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SHORT COMMUNICATION

Genomic and metabolomic profiling of endolithic *Rhodococcus fascians* strain S11 isolated from an arid serpentine environment

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Abstract

Genomic and metabolomic studies of endolithic bacteria are essential for understanding their adaptations to extreme conditions of the rock environment and their contributions to mineralization and weathering processes. The endoliths of arid serpentine rocks are exposed to different environmental stresses, including desiccation and re-hydration, temperature fluctuations, oligotrophy, and high concentrations of heavy metals. Bacteria of the genus *Rhodococcus* commonly inhabit endolithic environments. Here, we describe genomic and metabolomic analyses of the non-pathogenic wild-type *Rhodococcus fascians* strain S11, isolated from weathered serpentine rock at the arid Khalilovsky massif, Russia. We found that strain S11 lacks the virulence plasmid that functions in the phytopathogenecity of some *R. fascians* strains. Phenotypic profiling revealed a high pH tolerance, phytase activity and siderophore production. A widely untargeted metabolome analysis performed using an Orbitrap LC–MS/MS method demonstrated the presence of chrysobactin-type siderophores in the culture medium of strain S11. The natural variation of secondary metabolites produced by strain S11 might provide a practical basis for revealing antibacterial, fungicide or insecticidal activities. Finally, plant infection and plant growth stimulation studies showed no observable effect of exposure strain S11 bacteria on the aerial and root parts of *Arabidopsis thaliana* plants. Based on our findings, *R. fascians* strain S11 might be promising tool for investigations of organo-mineral interactions, heavy metal bioremediation, and mechanisms of bacterial mediated weathering of plant-free serpentine rock to soil.

Keywords *Rhodococcus fascians* · Avirulent strain · Biosynthetic gene clusters · Siderophores · Chrysobactin · Serpentinite · Endolithic bacteria

Introduction

Endolithic environments are ubiquitous habitats for microbial ecosystems and reservoirs for beneficial bacteria producing a variety of metabolites with diverse chemical

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structures and biological activities (Walker and Pace 2007). A weathered serpentinite deposit represents a lithic substrate characterized by heavy metal-containing minerals with a low moisture retention capacity and nutrient content (Rajkumar et al. 2009; Khilyas et al. 2019a, b). Bacteria have developed mechanisms that allow them to survive under extreme conditions and simultaneously mediate different processes, such as biomineralization, accumulation/detoxification heavy metals and colonization and weathering of hard rock minerals into soils that can support plant growth. Although rock-colonizing bacteria are widespread from arid desert to Arctic regions, information about members of the *Rhodococcus* genus inhabiting endolithic environments is limited. One study investigated the ability of two strains of *Rhodococcus opacus* to adhere/adsorb onto different mineral surfaces as calcite, serpentine and dolomite (Li et al. 2015) and we recently described a desiccation-tolerant, siderophore-producing *Rhodococcus qingshengii* strain isolated from serpentine rock (Khilyas et al. 2021).

Rhodococcus fascians causes leafy gall disease in a variety of mono- and dicotyledonous plants, adversely impacting agriculture worldwide (Dhaouadi et al. 2020). Virulence of *R. fascians* is attributed to the presence of a linear plasmid encoding *fas* and *att* genes responsible for the production and regulation of cytokinins, respectively (Crespi et al. 1992; Nikolaeva et al. 2009; Francis et al. 2012). The loss of this linear plasmid results in an avirulent, plant-growth promoting (PGP) phenotype (Francis et al. 2016). A combination of biochemical characteristics, such as secretion of phytohormones (cytokinin and auxin), biofilm formation and siderophore and trehalose production, were found to be responsible for the PGP activity of this plasmid-less strain.

The aim of the present work was to characterize an endolithic non-pathogenic *R. fascians* strain possessing features that adapt it to survival in a weathered serpentinite environment and potentially contribute to weathering processes that improve the bioavailability of serpentinite substrate for plant growth. Strain S11 was isolated from weathered serpentine rock sampled from the Khalilovsky massif, Russia (Latitude: N 51°30' Longitude: E 58° 11') (Khilyas et al. 2019a). Consistent with phenotypic features of *Rhodococcus* species (Goodfellow 2014), cells of strain 11 were rod-shaped (0.6–0.7 µm wide, 2–5 µm long; Fig. S1), Gram-positive, non-sporulating and non-motile and formed small (0.5–1.0 mm), round, orange-colored colonies with a smooth shape and glistening surface after 48–72 h of cultivation on LB agar at 30 °C.

