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Specific enumeration and analysis of the community structure of culturable pseudomonads in agricultural soils under no-till management in Argentina

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ABSTRACT

Members of the *Pseudomonas* genus have been isolated and identified worldwide as plant probiotic microorganisms. Little is known about the dynamics of pseudomonads population in extensive agricultural systems under the influence of biotic and abiotic factors. As part of the national consortium BIOSPAS devoted to study soil biology and its relationship with productivity, our lab began to characterize pseudomonad populations in agricultural soils under no-till management. We here report the set up and application of a combination of selective plating and PCR-RFLP analysis of the genus specific *gacA* and *oprF* genes, to quantify and study the community structure of culturable pseudomonads in bulk soil and rhizosphere samples. The specificity of both methods has been verified by 16S rDNA and *oprF* sequencing. The application of selective plating and *gacA*/*oprF* PCR-RFLP analyses revealed differences in the amount and predominant type of culturable pseudomonads among geographical locations, among treatments, and between rhizospheric and bulk soil. The results indicate that these simple methods prove useful to systematically monitor pseudomonads structure in bulk and rhizospheric soils along sampling periods covering crop rotations and agricultural practices at different locations.

1. Introduction

Bacteria are key players in soil ecosystems as participants in major nutrient cycles and interactions with other microorganisms and eukaryotes as components of the food web (Cho and Tiedje, 2000). In particular, bacteria have important roles in determining productivity of agricultural systems because certain species can cause crop diseases whereas other species can promote plant growth and/or health (Raaijmakers et al., 2009; Schippers et al., 1987). Among plant-beneficial bacteria, the pseudomonads have long been recognized as a group of plant probiotic microorganisms (Mercado-Blanco and Bakker, 2007), because of their good root-colonizing habit and a variety of mechanisms for plant growth stimulation or plant protection, including phytohormone production, phosphate solubilization, induced systemic resistance and production of antibiotics (Haas and Defago, 2005; Lugtenberg and Kamilova, 2009; Preston, 2004; Richardson et al., 2009). These traits make pseudomonads attractive microorganisms for development of agricultural inputs (Walsh et al., 2001). The genus *Pseudomonas* comprises motile Gram negative and oxidase positive bacilli that are almost ubiquitous in Earth, and it is characterized by its intrinsic genetic and physiological diversity (Peix et al., 2009). Representative species usually found in soil include the plant probiotic *P. fluorescens*, *P. putida*, *P. chlororaphis* and *P. stutzeri* (Haas and Defago, 2005), and the plant pathogens *P. syringae*, *P. cicchorii* and *P. savastanoi* (Peix et al., 2009). Specific plant probiotic strains appear to be enriched in the rhizosphere of different plants (Picard and Bosco, 2008).

No-till agriculture dominates cropping practices in Argentina (*ca.* 25 millions ha), representing more than 75% of the total cultivated area (source: AAPRESID; www.aapresid.org.ar). Compared to conventional tillage, this conservative practice tends to preserve soil carbon and water content, to reduce soil erosion and to improve soil aggregation (Derpsch et al., 2010). There are however, some concerns about the success of no-till practice related to the negative impact of market-driven monoculture (in particular, soybean) and to inadequate nutrient management. Consequently, agricultural producers recognized the need to promote good agricultural practices, including crop rotations and nutrient maintenance, in parallel to monitoring soil health to ensure long term productivity. Contrary to well established physical and chemical indicators of soil properties, biological indicators of soil quality and productivity are lacking. In this context, a national public–private research initiative (The BIOSPAS project) started in 2009 to identify biological indicators of soil quality and productivity in agricultural soils under no-till management in Argentina (Wall, 2011).

Despite the number of pseudomonads isolates characterized so far, their contribution to soil ecology is largely unexplored,

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particularly in extensive agricultural systems. Evidence gathered from various cropping systems at diverse geographical sites strongly suggest that the structure of pseudomonads communities in soil is influenced by the plant species, soil conditions, crop management system, and interactions with other biological agents like predators and competitors (Picard and Bosco, 2008). As part of the BIOSPAS consortium, we aim to characterize pseudomonad populations in soil and the influence that soil type, crop rotations and land management have on the community structure. To meet these goals, we rely on specific detection methods. For pseudomonads, three genes have been utilized for phylogenetic diversity studies at the genus levels: 16S rDNA, *gacA* and *oprF* (Bodilis et al., 2004; de Souza et al., 2003; Pesaro and Widmer, 2006). *gacA* encodes the cognate transcriptional regulator of the membrane associated sensor protein GacS. The GacS/GacA two-component system is involved in control of secondary metabolism, biocontrol and adaptation to environmental stresses (Heeb and Haas, 2001). *oprF* encodes the major outer membrane porin, possibly involved in the slow non-specific transmembrane diffusion of solutes (De Mot et al., 1994; Nestorovich et al., 2006). The *oprF* gene sequence has been utilized to characterize pseudomonads isolates (Bodilis et al., 2004; Bodilis et al., 2006), but it has not been exploited yet to characterize the structure of pseudomonads communities in environmental samples. We here report: (1) the procedure to study the size and community structure of culturable pseudomonads in bulk soil and rhizospheric soil samples from agricultural plots under no-till management in Argentina, based on a combination of selective plating and PCR-RFLP analyses using newly designed oligonucleotides targeting the genus specific *gacA* and *oprF* genes; (2) the results of the analysis of the first sampling of the BIOSPAS project that serve as a starting point of a future study of the dynamics of this bacterial group according to site and soil management.

2. Methods

2.1. Bacterial strains

The typed strains used to set up the PCR protocols are listed in Table 1. All strains were cultured on S1 medium (Gould et al., 1985) and on nutrient agar (NA; 40 g/L blood agar base; 5 g/L yeast extract), at 28 °C.

2.2. Primer design and PCR amplification

Oligonucleotides targeting conserved regions within *oprF* and *gacA* genes were designed manually, based on the alignment of reference sequences of both genes retrieved from GenBank database (April 2010; Supplementary Table S1). The alignments were performed with ClustalX version 1.64b (Thompson et al., 1994). Candidate primers were tested *in silico* using AmplifiX v.1.5.4 (available at http://ifrjr.nord.univ-mrs.fr/AmplifX), and the best primer pairs for each gene were evaluated *in vitro* by PCR, using a collection of target and non-target strains (Table 1). The properties of the newly designed primers are shown in Table 3.

PCR conditions were optimized to obtain a single amplified fragment. Typically, 25 μl-reaction mixtures contained 1× buffer (PB-L, Argentina), 0.2 mM dNTPs (PB-L, Argentina), 0.8 μM primers (Invitrogen, USA), 2.0 mM $MgCl_2$, 5% (v/v) DMSO (only for *gacA* PCR), and 1 U of Taq polymerase (PB-L, Argentina). The PCR cycle consisted in an initial denaturation step of 5 min at 94 °C, followed by 35 cycles of 1 min at 92 °C, 0.75 min at 57 °C (*oprF*) or 56 °C (*gacA*), and 0.75 min at 72 °C; and a final extension step at 72 °C for 5 min. After amplification, 2 μl of each reaction was run in 1% agarose gels in 0.5× Tris–borate–EDTA (TBE) at 10 V/cm for 50 min. The gel was stained with ethidium bromide and visualized under UV light.

2.3. PCR-RFLP

Restriction analysis of *oprF* and *gacA* genes was set up *in silico* with Clone Manager 7 v7.11 (Scientific & Educational Software, USA), using the expected amplicons generated with the designed primers (Table 3) on reference *oprF* and *gacA* sequences. A set of classical enzymes was tested to identify those producing differential RFLP patterns for each reference *Pseudomonas* species.

PCR-RFLP assays were carried out in a final volume of 20 μl, containing 10 μl of PCR mix and 2 U of the endonucleases *Hae*III (Promega) or *Taq*I (Fermentas) for *oprF* amplicons; and *Hae*III or *Mbo*I (Fermentas) for *gacA* products. Reactions were incubated at 37 °C (*Hae*III and *Mbo*I) or 62 °C (*Taq*I) for 3 h. The restriction products were separated by electrophoresis in 2% agarose gels in 0.5× TBE at 6 V/cm for 2 h. Gels were stained with ethidium bromide and DNA banding patterns were visualized under UV light.

2.4. Double check of Gould's S1 medium selectivity and oprF primer specificity

Fifty isolates (Table 2) from a variety of soils and plant root systems were randomly selected from S1 agar plates, trying to pick up different fluorescent or non fluorescent colony morphologies, to confirm the selective power of S1 medium and the specificity of the designed *oprF* primers. To this end, the 16S rDNA gene was PCR amplified with primers P0 and P6 (Picard et al., 2000) and the *oprF* gene was amplified with oprF-FW2 and oprF-Rev2 primers (Table 3). Partial DNA sequencing was carried out by Macrogen Inc. (Seoul, Korea) with primers P0 for 16S rDNA amplicons, and oprF-FW2 for *oprF* amplicons. The complete set of partial 16S rDNA and *oprF* sequences have been submitted to GenBank under Submission code 1461082. The obtained 16S rDNA sequences were used to query the Seqmatch tool of the Ribosomal database project II (Cole et al., 2009). Partial *oprF* sequences were analyzed with the BlastN tool, in the NCBI database.

Phylogenetic analyses were carried out with the partial 16s rRNA and *oprF* sequences of isolates. Sequences of reference strains obtained from GenBank were also included. Neighbor-joining trees were inferred from evolutionary distances calculated with the Kimura 2-parameter formula, using the software MEGA v4 (Tamura et al., 2007). Confidence analyses were undertaken using 1000 bootstrap replicates. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic analysis was also performed on sequence concatenation of conserved regions of the 16s rRNA (300 nt, from position 225 to 524 of the *P. fluorescens* Pf-5 16s rRNA gene; AJ417072), and *oprF* (276 nt, from position 282 to 558 of the *P. fluorescens* Pf-5 *oprF* gene; NC004129). This was done with the 50 isolates and some of the reference strains used for *oprF* gene phylogeny (when both sequences were available from GenBank), and following the same methodology.

