Article

Genome analysis of two bacterial strains isolated from diseased freshwater sponge reveals the probable cause of its joint domination in microbial community

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Abstract: Endemic freshwater sponges (Demosponges, Lubomirskiidae) dominate in Lake Baikal and are multicellular filter-feeding animals represent a complex consortium of many species of eukaryotes and prokaryotes. In recent years, mass disease and death of the *L. baicalensis* have been an urgent problem of Lake Baikal. The etiology and ecology of these events remain unknown. Bacteria in microbiomes of diseased sponges of the families *Flavobacteriaceae* and *Oxalobacteraceae* were dominant. Both species are opportunistic pathogens common for freshwater ecosystems. The aim of our study is to analyze the genomes of strains *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02, isolated from diseased sponges to identify the reasons for their joint dominance. The first one attacks the other cells using type VI secretion system, suppress gram-positive bacteria with violacein pigment and regulate its own activity via quorum sensing. It makes the floc and strong biofilm by exopolysaccharide biosynthesis and PEP-CTERM proteins expression. The second one utilizes the fragments of cell walls produced of polysaccharides. Named two strains have noticeable difference in carbohydrates acquisition. We described the possible way of joint occupation of ecological niche into freshwater sponge microbial community. This study expands understanding about symbiotic relationship of microorganisms with freshwater Baikal sponges.

Keywords: Symbiosis; Opportunistic pathogens; *Janthinobacterium* sp., *Flavobacterium* sp., Genomes; Floc formation; *Lubomirskia baikcalensis*

1. Introduction

Endemic freshwater sponges (*Demosponges*, *Lubomirskiidae*) dominate in Lake Baikal in the littoral zone. They cover up to 50% of the available surfaces [1] and represent a complex consortium of many species of eukaryotes and prokaryotes, including diverse chlorophyll-containing microalgae [2–4]. The first appearance of anomalously pink-colored *L. baicalensis* (Pallas, 1776) sponges was found in 2011. In recent years, significant changes in the ecological system of the coastal (littoral) zone, including mass death of the endemic representatives of the freshwater sponges of the *Lubomirskiidae* family, have been an urgent problem of Lake Baikal. Diseased and dying sponges have been observed in many areas of the lake [5–7]. The etiology and ecology of these events remain unknown.

Freshwater sponges are multicellular filter-feeding animals, so they are good indicators of the environmental state [8,9]. Sponges pump large quantities of water and have ability to concentrate a wide range of chemicals from both the suspended and dissolved phases of the water (Orani et al., 2018). The understanding of molecular mechanisms of the sponge stress response is not clear, most studies focus on the effect of stress on the sponge-associated microbial community [10–12].

In previous studies we reported a shift in microbial communities of the diseased Baikal sponges characterized by mass mortality of green symbionts (*Chlorophyta*) and increased abundances of several different opportunistic colonizers [7,13]. Microbes in diseased sponges belonged mainly to the phyla *Bacteroidetes* and *Proteobacteria* and were much more diverse at the family level. Among these, the families *Flavobacteriaceae* and *Oxalobacteraceae* were dominant. Further we observed the increase in relative abundance of *Flavobacteriaceae* and *Oxalobacteraceae* in the diseased sponges and the infected cell cultures of primmorphs [7,13], isolated and cultivated separately and performed whole genome sequencing two dominating strains named *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02 respectively [14,15].

