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ORIGINAL ARTICLE

Antifungal peptides produced by *Bacillus amyloliquefaciens* AG1 active against grapevine fungal pathogens

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Abstract Antifungal metabolites produced by *Bacillus amyloliquefaciens* AG1, previously isolated from wood of grapevine with "esca syndrome", were studied. The crude protein extract (CPE) obtained from culture supernatant fluid by precipitation with ammonium sulfate was assayed against many grapevine fungal pathogens. *B. amyloliquefaciens* strain AG1 showed a broad spectrum of antifungal activity, inhibiting mycelial growth in vitro of all tested fungal microorganisms. The metabolites contained in CPE were heat stable and remained active over a wide pH range (2–10). Their activity was not affected by proteolytic or glycolytic enzymes. Tricine-SDS-polyacrylamide gel electrophoresis revealed a single band within the range of 2,510–3,480 Da, that showed inhibitory activity when used in the antifungal assay. Mass spectrometry analysis of this band allowed the substances involved in antibiosis to be identified as two tryptic peptides that correspond to the N-terminal sequence of subtilisin BPN'. These results suggest a potential role of *B. amyloliquefaciens* AG1 as a biocontrol agent.

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Introduction

Grapevine (*Vitis vinifera* L.) is one of the oldest and most important perennial crops in the world. Several fungal pathogens have been in reported in grapevine. Recently, in Sicily, besides the already well-known grapevine diseases, two emerging diseases "esca syndrome" (Mondello et al. 2008) and "*botryosphaeria* canker" (Burruano et al. 2008) —have been reported. Several *Bacillus* species produce various antibiotic substances (iturin, fengycin and bacillomycin) that play a dominant role in the biological control of fungal plant pathogens (Romero et al. 2007). Some of these species have also been reported to have antifungal activity in vitro towards grape pathogens, i.e., *Armillaria mellea* (Baumgartner et al. 2006), *Eutypa lata* (Schimdt et al. 2001) and *Botrytis cinerea* (Paul et al. 1997), agents of root disease, "eutypa dieback" and grey mold, respectively. A few of the antibiotic substances produced by *Bacillus* spp. have been characterized. In particular, strains of *Bacillus amyloliquefaciens* and *Bacillus subtilis* have been shown to synthesize chitinase (Wang et al. 2002) and antifungal peptides (Hiradate et al. 2002; Pinchuk et al. 2002).

The endophytic bacterial strain AG1, identified presumptively as *Bacillus subtilis* and isolated from grape wood tissues affected by "esca syndrome", has been shown to be effective as a biocontrol agent of esca fungi in vitro (Alfonzo et al. 2008). Therefore, the objectives of present study were to identify this strain more accurately, to test its antagonistic activity against grapevine fungal pathogens,

and to purify and characterize the antifungal substances involved in the antibiosis.

Materials and methods

Strains and growth conditions

Strain AG1 (belonging to the Culture Collection of Dipartimento DEMETRA—Università degli Studi di Palermo, Italy), previously selected as an antifungal compound producer and preliminary classified as *Bacillus subtilis* (Alfonzo et al. 2008), was overnight grown in nutrient broth (NB, Oxoid) at 28°C with shaking. The production of active metabolites occurred in malt extract broth (MEB, Oxoid, http://www.oxoid.com) after overnight incubation at 37°C with shaking. The fungi used in antagonism assays were: *Alternaria alternata*, obtained from grapevine leaves showing necrotic concentric spots; *Aspergillus carbonarius*, *Aspergillus ochraceus* and *Penicillium verrucosum*, isolated from grapes affected by rot secondary; *Botrytis cinerea*, isolated from grapes with grey mold; *Cladosporium viticola*, agent of leaf spots of grapevine; *Fomitiporia mediterranea* (FOMED), obtained from grapevines with symptoms of white rot; *Fusarium oxysporum*, isolated from the grapevine rhizosphere; *Lasiodiplodia theobromae*, isolated from vine wood affected by "*Botryosphaeria* canker"; *Phaeoacremonium aleophilum* (PAL) and *Phaeomoniella chlamydospora* (PCH), both isolated from grapevine showing esca symptoms; *Phoma glomerata* from grape leaves showing necrotic spots; *Verticillium dahliae*, obtained from decaying vine. All fungi were incubated at 27°C for 2 weeks and maintained on potato dextrose agar (PDA, Oxoid).

