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Examination of Gould's modified S1 (mS1) selective medium and Angle's non-selective medium for describing the diversity of *Pseudomonas* spp. in soil and root environments

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Abstract

Studies on the diversity of environmental culturable *Pseudomonas* populations are dependent on the isolation procedure. This procedure includes the use of selective media which may influence the recovery of strains and thus the diversity described. In this study, we assessed the use of two agar isolation media for describing the diversity of soil- and root-inhabiting *Pseudomonas* associated with the perennial grass *Molinia coerulea*. A total of 382 *Pseudomonas* strains were recovered on either non-selective Angle's medium, or on Gould's modified S1 (mS1) *Pseudomonas*-selective medium. Their diversity was assessed by restriction analysis of PCR (polymerase chain reaction)-amplified 16S–23S rDNA internal transcript spacer sequences. The comparison of mS1- and Angle-recovered populations showed that the use of mS1 selective medium led to an underestimation of both *Pseudomonas* counts and diversity, especially in the soil environment.

Keywords: *Pseudomonas*; Isolation medium; Genetic diversity; 16S–23S ribosomal spacer DNA; PCR–RFLP; Rhizosphere; Perennial grass

1. Introduction

The genus *Pseudomonas* includes several species of general interest, such as human [1] and plant [2] pathogens, xenobiotic degraders [3], plant growth promoters [4] and biocontrol strains [5]. Because of these multiple roles and its wide distribution in the environment, this genus has become one of the best-studied bacterial taxa.

Whereas the soil is considered as an oligotrophic environment [6], plant-derived compounds released by the roots (rhizodeposition) provide abundant carbon and energy sources [7] as well as selective compounds for rhizosphere living microorganisms. Consequently, the rhizosphere is altogether an elective and selective environment, favouring specific populations, which best fit these conditions [8]. *Pseudomonas* are a significant component of the rhizosphere microflora [9–10], denoting their fitness in the rhizopheric environment [11–13].

Our perception of culturable *Pseudomonas* diversity depends strongly on the isolation procedure used [14–15]. Consequently, one requirement for such investigations is the development of culture media which permit the recovery of the largest (even exhaustive) diversity of culturable *Pseudomonas* and which are selective for this genus. The cultivation of microorganisms depends on their physiological and metabolic properties. The various culture media proposed for the selective isolation of *Pseudomonas* [15–18] are usually iron-deficient, thus enhancing siderophore production. Among them, King's B and Gould's S1 media are often used for the isolation, enumeration and diversity evaluation of fluorescent *Pseudomonas* [19–21]. The selectivity of Gould's S1 medium is based on an iron limitation, and high glycerol and sucrose contents. It is also based on other compounds such as sodium lauroyl sarcosine (which inhibits the growth of Gram-positive bacteria) and trimethoprim (an antibiotic limiting the growth of non-fluorescent *Pseudomonas*). This medium allowed a

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high recovery of fluorescent *Pseudomonas* phenotypes from soil samples. Fromin et al. [12] proposed Gould's modified S1 (mS1) medium, without thrimethoprim to allow the growth of non-fluorescent *Pseudomonas*.

Further studies have revealed limitations of these selective media. For instance, Kragelund et al. [9] has shown that *Pseudomonas* counts from barley rhizosphere soil were higher on a non-selective medium (10-fold-diluted tryptic soy agar) than on either Gould's S1 or King's B medium. Comparative investigations using Gould's S1 and King's B media have presented contrasting results. The use of Gould's S1 medium sometimes gave either higher [22] or lower [19] counts of fluorescent *Pseudomonas* than the use of King's B medium. Nonetheless, a wider genotypic variety of *Pseudomonas*, of both fluorescent and non-fluorescent types, could be recovered using S1 compared to King's B [19]. Furthermore, Aagot et al. [15] recovered different counts and diversity of *Pseudomonas* from the same soil sample by varying the concentration of casamino acids in NAA *Pseudomonas*-selective medium.

