Insights into Variability of Actinorhodopsin Genes of the LG1 Cluster in Two Different Freshwater Habitats

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Abstract

Actinorhodopsins (ActRs) are recently discovered proteorhodopsins present in Actinobacteria, enabling them to adapt to a wider spectrum of environmental conditions. Frequently, a large fraction of freshwater bacterioplankton belongs to the acl lineage of Actinobacteria and codes the LG1 type of ActRs. In this paper we studied the genotype variability of the LG1 ActRs. We have constructed two clone libraries originating from two environmentally different habitats located in Central Europe; the large alkaline lake Mondsee (Austria) and the small humic reservoir Jiřická (the Czech Republic). The 75 yielded clones were phylogenetically analyzed together with all ActR sequences currently available in public databases. Altogether 156 sequences were analyzed and 13 clusters of ActRs were distinguished. Newly obtained clones are distributed over all three LG1 subgroups - LG1-A, B and C. Eighty percent of the sequences belonged to the acl lineage (LG1-A ActR gene bearers) further divided into LG1-A1 and LG1-A2 subgroups. Interestingly, the two habitats markedly differed in genotype composition with no identical sequence found in both samples of clones. Moreover, Jiřická reservoir contained three so far not reported clusters, one of them LG1-C related, presenting thus completely new, so far undescribed, genotypes of Actinobacteria in freshwaters.

Introduction

Members of the phylum Actinobacteria are abundant, cosmopolitan and extremely successful inhabitants of freshwater ecosystems [1,2,3]. It is now widely accepted that the most abundant freshwater actinobacterial group is usually represented by the acl lineage [3]. acl bacteria are found widespread in diverse types of freshwater environments [4,5]. Moreover, they might be capable of both carbon fixing and rhodopsin-based phototrophy [6]. A draft genome sequence of a non-cultured member of the acl lineage has been published recently [7], however there are still pure cultures missing. It has been confirmed by the genome sequence, that the GC content of acl 1 is lower than usually found in Actinobacteria.

It has recently been discovered that Actinobacteria possess variants of rhodopsin genes, so-called actinorhodopsins (ActRs, [8]). Several recent studies performed globally highlighted the broad taxonomic and ecological distribution of microbial rhodopsin genes in diverse aquatic environments [8-12] suggesting that they might be important in the adaptation of these microbes to life on earth’s surface. The similarity of ActRs isolated from lakes in different parts of the world suggests that these genes are dispersed globally and that they may encode important functional capabilities enabling successful competition in a wide range of freshwater environments. However, their variability in freshwater habitats in Central Europe has so far remained rather undescribed.

Actinobacteria from freshwater habitats comprise three phylogenetic groups of actinorhodopsin genes named LG1, LG2 and PCL1 [8]. These three clades were also reported from estuaries and hypersaline lagoons, but are almost completely absent from marine environment and cluster outside of the major proteorhodopsin clade. The LG1 group, comprising mostly freshwater sequences, can be split into three subgroups, LG1-A, B and C [13]. The genes of the LG1-A group are encoded by the acl lineage [13,14] whereas genes of the LG1-B group of ActRs are carried by the Luna lineage of Actinobacteria. So far undescribed LG1-C group is represented solely by clone sequences [13] and no further information is currently available.

Previous studies on ActRs were largely focused on describing the overall diversity in all three gene clusters and studied places mainly outside Europe. As our goal was to deepen the knowledge on acl actinobacterial lineage, in this study we exclusively focused on the freshwater LG1 cluster and intended to describe the actinorhodopsin gene diversity solely within this cluster. Two new clone libraries originating from two different freshwater habitats and all actinorhodopsin sequences available in public databases were analyzed to reach this goal. More specifically, the aim of our study was to describe genetic variability of the ActR gene in two distinct freshwater habitats in Europe and to search for new ActR variants and clusters. We selected two habitats known for their high actinobacterial abundance, but unknown actinobacterial diversity, as two examples of different habitat types – a large alkaline lake and a small humic reservoir.
Figure 1. Maximum Likelihood tree of LG1 actinorhodopsin nucleotide sequences. ML best tree of LG1 actinorhodopsin nucleotide sequences (291 bp). Rooted with LG2 sequences (GS020_39, GS012_40, GS012_3). Bootstrap values from Maximum likelihood/Bayesian inference/Neighbor joining methods are depicted. Clones from Lake Mondsee are highlighted in blue and clones from Jílové reservoir in red. Star - reference sequence (16S rRNA gene sequence is available). Numbers on the side symbolize cluster numbers, color the group affiliation (yellow = LG1-A, rosa = LG1-C, green = LG1-B). Bold frames – new clusters.

