J. Microbiol. Biotechnol. (2005), **15**(1), 86–90

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Isolation and Characterization of a New Fluorescent *Pseudomonas* Strain that Produces Both Phenazine 1-Carboxylic Acid and Pyoluteorin

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Received: February 23, 2004
Accepted: July 8, 2004

Abstract Strain M-18 was isolated from the rhizosphere soil of sweet melon, using 1-aminocyclopropane-1-carboxylate (ACC) as a sole nitrogen source. Its phenotypic characteristics, metabolic tests, and 16S rDNA sequence were analyzed. The antibiotics secreted by strain M-18 were determined to be phenazine 1-carboxylic acid and pyoluteorin. These data showed that strain M-18 was a new fluorescent *Pseudomonas* strain that produced both phenazine 1-carboxylic acid and pyoluteorin, some features being similar to *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Therefore, the strain M-18 appears to be the first pseudomonad described to date that is capable of producing both phenazine 1-carboxylic acid and pyoluteorin.

Key words: Biocontrol, phenazine 1-carboxylic acid, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, pyoluteorin

Interest in biological control of plant pathogens has increased considerably over the past years as a response to public concern about the use of hazardous chemical pesticides. Fluorescent pseudomonads are ubiquitous inhabitants of plant surfaces, including roots, leaves, and floral parts, and certain strains are promising candidates for the biological control of plant diseases caused by phytopathogenic fungi and bacteria [5, 19]. These strains produce some secondary metabolites, such as 2,4-diaectylphloroglucinol (Phl) and pyoluteorin (Plt), and play a significant role in the suppression of soilborne diseases of important crop plants. In addition, effective root colonization and induction of systemic resistance in the plant contribute to their effectiveness in suppressing soilborne plant diseases [23].

In this paper, a new fluorescent *Pseudomonas* strain, named M-18, was isolated from the rhizosphere soil of sweet melon in Shanghai suburb in China using 1-aminocyclopropane-1-carboxylate (ACC) as a sole nitrogen source. It was found that M-18 could secrete phenazine 1-carboxylic acid (PCA), a kind of phenazine, and pyoluteorin (Plt), a kind of polyketide, to inhibit several phytopathogens. The strain M-18 appears to be the first bacterium described to date that is capable of producing both a phenazine and polyketide as antibiotics by a single strain. Moreover, the strain M-18 would be more effective for suppression of some specific or multiple plant diseases, because of the common function of PCA and Plt produced.

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Materials and Methods

Isolation of Strain M-18

The rhizosphere soil of sweet melon was collected from Songjiang, Shanghai, China. The screening procedure by using 1-aminocyclopropane-1-carboxylate (ACC) as a sole nitrogen source [4] was carried out at 28°C for pseudomonad isolation. According to the procedure, well isolated colonies were selected for the suppression activity test against *Fusarium oxysporum f.* sp. *cucumerinum* by a dual culture plate method. Thus, *F. oxysporum f.* sp. *cucumerinum* was cultivated on PDA (potato dextrose agar) plate for one week, and an 8 mm diameter agar plug with actively growing mycelia mats of *F. oxysporum f.* sp. *cucumerinum* was inoculated on one side of the Petri plate (9 cm) containing PDA. The selected strains were inoculated on the other side of the Petri plate. The plates were incubated at 28°C, and the suppression zone was recorded after 3 days. From more then one thousand selected colonies tested, the strain that had the strongest suppression activity against *F. oxysporum f.* sp. *cucumerinum* was designated M-18. Identification of the strain M-18, based on morphological, physiological, and biochemical characteristics, was performed by the described methods [9, 10]. *Pseudomonas fluorescens*

2-79 (NRRL B-15132, obtained from L.S. Thomashow, USDA Agricultural Research Service, Pullman, Wash., U.S.A.) and *Pseudomonas aeruginosa* ATCC 27853 (Oxoid Limited, Hampshire, England) were used as the control.

