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# No antibiotic and toxic metabolites produced by the biocontrol agent *Pseudomonas putida* strain B2017

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**One sentence summary:** The non-production of antibiotics / toxic metabolites by *Pseudomonas putida* strain B2017 makes it a promising biocontrol microorganism for plant protection without side effects on environment, non-target organisms, and human health.

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## ABSTRACT

*Pseudomonas putida* and closely-related species such as *Pseudomonas fluorescens* and *Pseudomonas brassicacearum* have been reported as potential biocontrol agents and plant growth-promoters. Recently, we have described the biocontrol activity of *P. putida* B2017 against several phytopathogens of agricultural relevance. In this study, its ability to produce potential antibiotic / toxic metabolites was assessed by functional, chromatography-mass spectrometry and genomic analysis. Our results show that B2017 is not able to synthesize surfactants and common antibiotics produced by *Pseudomonas* spp., i.e. pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin and pyocyanin, but it produces pyoverdine, a siderophore which is involved in its biocontrol activity. The non-production of other metabolites, such as cyanide, safracin, promysalin and lipopeptides between others, is also discussed. Our data suggest that the mode of action of B2017 is not mainly due to the production of antimicrobial / toxic metabolites. Moreover, these features make *P. putida* B2017 a promising biocontrol microorganism for plant protection without side effects on environment, non-target organisms and human health.

**Keywords:** secondary metabolites; siderophore; biosurfactant; agriculture; biological control agent; pseudomonads

## INTRODUCTION

Fluorescent pseudomonads are biological control agents (BCAs) commonly isolated from soil and with the ability to produce a wide spectrum of bioactive metabolites (Haas and Defago 2005; Couillerot et al. 2009; Deveau et al. 2016; Mishra and Arora 2018).

The most common antibiotics produced by *Pseudomonas* spp. are pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and phenazines that may play an important role in biocontrol (Howell and Stipanovic 1980; Shanahan et al. 1992; Pierson and Pierson 1996; Haas, Blumer and Keel 2000; Chin-A-Woeng, Bloembergen and Lugtenberg 2003; Neidig et al. 2011; Nandi et al.

2015; Mishra and Arora 2018). Pyrrolnitrin, synthesized by a four-gene cluster (*prnABCD*), is a phenylpyrrole with strong antifungal activity inhibiting fungal respiratory chains (Kirner et al. 1998). DAPG is toxic to a wide range of plant pathogenic fungi and its core biosynthetic pathway is encoded by genes *phlA*, *phlB*, *phlC* and *phlD* (Bangera and Thomashow 1999). Pyoluteorin shows strong toxicity against oomycetes, such as *Pythium ultimum*. A total of eight genes were identified for pyoluteorin biosynthetic pathway in *Pseudomonas fluorescens* Pf-5 (*pltA*, *pltB*, *pltC*, *pltD*, *pltE*, *pltF*, *pltG* and *pltM*) (Nowak-Thompson et al. 1999). Other low-molecular-weight metabolites with a broad-spectrum antimicrobial activity are phenazines. Five genes of the *phz*

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gene cluster—*phzE*, *phzD*, *phzF*, *phzB* and *phzG*—are strictly necessary for their biosynthesis (Blankenfeldt and Parsons 2014). In addition, all pseudomonads have a *phzA* gene encoding for a PhzB-paralogue that may be involved in the specific formation of phenazine-1-carboxylic acid (PCA) instead of phenazine-1,6-dicarboxylic acid (PDC) (Mavrodi et al. 2010). Furthermore, the action of PhzM and PhzS are also strictly necessary for the biosynthesis of pyocyanin using PCA as precursor (Mavrodi et al. 2001; Parsons et al. 2007).

In addition, other antimicrobial / toxic metabolites have been described in pseudomonads, including cyanide, promysalin, safracins and lipopeptides, between others (Kuiper et al. 2004; Velasco et al. 2005; Raaijmakers, de Bruijn and de Kock 2006; Roongsawang, Washio and Morikawa 2010; Li et al. 2011; Rokni-Zadeh et al. 2013; Mishra and Arora 2018). Lipopeptides are bio-surfactants with a wide range of biological activities, such as antagonism against pathogenic microorganisms, biofilm formation or triggering plant defense (Raaijmakers et al. 2010; Bonnichsen et al. 2015).

Other compounds involved in the biocontrol activity of *Pseudomonas* spp. are siderophores, small extracellular molecules that are able to sequester the limited iron pool in the rhizosphere competing with phytopathogens, which also require iron to grow (Cornelis 2010, Ahmed and Holmström 2014, Ringel and Brüser 2018). Pyoverdines, the primary siderophores synthesized by fluorescent *Pseudomonas* species, are composed of three parts: (i) a conserved dihydroxyquinoline chromophore, (ii) a variable peptide chain (6–12 amino acids) which differs substantially between species and even between strains of the same species and (iii) an acyl side-chain attached to the 3-amino group of the chromophore.

Although there are many studies regarding the production and regulation of antimicrobial metabolites and siderophores in fluorescent *Pseudomonas* spp., few works focus on *Pseudomonas putida*. Hassan and coworkers demonstrated the production of pyoluteorin and siderophores by the *P. putida* NH-50, although it was unable to produce pyrrolnitrin and DAPG (Hassan, Afghan and Hafeez 2011). By PCR detection using specific primers for genes involved in the biosynthesis of pyrrolnitrin, DAPG, pyoluteorin and phenazines, a diverse profile of antibiotic production was shown among isolates of *P. putida* (Agrawal et al. 2015).

