Article

Integrated into environmental biofilm *Chromobacterium vaccinii* survives winter with support of bacterial community

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Abstract: *Chromobacterium* species are common in tropical and subtropical zones in environmental water samples and believed to «tropical» species. Here we describe an environmental case of resident *Chromobacterium vaccinii* in biofilms associated with *Carex spp.* roots in Moscow region, Russia (warm-summer humid continental climate zone). We performed broad characterization of individual properties as well as surrounding context for better understanding the premise of *C. vaccinii* survival during winter season. Genome properties of isolated strains propose some insights into adaptation to habit and biofilm mode of life, including social cheaters carrying *ΔluxR* mutation. Isolated *C. vaccinii* differs with previously described strains in some biochemical properties and some basic characteristics like fatty acid composition as well as unique genome features. Despite potential to modulate membrane fluidity and presence of several genes responsible for cold shock response, isolated *C. vaccinii* didn`t survive during exposure to 4 °C, while in initial complex biofilm it was able to survive for months *in vitro* at 4 °C. Surrounding bacterial community within the same biofilm with *C. vaccinii* represented a series of psychrophilic bacterial species which may share resistance to low temperatures with other species within biofilm and provide *C. vaccinii* opportunity to survive during cold winter season.

Keywords: *Chromobacterium vaccinii*; biofilm; sharing goods; social cheater; bog microbiome; bacterial genome, violacein, cold adaptation, IDBac, QS mutant

1. Introduction

Biofilms and microbial mats are an important part of any ecosystem and one of the main biotic factors with a dramatic impact in metabolic processes and influence on other organisms' habits and species diversity (plants, insects, protozoans, and others). Bacterial species composition of biofilms both orchestrates and reflects variation in the ecological context: pollution, climate change, other direct and indirect anthropogenic influences as well as metabolic capacity and bioremediation processes. Despite awareness about importance of bacterial abundance and biogeography the worldwide distribution of bacterial species and their adaptation to different ecological niches remains pure explored [1,2]. While clinical cases of different bacterial species isolation and description are broadly appreciated in clinical microbiology, «environmental cases» in microbial ecology and biogeography are limited. Only a few reports describe environmental *Chromobacterium* species

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isolation in Europe: *Chromobacterium violaceum* in Poland (from *Ixodes ricinus* ticks) and an evidence of *C. vaccinii* isolation from bog in Russia [3,4]. Moreover, in cases of infections in Europe *Chromobacterium* species were discussed in a prism of global warming, but primary sources of infection were not identified, and natural reservoirs of *Chromobacterium* species in Europe remain unexplored [5]. While members of *Chromobacterium* genus are still considered as tropical/subtropical bacteria with poor viability at low temperatures, this is still questionable if changing environment and other processes affect worldwide distribution of 'tropical" *Chromobacterium* species or an abundance of these species are underestimated in temperate climate zone. Even though some species of the genus were isolated from complex communities like rhizosphere and root-associated biofilms, most of these evidences were associated with aquatic environment and some species were found in water samples [6–11]. Freezing water and ice coverage might provide significant stress to *Chromobacterium* species in natural reservoirs in case of climate zone with winter temperature below zero.

Members of *Chromobacterium* genus are known as producers of violacein, deoxyviolacein, cyanide, extracellular chitinase and some other active compounds. These metabolites might have high environmental significance due to a broad range of biological activities: antibacterial activity against both planktonic and biofilm form of gram-positive bacteria, insecticidal, antiprotozoal, possible antiviral, and fungicidal features [11–16]. Moreover, production of at least one of them – violacein, is higher in the biofilm mode of life and upregulated by intra- and interspecies quorum-sensing signals that rise a question about an existence within multispecies biofilms in natural ecosystems [17,18]. A broad spectrum of activities provides competition advantages for *Chromobacterium* species and might promote niche partitioning in their presence, but little is known about life of *Chromobacterium* species in non-optimal habits like climate zones with cold seasons.

Altogether this data suggests an importance of deeper understanding of biogeography, ecology, and adaptability of *Chromobacterium* species to their habits and surrounding communities.

Here we describe the case of *C. vaccinii* isolation from quaking bog rhizospheres' biofilms in European part of Russia (Df2b climate zone), provide characteristics of isolates and surrounding bacterial community and describe naturally occurring QS mutant proposed as an example of social exploitation of community goods and represent evolutionary pressure on social cheating within biofilm [19].

2. Materials and Methods

Samples collection and processing. Sediment samples, root-associated biofilms of *Carex spp.*, sphagnum moss and water samples were collected in triplicates. For sediment samples we scooped sediments directly into sampling plastic tubes (V=15 ml). Water samples were collected into sampling plastic tubes (V=15 ml) for microbiology observation and in 3 L glass bottles for water quality analysis. Sphagnum moss fragments were directly placed into sampling plastic tubes (V=50 ml). For root-associated biofilms we unearthed *Carex spp.* plants at the border of water and sphagnum moss, scraped rizodermis with root-associated biofilms using sterile scalpels and then returned plant in its initial place. All samples were immediately transferred to laboratory at +4 C. For dissociation of microbial aggregated we vortexed samples at high speed for 5 min and then ten-fold diluted samples processed with conventional microbiology plating on the following solid mediums: LB, TSA, M9 salts with 1% tryptone, Nutrient agar, BHI and blood agar. Plates were incubated at 25 C for 48 hours.

Water quality analysis was performed in MSULab company (Moscow, Russia).

Species identification and metabolic association network. MALDI-TOF MS identification: we picked single colonies from solid medium and processed with MALDI-TOF MS Sample Preparation and Data Acquisition. For MALDI-TOF MS analysis, proteins were extracted by using an extended direct transfer method that included a formic acid overlay as described in [20] [21]. In brief: bacterial colonies were applied as a thin film onto a MALDI ground-steel target plate (Bruker Daltonics, Billerica, MA, US). Over each bacterial smear, 1 µL of 70% Optima™ LC/MS Grade formic acid (Fisher

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Chemical, Hampton, New Hampshire, US) was added and allowed to evaporate, followed by the addition and subsequent evaporation of 1 µL of 10 mg/mL α-cyano-4-hydroxycinnamic acid solubilized in 50% acetonitrile, 2.5% trifluoroacetic acid and 47.5% water. All solvents were HPLC or MS grade.

Measurements were performed using an UltrafleXtreme mass-spectrometer (Bruker Daltonics, Billerica, MA, US) equipped with a smartbeam™-II laser (355nm). Detailed instrument settings are available in table 2. Natural products spectra were recorded in positive reflectron mode (2000 shots; RepRate:2000 Hz; delay: 8198 ns; ion source 1 voltage: 20 kV; ion source 2 voltage: 18.8 kV; lens voltage: 7.5 kV; mass range: 50 Da to 5,000 Da, matrix suppression cutoff: 50 Da). Protein spectra were recorded in positive linear mode (1200 shots; RepRate: 1000; delay: 29793 ns; ion source 1 voltage: 19.5 kV; ion source 2 voltage: 18.2 kV; lens voltage: 7.5 kV; mass range: 1.9 kDa to 22 kDa matrix suppression cutoff: 1.5 kDa). Protein spectra were corrected with an external Bruker Daltonics bacterial test standard (BTS). Natural products spectra were corrected with an external Bruker Daltonics peptide calibration standard and CHCA [2M+H]+ (379.0930 Da). Automated data acquisitions were performed using flexControl software v. 3.4.135.0 (Bruker Daltonics, Billerica, MA, US) and flexAnalysis software v. 3.4. Spectra were automatically evaluated during acquisition to determine whether a spectrum was of high enough quality to retain and add to the sum of the sample acquisition.

For species identification we used both conventional database from Biotyper® (Bruker Daltonics, Billerica, MA, US) and recently introduced automatic IDBac algorithm [22]. Identification was validated in selective manner through Sanger sequencing of *16S rDNA* amplicons obtained with 27F/1294R primers.

Sanger sequencing. 16S rDNA amplicons obtained with 27F/1294R primers were sequenced according to the protocol of BigDyeTerminator 3.1 Cycle Sequencing kit for the Genetic Analyzer 3500 Applied Biosystems (Waltham, Massachusetts, US). The electrophoretic DNA separation was performed in 50-cm capillaries with POP7 polymer.

16S rDNA bacteriome analysis. Total DNA was isolated from 1 ml of root-associated biofilm using ZymoBIOMICS DNA Kit (Zymo Research, Irvine, CA, US). Fragments of 16SrDNA gene containing V1-V4 hypervariable regions (max 753 bp) were amplified and used for preparation of paired-end libraries according to the KAPA HyperPlus (Roche, Basel, Switzerland) protocol. Libraries were checked with High Sensitivity DNA Chips on a 2100 Bioanalyzer System (Agilent, Santa Clara, CA, US) and sequenced on NextSeq 500 (Illumina, San Diego, CA, US) with NextSeq 500/550 High-Output Kit v2.5 (300 cycles). NGS data are available in GenBank: Bio Project PRJNA635774.