Genomic DNA from strain S11 was extracted from a 48-h LB-grown culture using a Fast DNA™ SPIN Kit (MP Biomedicals, USA). A paired-end DNA library was prepared using the TruSeq DNA HT Library Preparation kit (Illumina) according to manufacturer guidelines. Whole-genome sequencing was carried out by MiSeq sequencing platform (Illumina) with 2*250 bp read length. The quality of raw sequence reads was evaluated by FastQC package (v0.11.3) (Andrews et al. 2010). The genome had an average 246X sequencing coverage. Overrepresented and low-quality sequences were removed using Trimmomatic (v0.36) (Bolger et al. 2014). The whole genome was assembled using SPAdes (v3.14.1) software (Bankevich et al. 2012). The k-mer-based correctness is counted in the QUAST (v4.6.2) program (Gurevich et al. 2013). Genome annotation was performed by RAST 2.0 server, Prokka (v1.14.5) annotation pipeline and the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) version 5.2 (Tatusova et al. 2016). The size of the assembled near-complete genome of strain S11 was 5,129,227 bp, comprising 50 contigs, with a DNA G + C content of 64.7%. Strain S11 contained 4,861 predicted genes, 4,795 putative coding sequences (CDS), 66 rRNAs, 47 tRNAs and 3 ncRNAs (Table S1).

16S rRNA gene analysis. Pairwise sequence similarities calculated using 16S rRNA genes available via the GGDC web server (http://ggdc.dsmz.de/) showed that strain S11 clusters with the *R. fascians* species group (Fig. S2).

Whole-genome sequence comparisons. In silico DNA–DNA hybridization (DDH) comparisons with GGDC2.0 (Meier-Kolthoff et al. 2013) between the genome of strain S11 and other accessible *Rhodococcus* genome sequences in GenBank indicated the closest match to the *Rhodococcus fascians* type strain (NBRC 12,155) with an estimated DDH value (high-scoring segment pair length/total length, generalized linear model based) of 79.7% and to *R. fascians* strain LMG 3623 with an estimated DDH value of 79.6% (Fig. S3).

The Average Nucleotide Identity (ANI) of the strain S11 genome was analyzed using FastANI (Jain et al. 2018). ANI values ranging from 97.5% to 98.25% were obtained with genomes assigned to *R. fascians* (Fig. S4).

Comparisons of protein-encoding marker genes. Multi-Locus Sequence Analysis (MLSA) of strain S11, based on conserved marker genes (*ftsY, infB, rpoB, rsmA, secY, tsaD*, and *ychF*) within the subclass *Actinobacteridae* (Creason et al. 2014), was performed using the Molecular Evolutionary Genetics Analysis (MEGA X) software (Kumar et al. 2018). The resulting phylogenetic tree was consistent with the tree generated from whole-genome alignments (Fig. S5).

The gyrase B (*gyr*B) and catechol 1,2-dioxygenase (*cat*A)-based phylogenetic analysis showed that strain S11 clusters to *R. fascians* species (Fig. S6).

Pan-genome analysis of nine *R. fascians* genomes was carried out using Roary (Fig. S7) and a phylogenetic tree based on the core-genome alignments was generated (Fig. 1; Page et al. 2015). A total of 8285 gene clusters were found: 3874 core genes (46.8%), 2159 accessory genes (cloud; 26.1%) and 2252 genes found in two or more genomes (shell; 27.1%) (Fig. S7A). The total number of genes in the