*Pseudomonas putida* B2017 was previously described as effective BCA against *Fusarium oxysporum* f.sp. *radicis-lycopersici* in tomato, *Rhizoctonia solani* and *Pectobacterium atrosepticum* in potato and *Sclerotinia sclerotiorum* in lettuce (Oliver et al. 2019). The risk assessment of microbial BCAs is always required before their market authorization (Mudgal et al. 2013; OECD 2018). The aim of this study was to assess the potential risk of using B2017 as BCA in agriculture based on its ability to produce secondary metabolites including antibiotics or any other toxic compound.

## MATERIALS AND METHODS

### Bacterial strains

B2017 was isolated from an agricultural field in Gavà (Spain) and characterized as a strain of *P. putida* (Oliver et al. 2019). After identification, B2017 was deposited in the Type Culture Spanish Collection with the accession number CECT 8538. Moreover, *P. putida* KT2440 and *Lysobacter enzymogenes* B25 were used in some assays for comparison purposes.

### Cultivation conditions for antibiotic production and extraction procedure

To grow B2017, a fermentation process was done in an industrial bioreactor F3–100 (F3-industrial model, Bionet) with a working volume of 100 L of LB medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) for 6 h at 28 °C, pH 7 and agitation ramp from 250 to 400 rpm. The inoculum was prepared in a lab bioreactor F1–5 (F1-lab model, Bionet) for 24 h and the same parameters.

After fermentation, the cells were harvested by centrifugation at 5000 g for 10 min (Hitachi CR22N), the culture supernatant was stored at 4 °C and the cells were lyophilized (Heto PowerDry LL 3000 Freeze Dryer, Thermo Fisher Scientific, Waltham, MA USA).

Secondary metabolites were extracted from both cells and cell-free culture supernatant. Lyophilized bacterial cells were quantified by measuring colony forming units per milliliter (CFUs/mL). Bacterial cells ( $0.5\text{--}1.0 \times 10^{10}$  CFUs) were suspended in 8 mL of methanol and acidified to pH 2.5 with 1 N HCl. After 4 h-shaking at room temperature, the cell suspension was centrifuged (5000 g, 20 min) and the supernatant was called EPM-C (from Extracted Potential Metabolites from Cells).

Culture supernatant was filtered by passing through a 0.22 µm pore size cellulose acetate filter to get 200 mL of cell-free culture supernatant (CS). Then, CS was adjusted to pH 2.5 with 1 N HCl and extracted three times with the same volume of ethyl acetate (EtAc). The organic phases were combined, dried by rotary evaporation and dissolved in 5 mL of methanol; this extract was called EPM-S (from Extracted Potential Metabolites from Supernatant). As a control, the same organic extraction was done on the culture medium LB without microbial inoculation. All fractions were kept at 4 °C until their use.

### Surfactant activity

The surfactant activity or the ability to collapse a droplet of water was tested as described (Kuiper et al. 2004). In summary, 20 µL of sample CS were pipetted as a droplet onto parafilm. Subsequently, 3 µL of the dye Congo Red (which had no influence on the shape of the droplets) was added to stain the water and supernatants for photographic purposes. The spreading of the droplet on the parafilm surface was followed over hours until the droplet became dry and the diameter was measured.

### In vitro antimicrobial activity of metabolic fractions

Metabolic fractions CS, EPM-C and EPM-S were assessed against bacteria using a radial diffusion assay as previously described (Murakami et al. 2004). Bacteria were overnight cultured in LB medium at 28 °C and 200 rpm, and further refreshed for 4 h. From this exponential-phase culture, a bacterial work solution was adjusted to 0.01 of absorbance at 600 nm in agar media (1% agar and 1% tryptone) and plated. After solidification, 30 µL aliquots of the tested fractions were loaded in 4 mm-wells done into agar plates. Chloramphenicol (40 µg/mL) was applied as positive control. After 30 min incubation at 4 °C, plates were incubated at 28 °C overnight and the inhibition zone radius after each treatment was measured.

Antifungal activity of fractions was evaluated by placing 40 µL aliquots in 5 mm-wells cut into potato dextrose agar plates that had been inoculated at the centre with a agar disc (5 mm diameter) containing mycelium from the active growing edge

of 7 days-old cultures of the pathogen (Balouiri, Sadiki and Ibn-souda 2016). Cyclohexamide (10 µg/mL) was used as positive control. After different days of incubation at 26 °C, the mycelial growth near to the wells was measured as the radius.

Moreover, the antimicrobial activity of pure commercial metabolites was evaluated. Standard of pyrrolnitrin was purchased from Sigma-Aldrich and standards of DAPG, pyoluteorin and pyocyanin from Santa Cruz Biotechnology. The metabolites were tested at 250 µg/mL dissolved in H<sub>2</sub>O.

All antimicrobial assays were carried out twice against each pathogen, and each experiment included fractions from two B2017 cultures. The mean and standard deviation (SD) were then calculated for each treatment and Student t-test was applied (P-value < 0.05).

### Siderophore production and antifungal activity

Production of ferric ion chelates was detected using Chromeazurol S (CAS) methodology (Schwyn and Neilands 1987). Overnight bacterial cultures were grown at 28 °C for 24 h on King's B medium plates (20 g/L casein peptone, 1.5 g/L K<sub>2</sub>HPO<sub>4</sub>, 1.5 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 10 mL glycerol and 15 g/L agar) containing 1/10 of CAS solution.

