Biological Control of Grape Crown Gall by *Rahnella aquatilis* HX2

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ABSTRACT

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Crown gall induced by *Agrobacterium vitis* is a worldwide plant disease in grape-growing regions. *Rahnella aquatilis* HX2, a new isolate from vineyard soil in Beijing, showed a significant inhibition effect on the development of crown galls in grapevines. In field trials, immersion of the basal ends of grape cuttings with HX2 cell suspension inhibited or completely prevented crown gall formation caused by *A. vitis* K308 in the roots of the plants from the cuttings. The 3-year average disease incidence in grape plants treated with HX2 was 30.8% compared to 93.5% in plants without HX2. The culture supernatant of HX2 exhibited a stronger inhibition effect on disease development than did the cell suspension. HX2 could be found in the grape rhizosphere, grown under field conditions, for up to 90 days after inoculation. There was no significant difference in the mean population sizes of root microflora between plants treated and not treated with HX2. The inhibition effect of HX2 on crown gall in sunflower, caused by different *Agrobacterium* strains, varied between 30.7 and 100%, depending on strains. Our results showed that *Rahnella aquatilis* HX2 may be used as a biological control agent for crown gall disease of grapes.

Following apple, citrus, pear, and banana, grape production now ranks fifth in fruit production in China. According to data published by the China Ministry of Agriculture, in 2004, the area under cultivation to grapes was estimated to be 413,500 hectares and their production to be 5,675,000 tons. Crown gall disease is one of the most destructive diseases of grapevine in China. It seriously affects the quantity and quality of grape production. In some vineyards, the incidence of crown gall disease is as high as 30 to 70% (51). To date, no effective chemical treatments are available for grape crown gall control.

The causal organism of crown gall disease is *Agrobacterium vitis* (31). Tumors form on roots and trunks as well as on branches. Biological control of crown gall disease has been implemented since *Agrobacterium rhizogenes* K84 was used commercially to control peach crown gall in 1980 (22). However, K84 is only effective in controlling crown gall caused by nopaline-type strains of *A. tumefaciens* and *A. rhizogenes*, and has no effect on crown galls induced by *A. vitis* in grapevines (6,22,23,42).

Many avirulent strains of *Agrobacterium* have been reported as biological control agents of the pathogens resistant to K84,

whose active compound is agrocin 84 (7,10,48). In addition to those *Agrobacterium* antagonistic strains, *Bacillus subtilis* AB8 and several *Pseudomonas* strains were found to be potential biocontrol agents to reduce the incidence of crown gall (24,44,52). Bell et al. demonstrated

that certain grapevine endophytes can control population numbers of *Agrobacterium* in situ and provide significant biocontrol of crown gall (2).

This paper reports on *Rahnella aquatilis* HX2 and its antagonistic activity on crown gall of sunflower and grape.

MATERIALS AND METHODS

Bacterial strains and the plasmid used in this study. Bacterial strains and the plasmid used in this study and their relevant characteristics are presented in Table 1. Strain HX2, isolated from vineyard soil in the course of a screening program for antagonistic rhizospheric bacteria, was naturally resistant to ampicillin (Amp, 200 µg/ml). This antagonistic bacterium has not been previously isolated in China.

Culture and growth media. HX2 was grown on 1.5% (vol/wt) potato dextrose agar (PDA) medium (100 g potato, 10 g dextrose, and 15 g agar per liter) at 28°C. *Escherichia coli* DH5α was cultured at 37°C in Luria-Bertani broth (LB) (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) or on LB agar plates. The *Agrobacterium* strains and soil bacteria were