Most of these studies were performed only on soil environments and did not test the validity of these selective media for *Pseudomonas* populations inhabiting environments with contrasting properties, such as rhizosphere habitats. Moreover, studies on *Pseudomonas* diversity in the rhizosphere usually deal with annual crops, while studies on perennial plants are rare. These latter plants might induce a long-term selection of the most adapted populations due to continuous exchanges between soil and roots [23].

In this study, we investigated the culturable *Pseudomonas* populations in soil and root environments, in a natural meadow dominated by the perennial oligonitrophilic grass *Molinia coerulea*. We compared the influence of selective mS1 medium and non-selective Angle's medium [24] on both quantitative (colony-forming unit (CFU) numbers) and qualitative (genotypic diversity) recovery of *Pseudomonas*.

2. Materials and methods

2.1. Culture media

The *Pseudomonas* selective mS1 medium was similar to S1 medium [18], except that trimethoprim was omitted to allow the growth of non-fluorescent *Pseudomonas*, and that sucrose content was doubled to 20 g l^{-1} [12]. Total heterotrophic aerobic bacteria were enumerated on Angle's medium [24]. This medium was designed to have an ionic strength similar to that found in most non-saline soils. Ion concentrations of Angle's medium were (in mM): 2.5 NO_3^-, 2.5 NH_4^+, 0.05 HPO_4^{2-}, 2.5 Na^+, 4.0 Ca^+, 2.0 Mg^{2+}, 0.503 K^+, 4.0 Cl^-, 5.0 SO_4^{2-}, and 0.02 Fe^{2+}. Glucose (1 g l^{-1}) was used as carbon source.

2.2. Sampling and isolation

The study site consisted of an oligotrophic littoral meadow dominated by a genetically homogenous population of the perennial grass *M. coerulea* in a Gleysol, Typic Haplaquoll (4.7% clay, 9.5% silt, 85.8% sand, $pH_{[H2O]}$ 8.4) [25]. It is located in the Cudrefin preserved natural area on the southern shore of Lake Neuchâtel (Switzerland), at an elevation of 430 m above mean sea level. In July 2000, three undisturbed soil cores, 2–3 m apart, were collected from the upper 20 cm of soil and immediately analysed. These cores were pooled and then divided into two fractions: soil fraction constituted by soil devoid of root material. The root fraction, corresponding to the root of *M. coerulea*, was washed twice in sodium phosphate buffer 0.1 M pH 7.0 (SPB). For each fraction, 1 g of fresh material was finely crushed in 10 ml of SPB. Ten-fold serial dilutions of root and soil suspensions were prepared and 100 μl aliquots from the appropriate dilutions were spread out on eight mS1 and eight Angle plates. The same suspension was used to inoculate both media. CFU, at a proper dilution (20–200 colonies per Petri dish), were enumerated after 72 h of incubation at 24°C.

2.3. Strain handling and cultivation

From mS1 medium, colonies (noted as **S**) were randomly picked from the eight plates for soil and root fractions. From Angle's medium, all soil and root colonies (noted as **A**) from the eight plates were isolated. Angle and mS1 strains were streaked twice on Angle's medium for purity check and conserved on Angle plates at 4°C. Strains isolated on Angle were tested on mS1, and noted to be either $\mathbf{AS}^+$ or $\mathbf{AS}^-$ when able or unable to grow on mS1 respectively.

2.4. Colony hybridisation

All the isolated strains were tested for their affiliation to the genus *Pseudomonas* by colony hybridisation with the *Pseudomonas*-specific 16S rDNA PSM_G probe [26], as previously described [10]. The specificity of the hybridisation protocol to the genus *Pseudomonas* was confirmed by testing *Pseudomonas putida* (ATCC 17430) and *Pseudomonas fluorescens* (ATCC 17397) as positive controls, and *Escherichia coli* (DSM 2840) and *Enteroccocus faecalis* (ATCC 29212) as negative controls on each membrane. Strains giving a positive hybridisation signal were noted as **AH** (including $\mathbf{AS}^+\mathbf{H}$ and $\mathbf{AS}^-\mathbf{H}$), and **SH**.

