Phylogenetic Characterization of Phosphatase-Expressing Bacterial Communities in Baltic Sea Sediments

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Phosphate release from sediments hampers the remediation of aquatic systems from a eutrophic state. Microbial phosphatases in sediments release phosphorus during organic matter degradation. Despite the important role of phosphatase-expressing bacteria, the identity of these bacteria in sediments is largely unknown. We herein present a culture-independent method to phylogenetically characterize phosphatase-expressing bacteria in sediments. We labeled whole-cell extracts of Baltic Sea sediments with an artificial phosphatase substrate and sorted phosphatase-expressing cells with a flow cytometer. Their phylogenetic affiliation was determined by Denaturing Gradient Gel Electrophoresis. The phosphatase-expressing bacterial community coarsely reflected the whole-cell bacterial community, with a similar dominance of Alphaproteobacteria.

Key words: phosphatase; phosphorus biogeochemistry, eutrophication, flow cytometer, ELF labeling

Benthic phosphorus (P) fluxes are an important factor that determines the availability of P for primary producers in the water layer. Whether P is released or retained in sediments is largely controlled by the activity of benthic prokaryotes (6). Therefore, a full understanding of benthic P cycling is needed in order to mitigate the hypoxic conditions caused by anthropogenic influences in many aquatic systems.

Since the majority of P reaches the sediment in the form of organic matter (14), the remineralization of P is the first step potentially leading to P release to the water column. The liberation of phosphate from organic matter is catalyzed by phosphatases. Although this group of enzymes is expressed by many prokaryotes (7), the prokaryotic groups that express phosphatases in sediments have not yet been identified. Phosphatase-expressing benthic microorganisms have previously been characterized with culture-dependent techniques (e.g., 2, 5). However, these techniques may introduce a bias towards organisms that can adapt easily to the culturing conditions (16), and, thus, may not accurately reflect the phosphatase-expressing organisms in the original sediment.

We herein introduced a culture-independent method for determining the phylogenetic affiliation of phosphatase-expressing bacteria in sediments. We previously performed slurry incubations of Baltic Sea sediments, and showed that phosphatases were likely used to relieve the limitation of microbial activity by utilizable carbon (17). In the present study, we labeled whole-cell extracts of these sediment incubations with an artificial substrate for phosphatase and sorted labeled cells using a flow cytometer. We used a PCR-DGGE (PCR-Denaturing Gradient Gel Electrophoresis) analysis targeting bacterial 16S rRNA genes to determine the phylogenetic affiliation of the sorted cells. The results from this study were then compared to the non-sorted whole-cell bacterial community composition of the incubations (which will be referred to as “non-sorted whole-cell fraction”) determined in a previous study, which focused on the impact of redox conditions on the bacterial community structure of Baltic Sea sediments (18). The present study presents a phylogenetic characterization of phosphatase-expressing bacteria in Baltic Sea sediments.

The sediments used in this study were sampled at four stations in the Baltic Sea (LF1, LF1.5, LF3, and LF5) and were incubated for approximately 80 d at 5.2°C. A triplicate full factorial design was used, in which slurries were either incubated under oxic or anoxic conditions, and with or without amendments with glucose, ammonium, and phosphate (“CNP” and “control”, respectively). After being incubated, whole-cells were separated from the slurries by blending and subsequent density centrifugation. The whole-cell fraction was stained with ELF (ELF®97 Endogenous Phosphatase Detection Kit; an artificial substrate for phosphatase; Molecular Probes, Eugene, OR, USA). ELF-labeled cells were sorted from the whole-cell fraction with a modular flow cytometer/cell sorter. DNA was extracted from the sorted cells by three cycles of snap-freezing in liquid nitrogen and subsequent thawing, as described in Steenbergh et al. (18). The extracts were dialyzed using membrane filters (Millipore MF 0.025 μm VSWP; Millipore, Darmstadt, Germany) to lower the salt content of the samples. PCR targeting 16S rRNA genes, DGGE, sequencing, and a phylogenetic analysis were performed as described in (18). A full description of these methods is given in the supplemental material.

