Activity of bacteria isolated from bats against *Pseudogymnoascus destructans* in China

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**Summary**

White-nose syndrome, a disease that is caused by the psychrophilic fungus *Pseudogymnoascus destructans*, has threatened several North America bat species with extinction. Recent studies have shown that East Asian bats are infected with *P. destructans* but show greatly reduced infections. While several factors have been found to contribute to these reduced infections, the role of specific microbes in limiting *P. destructans* growth remains unexplored. We isolated three bacterial strains with the ability to inhibit *P. destructans*, namely, *Pseudomonas yamanorum* GZD14026, *Pseudomonas brenneri* XRD11711 and *Pseudomonas fragi* GZD14479, from bats in China. *Pseudomonas yamanorum*, with the highest inhibition score, was selected to extract antifungal active substance. Combining mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy analyses, we identified the active compound inhibiting *P. destructans* as phenazine-1-carboxylic acid (PCA), and the minimal inhibitory concentration (MIC) was 50.12 μg ml⁻¹. Whole genome sequencing also revealed the existence of PCA biosynthesis gene clusters. Gas chromatography-mass spectrometry (GC-MS) analysis identified volatile organic compounds. The results indicated that 10 ppm octanoic acid, 100 ppm 3-tert-butyl-4-hydroxyanisole (isoprenol) and 100 ppm 3-methyl-3-buten-1-ol (BHA) inhibited the growth of *P. destructans*. These results support that bacteria may play a role in limiting the growth of *P. destructans* on bats.

**Introduction**

Over the past several decades, fungal diseases have caused widespread declines in wildlife populations, including amphibians affected by chytridiomycosis, snakes impacted by snake fungal disease and bats affected by white-nose syndrome (WNS) (Skerratt et al., 2007; Langwig et al., 2012; Lorch et al., 2016; Scheele et al., 2019). The application of probiotics to treat fungal pathogens has achieved varying degrees of success in the fields of human and veterinary medicine, agriculture and aquaculture (Tan et al., 2016; Kerry et al., 2018; Markowiak et al., 2019; Xia et al., 2020). While beneficial bacteria are used less frequently to combat wildlife disease, there is evidence that naturally occurring microbes may contribute to differences in infection severity.

White-nose syndrome, an emerging infectious disease of hibernating bats in North America, is caused by the psychrophilic fungus, *Pseudogymnoascus destructans* (Gargas et al., 2009; Lorch et al., 2011; Warnecke et al., 2012). WNS has killed millions of bats in North America from 2006, with several species being severely affected (Frick et al., 2010; Langwig et al., 2012). Bacteria and fungi isolated from bats and the environment have been shown to inhibit the growth of *P. destructans in vitro* (Hoyt et al., 2015; Raudabaugh and Miller, 2015; Micalizzi et al., 2017; Rusman et al., 2020), and application of *Pseudomonas fluorescens* on bat skin over winter was recently shown to increase survival for one species of bats.
bat (Hoyt et al., 2019). *P. destructans* conidia have also been shown to be inhibited by the volatile organic compounds produced by *R. rhodochrous* (Cornelison et al., 2014) and trans-farnesol, a sesquiterpene made by the yeast *Candida* (Raudabaugh and Miller, 2015). In addition, *Oidiodendron truncatum* was shown to produce various compounds that reduced the growth of *P. destructans* at low concentrations (Rusman et al., 2020). These studies demonstrated that bacteria were likely to contribute to differences in infections among individuals and species in bats.

Hibernating bats across Europe and Asia have been shown to be infected with *P. destructans*, but the symptoms of this disease are greatly reduced, and fungal load and prevalence are lower than bats that suffer mortality in North America (Wibbelt et al., 2010; Hoyt et al., 2016a,b). While the timing of transmission has been found to be an important factor for bats surviving with this disease across Eurasia there is likely a multitude of factors that contribute to bats persistence with this disease (Hoyt et al., 2020). We hypothesized that skin microbiota may also contribute to the reduction of infection by *P. destructans*.