16S rRNA gene sequencing

Total DNA was extracted by InstaGene^{TM} Matrix (Bio-Rad Laboratories, Hercules, CA) according to the supplier's recommendations. The primers used for 16S rRNA gene amplification were fD1 and rD1described by Weisburg et al. (1991). The PCR mixture (50 μL total volume) included 50 ng of target DNA, 1 × *Taq* DNA polymerase buffer (Invitrogen, Carlsbad, CA), 2.5 mM $MgCl_2$, 250 μM of each dNTP, 0.2 μM of each primer and 2.5 U of *Taq* DNA polymerase (Invitrogen). PCR conditions were as follows: initial denaturing step at 95°C for 3 min; 30 cycles (1 min at 94°C, 45 s at 54°C, 2 min at 72°C); and an additional final chain elongation step at 72°C for 7 min. The amplification reaction was carried out in a T1 Thermocycler (Biometra, Göttingen, Germany). The PCR product was resolved by agarose (1.5% w/v) gel electrophoresis at 100 V for 2 h. The gel was stained with ethidium bromide and the band was visualized under UV illumination. The PCR amplification fragment was purified by a QIAquick gel extraction kit (Qiagen, Milan, Italy) and sequenced by the dideoxy chain termination method using a DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA) according to the manufacturer's instructions. The sequence was analyzed using MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe, Olivet, France) and compared to the GenBank nucleotide data library using the BLAST software at the National Centre of Biotechnology Information website (http://www.ncbi.nlm.nih.gov) (Altschul et al. 1997) in order to determine its closest phylogenetic relative. The determined sequence was deposited with GenBank. Phylogenetic analysis was performed using MEGA version 4.0 (Tamura et al. 2007) after multiple alignment of 16S rRNA sequences of strain AG1 and 18 type strains by ClustalW 1.8 (Thompson et al. 1994).

Extraction of secondary metabolites

The method reported by Wichitra et al. (2008) was modified as required and applied to extract secondary metabolites from crude protein extract (CPE) of strain AG1. The cell-free supernatant was obtained after centrifugation (10,000 *g* for 15 min) and then subjected to sonication at 20 KHz for 300 s. The culture supernatant was brought to 80% saturation with solid ammonium sulfate and, after stirring overnight at 4°C, the precipitate was collected by centrifugation (15,000 *g*, 20 min, 4°C). The pellet was dissolved in 1 M Tris-HCl buffer (pH 7.5) and desalted by dialyzing through a 12–14 kDa Spectra/Por molecularporous membrane tubing (Thomas Scientific, Swedesboro, NJ) in a 50 mM Tris-HCl buffer solution for 24 h. The dialyzed suspension was centrifuged at 13,500 *g* for 15 min at 4°C. The supernatant was filtered through a 0.2 μm membrane and tested for antifungal activity. This desalted active sample was lyophilized, stored at room temperature and labeled as CPE. Protein concentrations were determined by Lowry's method.

Characterization of secondary metabolites

The inhibitory metabolites were characterized for their antifungal activity, thermal and pH stability and susceptibility to several enzymes as reported below.

Antifungal activity

The activity of the CPE towards fungi was evaluated according to Villani et al. (1993) with some modifications. The fungal colonies were grown on PDA, until full invasion of the plate. Peptone solution (5 ml; 1 g/L) was added to sporulating and non-sporulating (FOMED) colonies, which were scraped to facilitate the dispersion of propagules. The fungal suspensions were collected and those of sporulanting fungi were sterile-filtered to separate the conidia from the

mycelial mass, obtaining a final concentration of approximately 10^6–10^7 propagules per milliliter. The FOMED suspension contained only mycelium. Aliquots (1 mL) of each fungal suspension were singularly placed in Petri dishes, covered and mixed with 14 mL malt extract agar (MEA) (T=43°C). After agar medium solidification, ten wells (Ø 6 mm) were cut and filled as follows: nine wells with 50 μL CPE serially diluted two-fold, and one well was used as negative control filled with Tris-HCl buffer (1 M, pH 7.5). CPE activity was expressed in arbitrary units (AU/mL) according to the following formula: AU/mL=radius of inhibition (mm)/[well capacity (mL) × concentration CPE (mg/mL)] (Villani et al. 1994).

