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Original article

The first detection of the sequence of bacteria from the *Simkaniaceae* family in surface waters in Poland

M. Pawlikowska-Warych[1,2], W. Deptuła[1,2]

[1] Department of Microbiology, Faculty of Biology, University of Szczecin, Poland
[2] Molecular Biology and Biotechnology Center, Faculty of Biology, University of Szczecin, Felczaka 3c, 71-412 Szczecin, Poland

Abstract

Bacteria from the *Simkaniaceae* family are intracellular parasites belonging to the *Chlamydiales* order, detected in surface waters, drinking water, chlorine water, and in wastewater. Its main representative, *Simkania negevensis*, is pathogenic to humans and animals, especially fishes, as it principally causes respiratory tract diseases. Bacteria from this family are also capable of surviving and existing in free-living amoebas, omnipresent in the natural environment, which makes them an additional risk for human and animal health. The aim of the present study was to search for representatives of this family in freshwaters from the Odra River and two municipal lakes (Rusałka and Goplana). Out of 100 water samples analysed, the sequence of bacteria of *Simkaniaceae* family was found just in 1 percent, because phylogenetic analysis revealed that the obtained OdraWCh30 sequence shows 93% similarity to *Simkania negevensis* strain Z as well as 87% similarity to *Candidatus* Syngnamydia salmonis isolate Ho-2008 and *Candidatus* Syngnamydia salmonis isolate VS10102006 and 84-85% similarity to endosymbiont of *Xenoturbella westbladi, Simkaniaceae* bacterium clone SM081012-5s and *Candidatus* Syngnamydia venezia strain Pi3-2. This is the first case of detecting sequence of bacteria of *Simkaniaceae* family in the aquatic environment in Poland.

Key words: Simkaniaceae, surface water, phylogenetic analysis, OdraWCh30 sequence, Poland

Introduction

Bacteria from the *Simkaniaceae* family with classic chlamydia (the *Chlamydiaceae* family) and 9 families referred to as CLO – chlamydia-like organisms or environmental chlamydia belong to the *Chlamydiales* order (Pierle et al. 2015, Bou Khalil et al. 2016, Pizzetti et al. 2016, Pawlikowska-Warych and Deptuła 2016). A prototype for this family is the identified in 1993 *S. negevensis*, isolated as contamination from cell cultures (Kahane et al. 1993). Its classification among the *Chlamydiales* order is conditioned by identical ribosomal genes, at the level of 80-90% (Everett et al. 1999) and characteristic of chlamydia two-phase but long

Correspondence to: M. Pawlikowska-Warych, e-mail: malgorzata.pawlikowska@usz.edu.pl

development cycle lasting even 12-15 days (Kahane et al. 2002, Yamaguchi et al. 2004). Another genus in this family is *Candidatus (Ca.)* Fritschea with two non-cultivated species *Ca*. Fritschea bemisiae and *Ca.* Fritschea eriococci (Everett et al. 2005), which are endosymbionts of silverleaf whitefly (*Bemisia tabacci*) and elm scale (*Eriococci spurius*), with similarity of their 16S-23S rRNA sequence to *S. negevensis* amounting to 91%.

Presently, the *Simkaniaceae* family has been ascribed with *Ca.* Syngnamydia venezia, pathogenic to fish, which has been isolated from gill epitheliocystis of Broadnosed pipefish (*Syngnathus typhle*) (Fehr et al. 2013), and *Ca.* Syngnamydia salmonis, detected in gill epitheliocystis of Atlantic salmons (*Salmo salar* L.) (Nylund et al. 2015). Furthermore, this family includes phylotypes identified in marine worms of the *Xenoturbella* genus (Israelsson 2007), corkwing wrasse (*Symphodus melops*) (quote: Nylund et al. 2015), as well as phylotypes (cvE6, cvE9, cvE38, cvE41) originating from fresh waters (Corsaro et al. 2002, Corsaro and Venditti 2009). Classification of species and phylotypes into this family is conditioned by the similarity of the 16S rRNA coding gene at the level of from 80% to 98.1% (Everett et al. 1999, Horn 2010).