In this study, we collected cutaneous bacteria from two bats species, *Rhinolophus ferrumequinum* and *Myotis petax*, in late hibernation from north-east and central China. We isolated and identified bacteria that inhibited the growth of *P. destructans* using in vitro challenge assays and analysed the antifungal metabolites produced by the bacteria isolate with the highest inhibitory effect. In addition, we examined the volatile organic compounds produced by this same isolate with the highest inhibition rate. Finally, we also examined the existence of gene clusters associated with these active substances. Our results provide evidence for a potential mechanism of disease resistance for *P. destructans* in East Asian bats by their skin microbes.

Results

Isolation, screening and identification of bacteria with anti-*P. destructans* activity

We isolated 90 total bacterial morphologies from the 25 samples collected from *R. ferrumequinum* (15) and *M. petax* (10). Agar plate challenge assays results showed that three bacterial isolates had the ability to inhibit the growth of *P. destructans*. One isolate, GZD14026, was from two individuals of *M. petax* in Ge-zi Cave. Two isolates, GZD 14479 and XRD11711, were from one individual of *R. ferrumequinum* in Ge-zi Cave and two individuals of *R. ferrumequinum* in Temple Cave, respectively (Table 1). An obvious inhibition zone appeared around isolate GZD14026. Concurrently, different inhibition modes were presented in the isolate GZD14479, while a small inhibition halo was observed surrounding isolate XRD14479 (Fig. 1A). These results suggest that bacterial isolate GZD14026 had the strongest inhibitory effect on *P. destructans*.

We further evaluated the inhibition rate of the above three bacterial isolates in cell-free supernatant assay. The results displayed that isolate GZD14026 had the strongest inhibition effect, with an average inhibition score of 71.33%, while isolate GZD14479 and XRD11711 were 33.27% and 22.90%, respectively. The inhibition score of isolate GZD14026 was more than double those of isolate GZD14479 and XRD11711 (Tukey’s tests: isolate GZD14026-isolate GZD14479, $P < 0.001$; isolate GZD14026–isolate XRD11711, $P < 0.001$; Fig. 1B), so we focused on the chemical mechanism underlying the strong inhibition produced by isolate GZD14026.

The 16S rRNA genes and three housekeeping genes *gyrB, rpoD* and *rpoB* were concatenated for a multilocus sequence analysis (MLSA) of these three bacterial strains, which were sequenced and compared with other strains in the GenBank database. The results revealed that the concatenated sequences of these three bacterial strains were all from the genus *Pseudomonas*, with greater than 99% gene sequence similarities with three different *Pseudomonas* spp., *Pseudomonas yamanorum*, *Pseudomonas fragi* and *Pseudomonas brenneri*. Therefore, the three bacterial strains were subsequently referred to as *P. yamanorum* GZD14026, *P. fragi* GZD 14479 and *P. brenneri* XRD11711, respectively (Table 1).

Morphological changes of *P. destructans* mycelium

Scanning electron microscopy (SEM) showed alterations of *P. destructans* mycelium when exposed to *P. yamanorum* GZD14026 cell-free supernatant after 14 days. The surfaces of the mycelium in the non-exposed group were

Table 1. Bacteria isolated from *R. ferrumequinum* and *M. petax* used in agar plate challenge assays.

| Label   | Bat species | Collection Province     | Bat numbers | Collection date (late hibernation) | Bacterial taxonomy            |
|---------|-------------|-------------------------|-------------|-----------------------------------|-------------------------------|
| GZD14026 | *M. petax*  | Ge-zi Cave, Jilin        | 2           | 9-Apr-18                          | *Pseudomonas yamanorum*       |
| GZD14479 | *R. ferrumequinum* | Ge-zi Cave, Jilin         | 1           | 9-Apr-18                          | *Pseudomonas fragi*           |
| XRD11711 | *R. ferrumequinum* | Temple Cave, Liaoning    | 2           | 6-Apr-18                          | *Pseudomonas brenneri*        |
smooth, slender, uniform thickness, plump and round (Fig. 2A–C). In contrast, the mycelium morphology of the group exposed to GZD14026 became malformed, short with uneven thickness, and many branches were fractured (Fig. 2D–F).

