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# A novel semi-selective medium for *Pseudomonas protegens* isolation from soil samples

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ABSTRACT

*Pseudomonas protegens* is a rhizosphere pseudomonad with a high agronomical potential (entomopathogenic and beneficial to plants) and bio-catalytic activities, but no selective medium has been described for its isolation. We developed a semi-selective minimum agar medium for the specific isolation and growth of *P. protegens*. We searched for both (i) a carbon source allowing the growth of *P. protegens* but potentially inhibiting the growth of other pseudomonads and (ii) an antimicrobial agent suppressing other members of the bacterial rhizosphere community. The M9-PP-agar medium consists of M9 base agar with adipic acid as the only carbon source and Irgasan® as an anti-bacterial agent. We tested the selectivity and sensitivity of M9-PP-agar by measuring the growth of 68 bacterial strains from 36 different species on this medium. Ten of the species tested were able to grow on M9-PP-agar medium: four species from the *Pseudomonadaceae (Pseudomonas aeruginosa, Pseudomonas protegens, Pseudomonas putida, Stenotrophomonas maltophilia*) as well as *Achromobacter xylosoxidans, Agrobacterium tumefaciens, Brevundimonas* sp., *Serratia liquefaciens*, *Serratia marcescens* and *Variovorax paradoxus*. All colonies were white, except for those of *P. protegens* (12 strains), which were typically brown. We demonstrated the efficiency of the M9-PP agar medium for *P. protegens* isolation, by inoculating two soils with the reference strain *P. protegens* CHAO$^T$ and then reisolating them. We also developed a *fitF*-PCR test targeting a regulator gene of the insecticidal *P. protegens fit* locus, for the rapid molecular detection of *P. protegens* colonies. We, therefore, developed a highly specific process for the routine isolation of new *P. protegens* strains from the soil environment, based on the use of a semi-selective medium and the specific color of colonies.

## 1. Introduction

*Pseudomonas* is a large and diverse genus that is ubiquitous in the environment and important for environmental processes. The bacteria of this genus have various lifestyles and live in a wide range of environments, including soil, water, plants and animals (Hesse et al., 2018). The genus *Pseudomonas* has a highly complex phylogeny, containing more than 190 species grouped into several phylogroups (Peix et al., 2018).

The *Pseudomonas fluorescens* phylogroup is very diverse and comprises plant beneficial strains from the rhizosphere (Mendes et al., 2013; Venturi and Keel, 2016). The plant-beneficial activities of rhizosphere pseudomonads have long been studied. They include the stimulation of plant growth and defense, the mobilization of soil nutrients and the suppression of phytopathogenic fungi, protists and bacteria through the production of antimicrobial compounds such as 2,4-diacetylphloroglucinol (Phl), phenazines (Phz), pyrrolnitrin (Prn), pyoluteorin (Plt), hydrogen cyanide (Hcn), cyclic lipopeptides (Clp) and rhizoxin (Rzx) analogs (Flury et al., 2017; Gross and Loper, 2009; Haas and Défago, 2005; Loper et al., 2008).

One distinct phylogenetic subgroup of *P. fluorescens*, sub-clade 1, containing strains of *Pseudomonas protegens* and *Pseudomonas chlororaphis*, has also potent insecticidal activities (Flury et al., 2016; Keel, 2016). The entomopathogenic activity of *P. protegens* and *P. chlororaphis* is associated with exoproducts and cell surface components such as chitinase, phospholipase C, O-antigen polysaccharide, Clp, Rzx and Hcn (Flury et al., 2017). Insecticidal activity is also dependent on the *fit* locus (Flury et al., 2016; Péchy-Tarr et al., 2013, 2008; Ruffner et al., 2013). FitF, a sensor-histidine kinase, detects the insect environment and induces the production of the FitD insecticidal toxin, specifically

*Abbreviations:* ADI, Adipic acid; CDS, Causse-De-La-Selle; CFU, Colony-forming-units; Clp, Cyclic lipopeptides; Hcn, Hydrogen cyanide; LB, Luria Bertani; MIC, Minimal inhibitory concentration; MTF, Montferriez-sur-Lez; PBS, Phosphate Buffered Saline; PCR, Polymerase Chain Reaction; Phl, 2,4-diacetylphloroglucinol; Phz, Phenazines; Plt, Pyoluteorin; Prn, Pyrrolnitrin; Rzx, Rhizoxin

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during insect infection but not on roots (Kupferschmied et al., 2014).

The species *P. protegens* was recently created after a polyphasic study based on molecular and phenotypic methods. This approach led to the requalification as *P. protegens* strains of strains previously classified as *P. fluorescens*, such as the CHAO$^T$ and the Pf5 strains (Ramette et al., 2011). Within the *P. fluorescens* group, *P. protegens* strains are the only strains that produce both the antimicrobial compounds Phl and Plt (Phl + Plt + *Pseudomonas* strains) (Flury et al., 2016; Ramette et al., 2011). The species *P. protegens* is of interest to several scientific communities focusing on the development of bacteriological control agents, plant growth promoting agents (Flury et al., 2019; Ryu et al., 2020; Vacheron et al., 2019) and new bio-catalytic activities (Mevers et al., 2019; Wilkes et al., 2019). Moreover, we recently showed that *P. protegens* is a member of microbiota associated with entomopathogenic nematodes (Ogier et al., 2020).

