PH.D. THESIS

ECOPHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF A BIOCONTROL *BACILLUS SUBTILIS* STRAIN

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INTRODUCTION

Plant and mushroom diseases caused by viruses, bacteria and fungi may result in serious losses in the agriculture. The use of chemical pesticides is more and more prevailing since the middle of the 20th century. The high usage rate infers many problems such as the appearance of secondary pests, development of resistance, environmental impact, furthermore the endangerment to human health. Therefore the biological control is an important scientific topic since the 1920's. Nowadays there is a growing need for alternative, chemical residue-free solutions such as the biocontrol strategies.

Biocontrol agents are microorganisms capable of suppressing the pathogens. Not just the microorganisms, but their secondary metabolites can also be applied for biocontrol.

The sporulation ability of the members of the genus *Bacillus* is beneficial for biological control, as it enables the survival of the bacteria under harsh conditions, furthermore, it is also facilitating the formulation of biological pesticides. *Bacillus subtilis* has an important role in the agriculture, food and pharmaceutical industries due to the production of extracellular enzymes (proteases, chitinases, lipases, cellulases) and antibiotics. About 4-5% of the genome of *Bac. subtilis* consists of genes responsible for antibiotic synthesis, thereby this species is capable of the production of more than two dozens of structurally diverse antibiotic compounds. The most studied non-ribosomal cyclic lipopeptide families are the surfactins, iturins and fengycins.

The members of the fengycin family are cyclic lipodecapeptides with 10 amino acids and a C14-C18 fatty acid chain. They possess a wide-spectrum of antifungal properties.

Iturins have seven major variants: iturin A and C, bacillomycin D, F, L and LC, and mycosubtilin. These compounds are built up from seven amino acids forming a cyclic lipopeptide ring and a C14-C17 fatty acid chain. Their strong *in vitro* antifungal activities are well known against yeasts and filamentous fungi however, their antibacterial properties are limited. Until today no antiviral activities have been reported.

The members of the surfactin family are heptapeptides with a cyclic lacton and a beta-hydroxy fatty acid chain. They have various biological effects such as hemolytic, antiviral, anti-mycoplasmatic and antibacterial activities. The anti-inflammatory effects of surfactins are realized by the inhibition of phospholipase A2. The antiproliferative effects on Ehrlich ascites cells are indicating their antitumor activities. The application of surfactins as thrombolytic therapeutic agents in the field of lung and heart diseases is also investigated.

Besides the above, *Bac. subtilis* is also playing a role in the stimulation of plant growth and the induction of plant resistance.

AIMS

The aims of our work were:

1. to demonstrate the possible role of *Bac. subtilis* strain SZMC 6179J isolated by our research group in biological control, to reveal the ecophysiological characteristics of this strain and to assess its *in vitro* antagonistic abilities,

2. to investigate the ability of the strain to produce extracellular enzymes and antibiotics forming the basis of its competitive abilities,

3. to isolatie spontaneous streptomycin-resistant mutants from the strain and investigate their the production of chymotrypsin-like proteases, as well as to determine the changes in the sequences of the *rpsL* genes of the streptomycin-resistant mutants,

4. to investigate the enzyme and antibiotic production of the isolate depending on various environmental factors,

5. to characterize the cyclic lipopeptides, in particular the surfactin-type compounds produced by the strain,

6. to sequence the full genome of the strain and perform its comparative genome sequence analysis with the genome of the type strain *Bac. subtilis* subsp. *subtilis* str. 168.

