Substrate Utilization Profiles of Bacterial Strains in Plankton from the River Warnow, a Humic and Eutrophic River in North Germany

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Abstract Bacteria are very important degraders of organic substances in aquatic environments. Despite their influential role in the carbon (and many other element) cycle(s), the specific genetic identity of active bacteria is mostly unknown, although contributing phylogenetic groups had been investigated. Moreover, the degree to which phenotypic potential (i. e., utilization of environmentally relevant carbon substrates) is related to the genomic identity of bacteria or bacterial groups is unclear. The present study compared the genomic fingerprints of 27 bacterial isolates from the humic River Warnow with their ability to utilize 14 environmentally relevant substrates. Acetate was the only substrate utilized by all bacterial strains. Only 60% of the strains respired glucose, but this substrate always stimulated the highest bacterial activity (respiration and growth). Two isolates, both closely related to the same *Pseudomonas* sp., also had very similar substrate utilization patterns. However, similar substrate utilization profiles commonly belonged to genetically different strains (e.g., the substrate profile of *Janthinobacterium lividum* OW6/RT-3 and *Flavobacterium* sp. OW3/15-5 differed by only three substrates). Substrate consumption was sometimes totally different for genetically related isolates. Thus, the genomic profiles of bacterial strains were not congruent with their different substrate utilization profiles. Additionally, changes in pre-incubation conditions strongly influenced substrate utilization. Therefore, it is problematic to infer substrate utilization and especially microbial dissolved organic matter transformation in aquatic systems from bacterial molecular taxonomy.

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Introduction

Bacteria in aquatic systems are an important component of the carbon cycle due to their ability to oxidize organic matter. This organic matter consists of relatively simple substances like amino acids, peptides, carbohydrates (mono- to polysaccharides), and carboxylic acids, as well as complex humic substances. Natural bacterioplankton are comprised of a continuum of cells with different physiological states and capabilities since fluctuating abiotic conditions and substrate availability dynamically favor different members of the community [23, 74]. Community composition is thought to control bacterial metabolic function since bacteria adapt to natural environmental changes (e.g., seasonality, algal blooms, mixing of contrasting water masses, leaf litter input) or anthropogenic incidents, such as toxic or oil spills. Thus, ecologically important prokaryotes have to be isolated and characterized to understand the essential role they play in (organic) geochemical cycles [55] and especially to

estimate and eventually predict bacterial reactions/substrate degradation in complex environmental systems.

While characterizing metabolic capabilities of specific types of environmental microorganisms based on isolation and cultivation and has commonly been employed (cf. [10, 21, 58, 71, 78, 85]), there are significant challenges in linking microbial community function and structure using this approach. The primary problem is that many novel phylotypes can still be isolated from aerobic pelagic environments [27, 75], indicating that many pelagic bacterial species are, at present, uncultured. While substrate utilization is routinely applied as a biochemical fingerprinting approach to characterize and identify isolates, this approach is most effective for pathogens and food-related bacteria [41, 50], for which commercially available biochemical profiling systems (e.g., Biolog) contain extensive databases (cf. [44]).

Nevertheless, molecular identification and phylogenetic assignment of bacteria in natural assemblages are often used to make conclusions regarding the potential carbon and energy flows they mediate. Additionally, in situ degradation of specific substrates is deduced from the presence of certain phylogenetic bacterial groups (e.g., [1, 34] and therein [86]). This approach may be suitable for metabolic specialists in sediments (like the obligate methanotroph *Methylococcus capsulatus*). Since aerobic and facultative anaerobic bacteria are thought to be more metabolically versatile, it is unclear whether bacterial species composition can be effectively linked to substrate utilization in bacterioplankton communities.

This study examined the congruence of metabolic and genomic fingerprints in 27 bacterial strains isolated from the eutrophic and humic River Warnow. Substrate utilization was assessed with recently developed fluorescence-based microplates based on the detection of oxygen consumption (cf. [25, 77, 84]). In contrast to typical characterization systems for biochemical isolates, the user can readily define substrate type and concentration. Moreover, bacterial oxygen consumption can be continuously monitored, allowing for multiple quantitative parameters (e.g., adaptation time and bacterial activity, as well as the duration and capacity of substrate respiration) rather than the quantitative binary response reported in common biochemical fingerprints. In addition, the effect of physiochemical conditions, such as temperature and substrate availability, on substrate utilization profiles can be readily assessed.

Methods

Isolation, Cultivation, and Basic Characterization

Bacterial strains were isolated on agar plates (nutrient agar) from the surface waters of the eutrophic and humic River Warnow (for detailed information of study site, cf. [23]) in northern Germany during 2004. Bacterial strains were named after the sampling location (OW = Oberwarnow), the sampling month (4 = April), and isolation temperature (15 = 15°C). Colonies of contrasting color and shape were chosen (Table 1) after samples were incubated at in situ and deviating temperatures for 1–35 days. Following incubation, colony form and color were recorded. Repetitive streaking of individual colonies was used to purify strains. Frozen stock cultures of all strains were stored in nutrient broth (M1) plus 50% glycerol at −80°C.

All bacterial strains were grown in M1 media. Gram reactions were estimated with 3% KOH [29] and microscopically checked with the Live *Bac*Light™ Bacterial Gram Kit (Molecular Probes, Inc.). Before applying the Gram kit, bacterial membrane integrity was checked with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes, Inc.). Both kits were applied in accordance with the manufacturer's product sheet. Briefly, equal volume mixtures of both components from each kit (viability: SYTO 9 and propidium iodide, Gram: SYTO 9 and hexidium iodide) were prepared. A 3-µl portion of each staining solution was added to 1 ml bacterial cultures, respectively, incubated for 5 min and filtered onto Irgalan black-stained 0.2-µm Isopore™ polycarbonate membranes (Sigma Aldrich, Co.). The filters were embedded in the corresponding *Bac*Light mounting oil and examined under an epifluorescent microscope at a ×1,000 magnification (Olympus BX51, blue excitation U-MWB2).

Strains were prepared for substrate utilization assays by streaking on M1 agar (except OW5/RT-4, which only grew successfully on R2A agar [65]), followed by transfer into liquid M1 or into the more substrate diverse, but lower concentrated, R2A (marked cases), and incubated at ~20°C over night.

Physiological Profiling of Bacterial Strains

Bacterial substrate utilization profiles were generated using a fluorescence-based microplate for assessing dissolved oxygen (BD Oxygen Biosensor System; BD Biosciences, Bedford, MA, USA). At the bottom of the microplate wells, an O_2-sensitive fluorophore, 4,7-diphenyl-1,10-phenathroline ruthenium (II) chloride, is absorbed into a silicone matrix, which is permeable to O_2 [84]. The ruthenium dye fluorescence is quenched by O_2, so the signal from the fluorophore-gel complex increases in response to the respiration in the overlying sample.

A 10-µl preculture aliquot (diluted with M1 to an OD of less than 0.35 at 590 nm, if necessary) was added to each well, which were preloaded with 140 µl of a substrate minimum medium (MM) mix (see below). Initial bacterial abundance in the wells ranged from 20 to 74×10^6 cells ml^{-1}. Counting was done for selected samples in Neubauer counting chambers with a Zeiss-Axioskop2 plus microscope. Substrates tested included amino acids (L-alanine, L-threonine,

Table 1 Description of investigated bacterial strains isolated from the River Warnow

Strain	Sampling date	In situ and isolation temperature (°C)	Gram reaction	Colony color
OW3/RT-1[a]	20 Mar		–	White
OW3/RT-2	20 Mar		+	Dark-yellow
OW3/RT-3	20 Mar		–	Beige
OW3/RT-4	20 Mar	7/20	–	Light-yellow
OW3/RT-5	20 Mar		+	Pinkish
OW3/RT-8	20 Mar		–	White-translucent
OW3/15-1	20 Mar		–	Orange
OW3/15-3-2[a]	20 Mar		–	Light-beige
OW3/15-5[a]	20 Mar	7/15	–	Orange
OW3/15-6	20 Mar		–	Yellow
OW3/15-7	20 Mar		–	Pink-orange
OW3/5-1	20 Mar		–	Light-beige
OW3/5-3	20 Mar	7/5	+?	Yellow
OW5/RT-2	4 May		–	Dark-yellow
OW5/RT-4	4 May	16/20	–	White
OW5/RT-8	4 May		–	Yellow
OVT1/RT-4[a]	4 May	19/20	–	White
OW5/19-1	4 May		+	Light-orange
OW5/19-2	4 May		–	Orange
OW5/19-4[a]	4 May	16/19	–	Dark-pink
OW5/19-6[a]	4 May		–	Beige-translucent
OW5/15-2	4 May	16/15	–	Beige
OW6/21-3	2 Jun	18/21	–	White
OW6/RT-2	2 Jun		–?	Light-orange
OW6/RT-3[a]	2 Jun	18/20	–	Violet
OW7/17-4	13 Jul	22/17	–	White, later violet
OW8/26-1	10 Aug	23/26	–?	Pink