2.5. Soil and plant root samples

The soil and root samples used to estimate pseudomonads counts were collected in agricultural fields located across a west-east transect in the most productive region in the Argentinean Pampas, specifically at Bengolea, Córdoba Province (33°01′31″S; 63°37′53″W); Monte Buey, Córdoba Province (32°58′14″S; 62°27′06″W); Pergamino, Buenos Aires Province (33°56′36″S; 60°33′57″W); and Viale, Entre Ríos Province (31°52′59″S; 59°40′07″W). In each location, three treatments were defined: (1) "Good Agricultural Practices" (GAP): sustainable agricultural management under no-till, subjected to intensive crop rotation, nutrient replacement, and minimized agrochemical use (herbicides, insecticides and fungicides); (2) "Bad Agricultural Practices"

Table 1
Strains used to set up PCR-RFLP protocols.

Strain designation	Origin	Reference or source
Pseudomonads		
P. fluorescens CHA0	Tobacco rhizosphere, Payerne, Switzerland	Stutz et al. (1986)
P. fluorescens Pf-5	Cotton rhizosphere, Texas, USA	Howell and Stipanovic (1979)
P. aeruginosa PAO1	Clinical isolate; human wound; Melbourne, Australia	Holloway (1969)
P. aeruginosa Hex1T	Hydrocarbon-contaminated soil	Pezza et al. (2002)
P. aeruginosa sp.	Clinical isolate; Hospital de Niños de La Plata, Argentina	Diego Noseda (CINDEFI – La Plata)
P. syringae pv. *maculicola* ES4326	Radish rhizosphere, USA	Dong et al. (1991)
P. putida KT2440	Grass rhizosphere, mt-2 derivative, Japan	Nakazawa (2002)
P. putida ATCC 17399	Psycrophilic; Western Utilization Research and Develop-ment Division of the US. Department of Agriculture, Albany, California	Stanier et al. (1966)
P. stutzeri AN10	Polluted marine sediments; western Mediterranean Sea	Rossello et al. (1991)
P. stutzeri ATCC 17588	Clinical isolate; human spinal fluid; Copenhagen, Denmark	Stanier et al. (1966)
P. stutzeri 2014	Clinical isolate, intrahospitalary infection, Buenos Aires, Argentina	Dr. D. Centrón (Facultad de Medicina, UBA)
P. stutzeri 2018	Clinical isolate, intrahospitalary infection, Buenos Aires, Argentina	Dr. D. Centrón (Facultad de Medicina, UBA)
P. mendocina 2013	Clinical isolate, intrahospitalary infection, Buenos Aires, Argentina	Dr. D. Centrón (Facultad de Medicina, UBA, Argentina)
P. mendocina 2019	Clinical isolate, intrahospitalary infection, Buenos Aires, Argentina	Dr. D. Centrón (Facultad de Medicina, UBA, Argentina)
Pseudomonas sp. LDe	Salina Grande, Jujuy, Argentina	Ordoñez et al. (2009)
Pseudomonas sp. N23	Laguna Negra, Catamarca, Argentina	Ordoñez et al. (2009)
Pseudomonas sp. CF5	Faeces from extreme high altitute wetland	Dr. M.E. Farías (LIMLA, Tucumán, Argentina)
Non pseudomonads		
Escherichia coli K12 MG1655	W1485 derivative, F^- λ^-	Bachmann (1987)
E. coli K12 TR1-5	BW3414 derivative, *csrA::kan*	Romeo et al. (1993)
Burkholderia cepacia	Environmental isolate	Diego Noseda (CINDEFI – La Plata)
Agrobacterium tumefaciens NTL4	Derivative of NT1, Δtet_{C58}	Luo et al. (2001)
Serratia marcescens AS-1	Soil isolate, AHL producer, Japan	Morohoshi et al. (2007)
Bacillus subtilis	Microbiología General, Universidad Nacional de Quilmes	Reference strain, lab stock.
Bacillus cereus	Microbiología General, Universidad Nacional de Quilmes	Reference strain, lab stock.
Shinorizobium meliloti 2011	Derivative of strain SU47, alfalfa root nodules, Australia	Meade and Signer (1977)
Azospirillum brasilense ATCC 29710	Mangrove rhizosphere	Tarrand et al. (1978)

(BAP): non-sustainable agricultural management under no-till with high crop monoculture, low nutrient replacement and high agrochemical use (herbicides, insecticides and fungicides); (3) "Natural Environment" (NE): as reference, natural grassland was selected in an area of approximately 1 ha close to the cultivated plots (less than 5 km), where no cultivation was practiced for (at least) the last 30 years. All sites were managed under no-till for at least the last 5 years, with the exception of 1 year (2004/2005) in Bengolea where the BAP site was chisel plowed. In the four localities GAP plots had on average 62% more maize in the crop rotation than the BAP plots. GAP had in the last 5 years 50% of the winters with crop whereas the BAP had only 20%. In addition, in the GAP, cover crops have been planted in winter in 3 out of 4 localities. The intensity of rotation expressed as the Rotation Index, calculated as the number of cultures per year, was always higher in GAP than in BAP in each location with average values of 1.58 and 1.17, respectively. Soils under BAP used in the last 5 years 36% more herbicides than GAP. Soybean yield was on average 24.7% higher in GAP than in BAP whereas maize yield was 149.9% higher in GAP. In terms of soil texture there is increasing clay and decreasing sand content from Bengolea to Viale. Values of soil organic matter follow the relation NE > GAP > BAP at all sites, except in Pergamino where GAP and BAP showed similar values. Soil N content also followed the same pattern with the exception of Bengolea where GAP had higher values than NE. The pH values varied between 5.5 and 6.7 in all treatments with no clear pattern.

Soil samples were collected in February 2010 (summer) as triplicates for each treatment-site in three 5-m^2 sampling points separated at least 50 m from other, taking care not to follow the sowing line in the field. Each sample of the top 10 cm of mineral soil was collected as a composite of 16–20 randomly selected subsamples. Soil subsamples were combined and homogenized in the field and transported to the laboratory at 4 °C. Within 3 days after collection, samples were sieved through 2-mm mesh to remove roots and plant detritus, and stored at 4 °C until processing. For root systems, a patch of *ca.* 300 cm^2 of natural pasture was collected per replicate in NE treatment, and 1–10 plant specimens per replicate of GAP and BAP treatments, depending on the plant species (maize, soybean) and its size. Care was taken to get the root system together with the surrounding soil to avoid root desiccation. Samples were transported to the lab at 4 °C and processed within 1 week after collection.

2.6. Processing of soil and root samples for counting plates

To estimate the population size of culturable *Pseudomonas* spp. we essentially followed a reported procedure (Raaijmakers and Weller, 2001). Approximately 1 g of sieved soil (2-mm mesh; wet weight) was suspended in 9 ml of saline solution (SS; 0.85%, w/v NaCl), in 15-ml screwed-cap tubes. Tubes were shaken vigorously for 1 min in a vortex, and immersed for 1 min in a sonication bath (40 kHz, 160 W, Testlab TB04, Argentina).

We operationally define rhizospheric soil as the biological material recovered from thorough washings of roots that have been previously shaken by hand to remove loosely adhered soil. Thus, it is expected that our "rhizospheric soil" suspensions contain bacteria from soil particles tightly adhered to roots as well as from microcolonies present on the root surface. To obtain rhizospheric samples, randomly selected plant roots were harvested from each replicate, and treated according to the definition described above. Approximately 2 g

Table 2
Phylogenetic assignment of *Pseudomonas* isolates based on partial 16S rDNA and *oprF* sequences.