METHODS

- Selective isolation of *Bacillus* strains
- Investigation of *in vitro* antagonistic properties of the isolated *Bacillus* strains
- Identification of the isolated *Bacillus* strains using PCR techniques
- Ecophysiological characterization of the isolated strain at various temperature, pH and water activity values
- Studying the *in vitro* antagonistic activities of the isolate via determination of biocontrol index (BCI) values
- Investigation of the effects of the isolated *Bacillus* strain on various plant- and mushroom-pathogenic fungi using RISA technique.
- Investigation of enzyme production
    - Siderophore, lipase, caseinase, gelatinase, chitinase activity assays and starch degradation on solid medium
    - Protease, chymotrypsin-like protease, trypsin-like protease, palmitoyl-esterase, β-glucosidase, N-acetyl-glucosaminidase (NAG-ase), chitobiosidase,

palmitoyl-esterase, cellobiohydrolase activity assays using chromogenic substrates

- Selective isolation of spontaneous streptomycin-resistant mutants, gene sequence analyses using BLAST method
- Optimization of growth and enzyme production
    - by Design-Expert 7.1 software
    - visualized by Box-Behnken
- Effect of different carbon and nitrogen sources as well as metal ions and pesticides on the chymotrypsin-like protease secretion and activity
- Investigation of surfactin production by TLC-direct bioautography assay
- Identification of the genes responsible for antibiotic biosynthesis using PCR tecniques
- Quantitative analysis of the surfactin isoforms by HPLC-MS
- Whole-genome analysis using various bioinformatic methods
    - Sequence assembly with Genomics Workbench 4.7.2 (CLC Bio) and Gapped SOLiD Alignment 1.2 plug-in (Omixon). Annotation with the NCBI Prokaryotic Genome Annotation Pipeline (PGAAP)
    - Read mapping with *Genomics Workbench 4.7.2*
    - Determination of the exact taxonomic position of the strain by multilocus sequence typing (MLST)

- Generation of Maximum likelihood (ML) inferences from the dataset with raxmlGUI 1.5b1 using the executables of RAxML 8.2.7
- Mining of SNPs and DIPs from the aligned reads with CLC Sequence Viewer v.6.5.3. and CLC Genomics Workbench 5.1
- Searching for gene clusters of putative antimicrobials by the web-based genome mining tool antiSMASH

  Analysis of the presence of prophage sequences in the *Bacillus* genomes with the PHAST search system, examination of the distribution of a specific prophage-like region in *Bacillus* strains by BLAST, visualization of the results with the Kablammo server

RESULTS

The ecophysiological characterization of the isolated *Bac. subtilis* SZMC 6179J strain revealed that the following parameters proved to be optimal for its growth: 40 °C, pH 7 – 7.6 and a_w = 0.995. The isolated strain showed the strongest inhibitory effect against *Botrytis cinerea* SZMC 14526 causing bunch rot, as well as *Bipolaris bicolor* SZMC 13055, *Curvularia spicifera* SZMC 13060, *Phytophthora infestans* SZMC 6246J as well as 3 *Armillaria* strains (*Armillaria gallica* SZMC 24095, *Armillaria ostoyae* SZMC 24129 and *Armillaria mellea* SZMC 24132). In the case of 4 *Trichoderma*

pleuroti strains the observed BCI values were above 50% Against *Aspergillus* strains we did not detect BCI values above 50%.

During the *in vitro* antagonism assays against plant pathogenic bacteria, outstanding inhibition was observed against *Pseudomonas syringae* pv. *panici* strain SZMC 16160. Summarizing the results of the *in vitro* antagonism assays against plant pathogenic bacteria, *Clavibacter michiganensis* SZMC 0016 completely disappeared at the 3 investigated pH-values on the second day. From the other investigated pathogenic strains only *Allorhizobium vitis* SZMC 21396 showed sensitivity to *Bac. subtilis* SZMC 6179J (Vágvölgyi et al. 2013).

Strain SZMC 6179J was found to be able to produce siderophores in biological assays, which may provide advantages in competition. The strain has no lipase, caseinase, chitinase or gelatinase activity, however, it is able to degrade starch.

The investigation of the extracellular enzyme production of strain SZMC 6179J was carried out in five different media based on our preliminary experiments, and the Besson-medium proved to be the most suitable. During the investigation of extracellular enzyme production, the secretion of chymotrypsin-like and trypsin-like proteases were found to be remarkable among the protease, β-glucosidase, N-acetyl-glucosaminidase, chitobiosidase, palmitoyl esterase, cellobiohydrolase and lipase enzymes.