Media and Culture Conditions

The strain M-18 was cultivated at 28°C in King's B medium [8], containing 2% glycerol (W/V), 2% tryptone, 0.15% K_2HPO_4, and 0.15% $MgSO_4$, for growth. King's B medium and PPM medium [12], containing 2% glucose, 2% peptone, and 1% KNO_3, were used for the production of antibiotics

Cell Growth and Antibiotics Production of PCA and Plt

The strain M-18 was precultured at 28°C for 12 h in a 150-ml conical flask containing a 15 ml aliquot of King's B liquid broth. A portion (8 ml) of this culture was inoculated to a 150 ml aliquot of King's B or PPM medium in 500-ml conical flasks for the production of Plt and PCA.

Culture broth was collected for measurements of total cell number, PCA, and Plt every 6 hours. For cell counting, samples were pre-treated as described [20], and cells were direct counted by microscopic observation. PCA was extracted from the supernatant of culture broth with chloroform after the pH was adjusted to 4.0, and then filtered (pore size 0.2 μm). Plt was extracted with ethyl acetate, desiccated in a vacuum, redissolved in methanol, and filtered (pore size 0.2 μm). Each sample was loaded on a HPLC (Shimadzu LC-8A, Kyoto, Japan) C_{18} reversed-phase column (Zorbax SB-C18, 5.0 μm, 4.6 mm×250 mm, Rockland Technologies Ind., Newport, DE, U.S.A.), and the column was eluted with methanol-water [70:30, v/v] at a flow rate of 2.0 ml min^{-1}. Plt and PCA were detected by UV at 308 nm and 248 nm, respectively. The retention times of Plt and PCA were determined to be 3.1 min and 1.78 min, respectively. Both Plt and PCA were quantified with authentic samples as standards. These antibiotics were collected, and analyzed by ultraviolet absorbent spectrum scanning (U-2001, Hitachi, Japan), and mass spectrum (Finnigan LCQ, ThermoFinnigan, San Jose, CA, U.S.A.) with electrospray ionization, and ^1H and ^{13}C nuclear magnetic resonance (Avance 500, Bruker, Switzerland).

16S rDNA Sequence Analysis

The total genomic DNA from the strain M-18 was isolated as described [18]. Polymerase chain reaction was performed in a total volume of 50 μl with forward primer (5'-TGG CTC AGA TTG AAC GCT GGC-3') and reverse primer (5'-GTT CCC AGG TTG AGC CCG G-3'). Both primers were designed according to the conserved sequence of 16S rDNA in *P. aeruginosa*. PCR amplification was carried out for 35 cycles, starting with a denaturation step at 94°C for 1 min, followed by primer annealing for 2 min at 50°C and primer extension for 3 min at 72°C. The final cycle was succeeded by an extension step at 72°C for 10 min.

Cloning of Gene Clusters Encoding PCA and Plt Producing Activity

A genomic DNA library of the M-18 was constructed by ligating partially digested *Sau*3AI fragments of 28-40 kb into a cosmid vector pLAFR-K [7]. The resulting DNA fragments were packaged with λ packaging protein (Promega) and transduced into *E. coli* LE392 [18]. The positive clones, containing *plt* genes or *phz* genes, were screened by colony hybridization with the digoxigenin (Roche)-labeled probes of *pltC* gene [14] or *phzF* gene [16], respectively.

RESULTS AND DISCUSSION

Characterization of Strain M-18

Strain M-18 was proved to be a Gram-negative bacterium. The strain was rod-shape (1.1-1.3 mm long and 0.3-1.0 mm wide) with one flagellium, thus motile. It produced water-

Table 1. Main characteristics of M-18.