Two approaches were used for the data analysis. 1) The Microbial Genomics Module of CLC Genomic Workbench v.20.0.4 (QIAGEN, Germantown, MD, US) was used with default settings to perform Operational Taxonomic Unit clustering. Greengenes database v.13_8 (Second Genome, Brisbane, CA, US) was used as reference with 97% threshold. 2) SPAdes v.3.13.0 (St. Petersburg genome assembler, Russia, URL: http://cab.spbu.ru/software/spades/) was used for assembling the contigs, which were identified by BLAST NCBI with rRNA/ITS databases [23]. Coverage of contigs of the same taxonomic units was summarized and served as an indicator of the taxon abundance. Diagram was created by Microsoft Excel (Microsoft, Redmond, Washington, US).

Phylogenetic analysis for the Proteobacteria fragments of 16S ribosomal RNA gene was performed in MEGA 6.0 [24]. The evolutionary history was inferred using of the Neighbor-Joining method [25]. The distances for phylogeny reconstruction were computed using of the Kimura 2-parameter [26], and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.25). The analysis involved 70 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1654 positions in the final dataset. Bootstrap analyses were performed with 1000 replicates [27].

Whole genome sequencing and annotation. DNA from bacterial cells of pigmented GIMC1602:ChrSima_v (ChrSV) and unpigmented GIMC1601:ChrSima_w (ChrSW) strains was isolated by Wizard Genomic DNA purification kit (Promega, Madison, Wisconsin, US). The KAPA HyperPlus (Roche, Basel, Switzerland) protocol was used for the libraries preparation. Sequencing was performed on NextSeq 500 (Illumina, San Diego, CA, US) with NextSeq 500/550 High-Output Kit v2.5 (300 cycles). CLC Genomic Workbench v.20.0.4 and SPAdes v.3.13.0 were used for genome assembling. CGView Server (http://stothard.afns.ualberta.ca/cgview_server/) was applied for the visualization of assembling results and for the genomes comparison [28]. The software Rapid Annotations Subsystems Technology (RAST) and SEED were used for genome annotation [29,30]. The conserved domains of the proteins were searched complementary by the services KEGG (http://www.genome.jp/kegg), KEGGOC (http://www.genome.jp/tools/oc), COGs (http://www.ncbi.nlm.nih.gov/COG). Prophage sequences were revealed with help of PHASTER (PHAge Search Tool Enhanced Release, https://phaster.ca/) [31].

WGS data are available in GenBank: Bio Project PRJNA597450. Accession Numbers of the chromosome and plasmid are CP060046 and CP060045 for GIMC1602:ChrSima_v, CP060044 and CP060043 for GIMC1601:ChrSima_w.

Whole genome-based taxonomic analysis was made by the Type (Strain) Genome Server (TYGS), a free bioinformatics platform of DSMZ, which is available under https://tygs.dsmz.de [32].

Biosynthetic gene clusters were predicted by antiSMASH v.5.1.2 available under https://antismash.secondarymetabolites.org/ [33]. This resource allows the rapid genome-wide identification, annotation, and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genomes.

Biochemical and growth properties characterization. Biochemical properties of *C. vaccinii* isolates were characterized using NEFERMtest 24 (Erba Lachema, Brno, Czechia) and ENDOtest (Erba Lachema, Brno, Czechia). For assessment of oxidase production, we used Microbact Oxidase Strips (Oxoid, Cheshire, England). Due to bacterial pigment interferences with color development we used: (a) early bacterial culture (before visible pigment production); (b) additional method for pigmented bacterial culture: a piece of filter paper was soaked one side in bacterial culture then placed on test strip soaked side up and removed after 3 min exposure.

For evaluation of pH effects on growing properties TSB medium was adjusted to pH in a range from 3,0 to 9,0 with step 1,0 and then inoculated with *C. vaccinii*. For evaluation of salinity effect, TSB medium w/o NaCl was supplemented with NaCl to 0-10 % with step 0,5% and then inoculated with *C. vaccinii*. We accessed growing properties through optical density of bacterial cultures at 600 nm after 48 h incubation.

For assessment of viability under different temperatures we divided stationary phase bacterial culture into 1 ml parts and incubated at 25 C, 4 C and on ice. After desired periods of incubation serial dilutions of bacterial culture were plated on TSA and incubated at 25 C for 48 hours for CFU counting.

Mixed colony biofilm was prepared by mixing of equal amounts of overnight cultures, 10 mkl of bacterial mixture was spotted on TSB. Plates were incubated at 25 C for 48 hours.

For analysis of membrane fluidity adaptation to different temperatures we grew up *C. vaccinii* on TSA at 25 C during 24 h, then changed cultivation temperature for the next 48 h and finally proceeded to fatty acid analysis.

Fatty acid composition and lipid A structure analysis. Fatty Acid Methyl Esters (FAME) were prepared as described previously with some modification [34]. Briefly, bacterial pellets were resuspended in solution for saponification (300 mkl) and incubated at 100°C for 30 min. After incubation, HCl-MeOH solution (600 mkl) was added and heated at 80°C for 10 min. Then FAME were extracted by 10 min mixing with 600 mkl Hexane-MTBE (600 mkl). The top phase was transferred into a new vial and used for analysis through GC-MS.

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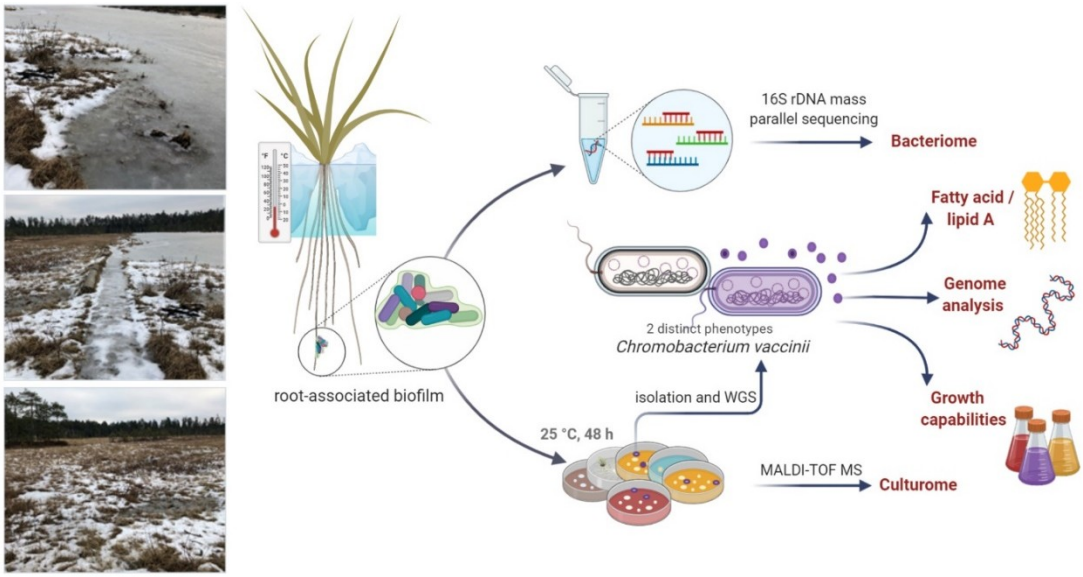
GC-MS analyses were carried out using Agilent 7820A (Agilent technologies, Santa Clara, CA, US) gas chromatograph with a Maestro MS detector (Interlab, Moscow oblast, Russia) with 30 m x 0.25 mm i.d. capillary column Rxi-5ms (Restek, Bellefonte, PA, US). The injection volume was 1 µl, with split ratio 10:1 splitting gas-carrier. Injector and interface temperatures were 250°C and 280°C, respectively. The temperature program for the column started at 125°C for 0.5 min, and then risen up to 280 at 5°C /min and then to 320°C at 20°C /min, the end temperature was held for 2 min. Electron impact (EI) spectra were obtained under 70 eV ionization voltage and 150°C source temperature. Registration of spectra was performed through a full scan at 40-550 Th mass range.

Post-run analysis was performed with the following software: Agilent Mass Hunter Unknown Analysis (Agilent Technologies, Santa Clara, CA, US), Enhanced Data Analysis (Agilent Technologies, Santa Clara, CA, US), NIST MS Search 2.2 (NIST, Gaithersburg, MD, US), and Microsoft Excel (Microsoft, Redmond, Washington, US). Equivalent chain-lengths of methyl ester derivatives of fatty acids were calculated as described [35].

Electron microscopy. For phase-contrast electron microscopy bacterial colonies were scraped off from plates, washed with sterile water three times and fixed in 10% neutral formalin. Samples were placed on silicon substrates that were fixed to aluminum stubs using double-sided carbon adhesive tape and sputtered with gold (gold layer thickness 5 nm) in the SPI-MODULE Sputter Coater (SPI Supplies, West Chester, PA, US). Scanning electron microscopy (SEM) was carried out in high vacuum mode at an accelerating voltage of 10 kV. dual-beam ion-scanning electron microscope Quanta 200 3D (FEI Company, Hillsboro, Oregon, US). Transmission electron microscopy of OMVs was carried out with negative staining with uranyl acetate with JEM-2100 200 kV analytical electron microscope (JEOL, Tokyo, Japan).

