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Bacterial Ghost Technology for Pesticide Delivery

TAMÁS HATFALUDI,*,† MARTINA LISKA,† DANIELA ZELLINGER,† JARJU PA OUSMAN,‡ MICHAEL SZOSTAK,§ ÁRPÁD AMBRUS,‡ KATRI JALAVA,§ AND WERNER LUBITZ†,§

Institute for Microbiology and Genetics, Section Microbiology and Biotechnology, University of Vienna, UZAII 2B522 Althanstrasse 14, A-1090 Vienna, Austria, Agrochemicals Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, A-2444 Seibersdorf, Austria, and BIRD-C GmbH & Co. KEG, Schönborngasse 12, A-1090 Vienna, Austria

Bacterial ghosts are nondenaturated empty cell envelopes of Gram-negative bacteria produced by E-mediated lysis. Such envelopes from the plant-adhering bacterium *Pectobacterium cypripedii* were tested for their ability to adhere to plant material and to be used as carriers for pesticide delivery. We show, using fluorescence-labeled *P. cypripedii* ghosts, that depending on the target plants 55 or 10% (rice or soya, respectively) of the applied bacterial ghosts was retained on the leaves after heavy simulated rain (84 mm). Furthermore, the bacterial ghosts could be loaded with the lipophilic triazole fungicide tebuconazole. In subsequent plant experiments in the glass house, the efficacy of the loaded bacterial ghost for resistance to rainfall and the protective and curative effects against the pathogens *Erysiphe graminis*, *Leptosphaeria nodorum*, and *Pyrenophora teres* on barley and wheat and against *Sphaerotheca fuliginea* on cucumber were tested. The bacterial ghosts were compared primarily with a commercial tebuconazole formulation, a wettable powder, as it has similar physical characteristics. The comparison revealed similar effects and showed consistently higher or comparable efficacy against the pathogens. The standard operational comparison with the most protective, cereal specific emulsion of oil in water displayed that the bacterial ghosts had equal to or lower efficacy than the emulsion. This study confirmed the potential of bacterial ghost platform technology as a new alternative carrier system for pesticides.

KEYWORDS: Bacterial ghosts; carrier; pesticide; formulation; plant; adhesion; delivery system

INTRODUCTION

Bacterial ghosts, which represent empty cell envelopes of Gram-negative bacteria, have been applied successfully as vaccine candidates (*1*) or as potential drug carriers (*2*, *3*). In this study, for the first time, bacterial ghosts were tested for their application as carrier and targeting vehicles for agricultural plants. Certain bacteria living on plant surfaces possess special adhesive capabilities. In this investigation, we used *Pectobacterium cypripedii*. This bacterium belongs to the previously described group of *Erwinia cypripedii* that were originally isolated from orchids (*Cypripedium* sp.) (*4*). They are ketogenic bacteria and members of the family *Enterobacteriacea* of the γ-subgroup of *Proteobacteria*. The original phylogenic position of the genera has been amended to *Pectobacterium* (*5*). Bacterial ghosts are intact, nondenaturated, bacterial envelopes, which have been produced from various Gram-negative bacteria (*6*). The ghosts are produced by the controlled expression of the plasmid-encoded lysis gene *E* of bacteriophage phiX174, which is under the control of either the temperature sensitive λ-promoter/cI857 repressor system (*7*) or the chemically inducible promoter system (*8*). After gene *E* expression, the cytoplasmic content is expelled through an E specific transmembrane tunnel that penetrates the inner and outer membranes of Gram-negative bacteria, resulting in empty, nondenatured, bacterial cell envelopes (*9*). These envelopes can be used as carriers for specific drug delivery, and the aim of the present study was to investigate the feasibility of their exploitation in pesticide application.

Reduction in pesticide use is a major goal in agriculture as it addresses increasing environmental, health, and consumer concerns (*10*, *11*). Much research is therefore directed toward new types of pesticides and formulations with properties of greater efficiency that allow reductions in the amount and frequency of application (*12*). The fungicide tebuconazole [1-(4-chlorophenyl)-4,4-dimethyl-3-[1,2,4]triazole-1-ylmethyl-pentan-3-ol] is characterized by a high level of efficacy against a wide range of pathogens causing, e.g., powdery mildew, leaf blotch, and rust diseases. It has a systemic mode of action and interferes with the metabolism of the fungal pathogen by inhibiting sterol biosynthesis (*12*). Tebuconazole is registered for use on more

* To whom correspondence should be addressed. Tel: +43-1-4277-54670. Fax: +43-1-4277-54674. E-mail: tamas.hatfaludi@univie.ac.at.
† University of Vienna.
‡ FAO/IAEA Agriculture and Biotechnology Laboratory.
§ BIRD-C GmbH & Co. KEG.

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than 70 crops including cereals, banana, and stone fruit and is one of the six most sold fungicides in the world (*13*). New formulations and general carrier systems in agriculture are applied to minimize the loss of activity caused by UV light or wash off by rain (*14*). Carrier systems under development include octadecyltrimethylammonium micelles (*15*), poly ϵ-caprolactone nanoparticles (*16*), lignin matrix granules (*17*), and microencapsulated formulations (*14*, *18*).

P. cypripedii, which adheres to plant leaves, was chosen as a bacterial ghost carrier for the lipophilic fungicide tebuconazole. This model system was used to test pesticide efficacy and phytotoxicity in experiments, carried out under glass house conditions.