Table 1. Bacterial strains and the plasmid used in this study

Strain or plasmid	Relevant characteristics ^a	Source
HX2	Amp ^r	This study
<i>Escherichia coli</i> DH5α		Provence (36)
<i>Agrobacterium tumefaciens</i>		
C58	Nop	LaPointe (27)
AC11	Nop	Kerr (23)
B6S3	Oct	Whatley (50)
MG14-2	Nop	This study
MG10-1	Nop	This study
CY4	Nop	This study
CY31	Nop	This study
CY32	Nop	This study
Pt12	Agr	This study
Y5-1	Nop	This study
MI4-6	Oct	This study
<i>Agrobacterium rhizogenes</i>		
K27	Nop	Kerr (23)
K75	Nop	This study
NL12-2	Nop	This study
HBT3-4	Nop	This study
B1	Nop	This study
D2	Nop	This study
<i>Agrobacterium vitis</i>		
K308	Oct	Kerr (22)
G11(3)	Oct	This study
G7(2)	Oct	This study
MI23-1	Oct	This study
MB26-1	Oct	This study
MB26-1	Oct	This study
Plasmid		
pSMC2	Km ^r , Amp ^r , GFP ⁺	Bloemberg (4)

^a Amp^r, Km^r, designate resistance to ampicillin and kanamycin, respectively; Nop, nopaline type; Oct, octopine type; Agr, agropine type; GFP⁺, green fluorescent protein positive.

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grown at 28°C on 1.5% (vol/wt) modified 523 agar (10 g sucrose, 2 g KH₂PO₄, 10 g yeast extract, 5 g CaCO₃, 0.2 g NaCl, and 0.3 g MgSO₄·7H₂O per liter). Soil fungal strains were cultured at 26°C on 1.5% (vol/wt) Martin broth (10 g dextrose, 5 g peptone, 1.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, and 0.03 g Bengal red per liter, and streptomycin sulfate at 30 µg/ml), and the actinomyces grew at 30°C on 1.5% (vol/wt) Gause's no. 1 synthetic medium (20 g starch soluble, 1 g KNO₃, 0.5 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl, 0.01 g FeSO₄, and 0.1 g K₂Cr₂O₇ per liter) (15).

Chrome Azurol S (CAS) agar medium was prepared according to Schwyn and Neilands (39). HX2 was inoculated onto the center of CAS medium plates and incubated at 28°C for 3 days. A yellow halo surrounding the colony indicated a positive reaction.

HCN production was assayed according to the method described by Castric and Castric (9) with the following modifications: a single colony of strain HX2 that is 18 h old was inoculated into the Eppendorf tube containing 0.5 ml of solid LB medium. A small piece of HCN-sensitive detection paper was hung over the medium. The tube was sealed with Parafilm and then incubated at 28°C for 48 h. The HCN-sensitive paper was Whatman chromatography paper soaked in the HCN detection reagent: copper (II) ethyl acetate (5 mg) and 4,4'-methylenebis-(*N,N*-dimethylaniline) (5 mg) in chloroform (1 to 2 ml). A blue color indicates the production of HCN of strain HX2.

Growth of sunflower seedlings and grapevine. Sunflower (*Helianthus annuus* L. cv. Frankasol) and grapevine (*Vitis vinifera* L. cv. Muscat Hamburg) were obtained from the Ministry of Agriculture, China. Sunflower seedlings were grown in pots (7 cm diameter) containing a mixture of sterile clay soil and vermiculite (1:1) and watered as required. Grapevine plants were grown in pots (26 cm diameter) containing the same soil mixture. All plants were kept at room temperature with a 16-h photoperiod.

Identification of strain HX2. The ability to utilize different carbon sources was tested using the Biolog identification system (Biolog Inc., Hayward, CA). The morphological, cultural, biochemical, and physiological characteristics of HX2 were examined according to the methods previously reported by Iimura and Hosono (20). Briefly, catalase production was detected by the production of bubbles in a 3% hydrogen peroxide solution. The oxidase test was carried out by use of cytochrome oxidase test paper. Phenylalanine agar and ferric chloride solution were used to detect the production of phenylpyruvic acid.