Sensitivity to different temperatures and pH

The thermal stability of CPE (1 mg/mL) was determined at 4, 20, 40, 60, 80, 100 and 120°C for 10 min; the remaining activity was assayed vs *V. dahliae* as above reported. The control test consisted of CPE kept at room temperature. The stability of CPE at different pH values (2, 4, 6, 8, 10) was determined after solution in distilled water (1 mg/mL). Samples of distilled water with pH adjusted to 2, 4, 6, 8 and 10 were used as control tests. The residual antifungal activity was assayed against *V. dahliae* as described above.

Effect of enzymes

To test enzymatic sensitivity, CPE was treated with the following enzymes (Sigma, St. Louis, MO), dissolved in distilled water at a concentration of 1 mg/mL: pepsin (pH 1.5), pronase (pH 7.5), α-chymotrypsin (pH 7.5), papain (pH 6.5), α-amylase (pH 6.1) and proteinase K (pH 8.5). The pH of distilled water was adjusted (by NaOH 1 M or HCl 1 M) to pH values indicated for the optimum activity of each enzyme. Antifungal metabolites (1 mg/mL) were added in enzyme solutions and incubated for 2 h at different temperatures, depending on the type of enzyme (pepsin, α-chymotrypsin, α-amylase and proteinase K at 37°C; pronase at 40°C; papain at 65°C; Munimbazi and Bullerman 1998). Controls consisted of CPE without enzymes and enzyme solutions alone. Residual CPE activity against *V. dahliae* was evaluated by the agar diffusion technique described above.

Tricine-SDS-PAGE gel electrophoresis and antifungal bioassay

The molecular mass of the antibiotic peptides was estimated in tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) according to Schägger et al. (1987). The gel was prepared with 4%, 10%, and 16.5% polyacrylamide for stacking, spacer, and separating portion, respectively. Molecular weight markers for peptides (Product No. MW-SDS-17S, Sigma) were used. After 4 h of electrophoresis at a constant voltage of 100 V, the gel was removed and cut into two vertical parts. One half containing the sample and molecular weight markers was stained with Coomassie Brilliant Blue R-250 (Sigma). The other gel half was fixed with a solution 50% methanol–10% acetic acid and washed in