MATERIAL AND METHODS

Bacteria, Plasmids, Transformation, and Growth Conditions. *P. cypripedii* strain HeP01 (*19*) was isolated from the surface of the fruit of *Vaccinium myrtillus* (bilberry). This strain was transformed with the lysis plasmid pFN12 (*20*) and the corresponding cloning vector pBluescript KSII+ (Stratagene, Cleveland, OH) as described by Sambrook (*21*). The transformants were grown in Luria broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl at pH 7) with ampicillin (200 μg/mL) at 28 °C with aeration. The competent cells were prepared with poly(ethylene glycol) (PEG 600, water content < 2%)−dimethyl sulfoxide (DMSO) using the method of Hanahan (*22*).

Ghost Production. Five hundred milliliters of an overnight *P. cypripedii* HeP01 culture, descending from a single pFN12 transformant colony, was used as a preculture for inoculation of 10 L of LB fermenter broth. Bacteria were grown in a laboratory-scale, stirred batch bioreactor (Meredos GmbH, Bovenden, Germany). Online measurement and temperature control, agitation, airflow, pH, pO_2, and antifoam sensing were monitored. *P. cypripedii* HeP01 harboring the corresponding cloning vector pBluescript KSII+ and a nontransformed strain served as negative controls. *P. cypripedii* HeP01 (pFN12) cultures were grown in LB−ampicillin medium with aeration (4.5 L/min) and agitation (350 rpm) at 28 °C to an optical density at 600 nm of 0.4−0.6 (OD_{600}). E-mediated lysis was induced by a temperature shift of the medium from 28 to 36 °C for 10 min, and the culture was then kept at 42 °C for 130 min. The efficacy of the bacterial culture lysis was determined and monitored by OD_{600} measurement until the maximal decrease was obtained, through the colony forming units (CFU) of the culture, at various time points and by visualization via phase contrast and electronic microscopy. At the end of the lysis process, bacterial ghosts were harvested by centrifugation (at room temperature) at 6000*g*, washed three times with phosphate-buffered saline (PBS), and subsequently lyophilized and kept at room temperature for long-term storage. The absence of viable cells in the lyophilized samples was ascertained by a sterility test (*23*).

Application and Detection of Fluorescein Isothiocyanate (FITC)-Labeled Bacterial Ghosts to Leaf Surfaces in Laboratory Rain Stability Tests. Lyophilized bacterial ghosts [6 mg (4.14×10^{10})/L] were suspended in 0.1 M sodium carbonate buffer (pH 9) and incubated with 25 nM FITC isomer I (Sigma-Aldrich, Steinheim, Germany) in DMSO for 2 h in the dark at room temperature. A 5 μL suspension of FITC-labeled ghost (2.09×10^8) with 0.05% Dobanol 91-1 (Brenntag N. V., Netherlands) as a surfactant was pipetted to the upper side of ca. 0.25 cm^2 leaf area of *Brassica oleracea* var. *capitat* (cabbage), *Gossypium herbacea* (cotton), *Hordeum vulgare* (barley), *Oryza sativa* (rice), *Soya hispida* (soya), and *Zea mays* (corn) (*24*, *25*). The leaves were dried in the dark under moderate air circulation for 2 h at room temperature. In these experiments, rain was simulated by subsequently rinsing the leaves seven times with 300 μL of water (tap) from a pipet. Each wash was collected, and the intensity of the light emitted was measured at 520 nm (excitation at 485 nm) in a Shimatsu RF 5001 PC fluorometer. Five microliters of FITC-labeled ghost suspension in 300 μL of water was set as a reference value of 100%; clear water served as a negative control.

Loading Bacterial Ghosts with the Fungicide Tebuconazole. Bacterial ghosts were loaded with tebuconazole (technical grade, Bayer AG, Leverkusen). The triazole fungicide tebuconazole (200 mg/mL) was dissolved in a 1:1 solution of ethanol (96%) and PEG 600 (water content < 2%). A 200 mg amount of lyophilized bacterial ghosts suspended in 200 mL of tebuconazole solution [1 mg (6.9×10^9)/mL ghost concentration] was incubated with vigorous shaking (1200 rpm) for 3 h at 28 °C. The loaded bacterial ghosts were collected by centrifugation at 10000*g* for 10 min, and the supernatant was discarded. Eleven loaded preparations were combined (altogether 2200 mg of lyophilized bacterial ghosts).

Gas Chromatography Measurements of the Fungicide Tebuconazole. The loaded ghost pellets were resuspended in a mixture of 250 μL of 0.9% saline and 750 μL of dichloromethane by vigorous vortexing. The samples were centrifuged at 10000*g* for 30 min, and the organic phase was collected for gas chromatographic measurements. The measurements were carried out using a Chrompach CP 9003 Autosampler CP9050, Software Mastro 2.5V, FID Detector, with a CP Sil 8CB-capillary column (0.32 mm diameter; 25 m long; film thickness of 0.25 μm). The detector temperature was 300 °C, the injector temperature was 260 °C, and the oven was heated from 200 to 260 °C at a rate of 15 °C/min. Helium was used as a carrier gas, and the flame gases were hydrogen and air (*26*). The injected volume was 1 μL. The analysis was done with an internal standard of chlorpyrifos (1 μL/mL in an acetone:2-propanol mixture) and an external standard of tebuconazole (diluted in acetone) with concentrations of 0.2, 0.5, 1, 2, and 3 mg/mL. The standard samples were distributed equally and measured between samples.