Characteristics	M-18	Pf 2-79	ATCC 27853
Voges-Proskauer test	-	-	-
Catalase	+	+	+
Facultative anaerobic growth	-	-	-
Obligatory aerobic growth	+	+	+
Acid from glucose	+	+	+
Hydrolysis of			
Esculin	-	-	-
Gelation	+	+	+
Starch	-	-	-
Utilization of			
Glucose	+	+	+
Mycose	+	+	-
Acetate	+	+	+
Arginine	+	+	+
Nitrate reduced to nitrite	+	+	+
Denitrification	+	+	+
Formation of			
Fluorescent pigment	+	+	+
H_2S	-	-	-
Indole	-	-	-
Levan	+	+	-
Pyocyanine	-	-	+
Growth in 5% NaCl	-	-	-
Growth at			
4°C	+	+	-
41°C	-	-	+
Growth at pH 5.5	-	-	+
Methyl red test	-	-	-
Arginine dihydrolase	+	+	+
Oxidation-fermentation test	oxidative	oxidative	oxidative

soluble, yellow fluorescent pigments. Other characteristics of M-18 are summarized at Table 1. The sequence of 16S rDNA was searched for homology in the GenBank with BLASTN program. Comparative analysis of a partial sequence (1,407 bp) of 16S rDNA (GenBank accession No. AY394845) from the strain M-18 showed that this strain was most closely related to members of *Pseudomonas aeruginosa*. The 99% identities were found with over 100 strains, and most of these strains were from *Pseudomonas aeruginosa*, including *P. aeruginosa* ATCC 27853 (No. AY268175), *P. aeruginosa* PAO1 (No. AE004844), and *P. aeruginosa* BHP7-6 (No. AY162139). There was no PCR result with primers from the conserved sequence of 16S rDNA in *P. fluorescens*. The antibiotic selection markers of the strain M-18 in PDA medium consisted of 100 μg ml^{-1} spectinomycin, 50 μg ml^{-1} ampicillin, and 10 μg ml^{-1} rifampicin. The strain M-18 was sensitive to tetracycline and kanamycin in the PDA medium.

The main characteristics of the strain M-18 suggested the strain as a fluorescent subgroup of the genera *Pseudomonas*. As shown in Table 1, most of the biochemical characteristics showed this strain to be closely related to *P. fluorescens* strain, according to the Bergey's Manual [2]. However, the characteristic of one flagellium and 16S rDNA sequence analysis could suggest a possibility that the strain M-18 was more closely related to *Pseudomonas aeruginosa*. Further evidences of close relationship between M18 and *P. aeruginosa* were obtained with PCA and Plt gene clusters cloned from the strain M-18. The PCA gene cluster of these cells was completely matched. The Plt gene cluster (GenBank accession No. AY394844) [6] from the strain M-18 shared a 60% to 92% sequence similarity of *pltLABCDEFGMR* [14] to those (GenBank accession No. AF081920) from *P. fluorescens* Pf-5. Interestingly, the Plt gene cluster was not observed in the genome sequence of *P. aeruginosa* [22]. In addition, a new gene *pltZ* in M-18 was identified in the downstream region of the *pltLABCDEFG* operon.

Of the fluorescent *Pseudomonas* spp. strains characterized to date for their biocontrol activity, almost all of them produce one or more of antibiotics, including PCA, Phl, Prn, and Plt [17, 23]. It was reported that plant-associated pseudomonads produce phenazine molecules via the shikimic acid pathway [23] and pyoluteorin via the polyketide pathway [1]. Furthermore, PCA has been shown to be produced by several strains of fluorescent *Pseudomonas* spp., such as *P. aureofaciens* 30-84, *P. fluorescens* 2-79, *P. aeruginosa* PNA1, and *P. aeruginosa* GC-B26 [11, 13, 16], and Plt by several strains of *P. fluorescens*, such as Pf-5 and CHA0 [14, 17]. However, Plt was not produced in *P. aeruginosa*. Nevertheless, there has been no report so far to demonstrate that both PCA and Plt are produced by a single strain, indicating that the strain M-18 would be unusual in sharing some distinct features of both