To improve siderophore production, *P. putida* strains B2017 and KT2440 were grown in King's B liquid medium with lower content of iron (KingBmod: 5 g/L acid casein peptone, 1.2 g/L K<sub>2</sub>HPO<sub>4</sub>, 0.25 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.03 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O). Moreover, the bacterial strains were also grown in King's B medium or KingBmod medium supplemented with 200 µM FeCl<sub>3</sub>. After incubation in darkness at 28 °C 170–200 rpm for 1 day, the production of siderophores in CS from each culture was confirmed by fluorescence; UV spectra were measured in a micro-volume spectrophotometer (DS-11 FX, Denovix). CS samples were also analysed by HPLC (eAlliance system, Waters) using an XBridge C18 column (5 µm 4.6 x 150 mm, Waters) eluted at 1 mL/min with solvents A (water + 0.05 % TFA) and B (acetonitrile + 0.05 % TFA). The gradient was 0–100 % B for 15 min, 100 % B for 2 min and an equilibration step with 0 % B for 8 min. Siderophores were detected by UV spectrometry and chromatograms were obtained by processing at 254 nm.

For evaluation of the activity of siderophores, fungal inhibition test was performed by Poisoned Food technique (Balouiri, Sadiki and Ibn-souda 2016). A solution of CS from B2017 grown in KingBmod, B2017 grown in King's B, or KT2440 grown in KingBmod was mixed aseptically with temperate PDA (1:1). Then, the medium was poured into 5.5 cm Petri plates and, after solidification, agar discs (5 mm diameter) containing mycelium from the active growing edge of 7 days-old cultures of the pathogen were placed on the center of each plate. Each treatment was done by triplicate. Plates were incubated at 26 °C in the dark and the radial mycelial growth of fungal colony was followed by measurement of the shorter and longer radius at different days. The effect of CS with/without siderophores was compared to control plates (PDA + control culture medium, 1:1).

### Search of homologues to antimicrobial metabolite biosynthetic enzymes in B2017

The whole genome shotgun sequence (WGS) of B2017 (DDBJ/ENA/GenBank accession QWEF00000000, version QWEF01000000) was annotated using the RAST server (Aziz et al. 2008). Homologues to the core pyrrolnitrin, DAPG, pyoluteorin and pyocyanin biosynthetic enzymes were searched among the B2017 predicted proteins. For this, the protein database from

NCBI was used ([www.ncbi.nlm.nih.gov/protein/](http://www.ncbi.nlm.nih.gov/protein/)), limited to *Pseudomonas*, *P. putida*, and/or Refseq entries to keep the number of hits below 300. Then, the accession numbers of the hits were retrieved and aligned against the B2017 proteins annotated by RAST using BLASTP (Zhang et al. 2000). As recommended by Pearson, the bit score was used as cut-off parameter, applying a threshold of 40 for the B2017 protein database since it includes less than 7000 items (RAST annotates 5676 coding sequences for the B2017 WGS) (Pearson 2013).

Similarly, a search of homologues to pyoverdine biosynthetic pathway was carried out by protein–protein alignments using the predicted B2017 proteins and the KT2440 pyoverdine biosynthetic genes (Supplemental Table 1). In addition, the B2017 genome sequence was uploaded to antiSMASH (version 4.2.0) to identify the pyoverdine biosynthetic genes cluster (Blin et al. 2017).

Additionally, homologues of the biosynthetic proteins of other, less widespread, antimicrobial compounds were studied (Supplemental Table 2). These compounds included cyanide, safracins, promysalin, mupirocin, viscosin, maladicins and splicostatin analogues. BLASTP searches were done as described before using reported biosynthetic proteins from *Pseudomonas* strains as query.

### Purification and amino acid analysis of siderophores

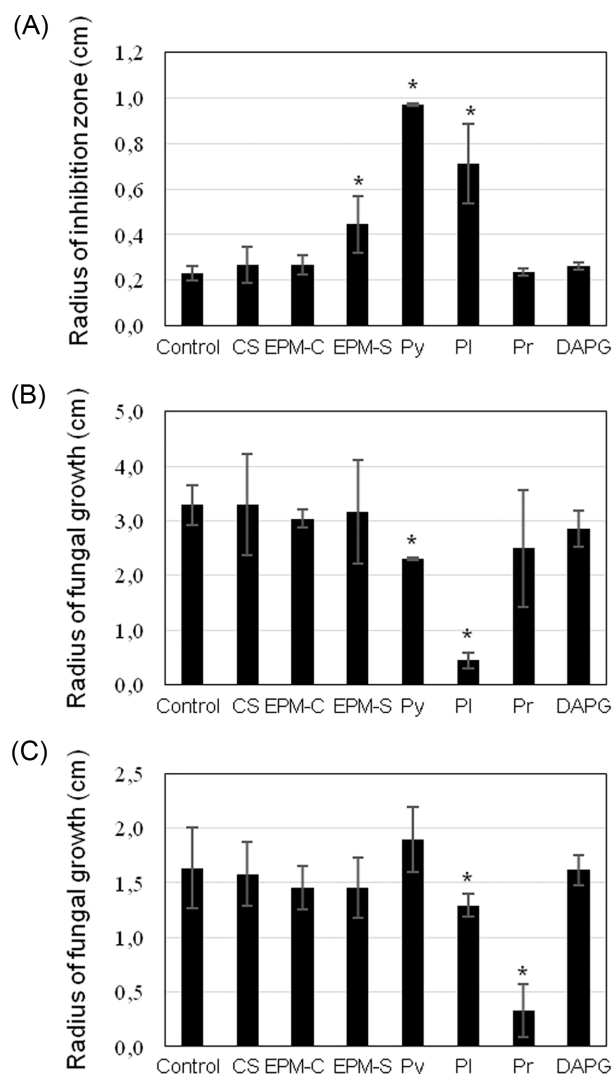
B2017 was grown in KingBmod medium as described above and siderophores were purified from CS sample by preparative HPLC (Delta Prep 400, Waters) with an OBD SunFire PrepC column (10 µm 19 x 250 mm, Waters) eluted at 15 mL/min with gradient 0–10 % B for 20 min, 10–15 % B for 2 min and an equilibration step with 0 % B for 3 min. Siderophores were detected by UV spectrometry with a 432 detector (Konron Instruments) at 254 nm.