Efficacy Trials of Tebuconazole-Loaded *P. cypripedii* Ghost Formulations on Barley, Wheat, and Cucumber with Rain Stability Test in a Glass House. The efficacy of fungicide-loaded bacterial ghosts and two commercial formulations of tebuconazole Folicur WP 25, a wettable powder (25 g tebuconazole per liter), and Folicur EW 250, an oil−water emulsion of 250 g tebuconazole per liter (Bayer), were compared and evaluated in plant pathogen experiments. Loaded bacterial ghosts (1.41×10^{10}) were used at a concentration of 1000 ppm tebuconazole, in a solution of 2% PS 16 in acetone, diluted with water at the ratio of 1:25. The two Folicur formulations were used according to the manufacturer's instructions at the same concentration (1000 ppm). Two monocots, *H. vulgare* cv. Villa (barley) and *Triticum aestivum* cv. Kanzler (wheat) were tested in protective and curative trials with these three formulations at a water application rate of 250 L/ha, simulating practical field spray conditions. A dicot *Cucumis sativus* cv. Hoffman Prodikto (cucumber) was tested only protectively with the tebuconazole-loaded bacterial ghosts, and the Folicur WP 25 at a standard operational water application rate of 600 L/ha. In the protective trials, the plants were treated homogeneously in a spraying cabin with the pesticides a day before pathogen inoculation. In the curative trials, the inoculation preceded the spraying by 1 day. Untreated control plants, infected with the pathogens, but not treated with fungicide, served as controls.

The plants were exposed to a rain stability test in two out of three experimental treatments, according to the method described by Hauser-Hahn (*27*). The plants received 20 mm of simulated rain in a 10 min event, at 1 or 24 h after inoculation, or pesticide application. In a third experimental treatment, the plants received no simulated rain.

Wheat was inoculated with the pathogens *Erysiphe graminis f.* sp. *tritici* (by shaking a highly infected plant over the uninfected plants) or with *Leptosphaeria nodorum* (10^6 spores/mL were sprayed on the plants and incubated for 48 h in the dark at 100% relative humidity). Barley was inoculated with *E. graminis f.* sp. *hordei* (in the same way as described above) or with *Pyrenophora teres* (10^4 spores/mL were sprayed on the plants and incubated for 48 h in the dark at 100% relative humidity). A random block design was used for each experimental treatment with four replicates, each consisting of 15 seedlings per pot (6 cm × 6 cm × 7 cm). The plants were grown under conditions of 18 °C, 80% relative humidity, with 12 h light and were used for testing after 7 days.

The cucumber plants were inoculated with *Sphaerotheca fuliginea* (by spraying with an emulsion of water and mildew spores from 10 to 12 day old highly infected plants). For each experimental treatment, two plants, representing two replicates, were grown at 20 °C, 80% relative humidity, with 14 h light and were used for the tests after 21

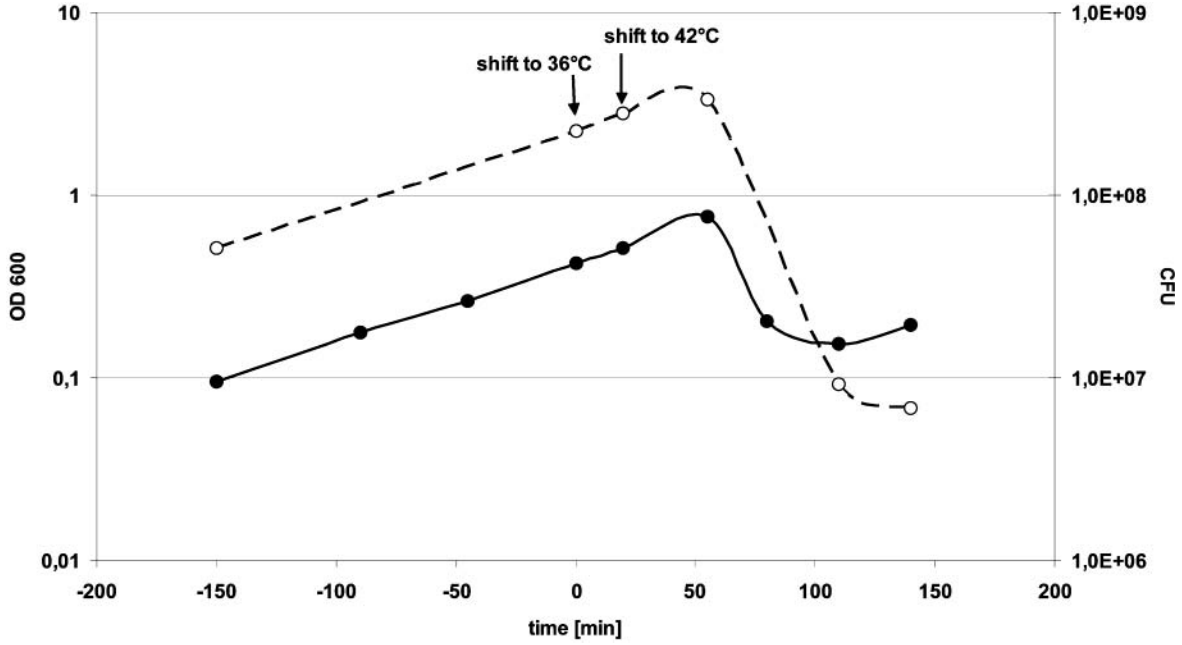

Figure 1. Bacterial ghosts production using E-mediated lysis of *P. cypripedii* HeP01 harboring the lysis plasmid pFN12. Growth and lysis profiles were monitored (●, OD_{600}; ○, CFU/mL) during fermentation. The bacterial culture was shifted from 28 to 36 °C for 10 min (at time point zero) and then kept at 42 °C, as indicated by the arrows.

days. The number of replication was determined by technical considerations: cucumber plants have large leaves, and the growth area was limited. All of the leaves except the two primary foliage leaves were removed before the tests (only primary leaves were fully developed at spraying time). Removal of the leaves reduced variation, i.e., it eliminated variation due to the application of different amounts of active ingredient applied to nonfully developed leaves.