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Figure 2. Maximum Likelihood tree of LG1 actinorhodopsin amino acid sequences. ML best tree of LG1 actinorhodopsin amino acid sequences (97 aa). Rooted with LG2 sequences (GS020_39, GS012_40, GS012_3). Bootstrap values from Maximum likelihood/Bayesian inference/Neighbor joining methods are depicted. Clones from Lake Mondsee are highlighted in blue and clones from Jilická reservoir in red.
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Actinorhodopsin Genes of the LG1 Cluster

Table 1. Amino acid sequence similarity.

| LG1 group | new clones | all sequences |
|-----------|------------|---------------|
| A1        | 0.083      | 91.70%        |
| A2        | 0.112      | 88.80%        |
| A         | 0.136      | 86.40%        |
| B         | 0.237      | 76.30%        |
| C         | 0.005      | 99.50%        |

Amino acid sequence similarity (97 aa) calculated as mean distance for the presented clones and for all 156 sequences in the analyses.

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Materials and Methods

No specific permissions were required for sampling of both habitats, since the habitats are not privately owned and are freely accessible.

We compared two habitats. First habitat was the Jiřická reservoir (also known as Pohořský pond; area 2.5 ha; max depth 8 m; pH 7.3) a humic dammed reservoir located in Nové Hrady area in the Czech Republic (48°36’56.88”N, 14°40’34.48”E). The second habitat was previously well described Lake Mondsee, an oligo-mesotrophic deep submontane lake in a prealpine region (area 14.2 km²; max depth 68 m; pH 8.5) located in the Salzkammergut area in Austria (47°49’N 13°23’E). The Jiřicka reservoir (Ježbera, pers. comm.) as well as Lake Mondsee [15] are known to host abundant populations of Actinobacteria. Both sampling campaigns were performed in July 2011. Water samples were taken from the water surface. 500 mL of water were filtered using a 0.2 μm membrane filters (Poretics) and stored at −20°C until further processed. DNA was isolated from the filters using a phenol-chloroform extraction protocol described in detail in [16].

CARD-FISH (Catalyzed Reporter Deposition Fluorescence in situ Hybridization) analysis to follow Actinobacteria. Both sampling campaigns were performed in July 2011. Water samples were taken from the water surface. 500 mL of water were filtered through 0.2 μm membrane filters (Poretics) and stored at −20°C until further processed. DNA was isolated from the filters using a phenol-chloroform extraction protocol described in detail in [16].

CARD-FISH (Catalyzed Reporter Deposition Fluorescence in situ Hybridization) analysis to follow Actinobacteria abundance was performed using the protocol of [17], employing the acf specific, horseradish-peroxidase labeled probe Acf1-6 [13] (5’-AAC GGG TTA GCT GCG TCG CA-3’). Samples from both habitats were counted in triplicates and the average value ±SD is presented. Bacteria were counted using DAPI staining as detailed in [18].

LG1 group specific PCR for the ActR gene was performed according to [15]. A combination of two forward primers 5’TAYMNTAYGTNGAYTGG-3’ and 5’MGNTAYATHGAYTGYYT-3’ and one reverse primer 5’ATNGGRTANACNCCCCA-3’ targeting the LG1 ActR clade were used. The clone library was constructed using pGEM-T Easy Vector System (Promega) with competent E. coli JM109 cells, according to the protocol supplied by the manufacturer. White colonies from the positive PCR products were sent for sequencing with M13 forward and reverse universal primers or M13 forward and 5’-MGNTAYATHGAYTGYYT-3’ primer.

Table 2. Between group mean distance (%) of LG1 actinorhodopsin sequences.

|       | A1 | A2 | B |
|-------|----|----|---|
| A2    | 75.8 |    |   |
| B     | 59  | 75.8| 59|
| C     | 64.5| 64.4| 59|

Between group mean distance (%) of LG1 actinorhodopsin sequences. Sequence lengths 291 bp.