Bacteria of the *Burkholderiales* is characterized by the presence of ecologically extremely diverse organisms and contains environmental saprophytic organisms, phytopathogens, and opportunistic pathogens, including those for freshwater ecosystems [16,17]. Bacteria of *Janthinobacterium* family are well-known for their antifungal effects most likely induced through a regulatory network in response to chitin [18]. Most of *Janthinobacterium* bacteria can produce violacein, known metabolite of *Janthinobacterium lividum* which has a broad bioactivity profile including antibacterial, antiviral, and antitumoral activity [19]. Its biosynthesis is a cell density dependent factor, controlled by quorum sensing [20,21]. Three key gens, encoding proteins associated with the quorum sensing are: the CAI-1/LAI-1 autoinducer synthase, two-component histidine sensor kinase and a two-component response regulator. All of them were discovered before in the closer specie *Janthinobacterium* sp. HH01 [21]

As reported before, in the stationary phase, *Janthinobacterium lividum* forms a strong biofilm that is rich in exopolysaccharides [22]. When cultivating *Janthinobacterium* sp. SLB01 we experimentally observed biofilms into the cell cultures of primmorphs of *L. baicalensis* (unpublished data). Exopolysaccharides (EPS) – the main component of biofilm produced by species of *Janthinobacterium* and *Flavobacterium* families [22–25]. PEP-CTERM-containing proteins generally contain an N-terminal signal peptide and exhibit high diversity and little homology to known proteins. All bacteria with PEP-CTERM have both an outer membrane and EPS production genes [26].

One essential strategy of Gram-negative bacteria is the secretion of virulence factors through the cell membranes of the target (victim) to achieve a potential target. In early studies the type VI secretion system (T6SS) was associated with bacterial virulence concerning eukaryotic host cells, but a scarce number of T6SSs are directly implicated in cell disruption [27]. There are few studies describing the T6SS for *Janthinobacterium* family, f.e. about HH01 strain [21,28].

The members of the genus *Flavobacterium*, which belongs to the phylum *Bacteroidetes*, are typical bacteria of saline and freshwater ecosystems that can be opportunistic pathogens [29,30]. In a number of previous works researchers have shown that some species of *Flavobacterium* contain proteolytic and collagenolytic enzymes [31,32]. These bacteria regulate a diverse array of activities, including symbiosis, antibiotic production, motility, virulence, and biofilm formation [33–35].

The aim of our study is to analyze the genomes of strains *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02, isolated from diseased sponges to identify the reasons for their joint dominance. The results of this study will help broaden our understanding about symbiotic relationships in microbial consortium during mass mortality freshwater Baikal sponges.

2. Results

2.1. Genome assembly, scaffolding and features

To unravel potential molecular mechanisms involved in symbiotic relationships in microbial consortium during mass mortality freshwater Baikal sponges, we established the genome sequences of the isolated strains. Violet-pigmented *Janthinobacterium* sp. SLB01 and yellow-pigmented *Flavobacterium* sp. SLB02 strains were isolated on Luria–Bertani (LB) broth medium agar plates (diluted 1/10, temperature 15 °C). Genomic DNA was isolated following standard bacterial DNA Isolation CTAB Protocol (http://www.jgi.doe.gov).

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After draft assembly with SPAdes we made the reference-assisted scaffolding with Ragout [36]. Genome completeness analysis with BUSCO showed results: for *Flavobacterium* sp. SLB02 96.2% complete, 1.1% fragmented, and 2.7% missing BUSCOs; for *Janthinobacterium* sp. SLB01 98.2% complete, no fragmented, and 1.8% missing BUSCOs.

Genomes were released into NCBI to further study and annotation. The final genome assembly statistics: raw reads count, genome size number of genes, pseudogenes, protein-coding sequences, tRNA and noncoding RNA presented in Table 1.

Table 1. Raw reads and genome features statistics of bacterial strains in this study.

Property	*Janthinobacterium* sp. SLB01	*Flavobacterium* sp. SLB02
Raw reads	12,099,942*	17,921,744*
GenBank accession number	VZAB00000000**	CP045928***
Genome size, bp	6,467,981	6,363,829
Number of contigs	2	1
GC content	62.63%	35,50%
Number of genes	6,023	4,964
protein-coding sequences	5,863	4,901
tRNAs	65	56
noncoding RNAs	4	3
pseudogenes	78	73

*The sequence library was generated from DNA using an Illumina Nextera XT DNA sample preparation kit. Whole-genome sequencing was performed using Illumina MiSeq platform with paired-end chemistry (2 x 250 bp).