Isolate	Origin[a]	Closest phylogenetic relative based on 16S rDNA sequence[b]	Closest phylogenetic relative based on *oprF* sequence[c]
6.2	Bulk soil, Monte Buey, Córdoba, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
6.7	Bulk soil, Monte Buey, Córdoba, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
8.1	Bulk soil, Monte Buey, Córdoba, Argentina	*P. reinekei* MT1 (AM293565)	*P. fluorescens* Pf0-1 (CP000094)
9.2	Bulk soil, Monte Buey, Córdoba, Argentina	*P. plecoglossicida* FPC951 (AB009457)	*P. putida* W619 (CP000949)
11.1	Bulk soil, Monte Buey, Córdoba, Argentina	*P. monteilii* CIP104883 (AF064458)	*P. putida* W619 (CP000949)
14.1	Bulk soil, Pergamino, Buenos Aires, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
17.3	Bulk soil, Pergamino, Buenos Aires, Argentina	*P. putida* IAM1236 (D84020)	*P. putida* LMG 2257 (AF117967)
18.2	Bulk soil, Pergamino, Buenos Aires, Argentina	*P. vancouverensis* ATCC 700688 (AJ011507)	*P. fluorescens* Pf0-1 (CP000094)
19.3	Bulk soil, Pergamino, Buenos Aires, Argentina	*P. moraviensis* CCM7280 (AY970952)	*P. fluorescens* Pf0-1 (CP000094)
26.9	Bulk soil, Viale, Entre Ríos, Argentina	*P. asplenii* ATCC 23835T (AB021397)	*P. putida* W619 (CP000949)
26.14	Bulk soil, Viale, Entre Ríos, Argentina	*P. asplenii* ATCC 23835T (AB021397)	*P. putida* W619 (CP000949)
2P	Grass rhizosphere, Bernal, Buenos Aires, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
2Pi	Grass rhizosphere, Bernal, Buenos Aires, Argentina	*P. reinekei* MT1 (AM293565)	*P. fluorescens* Pf0-1 (CP000094)
Trébol	Clover rhizosphere, Bernal, Buenos Aires, Argentina	*P. mediterranea* CFBP 5447 (AF386080)	*P. fluorescens* WCS365 (AF115336)
Black	Grass rhizosphere, Bernal, Buenos Aires, Argentina	*P. asplenii* ATCC 23835T (AB021397)	*P. putida* LMG 2257 (AF117967)
LPP	Grass rhizosphere, Bernal, Buenos Aires, Argentina	*P. umsongensis* Ps 3-10 (AF468450)	*P. putida* UW4 (EU514690)
RZ1a	*Discaria trinervis* rhizosphere, Bariloche, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
RZ1b	*Discaria trinervis* rhizosphere, Bariloche, Argentina	*P. cedrina* LMG 21467 P 515/12 (AJ492830)	*P. fluorescens* Pf0-1 (CP000094)
RZ2	*Discaria trinervis* rhizosphere, Bariloche, Argentina	*P. trivialis* LMG 21464 P 513/19 (AJ492831)	*P. fluorescens* OE 46.1 (L21202)
RP1a	*Discaria trinervis* rhizoplane, Bariloche, Argentina	*P. oryzihabitans* IAM 1568 (D84004)	*P. fluorescens* OE 46.1 (L21202)
RP1b	*Discaria trinervis* rhizoplane, Bariloche, Argentina	*P. vancouverensis* ATCC 700688 (AJ011507)	*P. fluorescens* Pf0-1 (CP000094)
RP2a	*Discaria trinervis* rhizoplane, Bariloche, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
RP2b	*Discaria trinervis* rhizoplane, Bariloche, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
SMAN1	Bulk soil, NE, Monte Buey, Córdoba, Argentina	*P. lutea* OK2 (AY364537)	*P. putida* GB-1 (CP000926)
SMAN5	Bulk soil, NE, Monte Buey, Córdoba, Argentina	*P. asplenii* ATCC 23835T (AB021397)	*P. putida* W619 (CP000949)
SMBP1	Bulk soil, GAP, Monte Buey, Córdoba, Argentina	*P. plecoglossicida* FPC951 (AB009457)	*P. putida* W619 (CP000949)
SMMP3	Bulk soil, BAP, Monte Buey, Córdoba, Argentina	*P. chlororaphis* NCIB 10068 (DQ682655)	*P. chlororaphis* subsp. *aureofaciens* LMG 1245 (AF117961)
SVAN1	Bulk soil, NE, Viale, Entre Ríos, Argentina	*P. rhodesiae* CIP 104664 (AF064459)	*P. fluorescens* SBW25 (AF117969)
SVBP9	Bulk soil, GAP, Viale, Entre Ríos, Argentina	*P. chlororaphis* DSM 50083T (Z76673)	*P. chlororaphis* subsp. *aureofaciens* LMG 1245 (AF117961)
SVMP9	Bulk soil, BAP, Viale, Entre Ríos, Argentina	*P. oryzihabitans* IAM 1568 (D84004)	*P. putida* W619 (CP000949)
SBAN2	Bulk soil, NE, Bengolea, Córdoba, Argentina	*P. umsongensis* Ps 3-10 (AF468450)	*P. fluorescens* CHA0 (EF592174)
SBBP5	Bulk soil, GAP, Bengolea, Córdoba, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. putida* BIRD-1 (CP002290)
SBMP6	Bulk soil, BAP, Bengolea, Córdoba, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. entomophila* L48 (CT573326)
SPAN5	Bulk soil, NE, Pergamino, Buenos Aires, Argentina	*P. chlororaphis* NCIB 10068 (DQ682655)	*P. chlororaphis* subsp. *aureofaciens* LMG 1245 (AF117961)
SPAN6	Bulk soil, NE, Pergamino, Buenos Aires, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
SPBP2	Bulk soil, GAP, Pergamino, Buenos Aires, Argentina	*P. mosselii* CIP 105259 (AF072688)	*P. entomophila* L48 (CT573326)
SPMP6	Bulk soil, BAP, Pergamino, Buenos Aires, Argentina	*P. monteilii* CIP 104883 (AF064458)	*P. putida* W619 (CP000949)
RBAN1	Grass rhizosphere, NE, Bengolea, Córdoba, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)
RBAN6	Grass rhizosphere, NE, Bengolea, Córdoba, Argentina	*P. extremorientalis* KMM 3447 (AF405328)	*P. fluorescens* SBW25 (AF117969)
RBMP2	Soybean rhizosphere, BAP, Bengolea, Córdoba, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. putida* UW4 (EU514690)
RPBP2	Maize rhizosphere, GAP, Pergamino, Buenos Aires, Argentina	*P. veronii* CIP 104663 (AF064460)	*P. putida* UW4 (EU514690)
RPBP5	Maize rhizosphere, GAP, Pergamino, Buenos Aires, Argentina	*P. rhodesiae* CIP 104664 (AF064459)	*P. fluorescens* SBW25 (AF117969)
RPBP7	Maize rhizosphere, GAP, Pergamino, Buenos Aires, Argentina	*P. koreensis* Ps 9-14 (AF468452)	*P. fluorescens* Pf0-1 (CP000094)

Table 2 (*Continued*)

Isolate	Origin[a]	Closest phylogenetic relative based on 16S rDNA sequence[b]	Closest phylogenetic relative based on *oprF* sequence[c]
SPSA1	Bulk soil, NE, Pergamino, Buenos Aires, Argentina	*P. chlororaphis* NCIB 10068 (DQ682655)	*P. chlororaphis* subsp. *chlororaphis* LMG 5004 (AF117962)
SPSA2	Bulk soil, NE, Pergamino, Buenos Aires, Argentina	*P. chlororaphis* NCIB 10068 (DQ682655)	*P. chlororaphis* subsp. *aureofaciens* LMG 1245 (AF117961)
SPSA5	Bulk soil, NE, Pergamino, Buenos Aires, Argentina	*P. tremae* CFBP 6111 (AJ492826)	*P. fluorescens* CHA0 (EF592174)
RMAN3	Grass rhizosphere, NE, Monte Buey, Córdoba, Argentina	*P. libanensis* CIP 105460 (AF057645)	*P. fluorescens* NRRL B-15132 (L21197)
RMAN4	Grass rhizosphere, NE, Monte Buey, Córdoba, Argentina	*P. libanensis* CIP 105460 (AF057645)	*P. fluorescens* NRRL B-15132 (L21197)
RMAN5	Grass rhizosphere, NE, Monte Buey, Córdoba, Argentina	*P. libanensis* CIP 105460 (AF057645)	*P. fluorescens* NRRL B-15132 (L21197)
RMAN6	Grass rhizosphere, NE, Monte Buey, Córdoba, Argentina	*P. libanensis* CIP 105460 (AF057645)	*P. fluorescens* NRRL B-15132 (L21197)

[a] NE, natural non-disturbed environment; BAP, field plot under bad agricultural practices; GAP, field plot under good agricultural practices.
[b] The closest type strain was identified with the SeqMatch tool of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp), using the following set up for the search: strain: type; source: isolate; size: >1200 bp; quality: good; taxonomy: nomenclatural. The GenBank entry of the closest match is indicated within parenthesis.
[c] The closest pseudomonad relative was identified with the BlastN tool (NCBI), using default parameters. The GenBank entry of the closest match is indicated within parenthesis.

of roots and associated rhizosphere soil were suspended in 18.0 ml of SS, subsequently shaken vigorously for 15 min on a rotary platform at 250 rpm to facilitate release of bacterial cells adhered to root surface, and then sonicated for 1 min. Thereafter, tubes with soil or root samples were centrifuged at $50 \times g$ at room temperature for 1 min, and the supernatant was recovered in a new clean 15-ml or 50-ml tube, respectively.

Serial dilutions of each soil or rhizosphere suspensions were plated in triplicate on Gould's S1 plates to count culturable pseudomonads, and on 10% TSA (tryptone-soy agar, Biokar) to count total heterotrophic mesophilic bacteria. Both media were supplemented with cycloheximide (100 μg/ml) to inhibit growth of fungi and yeasts. Colony counts were done after 48 h of incubation at 28 °C. The proportion of fluorescent colonies under UV light (290 nm) was determined on S1 plates.

2.7. Total DNA from culturable population

To study the community structure of culturable pseudomonads, all colonies from S1 agar plates (n = 100–200) were resuspended in a volume of deionized water proportional to the number of colonies grown on each plate, collected in 1.5-ml tubes, and treated at 100 °C in boiling water during 15 min to lyse the cells. Treated suspensions were centrifuged at 14,000 rpm for 2 min. The supernatants were recovered in new clean 1.5-ml tubes and conserved at −20 °C until used as a source of DNA for PCR reactions. Supernatants lysates from each site and treatment replicate were combined to get only one representative sample per treatment at each geographical site. Typically, 3 μl of 1000-fold diluted lysates were used as template. RFLP assays were done as described above. DNA banding patterns were analyzed with Bionumerics 4.0 (Applied Maths, Kortrijk, Belgium) by correlation based clustering (Rademaker et al., 1999).