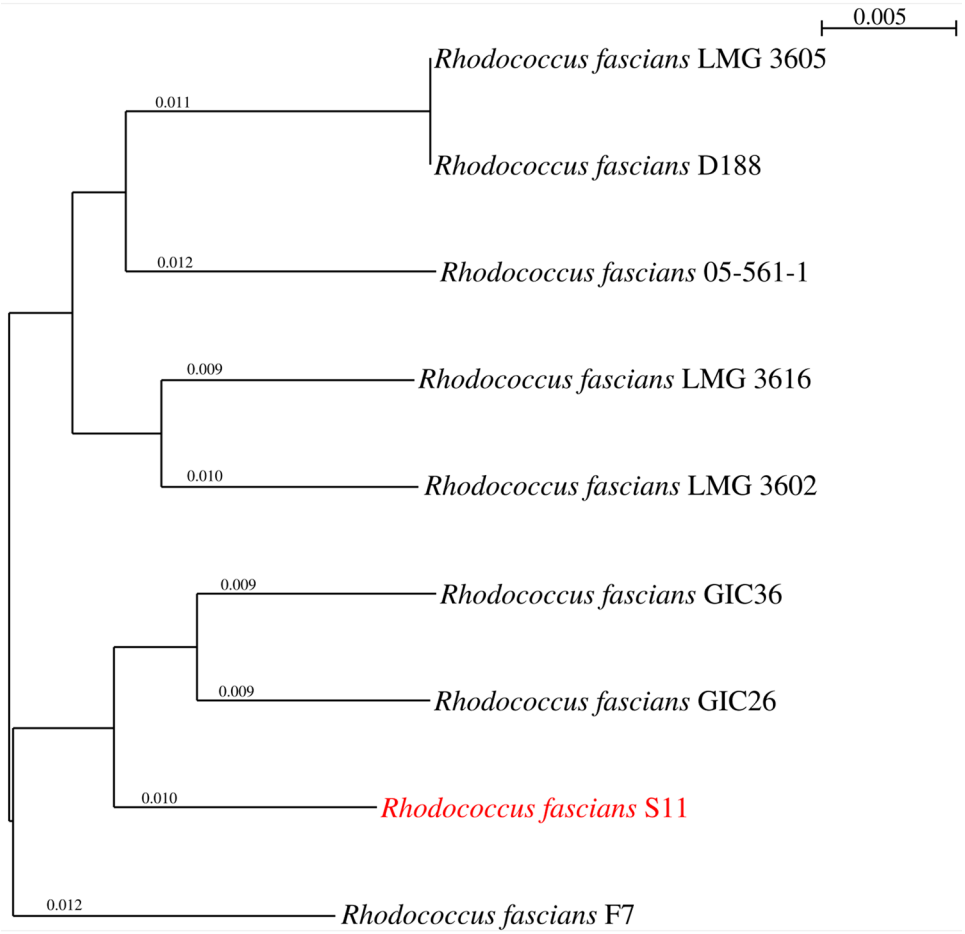

Fig. 1 Core-genome-based phylogenetic tree of nine *Rhodococcus* genomes visualized with the use of Roary software. The final alignment included the amino acid sequences of 3874 core genes per genome present in the core-genome of the selected bacteria. Conservation values are shown at the branches

pan-genome did not reach a plateau after adding a number of genomes of *R. fascians* strains (Fig. S7B). Thus, *R. fascians* strains have an open pan-genome with high genomic plasticity within this species (Sitto and Battistuzzi 2020).

Genome mining of virulence genes in the *R. fascians* strain S11 genome was performed. Plasmid-encoded *fas* and *att* genes were not detected (Crespi et al. 1992; Francis et al. 2012). Moreover, the PlasmidFinder confirmed that *R. fascians* strain S11 was plasmid-free (Carattoli et al. 2014). The chromosomally encoded *vic*A gene (malate synthase), which functions in but is not essential in *R. fascians* pathogenicity (Vereecke et al. 2002), was found in the genome of strain S11.

S*iderophore-related genes and siderophore production.* Fifteen biosynthetic gene clusters were predicted to be present in the genome of strain S11 by the antiSMASH software (Blin et al. 2019). Among those, one putative polyketide synthase (PKS) cluster, seven putative non-ribosomal peptide synthetases (NRPSs) clusters and one RiPP-like (ribosomally synthesized and post-translationally modified peptide product) cluster were identified. One NRPS cluster has sequence homology to a cluster known to encode for siderophore production. We also showed that *R. fascians* strain S11 can produce siderophores in chrome azurol S (CAS) agar and in liquid M9 medium under iron-limiting conditions (Arnow 1937; Atkin et al. 1970). The maximum concentration of siderophores was detected after 96 h and reached 19 ± 1.46 µM for catechol type and 43 ± 8.83 µM for hydroxamate type of siderophores.