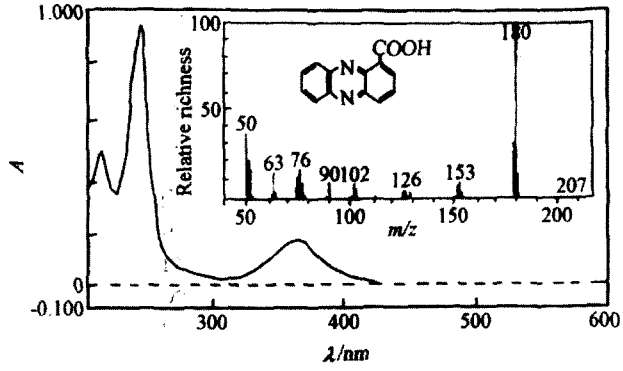

Fig. 1. The ultraviolet spectrum and mass spectrometric data of PCA.

P. aeruginosa and *P. fluorescens*. Further taxonomic and molecular biological characterizations of M-18 are in progress.

Confirming the Molecular Structure of PCA and Plt

PCA and Plt were isolated from M-18 culture broth and purified by using HPLC. The ultraviolet spectrum and mass spectrometric data of PCA and Plt are shown in Fig. 1 and Fig. 2, respectively. As shown in Fig. 1, the mass spectrum displayed a molecular ion peak at *m/z* 224 and a base peak at *m/z* 180. The base peak at *m/z* 180 and a strong absorption peak at 252 nm accompanied by a broad peak at 365 nm in UV spectral analysis indicated the presence of a phenazine moiety in the molecule. The molecular formula was thus deduced as $C_{13}H_8N_2O_2$ on the basis of analysis of mass spectroscopy at *m/z* 224.1 (M^+). In Fig. 2, the molecular ion peak at *m/z* 108 and 137 indicated the dihydroxy-benzene moiety and pyrrole moiety, respectively. Peaks at *m/z* 200, 236, and 271 suggested the presence of two chlorines. The molecular formula was deduced as $C_{11}H_7NO_3Cl_2$ on the basis of analysis of mass spectroscopy

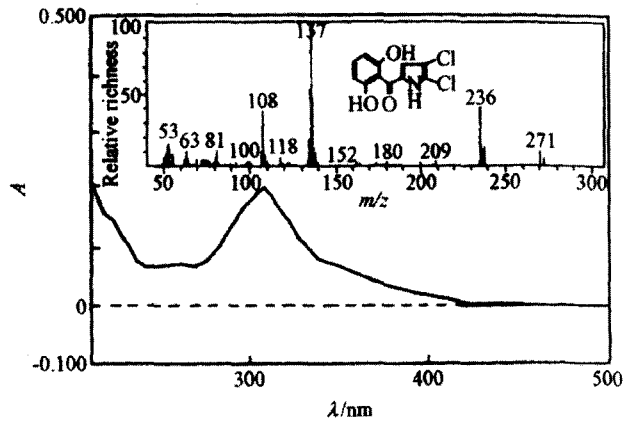

Fig. 2. The ultraviolet spectrum and mass spectrometric data of Plt.

at *m/z* 271(M^+). These results were also confirmed by ^1H and ^{13}C nuclear magnetic resonance (data not shown).

Time Course of Growth and Production of PCA and Plt

A typical time profile of cell growth, and PCA and Plt production by the strain M-18 in King's B and PPM media, is shown in Fig. 3 and Fig. 4, respectively. In cultures of King's B media, the amount of PCA increased linearly during the early period (0-24 h) up to 15.5 mg l^{-1} at 24 h and further increased slowly to 19.3 mg l^{-1} at 60 h. The amount of Plt increased slowly and reached about 24 mg l^{-1} at 60 h (Fig. 3). In cultures of PDM media, PCA began to accumulate after 2 h of culture and reached a maximum (108.3 mg l^{-1}) after 24 h of cultivation, and further increased slowly to 124.3 mg l^{-1} at 60 h. Plt began to accumulate in PPM medium after 18 h of cultivation and reached a maximum of 2.4 mg l^{-1} after 48 h of culture.