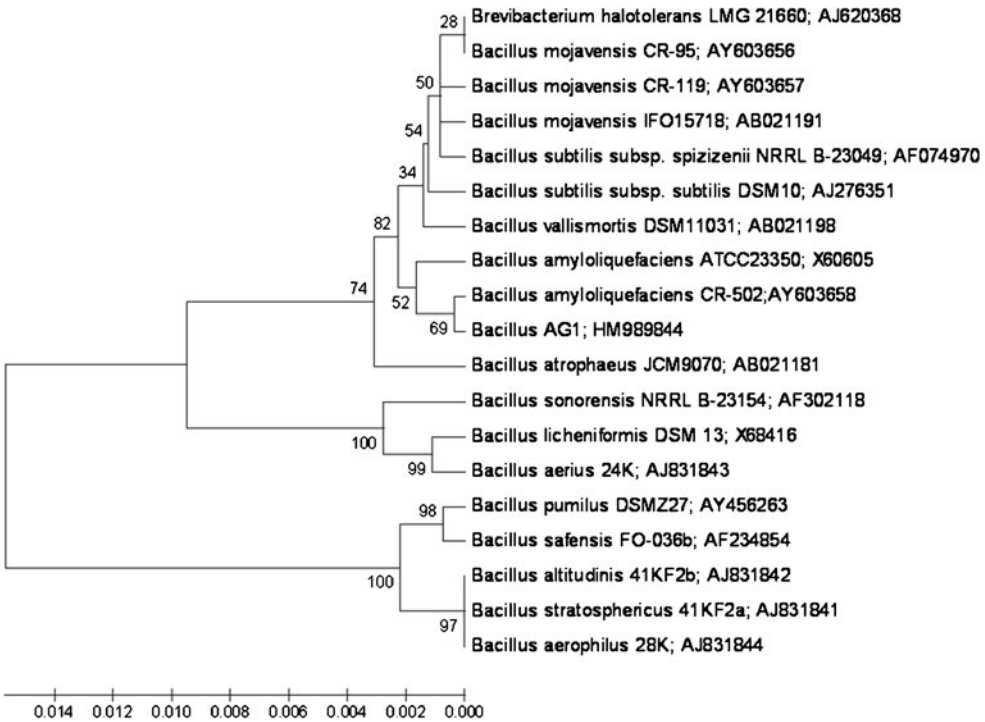

Fig. 1 Neighbor-joining tree based on comparison 16S rRNA gene sequences showing the relationships between *Bacillus* species. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. The *scale bar* estimates the number of substitutions per site

Table 1 Sensitivity of fungal strains to crude protein extract (CPE) of *Bacillus amyloliquefaciens* AG1, as determined by the critical dilution assay

Fungal strains	AU/mL
Alternaria alternata	7,500
Aspergillus carbonarius	5,000
Aspergillus ochraceus	7,500
Botrytis cinerea	7,500
Cladosporium viticola	1,250
Fomitiporia mediterranea	2,000
Fusarium oxysporum	3,750
Lasiodiplodia theobromae	5,000
Phaeoacremonium aleophilum	5,000
Phaeomoniella chlamydospora	5,000
Phoma glomerata	2,500
Penicillium verrucosum	10,000
Verticillium dahliae	12,500

distilled sterile water for 3 h. This gel was placed into a sterile Petri dish (Ø=20 cm) and overlaid with 10 mL MEB soft agar (MEB plus 0.8% agar) containing about 10^6 CFU/mL *A. carbonarius* used as an indicator organism. The plate was incubated at 25°C for 7 days and examined for inhibition (Bhunia et al. 1987).

Mass spectrometry analysis

Coomassie blue-stained spots were excised from SDS gels, de-stained twice with 50% acetonitrile (ACN) in 5 mM ammonium bicarbonate and dried for 30 min in ACN. Dried proteins were digested with trypsin (200 ng Sigma T6587) in 5 mM ammonium bicarbonate buffer, at 37°C for 15 h). Mass Spectrometry (MS) analysis was performed with a ESI Q-ToF Ultima (Micromass, Manchester, UK). Peaks were analyzed by means of Mascot software using Swiss-Prot database (SwissProt 2010_07; 517,802 sequences). Search settings allowed one missed cleavage with the trypsin enzyme selected, oxidation of methionine as potential variable modification, peptide mass tolerance of 0.6 Da, fragment mass tolerance: ± 0.3 Da, *taxa Firmicutes*.

Results

Molecular analysis of bacterial strain AG1

Sequencing of the 16S rRNA gene allowed strain AG1, previously identified as *B. subtilis*, to be reclassified. The whole gene sequence (1,491 bp) of this bacterial strain showed an identity of 99% with *B. amyloliquefaciens* using Blast software (GenBank ID: HM989844).

A phylogenetic tree was constructed including 18 *Bacillus* 16S rRNA gene sequences available at the ribosomal database (http://rdp.cme.msu.edu/). The results of neighbor-joining analysis of 16S rRNA sequences of the 19 strains are shown in the dendrogram depicted in Fig.1. The closest relative species was *B. amyloliquefaciens*, demonstrating that the strain AG1 can be ascribed to this species.