Gram reaction was identified with KOH test and Live *Bac*Light Bacterial Gram Kit (Molecular Probes)

[a] Isolates identified via the 16S rRNA gene

L-arginine, and L-leucine), monosaccharides (D-glucose, D-mannose, D-fructose, and 2-deoxy-D-ribose), a N-containing monosaccharide (*N*-acetyl-D-glucosamine or NAG), a disaccharide (D-cellobiose), low molecular organic acids (sodium acetate, DL-malic acid, and octanoic acid; all Sigma-Aldrich), and a high molecular organic acid (humic acid, Alfa Aesar). Each substrate was added to the MM separately to produce end concentrations of 50 and 500 mg l^{-1}. MM contained 0.03 M Na–K–phosphate buffer, pH 6.9 (1 l), NH_4Cl (1 g l^{-1}), $MgSO_4{\cdot}12H_2O$ (0.5 g l^{-1}), $CaCl_2{\cdot}6H_2O$ (5 mg l^{-1}), Fe–NH_4–citrate (0.5 mg l^{-1}), and a trace element solution (SL-6 [59]; 10 ml l^{-1}). All 27 strains were directly inoculated from the M1 preculture into the MM with different substrates. Therefore, a negative control containing MM and bacteria without an additional carbon source was measured to exclude effects from the residual M1 medium.

Plate Reading and Data Analysis

All microplates were read at time 0 on a Dynex MFX (20°C) or a Wallac Victor 2TM (27°C) microplate fluorometer at 485 nm excitation using the bottom-reading mode. The plates were incubated at 20°C or 27°C without shaking, with readings obtained every 15 min for 48 h. Fluorescence increased to a clear peak as oxygen was consumed in the wells and then decreased to baseline values as cell respiration decreased below the rate of oxygen diffusion (i.e., reaeration) into the wells [80]. Fluorescence readings were converted to a normalized relative fluorescence unit (NRFU) by dividing the fluorescence reading at each time point by the fluorescence at 1 h. A delayed time point for normalization was selected to allow for temperature equilibration given the temperature sensitivity of the ruthenium dye.

Several parameters can be used to describe the temporal pattern of the fluorescent response and to compare bacterial substrate respiration profiles [25]. The adaptation or lag period (*t*-lag in days) was calculated as the time necessary for the response value to increase 10% above the maximum value of the negative control (Fig. 1). The maximum response (max in NRFU) and the total response (area in NRFU × days) were defined. Area was calculated as the difference between the integrals of the response wells

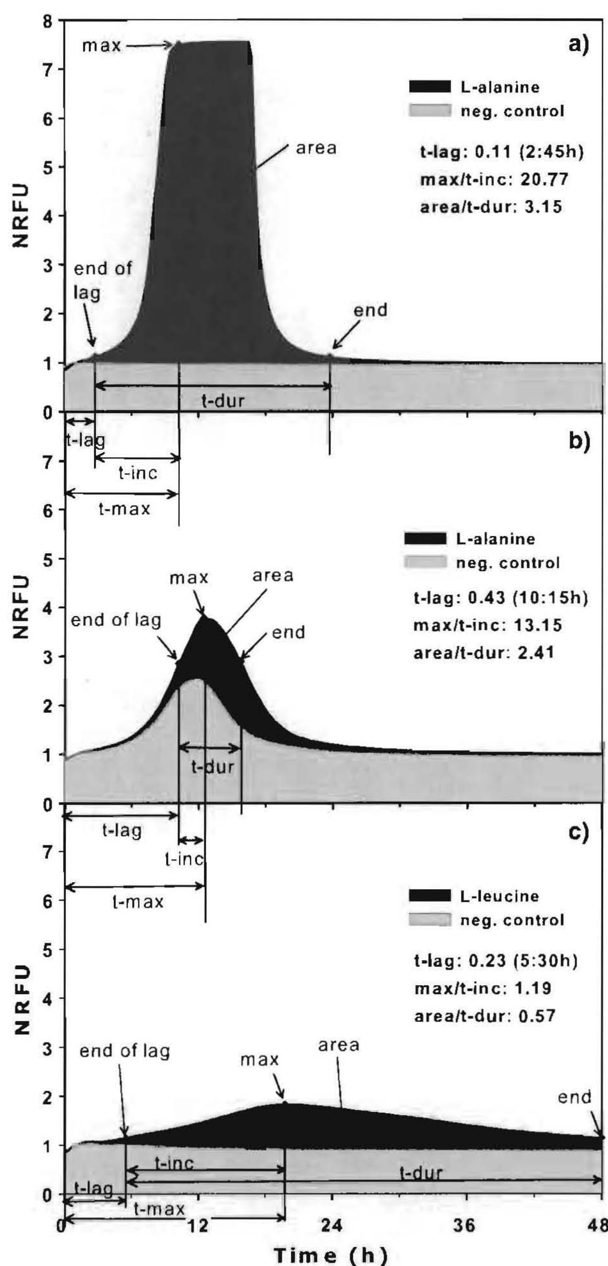

Figure 1 a–c Oxygen consumption, measured as the normalized relative fluorescence unit (*NRFU*), of bacterial strain OVT1/RT-4 in response to 500 mg l^{-1} L-alanine (**a**), OW3/15-5 in response to 50 mg l^{-1} L-alanine (**b**), and OW5/19-4 in response to 500 mg l^{-1} L-leucine (**c**) in comparison to a negative control without substrates over the indicated time period (hours). All estimated response parameters (end of adaptation period (*end of lag*), maximal value (*max*), time of maximal value (*t-max*), area of bacterial response (*area*), end of response (*end*), duration of substrate respiration (*t-dur*), and time of increasing oxygen consumption (*t-inc*)) were marked and the values of the adaptation time (*t-lag*), bacterial activity (*max/t-inc*), and substrate respiration capacity (*area/t-dur*) were inserted

versus the negative controls using the trapezoidal rule. A positive response was defined when a *t*-lag occurred or the peak area was >0.2 NRFU × days. For positive responses, additional parameters were calculated. The time to maximum response (either peak or the onset of a plateau; *t*-max in days) reflects the time to maximum utilization and the onset of respiratory and growth stagnation. The time of increasing oxygen consumption (*t*-inc in days), the difference between *t*-max and *t*-lag, was also calculated to estimate the length of the active period. *t*-dur (days), the total time the response was 10% higher than the negative control, reflects the overall substrate respiration duration. Max/*t*-inc (in NRFU day^{-1}) was calculated as the quotient of the maximal value (oxygen minimum; maximum minus the maximum of the negative control) and the time of increasing oxygen consumption (i.e., using the utilization duration to quantify substrate utilization dependent on the bacterial physiological state or activity). Rapid achievement of high maximal substrate utilization (max/*t*-inc was high; Fig. 1) is very likely due to cells with a high activity (fast reproduction and/or high respiration). For example, alanine stimulated at least an 8-fold higher activity in OVT1/RT-4 and OW3/15-5 than leucine did in OW5/19-4 (Fig. 1a–c). Finally, the absolute substrate respiration (area) was normalized to the response duration (*t*-dur) to estimate the bacterial substrate respiration capacity (area/*t*-dur in NRFU), which allows discrimination between short, intense, and low, long response dynamics.

The Effect of Bacterial Treatment on Physiological Substrate Utilization Profiles

To investigate the effects of temperature change on substrate utilization, the respiration of several bacterial strains (OW3/15-5, OW3/RT-1, OW3/RT-2, OW5/19-6) was measured at 27°C. The influence of slight temperature changes (from precultures incubated at ~18°C to measurements at 20°C) on substrate utilization (OW3/RT-5, OW3/15-3-2) was also determined. The influence of nutrition in the preculture phase on substrate utilization patterns was also checked. An OW5/19-6 preculture was additionally grown in R2A, measured at 27°C, and compared to M1 precultures measured at the same temperature. Comparisons were performed at higher temperatures given that this strains activity is greater at 27°C compared to 20°C.