Phenotypic analysis. Cells of *R. fascians* strain S11 were inoculated into Biolog GEN III microplates to test for consumption carbon sources, polymers, and amino acids, sensitivity to antibiotics and antiseptics, and growth under different pHs and salt concentrations (Bochner et al. 2001). Among all *GEN III* microplate tested substrates, strain S11 was able to utilize a range of sugars and carboxylic acids as carbon sources but was notable in its inability to catabolize polymers (i.e., dextrin, gelatin and pectin) (Table S2). Cells grew in the presence of up to 8% NaCl and at a pH range of 5–9. No hemolytic activity was observed on Columbia agar with 5% sheep blood (Bio-Rad).

Phytase activity was assayed on a screening medium containing 2% glucose, 0.5% NH_4NO_3, 0.2% $CaCl_2$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.05% KCl, 6 mM sodium phytate and 1.5% agar at a pH of 5.0 at 30 °C (Mittal et al. 2011). Colonies showed a

hydrolytic zone on this medium, indicating that strain S11 has phytase activity. Although no phytase homologs were identified in the genome of strain S11, other less specific phosphatases are known to possess phytase activity (Azeem et al. 2015).

Plant infection assays. Plant infection assays were performed using the seedling flood-inoculation method with modifications (Ishiga et al. 2011). In brief, *Arabidopsis thaliana* (Columbia ecotype) seeds were surface sterilized using 50% bleach and plated on ½ strength Murashige and Skoog medium with 2% sucrose and 0.7% agar. Seeds were incubated for 7 days in a growth chamber under a 16-h light / 8-h dark cycle at 20° C. Upon reaching the cotyledon stage, seedlings were transferred to new plates (6 plants per plate) with the same medium and grown for an additional 7 days under the standard conditions. *R. fascians* strain S11 was cultured in 50 ml of LB medium at 30° C on an orbital shaker with 250 rpm agitation for 72 h until the OD_{595} reached 1.5–2.0. The bacterial cells were pelleted at 7,000 g and washed twice with 1% sucrose. Cells were resuspended in 1% sucrose to final densities of 0.1, 0.5, 1.0 and 1.5 OD_{595}.

To conduct infection assays 40 ml of the bacterial suspension was added into a Petri plate with *A. thaliana* seedlings and left to sit for 10 min. Then the culture was decanted and plants were incubated for 7 days under a 16-h light / 8-h dark cycle at 20 °C. Plants treated with 1% sucrose were used as a control. The phenotypes of plants were visually checked at 3 dpi (day past infection), 5 dpi, 7 dpi.

The plant infection analysis of *A. thaliana* revealed that exposure of plant roots to *R. fascians* strain S11 did not confer any visually detectable negative effects on the plant growth and development (Figs. S8 and S9). Moreover, the additional analysis showed that the bacterial strain did not cause any developmental changes in the root formation of *A. thaliana* (Fig. S8). Additionally, *R. fascians* strain S11 did not exert any noticeable effect on the germination of wheat seeds (*Triticum aestivum* L., 'Yoldyz' spring wheat cultivar; Table S3). Therefore, we concluded that strain S11 does not negatively affect vascular plants, in accordance with genomic data confirming absence of the virulence genes in the strain S11 genome.

Isolation of siderophores from culture supernatant. R. fascians strain S11 was cultivated for 72 h in M9 medium under iron-limited conditions. After extraction of culture supernatant with XAD16 resin, the adsorbed compounds were eluted with methanol and subjected to Orbitrap LC–MS/MS.

