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Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from freshwater fish gut microbiota

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ABSTRACT

The diversity of Actinobacteria isolated from the gut microbiota of two freshwater fish species namely *Schizothorax zarudnyi* and *Schizocypris altidorsalis* was investigated employing classical cultivation techniques, repetitive sequence-based PCR (rep-PCR), partial and full 16S rDNA sequencing followed by phylogenetic analysis. A total of 277 isolates were cultured by applying three different agar media. Based on rep-PCR profile analysis a subset of 33 strains was selected for further phylogenetic investigations, antimicrobial activity testing and diversity analysis of secondary-metabolite biosynthetic genes. The identification based on 16S rRNA gene sequencing revealed that the isolates belong to eight genera distributed among six families. At the family level, 72% of the 277 isolates belong to the family *Streptomycetaceae*. Among the non-streptomycetes group, the most dominant group could be allocated to the family of *Pseudonocardiaceae* followed by the members of *Micromonosporaceae*. Phylogenetic analysis clearly showed that many of the isolates in the genera *Streptomyces*, *Saccharomonospora*, *Micromonospora*, *Nocardiopsis*, *Arthrobacter*, *Kocuria*, *Microbacterium* and *Agromyces* formed a single and distinct cluster with the type strains. Notably, there is no report so far about the occurrence of these Actinobacteria in the microbiota of freshwater fish. Of the 33 isolates, all the strains exhibited antibacterial activity against a set of tested human and fish pathogenic bacteria. Then, to study their associated potential capacity to synthesize diverse bioactive natural products, diversity of genes associated with secondary-metabolite biosynthesis including PKS I, PKS II, NRPS, the enzyme PhzE of the phenazine pathways, the enzyme dTGD of 6-deoxyhexoses glycosylation pathway, the enzyme Halo of halogenation pathway and the enzyme CYP in polyene polyketide biosynthesis were investigated among the isolates. All the strains possess at least two types of the investigated biosynthetic genes, one-fourth of them harbours more than four. This study demonstrates the significant diversity of Actinobacteria in the fish gut microbiota and it's potential to produce biologically active compounds.

1. Introduction

Among the 18 major lineages of bacteria, the phylum Actinobacteria are one of the largest taxonomic units and its divergence from other bacterial phyla is so ancient that it is currently not possible to identify their most closely related group (Ventura et al., 2007). Actinobacteria are enormously important in human medicine, agriculture and food production, and key to this is their proven ability to interact with other organisms, an area which has largely been overlooked in the last 150 years of their study (Seipke et al., 2012). For instance, it has been reported that Actinobacteria, especially *Streptomyces* spp. can be considered as promising potential probiotics in aquaculture (Das et al., 2008). Oskay et al. (2004) also reported that the ability of actinomycetes isolated from Turkey's farming soil possess the ability to inhibit *Erwinia amylovora*, a bacterium that causes Fire Blight to apple, as well as *Agrobacterium tumefaciens*, a causal agent of Crown Gall disease. Hence, owing to their ability to produce various natural drugs, enzymes and bioactive metabolites, Actinobacteria have been paid a great attention (Jiang, 2013). Already in 1988 Demain (1988) stated that '...it is clear that actinomycetes have served and are serving us well beyond the call of duty; however actinomycetes can never relax because we shall expect more from them in the future'. This statement is still valid. In this context, the list of novel actinomycetes and products derived from their poorly explored terrestrial habitats has stimulated researches focussing on new habitats (Sibanda

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et al., 2010). So far unexplored sources of Actinobacteria may contain new gene clusters synthesizing secondary metabolites, which may be of interest to current biotechnology (Jiang, 2013).

In recent years there has been a growing awareness of the potential value of freshwater habitats as a source of actinomycetes, as there they represent a large fraction (30–70%) of total bacteria (Warnecke et al., 2004; Holmfeldt et al., 2009; Newton et al., 2011). There is increasing evidence that they act as key players in nutrient and energy cycling (Holmfeldt et al., 2009). Given that discrete environmental niches contain unique distributions of this group of microorganisms and that vertebrates including fish hitherto have been largely ignored, it has become worthwhile to examine the freshwater fish microbiome. In addition, attempts to isolate and to characterize culturable Actinobacteria seems to be a first step towards a better understanding of the Actinobacteria community as well as the biosynthetic potential from this unexplored environment. In the present study, two candidate fish species, the Snow trout (*Schizothorax zarudnyi)* and the Anjak (*Schizocypris altidorsalis*) were selected for examining the presence of Actinobacteria strains from the fish intestinal flora using different selective media. As both possess herbivorous and detritivore feeding regimes, respectively, they are mainly dependent on the sediments and plant residues, which are known to be naturally occurring niches of Actinobacteria. To this end, several selective isolation media were used, and the isolates were subjected to phylogenetic analyses based on 16S rRNA genes, and were tested for antimicrobial activity. Also to study their associated potential capacity to synthesize diverse bioactive natural products, the presence of secondary metabolite genes encoding polyketide synthases (PKS I and PKS II), nonribosomal peptide synthetases (NRPSs), aminodeoxyisochorismate synthase (phzE), dTDP-glucose-4, 6-dehydratase (dTGD), halogenase (Halo) and cytochrome P450 hydroxylase (CYP) was investigated by using degenerate oligonucleotide primers.