Following the rain simulation, the plants were observed for symptoms of disease, after 10−12 days for barley and wheat and 7−8 days for cucumber, and the infestation, i.e., percent of infected surface area, was recorded. The efficacy was calculated according to Abbot (*28*) where untreated control as a baseline efficacy was considered as 100%, and the following formula was applied

$$\text{efficacy} = 100 - \frac{\%\ \text{infestation of treated plants} \times 100}{\%\ \text{infestation of untreated control}}$$

Testing Phytotoxicity of the Fungicide Formulations. Barley was cultivated as described in the efficacy trials, and four replicates were used for each experimental treatment. The plants were treated in parallel with 1000 or 2000 ppm tebuconazole-loaded bacterial ghosts or commercial tebuconazole (Folicur WP) at an application rate of 250 L/ha. The plants underwent the same rain stability test as described above in the three experimental treatments: no rain, rain after 1 h, and rain after 24 h. The plants were monitored at days 7 and 10. Two characteristics for the toxicity (necrosis and yellowing) were determined on a scale from 0 to 100 and summed for the two concentrations.

Electron Microscopy. Scanning electron micrographs were taken with a Hitachi S-800 field emission scanning electron microscope. Fixation of cells and preparation for electron microscopy were essentially the same as previously described (*9*).

RESULTS

Production of *P. cypripedii* HeP01 Ghosts. *P. cypripedii* HeP01 (pFN12) was grown by fermentation, and E-mediated lysis of the bacteria was induced by a temperature shift of the culture. The onset of culture lysis occurred 55 min after the temperature shift (time point zero), and the bacterial culture reached the minimum OD_{600} at 110 min (**Figure 1**). The CFU/mL at the onset of the lysis was 3.4×10^8 after 55 min and reached the minimum of 6.9×10^6 after 140 min of induction of gene *E* expression. Thus, 98% of the *P. cypripedii* was inactivated by the lysis process. A representative electron micrograph of *P. cypripedii* ghosts is given in **Figure 2**. The bacterial ghosts retained the original cell morphology, and because of the loss of the inner osmotic pressure, they appear as wrinkled empty envelopes. Lyophilization of the *P. cypripedii* ghosts resulted in complete inactivation, as the subsequent sterility test performed revealed no living cells. Under fermentation conditions, 389.5 mg (2.69×10^{12}) of bacterial ghosts was obtained from 10 L of culture volume. *P. cypripedii* HeP01 harboring the corresponding cloning vector pBluescript KSII+ and the nontransformed bacteria showed exponential growth with no reduction in cell viability when grown under identical conditions as the Hep01 (pFN12) culture.

Detection of FITC-Labeled Bacterial Ghosts on Leaf Surfaces. FITC-labeled bacterial ghosts were applied on leaves of agricultural crops (cabbage, cotton, barley, rice, soya, and corn) and subsequently washed off by simulated rain. Depending on the plant species, 61−85% of the bacterial ghosts remained attached to leaves after the first wash (**Table 1**). The second wash reduced the percentage of attached bacterial ghosts further to values between 14 and 68%. The additional washes did not significantly reduce the number of attached bacterial ghosts, as after the 7th wash (corresponding to heavy rain of 84 mm), and the values were similar as after the 2nd wash. Rice, corn, cabbage, cotton, and barley retained between 55 and 35% of the labeled *P. cypripedii* ghosts, whereas only 10% of ghosts were retained on soya leaves after 84 mm of simulated rain.

Efficacy Trials of Tebuconazole-Loaded *P. cypripedii* Ghost Formulations on Barley, Wheat, and Cucumber in Rain Stability Tests in a Glass House Experiment. In the rain simulation experiments of leaf attached *P. cypripedii* ghosts, barley leaves retained 35% of the applied bacterial ghosts after 84 mm of simulated rain (**Table 1**). This monocot crop plant and another, wheat, were then chosen to determine the efficacy of tebuconazole-loaded *P. cypripedii* ghosts for protective and curative effects against fungal infections with *E. graminis*,