The influence of bacteria's physiological state was estimated with the bacterial strain (OW6/RT-3). Substrate utilization profiles were determined with both overnight and 2-day pre-incubation periods. With OW3/15-3-2, the effect of cell washing was tested with a reduced substrate spectrum. Bacterial substrate utilization differences were detected for 11 out of the 14 substrates (without L-arginine, octanoic acid, and humic acid) at end concentrations of either 50 or 500 mg l^{-1}. Some of the cultures were washed

with NaCl (7 g l^{-1}), while other precultures were rinsed with phosphate buffered mineral salt (PBMS; K_2HPO_4 (7 g l^{-1}), KH_2PO_4 (3 g l^{-1}), $MgSO_4$ (0.1 g l^{-1}), $[NH_4]_2SO_4$ (0.5 g l^{-1}), $CaCl_2$ (0.01 g l^{-1}), $FeSO_4$ (0.005 g l^{-1}), $MnSO_4$ (0.0025 g l^{-1}), and Na_2MoO_4 (0.0025 g l^{-1})). Bacteria were centrifuged at 4,000 rpm for 5 min, the supernatant was discarded, and the cells were resuspended in NaCl or PBMS, respectively. After repeating this wash step, bacteria were diluted to gain cell numbers comparable to unwashed bacteria of $48 \pm 6.8 \times 10^6$ cells ml^{-1} in the microplate wells.

Adjustment of Setup and Statistical Analysis

Before bacterial substrate utilization profiles were generated, required substrate concentrations, as well as measurement and parameter reproducibility, were tested with the strain OW3/15-5. Therefore, bacterial responses to seven M1 dilutions (from 1:1 to 1:100) and five separate substrates (L-alanine, D-glucose, D-fructose, acetate, and DL-malic acid) at concentrations of 500, 100, and 25 mg l^{-1} were measured in triplicate (data not shown). The NRFU standard deviations of each reading among these triplicates were on average less than 1.4% of the respective mean. Because triplicates were nearly identical, substrate utilization was measured once per isolate. The above-mentioned response parameters were estimated and deviated by <10% of the mean (~6.7% on average). This was considered when comparing the influence of abiotic parameters on bacterial substrate utilization. A maximum (constant) standard deviation of 10% was applied for all response variables. A bacterial response was defined as distinctly different if the summed standard deviations of the respective variables (e.g., adaptation time) were less than the difference between the variable values.

To estimate the *t*-lag dependency on inoculation cell density, Spearman rank order analyses were performed between inoculation cell density and *t*-lag for each substrate and concentration (data not shown). This was done for each substrate separately because *t*-lag varied the greatest between different substrates inoculated with the same cell density. Cell density did not influence *t*-lag significantly. Only four analyses out of 28 showed a significant negative correlation, i.e., randomly higher initial abundances yielded in a shorter *t*-lag.

BOX-PCR Genomic DNA Fingerprinting and Computer-Assisted Cluster Analysis of Genomic Fingerprints

Genomic fingerprints were obtained by amplification with a BOX A1R primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) [63]. Polymerase chain reaction (PCR) was carried out in 25 µl reaction mixtures containing 12.5 µl 2× Taq PCR Master Mix (Qiagen), 9 µl sterile distilled water, 2.5 µl Box A1R primer (10 pmol/µl), and 1 µl of bacterial DNA templates. Negative control reaction mixtures without cell lysates were used for each amplified set. Cycling conditions included an initial denaturation at 95°C for 6 min, 35 cycles of 94°C for 1 min, 53°C for 1 min, and 65°C for 8 min, a single final extension cycle at 65°C for 16 min, and a final soak at 4°C. PCR products were separated by electrophoresis on 1.5% agarose gels in 1× Tris–acetate–ethylenediaminetetraacetic acid buffer at 90 V for 5 h. DNA-banding patterns were visualized by staining with ethidium bromide (0.01% in agarose gel) and were analyzed with the GelCompare II software (Applied Maths, Kortrijk, Belgium). Degrees of homology were determined by the Dice similarity coefficient, and dendrograms were constructed using the Ward clustering algorithm. Band position tolerance and optimization of 2.0% was used to compare the DNA patterns, whereby three replicates per bacterial strain had a similarity of 93%.

Amplification of 16S rRNA Gene and Sequencing

Nearly full-length 16S rRNA gene sequences were obtained from the seven most important bacterial strains by PCR amplification using the eubacterial primer 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and the universal primer 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′). The 27f primer was used in conjunction with 907r (5′-CCG TCA ATT CMT TTG AGT TT-3′) and the 533f primer (5′-GTG CCA GCM GCC GCG GTA A-3′) with 1492r [46]. PCR master mix contained 20 µl 2× Taq PCR Master Mix (Qiagen), 15 µl sterile distilled water, 1 µl of each primer (10 pmol μl^{-1}), and 1 µl of DNA template. Cycling conditions were initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 45°C for 45 sec, and 72°C for 1.5 min, a single final extension cycle at 72°C for 20 min, and a final soak at 4°C. PCR products were purified with the PerfectPrep Gel Cleanup kit (Eppendorf AG, Hamburg, Germany). Sequencing with sequencing primers 27f, 533f, 907r, and 1492r was performed with a CEQ Dye Terminator cycle and the sequencing quick start kit in the sequencer CEQ 2000 (Beckman Coulter). The sequences were automatically analyzed using the CEQ2000 XL software (Beckman Coulter), visually controlled, and finally aligned using the Bioedit software, version 7.0.5.3 [31]. The 16S rRNA gene sequences (lengths 1,255 to 1,422 bases) were compared to sequences in the GenBank database using the Basic Local Alignment Search Tool [2]. The sequence data were submitted to GenBank and were provided with GenBank accession numbers (OW6/RT-3: EF523603, OVT1/RT-4: EF523604, OW3/15-3-2: EF523605, OW3/15-5: EF523606, OW3/RT-1: EF523607, OW5/19-4: EF523608, and OW5/19-6: F523609).

Results

Genomic Diversity

Of the pelagic bacteria isolated from the River Warnow, half of the isolates were obtained in March (OW3) at a low temperature (7°C) and the other half at higher (>15°C) in situ temperatures (Table 1). Colony morphologies varied strongly from well-defined, bright white (OVT1/RT-4) to diffuse orange (OW3/15-5) and dark violet forms (OW6/RT-3). The majority of the bacterial strains were Gram negative (85%).

Genomic fingerprint cluster analysis divided the bacterial strains into two groups (I and II) with only 3% similarity (Fig. 2). Group II was comprised of fewer strains and was subdivided into three subgroups (IIa–c) with less than 60% similarity. The orange OW3/15-5 belonging to subgroup IIb was identified as a *Flavobacterium* sp., which affiliates with the *Bacteroides* (Table 2 and marked with an asterisk in Fig. 2). The biggest subgroup (IIa) contained the violet bacterial strain OW6/RT-3 identified as *Janthinobacterium lividum*, which is of the *Betaproteobacteria* class. Group I, which contained 20 strains, was considerably larger and also divided into different subgroups (Ia–e). Subgroup Ia, which differed at least 76% from other subgroups, included a *Psychrobacter* sp. (OW3/RT-1), a member of the *Gammaproteobacteria*. A subgroup (in subgroup Id) of four bacterial strains with similar genomic fingerprints (81%) was completely identified. All of these strains also belonged to the *Gammaproteobacteria*. One was identified as *Shewanella* sp. (OW19-4), an *Alteromonadales*, while the others were identified as *Acinetobacter* sp. (OVT1/RT-4) and *Pseudomonas* sp. (OW5/19-6 and OW5/15-3-2). These three, as well as OW3/RT-1 from the subgroup Ia, belonged to the *Pseudomonadales*, even though their genomic fingerprints were strongly different (Fig. 2; Table 2). The Gram-positive bacteria OW3/RT-5 and OW5/19-1, preliminary identified as *Actinobacteria* (*Arthrobacter* sp. (*Micrococcaceae*) and *Rhodococcus* sp. (*Nocardiaceae*), respectively), were also distributed into group I but in different subgroups (Ib and Ie1, respectively).