**Reference genome: *Janthinobacterium* sp. strain LM6 chromosome (GenBank accession number CP019510)

***Reference genomes: *Flavobacterium* sp. strain KBS0721 chromosome (GenBank accession no. CP042170) and the *Flavobacterium* piscis strain CCUG 60099 whole-genome sequence (GenBank accession no. MUHC01000000)

2.2. Violacein synthesis by Janthinobacterium sp. SLB01

Strain *Janthinobacterium* sp. SLB01 is able to produce violacein (see further in text culture photo in Figure 4). Its genome contains required violacein synthesis operon vioABCDE. Locus structure presented as the diagram in Figure 1, genes' coordinates and loci names presented in Table S1.

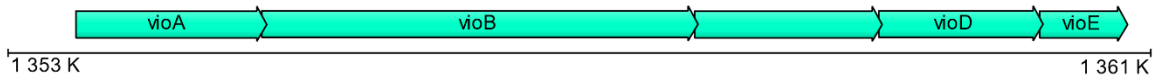

Figure 1. Violacein production loci diagram into *Janthinobacterium* sp. SLB01 genome.

2.3. Type VI secretion system genes identification

Genome of *Janthinobacterium* sp. SLB01 contains all three categories of the genes, required for function of type VI secretion system (listed above). That genes are allocated through genome by 10 clusters, the largest one – contains most of the genes – is shown in Figure 2. Name, locus, localization and annotation of each gene presented in Supplementary (Table S2).

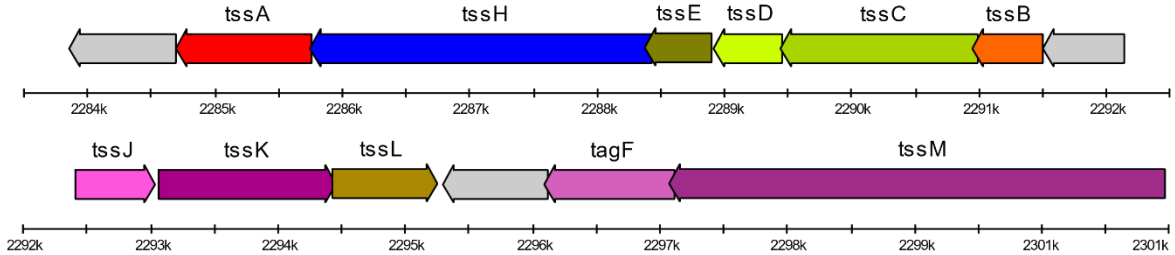

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Figure 2. Schematic diagram of the genetic organization of the type VI secretion system main gene cluster into *Janthinobacterium* sp. SLB01 genome.

2.4. Quorum sensing in Janthinobacterium sp. SLB01

We found genes associated with the quorum sensing in Janthinobacterium sp. SLB01 genome. Three key genes are: synthesis of the CAI-1/LAI-1 autoinducer synthase, two-component histidine sensor kinase and a two-component response regulator. We found homologous genes to all of them in *Janthinobacterium* sp. SLB01 genome. Localization and homology percentage of these genes are presented in Table 2.

Table 2. Quorum sensing genes description into *Janthinobacterium* sp. SLB01 genome.

***Janthinobacterium* sp. SLB01**		*Janthinobacterium* sp. HH01		
locus tag	annotation	locus tag	% ident	% simi-larity
F3B38_RS23475	quorum-sensing autoinducer synthase	Jab_2c24330*	68.6	81.0
F3B38_RS23480	HAMP domain-containing histidine kinase	Jab_2c24340	60.6	73.1
F3B38_RS23485	response regulator	Jab_2c24350	68.2	79.8

* CAI-1/LAI-1 autoinducer synthase at first time identified for *Janthinobacterium* specie [21]