The temporal profile of the strain M-18 in King's B medium showed that high cell accumulations were achieved but with low PCA and Plt productions. It is apparent from Figs. 3 and 4 that PCA production in PPM medium increased about 6-fold more than that in King's B medium, indicating that PCA accumulation could be modulated by the carbon source supplied. The highest productivity was obtained when glucose was used as carbon sources. The kinetics of PCA production by the strain M-18 was similar to that in *P. fluorescens* 2-79 [20, 21]. However, Plt productivity decreased in PPM medium compared to that in King's B medium. The inhibitory effect of glucose on Plt production was also found in some strains of *P. fluorescens* [3, 15].

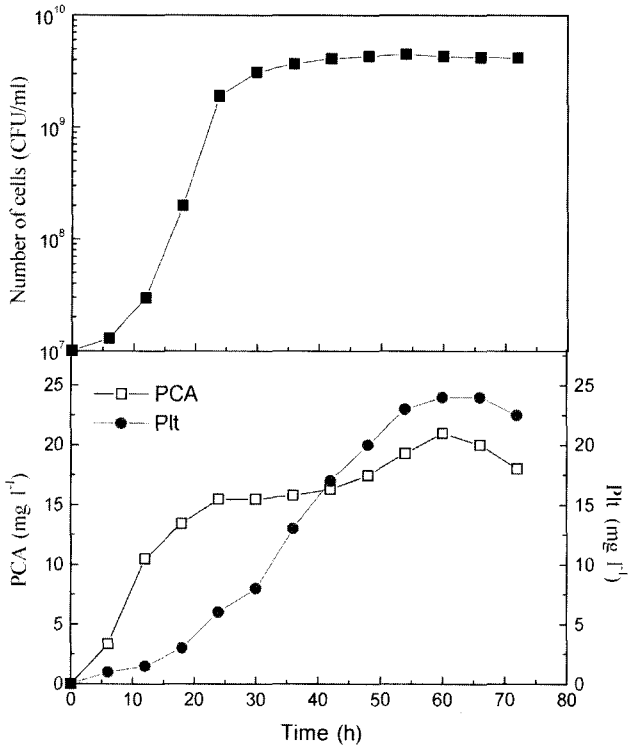

Fig. 3. Time course of strain M-18 and production of PCA and Plt in King's B medium.

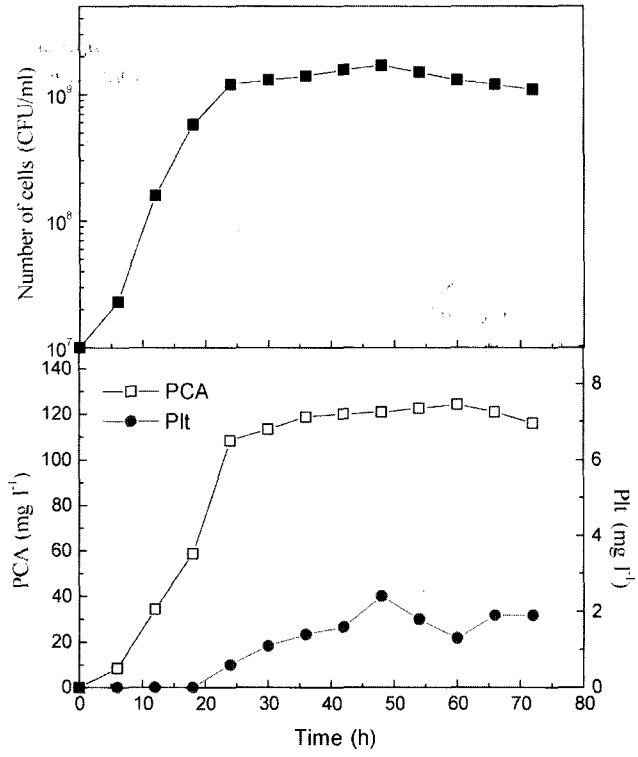

Fig. 4. Time course of strain M-18 and production of PCA and Plt in PPM medium.

Acknowledgment

This study was supported by a grant from the National Natural Sciences Foundation of China (No. 30370041).

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