2.8. Statistics

For every geographical site (four), and for every treatment (three) within each geographical site, the number of replicate samples was three (n = 3). These replicate samples were collected at a distance of *ca.* 50 m each, and each one corresponded to a composite of 16–20 soil cylinders. Plant roots were sampled in parallel to have 3 replicates per treatment and per site. So, the sampling consisted in a total of 4 sites × 3 treatments × 3 replicates = 36 soil samples, and an equivalent number of rhizosphere samples. Every sample was processed and plated in triplicate plates. Thus, the data shown in Tables 4 and 5 correspond to averages of 3 replicate plates × 3 replicate samples = 9 replicates per treatment and per site. CFU values were $\log_{10}$-transformed prior to statistical analyses. Analysis of variance (ANOVA) was followed, when appropriate, with Tukey "Honestly Significantly Different" tests with Statistix 8 (version 8.0, Analytical Software, St. Paul, MN) to evaluate significant differences between values. Statistics were done at $P < 0.05$.

3. Results

3.1. Selective power of the Gould's S1 medium and unbiased recovery of pseudomonads from soil and root samples

Among reported media, we chose the Goulds' S1 selective medium (Gould et al., 1985) based on previous publications reporting successful recovery of a wide range of pseudomonads from different habitats (Johnsen et al., 1999; Johnsen and Nielsen, 1999). As there are no commercial S1 formulations available, we verified the selectivity of the S1 medium formulated in our lab by

Table 3
Oligonucleotides targeting *Pseudomonas* spp. *gacA* and *oprF* genes for PCR-RFLP analysis.

Gene	Function	Amplicon size (bp)[a]	Primer	Sequence (5′ → 3′)	Melting temperature (°C)[b]	Annealing temperature (°C)
oprF	Non-specific porin	602–683	oprF-FW2	ATCGGYTACTTCHTBACHGA	52.3–60.5	57
			oprF-Rev2	CCNACGGAGTCRGTRTGRCC	60.5–68.7	57
gacA	Response regulator	480	gacA-FW	TGATTARGGKSYTRGTDGTCG	37.0–54.5	56
			gacA-REV	ATCATCARSGCRATCTGGAT	46.9–53.2	56

[a] The size of the *oprF* and *gacA* amplicons were estimated from the genomic sequences of available *Pseudomonas* spp. genomes in GenBank by 15 March 2011.
[b] Melting temperatures were calculated using Oligo Calculator v.3.26 with salt concentration adjusted to 50 mM.

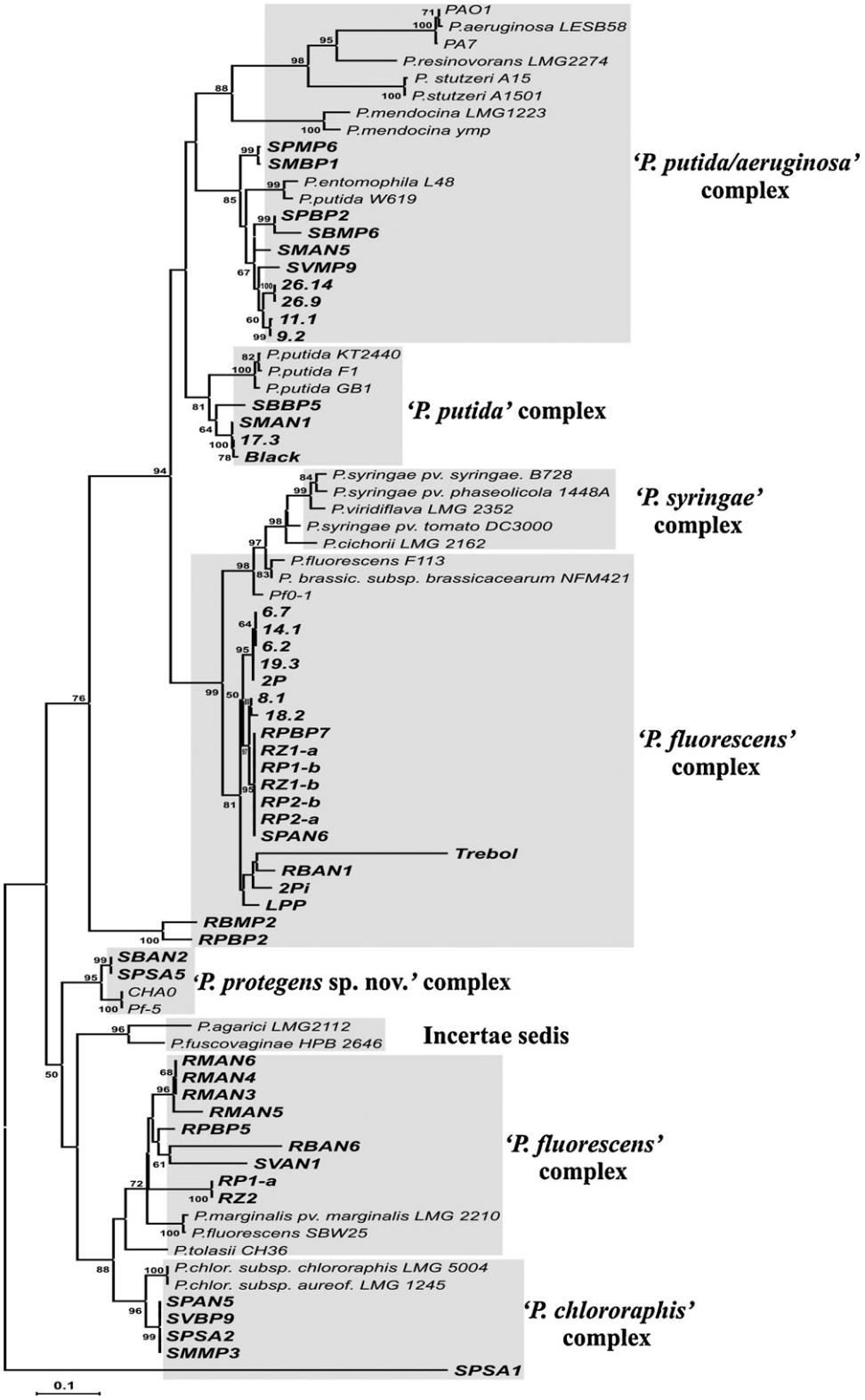

Fig. 1. Phylogenetic analysis of partial *oprF* gene sequences from 80 taxa, including the 50 isolates from Argentinean soil or root samples, and reference strains. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown at the nodes when values are higher than 50%. The scale bar represents 0.02 nt substitutions per site. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Taxonomic grouping was based on the work of Ramette et al. (2011).

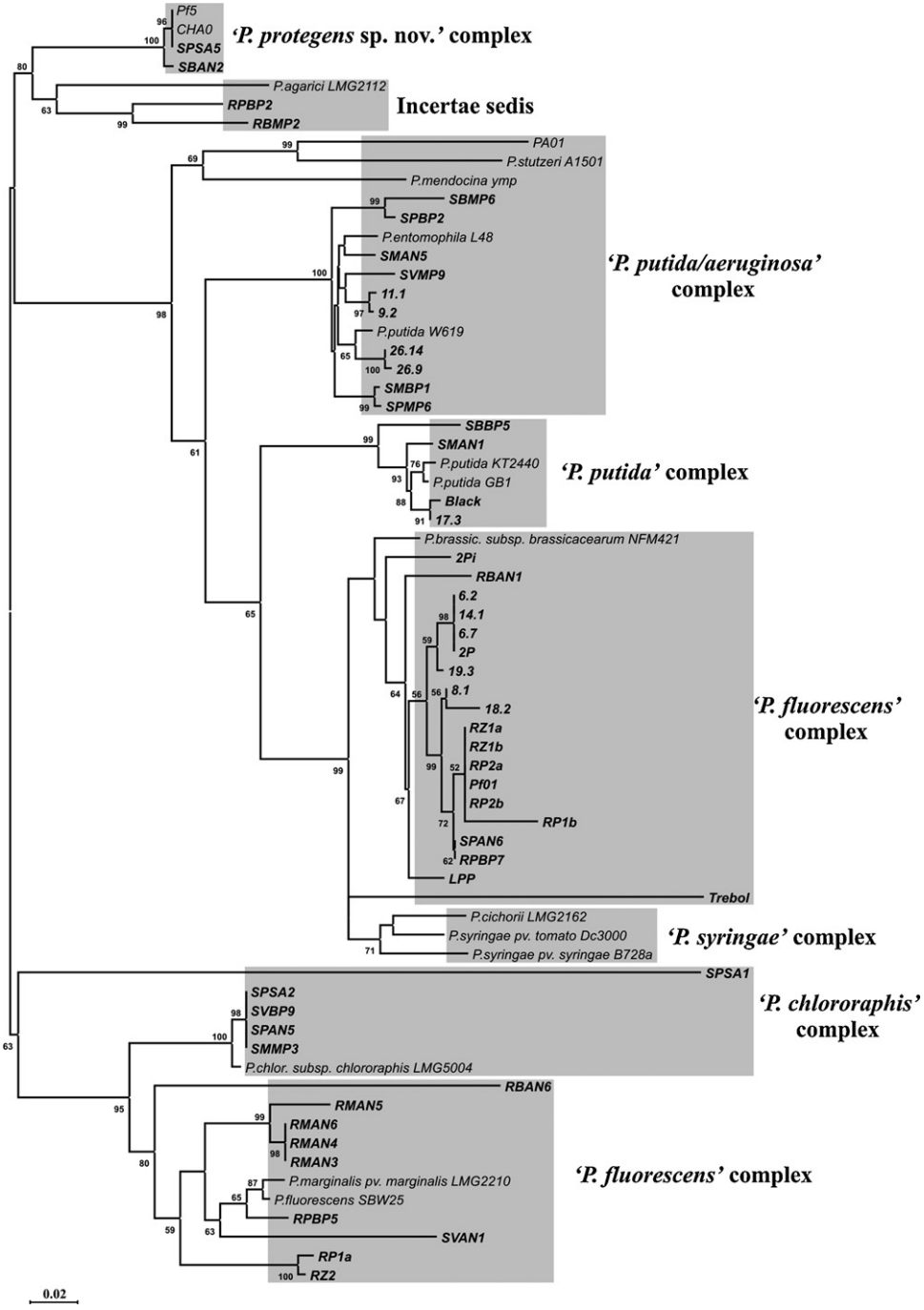

Fig. 2. Phylogenetic analysis of 16s rRNA-*oprF* concatenated genes from 63 taxa, including the 50 isolates and reference strains. See legend to Fig. 1 for details about the analysis.

