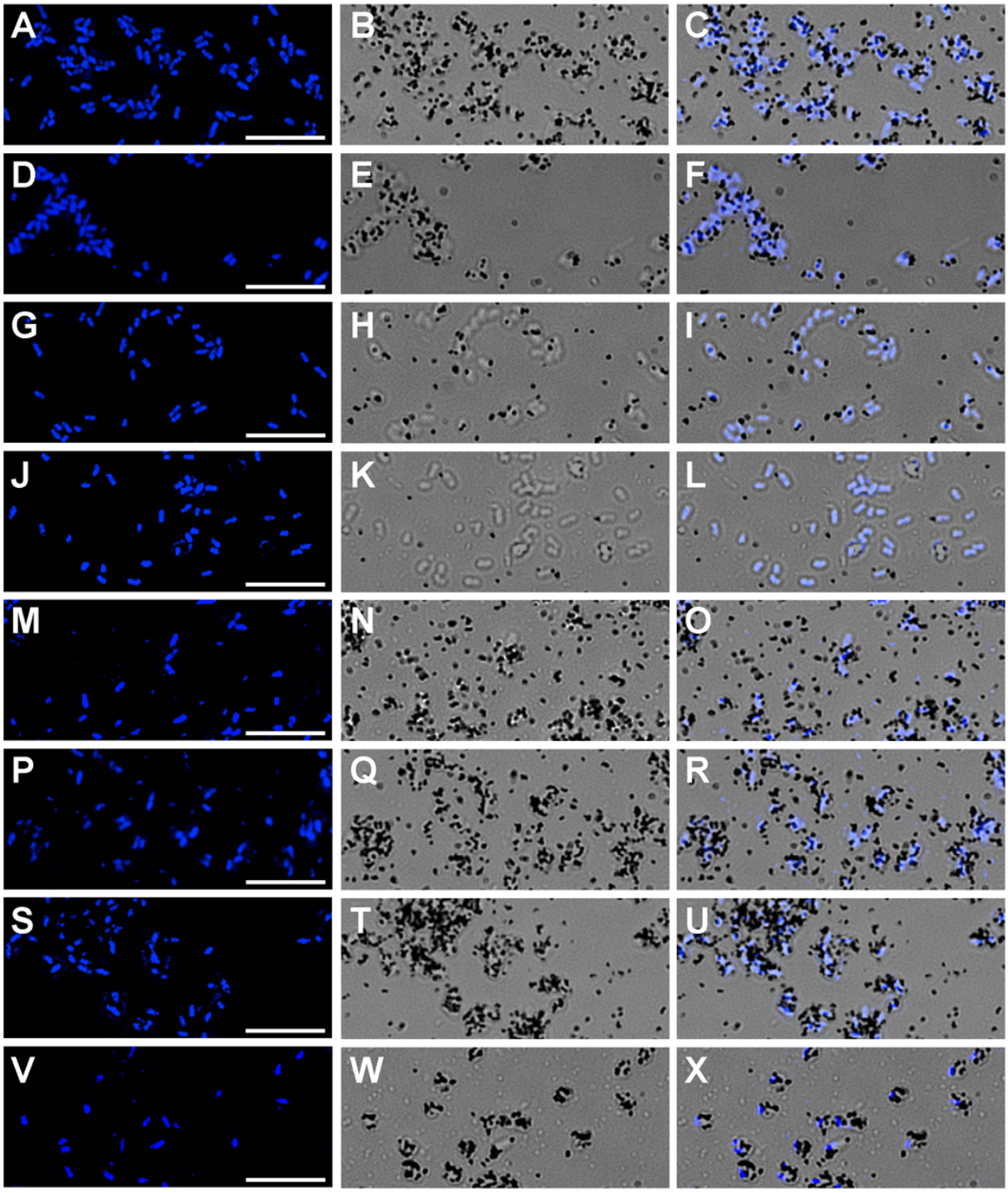

Figure S1

Table S1. Substrate uptake patterns of the four probe-defined populations under aerobic conditions – previous studies from full-scale EBPR plants.

Substrate	Microthrix	Caldilinea	Accumulibacter	Azoarcus
Formate	-	-	-	-
Acetate	-	-	+	+
Propionate	-	-	+	(+)
Butyrate	-	-	-	-
Lactate	-	ND	ND	-
Pyruvate	-	-	+	+
Oleic acid	+	-	-	-
Glucose	-	+	-	-
Mannose	-	(+)	-	-
Galactose	-	(+)	-	-
N-acetyl-glucosamine	-	+	-	ND
Glycine	-	-	ND	-
Leucine	-	-	-/+*	-/+*
Glutamate	-	ND	+	-
Methanol	ND	ND	ND	+
Ethanol	-	-	-	(+)
Glycerol	ND	ND	ND	ND
References	Andreasen and Nielsen, 2000 Nielsen et al., 2002	Kragelund et al., 2011	Kong et al., 2004	Thomsen et al., 2007

ND: Not determined

*: uptake of leucine only in presence of acetate

() a minor fraction of probe-defined cells active

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Table S2. Experimental overview of high, low, and multiple substrate concentration conditions. Incubation time was for all 2 h. In all incubations the added labeled substrate was 5 μCi ml^{-1}.