2.5. Polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA

AH and **SH** strains were submitted to genomic DNA extraction with a Wizard Genomic DNA Purification Kit

(Promega, Madison, WI, USA) according to the manufacturer's protocol, except that only 400 µl of Nuclei Lysis solution were used. A 1100–1300-bp fragment of the rDNA operon (named 16S-ITS1 rDNA), including the 3′ half of 16S rDNA and the whole 16S–23S rDNA internal transcript spacer sequence (ITS1), was amplified using a *Pseudomonas*-specific PCR protocol [27]. The PCR products were checked for size and yield on 0.8% Standard Agarose gel (Eurobio, Les Ullis, France) using Low DNA Mass Ladder (Gibco). Two aliquots of eight µl of the PCR product were digested using *Hae*III endonuclease on one hand and *Taq*I on the other hand (Macherey-Nagel GmbH, Düren, Germany). The digested products were resolved by electrophoresis in 2% STG Agarose (Eurobio) in TBE 0.5× at 3.5 V cm^{-1} for 1.5 h and visualised by staining with ethidium bromide. The ΦX174/*Hae*III Fragment Ladder (Gibco) was used as the size ladder. Isolates displaying identical restriction profiles with *Hae*III and *Taq*I enzymes were grouped in the same operational taxonomic unit (OTU) [10].

2.6. Cloning and sequencing of 16S rDNA fragments

The 3′ end of the 16S rDNA gene sequence was amplified with 907f and GM4r primers [28]. PCR products were purified using a Nucleotrap Extraction Kit for Nucleic Acids (Macherey-Nagel), and cloned into pGEM-T vector (Promega) and *E. coli* competent cells [29]. Transformants with an expected size insert were processed with NucleoSpin Plasmid Kit (Macherey-Nagel) for plasmid extraction. The corresponding inserts were sequenced (MWG Biotech, Ebersberg, Germany). The identification of the corresponding organisms was achieved using a BLAST analysis of the retrieved sequences [30]. As non-ambiguous affiliation of strains could not always be achieved, they were classified into clusters according to Anzai et al. [31]. These sequences were registered in the EMBL database under the accession numbers AJ512378 to AJ512408, and AJ517396 to AJ517410 (Table 3).

2.7. Data analysis

CFU counts were compared statistically using the Student's *t*-test. Proportions of **H** strains were compared using a χ^2-test. Shannon diversity index was calculated, based on OTUs, as $H' = -\Sigma p_i \log_2 p_i$, where p_i is the number of isolates in the OTU being analysed. Evenness was calculated as $J = H'/H'_{max}$, where H'_{max} is the value of H' when all isolates are evenly distributed among the OTUs. Differences between Shannon index values for both media and for both fractions were evaluated using Student's *t*-test, according to Magurran [32]. Differences in OTU distribution on both media, and in both fractions, were evaluated using Fischer's exact test. The statistical analyses were performed using S-Plus 6 Statistical Software (Insightful Corporation, Seattle, WA, USA).

3. Results and discussion

3.1. Quantitative evaluation of root and soil Pseudomonas using mS1 and Angle's media

CFU counts on Angle's and mS1 media are presented in Fig. 1A. The total heterotroph A counts were significantly higher (about one log) than the **S** and **AS**$^+$ counts for both soil and root fractions (*t*-test, $P < 0.001$). For the soil fraction, **AS**$^+$ and **S** counts were not significantly different. But for the root fraction, **AS**$^+$ count was significantly higher than **S** count (*t*-test $P < 0.001$). This result showed that more root bacteria could grow on mS1 when they were first isolated on Angle's medium.