plating either typed pseudomonads or bacterial strains belonging to other genera (Table 1). All typed pseudomonads grew in S1 medium but none of the non-pseudomonads strains developed colonies after 24–48 h at 28 °C. In order to confirm the utility of the S1 medium to recover pseudomonads, we built a collection of 50 untyped bacterial isolates able to grow in S1 medium, i.e., presumably *Pseudomonas* spp. (Table 2). The isolates were obtained from diverse bulk soil and rhizospheric soil samples, from different geographic locations in Argentina and from different plant species (Table 2). The partial sequences of the 16S rDNA gene and of the genus specific marker gene *oprF* confirmed that all isolates are indeed members of the *Pseudomonas* genus (Figs. 1 and 2; Table 2; Supplementary Fig. S1). The phylogenetic analyses of 16S rDNA and *oprF* genes sequences of the 50 pseudomonads isolates confirm the distribution of these isolates within different *Pseudomonas* clades (Figs. 1 and 2; Supplementary Fig. S1).

3.2. Specific detection of pseudomonads with improved PCR oligonucleotides targeting gacA and oprF genes

Both *gacA* and *oprF* genes are considered specific for the *Pseudomonas* genus (Bodilis and Barray, 2006; de Souza et al., 2003). For *gacA* we initially tested published oligonucleotides pairs designed to target the genus specific gene (Costa et al., 2007), but the PCR gave unspecific amplicons (data not shown). Thus, we generated new *gacA* primers to improve the PCR specificity. A set of

Table 4
Total culturable pseudomonads counts in soil samples from agricultural plots under no-till management in Argentina.

Geographical site	Agricultural treatment								
	NE			GAP			BAP		
	TH	TP[y]	FP[z]	TH	TP[y]	FP[z]	TH	TP[y]	FP[z]
Bengolea	6.81 ± 0.07a	5.57 ± 0.36[d] (3.6)	4.44 ± 0.4[g] (9.9)	6.63 ± 0.08b	4.17 ± 0.32[e] (0.7)	3.89 ± 0.19[h] (64.5)	6.56 ± 0.07b	3.89 ± 0.38[e] (0.3)	3.67 ± 0.56[h] (68.2)
Monte Buey	6.66 ± 0.13a	4.21 ± 0.13[e] (0.2)	3.85 ± 0.3[h] (50.1)	6.17 ± 0.18c	4.30 ± 0.61[e] (0.7)	3.91 ± 0.3[h] (69.5)	6.49 ± 0.12b	4.96 ± 0.27[d] (1.5*)	4.85 ± 0.28[g] (76.0)
Pergamino	6.61 ± 0.11a	5.03 ± 0.12[d] (2.8)	4.61 ± 0.2[g] (47.1)	6.27 ± 0.16c	3.96 ± 0.37[f] (0.7)	3.75 ± 0.38[i] (64.1*)	6.43 ± 0.03b	4.51 ± 0.27[e] (1.7*)	4.11 ± 0.22[h] (39.1)
Viale	6.60 ± 0.45a	4.57 ± 0.68[d] (1.2)	4.55 ± 0.6[g] (95.8)	6.26 ± 0.19b	3.54 ± 1.11[e] (0.6)	3.31 ± 0.9[h] (67.5)	6.42 ± 0.17ab	3.54 ± 0.48[e] (0.2)	3.24 ± 0.54[h] (66.1)

NE, natural environment; GAP, good agricultural practices; BAP, bad agricultural practices; TH, total heterotrophic mesophyles; TP, total pseudomonads; FP, fluorescent pseudomonads. Values are expressed in log CFU/g dry soil and correspond to averages from $n = 9$ replicate platings per site and per treatment, ±: its standard deviation. Different superscript letters indicate significant statistical difference ($P < 0.05$) between average CFUs for each site and bacterial group ([a,b,c] for TH; [d,e,f] for TP and [g,h,i] for FP). [(y)] The percentage of TP in relation to TH is indicated between parenthesis; [(z)] The percentage of FP in relation to TP is indicated between parenthesis. Asterisks indicate the higher % between GAP and BAP treatments, for $n = 3$ replicates per site ($P < 0.2$). For the statistical comparison, the % values were subjected to an angular transformation.

Table 5
Total culturable pseudomonads counts in rhizosphere samples from agricultural plots under no-till management in Argentina.

Geographical site	Agricultural treatment								
	NE			GAP			BAP		
	TH	TP[y]	FP[z]	TH	TP[y]	FP[z]	TH	TP[y]	FP[z]
Bengolea	Pasture 9.99 ± 0.05[a]	8.03 ± 0.16[e] (1.0)	7.95 ± 0.12[h] (83.7)	Soybean 9.63 ± 0.12[b]	8.61 ± 0.49[d] (14.1*)	8.51 ± 0.45[g] (81.4*)	Maize 8.15 ± 0.12[c]	6.19 ± 0.32[f] (1.2)	5.71 ± 0.67[i] (46.4)
Monte Buey	Soybean 8.89 ± 0.21[a]	7.63 ± 0.56[e] (6.1)	7.43 ± 0.6[h] (78.9)	Soybean 8.98 ± 0.11[a]	8.28 ± 0.34[d] (7.6)	8.18 ± 0.4[g] (80.2*)	Soybean 8.90 ± 0.11[a]	7.27 ± 0.40[e] (2.7)	7.09 ± 0.46[h] (64.7)
Pergamino	Pasture 8.48 ± 0.13[c]	7.38 ± 0.14[f] (6.1)	7.27 ± 0.18[i] (78.7)	Maize 8.95 ± 0.08[b]	8.05 ± 0.18[e] (7.6)	7.89 ± 0.28[h] (74.8)	Soybean 9.72 ± 0.07[a]	9.12 ± 0.14[d] (2.8)	8.92 ± 0.05[g] (66.1)
Viale	Soybean 8.80 ± 0.09[b]	7.19 ± 0.60[e] (6.9)	6.99 ± 0.63[h] (68.1)	Soybean 9.00 ± 0.28[a]	8.36 ± 0.39[d] (24.1*)	8.06 ± 0.4[g] (57.9*)	Soybean 8.89 ± 0.14[ab]	7.69 ± 0.59[e] (7.4)	6.57 ± 1.41[h] (42.2)

NE, natural environment; GAP, good agricultural practices; BAP, bad agricultural practices; TH, total heterotrophic mesophyles; TP, total pseudomonads; FP, fluorescent pseudomonads. Values are expressed in log CFU/g of fresh roots, and correspond to averages from $n = 9$ replicate platings per site and per treatment, ±: its standard deviation. The crop or vegetation present in the sampled plots is indicated for each site and treatment. Different superscript letters indicate significant statistical difference ($P < 0.05$) between average CFUs for each site and bacterial group ([a,b,c] for TH; [d,e,f] for TP and [g,h,i] for FP). [(y)] The percentage of TP in relation to TH is indicated between parenthesis; [(z)] The percentage of FP in relation to TP is indicated between parenthesis. Asterisks indicate the higher % between GAP and BAP treatments, for $n = 3$ replicates per site ($P < 0.2$). For the statistical comparison, the % values were subjected to an angular transformation.

sequences encoding *gacA* and *oprF* from a range of *Pseudomonas* species was obtained from GenBank (Supplementary Table S1) and used to identify highly conserved regions from multiple alignments that would serve as suitable targets for oligonucleotide annealing. Particular attention was paid to select primer pairs that would generate amplicons from different pseudomonads groups (Bodilis and Barray, 2006) that could be distinguished by their restriction pattern (RFLP). Based on the sequence of the available *Pseudomonas* type strains, the restriction enzymes *Hae*III, *Taq*I and *Mbo*I were selected to obtain the corresponding RFLPs, because *in silico* analysis generated distinguishable fragment patterns for both marker genes from different strains (Supplementary Fig. S2a).

The designed primer pairs and their features are shown in Table 3. The specificity of the primers was tested with typed pseudomonad isolates (Table 2), which produced the expected amplicons without unspecific bands (Fig. 3a). Note that the size of the *oprF* amplicon may differ in *ca*. 50–80 bp among certain pseudomonads due to the presence of an internal deletion in the central domain of the OprF polypeptide (Bodilis et al., 2006). A set of isolates belonging to bacterial genera other than *Pseudomonas* failed to amplify *oprF* and *gacA* gene fragments (Fig. 3a). The RFLP patterns obtained for a subset of tested strains matched the expected profiles deduced from the *in silico* analysis of *oprF* and *gacA* sequence fragments (Supplementary Fig. S2b). In addition, the RFLP banding patterns were able to distinguish different pseudomonas species for which the *gacA* and *oprF* genes were not available (Fig. 3b).