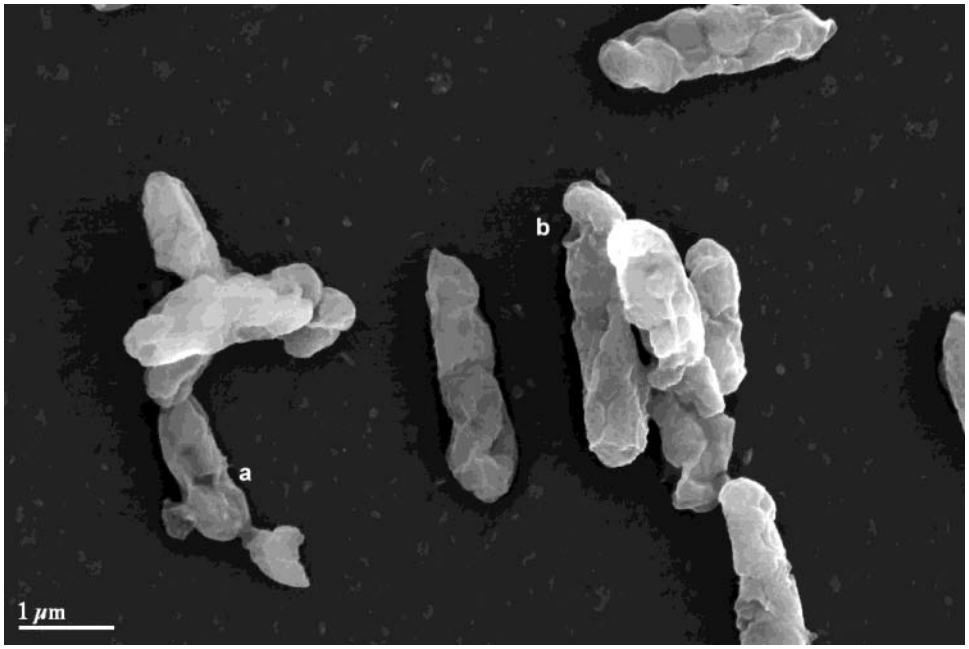

Figure 2. *P. cypripedii* HeP01 (pFN12) ghosts visualized by scanning electron microscopy. The E specific lysis hole can be seen either in the middle (**a**) or the polar (**b**) site of the ghost. The bacterial ghosts retained their original cell morphology, and because of the loss of the inner osmotic pressure, they have the appearance of wrinkled empty envelopes.

Table 1. Proportion of Fluorescence-Labeled Bacterial Ghosts on Leaf Surfaces after Rain Simulation[a]

	percent of remaining bacterial ghosts on leaf surface		
plants	after 1st wash	after 2nd wash	after the final (7th) wash
B. oleracea var. *capitat* (cabbage)	68	45	41
G. herbaceum (cotton)	85	51	39
H. vulgare (barley)	61	39	35
O. sativa (rice)	82	68	55
S. hispida (soya)	61	14	10
Z. mays (corn)	78	49	42

[a] FITC-labeled *P. cypripedii* ghosts (2.09×10^8) were applied to 0.25 cm^2 of leaf material. After they were dried for 24 h, seven time portions of 300 μL of water were used for rinsing. The numbers represent relative percentage values as compared to a nonwashed control. Each wash corresponds to 12 mm of simulated rain; altogether, it is 84 mm.

L. nodorum, and *P. teres*. Cucumber, as a contrasting dicot, was also used in experiments testing the efficacy against *S. fuliginea*.

The loading capacity of the *P. cypripedii* ghosts was determined through numerous loading trials. In the efficacy trials, the bacterial ghosts were loaded at a rate of 0.49 ± 0.03 mg tebuconazole/mg ghosts. The loading procedure did not change the overall structure of the bacterial ghost envelope, as **Figure 3** shows.

In the experiments, the efficacy of the pesticide-loaded bacterial ghosts was investigated in comparison with two commercial formulations of Folicur, the WP 25 and the EW 250, all at the same tebuconazole concentrations of 1000 ppm. The formulations were applied in protective and curative trials on barley and wheat in two series, and the rate of infestation was recorded (**Table 2**). Cucumber was tested only in protective trials with the Folicur formulation WP 25 and the loaded ghosts.

The efficacy comparisons of the tebuconazole-loaded bacterial ghost against the Folicur formulations, computed from the infestation data according to the Abbot formula, are averaged in **Table 3**. There was no difference in the efficacy between the loaded bacterial ghosts and the two Folicur treatments against the pathogen *E. graminis* on barley neither in the protective nor in the curative trials. Against the other pathogens, however, the loaded bacterial ghost results showed in a general higher efficacy as compared to the WP 25 formulation. Considerably higher values were achieved in wheat in protective trials against *E. graminis* and *L. nodorum* (rain after 24 h); the values in a relative comparison were 70−165% higher for the loaded ghosts. In the curative trials with *E. graminis* on wheat, the protection was on average 37% better for the loaded ghosts. Against *L. nodorum* on wheat, 14−24% better protection was achieved. Against the pathogen *P. teres* on barley, both the WP 25 and the loaded bacterial ghosts showed very low efficacy. In the protective trial with this pathogen (rain after 1 h), the WP 25 formulation was exceptional, showing higher efficacy.

The industrial Folicur EW 250 formulation was applied in the second series to test tebuconazole-loaded bacterial ghosts directly against a cereal specific formulation. For the EW 250, some comparisons showed considerably higher results, while others were in the same range as the loaded ghosts (**Table 3**). A relative difference of 4−16% could be seen between the two formulations in two experimental treatments: no rain and rain after 24 h with *E. graminis* and *L. nodorum*. In the case of the pathogen *P. teres*, in the same experimental treatments, the relative differences between formulations were 38−58% higher for the EW 250. If the rain application was after 1 h, the efficacies for the loaded ghosts differed in all cases substantially; they were at least 50% lower.

In the dicot cucumber, the tebuconazole-loaded bacterial ghosts showed 63% greater protection against *S. fuliginea* in the no simulated rain treatment. Furthermore, the bacterial ghosts gave similar protection at the experimental treatment, rain after 24 h as in the Folicur WP 25 treatment. As in the Folicur EW 250 comparison in the monocots, having simulated rain after 1 h, the bacterial ghost results were 50% lower than those of the WP 25 formulation.