From Angle plates, 1770 colonies were isolated and tested for growth on mS1 medium. From mS1 plates, 108 soil and 118 root colonies were randomly picked up. Strains hybridising with the *Pseudomonas*-specific PSMg probe, and consequently affiliated to *Pseudomonas*, were designated with a **H** (Table 1). About 23% of mS1 soil strains (**AS**$^+$ and **S**) were not affiliated to *Pseudomonas* (4% only for root strains). Consequently, mS1 medium permitted the growth of non-*Pseudomonas* strains. Some *Pseudomonas* strains isolated on Angle's medium (**AH**) were unable to grow after transfer on mS1 medium (**AS**$^-$**H**). They represented 42.4% and 6.7% of **AH** strains for soil and root strains respectively. Proportions of *Pseudomonas* isolated on Angle's medium (**AH**) were statistically equivalent in both soil (9.6%) and root (11.1%) fractions (Table 1). However, the proportion of mS1-growing *Pseudomonas* (**AS**$^+$**H**) among total Angle isolates (**A**) was lower in the soil fraction (5.5%) compared to the root

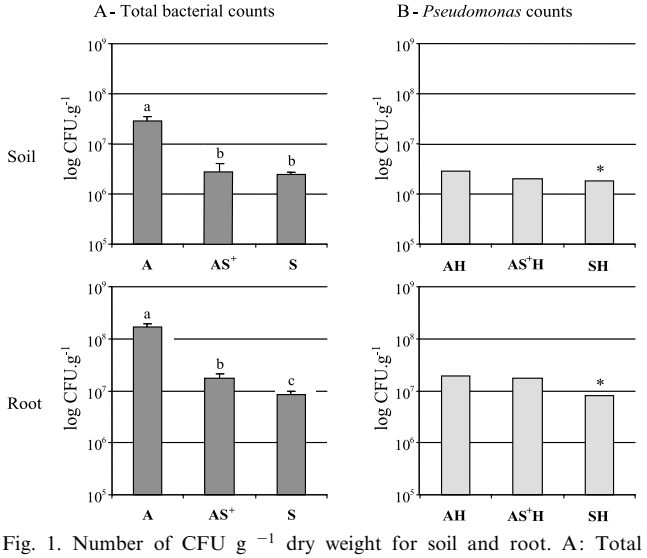


Fig. 1. Number of CFU g $^{-1}$ dry weight for soil and root. A: Total counts. B: *Pseudomonas* counts (calculated using **H** proportions, see Table 1). **A**: on Angle's non-selective medium, **S**: on mS1 *Pseudomonas* selective medium, and **AS**$^+$: after the two consecutive non-selective and selective media. * indicates an estimation from one hundred randomly picked colonies from mS1 medium. a,b,c: Different letters correspond to a highly significant statistical differences (*t*-test, $P < 0.01$).

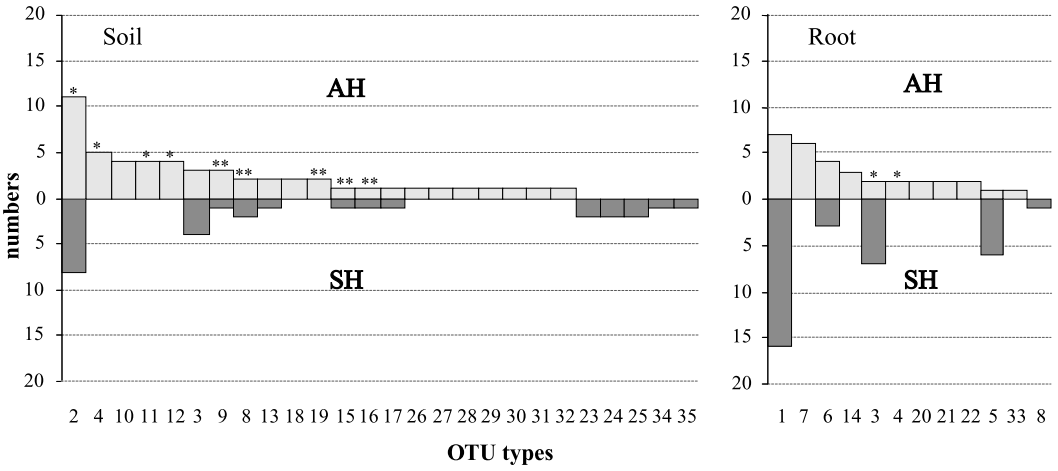


Fig. 2. Distribution of **AH** (isolated on Angle) and **SH** (isolated on mS1 medium) *Pseudomonas* strains among the different OTUs, for soil and root fractions. The name of each OTU is noted at the bottom. * indicates that Angle OTUs contained strains both capable (**AS$^+$H**) and incapable (**AS$^-$H**) of growing on mS1 medium. ** indicates Angle OTUs where all strains were unable to grow on mS1 (**AS$^-$H**). Other OTUs were composed of **AS$^+$H** strains.

