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Biological control of grapevine crown gall: purification and partial characterisation of an antibacterial substance produced by *Rahnella aquatilis* strain HX2

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Abstract Strain HX2 of *Rahnella aquatilis* has been previously reported as a potential biological control agent (BCA) of grapevine crown gall. The production of an antibacterial substance (ABS) was suggested to be an important factor in the biocontrol process. This study was undertaken to determine the antibacterial properties and mode of action of ABS. Isolation and purification of ABS from culture broth of strain HX2 was achieved by methanol exaction, chromatography on macroporous resin and silica gel, and high-performance liquid chromatography (HPLC). Physical and chemical characteristic analysis revealed that ABS may be a thermostable and alkali-sensitive substance containing sugar(s) and an unknown 285-nm absorbed substance. Antibacterial activity assays revealed that ABS displayed a broad activity spectrum against all the 13 test isolates of phytopathogenic bacteria including the genera of *Agrobacterium, Clavibacter, Pectobacterium, Pseudomonas,* and *Xanthomonas*. *Agrobacterium* spp. strains were more sensitive to ABS than other tested strains, with larger inhibition zones and lower minimal inhibitory concentration (MIC). ABS exhibited a bactericidal effect against *A. vitis* both *in vitro* and *in vivo*. This compound did not cause bacterial cell lysis, as determined by morphological observation with an electron microscope. Also, no leakage of cytoplasmic materials from cells of *A. vitis* occurred after treatment with ABS at concentrations equivalent to the MIC. However, an inhibition of the incorporation of radiolabelled precursors into RNA and protein was observed after treatment with ABS. These results suggest that ABS inhibits RNA and protein syntheses in tumorigenic *A. vitis*.

Keywords *Agrobacterium vitis* · Antibacterial substance · Biological control · Mode of action · *Rahnella aquatilis*

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Introduction

Grapevine crown gall, caused by tumorigenic *Agrobacterium vitis* (formerly *A. tumefaciens* biovar 3), is an economically important disease in grape-growing regions worldwide (Burr et al. 1998). Biological

control of crown gall disease has been implemented since *A. rhizogenes* strain K84 (formerly *A. radiobacter* strain K84) was used commercially to control peach crown gall in 1980 (Kerr 1980). However, K84 is not effective in preventing grapevine infections caused by tumorigenic *A. vitis* (Staphorst et al. 1985; Thomson 1986; Farrand 1990; Burr et al. 1998; Kawaguchi et al. 2005); therefore, several avirulent bacterial antagonists have been reported as biological control agents (BCAs) of the pathogens insensitive to K84 (Hendson et al. 1983; Staphorst et al. 1985; Webster et al. 1986; Chen and Xiang 1986; Liang et al. 1990; Burr and Reid 1994; Bell et al. 1995; Kawaguchi et al. 2005; Chen et al. 2007). The non-pathogenic bacterium *Rahnella aquatilis* strain HX2, isolated from vineyard soil in Beijing, China, was active against the growth of crown gall strains of *A. vitis*, *A. tumefaciens,* and *A. rhizogenes in vitro* (Chen et al. 2007). Greenhouse and field experiments indicated that HX2 could reduce the disease incidence of crown gall in grapevines by inhibiting or completely preventing tumour formation induced by *A. vitis* (Chen et al. 2007). The culture supernatant of strain HX2 exhibited a stronger inhibitory effect on disease development than cell suspension (Chen et al. 2007). Moreover, reduced biocontrol effect of Tn5-induced antibacterial substance (ABS)-deficient mutants compared to the wild-strain HX2 indirectly indicated the role of ABS in disease suppression (This laboratory, unpublished data).

Agrobacterium spp., acting as BCAs, were commonly reported to produce agrocins against pathogenic strains of *Agrobacterium* (Kerr 1980; Hendson et al. 1983; Staphorst et al. 1985; Webster et al. 1986; Chen and Xiang 1986; Liang et al. 1990; Burr and Reid 1994; Kawaguchi et al. 2005). The well-characterised Agrocin 84, produced by strain K84, exhibits specific toxicity towards *Agrobacterium* strains able to utilise the nopaline/agrocinopine-type opines (Kerr 1980), and is thought to be an important factor involved in the biocontrol process by strain K84 (Mcclure et al. 1998). Agrocin 84 was identified as a disubstituted analogue of adenosine (Tate et al. 1979) and was demonstrated to target protein synthesis in the pathogen (Kim et al. 2006).

Little information on antibiotics produced by strains of *R. aquatilis* exists, although several antagonists of this organism were found to inhibit growth of pathogenic bacteria *in vitro* and/or *in vivo* (Bell et al. 1995; Calvo et al. 2007; El-Hendawy et al. 2003, 2005; Hashidoko et al. 2002). Our previous work revealed that strain HX2 exhibited *in vitro* inhibitory activity against 11 isolates of *A. tumefaciens*, five isolates of *A. rhizogenes*, and five isolates of *A. vitis* (Chen et al. 2007), suggesting ABS production. In addition, two Tn5 mutants of strain HX2 that abolished the production of ABS displayed evidently reduced biocontrol effects, and the non-ABS-producing mutants which restored ABS production after the introduction a gene region responsible for ABS synthesis from strain HX2 were shown to be equal to strain HX2 in their ability to suppress *A. vitis* -induced crown gall of grapevine (This laboratory, unpublished data). These results indicated that the production of ABS has an important role in the ability of strain HX2 to control crown gall of grapevine by *A. vitis*. This paper describes the purification and partial characterisation of the antibacterial compound ABS. Additionally, ABS was analysed for its mode of action on sensitive tumorigenic *A. vitis*.