3. Results

Overall workflow of the current study presented on Figure 1.



(a) (b)

Figure 1. General description of the study design. (a) Photography of the location at the day of sampling. (b) Sample processing workflow. Created with BioRender.com

3.1. Quaking bog description

Quaking bog Volkovskoye with abandoned quarry Sima developed over a lake approximately 7000 years ago in Moscow region, Russia (55°40'09.2"N 36°42'44.4"E). According to Köppen climate

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classification this is Dfb (warm-summer humid continental) climate zone. Quarry Sima used to exploitation of peat in 18-19 centuries [36]. Nowadays Volkovskoye is a relatively small bog – approximately 90000 square meters in total with open-water region less than 10000 square meters (maximum depth 1,5 m), surrounded by fir-tree forest. The major genus of higher plants on the edge of sphagnum moss and open water - *Carex spp*. The bog is outlined by a stretch of birches and pines. Floating mat of sphagnum moss has a thickness around 40 sm. Analysis of water chemical properties and composition confirms low salt concentration which is a common characteristic for quaking bogs (Table 1).

Table 1. Water quality analysis.

General characteristics		
Turbidity	2,37	OD530nm
Chromaticity	36,9	°
Odour	0	grade 0-5
pH	5,68	pH units
Hardness	<0,060	mg-CaCO3/L
Chemical oxygen demand	22,2	mg/L
H2S	<0,002	mg/L
Petroleum products	0,048	mg/L
Free alkalinity	<0,1	mM/L
Total alkalinity	<0,1	mM/L
Sulfide minerals	<0,002	mg/L
Dry weight	7,37	mg/L
Conductivity	11	mkS/sm

Cations		
Mg2+	0,26	mg/L
Ca2+	0,66	mg/L
Fe2+	0,154	mg/L
K+	0,43	mg/L
Na+	0,66	mg/L
Al3+	0,051	mg/L
[NH4]+	0,38	mg/L
Li+	<0,001	mg/L

Anions		
[SO4]-	1,21	mg/L
[Cl]-	1,14	mg/L
[NO]-	0,419	mg/L
[HCO3]-	<6,1	mg/L
[CO3]2-	<6,0	mg/L
[NO2]-	<0,1	mg/L
[Br]-	<0,05	mg/L
[PO3]-	<0,1	mg/L
[F]-	0,159	mg/L

Heavy metals		
Hg	<0,00001	mg/L
V	<0,0001	mg/L
Ba	0,003	mg/L
Be	<0,0001	mg/L
B	<0,05	mg/L
Mo	<0,0001	mg/L
Co	<0,0001	mg/L
Ag	<0,0001	mg/L
Zn	0,01	mg/L
Ni	<0,0001	mg/L
Si	0,556	mg/L
Cr	<0,0001	mg/L
Sr	0,003	mg/L
Cd	<0,0001	mg/L
As	<0,0001	mg/L
Cu	0,002	mg/L
Pb	<0,0001	mg/L

3.2. Individual properties of Chromobacterium vaccinii

3.2.1. Isolation and species-level identification. C. vaccinii was found during investigation of biofilms composition in quaking bog Volkovskoye. Biofilm samples were collected in triplicates at winter season (in January 2020; average daytime temperature -7°C (20°F), night -13°C (9°F)) then bog was covered with ice and water temperature was 2-4 C. While previously we have already found *Chromobacterium* spp. at the same bog during summer season (August, 2019), that isolates didn`t survive neither at temperature 4-8 C during short (few days) storage on plates with solid mediums (BHI, TSA, LB) nor during deep freezing in storage medium supplemented with glycerol (data not shown). That was the premise to collect samples during cold season and exclude possibility of transient occurrence of "tropical" bacterial species carried by reservoir birds or insects during migration.

After collection and transportation sediment samples, root-associated biofilms of *Carex spp*., sphagnum moss and water samples bacteria were grown on the following solid mediums: LB, TSA, M9 salts with 1% tryptone, Nutrient agar, BHI and blood agar. After incubation at aerobic condition, 25 C for 48 hours we have isolated 18 circulars, smooth, raised colonies with entire margins and deep violet pigmentation from all root-associated biofilms of *Carex spp*., but neither from water samples nor from sphagnum moss. Single CFU with the same properties was detected in one sediment sample. Identification of isolated colonies by MALDI Biotyper™ (Bruker, Billerica, MA, US) resulted in probable genus identification with a score no more than 1.71 for *Chromobacterium spp.* At the time of identification number of specters in the database related to *Chromobacterium* genus were restricted with only two species - *C. violaceum* and *C. substugae*. Also 3 unpigmented colonies of *Chromobacterium spp.* were found in root-associated biofilms of *Carex spp*.

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To confirm belonging to *Chromobacterium* genus we performed *16S rDNA* sequencing for all isolates. Identification based on *16S rDNA* sequence analysis resulted in more than 99 % similarity to *C. violaceum*, *C. vaccinii* and *C. piscinae* species. Due to high homology between these *Chromobacterium* species *16S rDNA* sequence analysis was not enough for secure species identification. So we performed WGS (whole genome sequencing) for one pigmented and one unpigmented isolate assigned as isolates GIMC1602:ChrSima_v (ChrSV) and GIMC1601:ChrSima_w (ChrSW) respectively. Assembled WGS data were used for determination of the closest type strain genome with help of the TYGS [32]. The TYGS database includes type strains of the eight *Chromobacterium* species: *C. amazonense* DSM 26508, *C. haemolyticum* DSM 19808, *C. phragmitis* IIBBL 112-1, *C. phragmitis* IIBBL 112-1, *C. pseudoviolaceum* LMG 3953, *C. sphagni* IIBBL 14B-1, *C. subtsugae* PRAA4-1, *C. vaccinii* MWU205, *C. violaceum* ATCC 12472. The digital DNA-DNA hybridization (dDDH) value was the highest for the pairs ChrSV - *C. vaccinii* MWU205 and ChrSW - *C. vaccinii* MWU205: 87.5 % and 87.4% respectively with confidence intervals (C.I.) 84.0 - 90.3% and 83.9 - 90.2% (Supplementary Figure S1). The type *C. vaccinii* strain MWU205 was initially isolated from cultivated cranberry bogs in Cape Code, Massachusetts [6,7].

3.2.2. General phenotype characterization. In order to obtain a broad description of phenotype we performed electron microscopy imaging, biochemical characterization, growth properties and analysis of fatty acids contains, including lipid A structure of LPS. Phase-contrast microscopy of pigmented and unpigmented isolates showed coccobacillus with single polar flagella and significant amount of OMVs for both isolates (Figure 2a). TEM imaging of OMVs showed electron-dense material inside some of the vehicles (Figure 2b).

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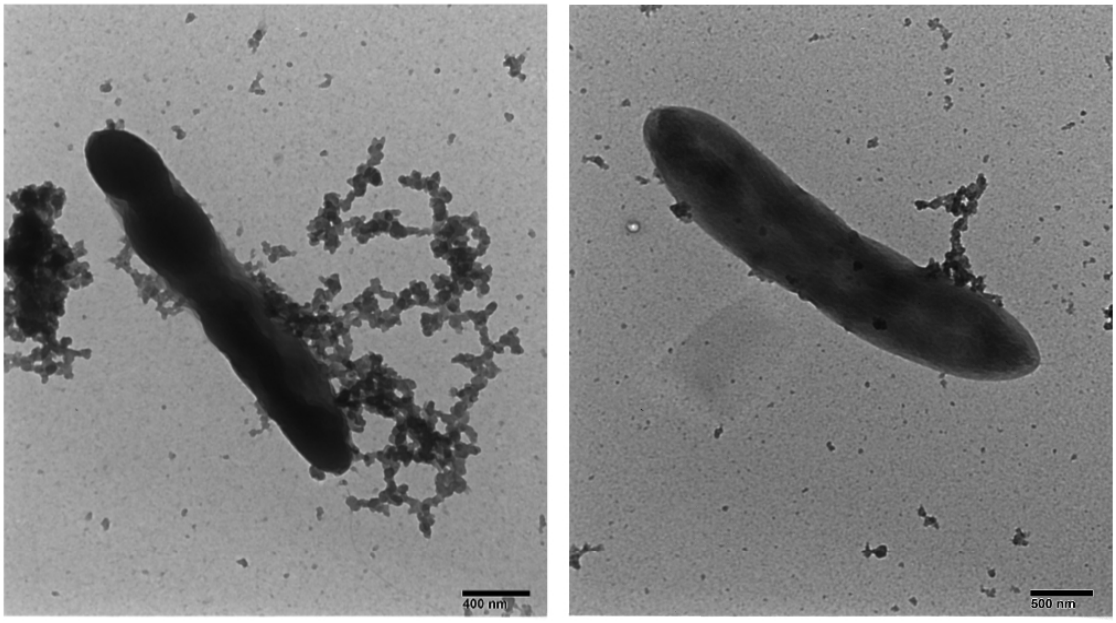
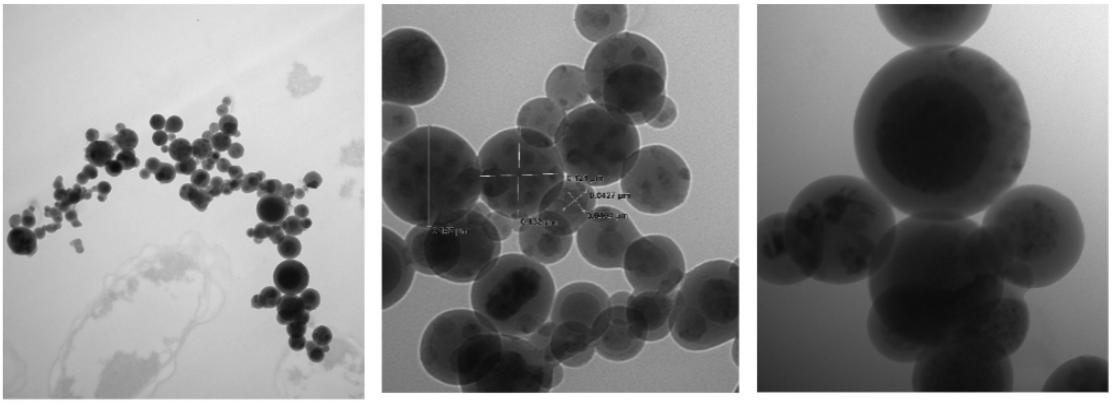