It is widely agreed that the success of culture-based techniques depends primarily based on the availability of a suitable culture medium. Most of culture media are selective for particular subsets of the total bacterial community and several different media are, thus, required to isolate microorganisms from different niches (Stevens, 1995). The development of selective media for *Pseudomonas* is a long-standing challenge. Members of the genus *Pseudomonas* are usually detected on the basis of their capacity to produce fluorescent pigments on the King media (King et al., 1954), but these media cannot distinguish between species. A selective medium has been developed for the isolation of several *Pseudomonas* species from water (Krueger and Sheikh, 1987). Efforts have mostly focused on the isolation of *P. aeruginosa*, a bacterial species pathogenic to humans that is frequently encountered as a contaminant of industrial processes and products (Weiser et al., 2014). Irgasan®, cetrimide and nalidixic acid are typically used as selective agents for isolation of *P. aeruginosa* (Fonseca et al., 1986; Lilly and Lowbury, 1972). Other media have also been designed to enhance pigment production, because the synthesis of pyocyanin is an unequivocal marker that can be used to identify *P. aeruginosa* (Smith and Dayton, 1972). However, despite growing interest in rhizosphere pseudomonads, particularly within agroindustry, there are currently no culture media for the specific isolation of these bacteria.

In the context of renewed interest in culturomics, an approach complementary to metagenomics for studying the functional biology of microorganisms in their ecosystems (Carini, 2019), we aimed to developed a selective medium for *P. protegens* strains. We described here the development of a new selective medium composed of a M9 minimal base agar supplemented with adipic acid as the carbon source and Irgasan® as a selective inhibitory agent. Based on both growth and the typical brown color of the colonies obtained, M9-PP agar medium was found to be highly sensitive and specific for *P. protegens*. We also assessed the efficacy of M9-PP agar medium for the isolation of *P. protegens* from soil matrices.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

The 75 strains used in this study belong to 39 species (12 species from the *Pseudomonadaceae*, and 27 are other Gram-negative species) isolated mostly from environmental sources (Table 1). Bacteria were isolated on nutrient agar medium (BD Difco™, Franklin Lakes, New Jersey, USA) plates incubated at 28 °C for 24 h. Bacteria were routinely grown at 28 °C in Luria-Bertani broth (LB) (Becton, Dickinson and Company, NJ, USA) or Mueller-Hinton broth (Biokar, Allonne, France) overnight (12*h*) in an orbital shaker at 120 rpm. For short-term bacterial storage (1 month), agar plates were stored at 4 °C. For long-term storage, bacterial suspensions in LB broth supplemented with 15% glycerol were stored in cryotubes (Nalgene®, USA) at −80 °C.

### 2.2. Carbohydrate source screening

Conventional determinations of carbohydrate assimilation were performed with the API®20 NE System, according to the manufacturer's instructions (bioMérieux S.A, Marcy l'Étoile, France). Briefly, overnight cultures of bacteria in LB broth were adjusted to a turbidity equivalent to a 0.5 McFarland standard. We diluted 200 µL of bacterial suspension in an ampule of API AUX medium. This suspension was dispensed immediately into the cupules of API test strips and incubated at 28 °C for 48 h.

### 2.3. Assessment of minimal inhibitory concentration (MIC)

*In vitro* susceptibility tests to determine the MIC of Irgasan® (5-Chloro-2-(2,4-dichlorophenoxy) phenol, Sigma-Aldrich, USA) were performed by the broth microdilution method. Irgasan® is not very soluble in water, and this problem was overcome by diluting a stock solution in absolute ethanol to a concentration of 20 mg/mL. The solution for MIC assessment was then prepared so as to obtain a concentration of 4 mg/mL of Irgasan® in 60% ethanol. Serial two-fold dilutions of Irgasan® were added directly to the wells of 96-well microtiter plates. A bacterial *inoculum* of $10^4$ colony forming units (CFU) grown overnight in LB broth was dispensed into Mueller-Hinton broth in each microdilution well. MICs were recorded relative to the positive growth control, a well without antibiotic. MICs values were determined, by eye, after incubation in microtiter plates at 28 °C for 48 h.

### 2.4. Preparation of M9-PP-agar medium

M9-PP-agar medium (pH 7) was prepared by mixing equal volumes of 2× basal M9-PP and preheated (45 °C) 3 g/L granulated agar (Difco™). 2× M9-PP consists of 2× M9 minimal salts (Difco™), 0.2 mM $CaCl_2$, 4 mM $MgSO_4$, 20 mM adipic acid (Sigma-Aldrich, USA) and 20 µg/mL Irgasan®. Stock solutions of the components of M9-PP were sterilized by heating at 120 °C for 20 min, with the exception of the adipic acid stock solution (0.1 M), which was passed through a filter with 0.22 µm pores (Millipore membrane PVDF, 0.22 µm-pore filters). We poured M9-PP-agar medium into Petri dishes and allowed it to cool under sterile laminar air-flow. Plates were stored at 4 °C until use, in accordance with standard practice.

