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Gluconic acid: An antifungal agent produced by *Pseudomonas* species in biological control of take-all

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Abstract

Pseudomonas strain AN5 (*Ps*. str. AN5), a non-fluorescent Australian bacterial isolate, is an effective biological control (biocontrol) agent of the take-all disease of wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*). *Ps*. str. AN5 controls *Ggt* by producing an antifungal compound which was purified by thin layer and column chromatography, and identified by NMR and mass spectroscopic analysis to be D-gluconic acid. Commercially bought pure gluconic acid strongly inhibited *Ggt*. Two different transposon mutants of *Ps*. str. AN5 which had lost take-all biocontrol did not produce D-gluconic acid. Gluconic acid production was restored, along with take-all biocontrol, when one of these transposon mutants was complemented with the corresponding open reading frame from wild-type genomic DNA. Gluconic acid was detected in the rhizosphere of wheat roots treated with the wild-type *Ps*. str. AN5, but not in untreated wheat or wheat treated with a transposon mutant strain which had lost biocontrol. The antifungal compounds phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol, produced by other Pseudomonads and previously shown to be effective in suppressing the take-all disease, were not detected in *Ps*. str. AN5 extracts. These results suggest that D-gluconic acid is the most significant antifungal agent produced by *Ps*. str. AN5 in biocontrol of take-all on wheat roots.

Keywords: *Pseudomonas*; *Gaeumannomyces graminis*; *Triticum*; Wheat; Take-all; Antifungal; Biological control; Gluconic acid; Root disease; Australia

1. Introduction

Pseudomonas bacteria produce different metabolites that can suppress fungal plant pathogens. The production of antibiotics (compounds which at very low concentrations, μg levels, inhibit microorganisms) such as phenazine and phloroglucinol by symbiotic fluorescent *Pseudomonas* bacteria has been shown to provide a natural defense to the plant against fungal diseases such as take-all (Weller, 1988; Keel et al., 1992; Cook et al., 1995). *Pseudomonas fluorescens* 2–79 and *Pseudomonas aureofaciens* 30–84 produce the novel antifungal secondary metabolite phenazine-1-carboxylic acid (PCA). PCA produced by *Pseudomonas* on wheat roots has been shown to be a crucial factor in take-all disease suppression (Turner and Messenger, 1986; Thomashow and Weller, 1990; Thomashow et al., 1990; Pierson and Thomashow, 1992). The antibiotic 2,4-diacetylphloroglucinol (DPG), which has also been shown to suppress the take-all pathogen on plant roots, is produced by several Pseudomonads including *Pseudomonas fluorescens* strain CHA0 (Keel et al., 1992, 1996). Other antibiotics produced by Pseudomonads include pyoluteorin (Maurhofer et al., 1994), Pyrrolnitrin (Ligon et al., 2000) and oomycin A (James and Gutterson, 1986) which can suppress a range of different plant pathogenic fungi. Novel bacterial antifungal metabolites produced by *Pseudomonas* species have also been identified: 3-(1-hexenyl)-5-methyl-2-(5H)-furanone (Paulitz et al., 2000); *N*-mercapto-4-formylcarbostyril (Fakhouri et al., 2001); aerugine

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(Lee et al., 2003); cyclic lipodepsipeptides (Pedras et al., 2003); phenazine-1-carboxamide (Kumar et al., 2005). It is noteworthy that almost all of the antifungal agents identified to date have complicated structures and are hydrophobic in nature. For example, PCA and DPG show medium polarity in nature.

The Australian continent contains unique soil microbes as it has been geographically isolated. Farming commenced in Australia approximately 200 years ago. The take-all disease of wheat is caused by the fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), which was first identified in South Australia in 1870 (Rovira et al., 1991). Large wheat yield losses over a long period of time due to the take-all disease have instigated significant Australian research in this area. There has been extensive characterization of various bacterial and fungal isolates that show biocontrol against take-all (Rovira et al., 1991). However, little is known about either the precise mechanisms involved in biocontrol, or the nature of the antifungal metabolites that Australian bacteria produce. *Pseudomonas* strain AN5 (*Ps*. str. AN5) is a non-fluorescent bacterial soil isolate from the Cowra region of New South Wales, Australia. *Ps*. str. AN5 is able to effectively protect against *Ggt* in agar plate bioassays and pot experiments (Nayudu et al., 1994). Furthermore, it has been demonstrated that *Ps*. str. AN5 biocontrol of take-all in field trials at dryland sites induces significant increases in wheat yield (Nayudu et al., 1994). Transposon mutants of *Ps*. str. AN5 which exhibited either decreased or abolished biocontrol against *Ggt* were isolated previously (Nayudu et al., 1994). We have since endeavoured to determine the antifungal compound(s) that *Ps*. str. AN5 produces and the nature of take-all biocontrol protection conferred by this unique Australian isolate. In this paper, we report the discovery that D-gluconic acid, a simple sugar acid, is the most significant antifungal metabolite produced by *Ps*. str. AN5 against the take-all fungal pathogen in biocontrol protection.

2. Results and discussion

Ps. str. AN5 extracts were made using methods developed for DPG (Keel et al., 1992) or PCA (Rosales et al., 1995) isolation for other Pseudomonads. These extracts were tested for their ability to suppress *Ggt*. DPG and PCA compounds are insoluble in water but soluble in organic solvents such as chloroform, methylene chloride, methanol and ethyl acetate (Gurusiddaiah et al., 1986). In the case of *Ps*. str. AN5, the biologically active compound(s) was soluble in water but insoluble in the organic solvents (Fig. 1a). *Ps*. str. AN5 extracts showed different *Ggt* inhibition patterns to those of PCA, DPG and pyoluteorin (Gurusiddaiah et al., 1986; Keel et al., 1992; Maurhofer et al., 1994) in agar overlay assays on thin layer chromatography (TLC) plates (data not shown). These assays were done using organic solvent systems that have successfully separated DPG, PCA or pyoluteorin previously. No organic solvent systems were able to move the biologically active compound(s) from the origin in *Ps*. str. AN5 extracts, as observed by *Ggt* inhibition in TLC agar overlay assays (Fig. 2a). The solvent systems previously used to identify hydrophobic antifungal agents which suppress plant pathogens (Gurusiddaiah et al., 1986; Keel et al., 1992; Maurhofer et al., 1994) could not resolve the biologically active compound(s) isolated from *Ps*. str. AN5. Solvent systems that have been previously used for isolation of hydrophilic compounds (Aszalos et al., 1968; Hellmut et al., 1990) were subsequently tested.