2.5. Floc formation by Janthinobacterium sp. SLB01

In the stationary phase, *Janthinobacterium* sp. SLB01 forms a strong biofilm that is rich in exopolysaccharides (EPS). In total, at least 27 genes encoding typical PEP-CTERM proteins had been identified in *Janthinobacterium* sp. SLB01 strain. Secretion of EPS, expression of PEP-CTERM proteins and exosortase forms the floc. Genome analysis of *Janthinobacterium* sp. SLB01 reveals all required gene clusters for floc formation. Its genome contains a large (F3B38_RS08235–F3B38_RS08375) and small (F3B38_RS15000–F3B38_RS15020) gene clusters of synthesis and export polysaccharides, which may be also involved in extracellular polysaccharide biosynthesis. These gene clusters include TIGR03013 family PEP-CTERM/XrtA system glycosyltransferase (previously called EpsH), PEP-CTERM system histidine kinase PrsK, PEP-CTERM-box response regulator transcription factor PrsR. In another study about floc formation two glutamine-dependent asparagine synthases asnB (F3B38_RS08405) and asnH were required [37] which also have orthologs in *Janthinobacterium* sp. SLB01 genome. Schematic diagram of the genetic organization of these gene clusters presented in Figure 3.

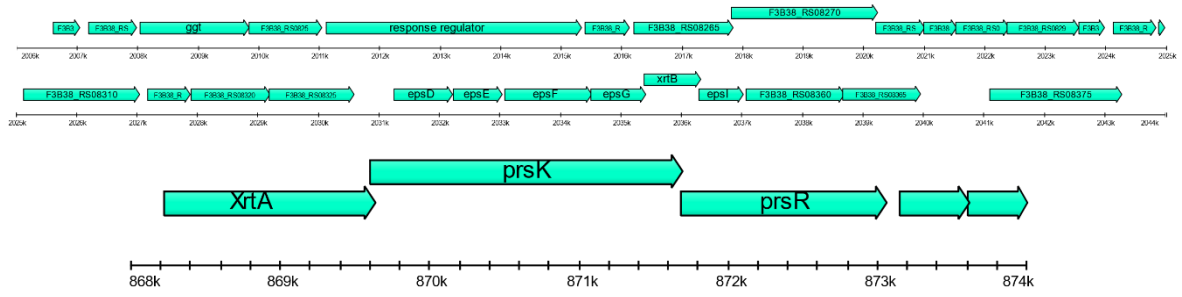

Figure 3. Schematic diagram of the genetic organization of *Janthinobacterium* sp. SLB01 gene clusters, required for floc formation: EPS synthesis, PEP-CTERM, exosortase. Genes are indicated by arrows and the direction of the arrows represent the direction of transcription of the genes in genome.

Floc formation and violacein pigment are visually observed into the cell culture of *Janthinobacterium* sp. SLB01 (see Figure 4).

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Figure 4. Visual observance of floc formation and violacein synthesis by *Janthinobacterium* sp. SLB01.

2.6. Polysaccharides utilization

We detected 45 predicted polysaccharide utilization loci (PULs) into *Flavobacterium* sp. SLB02 genome, which are annotated previously and available online in PULDB http://www.cazy.org/PULDB/index.php?sp_name=Flavobacterium+sp.+SLB02 [38]. Each PUL consists of SusC/D marker genes with various combination of glycoside hydrolases, carbohydrate-binding modules, carbohydrate esterases, polysaccharide lyases, extracytoplasmic function σ-factor, peptidases and transporters (all definitions are described at http://www.cazy.org/PULDB/tags.html).

Genomic comparisons showed that homologous loci to these PULs occur in other *Bacteroidetes* members, for some of which exists experimental data about utilized polysaccharides. PULs with numbers 4, 7, 10, 11, 13, 15, 18, 23, 24 and 36 have strong homology (according to PULDB reports) with *Flavobacterium johnsoniae* UW101. Map of each of these loci presented in Figure 5.