HX2 genomic DNA was extracted and purified according to the method described by Ausubel et al. (1). Briefly, 30 µl of so-

dium dodecyl sulfate (SDS) (10%) and 3 µl of Proteinase K (30 µg/ml) were added to 567 µl of bacterial cells suspended in TE buffer, and the mixture was incubated at 37°C for 1 h, followed by the addition of 100 µl of NaCl (5 M) and 50 µl of CTAB/NaCl. The mixture was incubated at 65°C for 10 min and extracted with phenol/chloroform/isoamyl alcohol (25:24:1 vol/vol/vol). The DNA was precipitated by adding 0.6 volume of isopropanol, separated by centrifugation (10,000 × g), washed with 70% ethanol, and dried. The 16S rDNA was polymerase chain reaction (PCR) amplified from HX2 genomic DNA with the primers 63F 5'-CAGGCC TAACACATGCAAGTC-3' and 1494R 5'-GGYTACCTTGTACGACTT-3' and then sequenced.

Inhibition test in vitro. Antagonist activity of the HX2 against pathogenic agrobacterial strains was studied according to Stonier's method (40) with the following modifications: a drop of 10 µl HX2 cell suspension was added on the center of PDA medium plates and incubated at 28°C for 48 h. Then the cells were killed with chloroform. After evaporation of the chloroform, 50-µl aliquots of agrobacterial strains (ca. 2×10^8 CFU/ml) mixed with 5 ml of 0.7% yeast extract broth (YEB) (46) soft agar was poured over the surfaces of the inoculated PDA plates. The plates were incubated at 28°C for 24 h, the inhibition zones around the killed HX2 colonies were observed, and the diameters were measured. Each strain was tested on five replicate plates at least twice.

Inhibition test in vivo. Sunflower seedlings with two true leaves grown in a greenhouse were used for in vivo tests. HX2 was cultured in liquid PDA medium and grown on a shaker at 180 rpm at 28°C for 28 h. Cells were pelleted and washed twice with sterile distilled water and then suspended in sterile buffered saline to get an OD₆₀₀ of 0.6 (ca. 2×10^8 CFU/ml). The supernatant was retained. The suspension of the agrobacterial strain (2×10^8 CFU/ml) was mixed with two volumes of (i) the supernatant, (ii) sterile buffered saline (0.85% NaCl), or (iii) the cell suspension of HX2.

Sunflower stems were wounded by making a 0.5-cm longitudinal cut with a sterile needle. A drop of the mixture (3 µl) was placed on the wounding site. Each of 30 plants per treatment was inoculated at one site that was immediately wrapped with Parafilm. Gall formation was observed, and the gall was excised and weighed 15 days after inoculation. The crown gall tumor inhibition activity was calculated as follows: Crown gall tumor inhibition (%) = $[(C - T)/C] \times 100$, where *C* is the average weight of the crown gall tumor of the control group, and *T* is the average weight of the crown gall tumor of the treated group.

The suspension of *A. vitis* K308 obtained as described above was mixed with

two volumes of (i) sterile buffered saline, (ii) cell suspension, or (iii) supernatant of HX2. Four intact grapevines were used in the experiments, and each plant contained four inoculation sites. The current-year stem of grapevine was wounded by making a 0.8-cm longitudinal cut with a sterile sharp scalpel, and a 10-µl drop was placed into each wound site. The inoculation site was wrapped with Parafilm. Gall formation was observed and tumor weight was measured 30 days after inoculation. The biological activity of the HX2 against gall agrobacterium K308 was evaluated based on the tumor weight as described above.

Field test. The ability of HX2 to inhibit gall formation caused by *A. vitis* K308 was also assessed under field conditions in Changli, Hebei province, China. One-year-old cuttings of Muscat Hamburg grapevine, free of crown galls and with two to three nodes each, were used in the field experiment. Prior to planting, the basal ends of the cuttings were soaked in a mixture of the HX2 and K308 cultures (1:1) for 10 min. Other cuttings were soaked only in K308 culture as a control treatment. Each treatment consisted of four rows and each row was applied to 20 plants. The rows were blocks and the experimental design was a randomized complete block. Crown gall formation was assessed and the weight of the tumor was measured 8 months after planting. Tumor inhibition activity was calculated as above. The experiment was conducted for three consecutive years.

