Distribution of proteobacteria in a freshwater water body influenced by seawater on Cape Cod

Katharina Porsch, Geomicrobiology Group, Centre for Applied Geosciences (ZAG),University of Tübingen, Germany

Individual Project, MBL Summer Course Microbial Diversity 2007, Woods Hole

Abstract

Alpha, beta, gamma and delta proteobacteria are found in all kinds of habitats including different aqueous environments. Although all four classes are present in both freshwater and seawater, the abundance of the four classes in these two water types differ. Alpha and gamma proteobacteria are dominant in seawater whereas beta proteobacteria are dominant in freshwater. Delta proteobacteria are more abundant in marine sediments than in the free water or freshwater sediment. In this study the distribution of the different proteobacteria classes in a shallow lake with increasing salinity towards the bottom of the lake was determined at three different water depths. A decrease in the beta proteobacteria and an increase in the alpha and gamma proteobacteria with depth could not be observed. Instead the seawater-preferring alpha and gamma proteobacteria were more abundant at all three depths. Clone library results indicate that some species of proteobacteria might be present in only one water depth, whereas others can be found in two or all three depths.

Introduction

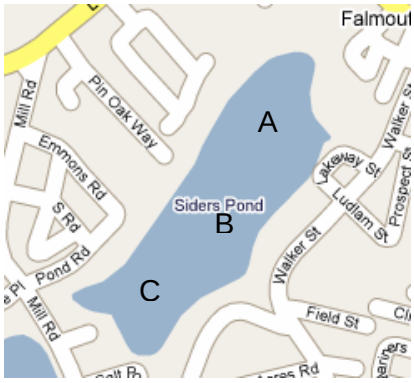
The proteobacteria are the largest group within the domain bacteria. In 2002 this group contained more than 460 genera and more than 1600 species. The proteobacteria are divided into 5 classes: the alpha-, beta-, delta-, epsilon- and gamma-proteobacteria. They are highly diverse according to their morphology and physiology (Kersters, 2006). Proteobacteria can be found in all kind of habitats, such as soil, freshwater, seawater, sediments and aquifers. Although proteobacteria can be found in fresh- and seawater, there are differences between the distribution of proteobacteria classes in these environments (Nold and Zwart, 1998). Alpha and gamma-proteobacteria can be found in freshwater and seawater, but are more abundant in seawater. Beta-proteobacteria are mainly found in freshwater. But nitrifying beta-proteobacteria can also occur in marine environments. Delta-proteobacteria are mainly found in marine sediments and rarely in oxygenated water columns.
It can be assumed that most of the freshwater bodies near the coast of Cape Cod are influenced by sea water. There are freshwater bodies which are directly connected to the sea by an outflow (e.g. Trunk River). An influence by mixing of groundwater and seawater at the bottom of freshwater bodies might be also possible, as well as occasional input of seawater during stormy weather. Since freshwater has a lower density than seawater, both water types should be separated in the water column, as long as there are no mixing processes like in estuaries or turnover events in lakes in spring and fall. Siders Pond in Falmouth on Cape Cod is such a shallow freshwater lake influenced by seawater, in which the salinity increases with water depth. The aim of this study was to elucidate if the distribution of the different proteobacteria classes follows the general observed distribution patterns dependent on the salt concentration as described above. Three samples from Siders Pond of different depth with different salinity were analyzed by CARD-FISH and a clone library was performed. As a

control CARD-FISH was also done for a water sample from School Street Marsh as an assumed freshwater body and for a seawater sample from Garbage Beach.

Methods

Sampling

Siders Pond was chosen as a freshwater lake influenced by saltwater. Siders Pond is located behind the town hall in Falmouth, Cape Cod, Massachusetts. The lake is ~660m long, ~200 m wide and ~10m depth. Samples were taken at sampling point C (Map 1) from 0.5m, 6m and 10m deep. Corresponding to their depth the samples are named in the following: C0.5m, C6m and C10m. The water from 10m smelled of sulfide. A water profile including dissolved oxygen, temperature, salinity and pH was taken with a YSI multiprobe (Figure1).



Map 1: Sampling points A, B and C in Siders Pond, Falmouth, Cape Cod, Massachussetts.