### 2.5. Preparation of soil suspension

Soils were sampled at a depth of 20 cm from riverside land around Montpellier in the South of France: on the banks of the River Hérault near Causse-De-La-Selle (CDS; N43°49.884′ E003°41.222′) and the banks of the River Lez near Montferrier-sur-Lez (MTF; N43°40.801′ E003°51.835′). At each sampling site, a soil matrix composed of 10 g of soil diluted in 5 mL of phosphate-buffered saline (PBS, GIBCO$^R$ Invitrogen, Carlbad, California, USA) was inoculated with a bacterial suspension of *P. protegens* CHAO$^T$ containing $1.2 \times 10^6$ CFU. The inoculated soil matrix was mixed vigorously in a Falcon tube for 1 min at room temperature. A working soil suspension was prepared in PBS (20% final concentration). We plated 100 µL of each 10-fold serial dilution of the working soil suspension on nutrient agar medium as a growth positive control, and on M9-PP-agar medium, and incubated the plates at 28 °C for 48 h.

### 2.6. Development of a specific PCR test for rapid identification of *P. protegens* isolates

We first searched for a gene specific to *P. protegens* genomes by *in silico* analysis with the Gene Phyloprofile tool on the MaGe annotation platform (http://www.genoscope.cns.fr/agc/mage). Using bidirectional best hit, a minimal alignment coverage of 0.8, and an amino acid

**Table 1**
Gram-negative species included in this study.

| Species | Strains | Source | References |
|---|---|---|---|
| *Pseudomonadaceae* | | | |
| *Pseudomonas aeruginosa* | DSM 1117 (ATCC27853) | DSMZ collection | Machreki et al. (2019) |
| *P. aeruginosa* | 23NWF | E. Bilak | This study |
| *Pseudomonas alcaliphila* | SLB27 | E. Bilak | This study |
| *Pseudomonas brassicacearum* | CFBP 5593$^T$ | O. Berge | Achouak et al. (2000), Delorme et al. (2002) |
| *Pseudomonas chlororaphis* | PcSg_SK39 ApoA | DGIMI collection | |
| *Pseudomonas chlororaphis* subsp. *aureofaciens* | LMG 1245$^T$ | C. Keel | Flury et al. (2016), Peix et al. (2007), Ramette et al. (2011) |
| *P. chlororaphis* subsp. *chlororaphis* | CFBP 2132$^T$ | C. Keel | Achouak et al. (2000), Delorme et al. (2002) |
| *P. chlororaphis* subsp. *piscium* | DSM 21509 | C. Keel | Burr et al. (2010), Flury et al. (2016) |
| *P. chlororaphis* subsp. *piscium* | PCL1391 | C. Keel | Flury et al. (2016) |
| *Pseudomonas corrugata* | CFBP 5307 | O. Berge | CFBP Collection |
| *Pseudomonas fluorescens* | CFBP 2102$^T$ | O. Berge | Achouak et al. (2000), Delorme et al. (2002), Ramette et al. (2011) |
| *Pseudomonas luteola* | 18 M | E. Bilak | This study |
| *Pseudomonas oryzihabitans* | 8 NUD | E. Bilak | This study |
| *Pseudomonas protegens* | CHAO$^T$ | A. Jousset | Flury et al. (2016), Jousset et al. (2014) |
| *P. protegens* | Pf5 | C. Keel | Howell (1979), Ramette et al. (2011) |
| *P. protegens* | PGNR1 | C. Keel | Flury et al. (2016), Keel et al. (1996), Ramette et al. (2011) |
| *P. protegens* | K94. 41 | C. Keel | Flury et al. (2016), Wang et al. (2001) |
| *P. protegens* | PF | C. Keel | Flury et al. (2016), Levy et al. (1992), Ramette et al. (2011) |
| *P. protegens* | PpSc_PP-SC-10 | DGIMI collection | Ogier et al. (2020) |
| *P. protegens* | PpSj_SJ1 | DGIMI collection | This study |
| *P. protegens* | PpSg_SG5APO | DGIMI collection | This study |
| *P. protegens* | PpSw_3Z | DGIMI collection | This study |
| *P. protegens* | PpSw_TCH07 2–2 | DGIMI collection | Ogier et al. (2020) |
| *P. protegens* | PpSw_SW3 | DGIMI collection | This study |
| *P. protegens* | PpSw_SW4 | DGIMI collection | Ogier et al. (2020) |
| *Pseudomonas putida* | CFBP 2066$^T$ | O. Berge | Achouak et al. (2000), Delorme et al. (2002), Ramette et al. (2011) |
| *P. putida* | PpuSg_SK39–3 | DGIMI collection | This study |
| *P. putida* | PpuSw_SW5 | DGIMI collection | This study |
| *Pseudomonas syringae* | CFBP 1392$^T$ | O. Berge | Ramette et al. (2011) |
| *Stenotrophomonas maltophilia* | StmSc_ALL5 | DGIMI collection | Ogier et al. (2020) |
| *S. maltophilia* | StmSc_B163 | DGIMI collection | Ogier et al. (2020) |
| *S. maltophilia* | StmSw_SW1 | DGIMI collection | Ogier et al. (2020) |
| *S. maltophilia* | StmHb_A3 | DGIMI collection | This study |
| *S. maltophilia* | 33 NUD | E. Bilak | This study |
| *S. maltophilia* | RT 257–5 | F. Aujoulat | This study |
| *Other Gram-negative species* | | | |
| *Achromobacter xylosoxidans* | AchxSc_DD44 | DGIMI collection | Ogier et al. (2020) |
| *A. xylosoxidans* | AchSc_D7–1 | DGIMI collection | Ogier et al. (2020) |
| *A. xylosoxidans* | AchxHb_B6 | DGIMI collection | This study |
| *A. xylosoxidans* | CECT 927 | F. Aujoulat | This study |
| *Acidovorax sp* | AciSx_173 | DGIMI collection | This study |
| *Acidovorax sp* | AciSx_175 | DGIMI collection | This study |
| *Acinetobacter baumanii* | 24 NW5 | E. Bilak | This study |
| *Acinetobacter calcoacaeticus* | RT 52–1 | F. Aujoulat | This study |
| *Acinetobacter lwoffii* | 25 OAT | E. Bilak | This study |
| *Aeromonas hydrophila* | ATCC 7966 | F. Aujoulat | Popoff and Véron (1976) |
| *Agrobacterium tumefaciens* | CFBP 2413$^T$ | F. Aujoulat | Kersters et al. (1973) |
| *A. tumefaciens* | AgrtSc_B10–2 | DGIMI collection | This study |
| *A. tumefaciens* | AgrtHb_AB 35–9 | DGIMI collection | This study |
| *Alcaligenes faecalis* | ATCC 8750 | F. Aujoulat | Yamamoto et al. (1992) |
| *Brevundimonas* sp. | BreSc_ALL3 | DGIMI collection | Ogier et al. (2020) |
| *Enterobacter cloacae* | 14 OAT | E. Bilak | This study |
| *Escherichia coli* | CIP 76.24 | I. Pasteur collection | Coyle et al. (1976) |
| *Klebsiella aerogenes* | RT 257–1 | F. Aujoulat | This study |
| *Klebsiella oxytoca* | RT 257–4 | F. Aujoulat | This study |
| *Ochrobactrum anthropi* | ATCC 49188$^T$ | E. Bilak | Holmes et al. (1988) |
| *O. anthropi* | OchaSc_ALL4 | DGIMI collection | Ogier et al. (2020) |
| *O. anthropi* | OchaSw_SW2 | DGIMI collection | Ogier et al. (2020) |
| *O. anthropi* | OchaHb_A2 | DGIMI collection | This study |
| *Ochrobactrum intermedium* | LMG 3301$^T$ | E. Bilak | Velasco et al. (1998) |
| *Pantoea agglomerans* | CIP A181 | F. Aujoulat | I. Pasteur collection 1949 |
| *Photorhabdus luminescens laumondii* | PholHb_TT01 | DGIMI collection | Duchaud et al. (2003) |
| *Proteus mirabilis* | RT 312–1 | F. Aujoulat | This study |
| *Rhizobium cellolosilyticum* | RT 322–3 | F. Aujoulat | This study |
| *Serratia liquefaciens* | T5–1 | DGIMI collection | This study |
| *Serratia marcescens* | RT 431–1 | F. Aujoulat | This study |
| *Serratia* sp. | SerSw_TUR03 2.4 | DGIMI collection | This study |
| *Sphingobacterium multivorum* | RT 35–3 | F. Aujoulat | This study |
| *Sphingomonas paucimobilis* | AP 172 | F. Aujoulat | This study |
| *Variovorax paradoxus* | VpSc_ALL2 | DGIMI collection | This study |
| *V. paradoxus* | VpSwb_1SWB | DGIMI collection | This study |
| *V. paradoxus* | VpHb_BS32–1 | DGIMI collection | This study |
| *V. paradoxus* | VpHb_AB33–2 | DGIMI collection | This study |