Root colonization on grapevine.

Preparation of HX2 competent cells and the plasmid pSMC2, which contained green fluorescent protein (GFP) gene, and electroporation were performed according to the protocols of Sambrook et al. (38): HX2 cells (ca. 2×10^{10} cells) were washed three times with ice-cold double-distilled water (ddH₂O) and then resuspended in a small volume (40 µl) of ice-cold 10 glycerol; 0.2 µg of plasmid DNA was mixed with 40 µl of these cells, added to a 2-mm-gap electroporation cuvette, and electroporated at 2.0 kV with a Gene Pulser (Bio-Rad, Melville, NY). The electroporated cells were diluted into 1 ml of PDA medium and grown for 1 h before being plated on modified 523 agar supplemented with Amp (200 µg/ml) and kanamycin (50 µg/ml). Stability of pSMC2 in the strain HX2 was analyzed by the method described by Van der Bij et al. (45): a single colony of strain HX2 harboring the plasmid pSMC2 was inoculated into PDA medium supplemented with Amp (200 µg/ml) and kanamycin (50 µg/ml). After 18 h of growth, bacterial suspension was centrifuged and washed twice with sterile water to remove the antibiotics. PDA medium was inoculated with approximately 10^8 CFU of HX2 per ml, and the culture was allowed to grow for 12 h at 28°C on a rotary shaker. Suspension was diluted

1,000-fold in 10-ml aliquots of fresh non-selective PDA medium. This procedure was repeated at least 40 generations without antibiotic pressure after the initial inoculation. During the course of the experiment, the sample was taken, diluted, and plated on modified 523 agar without or supplemented by Amp (200 µg/ml) and kanamycin (50 µg/ml). The stability of plasmid pSMC2 was determined by comparing the numbers of colonies present on selective and nonselective plates.

The modified HX2 strain harboring plasmid pSMC2 was used to analyze survival of HX2 in the grapevine roots as described by Vicedo et al. (47). Thirty-one-year-old grape plants were inoculated with HX2 by immersing the roots for 10 min in the cell suspension (1×10^8 CFU/ml). An additional 30 plants were treated with sterile water as controls. In order to detect the dynamic of population of HX2 in the grape root under natural conditions, inoculated plants were planted in nonsterile field soil. After various periods following planting (1, 15, 30, 60, 90, and 120 days), three plants of each treatment were uprooted and adhering soil was removed. The roots were placed in 100 ml of 0.25× Ringer's Solution (1× Ringer's Solution containing 2.25 g NaCl, 0.105 g KCl, 0.06 g CaCl₂, and 0.05 g NaHCO₃ per liter) and shaken for 10 min at 180 rpm. A series of dilutions of the root solutions were plated on the media of modified 523 agar containing Amp (200 µg/ml) and kanamycin (50 µg/ml). After incubation for 48 h at 28°C, fluorescent green pigment colonies were counted and

the numbers of CFU per gram of roots were calculated. For the purpose of confirmation, five randomly selected HX2 colonies were tested for antagonistic activity against *A. vitis* K308. The 16S rDNA were amplified from the five colonies and sequenced.

The population of rhizosphere microflora of grapevine was also analyzed at the same time. The rhizosphere soil dilutions were plated on the modified 523 agar, Martin broth, and Gause's no. 1 synthetic medium for obtaining bacteria, fungi, and actinomycetes, respectively.

Statistical analysis. An analysis of variance was performed on the data with SAS (version 8.2; SAS Institute, Inc., Cary, NC). The mean square error values were tested with a *t* test at *P* < 0.05.

RESULTS

Isolate characterization. The antagonistic strain HX2 was identified by the Biolog identification system as *Rahnella aquatilis*. The strain HX2 was small, rod-shaped, gram negative, and the biochemical test results showed that it was negative for cytochrome oxidase and positive for catalase (Table 2). It was typically a member of the Enterobacteriaceae family.

Siderophores were not produced on the CAS agar and it was HCN negative.

The 16S rDNA sequence was 99% identical to that of the genus *Rahnella*.