The solvent system comprising *n*-propanol:ethyl acetate:water (5:2:3), previously used for separating carbohydrates (Hellmut et al., 1990), separated the biologically active compound(s) in *Ps*. str. AN5 extracts with *Ggt* inhibition being observed at approximately 0.7 R_f value in TLC agar overlay assays (Fig. 2b). Crude extracts of *Ps*. str. AN5 were differentiated on a silica column using *n*-propanol:ethyl acetate:water (5:2:3). The column fractions were collected and separated on TLC plates and also tested for biological activity against *Ggt* on potato dextrose agar (PDA) overlay bioassays. Fractions from 13 to 26 with R_f about 0.7 on TLC were found to be active in the case of *Ps*. str. AN5. These biologically active fractions were pooled for further analysis. The pooled fractions from *Ps*. str. AN5 were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and mass spectroscopy. NMR spectra of pooled fractions were dominated by the presence of signals which could be assigned to α- and β-glucopyranose (Horton et al., 1983). None of the signals present in the NMR spectra of *Ps*. str. AN5 (Fig. 3a) corresponded to resonances characteristic of either PCA or DPG. Due to the presence of a large quantity of glucose in biologically active *Ps*. str. AN5 fractions (as seen in NMR spectra) GC/MS of the silylated extract was carried out to separate glucose and other impurities from the biologically active component(s). The total ion current (TIC) trace showed the presence of a significant chromatographic peak in *Ps*. str. AN5 silylated pooled fractions. The spectra observed in *Ps*. str. AN5 was consistent with the spectra of a glucopyranose, and in particular, gluconic acid (Horton et al., 1983; Milson and Meers, 1985; Tsai et al., 1995). This was confirmed by comparing with a library of mass spectra (Merkey et al., 1974) which identified this peak as the trimethylsilyl (TMS) derivative of gluconic acid (data not shown). A retention time of 13.47 min in GC/MS is the same as for pure D-gluconic acid. It also had an identical mass spectrum to that of the hexa-TMS derivative of a pure sample of D-gluconic acid which was obtained commercially (Fig. 4). After comparison of ^{1}H and ^{13}C NMR spectra of pure D-glucose and D-gluconic acid with the partially purified active fraction from *Ps*. str. AN5, it was possible to reject the signals assigned to glucose. The remaining resonances matched those of D-gluconic acid exactly (data not shown).

In agar plate bioassays, commercially bought pure D-gluconic acid strongly inhibited *Ggt* (Fig. 1b) but, as

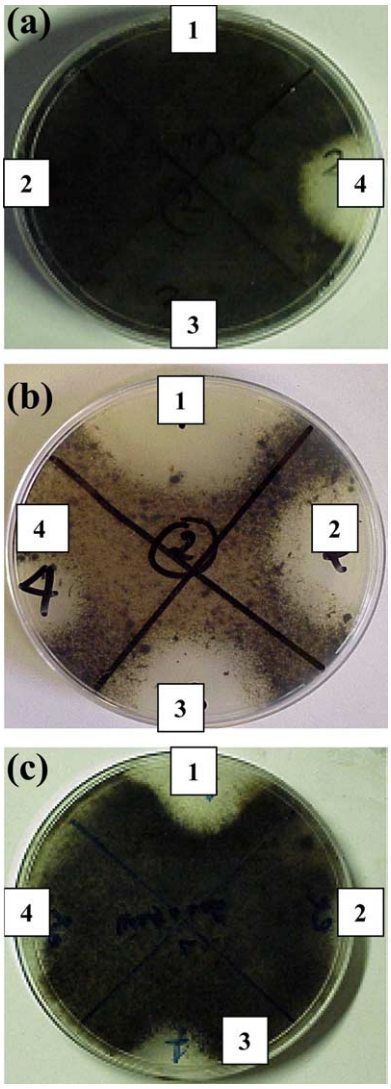

Fig. 1. Agar overlay plate bioassays showing antifungal activity of crude bacterial extracts and pure gluconic acid against the take-all fungus *Gaeumannomyces graminis* var. *tritici*. (a) Activity of crude extracts of *Pseudomonas* strain AN5 grown on PDA using for extraction: (1) methanol; (2) chloroform; (3) ethyl acetate; (4) water. (b) Activity of commercially obtained pure gluconic acid at concentrations of mg/ml: (1) 50; (2) 25; (3) 12.5; (4) 6.25. (c) Activity of crude ethyl acetate extracts derived from the following bacterial strains on MA (DPG extraction): *Pseudomonas fluorescens* strain Pf-5 (1,3); *Pseudomonas.* strain AN5 (2,4).