When analysing the representatives of the *Simkaniaceae* family in the epidemiological meaning, it must be stated that particularly *S. negevensis* is a factor pathogenic to humans, as it was isolated from bronchitis in children and adults in Israel (Negev) (Lieberman et al. 1997, 2002, Kahane et al. 1998, Friedman et al. 1999, Greenberg et al. 2003). Furthermore, among adult Inuit people in Canada (Lieberman et al. 2002, Greenberg et al. 2003), and among adults in Japan (Yamaguchi et al. 2005), in Jordan (Al-Younes and Paldanius 2014), and in European countries (Switzerland, England, Israel) (Vouga et al. 2018), high level of anti-*Simkania* sp. antibodies were found, which points to the presence of such bacteria in these societies. Morever, *S. negevensis* was also reported in people with rejected lung transplants (Jamal et al. 2015). It should be added that isolation not only from freshwaters, but also from drinking water, or chlorinated swimming pool water, as well as wastewater proves its high infectious potential (Kahane et al. 2005, 2007, Donati et al. 2015). This risk to humans in the epidemiological meaning also increases due to its capacity of infecting *Acanthamoeba polyphaga* which are omnipresent in the aquatic environment and soil, as well as on vegetable products (Kahane et al. 2001, Trabelsi et al. 2012), as they transmit many bacteria, including chlamydia (Leońska-Duniec 2011, Kebbi-Beghdadi et al. 2014). It was evidenced that amoebas transmit *S. negevensis* to mammal cells, as by introducing the *Acanthamoeba polyphaga* amoebas infected with this bacterium to human monocyte and macrophage cultures, after several hours, *S. negevensis* was found in them (Kahane et al. 2008).

Due to the presence of bacteria from the *Simkaniaceae* family in the natural environment, including in water in a few countries around the world, and no data on their prevalence in the environment in Poland, attempts were undertaken to search for *Simkania negevensis* and other phylotypes from the *Simkaniaceae* family in standing and flowing waters.

Materials and Methods

The material for the study involved water sampled from the Odra River, as well as Goplana and Rusałka Lakes located within the City of Szczecin. At the Odra River, three water sampling sites were established: on Kolumba Street at the level of the Main Railway Station, on Jana z Kolna Street at the exit from Trasa Zamkowa (The Castle Route, at the foot of the Wały Chrobrego), and on Parnica from the side of the Gdański boulevard. The sites have been selected because these are popular recreation areas, as well as fishing sites. The choice of the two lakes: Goplana and Rusałka was also made because they form recreation areas. Goplana Lake is situated on the walking route in the Arkoński Forest, popular as a place for recreation and a beach in the summer season. In turn, Rusałka Lake is located in the Kasprowicza Park almost at the heart of the City of Szczecin.

The tests involved 100 water samples of 1L each, pursuant to water sampling principles, transported within maximum one hour to the laboratory at the temperature of 4°C according to standards PN-ISO 5667--6:2003 and PN-ISO 5667-4:2003.

Sample concentration and DNA extraction

Each water sample (1L) was concentrated by filtration, directly after delivery, at nitrocellulose filters with pore diameter of 0.22 (Whatman, GE Healthcare, UK). The filters covered with water residue were placed in Falcon tubes and poured with 10mL sterile saline solution, and vortexed with the addition of 3 g glass balls (ø 3mm) for 30s. Next, 2mL obtained suspension were transferred to Eppendorf tubes and centrifuged (10000×g, 5min) to achieve densification and obtain a sample of 200 μL. DNA was extracted with the ExtractMe DNA Tissue Kit (DNA Gdańsk, Poland) according to the manufacturer's instruction. The isolated DNA was stored at the temperature of -20°C until material was collected from all 100 water samples, followed by PCR reactions.

