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Diversity among strains of *Pseudomonas aeruginosa* from manure and soil, evaluated by multiple locus variable number tandem repeat analysis and antibiotic resistance profiles

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Abstract

The results of a multiple locus variable number of tandem repeat (VNTR) analysis (MLVA)-based study designed to understand the genetic diversity of soil and manure-borne *Pseudomonas aeruginosa* isolates, and the relationship between these isolates and a set of clinical and environmental isolates, are hereby reported. Fifteen described VNTR markers were first selected, and 62 isolates recovered from agricultural and industrial soils in France and Burkina Faso, and from cattle and horse manure, along with 26 snake-related isolates and 17 environmental and clinical isolates from international collections, were genotyped. Following a comparison with previously published 9-marker MLVA schemes, an optimal 13-marker MLVA scheme (MLVA₁₃-Lyon) was identified that was found to be the most efficient, as it showed high typability (90%) and high discriminatory power (0.987). A comparison of MLVA with PFGE for typing of the snake-related isolates confirmed the MLVA₁₃-Lyon scheme to be a robust method for quickly discriminating and inferring genetic relatedness among environmental isolates. The 62 isolates displayed wide diversity, since 41 MLVA types (i.e. MTs) were observed, with 26 MTs clustered in 10 MLVA clonal complexes (MCs). Three and eight MCs were found among soil and manure isolates, respectively. Only one MC contained both soil and manure-borne isolates. No common MC was observed between soil and manure-borne isolates and the snake-related or environmental and clinical isolates. Antibiotic resistance profiles were performed to determine a potential link between resistance properties and the selective pressure that might be present in the various habitats. Except for four soil and manure isolates resistant to ticarcillin and ticarcillin/clavulanic acid and one isolate from a hydrocarbon-contaminated soil resistant to imipenem, all environmental isolates showed wild-type antibiotic profiles.

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Keywords: *Pseudomonas aeruginosa*; Antibiotics; Resistance; MLVA typing

1. Introduction

Pseudomonas aeruginosa, a ubiquitous Gram-negative bacterium, is known to be an opportunistic human pathogen and an important cause of infection in immunocompromised patients. It is responsible for severe nosocomial pneumonias among intensive care unit patients and for the chronic lung infections of patients with cystic fibrosis [26,27]. Community-

acquired infections in healthy individuals have also been reported, including keratitis [41], otitis [15] and dermatitis [44]. In addition, *P. aeruginosa* causes a wide variety of diseases in animals, such as ocular infections in dogs [25] and cattle mastitis [8]. This species is characterized by inherent resistance to numerous antimicrobial agents due to its low membrane permeability and the presence of several drug efflux systems and porins, as well as a remarkable ability to acquire further resistance mechanisms.

P. aeruginosa is a metabolically versatile bacterium capable of surviving in natural and human-associated environments. It has been frequently isolated from a wide range of aquatic

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environments including rivers [32], sea water [22], swimming pools [3], bottled water [17] and wastewater [11]. It has also been recovered from terrestrial environments such as agricultural soils [12] and hydrocarbon-polluted sites [10], but at a lower frequency [37].

Studies on the genetic structure of *P. aeruginosa* populations have been massively conducted on clinical isolates for ecological and epidemiological purposes [7,23,50] using various molecular typing methods. Pulsed field gel electrophoresis (PFGE), the “gold standard” method, showed high discriminatory power in epidemiology studies [13]. However, this technique does not allow easy comparison of results between different studies because of interlaboratory variations in the reproducibility and absence of an international database [46]. Multi-Locus sequence typing (MLST) is an increasingly used technique, but it remains expensive and is less discriminatory than PFGE for detecting genetic differences among related *P. aeruginosa* isolates [18]. In recent years, multiple locus VNTR analysis (MLVA), a PCR-based typing method in which isolates are characterized by the number of repeat units at each locus [47], has been developed and optimized, leading to several schemes validated in studies on *P. aeruginosa* clinical isolates [39,45,46,48]. Recently, [39] developed a high-throughput MLVA assay based on multiplex PCRs, followed by capillary electrophoresis to evaluate strain evolution during long-term colonization of cystic fibrosis patients. MLVA-based studies showed that this approach is highly discriminatory and reproducible, congruent with PFGE analysis, easy to standardize and applicable to a large number of samples at a low cost. Nevertheless, all these molecular typing methods have been primarily developed and used for surveillance of epidemic strains and for investigation into the global population structure of *P. aeruginosa*. Studies using these typing methods focused on clinical isolates and compared them to isolates mainly from aquatic environments but rarely from terrestrial ones. Furthermore, MLVA protocols have not yet been tested to type and infer genetic relatedness among environmental isolates or between environmental and clinical isolates.