Hydrolysis was done by subjecting around 2 mg of purified siderophores to 1 mL of 6 M HCl at 105 °C for 24 h. The amino acids were derivatized and quantified according to AccQ-Tag method (Waters) by HPLC (eAlliance system, Waters) with a photodiode array detector. L-2-aminobutyric acid (Acros Organics, Thermo Fisher Scientific) was used as internal reference standard. For each amino acid, the values were normalized to the aspartic acid content. Two independent hydrolysis were done and the results are shown as average of both assays.

## RESULTS

### In vitro antimicrobial activity of culture supernatant and extracts from B2017

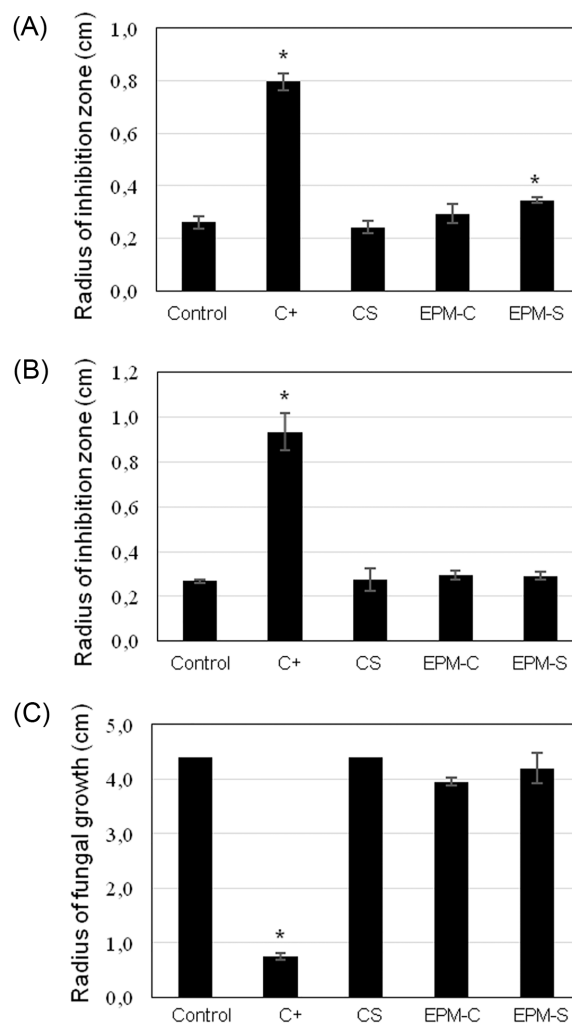
*P. putida* B2017 reduced the growth of eight selected fungal phytopathogens according to its activity as BCA previously published (Oliver et al. 2019) (Supplemental Figure 1). In order to find out if the mode of action of B2017 is due to the production of antimicrobial metabolites, the evaluation of the activity of its metabolic extracts was carried out and compared with the antimicrobial activity of the most relevant metabolites produced by *Pseudomonads*, i.e. pyrrolnitrin, DAPG, pyoluteorin and pyocyanin. No activity of the metabolic fractions from B2017 was detected against the phytopathogens *P. carotovorum* DSM 30168, *P. ultimum* CECT 20902 and *S. sclerotiorum* CECT 2822 (Fig. 1). Only a significant antibacterial activity was observed for EPM-S although same behaviour was detected for organic extract from the culture medium LB (0.45 ± 0.12 for EPM-S from



**Figure 1.** Antimicrobial activity of metabolic fractions from *Pseudomonas putida* B2017 and pure antibiotics. Activity against the bacterium *Pectobacterium carotovorum* DSM 30168 (A) and the fungi *Pythium ultimum* CECT 20902 (B) and *Sclerotinia sclerotiorum* CECT 2822 (C) of cell-free culture supernatant (CS), extracted potential metabolites from cells (EPM-C) and supernatant (EPM-S), pyocyanin (Py), pyoluteerin (PI), pyrrolnitrin (Pr) and 2,4-diacetylphloroglucinol (DAPG). Data show the mean  $\pm$  SD of at least two experiments and with B2017 fractions from two independent fermentations in each experiment. \* Statistically different compared with control sample without treatment (control) (Student t-test, P-value < 0.05).

B2017 vs  $0.41 \pm 0.07$  for EPM-S from LB). On the contrary, pyrrolnitrin, pyoluteerin and pyocyanin showed different antifungal profile against *P. ultimum* and *S. sclerotiorum*, with a major antifungal activity of pyoluteerin against *P. ultimum* and pyrrolnitrin against *S. sclerotiorum*. Regarding antibacterial activity, a clear inhibition zone was observed around the wells containing the metabolites pyoluteerin and pyocyanin, while no inhibition was detected with pyrrolnitrin. The metabolite DAPG did not show antimicrobial activity at the tested concentration (i.e. 250  $\mu$ g/mL).