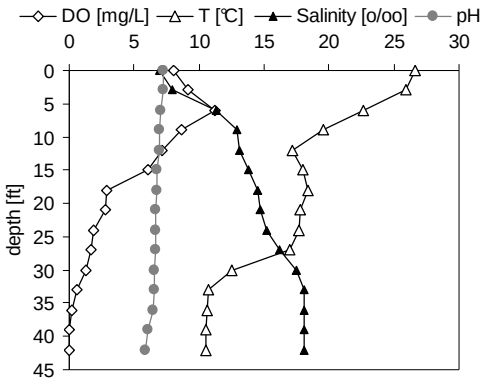


Figure1: Water profile in Siders Pond at sampling point C

Garbage beach (GB), Woods Hole, Cape Cod, Massachusetts was sampled at the end of the MBL pier at 3m depth. In School Street Marsh (SSM) the surface water was sampled.
The salinity of all water samples was determined in the lab with a refractometer.

CARD-FISH

CARD-FISH was performed for all water samples. In order to fix the microbial cells formaldehyde was added at a final concentration of 1% to 50ml of each of those water samples in the lab immediately after sampling. The treated samples were stored for <24h at 4℃. Different amount of fixed samples were filtere d on a membrane filter (0.2µm pore size, white polycarbonate, 47mm diameter). After air drying, a piece of each filter was stained with DAPI (4',6'-Diamidino-2'-phenylindole). 30µl of 1µg/ml DAPI was added on each filter piece and incubated for 5 min in the dark. Subsequently the pieces were washed for 1 min in autoclaved MiliQ-water and 1 min in 100% ethanol in the dark. The pieces were fixed on slides with 2µl of mounting solution, consisting of Citiflour:Vectashield 4:1. Filters with an appropriate number of cells were chosen for CARD-FISH. The following probes were used: EUBI-III for bacteria, NON338 as unspecific probe, Alf986 for alpha-proteobacteria, Bet42 with the competitor probe Gam42 for beta-proteobacteria, and Gam42 with the competitor probe Bet42 for gamma-proteobacteria. CARD-FISH was performed as described in chapter 10 of the course material (Microbial Diversity Course 2007). Subsequently a DAPI stain of the cells was performed. The DAPI stain was visible in the DAPI channel and the CARD-FISH signals in the FITC channel of the microscope. DAPI stained cells and CARD-FISH

signals were counted at ten different spots of the filter and the percentage of CARD-FISH signals in comparison to the DAPI counts was calculated.

Clone library

Clone libraries were created for the three water samples from Ciders Pond by amplifying parts of the 16S rRNA gene with reverse primer specific for alpha and beta proteobacteria.

DNA-Extraction

50-100ml of the 3 water samples from Siders Pond were filtered on a membrane filter (0.2µm pore size, white polycarbonate, 47mm diameter) in the lab directly after sampling in order to avoid a bottle effect (Ferguson et al, 1984). DNA was extracted from the filter with the MoBio Soil DNA Extraction Kit. Half of each filter was cut in very small pieces and added to the beat containing tube of the kit. Only the cell containing part of the half filter was used. The DNA was extracted as described in the kit instructions. Instead of vortexing the bead-containing tube for 10min, a bead beater was used for 3min. The amount of extracted DNA was determined by nanodrop.

PCR

During PCR a part of the 16S rRNA gene was amplified.
For the PCR the universal forward Primer 8F and the reverse primer 681R specific for alpha proteobacteria and the reverse primer 680R specific for beta proteobacteria, respectively, were used (Table1). For the alpha-proteobacteria the 25µl PCR-reaction mix contained 12.5µl Promega master mix 2x, 2µl 8F primer (end concentration 15 pmol), 2µl 681R primer (end concentration 15 pmol), 6.5µl nuclease free water, and 2µl DNA template. For the beta-proteobacteria the 25µl PCR mix contained 12.5µl Promega master mix 2x, 2µl 8F primer (end concentration 15 pmol), 4µl 680R primer (end concentration 30 pmol), 4.5µl nuclease free water, and 2µl DNA template. For the 680R primer a higher concentration was used to increase the amount of PCR product. The low amounts of PCR product might be due to the fact that the 680R primer contains a degenerate pyrimidine.

Table1: Primers used for the PCR assays

Primer name	Target Group	Primer sequence (5'-3')
8F	Bacteria, universal	AGAGTTTGATCMTGG
681R	Alpha Proteobacteria	ATTTCACCTCTACACT
680R	Beta Proteobacteria	TCACTGCTACACGYG

The PCR thermocycler profile was as follows: 5 min at 95℃ for the initial denaturation, 25 or 30 cycles of denaturation at 95℃ for 30 sec, annealing of primers at 46℃ or 50℃ for 30 sec, extension at 72℃ for 45 sec, followed by a final extension at 72℃ for 5 min and 4℃ holding temperature at the end. The number of cycles and the annealing temperature varied according to the used primer (see Table2). For the beta Primer a positive negative control at a high number of cycles, but weak products at low number of cycles could be observed. 5µl of the PCR products were checked on a 1% ethidumbromide containing agarose gel.