Materials and methods

Strains, media, and growth conditions

Rahnella aquatilis strain HX2 was isolated from the rhizosphere of grapevines grown in Beijing, China (Chen et al. 2007). The strain was cultured at 28°C with shaking (180 r.p.m.) in potato-dextrose broth (PDB). Test strains of phytopathogenic bacteria (Table 1) were obtained from our laboratory collection and were stored at −20°C in yeast extract broth (YEB) (Vervliet et al. 1975) containing 15% (vol/vol) glycerol, and grown on YEB agar medium at 28°C when required.

Preparation of crude extract and ABS purification

Strain HX2 was incubated for 2 days in PDB. The culture mixture was centrifuged at 15,300 *g* for 20 min at 4°C, and the supernatant retained. The supernatant was then concentrated to 1/50 volume in a rotary evaporator at 40°C. Two volumes of methanol were added to one volume of the concentrate and thoroughly mixed at room temperature (RT). Precipitate in the mixture was discarded by centrifugation (15,300 *g*, 10 min). The methanol in the supernatant was

Table 1 Antibacterial activity of the ABS produced by *R. aquatilis* strain HX2

Bacteria	Source/Reference	MIC (μg ml^{-1})	Diam of inhibition zone (mm)
Agrobacterium tumefaciens C58	Watson et al. (1975)	2.5	60.8±2.9 a[a]
Agrobacterium tumefaciens MG10-1	This laboratory	2.5	65.2±2.6 a
Agrobacterium rhizogenes K27	Shim et al. (1987)	2.5	60.6±1.7 a
Agrobacterium rhizogenes NL12-2	This laboratory	2.5	65.4±3.4 a
Agrobacterium vitis K308	Salomone et al. (1996)	2.5	62.4±1.2 a
Agrobacterium vitis MI23-1	This laboratory	2.5	61.0±1.3 a
Clavibacter michiganensis subsp. *michiganensis* B11	This laboratory	20.0	18.3±2.1 d
Pectobacterium carotovorum subsp. *carotovorum* H02	This laboratory	5.0	39.0±3.5 b
Pseudomonas syringae pv. *syringae* K3	This laboratory	10.0	22.5±2.3 d
Pseudomonas syringae pv. *lachrymans* X15	This laboratory	10.0	22.5±2.3 d
Xanthomonas campestris pv. *campestris* C21	This laboratory	10.0	27.6±2.5 c
Xanthomonas campestris pv. *malvacearum* Z9	This laboratory	10.0	28.9±0.8 c
Xanthomonas oryzae pv. *oryzae* N07	This laboratory	10.0	28.9±0.8 c

The experimental data were treated according to critical values of the Student-t test

[a] Data shown as mean ± standard error of three replications. Values followed by different letters are statistically significant ($P<0.05$)

completely removed with the rotary evaporator and the sample was dried to a solid form with a freezing dryer. The dried material was kept at −20°C and used as ABS crude extract samples in this experiment. For a bioassay of the antibacterial activity present in the crude extract, the residue was dissolved in methanol (1 ml of the solvent per residue from 500 ml supernatant) and a well-agar plate diffusion assay was used as described previously (Li et al. 2008). Briefly, 50 μl of the residue in methanol was added to a well formed by a small sterilised steel cylinder (10 mm× 8 mm×10 mm, outer diam × inner diam × height) placed in the centre of a YEB agar (1.5%, *w*/*v*) plate previously inoculated with 100 μl of cell suspension of *A. vitis* strain K308 (10^8 CFU ml^{-1}). After incubation for 4 h at RT to allow the sample to diffuse across the surface, the cylinder was removed and the plate incubated at 28°C for 48 h. The antibacterial activity of the sample was demonstrated by a clear zone of inhibition around the well.

The ABS crude extract was dissolved in ethyl acetate-chloroform (1:1, vol/vol), and absorbed into NKA-2 Macroporous Resin (Nankai Chemical Co., Tianjin, China) column (4.5×90 cm) and then eluted seriatim with ethyl acetate-chloroform (1:1,1.5:1, 2:1, 4:1, vol/vol), ethyl acetate, ethyl acetate-ethanol (5:1, 2:1, 1:1, vol/vol), and ethanol at a rate of 1.0 ml min^{-1}. The eluent was collected into tubes in fractions of 5 ml with a BSZ-100 automatic fraction collector (Shanghai Huxi Analytical Instrumentation Factory, Shanghai, China). After being bioassayed using the well-agar plate diffusion method with *A. vitis* strain K308 as the indicator, the active fractions obtained by elution with ethyl acetate-chloroforml (4:1, vol/vol) were combined and concentrated under vacuum at 40°C, then diluted with twice the volume of ethyl acetate-chloroform (1:1, vol/vol) and separated again by using a semi-preparative scale silica gel 60 column (2.6×40 cm, Beijing Chemical Reagent Co., Beijing, China) with the mobile phase consisting of ethyl acetate-chloroform (4:1, vol/vol). The eluent was collected, concentrated and bioassayed again. The main active fraction was picked out as the terminal sample and concentrated with a SpeedVac centrifuging concentrator (Thermo SPD1010, USA).