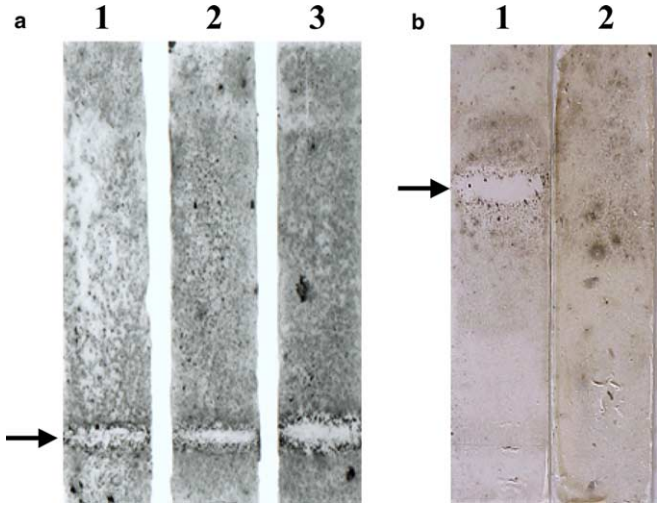

Fig. 2. Thin layer chromatography (TLC) agar overlay bioassays showing antifungal activity of separated crude fractions of *Pseudomonas* strain AN5 (*Ps*. str. AN5) and *Ps*. str. AN5MN1 against the take-all fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*). (a) Activity of crude extracts of *Ps*. str. AN5 separated on TLC plates using the following solvent systems: (1) methanol:chloroform, 1:9; (2) methanol:chloroform, 3:7; (3) methanol:chloroform, 5:5. (b) Activity of crude extracts separated on TLC plates of *Ps*. str. AN5 (1) and mutant *Ps*. str. AN5MN1 (2) with a *n*-propanol:ethyl acetate:water (5:2:3) solvent system. In all cases, a 100 μl suspension of crude extract was applied as a band at the origin of the TLC plate for development. The arrow indicates clear zones where *Ggt* growth has been inhibited.

expected, no inhibition was observed with D-glucose (data not shown). Mass spectroscopic analysis of pure D-gluconic acid showed the presence of D-gluconic acid and a small amount of D-gluconolactone. Gluconolactone is an intermediary in the conversion of glucose to gluconic acid (Goodwin and Anthony, 1998). In the isolation procedures used for *Ps*. str. AN5, the neutral extraction conditions would shift the D-gluconolactone/D-gluconic acid equilibrium towards the acid species (Milson and Meers, 1985). However, one would reasonably expect to detect D-gluconolactone, if present, as we observed it in the pure D-gluconic acid. D-Gluconolactone was not detected in any of the biologically active extracts from *Ps*. str. AN5, which suggested that it is not an important compound in biocontrol of take-all by *Ps*. str. AN5.

DPG or PCA producing *Pseudomonas* strains are known to produce coloured pigments in medium. *Pseudomonas* DPG producing strains are characterized by a red pigment in King's B medium (Keel et al., 1996). Turner and Messenger (1986) reported that PCA production leads to golden yellow crystals in pigment production medium (PPM) and PDA. *Ps*. str. AN5 did not produce red pigments on King's B agar or yellow crystals on PPM agar or PDA (data not shown). There is a good correlation between DPG and red pigment production in a large number of *Pseudomonas* species tested (Keel et al., 1996). PCA production by *Pseudomonas* is also consistent with golden yellow crystal formation (Gurusiddaiah et al., 1986; Turner and Messenger, 1986). *Ps*. str. AN5 extracts made from malt agar (MA), according to the methods of Keel et al. (1996) for isolation of DPG, did not suppress the take-all pathogen in agar overlay bioassays (Fig. 1c). As a control, DPG was also isolated from *P. fluorescens* strain Pf-5 (Nowakthompson et al., 1994) which did suppress take-all (Fig. 1c). There was no DPG detected in *Ps*. str. AN5 extracts but DPG was detected in *P. fluorescens* strain Pf-5 extracts, by ^{1}H NMR and mass spectroscopy (data not shown). *Ps*. str. AN5 extracts were made from PPM using established methods for PCA isolation (Rosales et al., 1995) but showed no take-all antifungal activity in bioassays. There was no PCA detected in *Ps*. str. AN5 extracts

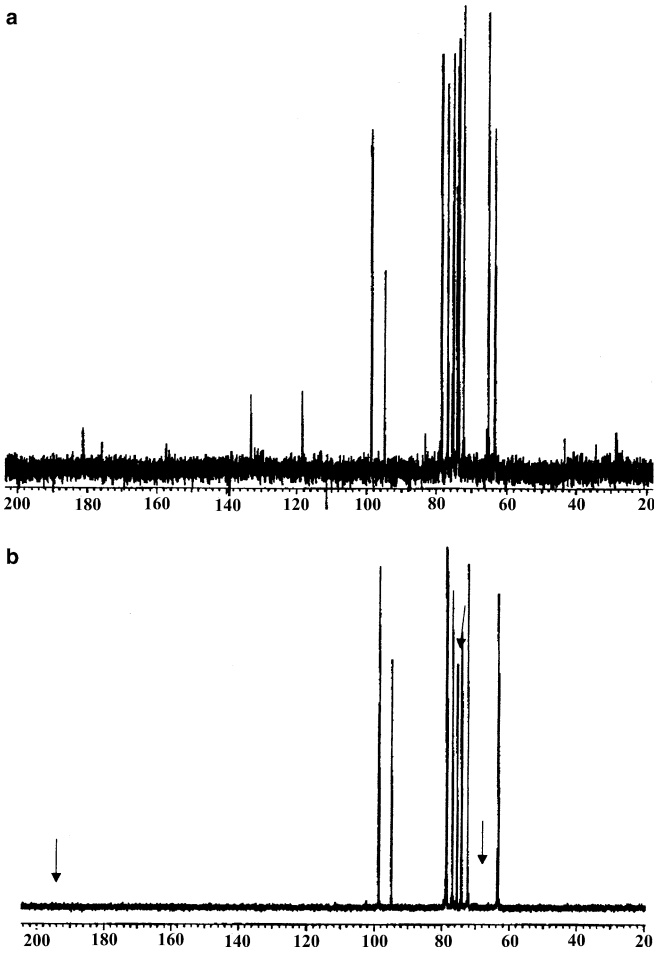

Fig. 3. Nuclear magnetic resonance (NMR) of *Pseudomonas.* strain AN5 (*Ps*. str. AN5) and *Ps*. str. AN5MN1. ^{13}C NMR spectra of semi-purified fractions from *Ps*. str. AN5 (a) and the mutant *Ps*. str. AN5MN1 (b). Arrows indicate gluconic acid peaks which are absent in the *Ps*. str. AN5MN1 but present at the same position in *Ps*. str. AN5. In the ^{13}C NMR of *Ps*. str. AN5, additional peaks at 181.2, 76.67, 73.54, 75.16, 73.76 and 65.2 were observed compared to the mutant *Ps*. str. AN5MN1. The peak at 181.2 is for C1 and the remaining peaks are for C2–C6 of D-gluconic acid.

by mass spectroscopy (data not shown). These results suggest that *Ps*. str. AN5 does not produce the antifungal metabolites DPG or PCA.