To prepare for untargeted LC–MS/MS analysis, extracts were loaded onto a 50 cm µPAC TM C18 column (Pharma Fluidics, Gent, Belgium) in 0.1% TFA (Fluka). Samples were eluted with a linear gradient of acetonitrile from 3 to 44% over 240 min followed by a wash with 72% acetonitrile at a constant flow rate of 300 nl/min (ThermoScientific™UltiMate™3000RSLCnano) and infused via an Advion TriVersa NanoMate (Advion BioSciences, Inc. New York, USA) into an Orbitrap Eclipse Tribrid mass spectrometer (ThermoScientific). The mass spectrometer was operating in positive-ionization mode with a spray voltage of the NanoMate system set to 1.6 kV and source temperature at 300 °C. Using the data-dependent acquisition mode, the instrument performed full MS scans every 2.5 s over a mass range of m/z 400–1600, with the resolution of the Orbitrap set to 120,000. The RF lens was set to 30%, auto gain control (AGC) was set to standard with a maximum injection time of 50 ms. In each cycle the most intense ions (charge state 2–6) above a threshold ion count of 5.000 were selected with an isolation window of 0.7 m/z for CID-fragmentation at normalized collision energy of 35% and an activation time of 10 ms. Fragment ion spectra were acquired in the linear IT with a scan rate set to rapid and mass range to normal and a maximum injection time of 100 ms. After fragmentation, the selected precursor ions were excluded for 20 s for further fragmentation. From each MS/MS cycle, up to ten fragment ions were selected with an isolation window of 3 m/z for HCD-fragmentation at normalized collision energy of 55%. MS3-fragment ion spectra were acquired in the Orbitrap with a resolution of 50.000. The mass range was set to 100–500 m/z, maximum injection time to 100 ms and AGC to 300.

The data obtained from HPLC–MS/MS analysis were converted to.mzXML format using the MsConvert software (Adusumilli and Mallick 2017). The LC–MS/MS converted data files (.mzXML) were uploaded to the global natural products social molecular networking (GNPS) platform (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) with Dereplicator + (Mohimani et al. 2018) and VarQuest (Analog Search) computational tools (Gurevich et al. 2018). Network parameters were default.

Among 1787 annotated compounds in culture liquid of *R. fascians* strain S11, the highest score was found for catechol-type siderophores related to linear and cyclic form trichrysobactin, and dichrysobactin (Table 1, Fig. S9). Production of different forms of chrysobactins has been shown for the plant pathogens *Dickeya chrysanthemi* EC16 and *Dickeya dadantii* 3937 (formerly *Erwinia chrysanthemi*), and for several strains of *Serratia marcescens* (Persmark et al. 1989; Ehlert et al. 1994; Sandy and Butler 2011; Khilyas et al. 2019b) but has not been previously reported for *Rhodococcus fascians*. The production of chrysobactin by those bacteria is attributed to *cbsF* gene-encoding NRPS (Sandy and Butler 2011). The amino acid sequences of two NRPSs in the genome of *R. fascians* strain S11 share 34% and 32% identity with the CbsF protein of *D. chrysanthemi.*

Table 1 The annotated compounds in cultural liquid of *R. fascians* S11 grown under iron-limited conditions

Name of identified compound	*p*-value	PepMass	SpecMass, *m/z*	Adduct
Trichrysobactin (linear)	4.1e–29	1071.44	468.72	M + 2H
Trichrysobactin (cyclic)	1.3e–27	1053.43	536.73	M + 2H
Dichrysobactin	1.4e–22	720.30	404.67	M + 2H

R. fascians strain S11 was deposited under the strain accession number Ac-2168 in the Russian National Collection of Industrial Microorganisms (VKPM).

Nucleotide sequence accession number. This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JAHXBW000000000.

Conclusion

In this study, we report the genome sequence and genomic analysis of endolithic strain S11 isolate, identified to be *Rhodococcus fascians*. No virulence genes or plasmids were found in the bacterium. Plant infection experiments did not reveal any visually negative effects on *A. thaliana* or wheat growth and development. Strain S11 demonstrates an ability to produce siderophores, has phytase activity, and displays pH and salinity tolerance. Metabolomic analysis of strain S11 shows a high number of annotated compounds related to different natural products, including siderophores of the chrysobactin type, which might be an indication of the competition for scarce resources and limited colonization space. Endolithic bacteria in extreme environments are pioneering colonizers that improve the availability of hard rock deposits for plant growth. The survival of *Rhodococcus fascians* strain S11 and possible role in altering its native arid rocky environment likely relies upon its complement of genes encoding for desiccation, pH and salinity tolerance and production secondary metabolites, such as non-ribosomal peptides and polyketides.

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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