(a)



(b)

Figure 2. Electron microphotography of isolated *C. vaccinii*. (a) Phase-contrast microscopy of *C. vaccinii* pigmented strain (left) and unpigmented strain (right) (b) TEM of OMVs produced by *C. vaccinii* pigmented strain.

Biochemical characterization of isolates was different from previously described features (Table 2).

Table 2. Biochemical properties of *C. vaccinii* isolated strains.

Characteristic	Fermentation or number of +/- isolates if variable
oxidase	-
indole production	-
bGL	-
NAG	16/18
SCI	16/18
LAC	-
MAN	1/18
TRE	+

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XYL	**5/18**
ARA	**3/18**
aGA	**-**
bGA	**-**
MAL	**-**
GAL	**1/18**
MLT	**-**
CEL	**-**
SUC	**2/18**
INO	**-**
GGT	**+**
PHS	**+**
ESL	**-**
H2S	**-**
MAL	**-**
ONP	**-**
SAL	**-**
SOR	**-**
MLB	**-**
GLP	**-**
DUL	**-**
ADO	**-**
ART	**-**
RAF	**-**
bXY	**-**
NaCl, % range	**0-3**
pH range	**4,0-8,0**
pigmentation	**3/18**

+ = all tested isolates were positive; - = all tested isolates were negative; numbers represent positive isolates from total number of tested isolates.

The most significant and valuable difference in terms of general species description was negative oxidase reaction for all isolates. We confirmed negative results of oxidase test with two different approaches – using early bacterial culture (before development of pigmentation) and using modified for pigmented strains method. Negative oxidase reaction is not common for members of Neisseriacea, but for a minor part of *Chromobacterium* isolates was previously mentioned [37]. Rather than cytochrome c oxidase other ferments might be responsible fot the aerobic respiration chain. Genome analysis of isolates *C. vaccinii* predicted cytochrome c oxidase operon (locus tags ChrSV_0681 -0685), cytochrome o ubiquinol oxidase operon (*cyoA, cyoB, cyoC, cyoD, cyoE* - locus tags ChrSV_1078-1082) and two copies of cytochrome d ubiquinol oxidase operon (*cydA, cydB*, locus tags ChrSV_1410 -1411 and ChrSV_3076-3077). The last one may enhance tolerance to oxidative and nitrosative stress in some bacterial species [38]. Fermentation tests of all isolates were positive for gamma-glutamyltransferase, arginine dehydrolase, phosphatase and fermentation of trehalose. Not all, but the most isolates were positive for N-Acetyl-beta-D-Glucosaminidase and Simmon`s citrate test. Some other infrequent biochemical features among isolates included utilization of ornithine, lysine, mannitol, xylose, arabinose, galactose and sucrose. Isolates were able to grow up at broad a range of pH and salinity.

We performed conventional fatty acid methyl ester (FAME) characterization of our isolates as the key features of microbial species characterization. FAME profile for our type isolate ChrSV in comparison with other characterized species is presented in Table 3 and FAME for different isolates in this study presented in Supplementary Table S1.

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Table 3. Fatty acid compositions of *Chromobacterium* species.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
11:0	**–**	–	0.2	–	–	–	–	–	–	0.2	–	–	–	–
10:0 3OH	**1.8**	3.2	3.4	4.3	4.7	2.9	3.7	1.5	3.2	4.6	3	2.2	5.1	2.4
12:0	**2.4**	3.8	3.8	5.0	3.9	4.0	4.2	9.7	3.3	8.8	3.2	3.1	4.9	3.3
11:0 3OH	**–**	–	–	–	–	–	–	–	–	–	–	–	0.4	–
13:0	**–**	–	–	–	–	–	–	–	–	–	–	–	0.4	–
12:0 2OH	**1.8**	1.9	2.0	2.9	2.4	1.4	2.3	–	1.9	0.2	1.9	1.6	3.3	1.7
12:0 3OH	**2.8**	3.3	3.4	4.0	3.6	2.5	2.9	1.4	2.9	4.4	2.8	2.5	4.8	2.6
13:0 2OH	**–**	–	–	–	–	–	–	0.4	–	–	–	–	–	–
14:1 w7c	**0.4**	–	–	–	–	–	–	–	–	–	–	–	–	–
14:1 w5c	**0.1**	0.2	0.2	0	0.4	–	0.3	0.4	–	–	0	0.2	–	–
14:0	**2.2**	2.3	2.1	3.2	2.5	2.3	3.3	4.0	2	2.6	3.1	2.5	3.5	2.0
15:0 iso	**–**	–	–	–	–	–	–	0.5	–	–	–	–	–	–
15:0 iso G	**–**	–	–	–	–	–	–	0.7	–	–	–	–	–	–
15:1 w8c	**0.1**	–	–	–	–	–	–	–	–	–	–	–	–	–
15:1 w6c	**0.2**	–	–	–	–	–	–	–	–	–	–	–	–	–
15:0	**1.0**	–	–	1.3	–	–	0.9		1	0.6	2.3	–	3.0	–
16:1 w7c	**43.7**	42.7	41.9	41.9	47.1	42.5	34.1	38.6	38.9	33.4	28.7	38.5	27.5	36.3
16:1 w5c	**0.4**	0.3	0.3	0.3	0.5	–	–	–	–	0.3	0	0.2	–	–
16:0	**28.4**	28.4	29.6	25.0	24.0	27.3	26.1	29.7	30.2	25.8	32	31.5	26.6	28.5
17:1 w6c	**–**	–	–	0.2	–	–	0	–	0.2	–	–		0.4	–
17:0 CYCLO	**0.2**	0.4	–	–	0.4	–	2.9	–	–	–	13.2	0.2	4.3	1.3
18:2 w6,9c	–	–	–	–	–	–	4.2	–	–	–	–	–	–	–
18:1 w9c	–	–	–	–	–	–	2	–	–	–	–	–	–	–
18:1 w7c /12t/9t	**12.5**	13.1	12.6	10.6	10.3	12.0	12.3	5.5	15.7	18.8	8.7	15.9	14.8	19.3
18:0	**1.8**	0.4	0.5	–	0.2	0.6	0.4	1.6	0.5	0	0.6	0.5	0.3	0.4
SFA/MUFA*	**0.6**	0.6	0.7	0.7	0.5	0.6	0.7	1.0	0.7	0.7	1.1	0.7	0.8	0.6
Hydroxy FA**	**6.4**	8.4	8.8	11.2	10.7	6.8	8.6	3.3	8	9.2	7.7	6.3	13.6	6.7

1, GIMC1602:ChrSima_v strain in this study; 2, MWU300-*C. vaccinii* [7]; 3, MWU205-*C. vaccinii* [7]; 4, PRAA4-1 - *C. subtsugae* [39]; 5, IIBBL 14B-1 *C. sphagni* [6]; 6, CCUG 53230 *C. haemolyticum* [40]; 7, ATCC 12472 *C. violaceum* [39]; 8, LAM1188 *C. rhizoryzae* [8]; 9, DSM 170043 - *C. subtsugae* [39]; 10, CC-SEYA-1 - *C. aquaticum* [41]; 11, LMG 3947- *C. piscinae* [42]; 12, IIBBL 112-1 *C. phragmitis* [43]; 13, LMG 3953 C. *pseudoviolaceum* [42]; 14, CBMAI 310T *C. amazonense* [44]. *saturated FA/monounsaturated FA; **summed hydroxy fatty acids

According to the FAME analysis, the predominant fatty acids (FA) were C16: 1ω7c (43.7%), C16 : 0 (28.4%) and C18 : 1ω7c (12.5%). The fatty acids profile of GIMC 1602 was different from any previously reported data. The close resemblance was found in C16 fatty acids contain between GIMC1602:ChrSima_v, *C. vaccinii* (MWU 300, MWU 205), *C. subtsugae* PRAA4-1, *C. sphagni* IIBBL 14B-1 and *C. haemolyticum* CCUG 53230. Some differences were found in hydroxy fatty acids content and other minor fatty acids. Namely, GIMC1602:ChrSima_v has a low level hydroxy fatty acid and contains 14:1 and 15:1 FA in contrast to other *C. vaccinii* strains (MWU 300, MWU 205). To determine if hydroxy fatty acid belongs to LPS or not we analyzed lipid A structure. Lipid A contained all 3 hydroxy fatty acids (Supplementary Figure S2) and its structure was the same as described for *C. violaceum* NCTC 9694 [45,46].