Table2: Annealing temperature and number cycles of PCR for clone library and colony PCR

		alpha reverse primer	beta reverse primer
Clone library	Annealing temperature	50℃	46℃
	Number of cycles	30	25
Colony PCR	Annealing temperature	50℃	46℃
	Number of cycles	30	25

As a positive control for the alpha proteobacteria, colonies of methylotrophs cultivated during the course were used, and for the beta primer, colonies of violacein producing bacteria. For both colonies a colony PCR were performed as described in chapter 11 of the course material (Microbial Diversity Course 2007). The PCR was performed as described above (see also Table2).

PCR products both with the alpha reverse and the beta reverse primer from the 10m sample produced a double band on the agarose gel. The lower bands had the size of the target fragment. The PCR products were obtained during the optimization of the primers. In this case the annealing temperature of the alpha primer was 46℃ and 30 cycles were performed. The PCR product with the beta primer derived from a gradient PCR with annealing temperatures of 46℃, 47.7℃ and 50℃ with 30 cycle s. The latter three PCR products were pooled. A 1% agarose gel electrophoresis was performed with the PCR products. The higher and lower bands were cut out and DNA extracted with a DNA Gel Extraction Kit from Qiagen.

Cloning

For cloning the pCR®2.1-TOPO vector from Invitrogen was used. The 6µl vector mix contained 1µl vector, 1µl corresponding salt solution, 0-3µl water and 1-4µl PCR product (see Table3).

Table3: Amount of water and PCR product added to the vector mix

	Alpha Primer		Beta Primer	
Sample	Water [µl]	PCR product [µl]	Water [µl]	PCR product [µl]
C 0.5m	2	2	2	2
C 6 m	2	2	2	2
C 10m	1	3	4	0
C 10m high band	1	3	2	2

The transformation reaction was performed as described in chapter 12 of the course material (Microbial Diversity Course 2007). 40 µl X-Gal and 40 µl IPTG were spread on the LB/Kan plates 1h before the transformed cells were spread. The plates were incubated at 37℃ over night. The insert in the vector could be detected by blue/white screening of the clones.

Results and Discussion

The five water samples cover a salinity range from 0.5% to 3.5%. The water samples of Siders Pond vary between 0.9% and 1.9%. Freshwater has a salinity of <0.5 % and seawater on average of 3.5% (http://en.wikipedia.org/wiki/Salinity). With exception of the Garbage Beach sample all water samples were brackish water.

Table4: Salinity of the different water samples determined with a refractometer

Sample	Salinity [%]
School Street Marsh	0.5
Siders Pond C0.5m	0.9
Siders Pond C6m	1.6
Siders Pond C10m	1.9
Garbage Beach	3.5

No fluorescent signals were visible in the FITC channel on filter pieces hybridized with unspecific NON338 probe. The EUBI-III probe bound to more than 70% of the cells in C0.5m, C6m and GB (Figure2). These results correspond with previous results obtained during the course, where different seawater samples were analyzed by CARD-FISH. EUBI-III signals are usually smaller than 100%, because on one hand the probe does not bind to the DNA of all bacteria, and on the other hand present archaea are also not detect by this probe. In the samples from SSM and C10m the CARD-FISH signals with the EUBI-III probe represent only 35-36% of the DAPI counts. In these two samples very small cells were visible in the DAPI channel but not in the FITC channel. It is possible that these were not cells but phages (Dagmar Woebken, personal communication). In this case the DAPI counts were too high and thereby the percentage of the CARD-FISH signals too low. This means that abundance of alpha, beta and gamma proteobacteria are probably underestimated in the SSM and C6m sample. Despite the underestimation of the CARD-FISH signals in the SSM sample, it can be concluded that the beta proteobacteria are more abundant in this sample that the alpha and gamma proteobacteria (Figure2). Although SSM is not a real freshwater body the results reflect the expected distribution of proteobacteria in a freshwater body as described in the introduction. The same is true for the seawater sample from BG in which the alpha and gamma proteobacteria are more abundant than the beta proteobacteria.
However, in Siders Pond the proteobacteria do not follow this distribution pattern. It was expected to see a decrease of beta proteobacteria and an increase of alpha and gamma proteobacteria with depth, because of the increasing salinity with depth. Since the abundance of bacteria is underestimated in the C10m sample the counts for the proteobacteria cannot be compared directly with the counts for the C0.5m and C6m sample. However, in all three samples the alpha proteobacteria, and in C0.5m and C6m the gamma proteobacteria, are more abundant than the beta proteobacteria. This lets us assume that a salinity of 0.9% in the C0.5m sample is already an inhibiting factor for the beta proteobacteria, and therefore a distribution as in seawater can be found in all three samples. In C0.5m and C6m the alpha and gamma proteobacteria together are 19% of the total cells, but with increasing depth there is a shift from the alpha proteobacteria to the gamma proteobacteria. This probably is due to the fact that not only the salinity, but also other conditions like light penetration and oxygen, play a role in bacterial distributions.