(*continued on next page*)

**Table 1** (*continued*)

| Species | Strains | Source | References |
|---|---|---|---|
| *V. paradoxus* | RT 306–2 | F. Aujoulat | This study |
| *Xenorhabdus nematophila* | XnSc_F1 | DGIMI collection | Akhurst and Boemare (1988) |
| *Xenorhabdus bovienii* | XbSw_CS03 | DGIMI collection | Bisch et al. (2015) |

sequence identity of 50% as homology constraints, we identified several genes, including the *fitF* gene encoding the histidine-kinase sensor of the insecticidal *fit* locus, which is present only in strains from subclade 1 of *P. fluorescens* (Flury et al., 2016). The nucleotide sequences of *fitF* of from *P. fluorescens* sub-clade 1 strains were aligned with the Multialin platform (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988). Based on this alignment, we manually designed primers PFL2985F (5′−CCT GGT GTT CTT GCT GTT GCT CT−3′; positions 891–913 of the *fitF* gene) and PFL2985R (5′−CGC TGC ATG ATC TTG CCG ATG T−3′; positions 1274–1295 of the *fitF* gene). Primer quality (control for self-dimerization, hairpin formation, and self-annealing of 3′ and 5′ ends) was checked with the OligonucleotideCalculator tool (http://biotools.nubic.northwestern.edu/OligoCalc.html) and specificity was checked *in silico* on a large panel of publicly available complete sequenced genomes with the "Blast and Pattern Search" web tool implemented on the MaGe annotation platform.