2. Methods

2.1. Fish sampling

In October 2013, fish samples representing *S. zarudnyi* (Snow trout) and *S. altidorsalis* (Anjak) were obtained from Chahnimeh reservoirs, Zabol, Iran (Fig. 1). Weight and length of the fish were measured before dissection. Forty-four adult fish (31 *S. zarudnyi* and 13 *S. altidorsalis*), with an average weight of approx. 500 g and 200 g, respectively, were sacrificed using high doses of anaesthesia. All instruments, surfaces, and the exterior of each fish were treated with 70% EtOH and instruments were flame-sterilized prior to dissection. The dissection was performed using flame-sterilized instruments. After opening the body cavity, the intestines were separated and the digesta from intestine were gently squeezed out, then transferred into a sterile 2 mL screw cap tube containing phosphate-buffered saline (PBS) and subsequently stored at −20 °C. Samples were transported to the laboratory at the University of Natural Resources and Life Sciences, Vienna, Austria, under deep-frozen conditions. The thawed content was transferred into sterile plastic bags followed by homogenization in a paddle mixer (Stomacher, Seward Laboratory, London, UK). Homogenates of the intestinal content were diluted in sterile 0.85% saline, and aliquots of 100 μL of the 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated onto freshly prepared agar media. The following three isolation media were used: (1) starch casein agar consisting of starch casein broth (US Biological S7968-25B) and agar (Oxoid Agar Bacteriological LP0011 18 g/L), pH 7 ± 0.2 at 25 °C, (2) actinomycete isolation agar (Difco, 212168) with final pH 8.1 ± 0.2 at 25 °C, and (3) Kuster agar (components in g/L; glycerol 10, casein 0.3, potassium nitrate 3.0, potassium phosphate 2.0, sodium chloride 2.0, magnesium sulfate 0.05, calcium carbonate 0.02, ferrous sulfate 0.01, agar 16.0; pH 7 ± 0.1 at 25 °C) (Baskaran et al., 2011). All isolation media were prepared with distilled water and amended with filter-sterilized (0.2 μm pore size) Nalidixic acid (50 mg/L) and Cycloheximide (50 mg/L) to inhibit the growth of Gram-negative bacteria and fungi (Sanchez et al., 2012). The isolation plates were incubated in the dark for 4 weeks at 28 °C. Isolates were obtained in pure culture after three successive transfers and loop-streaking on fresh agar media. Pure cultures were frozen at −80 °C in International Streptomyces Project Medium 2 (ISP2) broth (Oxoid, CM1135) containing 20% glycerol for long term storage.

2.2. DNA isolation, PCR amplification and sequencing

2.2.1. DNA extraction

From each isolate genomic DNA was extracted using the procedure reported by Cook and Meyers (2003) with some modifications. Briefly, the isolates were grown in 10 ml International Streptomyces Project Medium 1 (ISP1) with agitation at 30 °C for 18–24 h and examined by means of Gram-staining (Shirling and Gottlieb, 1966). Then bacterial cells of 2 mL broth were harvested by centrifugation (20,000 × *g* for 10 min at 4 °C), washed once with 500 μL of TE-buffer (pH 7.7), centrifuged (20,000 × *g* for 10 min at 4 °C) and re-suspended in 500 μL TE-buffer (pH 7.7). The samples were heated in boiling water for 20 min and allowed to cool for 5 min at room temperature. After centrifugation (7500 × *g* for 3 min), 300 μL of supernatant were transferred into a clean tube and stored at −20 °C.

2.2.2. PCR amplification

The DNA preparations were used as template for partial 16S rRNA gene PCR amplifications applying the primers S-C-Act-0235-a-S-20 and S-C-Act-0878-a-A-19 (Stach et al., 2003). Each PCR reaction contained 5 μL DNA template, 5 μL 5× KAPAHiFi fidelity Buffer (Kapa Biosystems Inc., Boston, MA, USA), 0.75 μL dNTP (10 mM stock mixture, Kapabio-systems, Boston, MA, USA), 0.5 μL of each primer (10 mM), KAPAHiFi HotStart DNA Polymerase (1 U/μL, Kapa Biosystems, Boston, MA, USA), made up to 25 μL with deionized sterile distilled water. Amplification was achieved using a Mastercycler Gradient cycler (Eppendorf AG, Germany) under the following conditions: initial denaturation 95 °C for 5 min, 30 cycles of denaturation at 98 °C for 20 s, annealing at 68 °C for 15 s, extension at 72 °C for 15 s and, and a final extension at 72 °C for 5 min.

2.2.3. Dereplication by repetitive sequence-based PCR (rep-PCR)

With the actinobacterial isolates repetitive DNA fingerprinting was performed using the BOX-A1R primer (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) (Stockmann and Piepersberg, 1992). The PCR reaction mixture (25 μL) contained 5 μL genomic DNA extract, 2.5 μL 10× buffer (Thermo Scientific, Rockford, IL USA), 0.5 μL dNTP mixture (10 mM), 2.5 μL BOX-A1R primer (15 μM) and 0.5 μL DyNAzyme II (2 U/μL) (Thermo Scientific, Rockford, IL, USA). PCR was performed by a Mastercycler Gradient cycler (Eppendorf, AG, Germany). The reaction started with pre-denaturation at 95 °C for 8 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min and 4 min of extension at 65 °C, with a final extension at 65 °C for 8 min. PCR products were separated by means of 2% agarose gel electrophoresis. Cluster analysis of the rep-PCR profiles was performed on similarity matrices, which were produced using Dice's coefficient (Dice, 1945) and subjected to the unweighted pair group method with arithmetic average (UPGMA) clustering algorithm, using Bionumerics v6.6 software (Applied Maths, Sint-Martens-Latem, Belgium). To assess the robustness of