Isolation, purification and identification of active compounds with anti-P. destructans activity

To obtain the active compounds that inhibited the growth of P. destructans from P. yamanorum GZD 14026, 8.3 g of crude extract was acquired from 30 l of LB liquid fermentation by extraction with dichloromethane. This was mixed with an equal weight of silica gel and then eluted. An anti-P. destructans compound was detected in a single band with an Rf of 0.78 by thin-layer chromatography (TLC) analysis, indicating the existence of a single compound. This antifungal compound was further detected by semi-preparative high-performance liquid chromatography (HPLC) and appeared as a single peak at 12.11 min (Fig. 3A), which was a lemon-yellow crystalline powder with inhibition rate against P. destructans of 60.23%.

We used the ultraviolet-visible spectra (UV/Vis), mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy to identify the active compound structure from P. yamanorum GZD 14026. Strong absorption peaks were observed at 206.7 nm, 251.4 nm and 364.1 nm using UV/Vis spectra analysis (Fig. S1A). MS results revealed two strong molecular ion peaks at m/z 225.21 [M + H]+ and m/z 207.21 [M - OH]+ (Fig. 3B). The results of 1H NMR and 13C NMR spectra were described in (Fig. S1B and Table 2).

All of the spectral detection results showed that the anti-P. destructans active compound of P. yamanorum GZD 14026 was phenazine-1-carboxylic acid (PCA), with the molecular formula C13H8N2O2 (Fig. 3C and Fig. S1C), which was consistent with the conclusions proposed by Lee et al., 2003. The minimal inhibitory concentration (MIC) of PCA against P. destructans in vitro was 50.12 μg ml⁻¹, and the half maximal inhibitory concentration (IC50) was 32.08 μg ml⁻¹.

Whole genome comparison involved in PCA biosynthesis

The whole genome of P. yamanorum GZD 14026 was 6 947 460 bp in length, with a GC content of 59.04%. A total of 6886 genes were identified from this genome, including 6797 genes encoding proteins. Additionally, genome analysis suggested that P. yamanorum GZD 14026 produced secondary metabolites of phenazine. We next investigated the genes involved in PCA biosynthesis from P. yamanorum GZD 14026, and we compared them with common PCA-producing bacterial strains, including P. chlororaphis GP72 (Shen, et al., 2012) and P. fluorescens 2-79 (Mavrodi, et al., 1998). The results revealed that, compared with the genes of the PCA biosynthetic operon from P. chlororaphis GP72, phzA to phzG had 88.73–94.36% and 87.80–93.03% homology with P. fluorescens 2-79 (Table 3). Therefore, this suggested that P. yamanorum GZD 14026 could produce PCA.

Evaluation, identification and verification of volatile organic compounds with anti-P. destructans activity

Pseudomonas yamanorum GZD 14026 was co-cultured with P. destructans, and after 14 days, we found that the
The number of *P. destructans* conidia was dramatically reduced and malformed when compared with control samples, suggesting that volatile organic compounds (VOCs) were also being released from *P. yamanorum* GZD 14026 and were impacting the growth of *P. destructans* (Fig. 4A).

We used gas chromatography-mass spectrometry (GC-MS) to identify the volatile organic compounds produced by *P. yamanorum* GZD 14026, and a total of 16 compounds were detected after removing controls (Table S1), of which three compounds were demonstrated to inhibit the growth of *P. destructans*, namely, octanoic acid, 3-methyl-3-butene-1-alcohol (isoprenol) and 3-tert-butyl-4-hydroxyanisole (BHA). Among the best inhibitory effect was octanoic acid, which generated a large inhibition circle at 10 ppm. BHA had similar results at 100 ppm, while almost no fungal growth was seen with 100 ppm isoprenol (Fig. 4B).