Figure 5. Polysaccharide utilization loci (PULs) of *Flavobacterium* sp. SLB02 genome which have homologous PULs into *Flavobacterium johnsoniae* UW101 genome.

According to previous study [39,40] these PULs give the ability to digest wide range of polysaccharides, which was predicted based on the genome analysis and confirmed experimentally. Digestible polysaccharides include chitin, starch, α-glucan, pectin and hemicelluloses: xylans, mannans, and xyloglucans. Predicted cell surface proteins related to *Bacteroides thetaiotaomicron* SusC

 doi:10.20944/preprints202009.0144.v1

and SusD, which are likely involved in binding of oligosaccharides and transport across the outer membrane, were also identified.

2.7. Carbon sources metabolism

Genome analysis made with SEED shows that *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02 use different carbohydrates as the carbon sources. As noted before, *Flavobacterium* sp. SLB02 can utilize the polysaccharides, but *Janthinobacterium* sp. SLB01 mostly cannot (have genes only for chitin degradation). We analyzed the feature counts for carbohydrate metabolism subsystem* and found out that the composition of carbon acquisition genes is rather different for many enzymes. In the Table 3 listed the pathways (or subsystems in SEED) with significant (more than two times) differences. The full list of subsystems is presented in Table S3.

Table 3. Carbon sources metabolism subsystem differences into *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02 genomes.

Carbon source group	Subsystem* name	SLB01**	SLB02***
Central carbohydrate metabolism	TCA Cycle	15	0
	Pentose phosphate pathway	0	9
Di- and oligosaccharides	Sucrose utilization	0	2
Organic acids	Methylcitrate cycle	7	0
	Propionate-CoA to Succinate Module	6	0
	Lactose and Galactose Uptake and Utilization	0	8
Fermentation	Mixed acid	0	7
Polysaccharides	Glycogen metabolism	0	4
Monosaccharides	2-Ketogluconate Utilization	4	0
	L-Arabinose utilization	0	9

*A subsystem is a set of functional roles that an annotator has decided should be thought of as related.

** Subsystem feature counts for Janthinobacterium sp. SLB01

*** Subsystem feature counts for Flavobacterium sp. SLB02

3. Discussion

Our study shows that strains *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02 may have symbiotic interaction in diseased sponge (host) microbial community. Bacteria of *Janthinobacterium* sp. SLB01 communicate with each other with quorum sensing and can make the floc from the EPS and PEP-CTERM proteins by exosortase XtrA (EpsH). They attack other neighboring cells with type VI secretions system (T6SS) and suppress the gram-positive bacteria with the violacein pigment. Cell walls, remaining from dead bacteria (made of polysaccharides) are the obstacle to T6SS function, because of its short range [41]. *Janthinobacterium* sp. SLB01 can utilize only the chitin, but *Flavobacterium* sp. SLB02 (as many of *Bacteroidetes*) have specific polysaccharide utilization loci (PULs) to digest the wide range of them. We observe the joint domination of these two strains into diseased sponges and the infected primmorphs [7,13].

Bacteria of *Janthinobacterium* sp. SLB01 colonizes the space and suppress the other bacteria (especially Gram-positive) with violacein: pigment production we observed in cell culture (Figure 4) and all required genes (operon VioABCDE) are present into its genome. Violacein also associated with quorum sensing (QS) and biofilm formation [20]. Three key gene clusters associated with the quorum sensing are: synthesis of the CAI-1/LAI-1 autoinducer synthase, two-component histidine sensor kinase and a two-component response regulator. There are very few studies describing the quorum sensing for *Janthinobacterium* family. We used the description of QS system for *Janthinobacterium* sp. HH01 strain [21,28] and found homologous genes to all required genes into *Janthinobacterium* sp. SLB01 genome. Localization, annotation and identity percentage of these genes are presented in Table 2.

 doi:10.20944/preprints202009.0144.v1

Janthinobacterium sp. SLB01 extracts the necessary nutrients from the eukaryotic and bacterial cell via T6SS. Its genome contains all three categories of the genes, required for function of type VI secretion system [42,43]. The first category includes genes encoding membrane-associated proteins, either integral membrane (TssL, TssM) or lipoproteins (TssJ). The second category of genes encodes proteins with relatedness to tailed bacteriophage components (Hcp or TssD, VgrG, TssB, TssC, TssE). The last category contains proteins for which no function can be inferred from in silico analyses (TssA, TssF, TssG, TssK).