A molecular analysis of phosphatase-expressing cells that were sorted with a flow cytometer resulted in detectable DGGE bands in more than 80% of the samples. The 31 samples that gave a PCR product of the correct size resulted in a total of 85 re-amplifiable DGGE bands. Although a single
bacterium can theoretically produce a detectable product on a
DGGE gel with the PCR protocol used, the effective detection
limit may be increased by several factors. For example, we
chose to use a DNA extraction protocol based on
snap-freezing and thawing, which is not the most efficient
method for lysing bacterial cells, but helps to prevent contami
nation of the samples. Suboptimal PCR amplification effi
ciency due to inhibition by co-extracted compounds may also
contribute to a higher detection limit (20), and a PCR-
DGGE analysis of complex communities only represents the
most abundant community members (8). Taken togeth-
er, an average of 2.7 bands per positive sample was likely
an underestimation of the actual number of bacterial phyla
types expressing phosphatases in the sediment samples. The
sequences, BLASTN results (1), SILVA (11), and RDP
(Ribosomal Database Project) classifier (21) results are given
in supplementary Table S1. Of 2 out of the 85 bands, the
closest match in BLASTN was related to bacteria found on
the skin of humans (see supplementary Table S1). However,
the possibility that the DGGE bands originated from benthic
bacteria with identical sequences cannot be excluded.

Abundance of Gram-positive and Gram-negative phosphatase-
expressing bacteria: The majority of the 85 DGGE bands
were affiliated to Gram-negative Proteobacteria (70%) and
Bacteroidetes (7%; Fig. 1 Panel a). Gram-positive bacteria
accounted for 12% of the DGGE bands and were represented by
Actinobacteria (8%) and Firmicutes (4%). In the non-
sorted whole-cell fraction that was used for flow-cytometric
sorting, Gram-positive bacteria accounted for only 5% of the
community (adapted from 18). In two studies using culture-
dependent methods, a high abundance of Gram-positive phosphatase-expressing bacteria was detected in aquaculture
ponds (64%; 2) and in sediment from the Arabian oxygen
minimum zone (~45%; 5). In contrast, a culture-independent
study of alkaline phosphatase sequences of near-surface
planktonic bacteria collected during the Global Ocean Survey
(GOS; 7) yielded hardly any phosphatase-coding gene se-
quences of Gram-positive bacteria, although Gram-positive
bacteria were abundant in the GOS samples on the basis of
16S sequences (>12%, 13). This difference in the abundance
of Gram-positive phosphatase-expressing bacteria between
studies may be attributed to differences in the detection
methods used, combined with phosphatase enzymes being
expressed at different locations within or outside the cells
(4, 7). In Gram-negative bacteria, phosphatases are present in
the periplasmic space or in the outer membrane of the cell
wall (4). Due to the lack of a periplasmic space in Gram-
positive bacteria, a higher percentage of phosphatases
expressed by these bacteria may be extracellular. The method
used in the present study labeled bacteria that expressed
phosphatases as ectocellular enzymes (i.e. attached to the
outer cell surface or present in the periplasmic space), but not
as exoenzymes. In contrast, both types of phosphatase
enzymes can be detected using the culture-dependent tech
iques of the two studies cited above (2, 5), which explains
the high abundance of Gram-positive phosphatase-expressing
bacteria in these studies. It is currently unclear why few
phosphatase-encoding sequences of Gram-positive bacteria
were detected in the GOS samples; it may have been due to
poor homology between Gram-positive and -negative phos-
phatase enzymes.