**Discussion**

In this study, we isolated three bacterial strains with the ability to inhibit the growth of *P. destructans* from *R. ferrumequinum* and *M. petax* from two localities (Table 1). This was similar to the number of active strains (*n* = 6) isolated in Hoyt and colleagues (2015), but less than others, such as (*n* = 28) from Micalizzi et al. (2017) and (*n* = 32) from Hamm and colleagues (2017). This numerical difference might be due to the method of our inhibition assays (McArthur et al., 2017). In this study, we used the same method to screen for anti-*P. destructans* bacteria as Hoyt and colleagues (2015), but this was different from others (Micalizzi et al., 2017; Hamm et al., 2017). In addition, the number of bats (*n* = 25) we collected was also less than those (*n* = 40) from Hoyt and colleagues (2015) and (*n* = 101) Hamm and colleagues (2017), which might result in the relatively low number of active strains isolated in this study. Actually, it is a real possibility that the growth of *P. destructans* is inhibited by the interactions of multiple microorganisms. In future work, we intend to optimize the method of inhibition experiments and expand sample sizes to screen more probiotic strains.

Coincidentally, the three bacterial isolates identified in this work were from the genus *Pseudomonas*. It is well-known that *Pseudomonas* is widely distributed in the environment and has been also found on mammals, amphibians, reptiles and plants (Gholami et al., 2017; Brunetti et al., 2019; Brockmann et al., 2020; Fernandez...
et al., 2020). *Pseudomonas* bacteria have been demonstrated to inhibit the growth of several relevant fungal diseases, including chytridiomycosis and white-nose syndrome in North America. *Pseudomonas* spp. from the skin bacterial communities of frogs have been shown to restrict *Batrachochytrium dendrobatidis* at an inhibition rate over 90% (Kruger, 2020), and Hoyt and colleagues (2015) demonstrated that most of the bacteria isolated from bats that could inhibit *P. destructans* growth came from the genus *Pseudomonas*.

*Pseudomonas* is well-known for its antibacterial activity and can produce a large number of secondary metabolites, such as phenazines, pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol and peptides (Schnider et al., 1995; Bender et al., 1999; Nielsen et al., 2002; Lee et al., 2003; Isnansetyo et al., 2003). Antibiotic-producing *Pseudomonas* can be highly effective biocontrol agents (Wang et al., 2020; Kamou et al., 2020). In this study, an anti-*P. destructans* active substance was separated from the culture medium of *P. yamanorum* GZD 14026. Using UV/Vis, MS and NMR detection, we revealed that phenazine compounds, namely phenazine-1-carboxylic acid (PCA), were responsible for this strong inhibitory activity. The whole genome of *P. yamanorum* GZD 14026 had PCA-producing gene clusters (Table 3).

Table 2. $^1$H and $^1$C spectral data of active compounds from *P. yamanorum* GZD 14026 against *P. destructans*.

| Carbon no. | $^{13}$C, δ ($^a$) | $^1$H δ ($^b$, $^c$, J in Hz) |
|------------|-------------------|-----------------------------|
| 1          | 125.2             | —                           |
| 2          | 130.5             | 8.57 (dd, 7.0, 1.5)         |
| 3          | 137.7             | 8.31-8.39 (m)               |
| 4          | 135.4             | 9.01 (dd, 8.7, 1.5)         |
| 4a         | 140.1             | —                           |
| 5a         | 140.3             | —                           |
| 6          | 132.0             | 7.99-8.08 (m)               |
| 7          | 128.2             | 8.31-8.39 (m)               |
| 8          | 130.3             | 8.31-8.39 (m)               |
| 9          | 133.5             | 7.99-8.08 (m)               |
| 9a         | 143.6             | —                           |
| 10a        | 144.3             | —                           |
| COOH       | 166.2             | 15.61(s)                    |

$^a$ 125 MHz, chemical shift in ppm.

$^b$ 500 MHz, chemical shift in ppm.