Partial characterisation of ABS

The ABS crude extracts and purified preparations were analysed by thin-layer chromatography (TLC) with silica gel 60-glass sheets (Nankai Chemical Co., Tianjin, China) and the mobile phase consisting of chloroform-methanol (5:1, vol/vol). Visualisation was performed by exposing to air for 10 min and monitoring at 315 nm with a UV lamp. The purity of ABS was examined using analytical HPLC (Shimadzu LC-10AT, Japan) with a

VP-ODS RP- C18 column (5 μm, 250×4.6 mm diam) and a gradient of methanol–water (20 to 100%) at a flow rate of 1 ml ml^{-1}. Effluent absorbance was monitored at 285 nm with a UV detector (JAI UV 3702, Japan).

The temperature stability of the purified ABS was tested at 20, 40, 50, 60, 70, 80, 90, 100, and 121°C as follows. ABS was dissolved in phosphate-buffered saline (pH 7.4) to a final concentration at 1 mg ml^{-1}, then incubated at different temperatures for 30 min, after which its antibacterial activity was tested by the well-plate diffusion method with strain K308 as the indicator. The diameter of inhibition zone of each treatment was measured after incubation at 28°C for 48 h. The treatment at 20°C was used as the control. Residual activity of each treatment was expressed as the ratio of the diameter of the inhibition zone to that caused by the control. All determinations were performed in triplicate and the experimental data were treated according to critical values of the Student-t test.

The stability of ABS at different pHs was determined by dissolving purified ABS in 50 mM Tris-hydrochloride (1 mg ml^{-1}); pH ranged from 2 to 12. The Tris-hydrochloride solution was incubated at 25°C for 1 h, and pH was checked before this solution was assayed for antibacterial activity using the well plate-diffusion method as described above. Residual activity of each treatment was expressed as the ratio of the diameter of the inhibition zone to that caused by the non-treated control. All experiments were done in triplicate and the data were treated according to critical values of the Student-t test.

The UV spectrum of ABS within 200–400 nm was monitored in methanol using a Unicam HeλIOSα spectrophotometer (Thermo Fisher Scientific, USA) according to the method of Hendson et al. (1983) with slight modification. Inactivation of this substance by DNase (10 μg ml^{-1}), RNase (10 μg ml^{-1}), or selected proteolytic enzymes (1 mg ml^{-1}) was studied. Reaction mixtures with or without enzyme were incubated at 37°C for 3 h before assaying for antibacterial activity. Enzymes included DNase I, RNase A, protease type X (thermolysin), trypsin type III, proteinase K and papain. Residual activity was assessed by comparing the diameter of the inhibition zone against strain K308 with that caused by untreated ABS. The experiments were repeated three times.

Determination of antibacterial activity and host spectrum of ABS

For determination of antibacterial activity in liquid culture, a broth-microdilution method was used as described previously (Li et al. 2008). The tests were conducted in YEB containing various concentrations of ABS. Bacteria (Table 1) were incubated overnight at 28°C in YEB and suspended in YEB broth to provide a concentration of approximately 10^8 CFU ml^{-1}. Serial 2-fold dilutions of ABS ranging from 1 to 100 μg ml^{-1} were prepared in 1 ml Eppendorf tubes with YEB. YEB broth was used as the negative control. Logarithmic phase cells of bacterium (Table 1) were introduced into the tubes to a final concentration at 10^6 CFU ml^{-1}. All tubes were incubated at 28°C with shaking for 24 h. Following incubation, 100-μl test bacterial suspensions from each concentration of ABS were separately spread onto YEB agar plates and incubated at 28°C for an additional 48 h. The numbers of colonies on each Petri plate were counted. The antibacterial activity was expressed as minimum inhibitory concentration (MIC). The MIC of ABS was defined as the lowest concentration that prevented the visible growth of bacteria on the agar plate. All determinations were performed in triplicate.

Effects of ABS against the growth of various phytopathogenic bacteria (Table 1) were also investigated using the well-agar plate diffusion assay as described previously. The diameter of inhibition zone was measured after incubation at 28°C for 48 h. All determinations were done in triplicate and the data were treated according to critical values of the Student-t test.

Effects of ABS on bacterial viability

For determination of bactericidal activity of ABS, bacterial viability assays were carried out in early logarithmic phase cells of *A. vitis* strain K308. These cells were obtained by culturing the bacterium in YEB in a 200-ml sterile flask and incubating the flask at 28°C on a shaker at 160 rpm for 12 h, then centrifugating at 10,000× *g* for 10 min. Bacterial cells were suspended in 100 ml YEB broth to a concentration at 10^8 CFU ml^{-1}. ABS was added into the suspension at different final concentrations ranging from the MIC to 8 × the MIC. Sterilised saline was used as the negative control. The cell suspension was incubated at 28°C with shaking. Samples (1 ml) were

withdrawn from the suspension at 2-h intervals and serially diluted in sterilised saline; 100-μl dilutions were plated onto YEB agar (1.5%, *w*/*v*). Colonies were counted after incubation at 28°C for 48 h. All determinations were performed in duplicate.