Using the same methods described above for isolating gluconic acid from *Ps*. str. AN5, we were unable to detect any biologically active fraction in *P. fluorescens* strain Pf5 extracts from TLC agar overlay assays using *n*-propanol:ethyl acetate:water (5:2:3) solvent system at 0.7 R_f value. Furthermore, mass spectroscopy analysis of the same fractions from *P. fluorescens* strain Pf5 extracts (where gluconic acid was detected in *Ps*. str. AN5 extracts) did not detect the presence of gluconic acid.

Two transposon mutants of *Ps*. str. AN5 (*Ps*. str. AN5MN1 and *Ps*. str. AN5MN2) which had lost biocontrol against the take-all disease were assayed for gluconic acid production. These two biocontrol deficient mutant strains each had a single Tn*5gus* insertion in a different region of the *Ps*. str. AN5 genome (Nayudu et al., 1994). There was no biocontrol activity detected against *Ggt* in any of the extracts from these strains. The crude extracts of these strains were separated on silica columns using an *n*-propanol:ethyl acetate:water (5:2:3) solvent system. Individual column fractions 13–26, with R_f about 0.7 on TLC (where the active compound was found for the parent strain) were found to be biologically inactive against *Ggt* in these mutants (Fig. 2b). Pooled fractions obtained for the two mutant strains were subject to ^{1}H and ^{13}C NMR, and mass spectroscopy. Glucose was present in these fractions but gluconic acid was not detected in ^{13}C NMR (Fig. 3b).

A cosmid containing the complementary wild-type region (pLAFR1-Mur 1) from the parent genome (Nayudu et al., 1994) was transferred into the mutant *Ps*. str. AN5MN1. This construct, *Ps*. str. AN5MN1(pLAFR1-Mur 1), inhibits the take-all fungus in agar plate bioassays (data not shown). Using the isolation method described for gluconic acid (with agar strips extracted from next to where the strain was growing on PDA plates) it was shown that *Ps*. str. AN5MN1(pLAFR1-Mur 1) produced a biologically active fraction. The active fraction had a R_f value (identical to that of gluconic acid) of about 0.7 using a *n*-propanol:ethyl acetate:water (5:2:3) solvent system in a TLC agar overlay bioassay. Analysis of a freeze dried biologically active fraction from this strain by mass spectroscopy confirmed the presence of gluconic acid. From the

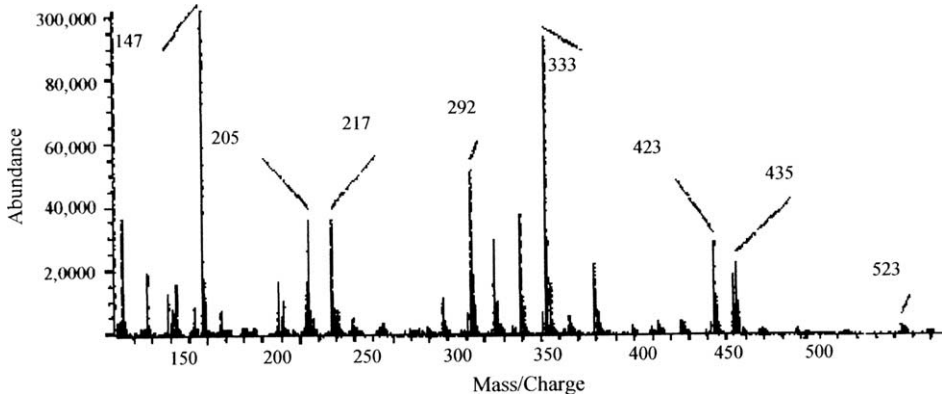

Fig. 4. Mass spectra of TMS derivative of commercially obtained pure D-gluconic acid. Characteristic peaks for gluconic acid are observed at 205, 217 and 292.

location of the Tn*5gus* transposon insertion site in *Ps*. str. AN5MN1 we were able to find a complementary open reading frame (ORF) region on pLAFR1-Mur 1. A polymerase chain reaction (PCR) product of approximately 3.0 kb containing the putative wild-type gene (ORFMN1) was obtained using pLAFR1-Mur 1 DNA as the template. ORFMN1 was cloned into the *Eco*RI site located in the chloramphenicol resistance gene of the broad host range vector pSUP106 (Priefer et al., 1985). This plasmid (pSUP 106::ORFMN1) was then transferred into *Ps*. str. AN5MN1. This *Ps*. str. AN5MN1 (pSUP 106::ORFMN1) construct produced gluconic acid and inhibited the take-all fungus in agar plate bioassays (data not shown). Although a recombinant deficient strain of *Ps*. str. AN5 was not used for these experiments, it was found that all of the plasmids used for complementation were relatively stable in the *Ps*. str. AN5MN1 background as they exhibited the same restriction enzyme pattern when transferred back to *Escherichia coli* K-12 and digested with a number of six base pair recognizing restriction enzymes (*Pst*I, *Sma*I, *Hin*dIII, *Sal*I). These experiments show that a *Ps*. str. AN5 mutant which is unable to produce gluconic acid concomitantly loses take-all biocontrol, but biocontrol and gluconic acid production is restored when this mutant is complemented by the corresponding wild-type gene. This strongly suggests that the ability to produce gluconic acid is directly correlated with the ability of the bacterial strain to suppress the take-all pathogen. In a similar manner, complementation experiments with other antifungal agents, such as DPG (Keel et al., 1992) and PCA (Thomashow et al., 1990), disclosed their essential role in take-all biocontrol.