When cultivating *Janthinobacterium* sp. SLB01 we experimentally observed biofilms and floc formation into the cell cultures of primmorphs of *L. baicalensis* (unpublished data). This bacteria make floc and strong biofilm also in the stationary phase. This process requires exopolysaccharide biosynthesis, but in recent study made clear: both widespread PEP-CTERM proteins and exopolysaccharides are required for floc formation [44]. For all required gene clusters for floc formation we've found the respective homologs into *Janthinobacterium* sp. SLB01 genome (Figure 3). Floc formation can affect negatively on breathing, nutrients acquisition and waste products removal of the host (sponge *L. baicalensis*) because of clogging the pores. Negative effect of biofouling for functioning of the filter-feeding sponge *Halisarca caerulea* is studied in [45].

Flavobacterium sp. SLB02 – Gram-negative, opportunistic bacteria of phylum *Bacteroidetes*, well-known fish pathogen [25]. As many of *Bacteroidetes* it has specific polysaccharide utilization loci (PULs). After release into GenBank its genome has been analyzed by PULDB [38]. The large number of annotated PULs (in PULDB) let us analyze their composition and compare it with other genomes of *Flavobacterium* family. We predicted some of the polysaccharides, which *Flavobacterium* sp. SLB02 can digest by comparing annotated PULs with the literature-derived data stored in PULDB [38]. Ten of 45 found PULs have strong homology (according to PULDB reports) with *Flavobacterium johnsoniae* UW101 (see Figure 5). According to study [39] *Flavobacterium johnsoniae* UW101 can digest polysaccharides including chitin, starch, α-glucan, pectin and hemicelluloses: xylans, mannans, and xyloglucans. Utilization of cell walls fragments clears the surrounding area, including for T6SS activity of *Janthinobacterium* sp. SLB01.

The named two strains have differences in carbon acquisition. We analyzed the feature counts for carbohydrates and other carbon sources metabolism subsystem using RAST SEED [46] and found out that the ratio of carbon acquisition genes is rather different for many enzymes. We listed the pathways (or subsystems in SEED) with significant (when one strain have genes, but second does not) differences in Table 3. Its almost half of total pathways number. We suggest that *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02 have little or no competition for simple sugars, organic acids and polysaccharides as the carbon sources.

There are lot of separate studies for each of named strains in freshwater niche: *Janthinobacterium* can live in cold condition [47–50]; *Flavobacterium* its well-known fish pathogen [23,25] and is component of activate sludge [51–53]. We see here that named two strains related in different phyla and have entirely different life style: virulence mechanism, digested polysaccharides and carbohydrates (feeding). But they probably act together and that's why dominate in the microbial community.

This study is the first step to understanding the role of microbial community in of *L. baicalensis* freshwater sponge disease. The results of this study will help broaden our understanding about symbiotic relationships in microbial consortium during mass mortality freshwater Baikal sponges.

4. Materials and Methods

4.1. Bacterial Strains, Growth Media, DNA Extraction

In this study, two strains were isolated from sample of diseased sponge *L. baicalensis* (collected in the Lake Baikal located at the Central Siberia, Russia). Violet-pigmented *Janthinobacterium* sp. SLB01 and yellow-pigmented *Flavobacterium* sp. SLB02 strains were isolated on Luria–Bertani (LB) broth medium agar plates (diluted 1/10, temperature 15 °C).