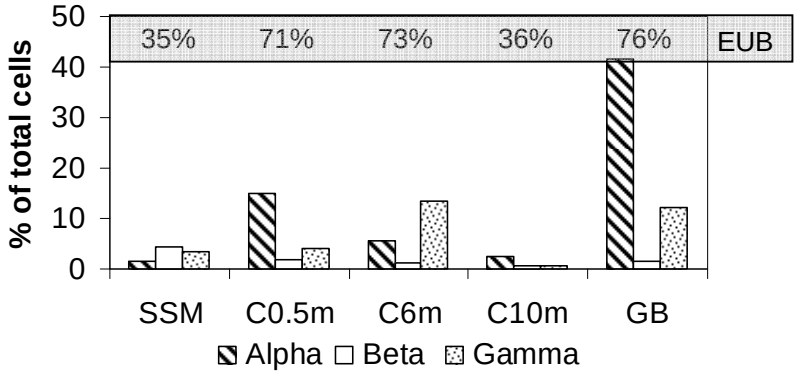
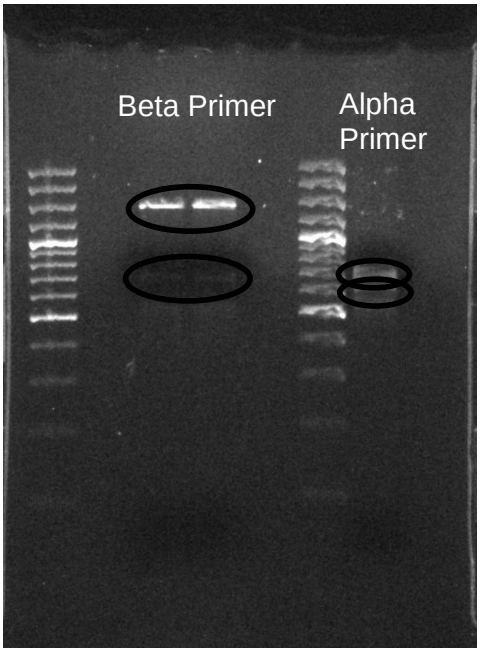


Figure2: Relative abundance of alpha, beta and gamma proteobacteria in different water samples determined by CARD-FISH. The total cell number was determined by counting DAPI stained cells at the same spot of the filter than the CARD-FISH signals. Relative abundance of bacteria determined by a universal bacteria CARD-FISH probe is given in % on top of the diagram.

A clone library was generated for the three samples from Ciders Pond. C10m produced a double band during the PCR both with the alpha and beta reverse primer (Picture 1). The fragments of lower bands had the same size as the fragments of C0.5m and C6m. The higher and lower bands of the C10m sample were cut out and the DNA extracted from the gel. The DNA from all four bands (alpha, beta primer, higher and lower band) was used for cloning.

The alpha and beta reverse primers were not specific for the target groups. Some of the clones obtained from PCR products with the alpha reverse primer fell also into the delta proteobacteria. The beta reverse primer also targeted 16S rRNA genes from organism of the gamma proteobacteria and Acidobacterium. A probe match analysis on the ribosomal database project webpage (http://rdp.cme.msu.edu/) reveals that the used alpha reverse primer targets 108 sequences of 16S rRNA genes outside of the proteobacteria, in the groups of Chloroflexi, Cyanobacteria, Firmicutes, Actionbacteria, Planctomycetes, Acidobateria, Bateriodetes and Gemmatimonadetes, as well as 740 sequences in Fusobacteria and 181 sequences of unidentified bacteria. In the group of proteobacteria the alpha reverse primer targets 32 sequences of the beta, 22 of the gamma, 457 of the delta and 606 sequences of unidentified proteobacteria. Within the alpha proteobacteria 64.6% of the sequences are target. The beta reverse primer targets outside the proteobacteria 39 sequences of 16S rRNA genes in Chloroflexi, Nirospira, Cyanobacteria, Firmicutes, Actionbacteria, Bacterioidetes, Verrucomicrobia and unclassified bacteria. Within the proteobacteria group 11 sequences of the alpha, 69 of the gamma, 4 of the delta, 1 of the epsilon and 49 sequences of unclassified proteobacteria are target. The primer binds to 60.0% of the sequences of the beta proteobacteria. Although the alpha reverse primer seems to be more unspecific than the beta primer, in this study more mismatches were obtained with the beta primer.



