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CHARACTERISATION OF STREPTOMYCIN RESISTANT MUTANTS OF BIOCONTROL *BACILLUS* STRAINS

BETTINA BÓKA, LÁSZLÓ MANCZINGER, ANDRÁS SZEKERES, CSABA VÁGVÖLGYI

Department of Microbiology, Faculty of Science and Informatics, University of Szeged,
6726 Szeged, Közép fasor 52., Hungary
boka.tina@gmail.com

ABSTRACT
A good antibiotic-producing *Bacillus subtilis* and an elevated extracellular enzyme-secreting *Bacillus amyloliquefaciens* biocontrol strain were investigated. The *B. subtilis* strain produces non-ribosomal oligopeptides: iturin, surfactin and fengycin. These cyclic lipopeptides have antifungal and antibacterial effects. The *B. amyloliquefaciens* strain secrets many extracellular enzymes (proteases, chitinases, cellulases and lipases); these have great significance in the antagonism. Kurosawa and co-workers reported elevated protease and β-amylase secretion from streptomycin-resistant *B. subtilis* mutants. We isolated twenty spontaneous streptomycin resistant mutants from our *B. subtilis* strain and four spontaneous streptomycin resistant mutants from the *B. amyloliquefaciens* strain. We investigated the extracellular enzyme and antibiotic production of the mutants. Some of the mutants showed elevated enzyme and antibiotic secretion. The *rpsL* gene in theses spontaneous streptomycin resistant mutants, were sequenced and point mutations were detected in it, so very likely the changes of the structure of the rpsL protein is responsible in some cases for the elevated enzyme and depsipeptide production.

Keywords: antibiotic, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, biocontrol, extracellular enzymes

INTRODUCTION

The proper control of the different pests (e.g. plant pathogenic fungi) is a basic requirement in the modern agriculture. Most of the common agricultural technologies rely on the extensive use of pesticides; this greatly contributes to the increased growth yields and the production of quality of food and feeds. At the same time, the widespread use of chemical pesticides considerably increased the environmental problems of the agricultural areas. Accordingly, it would be important to find new and effective biocontrol agents which do not have ecotoxicological risks but could replace the chemical pesticides.
Bacillus subtilis and *Bacillus amyloliquefaciens* strains are effective biocontrol agents, because they produce antibiotics and various pest-degradative extracellular enzymes. For example, *B. amyloliquefaciens* has excellent protease secreting capability (PRIEST, 1977). These proteases attack the proteins which are present in the cell-wall of many plant pathogenic fungi, and in this way they can suppress the pathogens. *B. subtilis* strains secrete non-ribosomal cyclic lipopeptides such as iturin, surfactin and fengycin (ONGENA AND JACQUES, 2008). These oligopeptides have excellent antagonistic effects against wide range of harmful microorganisms: compounds belonging to iturin family have strong antifungal effects on yeast, surfactin family have anti-bacterial and anti-viral activities and fengycin have excellent fungitoxic effects on filamentous fungi (KATZ AND DEMAIN, 1977).
KUROSAWA and co-workers (2006) reported the elevated protease and α-amylase secretion of streptomycin resistant *B. subtilis* strains. This phenomenon appeared in connection with spontaneous point mutations in the *rpsL* gene. This gene encodes the protein S12 of the 30S ribosomal subunit. In this work we report the successful isolation of a potent antagonistic *B. subtilis* and *B. amyloliquefaciens* strains from the rhizosphere of tomato

plants and the isolation and characterization of spontaneous streptomycin resistant mutants of these strains.

MATERIAL AND METHOD

Isolation of spontaneous streptomycin resistant *B. subtilis* and *B. amyloliquefaciens* strains

Bacillus cells from a 24 h liquid culture were suspended in 0.5 ml of 1% NaCl solution (5 x 10^7 cell ml^{-1}). Fifty µl of this suspension were spread on the surface of YEG medium supplemented with 100 µg ml^{-1} streptomycin. After one week the appearing colonies were picked and further subcultured (purified) on streptomycin-containing medium.