### 2.7. DNA extraction, PCR amplification and sequencing

Bacterial genomic DNA was extracted with the QIAamp DNA mini kit (QIAGEN, Germany) according to the manufacturer's instructions. For the molecular identification of bacterial isolates, a near full-length fragment of the 16S rRNA gene was amplified (hybridization temperature: 58 °C; theoretical size of the expected amplicon: 1500 bp) and sequenced as previously described (Tailliez et al., 2006) with the primers SP1 (5'−GAAGAGTTTGATCATGGCTC−3′, corresponding to the *Escherichia coli* 16S rRNA gene, positions 6–25, forward) and SP2 (5'−AAGGAGGTGATCCAGCCGCA−3′, corresponding to the *Escherichia coli* 16S rRNA gene, positions 1522–1540, reverse). For the specific molecular detection of *P. protegens*, the *fitF* gene was amplified with the primers PFL2985F and PFL2985R (theoretical size of the expected amplicon: 405 bp long) with the *Taq* G2 Flexi (Promega, Madison, WI, USA) according to the manufacturer's instructions. PCR amplifications were performed in a T100™ Thermal Cycler (BIO-RAD, USA). PCR products were subjected to electrophoresis in a 1% agarose gel in 1× Tris-Acetate-EDTA buffer. DNA Ladder Plus (Euromedex, France) was used as a size marker. Sequencing was performed by Eurofins, Germany.

## 3. Results

### 3.1. Carbon source assimilation

We used API®20 NE strips to assess the assimilation of 12 carbon sources by the 35 strains from the *Pseudomonadaceae* and the other 23 other Gram-negative. Adipic acid (ADI) was selectively used by *P. aeruginosa*, *P. protegens, Achromobacter xylosoxidans* and *Variovorax paradoxus* (Table 2). We therefore considered ADI to be a potentially interesting carbon source for the selective medium for *P. protegens*.

### 3.2. Anti-bacterial agent susceptibility

We determined the MICs of nalidixic acid and Irgasan®, two selective agents widely used for *P. aeruginosa* isolation, for a set of bacteria from the *Pseudomodanaceae* and other Gram-negative bacteria. The MICs of both antibacterial agents exceeded 1 mg/mL for *P. protegens*. However, more species were resistant to nalidixic acid than to Irgasan®. Moreover, the low solubility of nalidixic acid in aqueous solution made it difficult to use high concentrations of this agent. We then specifically assessed the MIC of Irgasan® for 30 bacterial species. In addition to *P. aeruginosa,* we found that the species *P. protegens, P. chlororaphis, P. putida*, *Stenotrophomonas maltophilia, A. xylosoxidans,* and *Agrobacterium tumefaciens* displayed resistance to Irgasan®, with a MIC between 100 µg/mL and 1 mg/mL. By contrast, *Variovorax paradoxus*, *Ochrobactrum anthropi, Escherichia coli, Xenorhabdus nematophila*, *X. bovienii* and *Photorhabdus luminescens* were susceptible to Irgasan® (MIC < 1 µg/mL). We therefore chose the Irgasan® as the antibacterial agent for the *P. protegens* selective agar medium.

### 3.3. Sensitivity and specificity of the M9-PP-agar medium

The *P. protegens* selective agar medium, which we named M9-PP-agar, consisted of M9 base, with 10 mM ADI as the only carbon source, 10 µg/mL Irgasan® as the antibacterial agent and 15 g/L agar. We evaluated the efficiency of the M9-PP-agar medium for the selective growth of *P. protegens* by patching a subset of 68 strains from 36 different bacterial species onto M9-PP-agar medium and onto nutrient agar as a growth positive control. Overall, 31 of the strains tested were able to grow on M9-PP-agar medium (Table 3). These strains belonged to the species *P. aeruginosa, P. protegens*, *P. putida, S. maltophilia, A. xylosoxidans, A. tumefaciens, Serratia liquefaciens, Serratia marcescens* and *V. paradoxus*. No growth was observed after 72 h of incubation at 28 °C for the other strains, which included the phylogenetically close species *P. chlororaphis*.

On the basis of bacterial growth alone, the M9-PP-agar medium had a sensitivity (number of true positives divided by the number of true positives + number of false negatives, multiplied by 100) of 100%, but a specificity (number of true negatives divided by the number of true negatives + number of false positives, multiplied by 100) of only 75%. However, the colonies of the 12 *P. protegens* strains developed a typical brown color after three days on the M9-PP-agar medium. We confirmed this feature by spotting 10 µL of cultures on M9-PP-agar medium and by incubating the plates for 72 h at 28 °C. None of the other species able to grow on this medium displayed this brown pigmentation (Fig. 1). The observation of both growth and a typical brown color of colonies therefore distinguishes *P. protegens* colonies from colonies of other strains growing on M9-PP-agar medium, rendering M9-PP-agar medium highly specific for *P. protegens* detection.

### 3.4. Assessment of the M9-PP-agar medium performance on soil samples

We evaluated the performance of M9-PP-agar medium on two complex matrices: two soil samples inoculated with *P. protegens* CHAO$^T$. After 48 h of incubation at 28 °C, significantly lower levels of fungal and bacterial growth were observed on M9-PP-agar medium than a on nutrient agar. Indeed, we counted 30 CFU for *P. protegens* for the MTF soil and 80 for the CDS soil on M9-PP-agar, whereas CFU were too numerous to count on nutrient agar at the same dilution (dilution $10^{-1}$). CFU only became countable on nutrient agar at dilution $10^{-3}$ (another two orders of magnitude). Photographs of the plates for CDS soil are shown in Fig. 2.