Twelve out of 169 spontaneous streptomycin-resistant mutants showed enhanced enzyme production. During the sequence analysis of the *rpsL* gene of spontaneous streptomycin-resistant

mutants, nucleotide transitions (thymine to adenine, adenine to guanine) were detected in the case of some mutants.

The growth and chymotrypsin-like protease production of the isolated *Bac. subtilis* SZMC 6179J strain were investigated in the presence of various carbon and nitrogen sources. Adonitol, D-xylose, erythritol, galactose, L-arabinose, L-rhamnose and sucrose induced enhanced biomass growth. Significant increase of chymotrypsin-like protease production was observed in the presence of cellobiose, D-xylose, fructose, glycerol, starch and sucrose.

During the investigation of the effects of various nitrogen sources, only L-alanine caused increase in the biomass, while in the case of L-aspartic acid and L-tyrosine remarkable cell number decrease was observed. Exchange of the original nitrogen source caused significant decrease in the chymotrypsin-like protease production in each case.

The effects of the concentrations of glucose and yeast extract and the cultivation time on the growth and protease production were also determined. The optimum cultivation time proved to be 5 days, while the optimal glucose and yeast extract concentrations were 11 and 5.5 g/l, respectively. The highest enzyme activity was observed on the first day on 2 g/l glucose and 5.5 g/l yeast extract.

During the investigation of the effects of glucose, sodium-glutamate and copper-sulfate on the growth and chymotrypsin-like protease production of strain SZMC 6179J, 30 g/l glucose and 10 g/l sodium-glutamate resulted in the highest cell density in the presence

of 1 mg/l $CuSO_4 \times 5H_2O$. This medium composition also favored the chymotrypsin-like protease production.

During the investigation of the effects of glucose, sodium-glutamate and $FeSO_4 \times 7H_2O$, the highest cell density was observed in the case of 15.5 g/l glucose, 10 g/l sodium-glutamate and 40 mg/l $FeSO_4 \times 7H_2O$. Interestingly, the highest protease activity was observed on 30 g/l glucose, 1 g/l sodium-glutamate and 40 mg/l $FeSO_4 \times 7H_2O$ in the cultivation media. During the investigation of the effects of metal ions, manganese sulfate enhanced the growth of *Bac. subtilis* SZMC 6179J significantly, the optical density was tripled in the presence of 0.1 mM manganese. $CuSO_4$, $FeSO_4$, $NiSO_4$ and $CdSO_4$ inhibited the growth of the strain, it showed tolerance only to 0.1 mM concentration values, while the higher concentrations notably inhibited growth. Increased chymotrypsin-like protease enzyme production was observed in the presence of $MnSO_4$, while the enzyme production was significantly decreased in the presence of 1, 0.5 and 0.1 mM $CuSO_4$. 1 mM $FeSO_4$ decreased the enzyme production significantly, 0.5 mM $FeSO_4$ caused no significant changes, while in the presence of 0.1 mM $FeSO_4$ the enzyme production increased. $NiSO_4$ inhibited the chymotrypsin-like protease production at all three examined concentrations. $CdSO_4$ showed strong inhibitory effect in the entire concentration range examined. The growth of the isolate has remarkable tolerance to $CuSO_4$ and $CdSO_4$, however, the protease secretion was completely inhibited by these metal salts even at low concentrations. During the investigation of the effects of metal ions on enzyme activity, 1 and 0.5 mM $MnSO_4$, $FeSO_4$, $CuSO_4$ and $CdSO_4$