fraction (10.4%) ($P < 0.01$). Our experimental set-up does not allow us to draw definite conclusions. Anyway, these results suggested that using the mS1 selective medium led to an underestimation of *Pseudomonas* proportions, as shown in previous studies [9,15]. This was particularly the case in the soil fraction, due to the occurrence of a large proportion of **AS$^-$H** strains. Furthermore, using both isolation media, *Pseudomonas* was a major component of the rhizosphere microflora, as already described for other grasses [9–10].

Using the proportion of **H** strains (Table 1), we could estimate the abundance of *Pseudomonas* in root and soil fractions from **A**, **AS$^+$** and **S** CFU counts (Fig. 1B). For the soil fraction, mS1-growing *Pseudomonas* counts were 30% lower than the **AH** count, using either direct isolation (**SH**) or prior cultivation on Angle (**AS$^+$H**). For the root fraction, the **SH** count was 57% lower than from **AH**, whereas the counts estimated from **AH** and **AS$^+$H** were similar. This result confirmed that isolation on mS1 medium led to an underestimation of *Pseudomonas* counts. Preliminary isolation on Angle improved the recovery of *Pseudomonas* strains capable of growing on mS1, mainly in the root fraction. Consequently root- and soil-inhabiting *Pseudomonas* strains did not have the same ability to grow on mS1.

There are two possible reasons for the differential recovery of *Pseudomonas* from mS1 and Angle's media. It is possibly because some *Pseudomonas* strains are inhibited by mS1 medium, even if transferred from fresh, active cultures on Angle's medium. This should be the main feature in soil. Alternatively, our results suggest that the physiological state of some cells do not allow them to form a colony by direct plating on mS1 at the time of isolation.

3.2. PCR–RFLP analysis

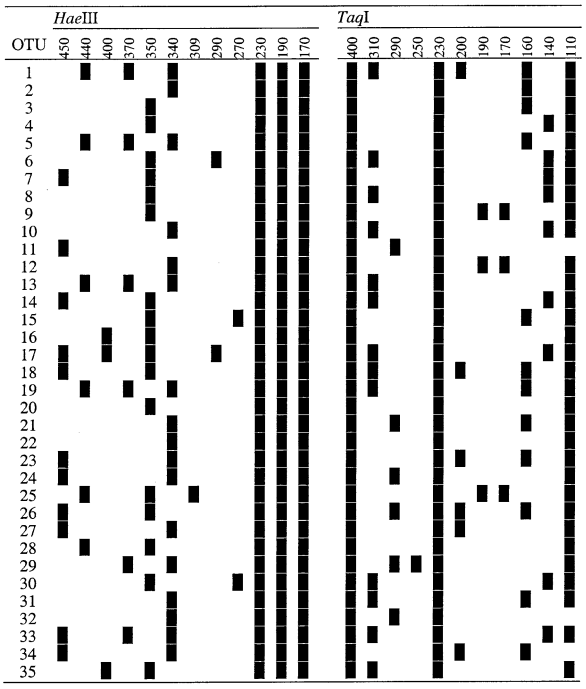
We then compared the genetic diversity of Angle and mS1 *Pseudomonas* strains for soil and root fractions. About 30% of all *Pseudomonas* strains were submitted to

Table 1
Numbers (**H** (*n*)) and proportions (**H** (%)) of root and soil *Pseudomonas* strains isolated on mS1 and Angle's media

		Isolates (*n*)	**H** (*n*)	**H** (%)
Soil	**A**	689	66	9.6
	AS$^+$	51	38	74.5
	AS$^-$	638	28	4.4
	S	108	83	76.9
Root	**A**	1081	120	11.1
	AS$^+$	114	112	98.2
	AS$^-$	967	8	0.8
	S	118	113	95.8

A: Total heterotroph bacteria isolated on Angle's medium.
AS$^+$: Angle isolates growing on *Pseudomonas* selective mS1 medium.
AS$^-$: Angle isolates unable to grow on *Pseudomonas* selective mS1 medium.
S: strains directly isolated on mS1 selective medium.