Bacterial Substrate Utilization

All strains had distinct substrate utilization profiles. For example, strain OVT1/RT-4 used 500 mg l^{-1} alanine as the

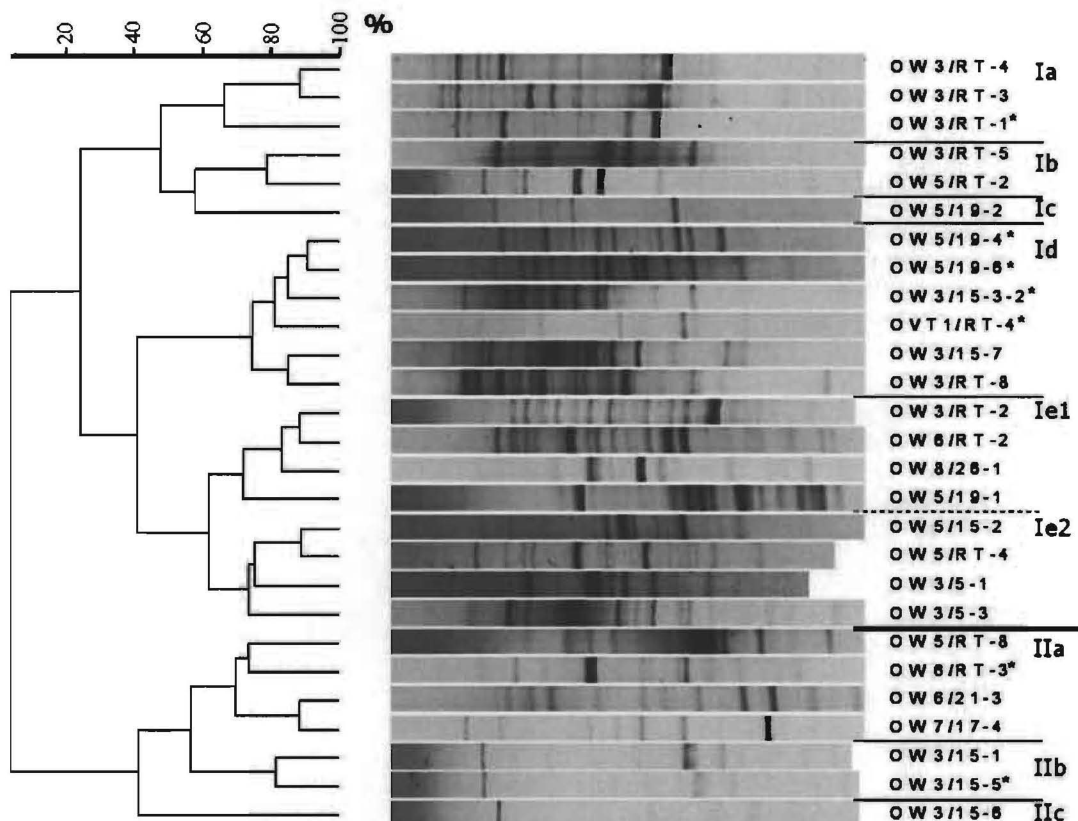

Figure 2 Cluster analysis of BOX-PCR genomic fingerprint patterns of bacterial isolates originating from the River Warnow. Using GelCompare II, homology degrees were determined by the Dice similarity coefficient, and dendrograms were constructed using the Ward clustering algorithm. Groups with less than 60% similarity were marked and an *asterisk* indicates isolates identified via the 16S rRNA gene

Table 2 Comparison of 16S rRNA gene sequences of bacteria isolated from the River Warnow with the most similar published match in NCBI database

Strain (1) GenBank A.N. (2)	Closest match (1) Closest published match (2)	GenBank A.N. of match	% of sequence similarity	Phylogenetic group	Publication
OW5/19-6	*Pseudomonas* sp. (K94.08)	AY456703.1	100.0	Bacteria, *Proteobacteria*, *Gammaproteobacteria*, *Pseudomonadales*, *Pseudomonadaceae*, *Pseudomonas*	
EF523609	*Pseudomonas rhodesiae* (CIP 104664)	AF064459.1	99.9		Coroler et al. [11]
OW3/15-3-2	*Pseudomonas syringae* (Lz4W)	AJ576247.1	99.9	Bacteria, *Proteobacteria*, *Gammaproteobacteria*, *Pseudomonadales*, *Pseudomonadaceae*, *Pseudomonas*	
EF523605	*Pseudomonas* sp. (AU2390)	AY486375.1	99.4		Spilker et al. 2004 [76]
OVT1/RT-4	*Acinetobacter* sp. (LUH4547)	AJ301674.1	99.8	Bacteria, *Proteobacteria*, *Gammaproteobacteria*, *Pseudomonadales*, *Moraxellaceae*, *Acinetobacter*	
EF523604	*Acinetobacter lwoffii* (DSM2403)	X81665.1	98.6		Rainey et al. 1994 [64]
OW3/RT-1	*Psychrobacter psychrophilus* (CMS 32)	AJ748270.1	99.7	Bacteria; *Proteobacteria*, *Gammaproteobacteria*, *Pseudomonadales*, *Moraxellaceae*, *Psychrobacter*	Shivaji et al. 2005 [72]
EF523607	*Psychrobacter maritimus* (Pi2-20T)	AJ609272.1	99.6		Romanenko et al. 2004 [69]
OW5/19-4	Bacterial species (Aspo5)	X95233.1	99.3	Bacteria, *Proteobacteria*, *Gammaproteobacteria*, *Alteromonadales*, *Shewanellaceae*, *Shewanella*	Pedersen et al. 1996 [57]
EF523608	*Shewanella putrefaciens* (LMG 2(6)268T)	X81623.1	99.1		Rossello-Mora et al. 1995 [70]
OW6/RT-3	*Janthinobacterium lividum* (BD17-1)	AF174648.1	100.0	Bacteria, *Proteobacteria*, *Betaproteobacteria*, *Burkholderiales*, *Oxalobacteraceae*, *Janthinobacterium*	
EF523603	*Janthinobacterium lividum* (CM37)	AY247410.1	99.8		Matz et al. 2004 [49]
OW3/15-5	*Flavobacterium* sp. (WB 3.1-22)	AM177612.1	99.0	Bacteria; *Bacteroidetes*, *Flavobacteria*, *Flavobacteriales*, *Flavobacteriaceae*, *Flavobacterium*	
EF523606	*Flavobacterium columnare* (ATCC 43622)	M58781.2	97.6		Gherna and Woese 1992 [26]

A.N. GenBank accession number

exclusive carbon source four times faster than OW3/15-5 50 mg l^{-1} (Fig. 1a–c). The absolute substrate respiration (area) and substrate utilization duration (*t*-dur) ranged from high and short, as in OVT1/RT-4 with alanine, and to small and long (e.g., OW5/19-4 with leucine). Thereby, OVT1/RT-4 had one of the highest bacterial activities (max/*t*-inc) and also substrate respiration capacity (area/*t*-dur) in contrast to OW5/19-4. OW3/15-5 gained lower activity (0.1 NRFU day^{-1}) with leucine than OW5/19-4, although its substrate respiration capacity was higher (2.5 NRFU).

Acetate was the substrate used by the most bacteria; all but one isolate respired it at the higher concentration and more than 80% at the lower (Table 3). However, this exceptional strain (OW3/5-3) could utilize acetate if measured for longer than 2 days (data not shown). At higher substrate concentrations, bacteria utilized a broader substrate range and mostly exhibited higher activities (data not shown). Octanoic acid was the only substrate which many more strains (up to 86%) used better at a lower concentration. A higher concentration of octanoic acid stimulated only three bacterial strains (*Pseudomonas* sp. OW3/15-3-2, OW5/15-2, and OW6/21-3) to high activity (max/*t*-inc; Table 4) as well as faster adaptation (*t*-lag, data not shown). The other low molecular organic acids stimulated the highest respiration activity (max/*t*-inc) in 56% of the strains (Table 4). *J. lividum* (OW6/RT-3)

Table 3 The percentage of bacterial strains from the Warnow using the different substrates tested, including the LMOA, HMOA, MS, MS + N, DS, and AA, at 500 or 50 mg l^{-1}; the concentration of millimoles of C per liter in 50 mg l^{-1} substrate; and the number of bacterial strains tested

Substrate	Sub. group	% using 500 mg l^{-1}	% using 50 mg l^{-1}	mmol C l^{-1} in 50 mg l^{-1}	Strains tested
Sodium acetate	LMOA	96	81	1.2	27
DL-Malic acid	LMOA	78	15	1.5	27
N-Acetyl-D-glucosamine	MS + N	70	48	1.8	27
L-Alanine	AA	67	41	1.7	27
D-Glucose	MS	63	48	1.7	27
D-Fructose	MS	59	33	1.7	27
Humic acid	HMOA	59	32		22
L-Arginine	AA	55	55	1.7	22
D-Mannose	MS	44	30	1.7	27
L-Leucine	AA	44	33	2.3	27
L-Threonine	AA	41	26	1.7	27
D-Cellobiose	DS	41	41	1.8	27
Octanoic acid	LMOA	36	86	2.8	22
2-Deoxy-D-ribose	MS	19	19	1.9	27

LMOA low molecular organic acids, *HMOA* high molecular organic acids, *MS* monosaccharide, *MS + N* monosaccharide with nitrogen, *DS* disaccharide, *AA* amino acids

showed the highest activity with the high molecular humic acid. Although humic acid was used by nearly 60% of the bacteria, it generated lower levels of activity on average.