The aim of this study was to understand the genetic diversity of soil- and manure-borne *P. aeruginosa* isolates and the relationship between these isolates and a set of clinical and other soil-related isolates mostly selected from international collections. For these purposes, an MLVA assay was used. Our strategy first relied on the selection of a set of 15 VNTR loci made of all the loci used in the MLVA₉-London [45] and MLVA₉-Utrecht [46] schemes, in order to evaluate whether one of these simplified schemes would be robust enough for genotyping of our soil-related isolates. This preliminary step enabled development of the MLVA₁₃-Lyon scheme, whose discriminatory efficiency was compared to that of PFGE typing using a subset of 26 previously studied snake-related isolates [5]. This typing scheme was then applied to isolates recovered either from agricultural and industrial soils or from horse and cattle manure sampled in various areas in France and Burkina Faso. As *P. aeruginosa* strains from manure can originate from animals exposed to antibiotics that might exert

a selective pressure on resistance emergence, the antibiotic susceptibilities of these strains were also evaluated.

2. Materials and methods

2.1. Bacterial isolates and DNA preparation

A set of 105 isolates was used in the present study (Table 1). Seventeen were from public international collections and were isolated from clinical ($n = 7$) and environmental ($n = 10$) sources. Among them, strains PAO1 and PA14 were included to be used as standards in each MLVA electrophoresis assay. Eighty-eight environmental isolates were from our laboratory collection (available from the EML Biological Resource Center at <http://www.eml-brc.org/>). They had been isolated on Cetrimide agar base medium supplemented with nalidixic acid from samples of various origins harvested between 2005 and 2011. Isolation and identification procedures were as described previously [6]. Twenty-three isolates were of soil origin: 12 were isolated from industrial sites contaminated with hydrocarbons (Neuves-Maisons in the Lorraine region and Paris in the Ile-de-France region, France), three from a vineyard field amended with mushroom manure (i.e. a compost of horse manure and straw that is used to grow mushrooms; Chinon, in the Burgundy region, France) and eight from a field planted with sorghum and amended with raw urban waste in the periphery of Ouagadougou in Burkina Faso. Thirty-nine isolates were from manure samples: 11 from horse manure collected from two sites in the Rhône-Alpes region (six from the National Veterinary School of Lyon, Marcy l’Etoile and five from a farm in Saint Olive), 13 from cattle manure collected at two farms in Feucherolles, Ile-de-France ($n = 8$) and Versailles, Rhône-Alpes ($n = 5$) and 15 from composted horse manure supplemented with either crude farm wheat straw (farm 1) or commercial wheat straw (Hippogold®) (farm 2) in Versailles (Ile-de-France). There were also 26 strains isolated from snake breeding facilities (healthy snake feces, cages, feeding samples), which had been previously characterized by PFGE SpeI profiles [5].

Isolates were grown at 28 °C in Luria-Bertani broth under agitation (160 rpm). Twenty ml of an overnight culture were used for DNA extraction. Extraction was carried out as previously described [34]. The DNA concentration was estimated with an ND-1000 spectrophotometer (NanoDrop; Labtech, Palaiseau, France).