Table 2
Schematic OTU pattern restriction profiles obtained by digestion of the PCR-amplified half 3′ end of 16S rDNA and whole ITS1 regions



OTUs were defined on the combined restriction patterns of *Hae*III and *Taq*I. The size of each band is given in base pairs.

PCR–RFLP analysis of 16S-ITS1 rDNA fragments. Fifty-two soil strains and 32 root strains from Angle (**AH**) and 27 soil strains and 33 root strains from mS1 (**SH**) were analysed. The 16S-ITS1 rDNA sequences of these 144 strains could be amplified with the *Pseudomonas*-specific PCR protocol, confirming their affiliation to the genus *Pseudomonas* [27]. For 29% of the strains, two or three PCR fragment sizes were generated (1050–1300 bp), as previously shown [33]. The amplicons of different sizes were due to the presence or absence of tRNA genes within the ITS1 region of different ribosomal operons within the same genome [34]. When ITS1 PCR products were digested, the sum of restriction fragment sizes was higher than undigested ITS1 fragments, even for a single-sized PCR product. Cho and Tiedje reported similar results and suggested the existence of at least two types of ITS1 regions in a PCR product of the same size [35].

Pseudomonas strains were grouped using restriction analysis of their PCR products. The corresponding restriction profiles are presented in Table 2. The endonucleases were equally discriminating, as 14 and 16 different restriction patterns were obtained with *Hae*III and *Taq*I enzymes respectively. When combining these profiles, the 144 strains were grouped in 35 different OTUs (Table 2). The clustering of strains into the different OTUs was not influenced by the isolation plate they were retrieved from (data not shown). Fig. 2 presents the repartition of **AH** and **SH** strains for soil and root fractions among the 35 OTUs.

3.3. OTU distribution between soil and root

The 79 soil strains were grouped in 26 OTUs. In contrast, the 65 root strains formed only 12 OTUs (Table 3). Twice as many *Pseudomonas* OTUs were described in the soil fraction compared to the root fraction for each culture medium. The overall distribution of *Pseudomonas* strains among OTUs was different between soil and root fractions. Such a difference was observed on mS1 ($P < 0.0001$) as well as on Angle ($P < 0.0001$). Twenty-three OTUs were recovered from soil alone, while nine OTUs were specifically found in the root fraction (Table 3). Previous studies for other plants [12,36–37] have shown that root environment was selective for some *Pseudomonas* types. Three OTUs (3, 4 and 8) were found among both soil and root strains. Nine soil OTUs and one root OTU were represented by a single isolate. Root and soil fractions harboured different major OTUs (Fig. 2). OTU1 grouped 35% of root strains exclusively, while OTU2 grouped 24% of soil strains exclusively. The OTU3 was the second most abundant in both soil and root fractions (9% and 14% of strains respectively).

3.4. Comparison of Angle and mS1 OTU distribution

The Shannon diversity index H' [32] and evenness J were calculated for root and soil *Pseudomonas* recovered on Angle and mS1 (Table 4). The comparison of Shannon index suggested that Angle's medium permitted a higher diversity to be recovered, compared to the mS1 medium, in both soil and root fractions (*t*-test non-significant). The evenness values for soil were similar using both media, whereas for root strains, the evenness was lower on mS1, indicating different population structures. The distribution of *Pseudomonas* strains among OTUs was different using Angle's and mS1 media, for both soil and root fractions ($P = 0.0002$ for root). Indeed, the 84 **AH** strains were grouped into 30 OTUs, while the 60 **SH** strains were grouped into 16 OTUs (Table 3). Eleven OTUs were retrieved on both Angle's and mS1 media, including the most abundant OTU1 (root), OTU2 (soil) and OTU3 (common). OTU1 and OTU2 displayed the highest strain numbers on both media. Five non-abundant soil types (OTU23, 24, 25, 34, and 35) were retrieved on the mS1 medium alone (Fig. 2). Nineteen OTUs were only retrieved on Angle's medium. These included some abundant OTUs, such as the OTU4 (common to soil and root), OTU7 (19% of root strains), and OTU10, 11 and 12 (23% of soil strains).