The determination of whether the effect of ABS was bactericidal or bacteriostatic for strain K308 was also made on YEB agar plates inoculated with strain K308 and diffused by ABS through the well formulated as described above. Two 6-mm YEB agar disks were taken from within inhibition zones and placed in 5 ml of YEB broth. Bacterial growth in YEB broth was monitored after shaking incubation (150 rpm) at 28°C for 24 h. Broth samples were then plated on a medium selective for *A. vitis* (Roy and Sasser 1983) to confirm the identity of the test strains. Experiments were repeated three times.

Scanning electron microscopy (SEM) of ABS-treated bacteria

To determine occurrence of morphological change of bacterial cells due to ABS, strain K308 was cultured in YEB broth with an ABS concentration equivalent to approximately 2 × the MIC at 28°C for 5 h. After incubation, the bacterial cells were harvested by centrifugation (10,000×g for 5 min) and observed under a scanning electron microscope (Hitachi Co, Japan, Model S-2400) after fixation in 2% glutaraldehyde-containing PBS for 1 h at RT.

Effect of ABS on cell membrane permeability

The effect of ABS on cell membranes was investigated by testing the cellular electrolyte leakage from *A. vitis* strain K308. ABS was added to a suspension of strain K308 (10^8CFU ml^{-1} in sterilised saline) at a final concentration equivalent to its MIC. Sterilised saline was used as the negative control. Samples (1 ml) were withdrawn from the suspension at 30 min intervals and diluted to 1:10 (vol/vol), then passed through a filter with a pore size of 0.2 μm. Filtrates were collected and conductivity was measured using a DDS-307 electrical conductivity meter (Shanghai Jingke Industrial Co. Ltd., Shanghai, China). The conductivities of three samples obtained at each time-point were measured. The ratio of the conductivity at each time-point to the initial conductivity at the zero time-point was calculated. The ratios for treatment samples were compared with those for the corresponding untreated samples. All determinations were performed in triplicate; the experimental data were treated according to critical values of the Student-t test.

Effect of ABS on the biosynthesis of macromolecules

Effects of ABS on bacterial DNA, RNA, and protein synthesis were tested by measurement of incorporation of radioactive precursors, according to the procedure of Li et al. (2008). Briefly, an overnight Luria-Bertani (LB) (Sambrook et al. 2001) broth culture of *A. vitis* strain K308 was diluted 1:10 (vol/vol) in LB to obtain an OD_{600} of 0.1 (10^8 CFU ml^{-1}). The culture was divided into two parts for use as the test and control cultures. ABS was added to one of the samples at a final concentration equivalent to approximately 2 × the MIC, and sterilised saline was added to the other sample, designated as the control. [^{3}H]Thymidine, [^{3}H] uridine, and [^{3}H]leucine were immediately added to the cultures at final concentrations of 1 μCi ml^{-1} ([^{3}H] thymidine or [^{3}H]uridine) or 2 μCi ml^{-1} ([^{3}H]leucine) to determine DNA, RNA and protein synthesis, respectively, in separate experiments. The cultures were then further incubated. Culture samples (0.5 ml each) were withdrawn every 30 min, added to ice-cold 10% (*w*/*v*) trichloroacetic acid (TCA) for 30 min, and passed through filters with a pore size of 0.2 μm. Following sequential washing in cold 5% (*w*/*v*) TCA and in 1% (vol/vol) acetic acid, the filters were dried and radioactivity was measured using a scintillation counter (BH1216, Beijing Nuclear Instrument Factory, Beijing, China). Each experiment was performed in triplicate.

Biological control of grapevine crown gall by ABS

A 10-μl drop of a suspension of *A. vitis* strain K308 (10^8 CFU ml^{-1} in sterile saline) and 10-μl drop of a purified ABS solution (in sterile saline, 1 mg ml^{-1}) were serially inoculated into a 1.0 cm longitudinal incision wound made with a sterile scalpel on shoots of potted grapevine (*Vitis vinifera* cv. Muscat Hamburg). The sterile saline alone was applied as a negative control, and strain K308 was mixed with sterile saline as a positive control. The experiment was repeated twice. For each treatment, three inoculations were performed on the stem of three plants. Inoculation sites were wrapped with Parafilm. Gall formation was observed 40 days after inoculation.

Results

Extraction and purification of ABS

Crude extract of the *R. aquatilis* strain HX2 growing in PDB was tested for antibacterial activity against *A. vitis* strain K308 *in vitro*. Bacterial growth suppression was achieved with the culture supernatant and the crude extract (Fig. 1a, b). The crude extract presented a stronger inhibitory activity than the culture supernatant against strain K308, as revealed by an increase in diameter of the inhibition zone. The average diameter of inhibition zones caused by crude extract (1.6 cm) was greater than that caused by the culture supernatant (1.1 cm).