Class level abundance: Although the number of DGGE
bands per sample was lower in the phosphatase-expressing
fractions than in the whole-cell fraction before sorting (aver-
age of 2.7 vs 9.8 bands per sample, respectively), the relative
abundance of bacterial classes was similar for these two
sample fractions (Fig. 1; 18). The majority of the DGGE
bands of phosphatase-expressing bacteria were affiliated to
the Alphaproteobacteria (35%). Betaproteobacteria were not
detected in the non-sorted whole-cell fraction, but accounted
for 5% of the phosphatase-expressing bacterial DGGE bands.
Betaproteobacteria in the GOS samples were also repre
sented more on the basis of phosphatase sequences than
on the basis of 16S sequences (approximately 7% compared
to 1.7%, respectively; 7, 13). This result suggested that
Betaproteobacteria made an above average contribution to P
liberation. All betaproteobacterial sequences detected in the
present study were affiliated to the Burkholderiales, and were
only present in the CNP-amended incubations. Burkholderiales
are generally not very abundant in surface sediments, but
have been identified as dominant members of bacterial
communities in deep sediments (10). The members of this
order exhibit high metabolic versatility (e.g., 23), which
benefits from the expression of phosphatases. Furthermore,
sequences affiliated to the Actinobacteria, Spingobacteria,
and Thermoleophilia classes were absent from the non-sorted
whole-cell fraction, but accounted for 5% (Actinobacteria)
and 3% (Spingobacteria and Thermoleophilia) of the DGGE
bands in the phosphatase-expressing fraction. This result
suggested that, similar to Betaproteobacteria, the contribu-
tion of these bacterial classes to P cycling in these sediments
was higher than was expected based on their abundance.

Although Gammaproteobacteria may also be highly abun-
dant in marine sediments (10, 24), only 5% of the sequences
of the phosphatase-expressing bacteria were affiliated to
this class. The low number of phosphatase-expressing
Gammaproteobacteria may have been due to the low abun-
dance of Gammaproteobacteria in the non-sorted whole-cell
fraction (Fig. 1; 18), and not to the low prevalence of
phosphatase expression among Gammaproteobacteria. In the
GOS samples, Gammaproteobacteria made a greater contri-
bution to the alkaline phosphatase gene pool than was
expected on basis of their abundance in the 16S RNA gene
pool, whereas the reverse was true for Alphaproteobacteria
(7). This was attributed to the higher occurrence of uptake
systems for glycerol phosphate (upg) in Alphaproteobacteria,
which reduced the need for ecto- or extracellular phosphatase
enzymes to fulfill their P demands. As the abundance of
phosphatase-expressing Alpha- and Gammaproteobacteria in
the present study corresponded with their abundance in the
whole-cell fraction, this difference in physiology between the
two classes was not detectable at the phosphatase expression
level for these Baltic Sea sediments.

Deltaproteobacteria made up 2% of the non-sorted whole-
cell bacterial community (18), but accounted for 7% of the
DGGE bands of the phosphatase-expressing bacteria. Of
these six phosphatase-expressing Deltaproteobacteria, five
were the most closely affiliated to the Desulfobacteriales.
Although sulfate-reducing bacteria are predominantly active
under anoxic conditions, their abundance in the phospha-
The abundance of phosphatase-expressing Epsilonproteobacteria was higher in the anoxic (16%, n=54) than in the oxic (2%, n=31) incubations, which was expected because Epsilonproteobacteria often occur in sulfidic habitats (3); however, this difference was not significant (t-test, p=0.10).

In summary, we herein linked the phosphatase activity of Baltic Sea sediments to bacterial phylogenetic taxa using a PCR-DGGE analysis of sorted ELF-labeled cells. The phosphatase-expressing community in these sediments coarsely reflected the non-sorted whole-cell bacterial community; however, a number of bacterial classes were only detected in the phosphatase-expressing sample fractions. The diversity in phosphatase enzymes precludes the use of one single method to phylogenetically characterize all phosphatase-expressing prokaryotes in a community. The method described in this study provided a culture-independent way to characterize bacteria expressing phosphatases as ectoenzymes. More detailed phylogenetic information can be gained using this method when more recently developed sequencing techniques are used instead of PCR-DGGE-based sequencing.

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