The average bacterial respiration activity (max/*t*-inc) was the highest with carbohydrates (Table 4). Glucose was used by many bacteria and also tended to stimulate the highest average activity. NAG, although used by many strains, sustained 2.6 times lower bacterial activity than glucose and required a longer bacterial adaptation time (*t*-lag, data not shown). The disaccharide cellobiose was used by fewer bacterial strains than the C6 monomers and stimulated lower bacterial activity (max/*t*-inc) than glucose. Although cellobiose must be first hydrolyzed enzymatically, its utilization led to higher average bacterial activities than fructose and mannose (Table 4) and some bacteria even adapted faster to it (*t*-lag, data not shown). The DNA component deoxyribose was very stable against degradation. Just five bacterial strains respired in its presence; however, when used, it stimulated the second highest average activity.

Amino acid utilization stimulated the lowest bacterial activity (max/*t*-inc). Alanine, even though it was respired by as many strains as glucose, stimulated a lower average bacterial activity than the humic acid, but the substrate respiration capacity (area/*t*-dur) was high. Leucine and threonine were utilized as sole carbon sources by few bacterial strains, and these showed the lowest activity and longest adaptation time (*t*-lag, data not shown). However, bacteria gaining rather low bacterial activities from a substrate, nevertheless, could have a high substrate respiration capacity (area/*t*-dur). For instance, OW3/15-1 and the *Pseudomonas* sp. OW3/15-3-2 were stimulated to a similar activity (max/*t*-inc) by alanine (Table 4) but showed substrate respiration capacities of 7.3 and 1.9 NRFU, respectively.

The Effect of Bacterial Treatments on Physiological Substrate Utilization Profiles

Culture age influenced substrate utilization. A stationary, older bacterial culture responded to fewer substrates than a log-phase culture within 48 h (Table 5). After 65 h, substrates were no longer used. Log-phase cells rapidly produced a more pronounced peak in fluorescence, and the distance between the double peaks of DL-malic acid utilization was shorter (Fig. 3). The stationary phase bacteria needed 20 to 38 h longer to adapt to all substrates. The utilization of acetate started so late that it was at least partly missed within the standard 48-h incubation period. Furthermore, the bacterial activity of older cultures was lower for all substrates.

Deviating temperatures significantly influenced substrate utilization patterns. Strain OW5/19-6, a *Pseudomonas* sp., responded significantly earlier to most substrates and had higher peaks at 27°C (Fig. 4). While only acetate response reached a saturating (i.e., plateau) response at 20°C, four of the five substrates reached that value at 27°C. Altogether, the number of substrates used increased and/or respiration began earlier when temperatures were elevated by only 2°C (Table 5). The isolate OW3/RT-1, a *Psychrobacter* sp., which responded to only four substrates at 20°C, increased its substrate spectrum to a rather normal substrate number of 15 responses (nine different substrates). The quality and quantity of the responses were distinctive at different temperatures for most of the strains tested. The activity

Table 4 Respiration activity (max/*t*-inc) of bacterial strains isolated from the River Warnow using high (500 mg l^{-1}) concentrations of carbohydrates (MS, MS + N, NAG), DS as well as AA and organic acids (LMOA, HMOA)

Strain	MS				MS + N	DS	AA				LMOA			HMOA
	D-Glucose	D-Fructose	D-Manose	2-Deoxy-D-ribose	NAG	Cellobiose	L-Alanine	L-Leucine	L-Threonine	L-Arginine	Acetate	DL-Malic acid	Octanoic acid	Humic acid
OW3/RT-4	0.1	–	3.8	1.7	5.6	–	1.3	0.1	0.1	0.6	23.7	1.4	0.1	2.2
OW3/RT-3	–	–	–	–	–	–	2.0	–	0.1	0.1	75.1	–	–	4.6
OW3/RT-1[a]	–	–	–	–	–	–	1.0	–	–	–	16.6	–	0.1	2.1
OW3/RT-5	–	6.1	–	–	3.8	–	–	2.8	–	–	4.0	6.6	–	–
OW5/RT-2	24.0	14.9	25.2	–	28.2	21.4	–	–	3.1	13.7	9.5	–	–	3.0
OW5/19-2	30.4	13.4	32.4	–	26.8	24.2	–	–	0.1	–	4.3	–	–	2.8
OW5/19-4[a]	–	–	–	–	18.3	–	0.1	1.3	–	–	3.9	17.0	–	6.1
OW5/19-6[a]	12.7	3.4	5.9	10.1	0.9	–	8.3	0.6	–	nd	21.0	1.0	nd	nd
OW3/15-3-2[a]	6.7	2.5	–	–	0.4	–	3.4	3.3	–	8.8	7.8	11.8	11.3	–
OVT1/RT-4[a]	–	–	–	–	–	–	20.8	–	–	–	40.7	0.6	0.1	–
OW3/15-7	0.6	–	–	–	–	0.6	–	–	–	nd	0.7	0.9	nd	nd
OW3/RT-8	–	5.8	–	–	3.7	–	–	2.7	0.1	nd	3.7	6.4	nd	nd
OW3/RT-2	8.0	8.2	6.2	–	9.4	5.9	11.3	7.4	10.0	1.6	4.8	10.0	–	–
OW6/RT-2	–	6.1	–	–	0.9	–	–	–	–	–	7.9	4.3	–	–
OW8/26-1	–	–	–	–	–	–	–	–	–	–	0.7	0.3	0.1	–
OW5/19-1	0.8	0.8	–	–	0.9	1.2	1.4	–	2.0	–	0.9	–	0.1	1.2
OW5/15-2	16.2	15.8	15.0	13.6	–	–	24.1	12.2	–	45.0	30.3	10.0	35.1	–
OW5/RT-4	–	–	–	–	–	–	0.8	–	–	nd	0.3	–	nd	nd
OW3/5-1	24.8	23.7	2.4	–	–	22.3	20.6	–	–	0.1	28.5	11.6	–	17.4
OW3/5-3	0.7	1.5	0.6	2.2	1.3	1.0	1.0	–	5.3	1.8	–	1.0	–	1.4
OW5/RT-8	1.0	–	–	–	1.3	2.5	–	0.6	–	–	0.4	1.0	–	–
OW6/RT-3[a]	18.1	16.7	0.3	–	16.2	16.2	16.6	–	–	0.1	19.0	5.9	–	60.5
OW6/21-3	22.6	0.4	–	–	5.3	–	8.7	9.9	–	12.5	26.5	16.5	56.9	–
OW7/17-4	–	–	–	–	0.8	–	0.1	–	–	nd	1.7	0.9	nd	nd
OW3/15-1	27.7	–	22.7	–	13.7	–	2.8	0.1	0.1	0.1	5.8	0.1	–	5.4
OW3/15-5[a]	31.6	9.2	28.6	13.1	17.9	12.7	0.6	0.1	16.6	5.9	18.8	11.0	–	10.2
OW3/15-6	15.3	10.8	15.6	–	13.4	15.3	–	–	0.1	–	0.8	19.3	–	1.6
Median	14.0	6.1	6.2	10.1	5.3	9.3	2.8	2.0	0.1	1.7	5.8	5.1	0.1	3.0

MS monosaccharide, *MS + N* monosaccharide with nitrogen, *NAG* *N*-acetyl-D-glucosamine, *DS*, *AA* amino acids, *LMOA* low molecular organic acids, *HMOA* high molecular organic acids, *Nd* not determined