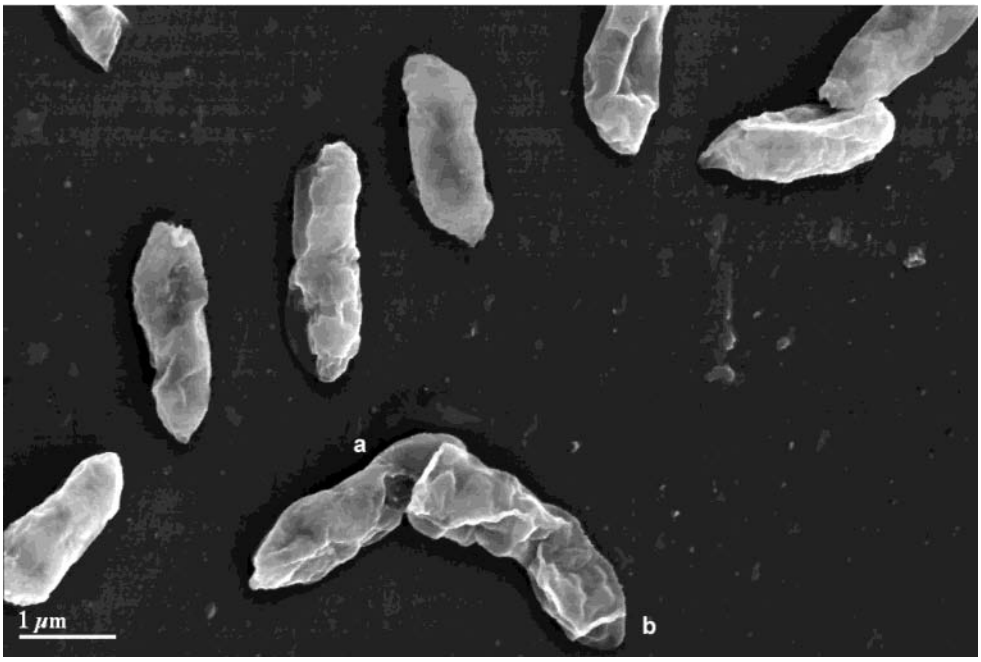

Figure 3. Tebuconazole-loaded *P. cypripedii* HeP01 (pFN12) ghosts visualized by scanning electron microscopy. The E specific lysis hole can be seen either in the middle (**a**) or in the polar (**b**) site of the ghost. There is no change in the structure caused by loading procedures.

Phytotoxicity Comparison of Folicur and Tebuconazole-Loaded Bacterial Ghosts Formulations. The toxic effects of the loaded bacterial ghosts and Folicur WP 25 formulations of tebuconazole are presented in **Table 4**. The values represent the sum of necrosis and yellowing of four replicates of barley on a rating scale (bonitur) from 0 to 100 (most toxic). Plants without pesticide application were used as controls and were set to zero. Slight yellowing and necrosis were observed in both no rain groups and in the loaded ghost group with rain after 1 h of treatment. In other treatment groups, no damage was observed. An overall comparison showed no expressed differences in phytotoxicity between the two formulations.

DISCUSSION

The present study showed that *P. cypripedii* ghosts adhere to different leaf surfaces of a range of plant species. In the first studies with fluorescence-labeled bacterial ghosts, we found that even after heavy rain simulation, 10 and 55% of the applied bacterial ghosts remained attached to leaves of soya and rice, respectively. Values for barley, corn, cotton, and cabbage varied from 35 to 42%. Furthermore, the bacterial ghosts were tested as a new carrier system for the lipophilic triazole fungicide tebuconazole. In a glass house experiment, we showed that the fungicidal activity of loaded bacterial ghosts was at least comparable to the commercial formulation. Advantages were observed in terms of rain stability and protection against fungal infection on a longer term. The experiment served as a proof of principal. The bacterial ghosts were compared in the first case with a formulation, WP 25, with similar physical characteristics (solid against solid), and differences in their mode of action were observed. Furthermore, the bacterial ghost system was tested directly with the EW 250 formulation, which was specially designed for use on cereals, as a standard operational procedure.

A *P. cypripedii* strain HeP01 has been isolated in our lab from bilberry, showing natural adhesion properties to plant tissue. Bacterial ghosts were successfully produced from this strain, revealing nondenaturated bacterial cell envelopes with intact surface structures.

Both di- and monocot plants of different genera and adapted to different climates were used in the adherence experiments with *P. cypripedii*. The variation observed in adherence, which could be further exploited for specific targeting of plants, was associated with plant species, the structures of the leaf surface, and the receptor recognition abilities of *P. cypripedii* ghosts. Leaf surface lectins, for example, play a role in plant−bacteria adhesion as plant defense proteins (*29*, *30*); epicuticular waxes affect the deposition, retention, and distribution of spray droplets (*31*); and different bacterial ghosts show different attachment characteristics (*3*, *32*). The amount of bacterial ghosts remaining on the leaves after the first wash ranged between 61 and 85%. Further rain simulation treatments gradually removed the bacterial ghosts from the leaves, resulting in lower pesticide residue levels.

P. cypripedii is a Gram-negative bacterium with an envelope composed of inner and outer membranes sandwiching the periplasmic space. The outer membrane is permeable to molecules smaller than 600 Da (tebuconazole 310 Da) giving access to the periplasmic space, whereas the inner membrane seals the cytoplasmic compartment of the bacteria. In bacterial ghosts, the inner membranes are fused at distinct areas of the envelope complex forming a hole through which the cytoplasmic contents had been expelled. The remaining cytoplasmic lumen has a maximal theoretical volume of 0.76×10^{-9} μL per bacterial ghost [calculated for a typical rod-shaped bacterial ghost with a length (l) of 2.5 μm, diameter of 0.7 μm, and an average membrane size of 21 nm, using the formula $V = 4r^3\pi/3 + r^2\pi l$]. The lumen is accessible for liquids.