were found to be inhibitory, while at a concentration of 0.1 mM these salts showed no significant changes in the enzyme activity. $NiSO_4$ inhibited the enzyme activity at all three examined concentrations. During the study of the effect of herbicides and fungicides on growth of strain SZMC 6179J, significant decrease was observed in the cell number in the presence of 25 μM 2,4-dichlorophenoxyacetic acid, chlortoluron; 6.25 μM linuron, 3.125 μM carbendazim and 12.5 μM mancozeb at the 9^{th} day of the experiment. The 2,4-dichlorophenoxyacetic acid, carbendazim and mancozeb proved to be inhibitory at 12.5 μM to the chymotrypsin-like protease production on the 6^{th} day of the investigation. Chlortoluron and linuron inhibited the enzyme production at 6.25 and 3.125 μM concentrations. In the case of 2,4-dichlorophenoxyacetic acid, carbendazim and mancozeb, the concentration of 3.125 μM resulted in no significant changes of the enzyme production. After the 9^{th} day of the experiment the pesticides had a negative effect on the enzyme production of strain SZMC 6179J. 25 μM 2,4-dichlorophenoxyacetic acid and chlortoluron, as well as 25, 12.5 and 6.25 μM linuron and mancozeb and all four concentrations of carbendazim resulted in significant decrease of the enzyme activity.

After confirmation of the haemolytic activity of strain SZMC 6179J we demonstrated its surfactin production using bioautographic assay. In PCR experiments using specific primers we identified the genes responsible for fengycin and surfactin biosynthesis.

During the mass spectrometric examination of the ferment broth extracts of *Bac. subtilis* SZMC 6179J, 26 surfactin isoforms

were identified and assigned into 3 known groups and a new one, [Val2]. The C14–C15 [Sur] and [Val7] were the four major surfactins produced by the examined strain, while the relative amount of each newly described isoform was below 1% of the total surfactin amount (Bóka et al. 2016).

The complete genome sequence of *Bac. subtilis* SZMC 6179J was determined and a comparative genome sequence analysis with the type strain *Bac. subtilis* subsp. *subtilis* str. 168 was carried out. During the investigation of the taxonomical position of the isolate, it is found to be the member of the *Bac. subtilis* subsp. *subtilis* group and is phylogenetically related to *Bac. subtilis* subsp. *subtilis* str. 168. 106 SNPs were found in the genome of strain SZMC 6179J. The most allelic variations were found in the *yqc*G gene encoding for a toxic ribonuclease. This gene plays a role in the successful competition, it can suppress the growth of other members of the genus *Bacillus* via contact-dependent inhibition. If the YqcG toxin of the bacterial cell population shows high N-terminal sequence variations, the bacteria will be able to suppress multiple competitor bacteria with different membrane protein surfaces. This might be the reason of the high sequence variability in the *yqc*G gene of strain SZMC 6179J. A single nucleotide deletion in the *sfp* gene might have reverted its function in *Bac. subtilis* SZMC 6179J enabling the synthesis of fengycins and surfactins, which resulted in better biocontrol properties than those of the reference strain. Regions of hypermutation detected in the genome of *Bac. subtilis* strain SZMC 6179J suggest that the fastest

evolutionary events happen in genes important for competition processes and cell wall lysis (Bóka et al. 2019).

SUMMARY

1. We have successfully isolated a *Bacillus* strain with good biocontrol properties.
2. The ecophysiological properties of the isolated strain were determined.
3. The *in vitro* antagonistic properties of the isolate were characterized against plant and mushroom pathogenic fungi and bacteria.
4. Extracellular enzyme production of the strain was investigated.
5. Spontaneous streptomycin resistant strains with increased chymotrypsin-like protease activities were isolated.
6. The effects of various carbon and nitrogen sources as well as metal ions and pesticides on chymotrypsin-like protease production were characterized.
7. Cyclic lipopeptides produced by the isolated *Bacillus* strain were identified.
8. The quantitative analysis of the surfactin isoforms produced by *Bac. subtilis* SZMC 6179J was carried out.
9. The taxonomical position of the isolated strain was determined during the whole-genome analysis.

10. 106 SNP and 23 DIP were identified. Most of the SNPs were found in the *yqcG* gene. A single nucleotide deletion in the *sfp* gene might have reverted its function in *Bac. subtilis* SZMC 6179J enabling the synthesis of fengycins and surfactins, which resulted in better biocontrol properties than those of the reference strain.
11. Regions of hypermutation detected in the genome of *Bac. subtilis* strain SZMC 6179J suggest that the fastest evolutionary events happen in genes important for competition processes and cell wall lysis.

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