[a] Isolates identified via the 16S rRNA gene

Table 5 Differences in substrate utilization profiles after conditions were changed

Treatment	Strain	Tested substrates	No. of positive answers	Positive after treatment	Adaptation after treatment		Activity after treatment		Capacity after treatment	
					Earlier	Later	Less	More	Less	More
temp-increase	OW3/15-5[a]	22	22	22	16	1	0	20	1	12
temp-increase	OW3/RT-1[a]	22	4	15	1	2	0	4	0	3
temp-increase	OW3/RT-2	22	16	20	13	0	12	1	8	3
temp-increase	OW5/19-6[a]	22	12	14	11	0	0	12	3	6
temp-slight inc	OW3/RT-5	14	7	9	7	0	0	4	0	5
temp-slight inc	OW3/15-3-2[a]	14	7	7	1	0	2	2	0	2
Media	OW5/19-6[a]	22	14	9	3	2	8	1	5	3
Age	OW6/RT-3[a]	22	14	9	0	9	8	0	6	2
Wash NaCl	OW3/15-3-2[a]	14	7	1	0	0	1	0	0	0
Wash PBMS	OW3/15-3-2[a]	14	7	5	1	2	0	4	0	4

The number of used substrates during normal and changed conditions, adaptation time (*t*-lag), bacterial activity (max/*t*-inc), and substrate respiration capacity (area/*t*-dur) in comparison to normal conditions are recorded

temp-increase temperature increase of 7°C, *temp-slight inc* a slight temperature increase of 2–3°C, *Media* different preculture medium, *Age* the use of stationary cultures, *Wash NaCl* washing of the preculture with NaCl before inoculation, *Wash PBMS* washing of the preculture with PBMS before inoculation

[a] Isolates identified via the 16S rRNA gene

(max/*t*-inc) of OW5/19-6 was higher for all substrates at elevated temperatures. Other strains also showed higher activity except strain OW3/RT-2, which had a contrary behavior with lower and longer lasting substrate responses and a reduced substrate respiration capacity (area/*t*-dur; Table 5). For most strains, substrate respiration capacity was higher at elevated temperatures. For example, the respiration capacity of the rarely used substrate mannose increased in *Pseudomonas* sp. OW5/19-6 by 95% compared to the standard conditions.

The preculture medium R2A, which had a lower but more diverse carbon composition than M1, did not influence bacterial adaptation time. However, incubation with R2A reduced the number of substrates respired. Bacterial activity (max/*t*-inc) was lower, but the substrate respiration capacity was less for only some substrates. For many procedures, it is necessary to wash the cells to remove media, substrates, or produced metabolites from the cells. However, washing with 0.7% NaCl changed the physiological abilities of isolate OW3/15-3-2 clearly. After the NaCl treatment, cells respired only one of the seven formerly used substrates (Table 5). Washing the same culture with PBMS inhibited use of just two substrate turnovers, fructose and NAG. In contrast to NaCl, PBMS treatment stimulated bacterial activity (max/*t*-inc) and substrate respiration capacity (area/*t*-dur; Table 5).

Comparison of Bacterial Substrate and Genomic Profile

Substrate utilization of the closest related identified strains (*Pseudomonas* spp. OW3/15-3-2 and OW5/19-6, 85% similarity) were also very similar, including the achieved bacterial activity (max/*t*-inc). However, OW5/19-6 utilized two more monosaccharides (mannose and deoxyribose) than OW3/15-3-2. OW3/15-1 and the *Flavobacterium* sp. OW3/15-5, which had a similar genomic fingerprint

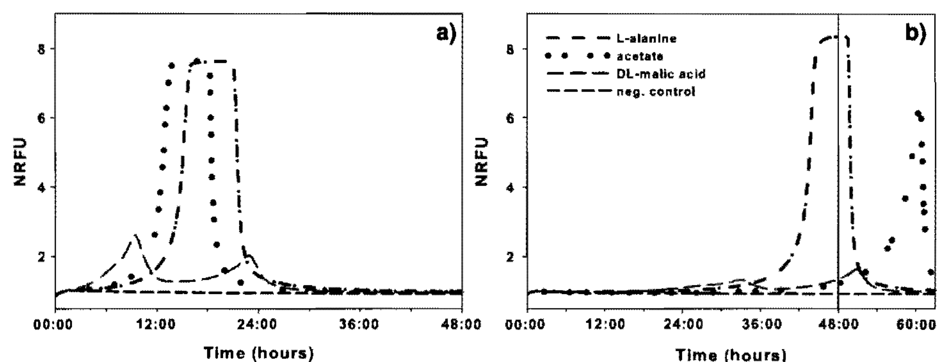

Figure 3 Oxygen consumption, measured as the normalized relative fluorescence unit (*NRFU*), of *J. lividum* OW6/RT-3, a bacterial isolate from the River Warnow, in the presence of three substrates (alanine, acetate, and malic acid (500 mg l^{-1})) under normal conditions (**a**) and a 2-day longer incubation (**b**) measured over the indicated time (hours)

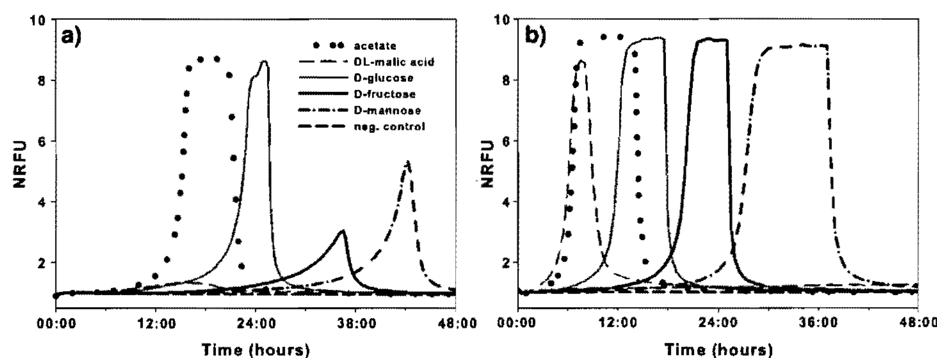

Figure 4 Oxygen consumption, measured as the normalized relative fluorescence unit (*NRFU*), of *Pseudomonas* sp. OW5/19-6, a bacterial isolate from the River Warnow, in the presence of five substrates (acetate, malic acid, glucose, fructose, and mannose (500 mg l^{-1})) at normal conditions (**a**) and measured at a higher temperature (**b**) over the indicated time (hours)

profile (Fig. 2) and colony morphology (Table 1), used the same amino acids and organic acids (at high concentration). However, OW3/15-1 consumed fewer carbohydrates (cellobiose, deoxyribose, and fructose). Interestingly, *Psychrobacter* sp. OW3/RT-1 has a similar genomic fingerprint to both OW3/RT-3 and OW3/RT-4, while it had a similar substrate profile to OW3/RT-3 but not OW3/RT-4. Concerning the substrate profile, the first two did not use any offered carbohydrate. However, the substrate profile of this same *Psychrobacter* sp. OW3/RT-1 included six carbohydrates at higher temperature (see above), which was more similar to that of the latter strain OW3/RT-4.

The ability to use deoxyribose did not seem to be typical for any investigated phylogenetic group. Strains OW3/15-5, identified as *Flavobacterium*, and OW5/19-6, a *Pseudomonas* sp., (Table 2) were representative deoxyribose users but belonged to totally different phylogenetic groups. Moreover, the genomic fingerprints of deoxyribose-consuming bacteria varied widely with less than 20% similarity (Fig. 2). Although these bacteria were all capable of consuming glucose, mannose, alanine, malic acid, and octanoic acid, their overall substrate profiles differed distinctly. *Pseudomonas* sp. OW5/19-6, for example, did not respire either cellobiose or threonine and six other substrates at the lower concentration. On the other hand, the *Flavobacterium* sp. OW3/15-5 consumed nearly all substrates (93%) and was more active than the other deoxyribose user. The strains (*Pseudomonas* sp. OW3/15-3-2, OW5/15-2, and OW6/21-3) utilizing the high octanoic acid concentration were also from different genomic fingerprint groups (Fig. 2) and were isolated at different times (Table 1). The consumption of another special substrate, cellobiose, which requires β-glucosidic activity, was also widely spread over nearly all genomic fingerprint groups. Even the substrate profile of *J. lividum* OW6/RT-3 and *Flavobacterium* sp. OW3/15-5, which belong to totally different phylogenetic groups (Table 2), varied just by three substrates (deoxyribose, leucine, and threonine).