Experiment	Labeled substrate	Unlabeled substrate (s) concentration (mM)
High concentration	[^{3}H]acetate	2
	[^{14}C]propionate	2
	[^{14}C]pyruvate	2
	[^{3}H]oleic acid	0.5
	[^{3}H]glycerol	2
	[^{3}H]glucose	2
	[^{3}H]mannose	2
	[^{3}H]galactose	2
	[^{3}H]glycine	2
	[^{3}H]leucine	2
	[^{3}H]ethanol	0.5
Low concentration	[^{3}H]acetate	0.02
	[^{14}C]propionate	0.02
	[^{14}C]pyruvate	0.02
	[^{3}H]oleic acid	0.005
	[^{3}H]glycerol	0.02
	[^{3}H]glucose	0.02
	[^{3}H]mannose	0.02
	[^{3}H]galactose	0.02
	[^{3}H]glycine	0.02
	[^{3}H]leucine	0.02
	[^{3}H]ethanol	0.005
Multiple substrate condition	[^{14}C]propionate	Acetate (2 mM) + Propionate (2 mM)
	[^{14}C]pyruvate	Acetate (2 mM) + Pyruvate (2 mM)
	[^{3}H]oleic acid	Acetate (2 mM) + Oleic acid (0.5 mM)
	[^{3}H]glycerol	Acetate (2 mM) + Glycerol (2 mM)
	[^{3}H]glucose	Acetate (2 mM) + Glucose (2 mM)
	[^{3}H]mannose	Acetate (2 mM) + Mannose (2 mM)
	[^{3}H]galactose	Acetate (2 mM) + Galactose (2 mM)
	[^{3}H]glycine	Acetate (2 mM) + Glycine (2 mM)
	[^{3}H]leucine	Acetate (2 mM) + Leucine (2 mM)
	[^{3}H]ethanol	Acetate (2 mM) + Ethanol (0.5 mM)
	[^{3}H]acetate	Oleic acid (0.5 mM) + Acetate (2 mM)
	[^{14}C]propionate	Oleic acid (0.5 mM) + Propionate (2 mM)
	[^{14}C]pyruvate	Oleic acid (0.5 mM) + Pyruvate (2 mM)
	[^{3}H]glycerol	Oleic acid (0.5 mM)+ Glycerol (2 mM)
	[^{3}H]glucose	Oleic acid (0.5 mM) + Glucose (2 mM)
	[^{3}H]mannose	Oleic acid (0.5 mM) + Mannose (2 mM)
	[^{3}H]galactose	Oleic acid (0.5 mM) + Galactose (2 mM)
	[^{3}H]glycine	Oleic acid (0.5 mM) + Glycine (2 mM)
	[^{3}H]leucine	Oleic acid (0.5 mM) + Leucine (2 mM)
	[^{3}H]ethanol	Oleic acid (0.5 mM) + Ethanol (0.5 mM)
	[^{3}H]acetate	Glucose (2 mM) + Acetate (2 mM)
	[^{14}C]propionate	Glucose (2 mM) + Propionate (2 mM)
	[^{14}C]pyruvate	Glucose (2 mM) + Pyruvate (2 mM)
	[^{3}H]oleic acid	Glucose (2 mM) + Oleic acid (0.5 mM)
	[^{3}H]glycerol	Glucose (2 mM) + Glycerol (2 mM)
	[^{3}H]mannose	Glucose (2 mM) + Mannose (2 mM)
	[^{3}H]galactose	Glucose (2 mM) + Galactose (2 mM)
	[^{3}H]glycine	Glucose (2 mM) + Glycine (2 mM)
	[^{3}H]leucine	Glucose (2 mM) + Leucine (2 mM)
	[^{3}H]ethanol	Glucose (2 mM) + Ethanol (0.5 mM)

Table S3. Experimental overview of starvation and induction conditions.

Experiment	Unlabeled substrate	Incubation time (h)	O_2 concentration in headspace (%)	Incubation with labeled substrate, [a] high (0.5-2 mM) or low (0.005-0.02 mM) concentration
Starvation under high O_2	None	12	20	All substrates, high and low concentration
Starvation under low O_2	None	12	1	All substrates, high and low concentration
Induction with glucose	Glucose (3 mM)	12	20	Glucose, mannose, or galactose, high concentration
Induction with mannose	Mannose (3 mM)	12	20	Glucose, mannose, or galactose, high concentration
Induction with galacose	Galactose (3mM)	12	20	Glucose, mannose, or galactose, high concentration
Induction with leucine	Leucine (3 mM)	12	20	Glycine, leucine, high concentration

[a] After starvation and induction, samples were incubated with labeled substrate as shown in Table S2.