Isolation of active metabolites and determination of antifungal activity

Antifungal metabolites produced by *B. amyloliquefaciens* AG1 were isolated successfully from cell-free supernatant culture fluid by precipitation with 80% ammonium sulfate. The technique based on ammonium sulfate extraction yielded approximately 64 mg/L CPE. The results of inhibition are reported in Table 1. The highest CPE activity was against *V. dahliae* (12,500 AU/mL) followed by *P. verrucosum* (10,000 AU/mL; Fig. 2).

Thermal and pH stability

The antifungal activity of CPE was subjected to stability tests in order to gain insight into the chemical nature of the responsible compounds. The antifungal activity of CPE was stable at high temperature (60–120°C). Autoclaved metabolites (120°C, 10 min, 100 KPa) retained full inhibitory activity against mycelial growth of *V. dahliae*. Furthermore, there was no difference between diameters of inhibition zones caused by metabolites treated at lower temperatures (4–40°C). Similarly, no difference in diameters of inhibition zones were found after treatment at different pH values, although the metabolites tended to precipitate at pH 2.0.

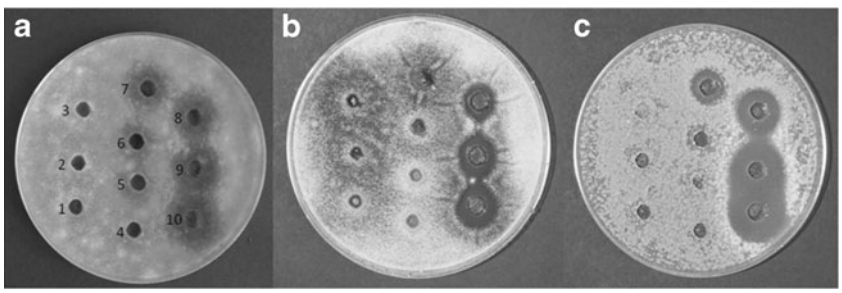

Fig. 2 Antifungal effect of crude protein extract (CPE) from *Bacillus amyloliquefaciens* on: **a** *Phoma glomerata*; **b** *Aspergillus carbonarius*; **c** *Penicillium verrucosum*. Wells 2–10 contained 50 μL CPE two-fold serially diluted; well 1 contained a control solution

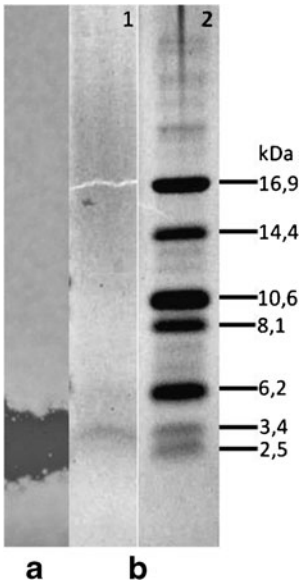

Fig. 3a,b Tricine-SDS-PAGE (**b**) and the associated antifungal bioassay (**a**) corresponding zone of inhibition. Lanes: *1* active substances, *2* protein molecular weight marker

Susceptibility to enzymes

The CPE treated with different enzymes (pepsin, pronase, α-chymotrypsin, papain, α-amylase and proteinase K) retained the antagonistic activity against *V. dahliae* in the agar diffusion assay. None of the seven enzymes altered the antibiotic properties of the secondary metabolites. Buffers and enzyme preparations alone had no effect on the growth of *V. dahliae*.

Gel electrophoresis and MS analysis

The inhibitory effect of the substance isolated from *B. amyloliquefaciens* strain AG1 against *A. carbonarius* was demonstrated by the zone of growth inhibition of *A. carbonarius* (Fig. 3a); this zone corresponded with the only band obtained in the gel stained with Coomassie Blue (Fig. 3b lane 1), with an approximate molecular weight in the range 2,510–3,480 Da (Fig. 3b, lane 2). The peptides were sequenced by ESI-Q-Tof. According to the Mascot searching results, some different substances were suggested and sorted by the total Mascot score based on the identified peptides. The active band in the gel showed the high presence of two tryptic peptides: SSLENTTTK and HPNWTNTQVR. These two peptides, compared with the entire amino acid sequence of subtilisin BPN′ (SUBT_BACAM, UniProtKB Number P00782), are located at the N-terminal of this sequence (Fig. 4). The subtilisin BPN′ is an extracellular alkaline serine protease produced by *B. amyloliquefaciens*.