Gluconic acid is produced by specialized bacteria such as *Gluconobacter* (Velizarov and Beschkov, 1994), and some filamentous fungi (Magnuson and Lasure, 2004). Production of D-gluconic acid has been generally reported to be transient in most cases in *Pseudomonas* bacteria (Haltrich et al., 1996). Schleissner et al. (1997) reported accumulation of low levels of D-gluconic acid in the extracellular medium in a *Pseudomonas putida* soil isolate. However, this *P. putida* isolate is not a biocontrol strain. This *P. putida* isolate requires D-glucose to be oxidized to D-gluconic acid for utilization in the glycolytic pathway, as it cannot use D-glucose. The reported gluconic acid production by this *P. putida* strain is a novel method used to metabolize glucose (which it cannot normally use as a carbon source). *Pseudomonas cepacia* isolates have been shown to produce gluconic acid. Gluconic acid production by these isolates has been linked to their ability to solubilize mineral phosphate (Babukhan et al., 1995). Gluconic acid producing bacteria or fungi have not been reported to be biocontrol strains.

Using the titration method of Schleissner et al. (1997) we estimated that *Ps*. str. AN5 produced approximately 5.0 mg/ml of acid when glucose was provided as the sole carbon source in water. We were only able to detect very low levels of acid production (approximately 0.12 mg/ml of acid) with the mutants *Ps*. str. AN5MN1 and *Ps*. str. AN5MN2. This suggests that most of the acid produced by *Ps*. str. AN5 is gluconic acid. There is significant production of D-gluconic acid by *Ps*. str. AN5 in medium where glucose is present, such as PDA. *Ggt* is inhibited by between 0.1 and 0.5 mg/ml concentration of pure D-gluconic acid when supplemented in PDA (data not shown). This indicates a good correlation between the concentration of D-gluconic acid that can be produced by *Ps*. str. AN5 and the concentration of gluconic acid required to inhibit *Ggt*.

Strikingly, there are no reports of D-gluconic acid production at such a high level in any other biocontrol bacteria that demonstrate antifungal activity. James and Gutterson (1986) reported that antifungal metabolite synthesis can be regulated by glucose in *P. fluorescens* strain HV37A which shows biological activity against *Pythium ultimum*. *P. fluorescens* strain HV37A produces oomycin A, a complex protein antibiotic of molecular weight 500–600 (James and Gutterson, 1986). The reported complexity of glucose-dependent regulation of antibiotic production in *P. fluorescens* strain HV37A (Gutterson et al., 1986; Gutterson et al., 1988) seems different to the simple production of gluconic acid by *Ps*. str. AN5.

It is important to know if *Ps*. str. AN5 in the rhizosphere of the wheat root is capable of producing gluconic acid. Wheat grown on Herridge's (H) agar plates were subject to one of the following three treatments: no treatment of wheat seed (control); treatment with *Ps*. str. AN5; treatment with *Ps*. str. AN5MN1. *Ps*. str. AN5 and *Ps*. str. AN5MN1 bacterial strains streaked alone on H agar plates supplemented with bromocresol purple pH indicator did not grow even after 10 days of incubation. As well, all plates were purple in colour (pH > 7) indicating that no acid was produced. This showed that H plates did not contain any carbon sources that *Ps*. str. AN5 could utilize for growth or gluconic acid production. For each of the three treatments, aqueous extracts were prepared using individual agar strips removed from plates with wheat roots growing on the surface of the agar. There was strong inhibition of *Ggt* observed in aqueous extracts obtained with *Ps*. str. AN5 treated wheat plants. However, there was no inhibition in aqueous extracts obtained with *Ps*. str. AN5MN1 treated wheat or untreated wheat. All of these extracts were freeze-dried and analyzed by mass spectroscopy. The mass spectra of aqueous extracts obtained using agar strips of *Ps*. str. AN5 treated plants disclosed peaks that are characteristic of gluconic acid (between 200 and 300 mass charge^{-1}). These were absent in aqueous freeze-dried extracts of *Ps*. str. AN5MN1 treated wheat or untreated wheat. This suggests that gluconic acid is being produced in the rhizosphere of wheat plants treated with *Ps*. str. AN5 but not untreated wheat or wheat treated with the mutant *Ps*. str. AN5MN1. Evidence that gluconic acid is produced in the rhizosphere of wheat roots colonized by the parent *Ps*. str. AN5 but not the mutant *Ps*. str. AN5MN1 (which has lost biocontrol) supports the hypothesis that this antifungal

metabolite is crucial for biocontrol protection of take-all on wheat roots.

In summary, *Ps*. str. AN5 was shown to produce the sugar acid D-gluconic acid. We were unable to detect any other putative antifungal agents (such as PCA or DPG) from *Ps*. str. AN5. Commercially bought pure gluconic acid strongly inhibited *Ggt*. Transposon mutants of *Ps*. str. AN5, which had lost take-all biocontrol, did not produce D-gluconic acid. However, when complemented with wild-type genomic DNA containing the corresponding ORF region, gluconic acid production was restored along with take-all biocontrol. Gluconic acid production was detected in the wheat rhizosphere with the wild-type parent strain but not with a transposon mutant strain which had lost biocontrol. These results suggest that D-gluconic acid produced by *Ps*. str. AN5 is a significant antifungal agent in the biocontrol of take-all on wheat roots. Gluconic acid is a very different antifungal metabolite to the antibiotics identified previously, such as PCA and DPG. Gluconic acid is known to be a strong chelating agent and a strong acid (Milson and Meers, 1985). This suggests that local changes in pH leading to a more acidic environment, caused by *Ps*. str. AN5 producing gluconic acid, is responsible for the ability of the bacteria to suppress the take-all pathogen in biocontrol. This is consistent with the results observed with PCA and DPG in inhibiting *Ggt*. The acidic form of PCA is active against *Ggt*. In contrast, the non-acidic form, phenazine-1-carboxylate, is inactive against *Ggt* (Brisbane et al., 1987; Chin-A-Woeng et al., 1998). DPG is more acidic in nature than monoacetylphloroglucinol, and also correspondingly shows significantly more antifungal activity against *Ggt* (Shanahan et al., 1993). The above proposition is also supported by the observation that the take-all disease is inhibited by low pH soils, and that incidence of take-all is high in alkaline soils (Murray et al., 1987). However, wheat root exudates do contain some acidic compounds (Wu et al., 2001), so further work is needed to determine the precise contribution that gluconic acid makes to pH of the wheat rhizosphere. Currently, the only known common property of the three take-all antifungal agents (PCA, DPG and D-gluconic acid) is that they are acidic and have the potential to lower pH in the environment they grow. Therefore, we suggest that the ability of these antifungal agents to inhibit the take-all fungus must be, at least in part, due to their ability to lower pH in the wheat rhizosphere.