2.2. MLVA genotyping

A set of 15 VNTR loci was selected for this study (Table 2). It included 12 minisatellites (ms77, ms127, ms142, ms172, ms211, ms213, ms214, ms215, ms216, ms217, ms222, ms223) and three microsatellites (ms61, ms207, ms209) previously described in [29] and [48].

To lower the number of PCR reactions, multiplex PCR was developed for nine of the 15 selected loci (multiplex 1: ms209, ms77, ms172; multiplex 2: ms214, ms61; multiplex 3: ms207, ms216; multiplex 4: ms127, ms222). Other loci were classically amplified in single PCR reactions. Single and multiplex

Table 1
List of strains and isolates, with their origin, sampling location, year of isolation and previously published labels.

Name	Origin	Location	Isolation year	Previous labels (respectively)
<i>Reference strains</i>				
PAO1	Wound, clinical	Melbourne (Australia)	1955	
PA14	Burn, clinical	UCBPP collection, Berkeley California, (USA)		
PA5	Intensive Care Unit, clinical	Brussels (Belgium)		
PA6	Urinary tract infection, clinical	Brussels (Belgium)		
PA12	Urinary tract infection, clinical	Brussels (Belgium)		
ATCC 15691	Urinary tract infection, clinical	Cape Town (South Africa)	1950	
ATCC 27853	Blood, clinical	Boston (USA)	1971	
ATCC 21776	Soil	Chiba (Japan)	1974	
ATCC 31479	Soil	Salem, Virginia (USA)	1981	
CIP 104590	PAH-contaminated soil	Munich (Germany)	1995	
LMD 50.34	Soil	Bogor (Indonesia)	1949	
LMD 68.7	Garden soil	Delft (Netherlands)	1968	
LMG 15153	Permian salt deposits	Nauheim (Germany)	1963	
7NSK2	Barley roots	Melle (Belgium)	1983	
CFBP 5036	Oil-contaminated soil	USA	1987	
CFBP 5037	Oil-contaminated soil	USA	1987	
ATCC 33988	Fuel storage tank	Ponca City, Oklahoma, (USA)		
<i>Team strain collection</i>				
Soil				
EML1316, EML1317, EML1319, EML1320, EML1321, EML1322, EML1323, EML1324, EML1325	Hydrocarbon- impacted soil	Neuves Maisons, Lorraine (France)	2005	bpoe1428, bpoe1429, bpoe1431, bpoe1432, bpoe1433, bpoe1444, bpoe1445, bpoe1447, bpoe1454
EML1331, EML1332, EML1333	Hydrocarbon- impacted soil	Paris, Île de France (France)	2005	bpoe1464, bpoe1465, bpoe1466
EML1326, EML1327, EML1328	Vineyard soil amended with mushroom manure	Chinon, Burgundy (France)	2005	bpoe1457, bpoe1459, bpoe1460
EML1257, EML1258, EML1259*, EML1260*, EML1261*, EML1262*, EML1263*, EML1264*	Raw urban waste- contaminated soil	Tabtenga (Burkina Faso)	2007 (EML1257) 2011	
Manure				
EML1318, EML1329, EML1330, EML1334, EML1335, EML1336	Horse manure	Marcy l'Etoile, Rhône-Alpes (France)	2004	bpoe1430, bpoe1461, bpoe1462, bpoe1474, bpoe1475, bpoe1479
EML1270, EML1271, EML1272, EML1273, EML1274	Horse manure	Saint Olive, Rhône-Alpes (France)	2012	
EML1275, EML1276, EML1277, EML1278, EML1279, EML1280, EML1281, EML1282	Cattle manure	Feucherolles, Île- de-France (France)	2006	
EML1265, EML1266, EML1267, EML1268, EML1269	Cattle manure	Versailleux, Rhône-Alpes (France)	2012	
EML1283, EML1284, EML1285, EML1292, EML1293, EML1294, EML1295, EML1296, EML1297	Compost of farm straw and horse manure	Farm 1 Versailles, Île-de- France (France)	2011	
EML1286, EML1287, EML1288, EML1289, EML1290, EML1291	Compost of commercial straw (Hippogold®) and horse manure	Farm 2 Versailles, Île-de- France (France)	2011	