Discussion

In the present study, strain AG1, previously recognized as *B. subtilis* based on a partial 16S rRNA gene sequence (Alfonzo et al. 2009), was reclassified as *B. amyloliquefaciens* on the basis of the whole 16S rRNA gene sequence. Antifungal metabolites produced by *B. amyloliquefaciens* strain AG1were purified and characterized. CPE was obtained from culture supernatant fluid by precipitation with ammonium sulfate and tested on grapevine fungal pathogens. The results demonstrated that *B. amyloliquefaciens* AG1, an endophyte in symptomatic wood of grapevine, has a wide spectrum of antifungal activity, inhibiting mycelial growth in vitro of all assayed fungi, although the latter exhibited different sensitivity to CPE.

The CPE of strain AG1 was heat stable and active under both acid and basic pH, in agreement with those reported for other antimicrobial metabolites produced by *Bacillus pumilus* (Munimbazi et al. 1998), *Bacillus licheniformis* (Esikova et al.

Fig. 4 Complete amino acid sequence of subtilisin BPN′. The two peptides identified in this work are highlighted in *gray*

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        10         20         30         40         50         60
MRGKKVWISL LFALALIFTM AFGSTSSAQA AGKSNGEKKY IVGFKQTMST MSAAKKKDVI

        70         80         90        100        110        120
SEKGGKVQKQ FKYVDAASAT LNEKAVKELK KDPSVAYVEE DHVAHAYAQS VPYGVSQIKA

       130        140        150        160        170        180
PALHSQGYTG SNVKVAVIDS GIDSSHPDLK VAGGASMVPS ETNPFQDNNS HGTHVAGTVA

       190        200        210        220        230        240
ALNNSIGVLG VAPSASLYAV KVLGADGSGQ YSWIINGIEW AIANNMDVIN MSLGGPSGSA

       250        260        270        280        290        300
ALKAAVDKAV ASGVVVVAAA GNEGTSGSSS TVGYPGKYPS VIAVGAVDSS NQRASFSSVG

       310        320        330        340        350        360
PELDVMAPGV SIQSTLPGNK YGAYNGTSMA SPHVAGAAAL ILSKHPNWTN TQVRSSLENT

       370        380
TTKLGDSFYY GKGLINVQAA AQ
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2002) and *Bacillus mojavensis* (Nair et al. 2002). Moreover, the results indicated that activity of CPE was not affected by proteolytic (pepsin, pronase, α-chymotrypsin, papain, proteinase K) and glycolytic (α-amylase) enzymes. However, failure of proteolytic enzymes to inactivate antifungal metabolites produced by *Bacillus* species or other bacteria is not unusual (Munimbazi et al. 1998).

The method described by Bhunia et al. (1987) enabled us to detected the specific fraction of protein associated with antifungal activity. MS analysis of this fraction allowed identification of the chemical compounds involved in antibiosis as two tryptic peptides that are part of the N-terminal sequence of subtlisin BPN′. The subtilisins are a prototypical group of bacterial serine proteases produced by various species of *Bacillus* (Rao et al. 1998). In particular, *B. amyloliquefaciens* is reported as producer of subtilisin BPN′ (Wells et al. 1983). Different serine proteases, such as subtilisin Carlsberg, subtilisin BPN′ and Savinase are used commonly as enzymes in the detergent industry (Gupta et al. 2002), while Bace16, produced by *Bacillus nematocide*, has been reported as active substance against nematodes (Niu et al. 2007).

This work reports for the first time the antifungal role of two tryptic peptides, secreted by *B. amyloliquefaciens* AG1, against several fungal pathogens of grapevine. Considering that the role of natural limiters is nowadays very important in agriculture, further studies are needed to understand the mechanism of antibiosis of these biologically active substances produced by *B. amyloliquefaciens* AG1, either under in semi-vivo or in vivo conditions, and characterize other substances that could be involved in antibiosis.

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