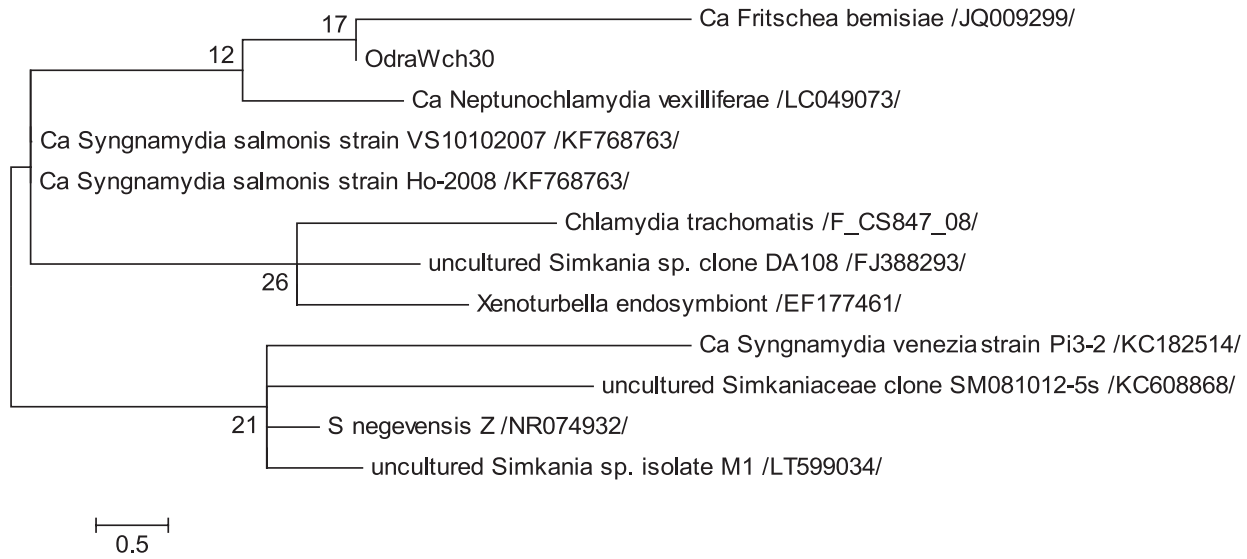

Fig. 1. The phylogenetic tree of obtained sequence OdraWCh30 compared to the sequences of bacteria of the *Simkaniaceae* family based on the Maximum Likelihood method (Kimura 2-parameter model).

PCR analysis, sequencing and phylogenetic analysis

The PCR reaction was performed on the basis of specific starters proposed by Ossewaarde and Meijer (1999), namely CHL16SFOR1 (3`-gtggatgaggcatgcgagtcga) and CHL16SREV1 (3`-ctctcagcccgcctagacgtcttag). Amplification was carried out in the Eppendorf vapo. protect thermocycler (Eppendorf, Germany). The reaction mix with a total volume of 30 μL comprised: 3 μL DNA, of 0.6μL starters each (Genomed, Poland), 3 μL dNTP (DNA Gdańsk, Poland), 0.75 μL Taq DNA polimerase (EURx, Poland), 3μL Taq buffer (EURx, Poland), 0.3μL 25mM $MgCl_2$ (EURx, Poland), and 18.75 μL water DEPC (EURx, Poland). The PCR reaction profile was determined on the basis of Taq DNA polymerase protocol (EURx, Poland), considering starter Tm. Preliminary denaturation was performed for 3 min at the temperature of 95°C, proper DNA amplification in 35 cycles in the profile: denaturation (95°C – 30s), annealing (56°C – 30s) and elongation (72°C – 90s), and final elongation 72° for 300s.

After the PCR reaction, the presence of the product was visually checked by electrophoresis on 2% agarose gel using Bio-Rad system equipment (Bio-Rad, USA). Positive results were obtained in 10 samples out of 100 (10%): 3 from Rusałka Lake, 1 from Goplana Lake, and 6 from the Odra River. The samples were sent for sequencing using Sanger method at a commercial company (Macrogen, the Netherlands). The DNA concentration of those samples was 180-210 ng/μl. After obtaining the sequence, homology analysis was performed using the BLASTn alignment (Altschul et al., 1997), and phylogenetic analysis in MEGA 6.0 software on the basis of the Maximum Likelihood (Kimura 2-parameter model). For comparison, sequence of the bacteria was used from the *Simkaniaceae* family and *Chlamydia trachomatis* strain F/CS847/08 collected from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Results

The analysis of the sequences obtained in the BLASTn (Altschul et al. 1997) alignment revealed that only the sequence marked as OdraWCh30, originating from a water sample from the Odra River, is identical with bacterial sequences from the *Simkaniaceae* family. The highest homology (93%) of the nucleotide sequence (224nt) was with the sequence of the *Simkania negevensis* – strain Z (NR074932). Next, the quite high homology (87%) was found in relation to *Ca*. Syngnamydia salmonis isolate Ho-2008 (KF768763) and *Ca*. Syngnamydia salmonis isolate VS10102006 (KF768762). However, when comparing the obtained OdraWCh30 sequence to the sequence of endosymbiont of *Xenoturbella westbladi* (EF177461), homology was found at the level of 86%, as well as *Simkaniaceae* bacterium clone SM081012-5s (KC608868) and *Ca.* Syngnamydia venezia strain Pi3-2 (KC182514) where homology was respectively 85% and 84%.