According to the manufacturer, tebuconazole shows good solubility with *n*-hexane, acetone, dichloromethane, dimethylformamide, and a mixture of ethanol and PEG. In this study, tebuconazole was dissolved in the ethanol/PEG mixture for the loading of *P. cypripedii* ghosts. This solvent affects the membrane integrity of the bacterial ghost envelope. The outer membrane was permeabilized by the ethanol/PEG solvent (data not shown), and this was evident by the release of the periplasmic marker enzyme β-lactamase (*33*). However, as can

Table 2. Percent of Infestation of Plants in Experiments with the Pathogens *E. graminis*, *L. nodorum*, and *P. teres* on Barley and Wheat, in Protective and Curative Trials[a]

		infestation (%): protective trial			infestation (%): curative trial		
pathogen and plant	formulation	no rain	rain after 1 h	rain after 24 h	no rain	rain after 1 h	rain after 24 h
			E. graminis on barley				
1st series	loaded ghost[b]	0	1.3 ± 1.3	1.3 ± 1.3			
	WP 25	0	2.5 ± 1.4	1.3 ± 1.3			
	control	83.8 ± 2.6	30.6 ± 5.3	22.5 ± 3.8			
2nd series	loaded ghost[b]	0	0	0	0	0	0
	WP 25	0	0	0	2.5 ± 1.4	3.8 ± 1.3	2.5 ± 1.4
	EW 250	0	0	0	0	0	1.3 ± 1.0
	control	90.0 ± 0	67.5 ± 2.5	58.8 ± 15.6	73.8 ± 3.7	35 ± 5.0	87.5 ± 3.0
			E. graminis on wheat				
1st series	loaded ghost[b]	30.0 ± 2.0	62.5 ± 2.5	16.3 ± 1.3	6.3 ± 1.2	30 ± 2.0	2.5 ± 1.0
	WP 25	32.5 ± 2.5	65.0 ± 5.0	37.5 ± 7.5	32.5 ± 6.6	45 ± 3.0	18.8 ± 1.0
	control	86.3 ± 1.8	77.5 ± 1.6	63.8 ± 3.8	77.5 ± 1.6	75 ± 3.0	52.5 ± 4.0
2nd series	loaded ghost[b]	28.8 ± 3.1	57.5 ± 4.8	13.8 ± 2.4	8.8 ± 1.2	37.5 ± 2.5	5.0 ± 0
	WP 25	33.8 ± 5.5	72.5 ± 2.5	38.8 ± 4.3	17.5 ± 2.5	41.3 ± 4.3	20.0 ± 2.0
	EW 250	23.8 ± 2.4	30.0 ± 4.1	8.8 ± 1.3	0	1.3 ± 1.3	1.3 ± 1.3
	control	87.5 ± 2.5	80.0 ± 0	80.0 ± 0	70.0 ± 2.7	63.3 ± 2.1	65.0 ± 2.9
			L. nodorum on wheat				
1st series	loaded ghost[b]	10.0 ± 0	45.0 ± 2.9	16.3 ± 1.3	21.3 ± 1.2		21.3 ± 1.3
	WP 25	18.8 ± 1.2	50.0 ± 4.0	31.3 ± 1.0	23.8 ± 2.4		26.3 ± 3.0
	control	62.5 ± 1.6	63.8 ± 2.6	46.3 ± 1.8	61.7 ± 3.1		65.0 ± 2.7
2nd series	loaded ghost[b]	8.8 ± 1.2	38.8 ± 4.3	8.8 ± 1.3	16.3 ± 1.2		18.8 ± 1.3
	WP 25	13.8 ± 2.4	42.5 ± 4.8	28.8 ± 2.4	25.0 ± 2.9		35.0 ± 2.9
	EW 250	6.3 ± 1.2	10.0 ± 0	6.3 ± 1.0	20.0 ± 2.0	22.5 ± 2.5	11.3 ± 1.3
	control	82.5 ± 2.5	48.8 ± 5.2	36.3 ± 2.4	66.7 ± 9.2	76.7 ± 6.7	80.0 ± 2.6
			P. teres on barley				
1st series	loaded ghost[b]	47.5 ± 2.5	52.5 ± 2.5	27.5 ± 1.4	67.5 ± 6.3	72.5 ± 2.5	62.5 ± 4.8
	WP 25	45.0 ± 2.9	45.0 ± 3.0	32.5 ± 3.0	77.5 ± 2.5	70.0 ± 0	77.5 ± 3.0
	control	71.3 ± 1.2	62.5 ± 1.6	50.0 ± 0	90.0 ± 0	70.0 ± 4.0	86.3 ± 2.0
2nd series	loaded ghost[b]	65.0 ± 5.0	85.0 ± 3.0	35.0 ± 3.0	31.3 ± 3.1	67.5 ± 2.5	36.3 ± 2.4
	WP 25	75.0 ± 5.0	77.5 ± 4.8	45.0 ± 6.5	75.0 ± 2.9	67.5 ± 2.5	67.5 ± 2.5
	EW 250	25.0 ± 2.9	25.0 ± 2.0	16.3 ± 1.0	15.0 ± 2.0	33.8 ± 4.7	20.0 ± 2.0
	control	90.0 ± 0	90.0 ± 0	72.5 ± 3.0	87.5 ± 2.5	77.5 ± 2.5	75.0 ± 2.9
			S. fuliginea on cucumber				
	loaded ghost[b]	64.6 ± 6.4	41.3 ± 2.8	40.8 ± 2.3			
	WP 25	78.3 ± 3.2	32.5 ± 3.5	42.1 ± 2.0			
	control	100.0 ± 0	51.0 ± 5.9	48.8 ± 3.0			

[a] The pathogen *S. fuliginea* was applied to cucumber in protective trials. The plants were exposed to rain simulation at different times. Untreated control plants, infected with the pathogens but not treated with fungicide, served as controls. For each experimental treatment with barley and wheat, four replicates were used consisting of 15 seed-derived plants each. In the case of cucumber, two plants, representing two replicates, were used. [b] Tebuconazole-loaded bacterial ghosts.