3.3. Counts of culturable pseudomonads in soil and rhizosphere samples from no-till plots

Upon confirmation of the selective properties of the S1 medium, the amount of culturable pseudomonads in samples from agricultural plots was estimated by plating appropriate dilutions of aqueous suspensions from bulk soil or rhizospheric soil samples. We introduced a low-speed ($50 \times g$) centrifugation step before dilution of the suspensions, to reduce the interference of soil particles and root material. We tested that both the sonication as well as the centrifugation steps do not affect CFU counts with cell suspensions of *P. fluorescens* CHA0. Sonication for up to 10 min did not significantly affect CFU counts (data not shown), whereas centrifugation at $50 \times g$ for 1 min did only reduce the CFU titers in the supernatant by 13% (data not shown). There was, on the other hand, a strong impact of soil drying on pseudomonads CFU counts and its population structure. We observed that air-dried soils suffered up to 2-$\log_{10}$ reduction in total pseudomonads CFU counts in comparison to fresh soils temporarily stored at 4 °C and processed without air-drying (Supplementary Table S2). In addition, we noted that upon a 24-h period of rehydration of air-dried soil, the soil suspensions resulted in S1 plates with colonies that were all similar in aspect, suggesting a strong selective effect of the dryness stress. For this reason, we have decided to process soil samples as soon as possible from the time of sampling. In the same vein, we sampled root systems taking care to bring soil patches around the roots to reduce humidity loss during sample transport.

Table 4 reports the CFU counts obtained for soil samples from the set of agricultural plots under study. Total mesophilic heterotrophic counts were quite similar for all sites and treatments, with an overall average of 6.2$\log_{10}$ CFU/g. Total pseudomonads load was on average 4.3$\log_{10}$ CFU/g, representing 1.2% of the culturable heterotrophic bacteria (range of 0.2–3.6%, depending on the site and treatment). There was, however, higher variability in total pseudomonads counts among samples compared to heterotrophic counts. The proportion of pseudomonads that produced fluorescent diffusible pigments under UV light (i.e., fluorescent pseudomonads) ranged 10–96% of total colonies in S1 medium. For the collected bulk soils, we did not detect a systematic difference in the number of total pseudomonads and fluorescent pseudomonads between GAP and BAP treatments for each geographical site (Table 4).

Independently of site and treatment, CFU counts of suspensions from rhizospheric soil samples (Table 5) had, on average, higher bacterial loads than bulk soil samples (9.0$\log_{10}$ CFU/g versus 6.2$\log_{10}$ CFU/g; $P < 0.05$). Total pseudomonads also were enriched in the root proximity, although the increment was proportionally higher than that of heterotrophic bacteria, rising from 1.2% (0.2–3.6%; Table 4) to 7.3% (1.0–24.1%) (Table 5). There was a generalized enrichment of pseudomonads associated with roots as the total pseudomonads counts were 2.3–4.8 $\log_{10}$-fold higher than in the corresponding bulk soil, independently of the sampling site, treatment and plant type (Tables 4 and 5). The proportion of fluorescent pseudomonads ranged from 42% to 84% of total pseudomonads from root samples. Interestingly, all samples from GAP plots showed higher % of total pseudomonads and % of fluorescent pseudomonads than in the corresponding BAP plots. These data were statistically different in Bengolea and Viale samples (Table 5).

The NE (natural environment) sampling sites of all locations had natural pastures, but in Monte Buey and Viale NE plots we deliberately introduced soybean in a limited portion of each plot, allowing the comparison of soybean root colonization by pseudomonads among different treatments. For both locations, the total pseudomonads counts were higher in soybean root samples from GAP plots, than those from NE and BAP plots ($P < 0.05$; Table 5). The same trend was observed for the % of fluorescent pseudomonads counts. For the other two sites (Bengolea and Pergamino), soybean root colonization by pseudomonads and fluorescent pseudomonads was higher than for maize, independently of the treatment (Table 5).

3.4. Community structure of culturable pseudomonads in soil and rhizosphere samples from no-till plots

The number of CFU per g of soil or root samples alone may not necessarily reflect the influence of agronomic practices or environmental factors. Moreover, similar CFU counts may be achieved with similar or different community structure. We thus applied the aforementioned *gacA* and *oprF* PCR-RFLP protocols and analyzed the banding patterns derived from the most abundant culturable pseudomonads present in the soil and root samples that were already used to quantify pseudomonad CFUs (Tables 4 and 5). For comparison of the treatments within sites, we analyzed the PCR-RFLP banding pattern of a pooled lysate prepared with colony suspensions from the three replicate platings of each site and treatment (see Section 2). The banding pattern from the pooled lysate was representative of the combination of banding patterns of the pseudomonads present in each replicate (Supplementary Fig. S3). In addition, the profiles were reproducible in independent PCR-RFLP reactions (Supplementary Fig. S4).

Both *gacA* and *oprF* RFLP patterns revealed changes in the pseudomonads population structure of bulk soil associated with cropping in all sites (Fig. 4). In some cases the differences were more evident between agricultural treatments as for *oprF* RFLP patterns from BAP and GAP samples in comparison to NE samples for Viale and Bengolea sites (Fig. 4). Clustering analysis of *gacA* banding patterns resulted in a preferential grouping according to the geographical origin of the soil samples (Fig. 4). In contrast, the *oprF* banding patterns from the same S1 platings did not follow the same trend, as we could not detect a clear grouping by sample location (Fig. 4). Soils from the GAP treatment had slightly higher diversity of pseudomonads based on the Shannon index of the RFLP profiles compared to the BAP treatment (Fig. 4), and also exhibited higher plant productivity. Any causal relationship between these two factors requires further study.

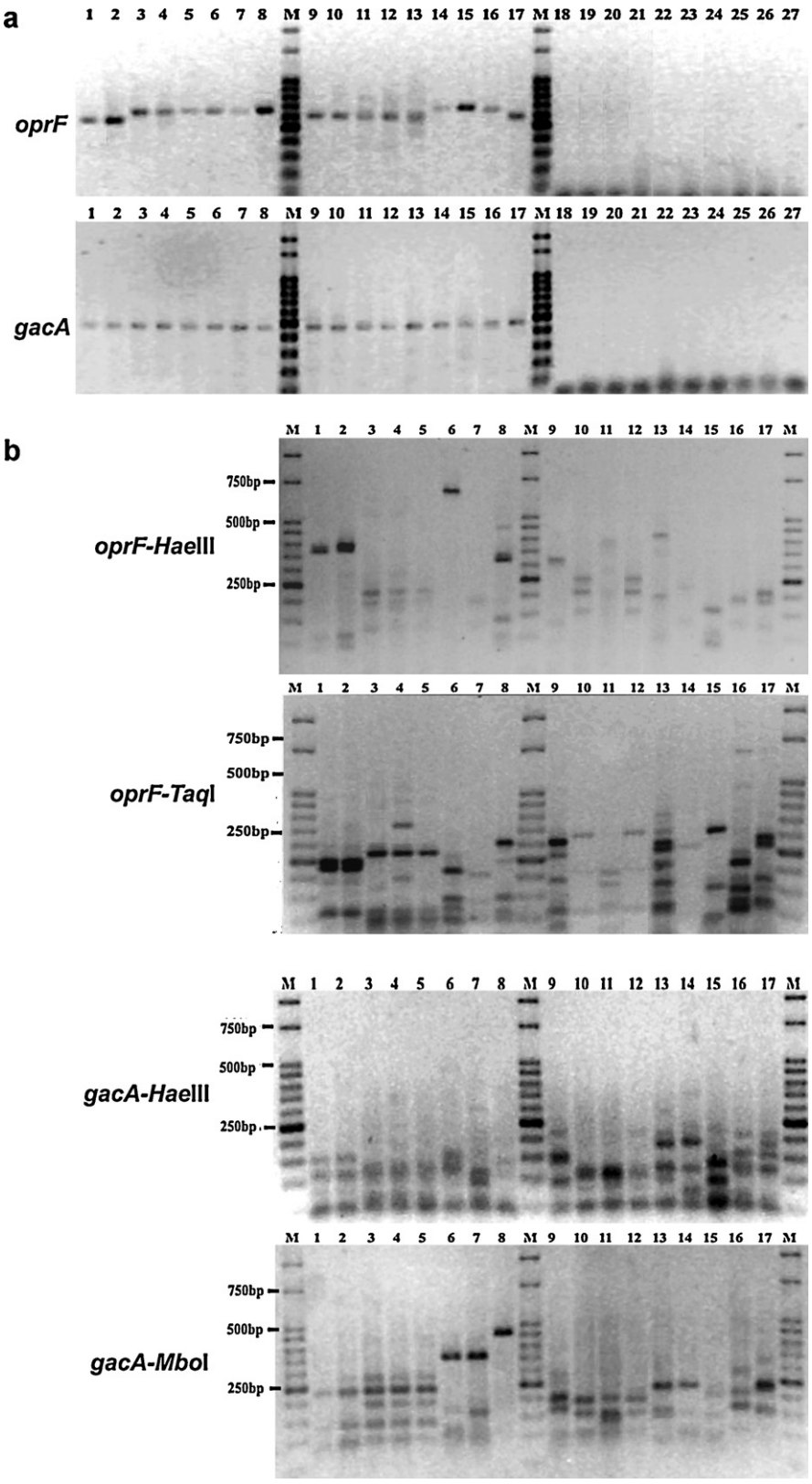

Fig. 3. Set up of PCR-RFLP protocols targeting *Pseudomonas oprF* and *gacA* genes. (a) PCR of *oprF* and *gacA* genes with positive and negative controls. Reactions were carried out with primers listed in Table 3; (b) RFLP patterns of positive controls. Strains were as follows: 1, *P. fluorescens* CHA0; 2, *P. fluorescens* Pf-5; 3, *P. aeruginosa* PAO1; 4, *P. aeruginosa* Hex1T; 5, *P. aeruginosa* sp.; 6, *P. syringae* pv. *maculicola* ES4326; 7, *P. putida* KT2440; 8, *P. putida* ATCC 17399; 9, *P. stutzeri* AN10; 10, *P. stutzeri* ATCC 17588; 11, *P. stutzeri* 2014; 12, *P. stutzeri* 2018; 13, *P. mendocina* 2013; 14, *P. mendocina* 2019; 15, *P.* sp. CF5; 16, *P.* sp. LDe; 17, *P.* sp. N23; 18, *E. coli* K12 MG1655; 19, *E. coli* K12 TR1-5; 20, *B. cereus*; 21, *B. subtilis*; 22, *S. marcescens* AS-1; 23, *B. cepacia*; 24, *S. meliloti* 2011; 25, *A. tumefaciens* NTL4; 26, *A. brasilense* ATCC 29710; 27, negative control (without DNA). Markers (M) are 100-bp DNA ladder (PB-L, Argentina) (a) or 50-bp DNA ladder (PB-L, Argentina) (b).