Picture1: Agarose gel with PCR product of DNA from C10m obtained with alpha and beta primer. Bands marked with black circles were cut out, DNA extracted from the gel and used for clone library.

Table5: Distribution of sequences found in clone libraries in target group

Reverse Primer	Clones	Clones per sample			
		C0.5m	C6m	C10m	C10m h[a]
Alpha	Alpha Proteo	22	21	18	0
	Delta Proteo	0	1	0	7
Beta	Beta Proteo	17	7	18	1
	Gamma Proteo	3	13	0	0
	Acidobacterium	0	0	2	0

a – clones from the higher double band produced by C10m during PCR

As positive control for the alpha reverse primer methylotrophic colonies cultured during the course were used. A colony PCR revealed that they were methylobacteria. The positive control for the beta reverse primer was violacein-producing colonies, assuming that these were Chromobacterium violaceum. However, the sequence obtained by colony PCR from one of the colonies felt into a group of Oxalobaceter and uncultured beta proteobacteria (Figure3).

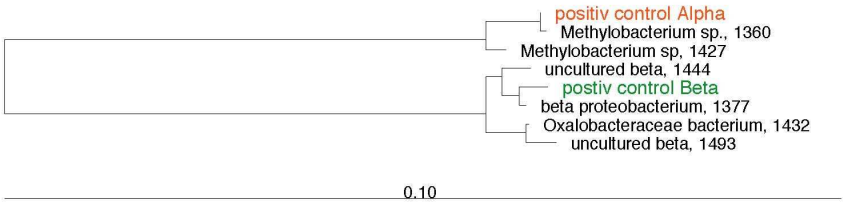


Figure3: Maximum likelihood tree of the colonies used as positive controls for the alpha and beta primer and their nearest neighbors.

From each sample clones were obtained that did not cluster together with other clones. In the case that clones clustered together, they formed a group with clones from the same water depth, with clones from the next water depth (clones from C0.5m with clones from C6m, and clones from C6m with C10m), or clones from all three depths. Although the clone library is not complete, the results let us assume that because of the different environmental conditions in the three different water depths, each depth contains proteobacteria that are only present in one water depth . But there are also proteobacteria that can live in two or in all three depths despite the different conditions. In order to get a better overview of the distribution and abundance of different subgroups of the proteobacteria, CARD-FISH with probes for those subgroups could be performed.

All sequences of the higher band of C10m amplified with the alpha primer felt into the group of delta proteobacteria. Six of the seven clones were closely related to Desulfobacterium anilini, a sulfate reducing microorganism. The origin of the salt water in Siders Pond is unknown, but it can be assumed that it originated from the sea. With an input of seawater, the sulfate concentration would increase. In the water from 10m deep, sulfide could be smelled. It is possible that the sulfide is derived from sulfate reduction.

Conclusion

The distribution of proteobacteria in Siders Pond, a freshwater lake influenced by seawater, was determined at three different water depths. Clones belonging to the alpha and beta proteobacteria could be found in all three water depth. However, the abundance of proteobacteria determined by CARD-FISH revealed that the beta proteobacteria were less abundant than alpha and gamma proteobacteria in all three depths. A salinity of 0.9% seems to be already too high for beta proteobacteria. Clone library results indicate that some species of proteobacteria might be present in only one water depths, whereas other can be found in two or all three depths.

Literature:

Course material of the microbial diversity summer course at the MBL 2007

http://en.wikipedia.org/wiki/Salinity

http://rdp.cme.msu.edu/

Kersters,K; Gillis,M; Swings,J; Vandamme,P; Stackebrndt,E (2006): Introduction to the Proteobacteria. The Prokaryotes, Springer, New York.

Nold,SC; Zwart,G (1998): Patterns and governing forces in aquatic microbial communities. Aquat. Ecol. 32:17-35 (Review)