$^c$ Abbreviations of signal multiplicity are (s): singlet, (dd): doublet of doublets, (m): multiplet.

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against plant diseases, as well as inducing systemic resistance in plants (Santos Kron et al., 2020; Jin et al., 2020). PCA is a very important compound widely present in microbial metabolites from Pseudomonas, Streptomyces and Bacillus spp. (Kim, 2000; Xiong et al., 2017). Additionally, PCA is used as a biocontrol agent against various plant pathogens in China (Zhou et al., 2010). The inhibitory effect of PCA on fungi may be a complicated process, which involves multiple complex physiological processes such as cell wall structure and synthesis, cell membrane integrity, intracellular redox system, protein sorting and vesicle transport, and chromatin remodelling (Xu et al., 2015; Zhu et al., 2017). Moreover, PCA is an ideal compound for the development of microbial metabolites because of its stable chemical structure, low toxicity and compatibility with the environment (Yuan et al., 2008), and represents a new type of microorganism-derived pesticide (Huasong et al., 2008).

### Table 3. Identification of the phz genes of *P. yamanorum* GZD 14026 with other *Pseudomonas* spp.

| Gene   | Accession number   | Homology to *P. chlororaphis* GP72 | Homology to *P. fluorescens* 2-79 | Description                                                                 |
|--------|--------------------|-------------------------------------|--------------------------------|----------------------------------------------------------------------------|
| phzA   | GZD14026_3940      | 92.07%                              | 87.80%                          | Phenazine biosynthesis protein                                              |
| phzB   | GZD14026_3941      | 94.36%                              | 93.03%                          | Phenazine biosynthesis protein                                              |
| phzC   | GZD14026_3942      | 90.72%                              | 88.44%                          | 3-Deoxy-7-phosphohexulonate synthase                                        |
| phzD   | GZD14026_3943      | 93.69%                              | 90.61%                          | Isochorismatase family protein                                              |
| phzE   | GZD14026_3944      | 91.38%                              | 88.26%                          | Anthranilate synthase                                                       |
| phzF   | GZD14026_3945      | 93.79%                              | 89.73%                          | Phenazine biosynthesis protein isomerase                                    |
| phzG   | GZD14026_3946      | 88.73%                              | 88.64%                          | Pyridoxamine-5-phosphate oxidase                                            |

**Fig. 4.** Evaluation and verification of volatile organic compounds from *P. yamanorum* GZD 14026 working against *P. destructans*. A. Co-cultured *P. yamanorum* GZD 14026 with *P. destructans* after 14 days (13°C). Control (only *P. destructans*). B. Four volatile compounds inoculated with *P. destructans* after 14 days (13°C). Control (100 µl 95% ethanol and *P. destructans*). The image of the front side (A) and the back side (B) of the culture medium.
Therefore, it could be used in conservation strategies combating white-nose syndrome in bats. The current studies on phenazines have primarily been in relation to the genus Pseudomonas, specifically P. fluorescens, P. chlororaphis and P. aeruginosa. P. fluorescens has been found to only synthesize PCA; however, P. chlororaphis and P. aeruginosa can also produce some other phenazine derivatives, such as phenazine-1-amide (PCN), pyocyanin (PYO), 1-hydroxypheanazine (1-OH-PhZ), 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA), which all have demonstrated antimicrobial activity (Mavrodi et al., 2006; Mossine et al., 2018). However, some phenazine compounds also have anti-cancer, anti-malarial and anti-parasitic biological activities, which has attracted increasing attention to these compounds (Haas, et al., 2000; Laursen and Nielsen, 2004; Rajkumar et al., 2020).