Table 3. Efficacy Comparison of Tebuconazole-Loaded Bacterial Ghosts against Folicur WP 25 (Wettable Powder) and EW 250 (Emulsion, Oil in Water) Formulations

		protective efficacy (%)			curative efficacy (%)		
pathogen and plant	formulation	no rain	rain after 1 h	rain after 24 h	no rain	rain after 1 h	rain after 24 h
E. graminis on barley	loaded ghost[a]	100.0	98.0	97.2	100.0	100.0	100.0
	WP 25	100.0	95.9	97.2	96.6	89.3	97.1
	EW 250	100.0	100.0	100.0	100.0	100.0	98.6
E. graminis on wheat	loaded ghost[a]	66.2	23.7	78.7	89.7	50.4	93.8
	WP 25	61.9	12.8	46.4	66.5	37.4	66.8
	EW 250	72.9	62.5	89.1	100.0	98.0	98.1
L. nodorum on wheat	loaded ghost[a]	86.7	25.0	70.4	70.6	-	71.9
	WP 25	76.7	17.2	26.6	62.0	-	57.9
	EW 250	92.4	79.5	82.8	70.0	70.7	85.9
P. teres on barley	loaded ghost[a]	30.6	10.8	48.4	44.6	4.7	39.6
	WP 25	26.8	20.9	36.5	14.1	6.5	10.1
	EW 250	72.2	72.2	77.6	82.9	56.5	73.3
S. fuliginea on cucumber	loaded ghost[a]	35.4	18.5	16.2	-	-	-
	WP 25	21.7	35.8	13.7	-	-	-

[a] Tebuconazole-loaded bacterial ghosts.

be seen in **Figure 3**, the envelope complex of *P. cypripedii* ghosts was not dissolved by the solvent and resembles the untreated control (**Figure 2**).

Table 4. Phytotoxicity Comparisons of Folicur WP 25 and Tebuconazole-Loaded Bacterial Ghosts on Barley Plants on a Rating Scale (Bonitur) from 0 to 100 (Most Toxic)

	no rain	rain after 1 h	rain after 24 h
Folicur WP 25	5	0	0
loaded ghosts[a]	15	5	0

[a] Tebuconazole-loaded bacteria ghosts.

Drug delivery studies showed by fluorescence microscopy and confocal laser scanning microscopy that the chemotherapeutical drug doxorubicin (anthracycline) was located and associated with the bacterial ghost membranes and in the inner lumen (*32*). This interaction with the membranes is due to the aromatic ring structures of the drug, and it can be assumed that the lipophilic pesticide tebuconazole has the same distribution as doxorubicin. In a test, comparing intact *P. cypripedii* cells with ghosts, both mixed with a tebuconazole solution, it was found that the intact cells had half the loading capacity of the bacterial ghosts.

In field conditions, the greatest pesticide runoff is correlated with the first significant rainfall after application (*34*). Experiments have shown that 25 mm of rain could wash off 67% of the active compound (*35*), and it was recommended, in the case of mancozeb, that the pesticide treatment is repeated if it rains the day after application (*36*). Furthermore, the analysis of

pesticide contamination in rainwater shows seasonal variation reflecting the weather conditions in the application periods (*37*). Simulated rain experiments are generally done with the intensity of up to 45 mm/h similar to that of natural rains when studying residues in the soil (*38*, *39*). In this study, the rain simulations were more intense; we tested the adherence of the fluorescence-labeled bacterial ghosts rather than the wash off. Despite a heavy rain simulation, the amount of attached labeled bacterial ghosts was decreased by only 20−40%. In the glass house experiment, a rain intensity of 120 mm/h was used. The highest comparative adhesion rates were reached using the pesticide-loaded bacterial ghosts when the rain simulation was done a day after treatment, i.e., this probably allowed time for the plant surface and bacterial ghosts interaction leading to adhesion. As previous experiments showed, the main advantages of bacterial ghosts are in long-term gradual release of loaded substances (*32*). To minimize the loss of activity, pesticide formulations have to contain UV protectants, stickers, or both (*14*). Although not tested, these requirements could be met using bacterial ghosts as a carrier system. The retention of active substances in bacterial ghost envelopes could provide protection from the outer environment facilitate higher stability for the formulation and the attachment properties, and reduce wash off.

The major emphasis in the development of new pesticide formulations is the improvement of the physical properties and the product performance of the formulations. The ability to deliver the pesticide to the site of action (plant, insect, or fungus) is a critical property determining the efficacy of the product. The wetting and the retention of the pesticide droplets on the foliar surface are key factors in product performance. Recently developed new formulations aim to incorporate these properties and include microencapsulation via interfacial polymerization or colloid particles (*12*, *14*), which mimic the bacterial ghosts.

The active ingredient tebuconazole is commercially available in EW (emulsion, oil in water), EC (emulsifiable concentrate), SP (suspension concentrate), WP (wettable powder), or WG (water dispersible granulates). The standard operational comparison of the new formulation with bacterial ghosts loaded with technical grade tebuconazole with the most protective, cereal specific emulsion (EW 250), which also contains adjuvants to improve the effectiveness of the pesticide, revealed that the bacterial ghosts had equal to or lower efficacy than the EW 250. However, the primary comparison with the wettable powder (WP 25), as it has similar physical characteristics, showed similar effects and consistently higher or comparable efficacy against the pathogens. The results of the current study confirmed the potential of bacterial ghost platform technology as an alternative carrier system for a lipophilic pesticide. In the next step, the specific field of application of such a formulation needs to be determined. This technology could be refined and extended to other target plants and active ingredients for plant protection.

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