Genomic DNA was isolated following standard bacterial DNA Isolation CTAB Protocol (http://www.jgi.doe.gov). The sequence library was generated from DNA using an Illumina Nextera XT DNA sample preparation kit. Whole-genome sequencing was performed using Illumina MiSeq platform with paired-end chemistry (2 x 250 bp).

4.2. Genome assembly and annotation

Draft assembly was built using SPAdes version 3.11.0 [54] with default settings, raw reads error correction and filtering with built-in BayesHammer module (quality threshold 98%). The resulting contigs were ordered with Ragout version 2.2 with default settings (https://github.com/fenderglass/Ragout) [36].

Genome completeness analysis made with BUSCO v. 3.1.0 and default settings using datasets: "proteobacteria_odb9" with 221 BUSCOs for *Janthinobacterium* sp. SLB01 and "bacteroidetes_odb9" with 443 BUSCOs for *Flavobacterium* sp. SLB02 [55].

Annotation made with NCBI Prokaryotic Genome Annotation Pipeline, PGAP [56], some genes were re-annotated with BLAST against Swiss-Prot database and protein sequences of closely related species.

4.3. In Silico Analysis of Type VI Secretion System Loci

A genome wide analysis was performed in this study to reveal the veil of T6SS in the *Janthinobacterium* sp. SLB01. The components and location of T6SS homologs in *Janthinobacterium* sp. SLB01 were determined by SecReT6 (http://db-mml.sjtu.edu.cn/SecReT6/, mode T6SS-HMMER) integrated database with default settings [57].

4.4. Genome subsystems

We analyzed the subsystems of *Janthinobacterium* sp. SLB01 and *Flavobacterium* sp. SLB02 by RAST SEED (http://rast.nmpdr.org/) with default settings [46]. Detailed reports are available upon request.

Violacein synthesis genes (VioABCDE operon) were annotated by NCBI PGAP and verified by BLAST against protein sequences from Swiss-Prot database.

Genes encoding PEP-CTERM proteins were partially annotated by NCBI PGAP. We used gene list from floc formation study [44] and found required homologs manually using UGENE [58].

4.5. Polysaccharides utilization loci analysis

Genome of *Flavobacterium* sp. SLB02 was released in NCBI in 2019. Maintainers of PULDB [38] added this genome to the database and analyzed it by fully automated pipeline for PUL prediction using genomic context and domain annotation [59]. Detailed report available at http://www.cazy.org/PULDB/index.php?sp_name=Flavobacterium+sp.+SLB02.

To compare 45 detected PULs into *Flavobacterium* sp. SLB02 genome with literature-derived data we performed the similarity search using PULDB build-in function. Hits with highest score were then analyzed manually.

Author Contributions: Conceptualization, S.B. and L.C.; methodology (bacterial culture), L.C.; software, I.P. formal analysis, S.B., I.P.; investigation, S.B., I.P. and L.C.; data curation, I.P.; writing—Original draft preparation, I.P., S.B., and L.C.; writing—Review and editing, I.P., S.B., and L.C.; visualization, I.P.; supervision, S.B. and L.C.; funding acquisition, S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research and article processing charge was funded by Russian Science Foundation, grant number 19-14-00088. Sample collection of diseased sponges was carried out within the framework of Siberian Branch, Russian Academy of Sciences basic budget funding from No 0345-2019-0002 (AAAA-A16-116122110066-1).

Acknowledgments: The bioinformatics data analysis was performed in part on the equipment of the Bioinformatics Shared Access Center, the Federal Research Center Institute of Cytology and Genetics of Siberian Branch of the Russian Academy of Sciences (ICG SB RAS).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

BUSCO	Benchmarking Universal Single-Copy Orthologs
CTERM	C-terminal
EPS	Exopolysaccharides
PGAP	Prokaryotic Genome Annotation Pipeline
PEP	Pro-Glu-Pro
PUL	Polysaccharides utilization loci
T6SS	Type VI Secretion System

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 doi:10.20944/preprints202009.0144.v1

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