Snake breeding facility	Healthy captive snake feces and breeding equipment	Lille, Nord-Pas de Calais, (France)	2004	bpoec600, bpoec602, bpoec603, bpoec605, bpoec610, bpoec612, bpoec616, bpoec618, bpoec626, bpoec662, bpoec667, bpoec674
EML59, EML61, EML62, EML64, EML67, EML68, EML71, EML73, EML80, EML100, EML117, EML124	Healthy captive snake feces and feeding rat feces	Loire (France)	2004	bpoec636, bpoec639, bpoec642, bpoec688, bpoec690, bpoec692, bpoec700, bpoec701
EML88, EML91, EML94, EML134, EML136, EML138, EML145, EML146	Healthy captive snake feces	Lyon, Rhône-Alpes (France)	2004	bpoec649, bpoec652, bpoec654, bpoec657
EML104, EML107, EML109, EML111, EML151, EML162	Healthy wild snake feces	Nzérékoré (Guinea)	2006	bpoec742, bpoec753

ATCC: American Type Culture Collection, Rockville, MD, USA. CFBP: Collection française de Bactéries phytopathogènes, INRA, Angers, France. CIP: Collection de l'Institut Pasteur, Paris, France. LMG: Laboratorium Microbiologie Ghent Culture Collection, Ghent, Belgium. NCBB: The Netherlands Culture Collection of Bacteria (in the past LMD and Phabagen), Utrecht, Netherlands.

EML isolates are from the Environment Microbiology Lyon Biological Resource Center Collection and were isolated by the author's research team.

Isolate names followed by * indicate that these isolates were isolated after an acetamide enrichment step.

PCRs were performed in 25 µl volumes containing 20–40 ng of DNA template, 1× PCR buffer, 200 µM of each dNTP, 0.5 µM of each primer, 1 U of Taq polymerase (MP Biomedical, Illkirch, France) and DMSO at a final concentration of 5% w/vol. Forward primers were differentially labeled with IRDye 700 and IRDye 800 to distinguish the PCR products after migration on acrylamide gels. Amplifications were performed in a PTC 200 thermocycler (MJ-Research, Marnes-la-Coquette, France) under the following conditions: an initial denaturation cycle for 5 min at 95 °C and 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 58 °C and elongation for 45 s at 72 °C followed by a final elongation step for 8 min at 72 °C.

Separation of the PCR products was performed in a LICOR® 4300 DNA analyzer (ScienceTec, Les Ulis, France) with 5.5% w/vol acrylamide gels under the following conditions: a pre-run of 25 min at 2000 V, 40 W, 45 °C and a run of 2 h 30 at 2000 V, 40 W, 45 °C. To minimize the number of wells needed for the migration, PCR products were pooled in five different combinations (Table 2). The following five pools were then diluted 50- to 100-fold to reduce the amount of fluorescence and to avoid saturation of the sensor. According to the manufacturer's recommendations, after the first migration run, the gels were reloaded for a second run without a pre-run step.

2.3. Data analysis

Gel data were collected and the different allele sizes were determined with SAGA^{GT} software (ScienceTec). The different genotypes of the samples were constructed by expressing the number of repeats for each locus according to the PAO1 sequence as a reference and by using the chart of the different allele sizes observed by [48] proposed on the website <http://bacterial-genotyping.igmors.u-psud.fr/pseudomonas/>.

When the allele size measured for an amplicon was not previously described, an allele number was attributed if the size of the amplicon matched a hypothetical number of copies for the locus. As previously described, intermediate-sized alleles resulting from intermediate-sized repeat units or small deletions in the sequence were reported as half sizes. Alleles of unexpected size with non-attributable allele number and loci that did not yield PCR products over 3 PCR reactions were assigned allele "99" to ascribe these isolates for further clustering analysis. These alleles of unexpected sizes or non-amplifiable alleles can be produced by insertion of IS elements as described in [48], but this was not confirmed.