At the same incubation and preculture conditions, bacterial strains used totally different numbers of substrates (18–93% of 14 or 9–93% of all 28 substrates and concentrations). Activity and adaptation time also varied strongly in respect to the different substrates offered to each isolate. The substrate consumption was sometimes totally different for genetically related isolates. For instance, OW5/15-2 consumed many substrates with very high activities, and OW5/RT-4 used only two substrates with one of the lowest activity (max/*t*-inc). Thus, bacteria with similar genomic fingerprint profiles did not have identical substrate profiles. Even *Acinetobacter* sp. OVT1/RT-4, a member of the *Pseudomonales*, was not capable of using one of the tested carbohydrates, which could be used by the genomically and phylogenetically similar *Pseudomonas* spp. OW3/15-3-2 and OW5/19-6.

Discussion

Response Parameters for Substrate Utilization

Bacterial substrate respiration profiles were generated using a fluorescence-based microplate assay for dissolved oxygen depletion rates. These time-dependent bacterial oxygen consumption rates could be compared using various response parameters describing adaptation time to the new carbon source or overall bacterial utilization [25]. Substrate utilizations of different bacterial strains were analyzed using four parameters. The first distinguished between positive or negative responses (yes or no). This response parameter characterized the strain-specific ability to use a substrate independently of the utilization rates. Therefore, the lag time (*t*-lag), after which significant oxygen consumption started, was detected. This delay covers the substrate uptake and the production of respective enzymes for channeling the substrates into the citric acid cycle. This lag time in culture is known to depend on the inoculated cell density. A smaller cell number normally takes longer to reach the threshold rate of oxygen consumption [25, 77]. However, here, high cell numbers (>10^7 ml^{-1}) varying by 3-fold were inoculated, and *t*-lag did not correlate significantly to the cell number. Therefore, the effect of inoculum density could be neglected for *t*-lag interpretation in this investigation. Thus, only bacterial metabolism (i.e., doubling time and enzymatic production) and its adaption capability

influenced t-lag. The adaptation time itself was prolonged if the bacteria came from different and probably more pessimal, cultivation conditions [47].

The maximal substrate utilization in combination with the duration of increasing oxygen consumption, i.e., substrate utilization, defines the bacterial activity (max/t-inc). If bacteria consume a large amount of oxygen within a short period of time and, therefore, reach a high maximum value fast, they were considered highly active. This activity is based on anabolic and catabolic reactions. If the strain-specific propagation and biomass production were high, the growing population used and respired more substrate. However, t-inc covered not only the logarithmic growth but also a part of the stationary phase, since cells do not have to grow to consume carbon. In contrast, it seems to be advantageous for bacteria to maintain the highest possible energy flow in the presence of growth constraints (cf. [14]). Thus, bacterial activity indicates a bacterial growth rate, respiration, and maintenance of cell metabolism in proportion to the energy and carbon content of the offered substrate. Some substrate response curves plateaued due to constantly low dissolved oxygen concentrations. For this result type, max and t-inc were both underestimated, and the quotient is reduced mathematically in max/t-inc if both terms are affected similarly. Another error source for the max value was an occasional max value in the substrate-free controls (cf. Fig. 1b). This response could be sustained by three different sources: (1) from the residues of the transferred preculture medium, (2) from bacterial storage material, and/or (3) from substrates released by living cells or originating from lysed cells. The max value was, therefore, always corrected for the max of the respective negative controls and the absolute substrate respiration (area) for the area of negative controls, respectively. Substrate utilization not only ceased when the substrate concentration was depleted (or strongly reduced) but might also have slowed down if inhibitory substances accumulated or by cell death (autolysis). It is unlikely that anoxic conditions caused the end of consumption even by asymptotic data (plateaus) because measurements with M1 showed a continuous plateau over 43 h until the end of the measurement (not shown). Additionally, oxygen may diffuse through the plastic plate material [5]. When bacteria have a low substrate respiration capacity (area/t-dur), the substrate utilization does not need to cease. Bacteria, which grow and respire slowly, barely supporting cell maintenance, utilize less substrate and do not deplete it. Thus, the response can go on if no above-mentioned "self-toxification" causing cell death occurs.

Bacterial Substrate Utilization

In aquatic systems, organic matter degradation and its role in the carbon cycle are of major importance. Bacteria, which are mainly responsible for this degradation, vary widely in their community composition [37], their physiological activity [23, 74], and local and temporal carbon turnover rates.

Substrate degradation depends strongly on species or even strain-specific abilities. Glucose often dominates the dissolved neutral monosaccharide in aquatic environments [35, 67]. This monosaccharide was not only used by many bacterial strains but stimulated high activity. In environmental bacterial communities, glucose also supported considerable bacterial growth and respiration [9, 67]. Glucose uptake is often (tacitly) assumed to be ubiquitous for bacteria, however, in contrast to acetate, not all isolates used it in this study. Less than half of the aerobic pelagic bacteria isolated from the Baltic Sea utilized glucose [24]. The fact that many bacteria did not utilize several substrates widely used for bacterial production and consumption assays in aquatic microbial ecology must be considered when interpreting, for instance, glucose uptake measurements. Environmental carbon consumption extrapolations based on glucose uptake measurements most likely underestimate carbon flow and bacterial activity. The consumption of other substrates, for instance leucine, was rather limited. Leucine is the model substrate to estimate bacterial production [38]. Therefore, low bacterial respiration, as observed in different pelagic communities [28], is a main prerequisite for the method to estimate biomass production. Even though some bacterial strains respired leucine when present as the sole carbon source, the long adaptation time and low activity indicate that leucine is not a preferred (catabolic) substrate and, from that point of view, suited for bacterial production measurements. However, the leucine uptake method requires bacteria to be able to incorporate the substrate, which is not true for all phylogenetic groups or species [12], but can be checked by microautoradiography in respective cultures.

Glucosamine, including NAG, the main component of bacterial cell wall peptidoglycan, was important for rivers entering the Baltic Sea, and NAG consumption by aquatic bacteria is common [36, 68]. Although many bacteria respired NAG, they needed a longer time period to adapt and reached lower activity than when utilizing glucose. This may be attributed to the preference of NAG as an anabolic substrate (channeled into bacterial biomass production) instead of its use for respiration. Additionally, unlike glucose, NAG is transformed via two more enzymatic reactions (deacetylation and deamination) into fructose-6-phosphate (cf. [3]) likely slowing down utilization.

Interestingly, malic acid and octanoic acid were also preferred by the bacterial strains originating from the River Warnow. Malic acid is otherwise utilized as an electron donor by sulfate reducing bacteria (e.g., [33]), favored by the bacterium *Oenococcus oeni* to deacidify berry juices and wines at low pH [81], or by other biotechnologically used bacteria. In natural environments, malic acid was only once detected as important for bacteria [28]. On the other

hand, it is ubiquitously present in organisms as an element of the tricarboxylic acid cycle and thus utilized (somehow) by many bacterial strains. Moreover, DL-malic acid repeatedly stimulated a two-peak utilization pattern (cf. Fig. 3), which may have been caused either by the bacterial adaptation to one ratiomere after the other, or by their growth on released substances of lysed/dead cells (cf. [20]). Octanoic acid, which occurs at lower concentrations in aquatic environments [48], was not previously considered as an important bacterial substrate. In contrast, antimicrobial activity associated with this organic acid was detected (e.g., [73]), which may explain the low responses to this substrate specifically at higher concentrations.

All other substrates triggered distinctly better utilization (more responses and higher substrate respiration capacity) at higher concentrations (500 vs. 50). The bacterial uptake rate depends on the substrate concentration below a certain concentration (saturation) and on maximal cellular metabolism above that concentration (cf. [43]). Thus, the substrate respiration capacity was elevated due to increases from nonsaturating to higher substrate concentrations. In bacterial cultures, cells can experience a growth limitation when grown on a single carbon source, probably due to cometabolism. Bacteria without a substrate response at low concentrations also often had low activity (e.g., <1 with acetate) with the higher concentration. At low concentrations, bacteria reduced their growth rate and changed into cells functioning with a different energy strategy (cf. [42]), thereby reducing substrate uptake below the detection limit.