These negative results might suggest that B2017 does not produce detectable levels of antibiotics or toxins during an industrial fermentation process. To confirm this hypothesis and assess the potential risk of using B2017 as biocontrol bacterium, a similar antimicrobial assay was done to test the



**Figure 2.** Evaluation of toxicity of metabolic fractions from *Pseudomonas putida* B2017 against not-target microorganisms. The tested microorganisms were the bacteria *Ensifer adhaerens* DSM 23677 (A) and *Lysobacter enzymogenes* C3 (B), and the soil fungus *Trichoderma harzianum* H999 (C). The evaluated samples from B2017 were cell-free culture supernatant (CS), extracted potential metabolites from cells (EPM-C) and supernatant (EPM-S). As positive control (C+), 40  $\mu$ g/mL of chloramphenicol or 10  $\mu$ g/mL of cyclohexamide was used for bacteria or fungi, respectively. Data show the mean  $\pm$  SD of at least two experiments. \* Statistically different compared with control sample without treatment (control) (Student t-test, P-value < 0.05).

effect of its metabolic extracts against non-pathogenic rhizospheric microorganisms. No activity was observed for the B2017 metabolic extracts against *L. enzymogenes* C3 and *Trichoderma harzianum* H999 comparing with control sample without treatment (Fig. 2). As described above, only significant activity against *Ensifer adhaerens* DSM 23677 was detected for the fraction EPM-S from B2017 although its control fraction from LB medium had similar activity ( $0.35 \pm 0.01$  for EPM-S from B2017 vs  $0.36 \pm 0.01$  for EPM-S from LB).

### Genomic analysis to determine the ability of B2017 to synthesize antimicrobials/ toxic metabolites

Secondary metabolism of *Pseudomonas* spp. and other microorganism is activated when there is a nutrient limitation such as during the late logarithmic or stationary phase (Haas, Blumer

and Keel 2000; Chin-A-Woeng, Bloemberg and Lugtenberg 2003).

The analysis of the different extracts from B2017 by HPLC and LC-MS/MS comparing with commercial metabolites pyrrolnitrin, DAPG, pyoluteorin and pyocyanin was carried out. However, none of these metabolites were detected in CS, EPM-C and EPM-S samples from two independent B2017 fermentations (Supplemental Figure 2), indicating that B2017 does not produce these metabolites under our regular growth conditions by fermentation process.

A complementary genomic study was carried out to determine whether B2017 bears the genetic machinery to synthesize these metabolites under any other growth/storage conditions.

Using the WGS of B2017 and by protein-protein alignment, a search of homologues to pyrrolnitrin, DAPG, pyoluteorin and pyocyanin biosynthetic enzymes was conducted (Table 1). B2017 has no PrnA and PrnB homologues but it has a PrnD homologue. It remains unclear whether B2017 has a homologue of PrnC, since only one of the 27 PrnC accessions analyzed has a homologue among the B2017 proteins. Nevertheless, it is deduced that B2017 is not able to produce pyrrolnitrin due to the lack of, at least, PrnA (Trp halogenase) and PrnB (monodechloroaminopyrrolnitrin synthase), the two enzymes that catalyze the first two committed steps in pyrrolnitrin biosynthesis. Similarly, B2017 may not be able to produce DAPG due to the lack of, at least, PhlA, PhlB and PhlC, the enzymes needed to transform phloroglucinol into DAPG, even though it remains unclear whether a homologue of PhlD exists in this strain.

Regarding the pyoluteorin biosynthetic pathway, B2017 has no homologues of PltM and PltD, two halogenases essential for its biosynthesis. Thus, B2017 is not expected to produce pyoluteorin even though it has PltA, PltB, PltC, PltE, PltF and PltG homologues.

Similarly, B2017 has no homologues of PhzB/A, or PhzM, which suggests that it cannot produce PCA or PDC, or transform PCA into pyocyanin, even though it has homologues of the remaining phenazine and pyocyanin biosynthetic proteins (PhzD, PhzE, PhzF, PhzG and PhzS).

In addition, due to the lack of homologues of essential biosynthetic enzymes, B2017 is not able to synthesize cyanide, maladins, mupirocin, promysalin, safracins or splicestatin analogues (Supplemental Table 2). Although B2017 has homologues for the viscosin biosynthetic proteins, these biosynthetic NRPSs were not recognized by AntiSMASH (data not shown), suggesting that viscosin synthesis is unlikely. The absence of surfactant activity by B2017 supports this fact.

### Siderophore and biosurfactant production

Following CAS assay in agar media, the production of siderophores by B2017 was determined by the change of dark blue to orange color after bacterial incubation; no change was observed after incubation with *L. enzymogenes* B25 that does not produce siderophores (de Bruijn et al. 2015) (Fig. 3A). King's B medium is routinely used to evaluate the production of fluorescent pyoverdine (King, Ward and Raney 1954). However, we detected a higher production of pyoverdine-type siderophores in a King's B modified medium, called KingBmod, than in the original King's B medium. Fluorescence of the liquid culture medium under UV exposure and a maximum of absorbance around 400 nm confirmed the production of pyoverdine-type siderophores by B2017 after incubation in a specific medium with lower content of iron (Fig. 3A). When

the bacterium was incubated in KingBmod supplemented with iron, fluorescence and peak of absorbance were not observed either (data not shown).