Investigation of the extracellular enzyme production of *B. subtilis* and *Bacillus amyloliquefaciens* strains

Medium for enzyme production was used as reported by BESSON ET AL. (1987): (constituents in g l^{-1}): glucose 10, glutamic acid 5, KH_2PO_4 1, K_2HPO_4 1, $MgSO_4$ x $7H_2O$ 0.5, KCl 1, $FeSO_4$ x 7 H_2O 0.005, $CuSO_4$ x $5H_2O$ 0.00016. Non-inductive enzyme production was measured after 7 days with chromogenic protease and chitinase substrates. Bz-Phe-Val-Arg-pNA, Suc-Ala-Ala-Pro-Phe-pNA and 4-nitrophenyl-N-acetyl-glucosaminide were used for trypsin-type protease, chymotrypsin-type protease and for exochitinase measurements, respectively.

Investigation of the antibiotics producing capabilities

The medium used for antibiotic production was reported by BESSON ET AL. (1987). The antibiotics were precipitated from the ferment broth by lowering the pH to 2 with HCl. The pelleted antibiotics were dissolved in ethanol and separated by thin layer chromatography (TLC) on Kieselgel 60 plates with chloroform:methanol:water (65:25:4) eluent. The separated antibiotics were visualized with a color reagent containing 3 g phenol, 94.98 ml ethanol, 5 ml sulfuric acid and 20 µl anisaldehide. The amount of the secreted, tyrosine containing antibiotics was measured spectrophotometrically at 280 nm.

Amplification of the *rpsL* gene

The *rpsL* gene from the wild-types and from the streptomycin resistant mutants were amplified by polymerase chain reaction (PCR) and sequenced. PCR was carried out in a final volume of 50 µl containing 5 µl of *Taq* polymerase 10× buffer, 1.6 mM $MgCl_2$, 200 µM for each of the dNTPs, 10 pM primers, 2 µl of template DNA in distilled water and 1 U of *Taq* DNA polymerase. The following primers were used BF-1 5' ATGCCTACAATTAATCAGCTAATTC 3' and UP BR-1 5' TACGGATGTTAATTAGTCGATTAAG 3'. Amplification was performed in a T3 thermocycler as follows: 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 30 s, 50°C for 40 s, and 72°C for 1 min and a final elongation step at 72°C for 3 min. PCR products were separated by electrophoresis (1.5% agarose gel prepared in TAE buffer containing ethidium bromide) and investigated under UV light.

RESULTS AND DISCUSSION

Isolation of streptomycin-resistant *B. subtilis and B. amyloliquefaciens* strains

Twenty spontaneous streptomycin resistant *B. subtilis* mutants were isolated. Ten of them belonged to morphotype-1; these were characterised with about 1 mm colony diameter with regular colony edge. Ten of them belonged to morphotype-2; these were characterised with 2-4 mm colony diameter with irregular edges.
From the *B. amyloliquefaciens* strain four spontaneous streptomycin-resistant mutants were isolated. All of these mutants showed morphotype-1 character.

Investigation of the extracellular enzyme production of *B. subtilis* and *B. amyloliquefaciens* strains

Trypsin-type protease, chymotrypsin-type protease and exochitinase activities in the ferment broths of wild-type and of the streptomycin-resistant mutants were measured. Two of the streptomycin-resistant mutants of *B. subtilis* had increased trypsin-type protease and three had increased chymotrypsin-type protease activities (*Figure 1* and *Figure 2*). The K2 mutant had nearly fourfold increase in chymotrypsin activity. We did not detect elevated exochitinase activity of the streptomycin resistant mutants.

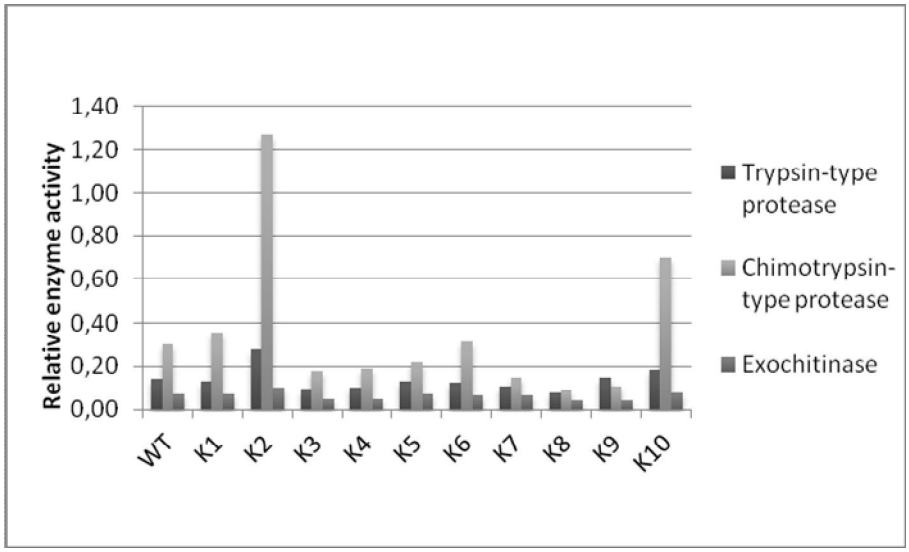

Figure 1. Enzyme profiles of the morphotype-1 streptomycin resistant *B. subtilis* mutants compared with those of the wild type strain (WT)