The results of the bioassay showed that the active constituents were only collected from the mobile phase of ethyl acetate-chloroform (4:1, vol/vol) in the case of the macroporous resin separation. Resultant data of TLC analysis revealed that the active fraction displayed an intense fluorescent orange spot on developed glass sheets when viewed under UV-irradiation, with an Rf of 0.28. The active fraction was separated again by a semipreparative scale silica gel column. A fraction with higher antibacterial activity was obtained as the terminal constituent. Only one peak with an absorption maximum at 285 nm at the retention time of approximately 2.82 min was detected for the terminal constituent by analytical HPLC, which revealed that the constituent contained a single compound (Fig. 2).

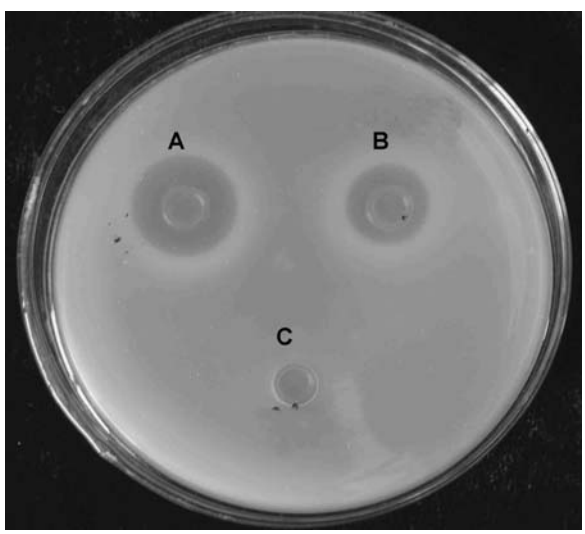


Fig. 1 Effects of the ABS produced by *R. aquatilis* strain HX2 on the growth of *A. vitis* strain K308 plated on YEB agar. Inhibition of bacterial growth at 28°C was estimated after 2 days. The treatments were **A** the crude extract of ABS in sterilised saline (0.5 mg ml^{-1}), **B** the culture supernatant of strain HX2, and **C** sterilised saline

Partial characterisation of ABS

The purified ABS was stable at −20°C and 4°C for at least 120 days and stable at 20°C for at least 7 days. The purified ABS was resistant to boiling for at least 30 min and to autoclaving at 121°C for 30 min (Table 2), and its antibacterial activity was not destroyed by 1 h of sunlight irradiation. The purified ABS was stable at pH 2 to 7 after 1 h of incubation. However, ABS was partially inactivated after 1 h at pH 8 to 9 and completely inactivated after 1 h at pH 10 to 12 (Table 2). The ABS preparations were resistant to DNase, RNase, and all proteolytic enzymes; the purified ABS showed maximal UV absorption at 285 nm and its chemical components showed a negative reaction to ninhydrin or ferric chloride and a positive reaction to Molish reagents (data not shown). These results indicated that the purified ABS may be a substance with sugar(s) and an uncharacterised 285 nm absorbed substance.

Antibacterial activity of ABS

The purified ABS exhibited a broad spectrum of activity against many phytopathogenic bacteria, even at low concentrations, depending on the organism tested (Table 1). The antibacterial activity was most efficient against *A. tumefaciens*, *A. rhizogenes*, *A. vitis,* and *Pectobacterium carotovorum* subsp. *carotovorum* (MIC$\leq$5.0 μg ml^{-1}). *Xanthomonas campestris* pv. *campestris, X. campestris* pv. *malvacearum, X. oryzae* pv. *oryzae, Pseudomonas syringae* pv. *syringae,* and *P. syringae* pv. *lachrymans* were suppressed at a higher concentration (MIC=10.0 μg ml^{-1}), whereas *Clavibacter michiganensis* subsp. *michiganensis* was only partially suppressed at MIC=20.0 μg ml^{-1}.

The growth inhibitions of these bacteria were also evaluated by using well plate testing. Results obtained (Table 1) appeared consistent with the sensitivity of the strains to ABS as determined by the broth microdilution method. Thus, *A. tumefaciens*, *A. rhizogenes*, *A. vitis,* and *P. carotovorum* subsp. *carotovorum* were more sensitive than *X. campestris* pv. *campestris, X. campestris* pv. *malvacearum, X. oryzae* pv. *oryzae,*

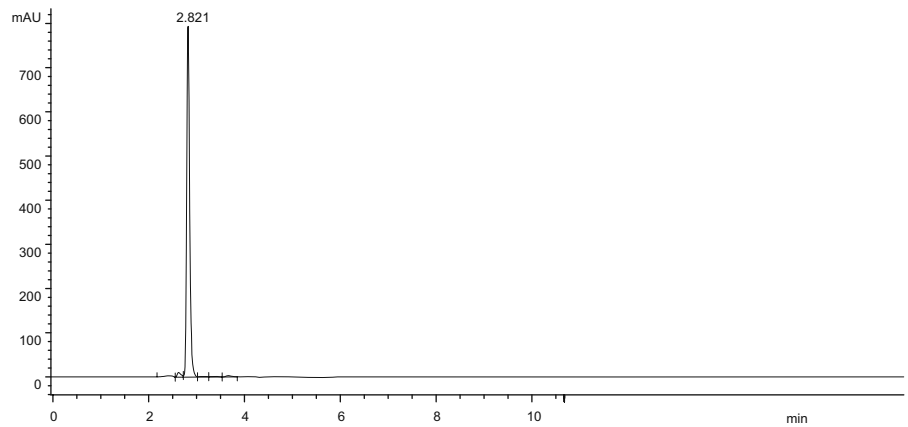


Fig. 2 HPLC elution profile of the purified ABS produced by *R. aquatilis* strain HX2 (absorbance at 285 nm versus retention time in min)