Moreover, the surfactant activity of CS fractions obtained from three independent cultures of B2017 was measured although no significant differences were observed compared with water (*Student t*-test, *p*-value < 0.05) (Fig. 3B). Our results showed that B2017 is not able to produce any compound with surfactant activity while positive activity was observed with CS from *L. enzymogenes* B25 that produced biosurfactants (de Bruijn et al. 2015).

### Characterization of the pyoverdine produced by B2017

Potential siderophores produced by B2017 were characterized by HPLC analysis of CS obtained by incubation of bacteria in KingBmod medium compared with the same medium supplemented with iron (supplemental Figure 3A). Three peaks with retention time ( $t_r$ ) of 4.4 min, 4.5 min and 4.8 min were only produced by B2017 growing in medium without iron suggesting their nature as siderophores. Moreover, the UV spectra of these compounds with peaks at 200, 250, 310 and 360–380 nm confirm that they are pyoverdine-type siderophores (Supplemental Figure 3A-inset) (Hoegy, Mislin and Schalk 2014).

To compare these pyoverdines with those produced by another *P. putida* strain, CS of KT2440 was obtained after incubation in KingBmod and KingBmod + Fe. After HPLC analysis of CS from two independent cultures, we observed that potential siderophores produced by both strains eluted at different  $t_r$  (Supplemental Figure 3), suggesting that B2017 is able to produce a different pyoverdine or different isoforms of the same pyoverdine than KT2440.

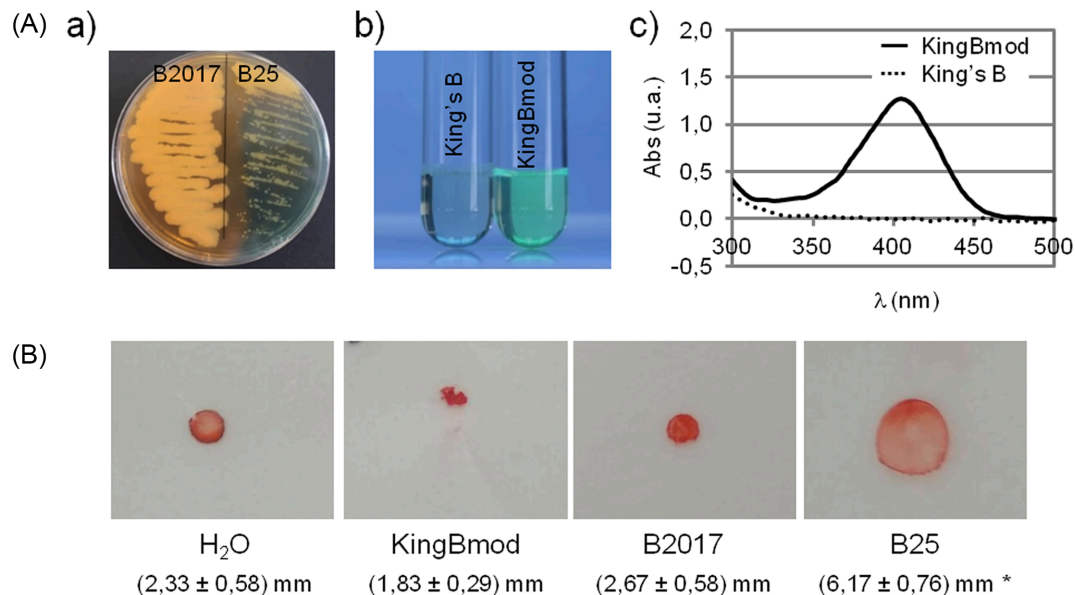
The annotation of the B2017 genome shows that this strain bears homologues of all pyoverdine biosynthetic proteins as compared to KT2440 (Supplemental Table 1). Moreover, antiSMASH predicts three regions as non-ribosomal peptide synthase (NRPS) with homology to the pyoverdine cluster; the different predicted domains and the adenylation domain substrates are shown in Fig. 4A. The first region contains the modules of a NRPS with high homology to PvdL, which incorporates the first three amino acids of ferribactin, the precursor of the pyoverdine. These three amino acids are L-glutamic acid, D-tyrosine and L-2,4-diaminobutyrate, considering the antiSMASH prediction and previous published results (Ringel and Brüser 2018) (Fig. 4A-a). The dihydroxyquinoline chromophore will be generated from the D-tyrosine and L-2,4-diaminobutyrate residues. The other two regions with homology to pyoverdine cluster may be involved in the incorporation of the residues in the peptide chain (Fig. 4A-b,c).

The main peaks corresponding to pyoverdines from B2017 ( $t_r$  of 4.5 min and 4.8 min, Supplemental Figure 2A) were purified by preparative HPLC and amino acid analysis of each peak was done to confirm our previous results. Both samples were identical in the amino acid composition, indicating that they are isoforms of the same pyoverdine. The analysis revealed the presence of one residue of aspartic acid, two serines, one glycine, two 2,4-diaminobutyric acid, one glutamic, two threonines and one ornithine (Table 2). These results were in part consistent with the antiSMASH prediction although we were not able to discern between ornithine and hydroxyornithine. In addition to the amino acids predicted by antiSMASH, two threonines were also identified.