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Table 3. GC content (%) of actinorhodopsin sequences.

|       | new clones | other (GOS, GenBank) |
|-------|------------|----------------------|
|       | average    | (min-max)             | taxa |
|       |            | (min-max)             |
| A1    | 45         | (43–47)               | 24  | 47  | (43–48) | 15 |
| A2    | 46         | (44–48)               | 36  | 47  | (44–53) | 47 |
| B     | 52         | (46–60)               | 7   | 49  | (40–54) | 13 |
| C     | 47         | (46–47)               | 8   | 65  | (65–66) | 2  |

GC content (%) of actinorhodopsin sequences in various LG1 groups. Sequence lengths 291 bp.

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Figure 3. Proportions of LG1-A, B and C actinorhodopsin genotype groups. Proportions of LG1-A, B and C actinorhodopsin genotype groups in the clone libraries of Jiřická reservoir and lake Mondsee.

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Newly described second part of the LG1-A group, the LG1-A2 representing a freshwater actinobacterium in a mixed culture [25], Candidatus Planktophila limnetica. Actinobacteria are bearers of the LG1-A actinorhodopsins. Furthermore, L06) mentioned above, clustered within the LG1-A1 group, which sequence obtained from the acI draft genome (genome AAA027-L06) and NA sequence similarity between these two sub-groups as they markedly differ in their positions in the nucleotide tree (Fig. 1) and NA sequence similarity as high as 99.7% (AA) and 98.9% (NA) similarity, respectively and clusters together with the LG1-C group. This ActR genotype represents almost one quarter of our clone library from the Jirˇicka reservoir (21%). The substantial presence of this genotype suggests its bearer represents an important freshwater Actinobacterium in the Jirˇicka reservoir. Since they show on average only 66% similarity to cluster of sequences from the GOS metagenome originating from a hypersaline lagoon (the only LG1-C representatives currently available [13]), and taking into account their tremendous GC content difference (Table 3), it makes the assignment to the LG1-C rather debatable. Both of the studied lakes differ markedly in the ActR composition (Fig. 3). Jirˇicka contained 66% of LG1-A, 13% LG1-B and 21% clones affiliated with cluster LG1-C. In contrast, no LG1-C genotype was observed in Mondsee. Mondsee comprised 95% of LG1-A and only 5% of LG1-B. ActR sequence similarity of all Jirˇicka clones was 75% (AA) and 72% (NA), and Mondsee clones 85% (AA) and 79% (NA), respectively. The Jirˇicka reservoir shared three clusters with Mondsee (Fig. 4), yet accompanied by clones from other habitats (Fig. 1 – cluster 2, 4 and 8). Three clusters out of thirteen contained exclusively Jirˇicka clones (cluster 3, 10, 13), whereas no cluster from exclusively Lake Mondsee clones was observed. All Lake Mondsee clones were always accompanied by GenBank clones from other habitats. The fact that two studied actinobacterial communities encoded such different genotypes suggests some polemic why this may happen. (i) The two actinobacterial communities are not so different but were investigated in two completely different stages of their seasonal succession. (ii) The two habitats are colonized by completely different acI groups, which diverged a long time ago. The different actR genotypes simply reflect this long-term divergence (but the genes do not differ in their function). (iii) Differences in actR genotypes reflect different functional adaptations of the genes in these two distinct habitats.

Conclusions

Our study evaluated overall LG1 actinorhodopsin gene variability and presented new genotypes and clusters. On two temperate freshwater habitats and sequences available from public
databases we demonstrate high variability of this gene and illustrate uniqueness of each place of origin. The two actinorhodopsin databases we demonstrate high variability of this gene and showed completely different ActR gene composition. We discovered three unique ActR gene clusters in Jirˇickaˇ that were so far not reported from the literature. One of them related to the LG1-C Actinobacteria, presenting completely new genotype of ActR gene inherent in freshwaters. In the two studied habitats, most of the ActR diversity is formed by LG1-A (acI) Actinobacteria, not by LG1-B (Luma) Actinobacteria. Based on newly introduced sequences we suggested to further split LG1-A in two subclusters.

Author Contributions
Conceived and designed the experiments: J. Jezberová J. Ježbera MH. Performed the experiments: J. Ježbera J. Jezberová MH. Analyzed the data: J. Ježbera MH. Contributed reagents/materials/analysis tools: J. Ježbera. Wrote the paper: J. Jezberová J. Ježbera MH.

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