Challenge assays results showed that the P. yamanorum GZD 14026 isolated from the skin of M. petax during hibernation showed the strongest inhibitory effect, with an inhibition rate of 71.33%. SEM further demonstrated that P. yamanorum GZD 14026 caused P. destructans mycelium morphology to undergo significant changes, indicating that the growth of mycelium was seriously affected. The P. destructans mycelium was affected by direct contact with the yellow active substance (PCA) secreted by P. yamanorum GZD14026 in SDA. PCA played a role in changing the P. destructans mycelium morphology. Similarly, Vibrio anguillarum appeared to have deformed mycelia when this fungus was treated with PCA (Zhang et al., 2017). However, there may be other factors influencing mycelium morphology, such as volatile organic compounds (VOCs). P. destructans mycelium growth and conidiation germination were persistently suppressed by the VOCs produced by R. rhodochrous (Cornelison et al., 2014). Therefore, the effect of P. yamanorum GZD14026 on the inhibition of P. destructans mycelium was a multiple action process. Other studies have shown that P. yamanorum showed strong inhibitory effects on other fungal pathogens, including Botrytis cinerea (Vignatti et al., 2020), Fusarium solani (Bahroun et al., 2018) and other Gram-negative and Gram-positive bacteria (Arnau et al., 2015).

In this study, it was proposed for the first time that P. yamanorum GZD 14026 produced not only PCA but also a series of VOCs. Specifically, 10 ppm octanoic acid, 100 ppm BHA and 100 ppm isoprenol significantly inhibited the growth of P. destructans. Octanoic acid was also isolated from Megasphaera (Jeon et al., 2017). Studies have shown that octanoic acid and its derivatives can effectively kill aquaculture pathogens and a variety of food-borne pathogens, including Escherichia coli O157:17, Enterobacter sakazakii and Listeria monocytogenes (Annamalai et al., 2004; Nair et al., 2004; Kollanoor et al., 2007). The volatile compounds extracted from the vegetative phase of Polygonum bistorta were majorly isoprenol, and illustrated their abilities against Paenibacillus larvae and Bacillus subtilis (Cecotti et al., 2012). Isoprenol is also found in Escherichia and Corynebacterium (George et al., 2015; Sasaki et al., 2019). BHA is an antioxidant that can be added to foods, and also kills a wide range of pathogenic bacteria, such as Salmonella typhimurium, Staphylococcus aureus, Vibrio parahaemolyticus and Aspergillus parasiticus (Chang and Brainen, 1975; Pierson et al., 1980). One study isolated BHA from Chlorococcus (Olasehinde et al., 2020). The mechanism of action of VOCs was to inhibit the growth of fungal mycelium, spore formation and germination (Elkahoui et al., 2015). At present, VOCs have been used in P. destructans hibernaculum sediment, and they have the characteristics of fast action and good killing effect (Micalizzi and Smith, 2020). These VOCs can be used as an effective management strategy to reduce the environmental exposure of bats to P. destructans in hibernacula.

Based on our results, the bacteria isolated from the bat skin can inhibit the growth of P. destructans under laboratory conditions, and secrete a variety of metabolites and volatile compounds. In future work, it is necessary to inoculate bats with either the probiotic P. yamanorum GZD 14026 or those active substances produced by this strain, such as PCA, octanoic acid, isoprenol or BHA, to test the inhibitory effect on P. destructans growth on living hibernating bats in vivo, which will have important significance for protecting bats from white-nose syndrome.

**Experimental procedures**

**Pseudogymnoascus destructans**

Pseudogymnoascus destructans JHCN111a (Hoyt et al., 2016b) was isolated from M. petax in north-eastern China during March 2015, and was cultured on Sabouraud dextrose agar (SDA) at 13 ± 1°C, which was within the optimum temperature range. P. destructans cultures were stored in Sabouraud dextrose broth (SDB) amended with 20% sterile glycerol at −80°C. To obtain conidiospores of P. destructans, cultures were incubated at 13 ± 1°C for 21 days on SDA. Fresh spore suspensions were obtained each time for subsequent experiments.