Typability criteria defined by [28] were calculated as described in [46] as the number of isolates for which repeat number could be inferred for all loci of the MLVA set divided by all isolates tested.

The discriminatory indices (DIs) of each VNTR locus and MLVA typing scheme were calculated by use of the Hunter-Gaston Discriminatory Index (HGDI) [16], an application of Simpson's index of diversity [38]. The 95% confidence intervals (CI) were calculated as described in [14].

MTs obtained with the different MLVA typing schemes were clustered with the R software using a categorical

Table 2
VNTR markers used in this study, characteristics, typability and discriminatory indexes and presence in the published MLVA schemes.

Locus name	Repeat unit size (bp)	Multiplex PCR	Migration pool	No. of observed alleles (alleles)	New alleles	Typability (%)	HGDI index	MLVA ₁₅ -Lyon	MLVA ₁₃ -Lyon	MLVA ₁₅ -Orsay	MLVA ₁₆ -Orsay	MLVA ₉ -London	MLVA ₉ -Utrecht
ms61	6	2	1	12 (4, 6–15, 17)	no	100	0.896	X	X	X	X	X	X
ms77	39	1	2	8 (1–6 + 1.5, 2.5)	no	100	0.625	X	X	X	X	X	X
ms127	15	4	3	2 (8–9)	no	100	0.455	X	X	X	X	X	X
ms142	115	no	4	9 (1–7, 12 + 2.5)	12	100	0.833	X	X	X	X	X	X
ms172	54	1	2	7 (8–13 + 8.5)	no	77	0.797	X	X	X	X	X	X
ms211	101	no	5	9 (2–9 + 1.5)	1.5	97	0.794	X	X	X	X	X	X
ms212	40	Not used in this study											
ms213	103	no	5	11 (0–7, 9–10 + 4.5 + 5.5)	0, 5.5, 10	98	0.858	X	X	X	X	X	X
ms214	115	2	1	8 (2–6, 12 + 2.5 + 5.5)	12	98	0.812	X	X	X	X	X	X
ms215	129	no	2	8 (1–7 + 2.5)	7	97	0.861	X	X	X	X	X	X
ms216	113	3	1	5 (1–5)	no	98	0.645	X	X	X	X	X	X
ms217	109	no	4	7 (1–6, 9)	9	100	0.732	X	X	X	X	X	X
ms222	101	4	3	7 (1–5, 7 + 5.5)	5.5, 7	99	0.764	X	X	X	X	X	X
ms223	106	no	3	6 (2–7)	no	100	0.697	X	X	X	X	X	X
ms207	6	3	1	10 (4–13)	no	100	0.872	X	X	X	X	X	X
ms209	6	1	2	5 (3–6, 9)	no	100	0.649	X	X	X	X	X	X

coefficient (also called Hamming's distance) and the unweighted pair group method with arithmetic mean (UPGMA) clustering method [40]. An MLVA type (MT) is a genotype obtained by MLVA typing. An MLVA clonal complex (MC) is defined as the clustering of MTs that differs at a maximum of two VNTRs, which corresponds to an interval of 85–100% similarity between MTs.

The quantitative level of congruence between the typing schemes was calculated using adjusted [35] and [49] coefficients available at <http://www.comparingpartitions.info/>. The adjusted Rand coefficient defines the global agreement between two methods or two protocols, whereas the adjusted Wallace coefficient indicates the probability that two isolates classified as the same type following a given protocol are also classified as the same type using another protocol [4].

2.4. Antibiotic resistance test

The in vitro antimicrobial resistance patterns of *P. aeruginosa* were determined using the Vitek®2 system with a card (NO93) dedicated to non-fermenting Gram-negative bacteria (Biomérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. *P. aeruginosa* ATCC 27853 (wild-type) was used as a control strain. Minimum inhibitory concentration (MIC) results were analyzed using the AES™ (Advanced Expert System) software incorporated in this system. Snake-related isolates and part of our set of soil and manure isolates were previously tested in [5] and [9], respectively.