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3.2.3. Genome characterization and comparison with known strains of C. vaccinii. Genome analysis of the pigmented (ChrSV) and unpigmented (ChrSW) *C. vaccinii* strains demonstrated, that both genomes consist of chromosome (5278675 bp and 5273834 bp, respectively) and plasmid (45365 bp). Alignment of ChrSV and ChrSW chromosomes with help of the BLAST NCBI with Genome Data Base revealed the highest homology (99.27% identity and 90% coverage) with *C. vaccinii* strain 21-1 genome, Accession Number CP017707.1. This strain was isolated from bog in Beltsville, Maryland, USA, the place with humid subtropical climate. The chromosome of *C. vaccinii* strain 21-1 is less then chromosome of ChrSV by 237445 bp.

The ChrSV/ ChrSW genomes have the biggest number of prophages – 12 and 7 of them are intact. The genome of *C. vaccinii* strain 21-1 has only 6 prophages (4 intact), 5 of which are similar to prophages of ChrSV/ ChrSW genomes. Second *C. vaccinii* complete genome – genome of strain XC0014 (Accession Number CP022344.1) is even smaller, and has 6 prophages (5 intact), but only 2 of them are similar to prophages of ChrSV/ ChrSW genomes. It should be noted that if the first two strains were isolated from the bogs, so *C. vaccinii* XC0014 strain had another source of isolation: the soil in Zhejiang Province of China. However, the climate in Zhejiang is humid subtropical as in Beltsville, in contrast to the climate in the Moscow region.

The next difference between the ChrSV/ChrSW genomes and genomes of 21-1 and XC0014 is the presence of the plasmid (45365 bp). In the Microbial Genome Data Base of NCBI only 3 *Chromobacterium* genomes from 58 genomes with different level of assembling have plasmids: *C. violaceum* FDAARGOS_635 (CP050991.1, 42965 bp), *Chromobacterium sp.* IIBBL 112-1 (NZ_CP029496.1, 17589 bp), and *Chromobacterium. sp.* IIBBL 274-1 (NZ_CP029555.1, 74363 bp). The plasmid of the type strain of *C. violaceum* ATCC 12472 was submitted in GenBank separately (MG651603.1., 44212 bp) [47].

The ChrSV/ ChrSW plasmids had not similarity with the *Chromobacterium sp.* plasmids but were homologous to plasmids of both *C. violaceum* strains with coverage 83%: FDAARGOS_635 - 93.05% identity, ATCC 12472 - 92.51%. Note, that *C. violaceum* strains were isolated from different sources: FDAARGOS_635 is clinical isolate (University of Louisville, US), and ATCC 12472 is freshwater isolate (Malaya, Malaysia).

So ChrSV/ ChrSW genomes are the biggest from known *C. vaccinii* genomes and differ in the presence considerable number of prophages (including intact prophages) and plasmid homologous to the plasmids of *C. violaceum* strains.

3.3. Regulatory nature of unpigmented isolate

We have isolated 3 unpigmented strains of *C. vaccinii* and performed whole genome sequencing for one of them - ChrSW. We identified full *vioABCDE* operon in genome sequence of unpigmented isolate that gave us a reason to hypothesize regulatory nature of absence pigmentation in ChrSW isolate. Regulation of violacein production depends on quorum-sensing (QS) signals [48]. Closer investigation of genome region responsible for LuxI/LuxR regulatory QS pathway revealed deletion of large DNA fragment including *luxR* sequence that made impossible classical positive-feedback regulation of LuxI/LuxR system in response to AHL (Figure 3).

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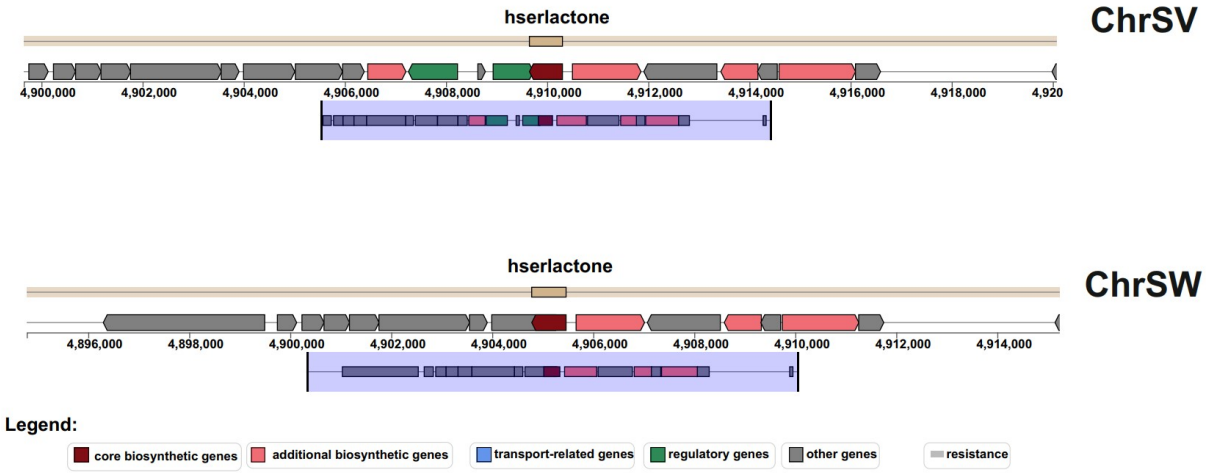

Figure 3. Biosynthetic gene cluster for homoserine lactone in natural pigmented (ChrSV) and unpigmented (ChrSW) isolate of *C. vaccinii.* The resource antiSMASH v.5.1.2 was used for prediction.

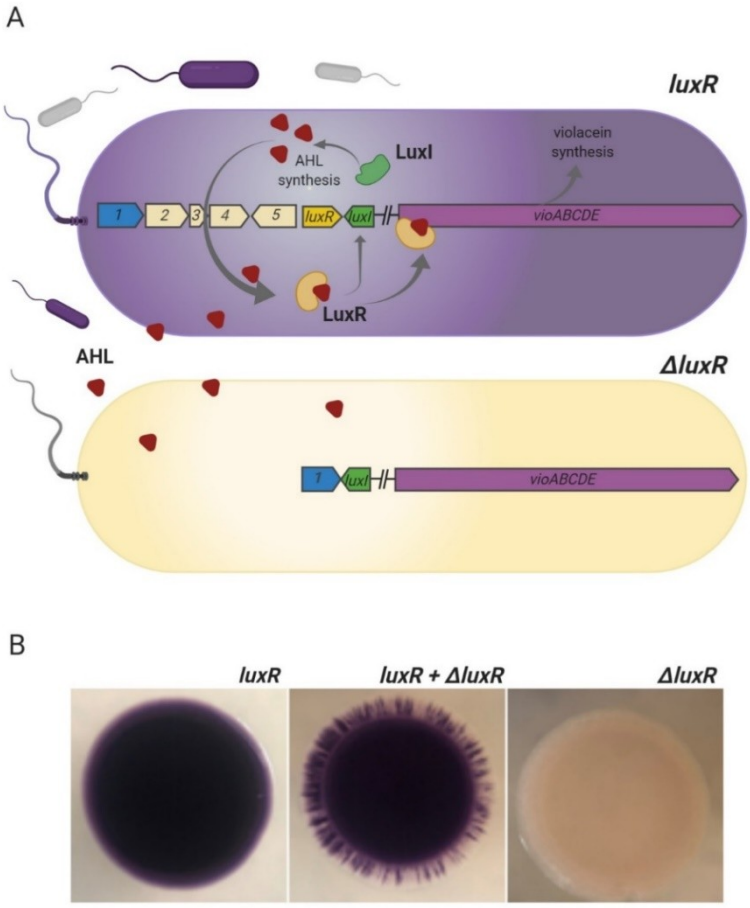

Figure 4. Nature of unpigmented strain. (a) Mechanism of luxI/luxR regulation of violacein production and inability to develop pigmentation in natural *ΔluxR* isolate. LuxR binds with exogenous AHL and activates LuxI production; LuxI syntheses endogenous AHL; increasing AHL concentration amplify AHL-QS signaling loop; increasing concentration of LuxR-AHL complex activates vioABCDE operon and bacteria produce violacein. In case of *ΔluxR* strain AHL signaling is interrupted and vioABCDE is not active. Unpigmented natural isolate of *C. vaccinii* has deletion of luxR and 1-5 protein-coding genes. (b) Inability of natural *ΔluxR* isolate to produce violacein in mixed biofilm colony model. Biofilm colonies formed by pigmented strain (*luxR)*; mix of pigmented and unpigmented strains *(luxR + ΔluxR);* unpigmented strain (*ΔluxR).* Mixed biofilm colony has a clear patterns