### 3.5. Rapid molecular identification of P. protegens colonies with a fitF-PCR test

We developed a specific *fitF*-PCR test for rapid identification *of P.*

**Table 2**
Utilization of 12 carbon sources by members of the *Pseudomonadaceae* and other Gram-negative species (1–12 strains per species), as assessed with the API 20NE identification kit.[a]

| | GLU | ARA | MNE | MAN | NAG | MAL | GNT | CAP | ADI | MLT | CIT | PAC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species (number of strains) | D-glucose | L-arabinose | D-mannose | D-mannitol | *N*-acetyl-glucosamine | D-maltose | Potassium gluconate | Capric acid | Adipic acid | Malic acid | Trisodium citrate | Phenylacetic acid |
| *Pseudomonas aeruginosa* (2) | + | − | − | + | + | − | + | + | + | + | + | − |
| *Pseudomonas alcaliphila* (1) | + | − | + | + | + | − | + | + | − | + | + | − |
| *Pseudomonas brassicacearum* (1) | + | + | + | + | + | + | + | + | − | + | + | − |
| *Pseudomonas chlororaphis* (5) | + | v | + | + | + | v | + | + | − | + | + | v |
| *Pseudomonas corrugata* (1) | + | + | + | + | + | − | + | + | − | + | + | − |
| *Pseudomonas fluorescens* (1) | + | + | + | + | + | − | + | + | − | + | + | − |
| *Pseudomonas luteola* (1) | + | + | + | + | − | + | + | + | − | + | + | − |
| *Pseudomonas oryzihabitans* (1) | + | + | + | + | + | + | + | + | − | + | + | − |
| *Pseudomonas protegens* (12) | + | − | + | + | + | v | + | + | + | + | + | + |
| *Pseudomonas putida* (3) | + | − | + | v | − | v | + | + | − | + | + | v |
| *Pseudomonas syringae* (1) | + | + | + | + | − | − | + | + | − | + | + | − |
| *Stenotrophomonas maltophilia* (6) | + | − | + | − | + | + | v | v | − | + | + | − |
| *Achromobacter xylosoxidans* (4) | v | − | v | − | − | − | + | + | + | + | + | + |
| *Agrobacterium tumefaciens* (3) | + | + | + | + | + | + | + | − | − | + | v | − |
| *Brevundimonas* sp. (1) | + | − | − | − | + | + | − | − | − | + | − | − |
| *Ochrobactrum anthropi* (4) | + | + | v | − | + | v | v | v | − | + | v | − |
| *Ochrobactrum intermedium* (1) | + | + | + | + | + | + | + | + | − | + | + | − |
| *Photorhabdus luminescens* (1) | + | − | + | − | + | + | + | − | − | − | v | − |
| *Serratia liquefaciens* (1) | + | + | + | + | + | + | + | + | − | + | + | + |
| *Serratia marcenscens* (1) | + | + | + | + | + | + | + | + | − | + | + | + |
| *Variovorax paradoxus* (5) | + | + | + | + | v | − | + | v | + | + | v | + |
| *Xenorhabdus nematophila* (1) | + | + | + | + | + | − | − | − | − | + | − | − |
| *Xenorhabdus bovienii* (1) | + | − | + | − | + | + | + | − | − | − | − | − |

+, positive; −, negative; v, variable.
[a] API 20NE results were read after 48 h of incubation at 28 °C.

**Table 3**
Specificity of M9-PP-agar medium for growth of *Pseudomonas protegens* and few other Gram-negative bacteria.

| Species | Number of strains tested | Number of strains displaying growth | | |
|---|---|---|---|---|
| | | Nutrient agar medium | M9-PP-agar medium | |
| | | 24 h | 24 h | 72 h |
| *Pseudomonadaceae* | | | | |
| ***Pseudomonas aeruginosa*** | **2** | **2** | **1** | **2** |
| *Pseudomonas alcaliphila* | 1 | 1 | 0 | 0 |
| *Pseudomonas brassicacearum* | 1 | 1 | 0 | 0 |
| *Pseudomonas chlororaphis* | 5 | 5 | 0 | 0 |
| *Pseudomonas corrugata* | 1 | 1 | 0 | 0 |
| *Pseudomonas fluorescens* | 1 | 1 | 0 | 0 |
| *Pseudomonas luteola* | 1 | 1 | 0 | 0 |
| *Pseudomonas oryzihabitans* | 1 | 1 | 0 | 0 |
| ***Pseudomonas protegens*** | **12** | **12** | **11** | **12** |
| ***Pseudomonas putida*** | **3** | **3** | **3** | **3** |
| *Pseudomonas syringae* | 1 | 1 | 0 | 0 |
| ***Stenotrophomonas maltophilia*** | **5** | **5** | **1** | **3** |
| Total | 34 | 34 | 16 | 20 |
| *Other Gram-negative species* | | | | |
| ***Achromobacter xylosoxidans*** | **3** | **3** | **0** | **3** |
| *Acidovorax* sp. | 2 | 2 | 0 | 0 |
| *Acinetobacter baumanii* | 1 | 1 | 0 | 0 |
| *Acinetobacter calcoaceticus* | 1 | 1 | 0 | 0 |
| *Acinetobacter lwoffi* | 1 | 1 | 0 | 0 |
| *Aeromonas hydrophila* | 1 | 1 | 0 | 0 |
| ***Agrobacterium tumefaciens*** | **3** | **3** | **1** | **1** |
| *Alcaligenes faecalis* | 1 | 1 | 0 | 0 |
| ***Brevundimonas* sp.** | **1** | **1** | **1** | **1** |
| *Enterobacter cloacae* | 1 | 1 | 0 | 0 |
| *Escherichia coli* | 1 | 1 | 0 | 0 |
| *Klebsiella aerogenes* | 1 | 1 | 0 | 0 |
| *Klebsiella oxytoca* | 1 | 1 | 0 | 0 |
| *Ochrobactrum anthropi* | 2 | 2 | 0 | 0 |
| *Pantoea agglomerans* | 1 | 1 | 0 | 0 |
| *Photorhabdus luminescens* | 1 | 1 | 0 | 0 |
| *Proteus mirabilis* | 1 | 1 | 0 | 0 |
| *Rhizobium cellolosilyticum* | 1 | 1 | 0 | 0 |
| ***Serratia liquefaciens*** | **1** | **1** | **0** | **1** |
| ***Serratia marcescens*** | **1** | **1** | **0** | **1** |
| *Sphingobacterium multivorum* | 1 | 1 | 0 | 0 |
| *Sphingomonas paucimobilis* | 1 | 1 | 0 | 0 |
| ***Variovorax paradoxus*** | **5** | **5** | **0** | **4** |
| *Xenorhabdus nematophila* | 1 | 1 | 0 | 0 |
| Total | 34 | 34 | 2 | 11 |