3. Results

3.1. MLVA schemes: efficiency and comparison

The MLVA typing method has not yet been used on environmental isolates. An MLVA scheme (MLVA₁₅-Lyon) made up of a set of 15 VNTRs previously used in the two published and simplified MLVA₉-London and MLVA₉-Utrecht MLVA schemes (Table 2) was first tested. The rationale behind this strategy was to evaluate whether one of these simplified schemes or a new scheme combining the lowest number of VNTRs would enable efficient discrimination and clustering of soil-related isolates.

The 15 VNTR markers selected for MLVA₁₅-Lyon showed typability superior to 97% except for the ms172 marker (Table 2), where an absence of amplification was frequently observed when typing manure isolates from miscellaneous sampling sites (Table 3). This lack of amplification can be explained by mismatches in the primer target regions (Fig. 1) or by rearrangements in the genomic sequence of the locus (large insertion sequences, partial or complete deletion of the VNTR flanking sequence) but this was not confirmed.

The HGDI of each marker considered separately was superior to 0.625 except for the ms127 marker, that showed an HGDI of 0.455. Alleles not previously described in the literature were observed for VNTRs ms142, ms211, ms213, ms214, ms215, ms217 and ms222 (Table 2). These new alleles occurred among isolates from both soil and manure.

Table 3
Number of repeats for each marker, MLVA type (MT) and MLVA clonal complex (MC) for the studied *P. aeruginosa* isolates.