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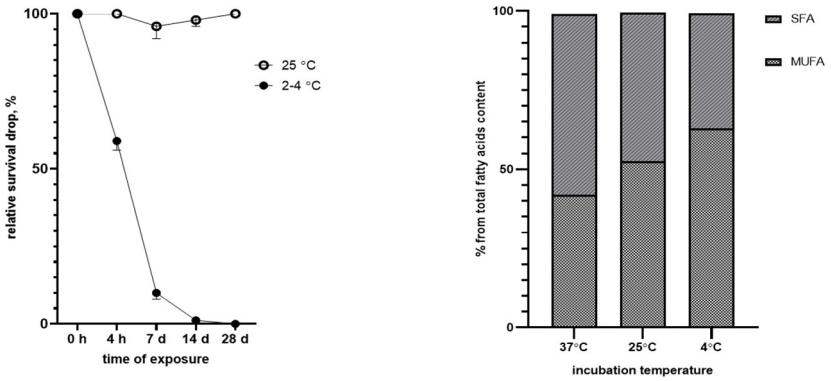
of pigmented and unpigmented zones – that indicates inability of *ΔluxR* strain to respond and amplify AHL-QS signal and produce violacein in mixed bacterial population. Created with BioRender.com

Lack of *luxR* expression due to deletion in the genome of ChrSW strain resulted in insensitivity to external AHL and inability to increase expression of endogenous AHL synthase LuxI, so all underlying modulation of gene expression remains intact, including expression of *vioABCDE* operon (Figure 4a). Clear pattern of unpigmented cells in mixed (equal mix of pigmented and unpigmented isolates) colony biofilm model confirmed inability *ΔluxR* mutant to respond through AHL - LuxI/LuxR pathway (Figure 4b).

Interesting to note that clear patterns at the edge of mixed colony biofilm indicated zip-like meso-scale structures (Supplementary Figure S3). Appearance of these structures was similar to recently observed intra-colony channels in *E. coli* [49].

3.4. Adaptability to low temperatures

Even though *C. vaccinii* was isolated during cold season, loss of viability was described during *C. violaceum* exposure to low temperatures [50]. During storage of plates (TSA, LB and Blood agar) with isolated *C. vaccinii at* 4 °C we observed complete loss of bacterial culture recovery from single colony after several days (within 1 week), while recovery during storage at 25 °C was restricted by drying of solid medium (4 weeks). In the genome of isolated *C. vaccinii* we revealed some genes responsible for temperature adaptation: ABC transporters for the putrescine import; genes for spermidine synthesis and export, the gene of cold shock protein, the operon for trehalose transport. To investigate ability to survive at low temperature in liquid LB medium we incubated stationary phase cultures of ChrSV at 4 °C or at 25 °C for different periods of time. After at least 4 h of incubation at 4 °C we observed twice decrease in viability in comparison with 25 °C incubation; by 1 week of incubation viability difference was more than 20 times lower and by the 4th week of incubation we observed complete loss of viability for sample at 4 °C storage condition (Fig. 5a). At the same time recovery of *C. vaccinii* from initial root-associated biofilm samples placed in the same LB medium was possible at least after 6 months of storage at 4 °C (due to numerous numbers of *C. vaccinii* colonies we evaluated recovery in a qualitative manner).



Recovery from initial biofilm sample during storage at 2-4 °C	7 d	14 d	28 d	180 d
	+	+	+	+

(a) (b)

Figure 5. Influence of low temperatures on *C. vaccinii*. (a) Survival under exposure of liquid culture of *C. vaccinii* to sub-zero (2-4 °C) temperatures presented in %% relatively exposure to 25 °C (presented as 100 %) and recovery from initial biofilm sample during storage at 2-4 °C. (b) Membrane fluidity adaptation through changing saturation of fatty acids during exposure to different temperatures.

Changing membrane fluidity through balancing saturated/unsaturated fatty acid content is an important part of bacterial cold adaptation [51]. To explore ability to change SFA/MUFA content during exposure to different temperatures we performed FAME analysis for bacterial culture grew

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up at 25 °C and then shifted to 4 °C or 37 °C for 48 h. After 48 h of exposure to 4 °C we observed significant shift to monounsaturated fatty acids (MUFA) and opposite in case of 37 °C exposure (Figure 5b). So isolated *C. vaccinii* has at least one mechanism of low-temperature stress reaction – changing membrane fluidity, but this attempt did not prevent loss of viability in monoculture, while multi-species environmental biofilm sample provides opportunity to survive for at least half a year at 4 °C *in vitro*.

3.5. Surrounding bacterial community

Bacteria grown on the solid mediums from the root-associated biofilms of *Carex spp.* were defined as culturome. Conventional Biotyper® identification of culturable bacteria was significantly restricted due to low number of environmental species in database, so we used IDBac approach to build a phylogenetic grouping based on MALDI-TOF MS small molecules data using algorithm described by Laura M. Sanchez and Brian T. Murphy [22,52]. For evaluation of this approach we performed *16S rDNA* sequencing for selected isolates from different groups and confirmed their correct classification at least on a group level. That gave us a general understanding of prevalent bacterial groups in culturome. In root-associated biofilms of *Carex spp.* we found members of Pseudomonadaceae accountant for more than 80% of culturable bacterial species (Figure 6a).

For description of the whole microbial community within the root-associated biofilm we performed massive parallel sequencing of *16S rDNA* amplicons. Microbiome was presented by the eight phyla: Acidobacteria, Actinobacteria, Armatimonadetes, Bacteroidetes, Cyanobacteria, Firmicutes, Nitrospirae, Proteobacteria (Figure 6b). The most abundant were Firmicutes (75,7%), followed by Proteobacteria (18,6%). Firmicutes included the five genera, among which *Clostridia* predominated (99,9%). *Clostridia* revealed in the root-associated microbiome were phylogenetically most closely related to a psychrophilic species *Clostridium estertheticum* and other species of the cluster I clostridia isolated from an Antarctic microbial mat [53]. Proteobacteria were presented by the five classes, including 73 genera (Figure 7).

The most abundant were Alfa- and Gammaproteobacteria, but taxonomic diversity of Deltaproteobacteria was as great as the Gammaproteobacteria. *Pseudomonas* predominated in Gammaproteobacteria (64%), which was partly consistent with the data of the culturome. However, *Serratia* was revealed only in trace amount, so as *Chromobacterium*. It should be noted that in the microbiome, few abundant Betaproteobacteria were represented mainly by the order Burkholderiales and only one genus of Neisseriales was detected. The amount of *Serratia* and *Chromobacterium* revealed in microbiome is in contrast to the fact that these genera did appear in culturome.

The abundance of the phylum Acidobacteria (2,6%) was an order of magnitude lower than Firmicutes and Proteobacteria, but agreed with the data of Pankratov et al. on the quantification by FISH of acidobacteria in native peat sampled from Sphagnum-dominated wetlands of different geographic location (0.1–4.1%) [54]. The phylum Bacteroidetes was even less abundant (1,8%). And as demonstrated Li et al. the presence of Bacteroides in the root-associated biofilm may depend on the type of the plant: Bacteroides were detected in samples of narrow-leaved cattail roots, but were absent in common reed root samples [55].

Analyzing 20 the most abundant genera we found they belonging to the next phyla: 1 – Firmicutes, 10- Proteobacteria, 2 – Bacteroidetes, 5 - Acidobacteria, 1 – Actinobacteria, 1 – Armatimonadetes. Thus, the microbial community of the root-associated biofilm demonstrated amazing diversity even in cold winter season.

Pseudomonas genus was the third according to *16S rDNA* microbiome data and the most prevalent according to low throughput culturome data. Important to note that diversity of culturable bacterial species obtained from IDBac protocol were dramatically distinct from the sequencing data.