**Bacterial isolation**

We collected cutaneous bacteria samples in late winter from 15 individuals of R. ferrumequinum and 10 individuals of M. petax in the Jilin, Liaoning, Beijing and Henan provinces of China. A total of 15 bacterial samples from R. ferrumequinum were collected in Jilin (Da-lazi Cave:
A small piece of *P. destructans* mycelium was cut from the inhibition zones on agar plates under the action of *P. yamanorum* GZD14026 at 14 days to make electron microscope samples. Non-treated mycelium was used as control. The samples were fixed in 3% glutaraldehyde for 24 h at room temperature and were then washed with deionized water for 30 min. Next, the samples were dehydrated for 30 min with 30%, 50%, 70%, 90% and 100% ethyl alcohol. Subsequently, samples were dried for 24 h at room temperature. The prepared samples were coated with gold particles and observed with a scanning electron microscope (SEM, FEI XL-30 ESEM-FEG, United States).

**Identification of anti-*P. destructans* active compounds**

*Pseudomonas yamanorum* GZD14026 was grown in LB liquid medium for 3 days at 13°C on a rotary shaker.
at 200 rpm. The liquid culture was centrifuged at 10,000 rpm for 10 min, and the cell-free culture supernatant was collected, followed by extraction with an equal volume of dichloromethane. The dichloromethane extracts were condensed in vacuo at 45°C to obtain crude extract. The crude extract was mixed with silica gel, and mixtures were eluted with a gradient from 100% dichloromethane to 100% methanol as a solvent system. The eluate was collected in bottles and detected by thin-layer chromatography (TLC). The fractions with similar results on TLC plates were then combined. These combined fractions were tested for anti-
P. destructans activity with cell-free supernatant challenge assays. The active fraction then was crystallized under reduced pressure.

The active fraction was analysed by semi-preparative high-performance liquid chromatography (HPLC, Shimadzu LC8A, Japan). The mobile phase consisted of acetonitrile and 0.2% ice acetic acid, with a linear gradient from 10% to 100%, on a C18 reverse-phase column (Agilent ZORBAX SB-C18, 5 µm, 4.6 mm × 250 mm, United States) at a flow rate of 1.0 ml min⁻¹ and detected at a wavelength of 250 nm. Meanwhile, the active fraction was measured by ultraviolet-visible spectroscopy (UV/Vis, Perkin Elmer Lambda 900, United States), mass spectrometry (MS, Waters ZQ2000, United States), and nuclear magnetic resonance (¹H NMR and ¹³C NMR, BRUKER AVANCE III HD 500, Germany) to detect the pure active compound that was dissolved in deuteriated chloroform (CDCl₃) at room temperature.

Evaluation of purified phenazine-1-carboxylic acid (PCA) for anti-P. destructans activity in vitro

Based on our cell-free supernatant challenge assays, the minimal inhibitory concentration (MIC) and the half maximal inhibitory concentration (IC₅₀) of PCA to P. destructans were determined. Briefly, different concentrations of PCA and 2 × 10⁵ P. destructans conidia suspension were transferred to a 96-well microplate for each reaction. DMSO (1%, v v⁻¹) was used as a solvent to dissolve PCA and as a negative control. Each microplate was incubated at 13°C for 7 days, and the absorbance at 492 nm was then recorded. All assays were conducted in triplicate.

DNA extraction and whole genome sequencing

Pseudomonas yamanorum GZD 14026 was grown in LB liquid medium for 3 days at 13°C on a rotary shaker at 200 rpm. The genomic DNA of strain GZD 14026 was extracted using the sodium dodecyl sulphate (SDS) method (Lim et al., 2016). The whole genome of P. yamanorum GZD 14026 was sequenced using the Pacific Biosciences (PacBio) Sequel platform.

Gene assembly and functional annotation

Filtered genome data were de novo assembled using the Single Molecule Real Time (SMRT) Link v. 5.0.1 program (Ardui et al., 2018). Six databases were used for gene functional annotation, including the Non-Redundant Protein (Li et al., 2002), Pfam, SwissProt (Bairoch and Apweiler, 2000; Finn et al., 2014), Gene Ontology and the Cluster of Orthologous Groups of proteins (Ashburner et al., 2000; Tatusov et al., 2003), the Kyoto Encyclopedia of Genes and Genomes databases (Kanehisa et al., 2004). Simultaneously, we utilized anti-SMASH 4.0 (Blin et al., 2017) to analyse secondary metabolic gene clusters to verify the presence of active substance. The whole genome sequence of P. yamanorum GZD 14026 was submitted to the GenBank database with the accession number CP058644.