P. syringae pv. *syringae, P. syringae* pv. *lachrymans,* and *C. michiganensis*, with a larger clear inhibition zone formation on the agar plate.

Mode of action of ABS

The addition of 2.5 μg ml^{-1} of ABS (equivalent to the MIC determined in liquid culture by the dilution method) to early logarithmic phase cultures of *A. vitis* strain K308 in YEB broth caused partial suppression of growth over the beginning of a 4-h period and subsequent complete cessation of growth (Fig. 3). This was demonstrated by small increases in viable cell numbers. The population of strain K308 was about 10^6 CFU ml^{-1} at the beginning of the experiment. Incubation over the beginning of the 4-h period raised

Table 2 Inhibitory effect of the heat-treated or pH-treated ABS produced by *R. aquatilis* strain HX2 on the growth of *A. vitis* strain K308 on YEB agar in a Petri dish at 28°C

Temperature (°C)	Ratio of inhibitory activity [a] (%)	pH	Ratio of inhibitory activity [b] (%)
20	100.0 a	2.0	98.4 a
40	103.1 a	3.0	100.7 a
50	98.4 a	4.0	99.1 a
60	101.5 a	5.0	100.0 a
70	99.2 a	6.0	98.5 a
80	102.4 a	7.0	98.2 a
90	101.8 a	8.0	71.4 b
100	99.1 a	9.0	55.8 c
121	100.7 a	10.0	0.0 d
		11.0	0.0 d
		12.0	0.0 d

[a] Ratio of the diameter of inhibition zone of each treatment to that treated at 20°C

[b] The ratio of the diameter of inhibition zone of each treatment to that of non-treated control. Data are means of three independent experiments and treated according to critical values of the Student-t test. Data with the same letter in the same column were not significantly different ($P<0.05$)

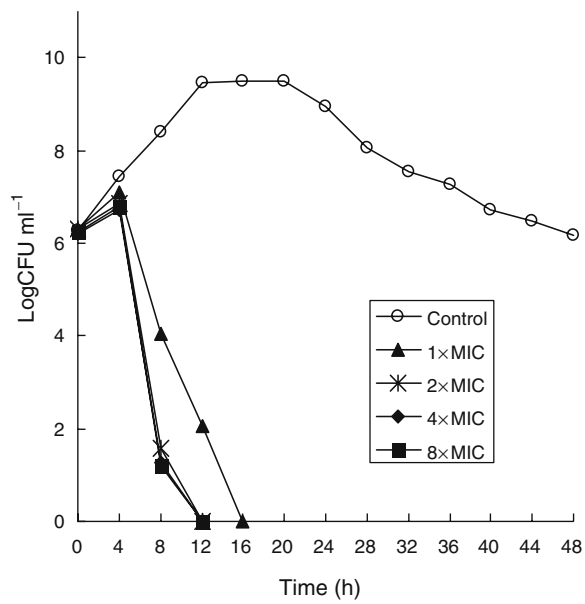


Fig. 3 Effects of the ABS produced by *R. aquatilis* strain HX2 on the growth of *A. vitis* strain K308 during exposure to this compound for 48 h. Every 2 h, 100-μl bacterial suspension was dilution plated on YEB. Colony counts were averaged in this graph. The data are representative of two independent experiments

the cell numbers of both the control and ABS treatments to about 10^7 CFU ml^{-1}. From then on, the population of strain K308 with ABS treatment reduced rapidly; however, the viable cell numbers of the control remained increasing. Addition of higher ABS concentrations (2 to 8 × the MIC equivalents) led to complete cessation of growth (Fig. 3). This may result from a bactericidal effect, since the number of viable cells declined rapidly after exposure to ABS for 4 h (Fig. 3). The inhibitory effect of ABS was bactericidal on solid medium. Strain K308 was not able to grow from agar disks taken from within the inhibition zones of the YEB plates.

In order to investigate the effect of the ABS on the external structures of *A. vitis* strain K308, morphology of the cells treated by the ABS was examined by SEM. The cells treated with ABS maintained a normal rod-like shape similar to the untreated cells and had no amorphous appearance.

ABS was determined not to affect bacterial cell membrane permeability. At each time-point, the proportion of the initial electrolyte leakage of the ABS treatment samples did not significantly differ from that of the control (Fig. 4), although a gradual increase in conductivity was observed both in filtrates from control suspensions (without the addition of ABS) and in filtrates from ABS-treated samples. Moreover, there was no significant difference between the cellular electrolyte leakage kinetics of the ABS treatment and that of the control (Fig. 4). RNA and protein syntheses in strain K308 cells, as measured by [^{3}H]uridine and [^{3}H]leucine incorporation, respectively, were inhibited after ABS was added at final concentrations equivalent to approximately 2 × the MIC, while DNA synthesis was not inhibited (Fig. 5).