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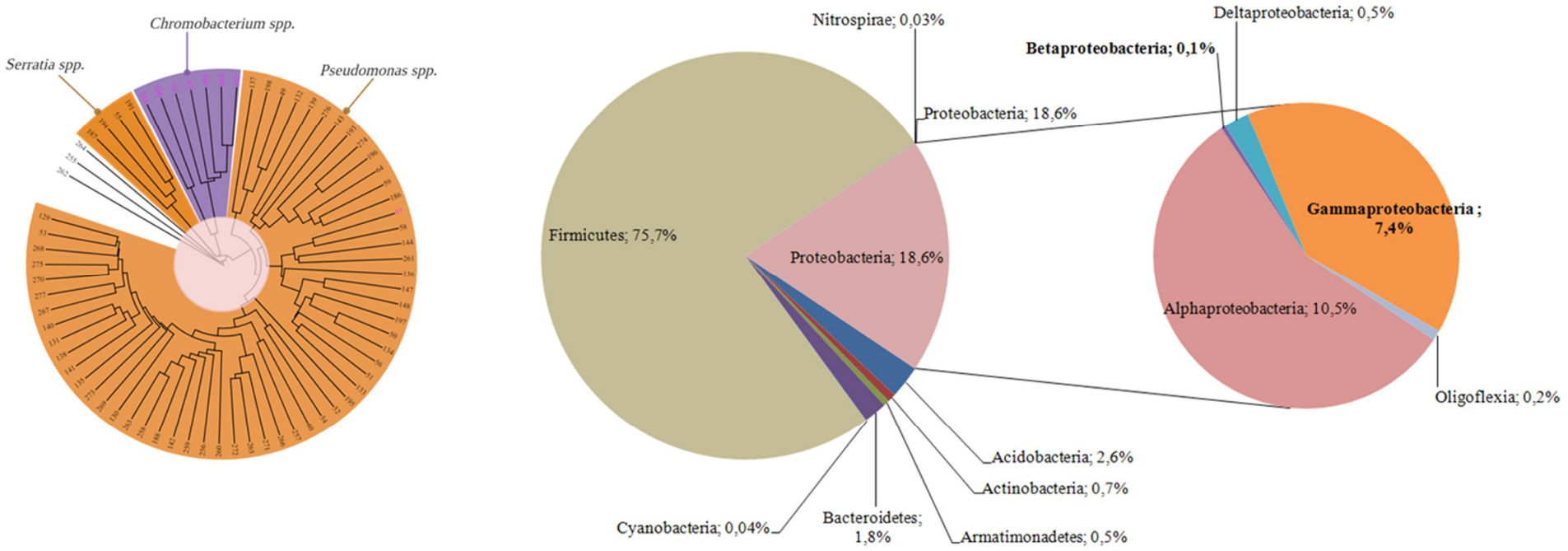

Figure 6. Surrounding bacterial community in biofilm. (a) Culturome of root-associated biofilm obtained through IDBac fingerprint grouping. Numbers represent colony identification number on plates. Mixed species colonies excluded from analysis; (b) Proportion of different bacterial taxonomic groups in *16S rDNA* microbiome of the root-associated biofilm. Inset in the diagram reveals the phylum of *Proteobacteria.*

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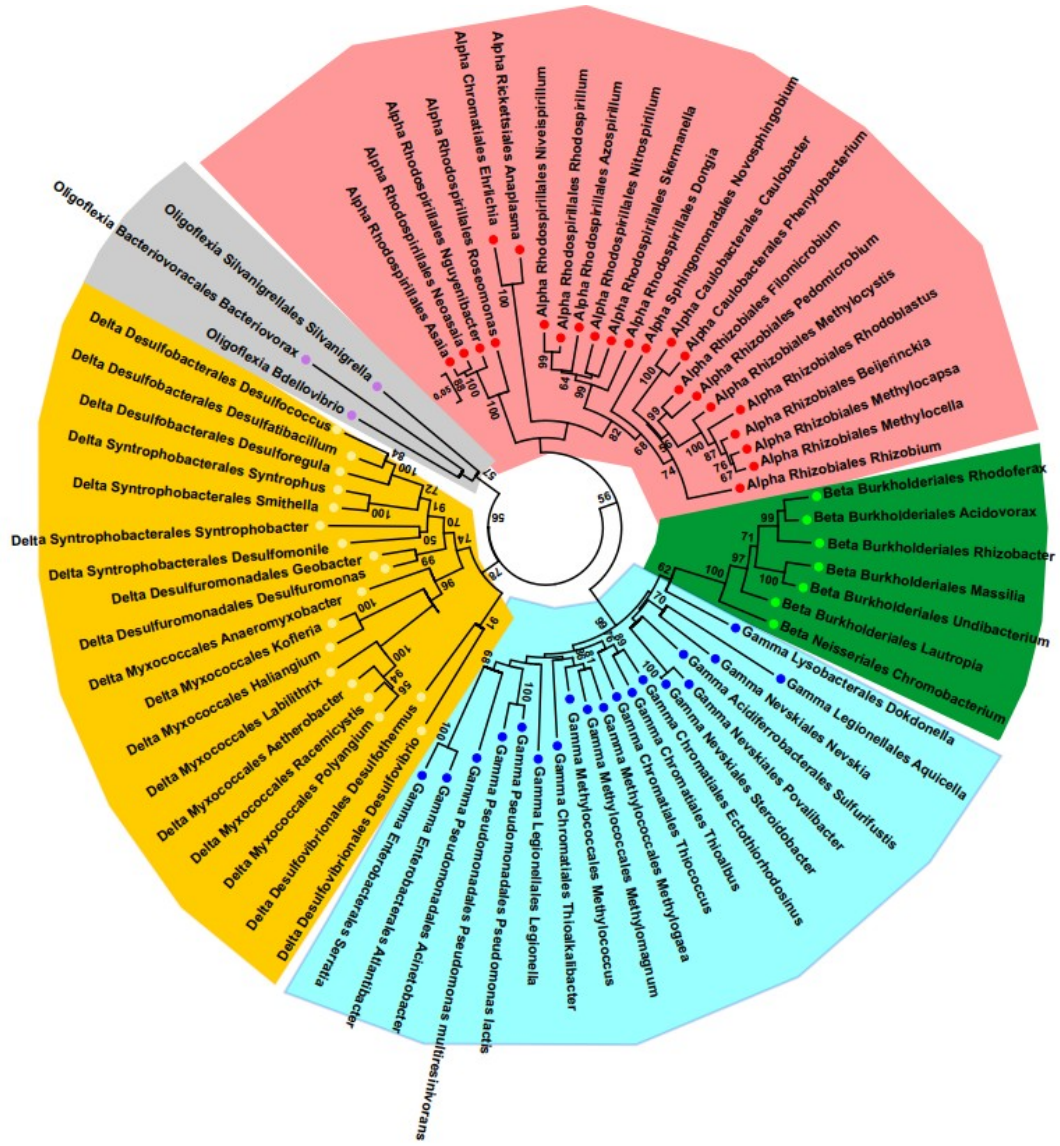

Figure 7. Taxonomic diversity of Proteobacteria in microbiome of the root-associated biofilm. Red circles – Alphaproteobacteria, blue circles – Gammaproteobacteria, yellow circles – Deltaproteobacteria, green circles – Betaproteobacteria, purple circles – Oligoflexia.

Disagreement between IDBac approach and sequencing data was expected and might be partially explained by unculturable state of some species and inappropriate culture conditions for growing up - like aerobic condition, inappropriate temperature, and medium composition. Meanwhile we noted unexpected prevalence of some species among colonies on solid medium despite their low abundance according to NGS data: Serratia was accounted for less than 0,003 % from all *16S rDNA* bacteriome and was presented >1500 times lower than *Pseudomonas* spp., but still grown up and was randomly picked for low throughput IDBac analysis. The same is for *Chromobacterium* genus. Significant limitation in culturome data collection is not only well-known growth competition but also growth cooperation in favorable conditions for some species in closer proximity on Petri dish in laboratory settings. Cooperation in terms of support during growing requires crossing of metabolic pathways between different species.

While it is difficult to predict cross-species metabolic interaction in natural multispecies communities IDBac approach estimated small metabolites fingerprint [52]. For the general understanding of possible *C. vaccinii* metabolism crossing with other bacterial species we created metabolic associated network (MAN) with all culturable bacteria within the same biofilm (Figure 8).

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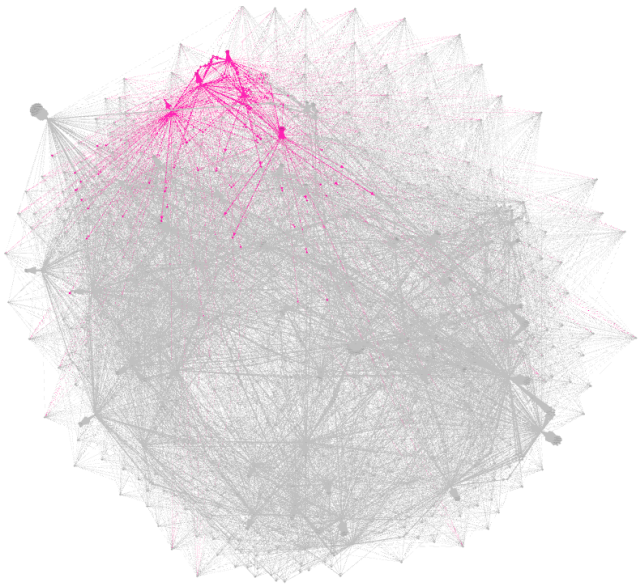

Figure 8. Metabolic associated network (MAN) of all culturable bacteria from the same with *Chromobacterium* biofilms. Small molecules metabolites from *C. vaccinii* are colored in pink, metabolites from other species are colored in grey. Cumulative distance was built via modularity analysis with default thresholds in Gephi after matrix and media signals were subtracted automatically from the network in IDBac.