3. Experimental

3.1. Media and growth conditions

The following media were used for growth of bacterial strains. Luria agar (L agar) – 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar in 1000 ml of distilled water. Pigment production medium (PPM) – 20 g peptone, 20 g glycerol, 5 g NaCl, 1 g KNO_3, 15 g agar in 1000 ml of distilled water at pH 7.0. King's B medium (KBM) – 20 g proteose peptone, 15 ml glycerol, 1.9 g $K_2HPO_4 \cdot 3H_2O$, 1.5 g $MgSO_4 \cdot 7H_2O$ in 1000 ml of distilled water. Potato dextrose agar (PDA) – 32 g of potato dextrose agar, 5 g agar in 1000 ml distilled water. Malt agar (MA) – 15 g of malt extract, 17 g of agar in 1000 ml distilled water. Nutrient agar (NA) – nutrient agar 20 g, yeast extract 5 g, agar 5 g in 1000 ml distilled water. Potato dextrose broth (PDB) – 24 g of potato dextrose broth in 1000 ml distilled water. Pontiac Broth (PB) – 400 g pontiac potatoes were washed. The potatoes were cut into approximately 1 cm^2 cubes without peeling, and boiled in distilled water till soft. The solution was allowed to cool down slightly and then strained through four layers of cheesecloth and the extract was made up to 1 L. The filtered solution was then autoclaved before use. Bromocresol purple was added to media at a concentration of 0.0075% and pH adjusted to pH 7 by addition of 1 M Potassium hydroxide solution before autoclaving. Top agar – 5 g potato dextrose agar, in 300 ml distilled water was solidified with 1.3% agar for the agar overlay bioassay on PDA plates and thin layer chromatographic (TLC) plates.

E. coli bacteria used in this study (refer to Section 3.2) were grown overnight in solid medium, or liquid medium with vigorous shaking, at 37 °C. *Pseudomonas* bacteria used in this study (refer to Section 3.2) were grown for 2 d in solid medium, or liquid medium with vigorous shaking, at 25 °C. The take-all fungi used for this study were Australian isolates C3 and QW1 (P.T.W. Wong collection). For identification of the antifungal compound, take-all strain C3 was used in agar overlay bioassays on plates and TLCs. For plate bioassays both C3 and QW1 strains were used independently and the results observed were identical so they are not differentiated in the results section. Take-all fungus was grown on PDA or PDB at 18 °C for 8–10 d and stored at 4 °C. Media components were purchased from DifcoBacto Laboratories and Sigma–Aldrich.

3.2. Bacteria, fungi and plasmids

Nayudu et al. (1994) previously generated a spontaneous rifampicin-resistant derivative of *Ps*. str. AN5 (AN5rif) and a spectinomycin-resistant derivative of *Ps*. str. AN5 (AN5sp) which were used for isolation of the antifungal agent in this study. *Ps*. str. AN5rif was grown on NA supplemented with 100 μg/ml rifampicin. *Ps*. str. AN5sp was grown on NA supplemented with 250 μg/ml spectinomycin. The Tn*5gus* mutant strains used in this study, *Ps*. str. AN5MN1 and *Ps*. str. AN5MN2, were derived from *Ps*. str. AN5 by suicide mutagenesis in tri-parental mating with *E. coli* K-12 using the suicide vector pRK600 Tn*5gusA*1 (Sharma and Signer, 1990). These strains have completely lost biocontrol against the take-all disease in plate assays and pot trials (Nayudu et al., 1994). Transposon Tn*5gus* mutants were grown on NA supplemented with kanamycin hydrochloride 250 μg/ml. The plasmid pLAFR1 (Friedman et al., 1982) encodes tetracycline resistance. Therefore,

selection for the cosmid was made by growing these strains on medium supplemented with tetracycline (*E. coli* K-12–20 μg/ml oxytetracycline hydrochloride in L agar). The cosmid (present in *E. coli* K-12 DH5α) was transferred into *Ps*. str. AN5MN1 using triparental mating with *E. coli* K-12 SM10 (helper strain) with selection being imposed for tetracycline resistance (*Ps*. str. AN5 – 60 μg/ml oxytetracycline hydrochloride in NA). Microbial genetic methods have been described previously (Nayudu and Holloway, 1981; Moore et al., 1983; Nayudu and Rolfe, 1987). Antibiotics were purchased from Sigma–Aldrich.