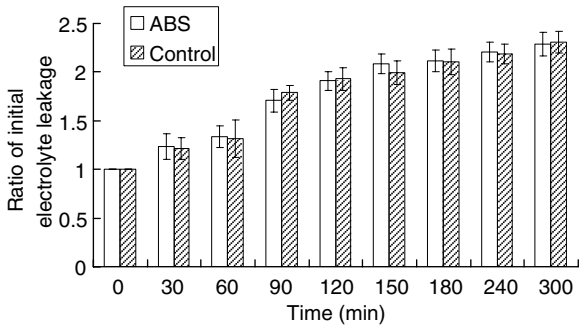


Fig. 4 Electrolyte leakage from *A. vitis* strain K308 cells treated with the MIC (2.5 μg ml^{-1}) of the ABS produced by *R. aquatilis* strain HX2 and the control (sterilised saline). The ratio of the conductivity at each time-point to the initial conductivity at the zero time-point was calculated. The ratios for treatment samples were compared with those for the corresponding untreated samples. Bars indicate standard deviation of the mean

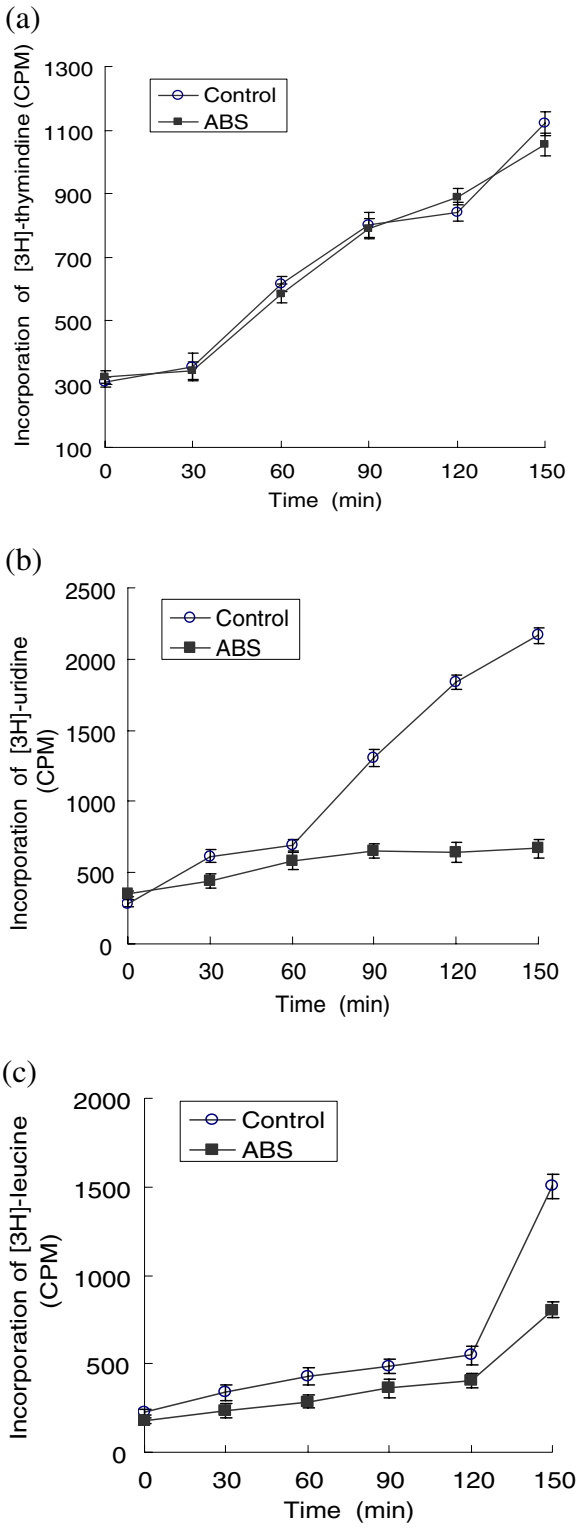
Biological control of grape crown gall by ABS

Application of the ABS in grapevines completely suppressed infection by *A. vitis* strain K308. No tumour formation was observed with both the ABS and the sterile saline treatments, while strain K308 induced evident crown gall symptoms (Fig. 6).

Discussion

Our research demonstrated that *R. aquatilis* strain HX2 produced an ABS that showed a broad spectrum of activity against a number of plant pathogenic bacteria, including strains of the genera *Agrobacterium*, *Pectobacterium*, *Xanthomonas*, *Pseudomonas*, and *Clavibacter,* respectively. This broad-activity spectrum is encouraging for the potential use of strain HX2 as a BCA of bacterial plant diseases.