Eight OTUs (as OTU3, 4, 11, and 12) contained strains both capable and incapable of growing on mS1 (Fig. 2). Consequently, the ability of strains to grow on mS1 was not related to their clustering using PCR-digestion of the rDNA sequences. Among the 30 OTUs found on Angle, 25 contained **AS$^+$H** strains, i.e. they could grow on mS1. Four OTUs comprised **AS$^-$H** strains, though these were

Table 3
Isolate numbers in each OTU, spread out between media (Angle and mS1) and fractions (soil and root), and phylogenetic affiliation based on partial 16S rDNA sequence

OTU	Global			Angle			mS1			*Pseudomonas*-related group*	Accession numbers
	n	soil	root	*n*	soil	root	*n*	soil	root	(*n*, category of sequenced strains)	
1	23	–	23	7	–	7	16	–	16	*fluorescens/aeruginosa* (2, **AS$^+$H-AS$^+$H**)	AJ512378-AJ512379
2	19	19	–	11	11	–	8	8	–	*aeruginosa/putida* (2, **AS$^-$H-AS$^+$H**)	AJ512380-AJ512381
3	16	7	9	5	3	2	11	4	7	*fluorescens* (2, **AS$^+$H-AS$^-$H**)	AJ517399-AJ517400-
										putida/graminis (1, **AS$^+$H**)	AJ512382
4	7	5	2	7	5	2	–	–	–	*fluorescens* (2, **AS$^+$H-AS$^-$H**)	AJ512383-AJ512384
5	7	–	7	1	–	1	6	–	6	*fluorescens* (2, **AS$^+$H-SH**)	AJ517398-AJ517397
6	7	–	7	4	–	4	3	–	3	*chlororaphis/fluorescens* (1, **AS$^+$H**)	AJ517396
7	6	–	6	6	–	6	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512385
8	5	4	1	2	2	–	3	2	1	*fluorescens* (2, **AS$^+$H-SH**)	AJ512386-AJ512387
9	4	4	–	3	3	–	1	1	–	*aeruginosa*(2, **AS$^-$H-SH**)	AJ517403-AJ517404
10	4	4	–	4	4	–	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512388
11	4	4	–	4	4	–	–	–	–	*aeruginosa* (2, **AS$^+$H-AS$^-$H**)	AJ512389-AJ517410
12	4	4	–	4	4	–	–	–	–	*aeruginosa* (2, **AS$^+$H-AS$^-$H**)	AJ512390-AJ517408
13	3	3	–	2	2	–	1	1	–		
14	3	–	3	3	–	3	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512391
15	2	2	–	1	1	–	1	1	–	*aeruginosa/fluorescens* (2, **AS$^-$H-SH**)	AJ517401-AJ517402
16	2	2	–	1	1	–	1	1	–	*aeruginosa* (2, **AS$^-$H-SH**)	AJ517405-AJ517406
17	2	2		1	1	–	1	1	–	*putida* (1, **AS$^+$H**)	AJ517409
18	2	2	–	2	2	–	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512392
19	2	2	–	2	2	–	–	–	–	*fluorescens* (1, **AS$^-$H**)	AJ512393
20	2	–	2	2	–	2	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512394
21	2	–	2	2	–	2	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512395
22	2	–	2	2	–	2	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512396
23	2	2	–	–	–	–	2	2	–	*fluorescens* (1, **SH**)	AJ512397
24	2	2	–	–	–	–	2	2	–	*fluorescens* (1, **SH**)	AJ512398
25	2	2	–	–	–	–	2	2	–	*fluorescens* (1, **SH**)	AJ512399
26	1	1	–	1	1	–	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512400
27	1	1	–	1	1	–	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512401
28	1	1	–	1	1	–	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512402
29	1	1	–	1	1	–	–	–	–	*fluorescens* (1, **AS$^+$H**)	AJ512403
30	1	1	–	1	1	–	–	–	–	*putida* (1, **AS$^+$H**)	AJ512404
31	1	1	–	1	1	–	–	–	–	*aeruginosa* (1, **AS$^+$H**)	AJ512405
32	1	1	–	1	1	–	–	–	–	*tolaasi* (1, **AS$^+$H**)	AJ517407
33	1	–	1	1	–	1	–	–	–	*syringae* (1, **AS$^+$H**)	AJ512406
34	1	1	–	–	–	–	1	1	–	*fluorescens* (1, **SH**)	AJ512407
35	1	1	–	–	–	–	1	1	–	*fluorescens* (1, **SH**)	AJ512408
Strains (*n*)	144	79	65	84	52	32	60	27	33		
OTUs (*n*)	35	26	12	30	21	11	16	13	5		