Inhibition of agrobacterial strains in vitro. Strain HX2 exhibited inhibitory activity against 11 isolates of *A. tumefaciens*, 5 isolates of *A. rhizogenes*, and 5 isolates of *A. vitis* (Table 3.). The inhibitory effect of HX2 on the growth of *A. vitis* K308 was clearly demonstrated in Figure 1.

In vivo tests. Application of strain HX2 in sunflower plants resulted in reduced infection by agrobacterial strains (Table 3, Fig. 2). When the culture supernatant and cell suspension of the HX2 were applied, the crown gall symptoms caused by phytopathogenic agrobacteria decreased by 39.8% ~ 100% and 30.7% ~ 92.9%, respectively, depending on pathogenic strains. The inhibitory efficacy of the supernatant was generally higher than that of the cell suspension except for controlling *A. tumefaciens* PT12.

Application of strain HX2 in grapevines resulted in reduced infection by *A. vitis* K308. Both HX2 cell suspension and the supernatant reduced crown gall symptoms as shown in Figure 3. The inhibition effects of supernatant and cell suspension were 100 and 63%, respectively.

Field experiment. In the first year, incidence of crown gall on plants treated and nontreated with HX2 were 31.3 and 93.8%, respectively, and the crown gall tumor inhibition was 81.8%. In the second year, incidence of crown gall on plants

Table 2. Reaction of *Rahnella aquatilis* HX2 in routine biochemical tests

Biochemical reaction	Result
Growth on 2% NaCl	+
Catalase	+
Citrate utilization	+
Methyl red	—
Voges-Proskauer	+
Growth at 4°C or 41°C	+
Phenylalanine deaminase	—
Arginine dihydrolase	+
Lysine decarboxylase	—
Ornithine decarboxylase	—
Motility	+
Gelatin liquefaction (20°C)	—
Cytochrome oxidase	—
Cellulose decomposition	—
Amylase	—
H ₂ S production	—
Malonate	+
Gas from D-glucose	+
Utilization of tartrate	—
Utilization of D-acetate	—
Utilization of L-phenylalanine	+
Utilization of Urethane	—
Acid from:	
D-Arabitol	+
D-Mannose	+
D-Sorbitol	+
Sucrose	+
D-glucose	+
Ethanol	—
Inositol	—

Strain	Inhibition zone (dia.) (mm)	Efficiency of biocontrol (%) ^a	
		Supernatant	Cells
<i>Agrobacterium tumefaciens</i>			
C58	28.8 ± 3.5 ^b	82.6 ± 7.5	60.9 ± 11.2
AC11	29.7 ± 3.6	80.6 ± 4.8	61.1 ± 9.6
B6S3	35.9 ± 2.1	39.8 ± 9.7	30.7 ± 0
MG14-2	27.7 ± 2.1	96.1 ± 3.3	84.6 ± 8.8
MG10-1	42.9 ± 5.2	66.7 ± 2.5	63.8 ± 2.5
CY4	30.0 ± 3.8	100 ± 0	92.9 ± 7.1
CY31	24.3 ± 1.9	100 ± 0	89.7 ± 2.5
CY32	28.5 ± 2.6	80.0 ± 6.9	78.0 ± 9.2
Pt12	30.5 ± 3.7	80.0 ± 8.8	86.5 ± 2.9
Y5-1	39.8 ± 2.1	92.1 ± 5.5	82.6 ± 2.7
MI4-6	38.0 ± 2.8	100 ± 0	66.7 ± 4.4
<i>A. rhizogenes</i>			
K27	26.4 ± 1.9	64.6 ± 8.8	63.3 ± 7.9
K75	39.1 ± 3.4	64.6 ± 8.8	62.1 ± 3.8
NL12-2	41.3 ± 2.5	97.1 ± 4.8	84.6 ± 4.8
B1	40.8 ± 2.7	96.1 ± 3.9	83.1 ± 4.5
D2	30.8 ± 3.7	100 ± 0	81.5 ± 6.4
<i>A. vitis</i>			
K308	33.8 ± 3.3	93.9 ± 6.1	53.1 ± 4.1
G11(3)	39.1 ± 3.6	81.6 ± 2.9	50.1 ± 4.0
G7(2)	25.8 ± 3.1	84.0 ± 3.5	60.1 ± 6.9
MI23-1	31.5 ± 2.1	66.7 ± 1.4	33.3 ± 4.4
MB26-1	27.3 ± 1.5	97.4 ± 2.1	58.3 ± 7.6