Co-culture assays P. yamanorum GZD14026 with P. destructans

A single-compartment Petri plate (200 mm × 30 mm) was used as a shared-airspace to assess P. yamanorum GZD14026 inhibition of the growth of P. destructans via volatile organic compounds. A total of 100 µl 2 × 10⁵ P. destructans conidia ml⁻¹ was spread on an SDA agar plate (90 mm × 18 mm). A 20 µl P. yamanorum GZD14026 suspension (suspended in LB liquid medium) was inoculated on an LB agar plate (90 mm × 18 mm) and cultured in this shared-airspace for up to 14 days at 13°C. All assays were conducted in triplicate.

Evaluation the ability of volatile organic compounds anti-P. destructans from P. yamanorum GZD14026

Volatile organic compounds from P. yamanorum GZD 14026 were collected using the headspace solid-phase micro-extraction (HS-SPME) technique that resulted in a shared-airspace inhibition of the growth of P. destructans after 14 days. P. yamanorum GZD 14026 was inoculated on LB agar (90 mm × 18 mm) for 14 days at 13°C. The blank control was an LB agar plate. All assays were conducted in triplicate. The headspace jars were added to cultured P. yamanorum GZD 14026 medium and heated at 40°C for 30 min. A SPME syringe containing 50/30 divinylbenzene/carburene in polydimethylene/oxane on a stable fibre (65 µm) was inserted into these headspace jars and exposed for 20 min. Then, the syringe was pulled out from the headspace jar and inserted into a gas chromatography-mass spectrometry (GC-MS, Agilent 5975, United States). A capillary
Column (30 m × 0.25 mm × 0.25 μm, Agilent DB-5MS, United States) was used to spread these volatile organic compounds. The oven temperature was 40°C and was held for 3 min, ramped at 5°C min⁻¹ to 180°C and then ramped at 20°C min⁻¹ to 230°C, where the temperature was then held for 5 min. The temperatures of the transfer line and ion trap were 250°C and 200°C, respectively. Identification of volatile organic compounds was based on the National Institute of Standards and Technology (NIST) spectra database.

To test the inhibition of *P. destructans* effect of volatile organic compounds, sterile filter paper containing 10 or 100 ppm (μg mL⁻¹) octanoic acid, 3-methyl-3-buten-1-ol or 3-tert-butyl-4-hydroxyanisole was placed on one side of a Petri plate (90 mm × 18 mm), while the other side was inoculated with 100 μL of 2 × 10⁶ *P. destructans* on SDA. The 3-tert-butyl-4-hydroxyanisole was diluted in 95% ethanol. Dilute sterile filter paper with 95% ethanol was used as a control. Plates were incubated at 13°C and monitored every day until 14 days observing the growth of *P. destructans*. The identified volatile organic compounds were purchased from Sigma-Aldrich and Aladdin.

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Conflict of interest

The authors declare no conflict of interests.

Author contributions

ZL performed laboratory work and data analysis, and drafted the manuscript. ZL, AL, WD, JRH, HL, LJ and SL collected the samples. YL and WL guided the laboratory work. KS and JF developed the study concept, design, and supervised the project. KS and JRH reviewed and revised the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Identification of the structure of the active compound working against *P. destructans* from *P. yamanorum* GZD 14026. A. UV/Vis detection of the active compound. Absorbance × Wavelength. B. (a) $^1$H spectral and $^{13}$C spectral (b) of the active compound. C. The chemical structure of phenazine-1-carboxylic acid (PCA).

**Table S1.** Volatile organic compounds produced by *P. yamanorum* GZD 14026 after 14 days.