3.3. PCR and plasmid construction

Nayudu et al. (1994) showed that there are two regions involved in antifungal agent production in *Ps*. str. AN5. They constructed a cosmid bank of *Ps*. str. AN5 and were able to complement transposon mutants in each of these regions with *Ps*. str. AN5 cosmids carrying wild-type regions leading to restoration of biocontrol. Cosmid pLAFR1-Mur1 has an approximate 23 kb insertion of the *Ps*. str. AN5 genome wild-type region corresponding to the site of insertion of the transposon in *Ps*. str. AN5MN1 (Nayudu et al., 1994). A 3.0 kb fragment encompassing an ORF region (corresponding to the site of insertion of *Ps*. str. AN5MN1) was generated by PCR using cosmid pLAFR3-Mur1 DNA with the primers 8 forward 1 (5′-AGCGGGTCAGCTTTTTACTG-3′) and 8 reverse 1 (5′-GGAACGATCAACAAGCTC-3′). Amplification was carried out using a Qiagen Mutiplex PCR kit (Cat. No. 206143) from Qiagen. There was an initial denaturation step at 95 °C for 15 min. Amplification occurred at 95 °C (30 s) for denaturation, 57 °C (45 s) for annealing and 72 °C (240 s) for extension. This was repeated for 30 cycles. A 3.0 kb PCR product was separated using 1.0% agarose gel electrophoresis, then the product was isolated from the gel and purified using a QIAquick gel purification kit (Cat. No. 28704) from Qiagen. The isolated 3.0 kb fragment was then cloned into a pGEM T-easy vector (Promega Corporation). This cloned insert did not have any *Eco*RI sites, so the *Eco*RI sites on either side of the 3.0 kb PCR cloned fragment (located on the pGEM T-easy vector) were used to liberate the fragment. *Eco*RI digestion products were separated by agarose gel electrophoresis. The 3.0 kb *Eco*RI–*Eco*RI digest product was isolated from the gel, purified using a QIAquick gel purification kit, and cloned into the *Eco*RI site, (located in the middle of the chloramphenicol gene) of plasmid pSUP106 (Priefer et al., 1985). Ligation was carried with T4 DNA ligase (Promega Corp.) at 16 °C for 12 h and transformed into DH5α-E competent cells (Invitrogen). This plasmid, pSUP106::ORF MN1, was transferred into *Ps*. str. AN5MN1 using the same methods described above (i.e., triparental mating) using tetracycline resistance for selection. Molecular biology methods have been described previously (Nayudu and Holloway, 1981; Nayudu and Rolfe, 1987; Nayudu et al., 1994).

3.4. Plate bioassays to determine fungal inhibition

Inhibition of *Ggt* by different *Pseudomonas* strains was determined in agar plate bioassays using PDA (Poplawsky et al., 1988). Antifungal activity of pure compounds was tested by supplementing PDA with each compound and then carrying out an agar plate bioassay. Growth was observed after incubation at 18 °C for approximately 8–10 d.

3.5. Agar overlay bioassays to test fungal inhibition

This bioassay was specifically developed in this study to determine *Ggt* inhibition by bacterial extracts and pure compounds. *Ggt* was grown in 100 ml PDB for 10 d at 18 °C without shaking. The resultant fungal growth was macerated in a Waring blender and then diluted 1 in 4 using potato dextrose top agar cooled to 42 °C. For plate agar overlay bioassays, this top agar was poured on top of a normal PDA plate. A standard 10 μl aliquot of bacterial extract or pure compound was spotted onto the agar overlay bioassay plates and dried. Plates were incubated for 3–4 d at 18 °C. Incubation of uninoculated plates for 3–4 d at 18 °C led to confluent growth of the fungus in the top agar. Clearing zones where the extract or pure compound was spotted on the agar overlay plate indicted inhibition of the fungus. For TLC agar overlay bioassays, this top agar was poured on top of a TLC plate which had tape around it to hold the top agar. This was done on developed TLC plates which had been air dried to evaporate the solvent. TLC plates were incubated at 18 °C for 3–4 d. Inhibition of *Ggt* led to a clearing zone on the TLC plate, compared to confluent growth of *Ggt* over the rest of the plate.

All assay treatments were replicated. Each assay has been repeated independently on at least two separate occasions. The results reported were consistently observed on all occasions.

3.6. Quantification of acid production

The amount of acid produced by *Ps*. str. AN5 and its mutant derivative strains was estimated using titration according to the methods of Schleissner et al. (1997). *Ps*. str. AN5 and the mutants *Ps*. str. AN5MN1 and *Ps*. str. AN5MN2 were grown in 100 ml PB for 2 d with shaking at 25 °C. Then 100 ml of fresh PB broths were inoculated with 100 μl from these stationary phase PB cultures and grown for a further 15–18 h under the same conditions. Bacterial cells were pelleted in a bench centrifuge at 6000 rpm for 10 min. The bacterial pellets were washed twice with sterile water and resuspended in 100 ml glucose medium containing 5.0 g/L glucose at pH 7. This was incubated on a shaker at 25 °C. Aliquots of 5 ml of the culture were collected at different time intervals and cells pelleted. The supernatant was used for titration. After measuring the initial pH of solutions, 5 ml of the supernatant

was titrated with 0.001 N sodium hydroxide (NaOH) using phenolphthalein as an indicator (Whistler and Schweiger, 1959; Vogel, 1978). The titration was repeated three times, and the amount of acid detected in each case is presented as an average of these results.

3.7. Extraction of antifungal compounds

Extractions of antifungal metabolites from bacterial strains were from agar plates. Each extraction was done in duplicate and repeated at least twice. The results reported were consistently observed on all occasions. A modification of the method of Aszalos et al. (1968) was used to determine if *Ps*. str. AN5 produced hydrophilic antifungal compounds. *Ps*. str. AN5rif , *Ps*. str. AN5sp and its transposon mutant derivatives *Ps*. str. AN5MN1 and *Ps*. str. AN5MN2 were grown on PDA for 5 d at 25 °C for extraction of antifungal compounds. Twenty agar plates of each strain (including bacterial growth) were cut into pieces of approximately 1 cm^2 and extracted by shaking on a rotary shaker for 1 h with 1 L 60% aqueous isopropanol (60:40 water:isopropanol). The extracts were filtered through cheesecloth and reduced in vacuo to evaporate isopropanol. The reduced extract was centrifuged at 3832*g* for 20 min. An equal volume of acetone was added to the supernatant in a sealed bottle and left overnight at 4 °C to precipitate proteins. The solution was again centrifuged and the supernatant was then reduced in vacuo to evaporate acetone. The remaining aqueous solution was freeze-dried and used for further analysis.