Bacterial isolation on nutrient-rich medium could have selected strains better adapted to higher substrate concentrations. Page et al. [55], for instance, tested isolated oligotrophic bacteria on solid nutrient rich medium (R2A), and just one third were able to grow at all. However, the River Warnow is not characterized as oligotrophic, with nearly 10 mg dissolved organic carbon per liter. Moreover, preculture conditions influence bacterial substrate utilization (cf. above). Precultivation with lower concentrated R2A rather than M1 reduced the substrate utilization of an exemplary strain by more than one third.

In summary, bacterial strains isolated from the environment had distinctly different substrate utilization profiles. Some substrates were used by nearly every strain, but the degradation of other substrates (e.g., deoxyribose) was only mediated by a few strains.

Implications of Substrate Profiles for Natural Communities

Overall, bacterial metabolic capabilities in culture and in the environment always have to be compared carefully. On one hand, bacteria in cultures do not always behave as they do in situ. Bacterial growth in pelagic environments is rarely in the exponential phase but mostly in the stationary phase or rather at starvation under limiting or stress conditions [61, 82]. However, dissolved organic matter pulses rapidly stimulated exponential growth of environmental bacterial communities [16]. Additionally, limiting conditions in the environment causes uncoupling between catabolism and anabolism compared to culture conditions, resulting in enhanced consumption of substrates and oxygen (i.e., respiration) but less growth (cf. [14, 40]). Thus, even though bacteria may not grow with the substrates in the pelagic environment, they presumably use them for respiration or energy maintenance.

On the other hand, cultivation success of environmental heterotrophic bacteria ranges between <0.1% and 15% [4, 8, 51]. However, the cultivable fraction (even cultivated on nutrient high solid medium) repeatedly represented the active or dominant populations in (aquatic) environments [18, 56, 60, 66]. Thus, the isolated bacteria may be at least partly important for substrate turnover in the environment. Statistical random isolation of colonies may have selected for more abundant cultivable bacteria (cf. [24]) but would not obtain a higher species richness with highly diverse substrate utilization profiles, which was preferred for this investigation.

In aquatic environments, especially in rivers, bacteria must cope with frequently changing conditions. Of course, temperature, which is one of the main factors influencing bacteria [39, 62], varies over the whole year. Temperature increases reduced the bacterial adaptation time (lag-phase) [79, 83]. Furthermore, temperature shifts increased the number of substrates (especially carbohydrates) used by the bacteria. In contrast, Haack et al. [30] found almost no substrate utilization changes due to temperature or media. Short-term temperature changes affected substrate utilization profiles, but these patterns may converge under constant conditions and, moreover, if followed for a longer time. In the environment, temperature enhancements may not only activate inactive bacteria but also stimulate a higher substrate utilization spectrum in already active cells.

Additionally, natural bacterial assemblages react to changes in the dissolved organic matter pool. Substrate input, due to inflow of terrestrial organic matter or phytoplankton blooms, often causes clear shifts in the bacterial community composition [13, 19, 37]. However, even bacterial isolates reacted differently to substrates depending on their preculture composition (i.e., of their status prior to the new substrate supply). The substances in the precultures even influenced the bacterial substrate utilization profiles. Sugars, for example, are transferred via phosphotransferase systems through the cell membrane. Therefore, compartments of the transport system (enzyme II) are highly substrate specific, especially for glucose [15]. The specificity can be induced by the preculture growth medium, for instance, glucose inactivated fructose or mannose uptake

[15, 45]. In media containing different substrates, uptake rates may have also been limited by the presence of similar substrates [3, 15]. In diverse compositions of dissolved organic matter, bacterial reaction to substrate input can be delayed due to an uptake adaption/inhibition. Thus, predicting bacterial reactions from substrate utilization patterns to environmental substrate changes would be rather difficult.

The influence of bacterial culture conditions on substrate utilization profiles must also be considered for comparisons and predictions. To estimate the absolute bacterial substrate utilization range, many tests with different abiotic and preculture conditions are most likely necessary. Bacterial substrate utilization profiles probably differ less under continuously varying conditions than between different constant conditions. However, at the moment, these tests do not occur. Environmental conditions are not even constant between different investigators, which should also be required for bacterial strain identification via substrate utilization profiles (cf. [44]). Additionally, the impact of washing steps on bacterial activity must be estimated and considered. A database, which also includes culture conditions and genetic information, would improve and verify species-specific abilities and identity.

Comparison of Genetic and Substrate Consumption Profiles

Bacterial species have a specific substrate utilization profile, which allows growth only under specific conditions, i.e., within the tolerance limits. Consequently, community composition may change due to fluctuating substrate availability (e.g., [37]). This idea often leads to a conclusion on potential prospective substrate utilization in natural assemblages from the molecular species identification (e.g., [1, 34] and therein), which is mostly true for higher organisms. The substrate utilization profiles of some genetically related bacterial strains (e.g., OW5/19-6 and OW3/15-3-2, identified as *Pseudomonas* sp.) were similar, which supports the idea of estimating substrate utilization from the genetic determination of species. For parallel tested substrates, the pattern of OW5/19-6 was also the same as determined for *Pseudomonas rhodesiae* CIP 104664 (99.9%, AF064459.1) [11] and *Pseudomonas grimontii* CFML 97-514 (AF268029.1; 99.8% to OW5/19-6 in NCBI), which was closely related to *P. rhodesiae* CIP 104664 [6]. Phenotypic and genotypic characterizations suggest that there may be a correlation between certain phenotypic properties (e.g., growth rate on certain substrates) and variation in genome organization determined by rep-PCR [54]. However, similar substrate profiles were not always an indication of close phylogenetic relationships. Moreover, the substrate utilization profiles of the environmentally relevant substances chosen here were mostly not congruent with the genomic fingerprint profiles of the bacterial strains. In natural communities, the abundance of phylogenetically defined bacterial groups also did not correlate with their substrate uptake (e.g., [17]). In addition, these phylogenetically identified groups consist of many different species, probably showing a much larger variability in substrate preferences than species and genera. Moreover, even closely related species can have very different substrate utilization patterns, so that a correlation between species occurrence and substrate turnover should not be expected per se. Additionally, the degradation of rarely used substrates was widespread over the genomic and phylogenetic groups included in this study probably because all the isolates originated from a humic river.

In (stratified) sediments, bacteria are specialized in the utilization of certain (restricted) substrates. For instance, *M. capsulatus* and the whole *Methylococcaceae* group are obligate methylotrophs. For these organisms, molecular identity, as well as the abundance of active cells, can lead to conclusions on carbon and energy flow (cf. [32]). However, this was not true for aerobic pelagic strains. These species have wide and diverse substrate spectra (this work) [53] most likely because they are exposed to diverse and changing organic matter in their natural environment [22, 52]. Additionally, active cells have highly varying genetic origins in pelagic environments [7]. Thus, relevant substrates are utilized by a diverse group of different planktonic species. Haack et al. [30] also concluded that interpretations of community substrate utilization profiles with regard to community taxonomic composition would be complicated. Since bacterial strains/species can adjust their substrate utilization profiles due to condition changes, the phylogenetic origin seems to be a less important structuring component of pelagic bacterial communities. Thus, species or especially phylogenetic group identification in aerobic pelagic communities does not necessarily allow conclusions on bacterial induced substrate turnover in situ. This may be different for more complex or special substrates, like polymers or xenobiotics. Since substrates can be chosen with the BD oxygen system and substrate utilization can be analyzed in detail, further work should be conducted with these substrates. Additionally, it remains to be investigated how bacterial genetic capabilities actually determine activity and the utilization of environmental substrates. Therefore, catabolic genes of isolates should be investigated simultaneously to determine if closely related strains have the same metabolic equipment, especially if they use different substrates.

Acknowledgments This work was funded by the NASA Planetary Biology Internship Program and a grant from the Ministry of Education, Science and Culture Mecklenburg-Vorpommern, Germany to H.M. Freese. For technical help, we thank M. Hummerick. We thank the Department of Medical Microbiology and Hospital Hygiene, University Rostock, Germany, for the opportunity to use their GelCompareII software and Dr. Bastrop for sequencing the bacterial strains, as well as Dr. Buck (Max-Planck-Institute for Marine Microbiology) for prelimi-

nary identification of the Actinobacteria. We thank the anonymous referees for their valuable and detailed comments.

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