^a HX2 was cultured in liquid PDA medium and grown on a shaker. Cells were pelleted and washed twice with sterile distilled water and then suspended in sterile buffered saline to get an OD₆₀₀ of 0.6 (2×10^8 CFU/ml). Supernatant was retained. Suspension of the agrobacterial strain (2×10^8 CFU/ml) was mixed with two volumes of the supernatant, sterile buffered saline, or cell suspension of HX2. Crown gall tumor inhibition activity was calculated. Crown gall tumor inhibition (%) = $[(C - T)/C] \times 100$, where *C* is average weight of crown gall tumor of control group, and *T* is average weight of crown gall tumor of treated group.

^b Standard error.

treated and nontreated with HX2 were 32.5 and 96.3%, respectively, and the crown gall tumor inhibition was 77.9%. In the last year, incidences of crown gall on plants treated and nontreated with HX2 were 28.8 and 90%, respectively, and the crown gall tumor inhibition was 81.0%. Weight of tumors of plants treated with HX2 was greatly reduced, and disease incidence in grape plants treated with HX2 decreased from 93.5 to 30.8%.

HX2 colonization in grapevine roots. The HX2 cell line transformed with pSMC2 plasmid containing *gfp*, and des-

ignated as *gfp*-HX2, showed the same characteristics of growth, motility, and production of antimicrobial substances as the wild-type cell lines. Population sizes of the *gfp*-HX2 were determined up to 120 days after root inoculation. The colonization dynamics of *gfp*-HX2 on grapevines were shown in Figure 4. Randomly selected *gfp*-HX2 colonies showed similar antagonistic activity against *A. vitis* K308. The 16S rDNA amplified from these colonies was the same as that of HX2. No fluorescent colony was detected when plants were sampled on the 120th day after inoculation.

Effects of HX2 on the root microflora population of grapevine. No significant difference was found in the mean population sizes of root microflora between control plants and plants treated with HX2 when we sampled the plants on the 15th, 30th, 60th, and 90th day after inoculation. A significant impact of HX2 on the total cell density of bacteria was observed on the first sampling time ($P < 0.05$). The population of root bacteria of plants treated with HX2 and control plants were 1.3×10^5 CFU/g and 2.3×10^4 CFU/g, respectively.

DISCUSSION

The biological control assays showed that the strain HX2 efficiently controlled the pathogenic agrobacterial strain *A. vitis* K308 under field conditions. The antibiotic substances produced by HX2 may play an important role. Treatment with the antagonistic bacterium not only decreased the proportion of plants with visible symptoms of crown gall but also reduced the disease severity of affected plants.

Gavini et al. (17) used the name the Group H₂ for phenotypically similar strains during a taxonomic study of the genus of *Enterobacter*. Izard et al. (21) named the organisms *Rahnella aquatilis*. Biochemically, *Rahnella* sp. is closely related to *Enterobacter agglomerans*–*Pantoea agglomerans* complex. *R. aquatilis* can grow at 4°C and 10°C and does not produce yellow pigment. It is negative for lysine and ornithine decarboxylase reactions. Therefore, it can be differentiated from other members of the former complex (26). In this study, the biochemical results of HX2 were consistent with the characterizations of *R. aquatilis*. HX2 16S rDNA sequence was 99% similar to that of the genus *Rahnella*.

Fig. 1. Inhibitory effect of *Rahnella aquatilis* HX2 against *Agrobacterium vitis* K308. Inhibition zone production by HX2 on potato dextrose agar medium plate (incubation at 28°C for 48 h).