**Pseudomonas* groups are defined according to Anzai et al. [31].

also retrieved on mS1 medium (OTU8, 9, 15 and 16; Fig. 2). More generally, OTUs including **AS$^-$H** were more frequent in soil (nine among 21 OTUs) than in root (two among 11) fractions. The soil OTU19 was the only one composed exclusively of **AS$^-$H** strains, i.e. it was never detected on mS1.

Eighteen OTUs were never detected by direct isolation on mS1. This was the case for about 50% of OTUs recovered from soil as well as from root fractions. For example, the root OTU7 was abundant among Angle strains and all its representatives were **AS$^+$H**. In contrast, none of these strains were directly isolated on the mS1 medium. This suggested that the physiological state of bacteria at the time of isolation influenced their direct recovery on the mS1 medium.

Angle's medium attempts to approximate the composition of the soil solution [24]. Its use should enhance the recovery of soil-inhabiting bacteria, as shown in this study. In contrast, high nutrient levels in the mS1 medium may affect the capacity of environmental *Pseudomonas* to grow and form colonies. This could explain in part the lower recovery of soil *Pseudomonas* strains on mS1. Aagot et al.

Table 4
Diversity (Shannon index H') and evenness (J) indexes among OTUs isolated from soil and/or root fractions, as obtained using Angle's or mS1 medium for the isolation of strains

	Soil		Root	
	Angle	mS1	Angle	mS1
H'	3.95	3.27	3.19	1.90
J	0.69	0.69	0.64	0.38

[15] also showed that media with high nutrient concentrations can have a negative effect on *Pseudomonas* colony formation in samples collected from soil. They suggested that *Pseudomonas* isolated on nutrient-poor media might occupy different ecological niches to those *Pseudomonas* recovered on traditional nutrient-rich isolation media. More generally, bacteria with different physiological states might be present simultaneously in the rhizosphere [38]. Some aspects of plant–microbe environment could also affect the differential recovery using the two media. When comparing mS1 and Angle for root *Pseudomonas* populations, we noticed that OTU3 and OTU5 displayed high numbers on the mS1 medium, whereas these were poorly represented among Angle strains. Such a result was also observed, to a lesser extent, for the major root OTU1. The corresponding strains could display a certain tolerance to high osmotic levels [39] or adaptation to high nutrient levels [15], and their relative abundance may be overestimated on mS1 isolation medium. As suggested by Wilson and Lindow [40], the culturability of environmental bacteria may be affected by components of the medium required for their selective isolation from environmental samples. In conclusion, there are two features which may explain the failure to isolate environmental *Pseudomonas* strains on mS1 medium. This may be due to the intrinsic inability of some strains to grow on mS1 medium. Alternatively it may be due to the inability of other strains to be revived on the mS1 medium even if they are able to grow on mS1 after being transferred from active culture.

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