**Table 1.** Homologues to pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and phenazines biosynthetic enzymes.

Metabolite	Proteins	Accessions analyzed	Hits score > 40	Conclusion
Pyrrolnitrin	PrnA	30	0	No
	PrnB	85	0	No
	PrnC	27	1	?
	PrnD	136	136	Yes
DAPG	PhlA	49	0	No
	PhlB	54	0	No
	PhlC	69	0	No
	PhlD	4	1	?
Pyoluteorin	PltA	214	213	Yes
	PltB	15	15	Yes
	PltC	23	19	Yes
	PltD	27	0	No
	PltE	21	21	Yes
	PltF	29	29	Yes
	PltG	20	20	Yes
	PltM	57	0	No
Phenazines/Pyocyanin	PhzB/A*	437	0	No
	PhzD	9	7	Yes
	PhzE	31	31	Yes
	PhzF	197	197	Yes
	PhzG	15	15	Yes
	PhzM	2	0	No
	PhzS	88	85	Yes

\*PhzA is the result of *phzB* gene duplication, a phenomenon observed in pseudomonads, so protein-protein alignment may not discriminate among the two gene products.

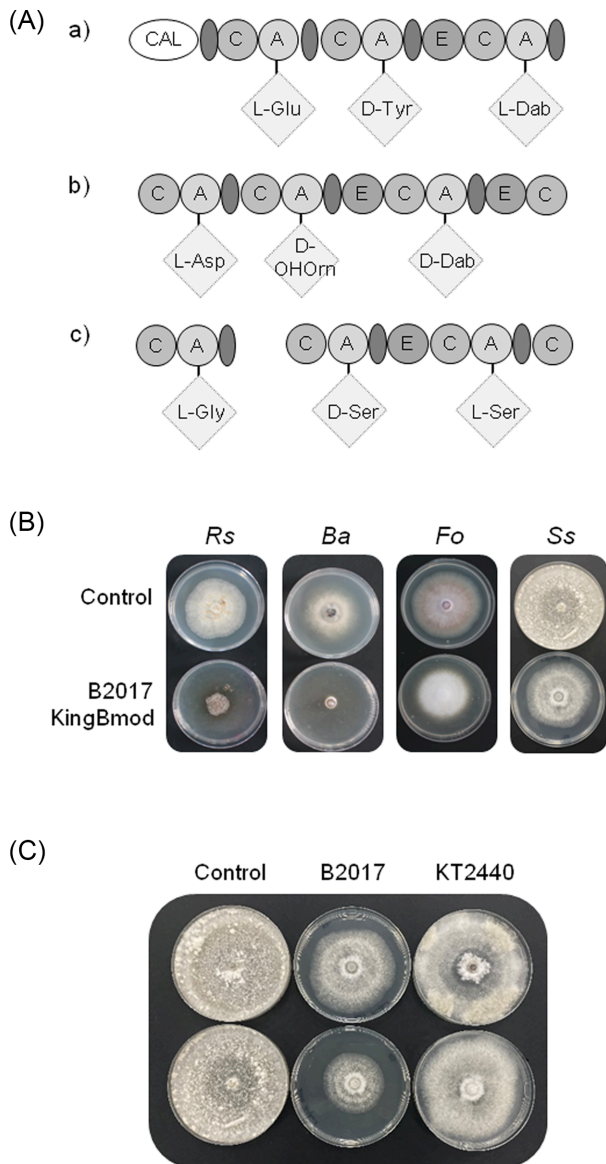


**Figure 3.** Production of siderophores and surfactants by B2017. (A) Siderophore production by B2017: (a) Incubation of B2017 and B25 in agar CAS medium; (b) Culture medium of B2017 grown in King's B and in KingBmod; (c) Absorbance of culture medium of B2017 grown in KingBmod (solid line) compared with B2017 grown in King's B (dotted line). (B) Surfactant activity of the samples CS from B2017 and B25 compared with the effect of H<sub>2</sub>O and the culture medium KingBmod. The mean  $\pm$  SD of the droplet diameter (mm) from three independent assays is shown in brackets and images correspond to a representative experiment. \* Statistically different compared with water (Student t-test, P-value < 0.05).

### Pyoverdine produced by B2017 may be involved in its biocontrol mode of action

The antifungal activity of pyoverdine produced by B2017 was tested against *R. solani* DSM 63010, *Botrytis aclada* CECT 2851, *F. oxysporum* H828 isolated from tomato and *S. sclerotiorum* H866 isolated from lettuce (Fig. 4B). The results showed differences in

growth area between fungus growing in PDA containing supernatant of B2017 with pyoverdine and control (PDA containing control medium). These data revealed that pyoverdine produced by B2017 is partly responsible for its biocontrol activity reducing the growth of the fungal pathogens. In this experiment, less antifungal activity was observed by the pyoverdine produced by KT2440 compared to the pyoverdine of B2017 (Fig. 4C).



**Figure 4.** Characterization of the pyoverdine produced by *Pseudomonas putida* B2017. **(A)** AntiSMASH prediction of nonribosomal peptide synthetases (NRPSs) with homology to the pyoverdine cluster in B2017. The three predicted regions are shown in (a), (b) and (c). The different NRPSs domains are Co-enzyme A ligase domain (CAL), condensation domain (letter C), adenylation domain (letter A), epimerization domain (letter E) and peptidyl carrier protein (ellipse). The amino acid abbreviations under each adenylation domain represent the substrate of this domain (Glu: glutamic acid, Tyr: tyrosine, Dab: 2,4-diaminobutyric acid, Asp: aspartic acid, OHOrn: hydroxyornithine, Gly: glycine; Ser: serine). L- and D- indicate the specific configuration of each amino acid. **(B)** Effect of pyoverdine of B2017 on the growth of the pathogenic fungi *Rhizoctonia solani* DSM 63010 (Rs), *Botrytis aclada* CECT 2851 (Ba), *Fusarium oxysporum* strain H828 isolated from tomato (Fo) and *Sclerotinia sclerotiorum* strain H866 isolated from lettuce (Ss). Tested samples were culture supernatant of B2017 containing pyoverdine compared with the culture medium KingBmod as control. Photos were taken after 5 days (for Ba and Fo) or 6 days (for Rs and Ss) post-inoculation. **(C)** Comparison of the antifungal activity of the pyoverdine produced by B2017 with the pyoverdine of KT2440 against *Sclerotinia sclerotiorum* strain H866. Photos were taken at 6 days post-inoculation.