Fig. 2. Inhibition by *Rahnella aquatilis* HX2 of sunflower tumor development by *Agrobacterium vitis* K308. **A**, inoculated with HX2 supernatant and K308 mixture (2:1); **B**, inoculated with K308 only; **C**, inoculated with HX2 cell suspension and K308 mixture (2:1); **D**, sterile buffered saline inoculation as negative control.

The genus of *Rahnella* is widely distributed in water and soil (3,5,19), as well as in foods and seeds (8,30). Some strains of *R. aquatilis* were rhizosphere-associated bacteria and nitrogen fixers (3) and have the ability to solubilize mineral phosphate (25). It has been reported that *Rahnella aquatilis* JC577 has in vitro antagonistic activity against *A. vitis*, whereas this strain showed no biocontrol effect on grapevine crown gall disease (2). Furthermore, two strains of *Rahnella* can effectively suppress bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* (14).

Some strains of *Enterobacter*, which are closely related to *Rahnella*, have been described as potential biological control agents for different fungal phytopathogens (29,32). Strain JC1270 of *Enterobacter agglomerans*, which can produce a complex of chitinolytic enzymes and an antibiotic, has been reported as an antagonist of many fungal phytopathogens (11,12). The ability to produce an antibiotic-like substance was considered to be an important factor for inhibition of *Phytophthora cactorum* by *Enterobacter aerogenes* strain B8 (43). Moreover, it is known that K84 is able to produce a highly specific antibiotic, agrocin 84, which is effective against many pathogenic *Agrobacterium* strains (22).

The possible roles of the different antibiotics produced by strain K84 during pathogen infection of plant cells had been investigated before (13,37,41). In a previous study, we observed that when HX2 strain was cultured in media containing dextrose, the amount of antibiotic substances was higher than in media with other carbon sources (data not shown). In this paper, we have shown that HX2 inhib-

ited the growth of pathogenic agrobacterial strains in vitro. The inhibition effect of HX2 is likely due to antibiotic substances that prevented tumor formation, since the effect of the culture supernatant on crown gall was stronger than that of the cell suspension.

Production of siderophores and accumulation of toxic metabolites (such as HCN) have been considered important factors in biological control (34,49). Strain HX2 did not show any evidence that it could produce siderophore or HCN.

Our data demonstrated that HX2 had colonized and survived in the rhizosphere of grapevine for only 3 months, which indicated that the colonization ability

may play a minor or dispensable role in the mechanism of biological control of crown gall disease by HX2. Rhizosphere colonization ability was considered as a possible mechanism of antifungal activity in *Enterobacter agglomerans* (18). K84 could control the crown gall caused by agrocin 84 resistant pathogens, suggesting that other traits of the strain K84 are involved in its biological control mechanisms (33). Farrand and Wang (16) considered that colonization played a role in crown gall biological control by the strain K84 but was not the most important parameter. The strain of K84 could inhibit the formation of tumors by different mechanisms including the physical block-

Fig. 4. Population size of *Rahnella aquatilis* HX2 on grapevine roots 120 days after inoculation. y-axis: population size (CFU per gram [fresh weight] of root) of HX2; x-axis: numbers represent days after inoculation.

Fig. 3. Inhibition by *Rahnella aquatilis* HX2 of *Agrobacterium vitis* K308-induced grape tumor development. **A**, inoculated with K308 only; **B**, inoculated with HX2 cell suspension and K308 mixture (2:1); **C**, inoculated with HX2 supernatant and K308 mixture (2:1); **D**, inoculated with sterile buffered saline as the negative control.

ing of the infection sites (28) and/or anti-biosis (13,35) or a combination of these or other mechanisms at the primary sites of infection.

We have demonstrated that the supernatant and cell suspension of HX2 had no detrimental effect on the total cell density of three microfloral components of rhizosphere: bacteria, fungi, and actinomycetes. However, it is not known if the supernatant or cell suspension will affect other beneficial microorganisms under other conditions.

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