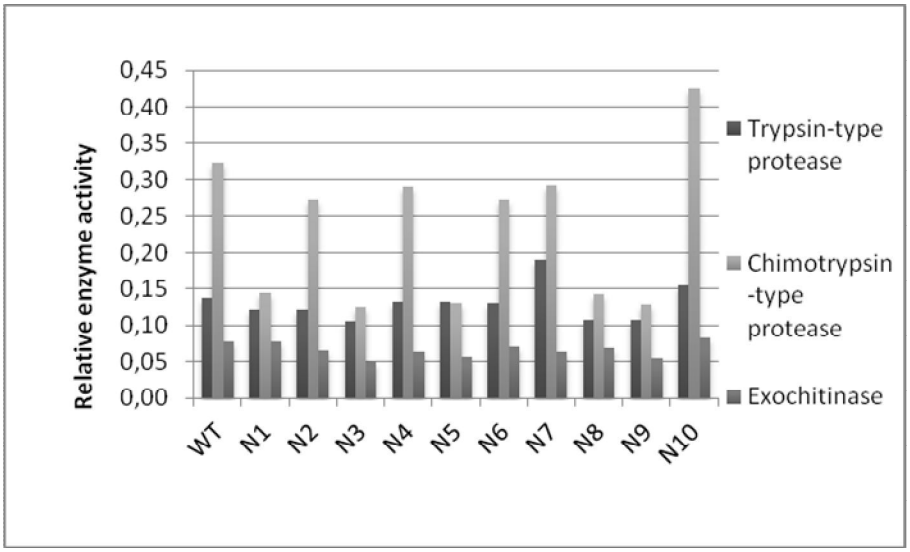

Figure 2. Enzyme profiles of the morphotype-2 streptomycin-resistant *B. subtilis* mutants compared with those of the wild type strain (WT)

The mutant *B. amyloliquefaciens* strain *SR5* had elevated chymotrypsin-type protease activity compared with the wild type (*Figure 3*).

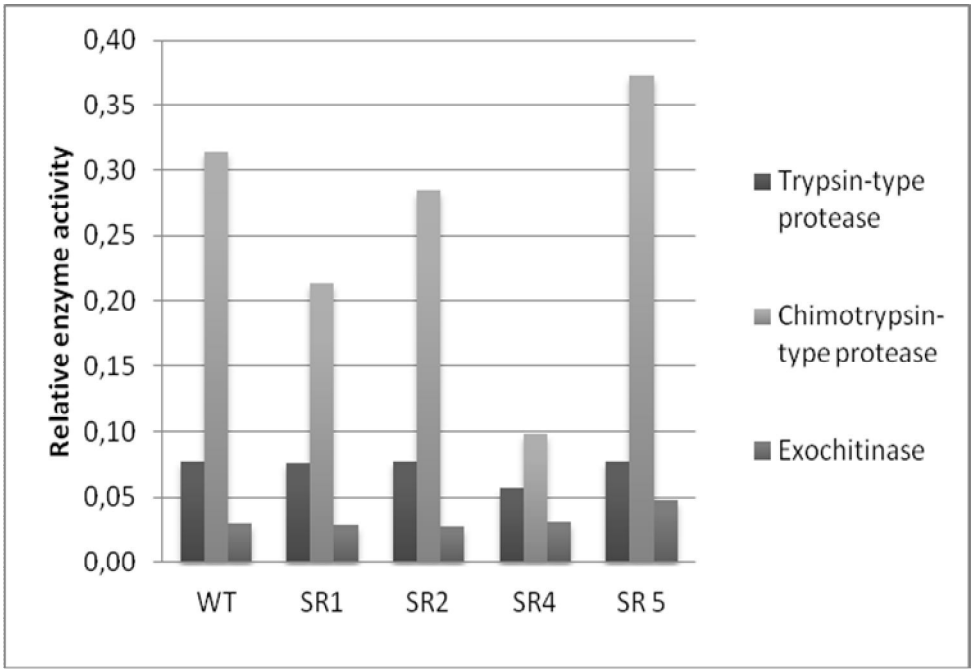

Figure 3. Enzyme profiles of streptomycin-resistant *B. amyloliquefaciens* mutants compared with those of the wild type strain (WT)