Species growing on M9-PP-agar medium are indicated in bold.

*protegens* isolates. We tested the *fitF*-PCR assay on 18 bacterial species. We observed an intense 400 bp band for the DNA of six *P. protegens* strains (data not shown). No amplicon could be detected for the DNA of three Gram-negative strains and six other members of the *Pseudomonaceae*, including *P. aeruginosa*, *P. putida* and *S. maltophilia*, all of which were able to grow on M9-PP-agar medium. We detected an amplicon of variable intensity with DNA from *P. chlororaphis* strains. However, we had already found that this species did not grow on M9-PP-agar medium.

We used this *fitF*-PCR test to validate the taxonomic identity of the colonies isolated from soil on M9-PP-agar medium (see above, Section 3.4). The colonies were picked from M9-PP-agar plates and subjected to the *fitF*-PCR assay. We found that 100% of the brown colored colonies were *fitF* positive. Genomic DNA was isolated from the colonies giving negative results on *fitF*-PCR and the 16S rRNA gene was sequenced. The colonies testing negative by *fitF*-PCR were identified as *A. xylosoxidans, Pseudomonas* sp. and *S. maltophilia*.

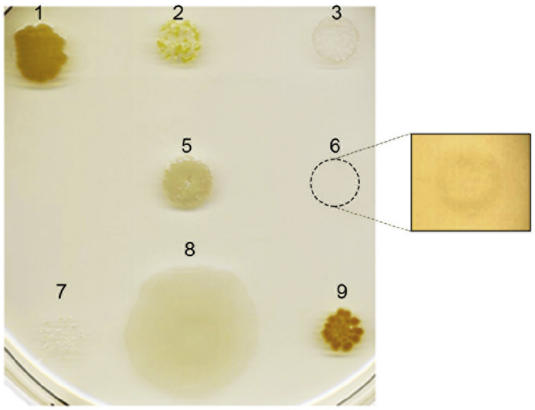


**Fig. 1.** Appearance of spots from different bacterial cultures on M9-PP-agar medium.
We deposited 10 µL of exponentially growing culture on M9-PP-agar plates, which we then incubated for 72 h of incubation at 28 °C. Colonies of *Pseudomonas protegens* PpSc_PP-SC-10 (1) and CHAO$^T$ (9) were brown whereas those of *Variovorax paradoxus* VpSc_ALL2 (2), *Serratia liquefaciens* T5-1 (3), *Achromobacter xylosoxidans* CECT 927 (5), *Stenotrophomonas maltophilia* StmSw_Sw1 (6), *Pseudomonas putida* CFBP2066$^T$ (7) and *Pseudomonas aeruginosa* DSM1117 (8) were white, yellow or beige. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 4. Discussion

We describe here a new selective medium, M9-PP-agar, for isolation of *P. protegens*. We took two main factors into account: (i) the composition of the basal medium, so as to allow the growth of *P. protegens* and potentially inhibit the growth of other members of the *Pseudomonadaceae* and (ii) the ability of selective agents to suppress the growth of other members of the bacterial community in a complex matrix. We supplemented M9 base agar with adipic acid, having shown that this carbon source was able to select against most members of the *Pseudomonadaceae* and Gram-negative bacteria. The assimilation of adipic acid by bacteria has been little investigated, but pseudomonads are generally able to utilize this dicarboxylic acid (Shih et al., 1972; Stieglitz and Weimer, 1985). M9-PP-agar medium also contains Irgasan®. At a concentration of 10 µg/mL, this antimicrobial agent inhibited the growth of a diverse range of environmental bacteria from outside the family *Pseudomonadaceae*. Irgasan® is typically used in selective media for *P. aeruginosa* (Fonseca et al., 1986; Lilly and Lowbury, 1972), but it is also included in CIN agar (Cefsulodin Irgasan® Novobiocin) for the specific isolation of *Yersinia* from foods or clinical samples (De Boer et al., 1982).