To determine if *Pseudomonas* strains produced phenazine they were grown on PPM media and then extracted according to the method of Rosales et al. (1995). To detect phloroglucinol based metabolites, the bacterial strains were grown on MA and a similar method of extraction was carried out to that reported by Keel et al. (1992). *P. fluorescens* strain Pf-5 (Nowakthompson et al., 1994) was used to obtain DPG. PCA was purchased from Sigma–Aldrich (Cat. No. S998907). Comparison of the extracts in both cases was done with TLC, NMR and mass spectroscopy analysis.

3.8. Differentiation of antifungal compound(s) from crude extracts using thin layer chromatography

Ten microliter of suspensions of crude extracts were applied to silica gel GF_{254} TLC plates at 2 cm intervals. TLC glass plates with silica gel F_{254} (0.25 mm thickness) used in this study were purchased from Merck (Cat. No. 1.05715). For DPG a chloroform:methanol solvent (9:1) and toluene:acetone (4:1) solvent system was used (Strunz et al., 1978). *Ps*. str. AN5 extracts were trialed with chloroform:methanol (19:1) and acetonitrile:methanol:water (1:1:1) solvent systems. Reverse-phase C_{18} TLC for PCA separation used an acetonitrile:methanol:water (1:1:1) solvent system (Pfender et al., 1993; Rosales et al., 1995). The solvent system *n*-propanol:ethyl acetate:water (5:2:3) was able to separate the biologically active compound(s) of *Ps*. str. AN5. TLC plates were run until the solvent was about 1 cm from the upper rim. The plates were dried at room temperature and visualized under UV light. For isolation of antifungal compounds, the bands were scratched from the TLC plate and the compounds extracted using 50% aqueous methanol. The biological activity of extracted compounds was determined in agar overlay bioassays. Individual TLC runs were always done in duplicate and the experiment repeated at least twice in the initial screening experiments to determine the appropriate solvents. Further experiments were done at least five times on separate occasions. The results reported were consistently observed on all occasions.

3.9. Purification of antifungal compound(s) from crude extracts using silica columns

Silica columns were prepared according to the method described by Still et al. (1978). 100 mg of freeze-dried crude extracts from *Ps*. str. AN5sp, *Ps*. str. AN5rif, *Ps*. str. AN5MN1 and *Ps*. str. AN5MN2 were dissolved in 5 ml *n*-propanol:ethyl acetate:water (5:2:3) and applied to silica columns separately. Fractions of 5 ml at a flow rate 1 ml/min were collected. The column fractions were analyzed by spotting a 10 μl sample from each fraction on 20 cm × 20 cm TLC plates. Biological activity of these fractions was determined using agar overlay bioassays. The fractions containing similar active bands on TLC plates (i.e., *Ps*. str. AN5) were pooled for further analysis. This was repeated independently on two separate occasions. The results reported were consistently observed on all occasions.

3.10. Extraction of antifungal metabolite from wheat rhizosphere

A sterile agar plate assay was used for growth of wheat plants. Herridge's (H) medium was prepared as described by Delves et al. (1986). H medium does not contain any carbon source for normal bacterial growth. Wheat seeds were surface sterilized using 12% sodium hypochlorite solution with gentle shaking for 5 min. Seeds were thoroughly washed with sterile water and then arranged on H agar plates. The plates were incubated vertically in the dark at 25 °C for two days. Germinated wheat seedlings were carefully removed and treated with the bacterial inoculum grown in PB, or left untreated. There is no gluconic acid (or other antifungal agents) produced by *Ps*. str. AN5 in PB (data not shown). Bacterial treatments used were *Ps*. str. AN5rif and the mutant strain *Ps*. str. AN5MN1. Four treated seedlings were fixed with 2% agar on H agar on a large Petri dish (20 cm diameter). These plates were then incubated vertically in a 12 h light/dark cycle and a 18 °C/12 °C regime. The wheat seedlings were grown for two weeks. The agar directly below and adjacent to where the wheat roots were growing was cut into strips and

extracted with 1 L 60% aqueous isopropanol as described above. The extracts from plates inoculated with *Ps*. str. AN5, *Ps*. str. AN5MN1 and uninoculated wheat were tested for take-all fungal inhibition in agar overlay bioassays. The extracts were freeze-dried, concentrated and subjected to further analysis by mass spectrometry. The results presented are from duplicate analyses.

3.11. Structure elucidation using NMR spectroscopy

^{1}H NMR spectra were obtained using a Varian Inova 500 MHz spectrometer in D_2O. 3-Trimethyl silyl propane sulphonic acid (TSP) was used as an internal standard at 0 ppm. ^{13}C NMR spectra were recorded on the same spectrometer at 100 MHz in D_2O.

3.12. Structure elucidation using GC/MS analysis

To a small amount of freeze-dried extract in a Reactivial was added dry pyridine (25 μl) and Regisil (10% chlorotrimethylsilane in *N*,*O*-bis(trimethylsilyl)trifluoroacetamide, Regis Chemical Company, USA; 25 μl). This was done in a dry incubator at 25 °C. The vial was heated to 80 °C for 30 min after sealing, then cooled and centrifuged. The same silylating procedure was used for pure compounds. The solution of the silylated compounds (1 μl) was injected directly onto the GC/MS system. GC/MS analyses were performed on a Hewlett-Packard 5890 gas chromatogram interfaced to a HP 5970 mass selective detector. Gas chromatography was carried out using a HP-1 fused silica capillary column (12.5 m × 0.20 mm i.d., 0.33 μm film thickness bonded methyl silicone stationary phase) in the splitless mode. Conditions used were: injector, 250 °C; transfer line, 250 °C; oven programme, 100–250 °C at 10 degrees/min, hold 10 min. Mass spectra were obtained by electron impact (EI) ionization at 70 eV. Full scan spectra were recorded by scanning from *m*/*z* 100 to *m*/*z* 600.

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