Metabolic associated network demonstrated clear clustering of *C. vaccinii* within culturable bacteria and showed compounds with unique mass (at least 20 masses were not found in any other bacteria within culturome), probably there were specialized metabolites by *C. vaccinii*. At the same time, a lot of crossing with other species was observed as a sign of integration into bacterial community. Mass list of small molecule metabolites available in Supplementary Table S2.

4. Discussion

Chromobacterium species demonstrate significant biological activity against other microbes and insects in laboratory settings, but little is known about overall context of their natural habits and their environmental adaptation. They are widely distributed in tropical and subtropical zones and still believed being «tropical bacterial species». Investigation of environmental cases of *Chromobacterium* species isolation in temperate climate zone and analysis of their adaptation potential, surrounding microbiome and description of natural niche may shed a light on system biology of complex environmental communities and microbial biogeography at the time of discuss around global warming.

We described a case of a resident *C. vaccinii* in root-associated biofilm in quaking bog in Dfb climate zone. Nowadays there are numerous cases of environmental *Chromobacterium* isolation in Europe and one of them is also related to bog, that is in line with the first reported case of *C. vaccinii* isolation from cranberry bogs in Cape Code, USA [3,7]. Additionally, ability to grow *in vitro* at low salinity and water composition from cite of sample collection support existence of *C. vaccinii* in oligotrophic environment. Biofilm formation in environment with low level of nutrients and significant climate variations around the year helps to create sustainable surrounding due to accumulation of nutrients in biofilm matrix and create network for metabolic cooperation to degrade xenobiotics and resist to threats [56].

In support of unique sing of biofilm mode of cooperation, we found QS-deficient mutant among *C. vaccinii* isolates, who has mutation in the key regulatory system LuxI/LuxR. QS-deficient mutant had no visible pigmentation due to inability to respond and amplify AHL signaling essential for vioABCDE operon induction. At the same time OMVs production was not interrupted. This observation is in line with recently published data: OMVs and violacein biosynthesis depends on LuxI/LuxR quorum sensing system and as was recently published Δ*cviR* (homolog of *luxR*) mutant of *C. violaceum* loss visible pigmentation due to dramatical drop in violacein production,

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while vascularization per cell in Δ*cviR* mutant reduced by only half because of alternative OMVs regulation [48]. Absence of pigmentation is the most noticeable phenotype of Δ*luxR*, while it is well-known that inability to respond through LuxI/LuxR system leads to significant changes in other important processes and minimization of the cooperative traits. Interruption of QS-mediated regulation makes complicated crosstalk with other bacteria and might lead to minimization of production, but not consumption of public goods [57]. Occurrence and persistence of irresponsible to quorum sensing bacteria represent social cheaters within biofilm and stress the general idea of public goods in bacterial social community. Important to note that in our study QS-deficient mutant was isolated from natural complex biofilm so this is an additional point to support laboratory-proved theory around social cheaters in biofilms. One of the costly social good might be polysaccharides or other molecules for bacterial coating and providing protection from temperature perturbations. That`s could explain viability of «tropical» *Chromobacterium* during cold winter season.

While isolated *C. vaccinii* showed in vitro some adaptability to sub-zero temperature through changing membrane fatty acid saturation, after long-term storage recovery was dramatically better from complex initial biofilm samples rather than from pure bacterial suspension. Such cold resistance in a prism of biofilm lifestyle might be related to extracellular matrix composition that serves as a social good [58]. This is consistent with the previous observations that environmental parameters rather than phylogeny determine the composition of biofilm matrix in microbial mats from extreme environments [59]. It might be proposed that ability of *Chromobacterium* to survive in the bog during cold winter season was due to sharing goods provided by a diverse microbial community of the root-associated biofilm of *Carex spp*.

Analysis of surrounding bacterial community by *16S rDNA* microbiome showed prevalence of psychrophilic anaerobic *Clostridium* species, previously described as members of microbial mat in Antarctic freshwater lake [53]. The second prevalent bacterial belong to *Rhodoblastus* genus, which is freshwater bacteria and might be associated with sphagnum peat [60,61]. The vast majority of other bacteria are also known as psychrophilic or psychrotolerant. Besides the psychrophilic clostridia (Firmicutes) mentioned above, Acidobacteria are adapted to growth at low temperatures, as demonstrated Pankratov et al., who isolated the acidobacteria from nine Sphagnum-dominated wetlands of West Siberia and European North Russia [54]. *Steroidobacter agariperforans* (Gammaproteobacteria, Nevskiales) may be characterized as psychrophilic so far as ATCC recommended growing the type strain BAA2459 at 3°C (https://www.lgcstandards-atcc.org/). Another representative of Nevskiales, *Povalibacter*, was revealed with high abundance from natural and constructed wetlands, demonstrating the stability in the different geographic zones [62]. From the two genera of Bacteroides *Mucilaginibacter* was characterized as psychrotolerant by Pankratov et al., who isolated this bacterium from *Sphagnum* peat bog Bakchar, in the Tomsk region of western Siberia [63], and *Flavobacterium was described as* "cool to cold environments" by Van Trappe et al., investigated bacteria from Antarctic lakes [64].

The four genera of Proteobacteria belonged to methanotrophic bacteria, which could be characterized as psychrotrophs (facultative psychrophiles, or psychrotolerants) according to the data of Kevbrina et al., who demonstrated that some methanotrophic species could grow at 10°C [65]. *Silvanigrella aquatica* (Oligoflexia) can be recognized as psychrotrophs too on the basis of growth in culture in the temperature range of 10-32 °C and habitat in the freshwater lake located in the Black Forest Mountains (Schwarzwald), Germany [66]. *Pseudomonas*, the third abundant genus in biofilm community is known as cold tolerant due to wide geographic distribution and ability to grow even at high altitude location in the North Western Indian Himalayas [67].

However, in relation to some bacteria from the list of the most abundant in the root-associated biofilm, the same question arises as in relation to *Chromobacterium*: how a bacterium which has a temperature optimum in a monoculture at 25-30 °C and quickly dies at 4°C can accumulate in large quantities at low temperatures? If *Fimbriimonas* (Armatimonadetes) is mesophilic with a growth temperature range of 15–30°C [68], then *Rhodoblastus* (Alphaproteobacteria) was described as mesophilic with optimum growth at 25–30 °C, when cultivated in laboratory, but has been isolated

from the Sosvyatskoe ombrotrophic bog located in Tver Region with cold winter temperatures [69]. Moreover the type strain IC-180T of the genus *Aciditerrimonas* has growth temperature 35–58 °C [70], but at the same time *Aciditerrimonas* is known as abundant genus of Actinobacteria in a worldwide range of samples from the wetland soil and sediment according to the *16S rDNA* microbiome analysis [71], and Oloo et al. revealed that *Sphagnum* interstitial water samples were enriched in genera such as *Aciditerrimonas*, on the base of *16S rDNA* sequencing data too [72].

The most intriguing was *Ehrlichia* which is granulocytic ehrlichiosis agent of humans and other vertebrates, and tick-borne pathogen. The abundance of *Ehrlichia* was comparable with abundance of some Acidobacteria and methanotrophic bacteria in microbial community of the root-associated biofilm. Since culturing *Ehrlichia* species requires canine macrophage cell line or tick cell line, it is complicated by temperature (34-37 °C) and aerobic conditions for eukaryotic cells growth [73]. All finds of *Ehrlichia* in the environment are associated with ticks. As revealed Zintl et al. tick density in marsh/bog sites was even slightly higher than in woodland sites [74]. The appearance of *Ehrlichia* in the Moscow region bog could be connected with the change in distribution of ticks and with the fact that bog is comfortable place for prolonged nonparasitic phases of ticks, required a microclimatic relative humidity of at least 80% to avoid fatal desiccation [75].

Such diverse and complex cultivating conditions of representatives of the root-associated biofilm members support the idea of cooperation within microbial community to provide survival opportunity for broad range of requirements.

5. Conclusions

Altogether we described isolation of resident *C. vaccinii* from environmental complex biofilm in temperate climate zone which is not common for members of *Chromobacterium* genus. This *C. vaccinii* has several genotype and phenotype unique properties in comparison with all other members of the genus. Also unpigmented isolate with interrupted QS-mediated signaling was represented social cheaters within biofilm and might be a sign of adaptation to community lifestyle through minimization of costly production of social goods, while survival during exposure to sub-zero temperatures (i.e. winter season) was completely rely on surrounding microbial community and factor serving as a sharing good produced by other biofilm members will be in focus for the future research.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,

Figure S1: Tree inferred with FastME 2.1.6.1 from GBDP (Genome BLAST Distance Phylogeny) distances calculated from genome sequences.

Figure S2: Lipid A structure analysis.

Figure S3: Channel-like structures at the edge of the mixed colony biofilm.

Table S1: Fatty acid composition of several *Chromobacterium vaccinii* isolates.

Table S2: Mass list of small molecule metabolites of culturable bacterial species from root-associated biofilm.

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