Investigation of the antibiotic production

Six streptomycin-resistant mutants of *B. subtilis* showed elevated antibiotic secretion (*Table 1*). From the streptomycin resistant mutants of *B. amyloliquefaciens* revealed increased tyrosine containing antibiotic secretion (*Table 2*).

Table 1. *B. subtilis* tyrosine-containing antibiotics in the ferment broths determined by optical density measurement at 280 nm

Strain name	OD 280nm	Strain name	OD 280nm
WT	0.125	WT	0.125
K1	0.076	N1	0.125
K2	0.102	N2	0.193
K3	0.134	N3	0.219
K4	0.169	**N4**	**0.467**
K5	0.035	N5	0.167
K6	**0.495**	**N6**	**0.409**
K7	0.051	**N7**	**0.324**
K8	**0.829**	N8	0.153
K9	0.064	**N9**	**0.314**
K10	0.12	N10	0.075

Table 2. *B. amyloliquefaciens* tyrosine-containing antibiotics in the ferment broths evaluated by optical density measurement at 280 nm

Strain name	OD 280nm
WT	0.337
SR1	0.319
SR2	0.272
SR4	**0.707**
SR5	0.361

The secreted antibiotics were visualized by TLC (*Figure 4*). *B. subtilis* strains showed outstanding surfactin producing abilities. This surfactin production slightly varied in the mutants. Surprisingly, the iturin production disappeared in the streptomycin-resistant strains. Fengycin production was very high in *B. amyloliquefaciens* strains. As in the case of SR4 mutants the fengycin disappeared from the ferment broth, the measured high optical density at 280 nm could origin from secreted free tyrosine or tyrosine containing proteins.

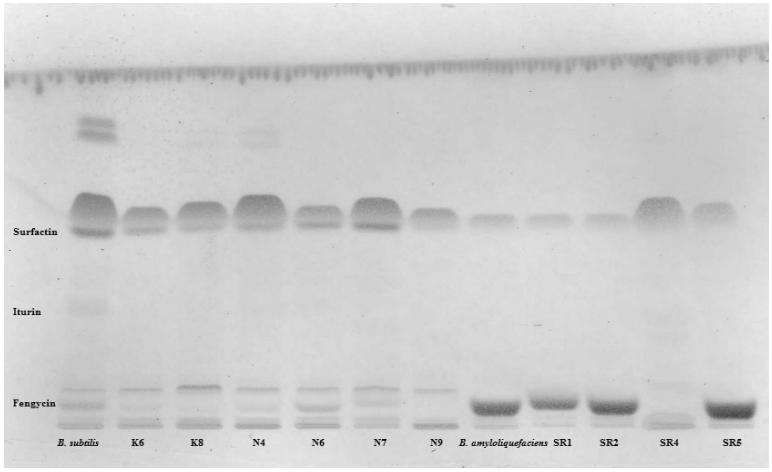

Figure 4. The secreted antibiotic profiles of the *B. subtilis* and *B. amyloliquefaciens* strains

Investigation of the *rpsL* gene

Investigation of *B. subtilis* mutants revealed point mutations in the sequence of the *rpsL* gene: in K9 **(**morphotype-1) adenine changed to guanine in the nucleotide 214 (transition). In the N1 strain (morphotype-2) guanine changed to thymine in the position of 103 (transversion). Sequence analysis of the *rpsL* gene did not reveal differences between *B. amyloliquefaciens* wilde-type and its streptomycin-resistant mutants strains.

CONCLUSIONS

Spontaneous streptomycin-resistant *B. subtilis and B. amyloliquefaciens* strains were isolated. Some of them showed elevated extracellular enzyme and antibiotic secretion. These phenomenon did not correlated with sequence changes in the *rpsL* gene. These findings suggest that the frequent abundance of strains with elevated secretion abilities amongst the spontaneous streptomycin-resistant mutants is not always related to genetic changes in the *rpsL* gene, but the method is useful for the breeding of biocontrol *Bacillus* strains.

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