**Table 2.** Amino acid composition of pyoverdine isolated from *Pseudomonas putida* B2017.

Amino acid <sup>a</sup>	Ratio <sup>b</sup>	
	Peak (t <sub>r</sub> 4.5 min)	Peak (t <sub>r</sub> 4.8 min)
Asp	1.0	1.0
Ser	2.3	1.8
Glu	0.8	0.9
Gly	1.5	1.2
Thr	2.2	1.8
Dab	1.7	2.2
Orn	nd	1.0

<sup>a</sup>Asp: aspartic acid, Ser: serine; Glu: glutamic acid; Gly: glycine; Thr: threonine; Dab: 2,4-diaminobutyric acid; Orn: ornithine.

<sup>b</sup>The values are refer to the analysis of the HCl hydrolysates of both peaks. Values are normalized to the aspartic acid content. The ratio are the average of two independent analyses. nd: undetermined

## DISCUSSION

A relevant feature for the biocontrol activity of *Pseudomonas* species is the production of antibiotics and other secondary metabolites with antagonistic activity. Based on the evidence obtained in this study by HPLC-mass spectrometry analysis and antimicrobial activity assays, B2017 does not produce the common antibiotics produced by *Pseudomonas* spp., i.e. pyrrol-nitrin, DAPG, pyoluteorin and pyocyanin, when it is grown in large-scale fermentation to get the active ingredient of a potential commercial biocontrol product. Moreover, genomic analyses suggest that B2017 is not able to produce these metabolites under any growth/storage conditions. In agreement with previous work carried out with *Pseudomonas* spp. DF41, the production of the aforementioned antibiotics does not contribute to the antagonistic activity of our strain (Zhang et al. 2006).

Moreover, it is required to assess the risk associated to a specific bacterial strain before its use as BCA regarding the production of antibiotics and other relevant metabolites with potential toxic effects on non-target organisms and the environment (Mudgal et al. 2013; OECD 2018). The lack of toxic metabolite production by B2017 reduces the risk of its use as biocontrol bacterium being an advantage for its future application in agriculture. It has been demonstrated that high concentrations of DAPG or pyoluteorin exhibit phytotoxic properties (Maurhofer et al. 1992). The cytotoxic metabolite pyocyanin is a virulence factor in *Pseudomonas aeruginosa* which stimulates a neutrophilic inflammatory response and up-regulate chemokines (IL-8) and adhesion molecules (ICAM-1) (Look et al. 2005). In addition, B2017 is not able to synthesize other antibiotics reported in *Pseudomonas*, namely cyanide, maladicins, mupirocin, promysalin, safracins or spliceostatin analogues.

The negative surfactant activity of B2017 supernatant also suggests that it does not produce any biosurfactant compound, e.g. the lipopeptide viscosin, at relevant quantity to observe an effect on surface tension between water and hydrophobic surfaces. By contrast, it is able to synthesize fluorescent siderophores that are partly responsible for its biocontrol activity by competing for available iron (Ahmed and Holmström 2014).

By amino acid analysis of this pyoverdine, we identified a glutamic acid residue. This residue could be part of the peptide chain or the side chain of the pyoverdine, being both uncommon options. The amino acid sequence of peptide chain in

*P. putida* pyoverdines is already known or predicted by bioinformatics analysis (Meyer et al. 2007; Ye et al. 2013). Among all known pyoverdines produced by *P. putida*, only one produced by strain DSM 50202 bears glutamic acid in their peptide chain (Persmark, Frejd and Mattiasson 1990). On the other hand, the identified glutamic acid could be the side chain of the pyoverdine, i.e. the first amino acid at the N-terminus without modifications. As previously reviewed, although L-glutamic acid is always the first residue in ferribactins, mature pyoverdines usually do not contain it any more (Ringel and Brüser 2018). Instead, the acyl side chain is modified to succinamide, succinic acid or  $\alpha$ -ketoglutarate, among others. Most frequently, the variation of this acyl side chain results in several isoforms of a pyoverdine produced by a given strain (Meyer et al. 2007; Meyer et al. 2008). Both possibilities are unusual in pyoverdines and therefore further studies will be conducted to elucidate the structure of B2017 pyoverdine in detail and its functional advantage.

By conclusion, our results show that *P. putida* strain B2017 is not able to produce antibiotics/toxic metabolites but produces a pyoverdine that may contribute to explain the mode of action of B2017, being a promising biocontrol microorganism for plant protection.

## SUPPLEMENTARY DATA

Supplementary data are available at [FEMSLE](https://femsle.onlinelibrary.wiley.com/doi/10.1111/femsle.13677) online.

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**Conflict of Interest.** The authors declare that *P. putida* B2017 is pending an European patent application (priority date May 6, 2017).

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