Based on growth alone, M9-PP-agar medium had a sensitivity of 100% for *P. protegens* isolation. Interestingly, species from the *Pseudomonadaceae* other than *P. protegens* (*P. aeruginosa*, *P. putida* and *S. maltophilia*) were also able to grow on the M9-PP-agar medium. M9-PP-agar medium is therefore likely to be of general interest to scientists and clinicians working on pseudomonads in medical contexts. Moreover, the typical brown color of *P. protegens* colonies observed after three days on M9-PP-agar medium increased the specificity for the detection of this species to 100%. A PflQ2_2232 locus closed to the *phl* cluster (2,4-diacetylphloroglucinol biosynthesis) and involved in the biosynthesis of a red pigment has been described in *Pseudomonas fluorescens* Q2–87 (Bangera and Thomashow, 1996). The function of the protein encoded by the PflQ2_2232 locus is unknown. This locus is conserved in all available *P. protegens* genomes (ortholog in *P. protegens* CHAO$^T$ strain: *phlI* with label PFLCHAO_c59130), but it is absent from the genomes of *P. chlororaphis*, *P. putida* and *P. aeruginosa*, which do not yield brown colonies on M9-PP-agar medium.

In the course of this study, we also developed a simple rapid

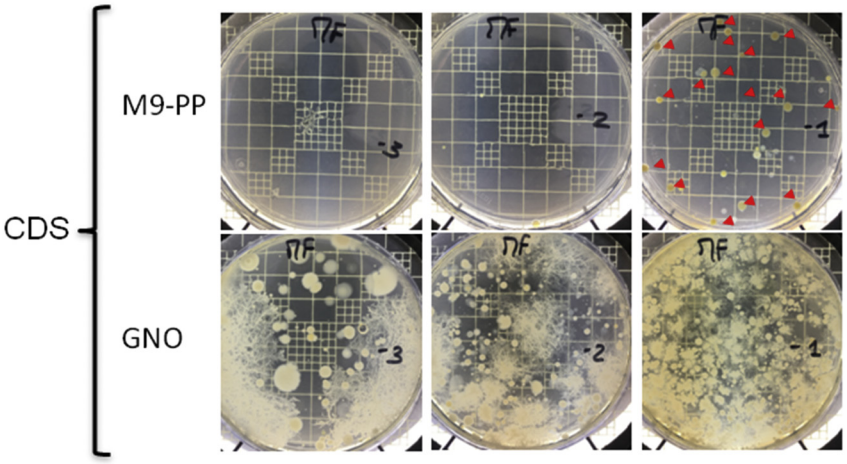


**Fig. 2.** Comparison of M9-PP-agar and nutrient agar plates after the spreading of diluted soil matrice containing *P. protegens* CHAO$^T$.
Soil sampled from the banks of the River Hérault river near Causse-De-La-Selle (CDS) was inoculated with $10^4$ CFU of *P. protegens* CHAO$^T$/g of soil. We plated $10^{-1}$ to $10^{-3}$ dilutions on M9-PP-agar and nutrient agar. Two orders of magnitude fewer CFU were counted on M9-PP-agar than on nutrient agar. On M9-PP-agar, brown colonies indicative of *P. protegens* are clearly distinguishable for the $10^{-1}$ dilution (red arrowheads). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

molecular tool for the specific identification of *P. protegens* colonies on the basis of *fitF* gene amplification. Within the *fit* insecticidal locus, the *fitF* gene encodes a sensor histidine kinase (Kupferschmied et al., 2014). This *fitF* gene has been shown to be present only in strains of *P. fluorescens* sub-clade 1 (Flury et al., 2016). This gene can, thus, be used to distinguish *P. protegens* from *Photorhabdus* and *Xenorhabdus* strains harboring the *fitD* gene but not the entire *fit* locus. This molecular method is less reliable for distinguishing *P. protegens* from *P. chlororaphis* because the amplicon detected for this latter species is of variable intensity. However, *P. chlororaphis* was unable to grow onto M9-PP-agar medium.

This work has many potential applications in biocontrol strategies for controlling plant pests. The new European directives require Member States to minimize the risks to human health and the environment associated with the use of chemical pesticides (Article 14 of Directive 2009/128 / EC). This may necessitate changes in agricultural practices, such as a decrease in the use of chemical pesticides and the development of new biological control agents. The properties of *P. protegens* renders this species of great potential interest as a “toolbox” for the development of a sustainable agronomy (Keel, 2016). Many academic studies described the plant protection properties of the *P. protegens* Pf5 and CHAO$^T$ strains (Flury et al., 2017), but this species is not yet commercially exploited. We are aware of two only strains of *Pseudomonas* being used to prevent and treat fungal infection of cereal seed in Europe: *P. chlororaphis* MA342 (trade name: Cerall) and a *Pseudomonas* sp. (trade name: Prorodyx) (Deravel et al., 2014; Tombolini et al., 1999). The isolation of new *P. protegens* strains with different biological properties of interest (ecological competence in the rhizosphere, strains that can be used in bacterial cocktails, *etc.*) is therefore a challenge for the development of novel bacteriological control agents. We show here that the M9-PP-agar medium is highly suitable for the screening required to identify such strains, because it can be used to both isolate and identify *P. protegens* from soils, the complex matrices that form the natural reservoir of *P. protegens*.

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## Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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