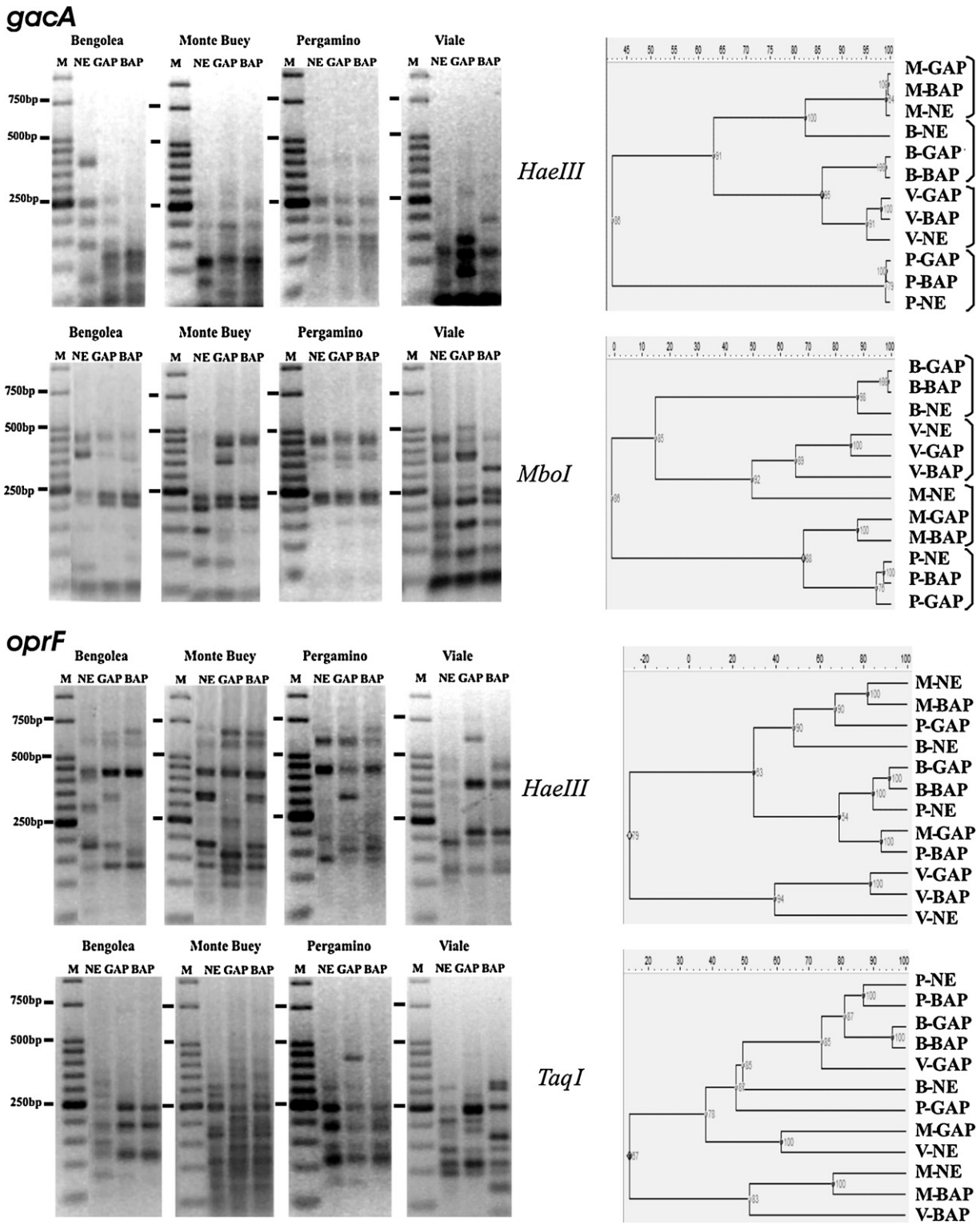

Fig. 4. *gacA* and *oprF* PCR-RFLP analysis of bulk soil samples from agricultural plots under no-till management in Argentina. A 50-bp DNA size standard (M) indicates the size of the amplified products and allows the comparisons among different gels. Cluster analyses were made with Bionumerics 4.0 (Applied Maths). The calculation of the similarities is based on the Pearson correlation coefficient, an objective coefficient that does not suffer from typical peak/shoulder mismatches as often found when band-matching coefficients are used, with tolerance and optimization set to 1% and 0.5%, respectively. The clustering algorithm of Ward was used to calculate the dendrograms of each RFLP gel and a combination of all gels.

Rhizosphere samples were also collectively distinguished by geographical location based on their *gacA* RFLP pattern, whereas clustering of *oprF* RFLPs was not clear (Supplementary Fig. S5). On the other hand, within the two sampling locations having the same crop species in the three treatments (Monte Buey and Viale, with soybean; Table 5), RFLPs suggest a rhizosphere selection of different pseudomonads groups among treatments (Supplementary Fig. S5). The most abundant pseudomonad groups present in bulk soil samples from those sites were already different (illustrated for Viale in Fig. 5).

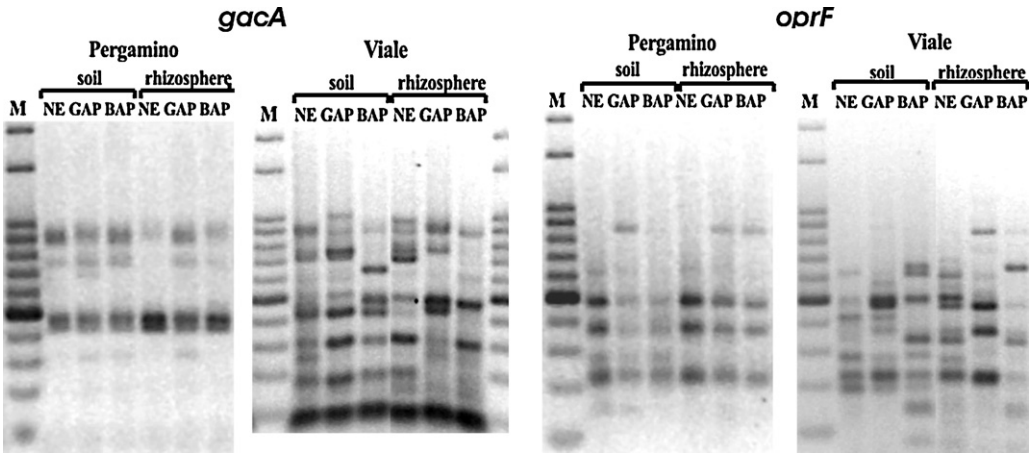

Fig. 5. A comparison of *gacA*/*Mbo*I and *oprF*/*Taq*I PCR-RFLP analysis of soil and rhizosphere samples from agricultural plots under no-till management in Argentina. A 50-bp DNA size standard (M) indicates the size of the amplified products and allows the comparisons among different gels.

When the RFLP patterns of root and soil samples were compared for each location and treatment, a rhizospheric effect on the composition of the most abundant pseudomonads could be clearly observed in Viale for each treatment (Fig. 5), suggesting that in addition to the quantitative enrichment of rhizospheric pseudomonads (Table 5), there was a specific selection of certain groups by soybean roots. In contrast, Pergamino had the least variable patterns between rhizospheric and bulk soil samples of all treatments (Fig. 5), being the rhizospheric effect restricted to a quantitative enrichment of the most abundant soil pseudomonad groups (Table 5 and Fig. 5). For Monte Buey and Bengolea, the rhizospheric effect on the community structure of culturable pseudomonads was clearer for NE plots than for GAP or BAP sites (Fig. 4 and Supplementary Fig. S5).

4. Discussion

4.1. Platings on S1 medium and oprF PCR for specific counts

In order to get an accurate picture of the amount and diversity of a specific taxonomic microbial group, appropriate culturing media and reliable genetic markers are required. Plate counts are a routine procedure to estimate the size of bacterial populations in biological samples, and selective media must be used to narrow down growth of competitive bacteria present in complex substrates. In this work, the density of culturable pseudomonads in soil and rhizosphere samples from agricultural plots under no-till management was determined by plating suspensions on the selective medium Gould's S1 (Gould et al., 1985). 16S rDNA sequencing of 50 colonies randomly picked up from S1 plates from different soil and rhizosphere sources (Table 2), confirmed that the S1 medium is strictly selective for *Pseudomonas* spp. (Supplementary Fig. S1). In addition, all the 50 randomly selected S1 colonies gave a single PCR amplicon for the genus marker gene *oprF* (Fig. 1). This is the first correlation of growth in S1 medium and PCR detection of the *oprF* allele, thus demonstrating a simpler procedure for confirming the presence of pseudomonads compared to colony blotting with an OprF antiserum (Kragelund et al., 1996). Moreover, as *Pseudomonas* genomes contain only one *oprF* allele, qRT-PCR protocols targeting *oprF* would be more appropriate to estimate total pseudomonads genomic units in soil and rhizosphere than methods based on 16S rDNA alleles (Lloyd-Jones et al., 2005). Soil and rhizosphere platings on Gould's S1 medium were not biased to particular subpopulations because we isolated both fluorescent and non fluorescent representatives from most phylogenetic pseudomonad clusters defined by 16S rDNA sequence (Supplementary Fig. S1). The only exception is the *P. syringae* clade, which may be related to the fact that these species are plant pathogens usually colonizing the phyllosphere (Hirano and Upper, 2000). Our data on partial sequencing of the *oprF* allele ($n = 119$ isolates) revealed that 89% of the isolates originated from bulk soil belongs to the *oprF* type I and 11% belongs to the *oprF* type II (Bodilis et al., 2006), whereas 22% of the rhizospheric isolates possess type II *oprF* alleles. This matches previous reports on niche preference (soil versus rhizosphere) of pseudomonad strains (Bodilis et al., 2006). Another interesting observation derived from the comparative phylogenetic analysis, is that the *oprF* phylogeny could detect the recently proposed group *P. protegens* (Ramette et al., 2011) as a separate clade distinct from members of the *P. fluorescens* complex (Fig. 1). This fact reinforces the taxonomic power of the *oprF* gene (Figs. 1 and 2).