Phylogenetic analysis performed using the Maximum Likelihood method (Kimura 2-parameter model) points to a clear division into two main branches (Fig. 1). The first branch contains four sequences, *Ca*. Syngnamydia venezia strain Pi3-2, uncultured *Simkaniaceae* clone Sm081012-5s, *Simkania negevensis* strain Z and uncultured *Simkania* sp. isolate M1. The second branch includes eight sequences in three groups. The first group includes the sequence we have

obtained, OdraWCh30, together with *Ca.* Fritschea bemisiae and *Ca*. Neptunochlamydia vexilliferae. Group two includes strains of *Ca.* Syngnamydia salmonis, the third contains uncultured strain DA108 *Simkania* sp. with endosymbiont of *Xenoturbella westbladi* and *C. trachomatis*.

When analysing the results using the Maximum Likelihood, one can state that our OdraWCh30 sequence, originating from water samples from the Odra River, is affiliated with *Ca.* Neptunochlamydia vexilliferae, and slightly distanced from *Simkania negevensis* strain Z (Fig. 1). It proved to be novel sequence, that we submitted to the GenBank database under accession number MF121683.

Discussion

In this study, using genetic markers allowing detection of sequences of bacteria from the *Simkaniaceae* family (Ossewaarde and Meijer 1999), for 100 river and lake water samples, only one was given a positive result (1%). The comparative analysis of the genetic sequence of the obtained sequence OdraWCh30 revealed that it is 93% identical to the *Simkania negevensis* strain Z sequence, and exhibits 87% similarity to *Ca*. Syngnamydia salmonis isolate Ho-2008 and *Ca*. Syngnamydia salmonis isolate VS10102006 and 84-85% similarity to endosymbiont of *Xenoturbella westbladi, Simkaniaceae* bacterium clone SM081012-5s and *Ca.* Syngnamydia venezia strain Pi3-2.

The results are confirmed by many studies, as many new chlamydia-like organisms have been isolated, determining that the new strains, isolates or phylotypes showed identity to the *Simkaniaceae* family at the level of 87.9-98% (Corsaro et al. 2002, Everett et al. 2005, Corsaro and Venditti 2009, Fehr et al. 2013, Nylund et al. 2015, Pizzetti et al. 2016). According to the requirements and principles adopted in chlamydia classification on the basis of 16S rRNA (Everett et al. 1999), phylotypes or isolates classified into a family must show identity of the nucleotide sequence at the level of >90%, although Kostjansek et al. (2004) included into the *Simkaniaceae* family the bacteria revealing identity at the level of just 84%. Therefore, as regards the results obtained, it must be stated that they are consistent with those obtained by Corsaro et al. (2002), who found that phylotypes cvE6 and cvE9 obtained from fresh water showed 87.9% identity to the bacteria from the *Simkania* genus. However, considering studies by Bou Khalil et al. (2016), evidencing the homology of 73% between *Rubidus massiliensis* and the *Parachlamydiaceae* family, it seems that the homology of the OdraWCh30 sequence at the level of 84-93% with members of the *Simkaniaceae* family, as obtained in the present study, points to its classification into this family. In the phylogenetic analysis, regardless of the research model applied, the analysed sequence OdraWCh30 remains close to the sequence of *Ca.* Neptunochlamydia vexilliferae, as well as in one group to *Ca.* Fritschea bemisiae. Therefore, it can be stated that the results of the analysis confirm that obtained sequence OdraWCh30 could belong to the bacteria of *Simkaniaceae* family, although it must be added that, according to Horn et al. (2004), phylogenetic analysis alone cannot definitively point to relations among isolates.

Conclusions

Considering the results obtained in the BLASTn and phylogenetic analyses, it must be stated that the obtained OdraWCh30 sequence originating from the Odra River waters shows very high similarity to the *Simkaniaceae* family from the *Chlamydiales* order. This is the first case of detecting sequence of bacteria of *Simkaniaceae* family in the aquatic environment in Poland, which would point to the fact that this bacteria must be accounted for as an infectious factor for humans in Poland.

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