Data obtained regarding physical and chemical characteristics of ABS indicated that the purified ABS may be a substance with unidentified sugar(s) and an uncharacterised 285 nm absorbed substance and needs further investigations. It remains uncertain whether the unknown substance and/or the sugar(s) in ABS play an important role in antibacterial activity. Agrocin 84, which exhibits specific toxicity towards *Agrobacterium* strains able to utilise the napoline/agrocinopine-type opines, is a disubstituted, fraudulent adenine nucleoside analogue with a toxic moiety that contains a 3'-Deoxy-D-arabinose substitute for deoxyribose (Murphy et al. 1981). The glucofuranosyl substituent at the N^6 position is required for specific uptake (Murphy et al. 1981), but not for toxicity. Ryu et al. (2006) reported that *R. aquatilis* strain AY2000 produces an anti-yeast substance (AYS) with heterologous sugars and an uncharacterised 230-nm absorbed substance. The role of the heterologous sugars of the AYS has not been identified yet (Ryu et al. 2006). *Rahnella aquatilis* strain bSL1 was antagonistic against *Penicillium expansum* and *Botrytis cinerea* on cv. Red Delicious apple fruit, but did not produce extracellular antibiotic substances (Calvo et al. 2007). Two *R. aquatilis* strains isolated from the phyllosphere of leaves of ramanas rose (*Rosa rugosa*),



◀ **Fig. 5** Effects of the ABS produced by *R. aquatilis* strain HX2 on macromolecular synthesis in *A. vitis* strain K308. **a** DNA synthesis determined by incorporation of [^{3}H]thymidine; **b** RNA synthesis determined by incorporation of [^{3}H]uridine; **c** protein synthesis determined by incorporation of [^{3}H]leucine. The data are means of three independent experiments. Bars indicate standard deviation of the mean. Sterilised saline was used as a control

can convert benzoic acid derivatives to decarboxylated simple phenolics with a higher level of antimicrobial activity than that of the substrates by the production of decarboxylase (Hashidoko et al. 2002). Other two *s*trains of *R. aquatilis* isolated from soil in Egypt were reported as antagonistic strains that produce siderophores as an inhibitory substance against bacteria (El-Hendawy et al. 2003). In addition, *R. aquatilis* strain JC577 has been reported to be antagonistic to *A. vitis in vitro* (Bell et al. 1995). However, this antagonistic activity remains unexplained.

In relation to the effect of ABS on early logarithmic phase strain K308 cells, the data obtained showed cessation of bacterial growth (Fig 3). Furthermore, prolonged incubation resulted in the decline in viable cell numbers either at concentrations of the MIC or up to eight-fold higher than the MIC (Fig 3).Therefore, the ABS seems to have a bactericidal rather than a bacteriostatic action. Subsequently, this hypothesis was supported by the biological control experiment in planta, where ABS completely inhibited crown gall symptoms (Fig. 6). Moreover, *A. vitis* strain K308 was not able to grow from agar disks taken from within the clear inhibition zones on YEB plates, which also indicated the bactericidal activity.

Fig. 6 Inhibition of tumour formation on grapevine shoots by the ABS produced by *R. aquatilis* strain HX2. *left (three shoots)* inoculated with sterile saline as the negative control; *middle (three shoots)* inoculated with *A. vitis* K308 only; *right (three shoots)* inoculated serially with *A. vitis* K308 and ABS

To further investigate this hypothesis, we attempted to isolate *A. vitis* strains from the grapevine shoots inoculated with strain K308 and ABS simultaneously following the method described by Moore et al. (2001) using Roy and Sasser's medium, which is selective for *A. vitis* (Roy and Sasser 1983). No *A. vitis* cells were isolated from the inoculation sites of grapevine shoots treated with ABS, suggesting that the effect of ABS was bactericidal for *A. vitis*.

Our data revealed that the bactericidal effect of ABS was mainly attributed to inhibition of RNA and protein synthesis in *A. vitis* cells (Fig. 5) as ABS did not cause whole-cell lysis leading to altered morphology and marked increase in electrolyte leakage, indicative of gross and irreversible damage to the cytoplasmic membrane, as shown by Bechinger (1997).

It was suggested that agrocin 84 targets protein synthesis in *A. tumefaciens* by specifically inhibiting the activity of leucyl-tRNA synthetase (Kim et al. 2006). We recently demonstrated that the agrocin produced by *A. vitis* strain E26 exerts a bactericidal effect on strain K308 by inhibiting DNA, RNA, and protein syntheses (Li et al. 2008). Strain JC1270 of *Enterobacter agglomerans,* a close relative of *R. aquatilis,* was observed to produce the antibiotic pyrrolnitrin, which has a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens (Chernin et al. 1996). Pyrrolnitrin's mode of action is still to be completely elucidated; however, the inhibition of fungal respiratory electron transport system by pyrrolnitrin has been demonstrated (Tripathi and David 1969; Leroux 1996), and it was also found that pyrrolnitrin was involved in inhibition of DNA and RNA synthesis, but not in protein synthesis (Tripathi and David 1969). Thus, the ABS produced by strain HX2 might use a different mode of action on *Agrobacterium* spp. than do these reported antibiotics.

The nature of RNA and protein synthesis inhibition in the ABS-treated *A. vitis* cells remains to be investigated. We are currently further investigating the nature of the ABS, its action mechanism, and the genetic bases for its production, together with development of suitable applications.

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