4.2. Pseudomonads CFU counts in bulk soil and rhizospheric soil samples

The total pseudomonads load of sampled sites ranged from 3.5 to 5.6 $\log_{10}$ CFU/g with higher average counts in non cultivated control soils (NE) than in the corresponding plots under no-till cropping management (GAP and BAP) (Table 4). Overall, the number of culturable pseudomonads in bulk soil (Table 4) is comparable to what has been reported for agricultural soils in other geographical locations (Funnell-Harris et al., 2010; Garbeva et al., 2004a; Johnsen and Nielsen, 1999; Kwon et al., 2005; Meyer et al., 2010; Thirup et al., 2001). Within each sampling location, cropping seems to be associated with a reduction in the % of pseudomonads in relation to total heterotrophic mesophyles in NE plots (Table 4). Plate counts confirmed the "rhizosphere effect" for pseudomonads (Heijnen et al., 1993; Meyer et al., 2010), i.e., the total number of S1 CFU/g of rhizospheric soil material of different crops is higher by at least two orders of magnitude compared to the respective bulk soil (Tables 4 and 5). As previously described (Kragelund et al., 1996), our results support the idea that pseudomonads are relevant components of the rhizosphere community, since they represent on average nearly 10% of the heterotrophic culturable population (Table 5). Furthermore, the data indicate that the rhizosphere effect is stronger for pseudomonads than for other culturable groups, because there was a generalized increase in the % of pseudomonads in relation to total heterotrophs for rhizosphere compared to bulk soil, for all sites and treatments (Tables 4 and 5). In two sampling locations (Monte Buey and Viale), we could compare the rhizospheric load of the same crop species (soybean) in the three treatment plots, and observed that in both locations the rhizospheric counts of total and fluorescent pseudomonads

from GAP plots were significantly higher than those from NE and BAP plots (Table 5), suggesting a correlation between GAP and pseudomonad rhizosphere colonization. Platings also revealed a significantly higher bacterial load in the soybean rhizosphere compared to maize, despite the plot treatment in which plants were sampled (Table 5). This may be related to the composition of plant root exudates of both crops (Berg and Smalla, 2009; Garbeva et al., 2008).

4.3. *gacA* and *oprF* PCR-RFLP patterns of bulk soil and rhizospheric soil samples

Our results on platings from bulk soil and rhizospheric soil samples indicate that the colonies collected from S1 plates are a *bona fide* source of DNA to analyze the structure of the culturable pseudomonad community. To approach the analysis of pseudomonads community structure of those samples, we set up PCR-RFLP protocols targeting internal fragments of two genus markers, *gacA* (encoding the response regulator GacA) (de Souza et al., 2003) and *oprF* (encoding the major outer membrane porin OprF) (Bodilis and Barray, 2006), with new primers (Table 3) to ensure single amplicons, coverage of different clades, genus specificity, and the possibility to distinguish between species by RFLP of both amplicons. The results show that both PCR-RFLP protocols (Fig. 3; Supplementary Fig. S2) proved useful to study changes in the composition of pseudomonads in bulk soil and rhizospheric soil samples (Figs. 4 and 5, Supplementary Fig. S5). The application of these PCR-RFLP analyses to the first set of soil and root samples collected by the BIOSPAS consortium (Wall, 2011), revealed different patterns of the most abundant pseudomonads in soil and rhizosphere samples among different geographical locations, and in some cases, among treatments.

The comparative analyses of *gacA* PCR-RFLP patterns preferentially grouped bulk soil and rhizosphere samples according to geographic sites, and not by treatments (Fig. 4 and Supplementary Fig. S5). On the other hand, *oprF* PCR-RFLP results did not parallel the geographical grouping pattern of *gacA*. It has been previously reported that the evolutionary rate of the *gacA* gene is slower than expected and that the protein is under selective constrains (de Souza et al., 2003). Thus, the different clustering power of the *gacA* and *oprF* PCR-RFLP might reflect local different evolutionary rates for each gene, with *gacA* being more able to detect the endemicity of pseudomonads (Cho and Tiedje, 2000).

In most of the sampled sites, there was a marked effect of cropping (GAP and BAP) on the structure of the pseudomonads community in bulk soil, when compared to the non-disturbed NE treatment. This was reflected in the PCR-RFLP profiles of both marker genes (Fig. 4). This effect is consistent with previous reports of higher microbial diversities measured in permanent grasslands than in arable land under monoculture or under rotation (Garbeva et al., 2004b). Nevertheless, when comparing the community structure of GAP versus BAP at each particular site, there was a slight trend in GAP samples to have a more diverse PCR-RFLP profile than those from BAP sites.

The PCR-RFLP patterns of the pseudomonads community from bulk soil samples having comparable CFU counts revealed a different composition of predominant pseudomonad groups (Table 4). This was the case for Viale (GAP and BAP samples) and Monte Buey (NE and GAP samples) (Fig. 4). Moreover, the rhizosphere samples of NE and BAP in Viale from the same crop plant (soybean) and with similar CFU counts (Table 5) had distinct PCR-RFLP patterns for both analyzed genes (Supplementary Fig. S5). It thus appears that the different community structure already present in Viale bulk soils influenced the group of selected pseudomonads that were revealed in the PCR-RFLP analysis of soybean rhizospheres. In another site (Pergamino), the community structure detected by PCR-RFLP of both marker genes was only marginally influenced by the agricultural practice or by the crop species (Fig. 5); there was, however, a quantitative enrichment of the predominant pseudomonad groups (Table 5). The data highlight the importance of considering both the size (CFU counts) and the composition of the pseudomonad community (PCR-RFLP patterns).

We are currently monitoring CFU counts and the culturable community structure in bulk soil and rhizosphere samples along a sampling period covering seasonal fluctuations, crop rotations and agricultural practices. We aim to detect correlations between pseudomonads abundance, community structure, and soil productivity, and to estimate the relative contribution of each factor in shaping the pseudomonads community structure. The results presented in this work support future use of *oprF* and *gacA* to monitor total (culturable plus unculturable) pseudomonads load and diversity by means of molecular methods applied directly on total environmental DNA from soil and rhizosphere samples. There are, however, severe limitations to reveal the diversity of total pseudomonads in bulk soil samples due to the relatively low number of genomic units present in the samples (Table 4), which seem to be at the detection limit of our PCR protocols. We do amplify the *Pseudomonas* specific 16S rDNA but we fail to obtain *oprF* and *gacA* amplicons using nested PCR for all bulk soil samples. In fact, to the best of our knowledge, only two works reported on the diversity of pseudomonads in bulk soil using culture-independent methods: Costa and colleagues utilized a taxon-specific, nested PCR protocol targeting the 16S rDNA (>4 copies per genome on average) to analyze soil DNA samples by DGGE (Costa et al., 2006), whereas Frapolli and colleagues amplified a *phlD* gene fragment (one copy per genome) directly from soil DNA but after inoculation of the soil with pure cultures at densities of at least 10^5–10^7 CFU/g (Frapolli et al., 2008). All other works describing the diversity of pseudomonads in soil have actually extracted total DNA from rhizospheric soil, in which the number of pseudomonads cells is significantly higher. For these reasons, we have started to assess the study of the community structure of culturable pseudomonads in bulk soils, and keep working to improve the detection limit of the PCR protocols to access the whole pseudomonad community of soils.

5. Conclusions

We here report the setup of simple methods to study the density and community structure of culturable pseudomonads in soil and rhizosphere samples from agricultural plots under no-till management in Argentina. We have verified the power of selective plating and the utility of genus-specific *gacA* and *oprF* PCR-RFLP to study pseudomonads community structure. In fact, this is the first report in which the genus marker gene *oprF* is used to analyze pseudomonads community structure. Both CFU counts as well as PCR-RFLP analyses of culturable pseudomonads from a set of soil and rhizosphere samples revealed that: (1) the abundance of pseudomonads in bulk soil was lower in cropped soil than in non-disturbed soil, with no significant effect of the agricultural practice; (2) crop plants increased the abundance of pseudomonads in the rhizosphere, with a stronger enrichment of fluorescent pseudomonads in no-till plots under conservative management (GAP); (3) the *gacA* PCR-RFLP profiles evidenced an endemic grouping of bulk soils and rhizosphere samples, which was not detected by *oprF* profiling; (4) the PCR-RFLP profiles insinuated a dissimilar pseudomonad community structure in the GAP soils with respect to BAP soils; (5) bulk soil samples with similar pseudomonads abundance could be distinguished by their genetic composition; (6) for the same crop plant (soybean), the pseudomonad groups enriched in the rhizosphere depend on the community composition of the bulk soil.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.apsoil.2011.11.016.

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