Sample	MT	MLVA ₁₃ -Lyon	MC	VNTR														
				61	207	209	77	127	142	172	211	213	214	215	216	217	222	223
PAO1	1			12	7	6	4	8	7	12	5	5	3	4	3	2	2	4
PA14	2			12	5	6	2	9	1	12	2	1	5	2	1	5	2	4
PA5	3			8	12	6	4	9	2	12	2	5	3	2	1	2	2	2
PA6	4			8	6	5	2.5	8	4	11	3	4.5	3	2	2	2	2	2
PA12	5			8	11	5	2	8	12	99	5	2	5.5	5	3	9	3	3
ATCC 15691	6			9	7	5	2.5	8	4	11	2	3	4	5	1	3	3	7
ATCC 27853	7			9	9	5	2.5	8	5	11	4	5	2	4	2	3	2	2
ATCC 21776	8			8	5	4	2.5	8	4	8	7	4	5	6	2	3	3	2
ATCC 31479	9			11	7	3	4	9	1	10	2	4	2	2	1	4	3	2
CIP 104590	10			9	4	4	2.5	8	2.5	8.5	8	5	2	1	2	1	2	2
LMD 50.34	11			8	10	4	2.5	8	1	13	3	4	5	1	1	2	1	2
LMD 68.7	12			14	9	3	2.5	8	3	11	6	5	4	2	2	3	3	2
LMG 15153	13			13	6	3	2.5	8	3	12	3	3	5	5	2	1	4	4
7NSK2	14			8	5	4	2.5	8	4	12	3	4	2	6	2	2	1	5
CFBP 5036, CFBP5037	15			13	10	3	2.5	9	1	12	3	4.5	5.5	4	3	4	3	4
ATCC 33988	16			10	7	3	2.5	8	4	11	2	4.5	5	5	2	2	2	5
EML59	17			10	9	3	4	9	12	10	2	1	2	2	1	1	1	3
EML61, EML62, EML68, EML124, EML136	18	2		10	4	4	2.5	8	3	8.5	8	5	2	1	2	1	2	3
EML64, EML100	19	16		7	7	4	2.5	9	7	11	2	4	5	5	2	2	2	5
EML67, EML117	20	5		7	8	4	6	9	1	10	2	1	2.5	2	1	4	4	3
EML71	21	5		7	8	4	6	9	1	10	2	1	4	2	1	4	4	3
EML73	22	5		7	9	4	6	9	1	10	2	1	2.5	2	1	4	4	3
EML80	23		15	8	4	6	9	1	10	2	4	3	1	2	3	3	2	
EML88, EML134, EML146	24	15	14	11	3	2.5	8	5	12	2	1	5	4	1	2	2	5	
EML91	25	7	14	6	3	2.5	9	1	12	5	6	2	2	1	4	1	2	
EML94	26		8	5	6	2.5	8	6	10	3	5	4	6	2	4	4	2	
EML104	27		10	8	5	2.5	8	5	11	2	3	5	6	1	2	2	2	
EML107, EML111	28	8	12	7	4	2	9	1	12	2	5	3	1	2	2	3	6	
EML109	29	16	4	7	4	2.5	8	7	11	2	0	5	5	2	2	2	5	
EML138	30	7	9	6	3	2.5	9	1	12	5	6	99	2	1	4	1	2	
EML145	31	7	15	6	3	2.5	9	1	12	5	6	2	2	1	4	1	2	
EML151, EML162	32	4	6	9	4	2.5	9	1	10	3	5	12	1	1	4	4	4	
EML1257	33		7	11	3	2.5	8	12	11	99	3	4	99	3	4	1	2	
EML1258	34		6	9	3	1	8	6	99	4	1	5	3	3	6	1	5	
EML1259	35		12	10	4	3	8	5	13	3	4	5	1	1	3	1	3	
EML1260	36		12	6	3	2.5	8	5	8	3	4	5	4	2	2	3	2	
EML1261	37		6	4	4	2.5	9	5	12	6	1	3	6	2	4	4	4	
EML1262	38		7	4	4	2.5	8	2	10	7	5	5	5	3	2	3	2	
EML1263	39		9	8	3	2.5	8	6	11	4	99	5	4	2	4	3	2	
EML1264	40		9	4	3	2	8	2	11	3	4	5	2	2	2	1	3	
EML1265	41	6	14	10	6	2.5	9	1	12	1.5	5	2	1	99	5	2	2	
EML1266	42	14	14	9	6	2.5	8	5	12	3	5	5	4	2	2	5	2	
EML1267	43	14	10	9	6	2.5	8	5	12	3	5	5	4	2	2	5	2	
EML1268	44		10	12	3	2	8	6	99	4	5.5	5.5	6	2	2	7	3	
EML1269	45	6	12	10	6	2.5	9	1	12	1.5	5	2	1	99	5	2	2	
EML1270	46		8	10	3	2.5	9	1	99	3	99	5	2.5	1	4	1	2	
EML1271, EML1272, EML1273	47	12	8	8	4	2.5	8	2	12	3	9	3	6	2	1	4	2	
EML1274	48	12	12	8	4	2.5	8	2	12	3	9	3	6	2	1	4	2	
EML1275, EML1283	49	3	13	6	4	1	8	5	99	5	10	4	5	2	1	3	4	
EML1276	50		15	6	5	2.5	8	4	8	3	5	5	3	4	3	1	2	
EML1277, EML1282	51	1	9	10	4	3	8	5	99	99	7	5.5	7	5	2	2	3	
EML1278	52		11	11	5	2.5	8	4	11	8	3	3	5	3	2	3	5	
EML1279, EML1281	53	1	9	10	4	3	8	5	99	9	7	5.5	7	5	2	2	3	
EML1280	54		17	10	4	2.5	9	1	10	4	5	99	5	1	2	99	5	
EML1284	55	10	13	6	3	2.5	9	2	99	3	5	3	2.5	1	1	4	2	
EML1285	56	10	7	6	3	2.5	9	2	99	3	5	3	2.5	1	1	4	2	
EML1286, EML1287, EML1288, EML1289, EML1290, EML1291	49	3	13	6	4	1	8	5	99	5	10	4	5	2	1	3	4	
EML1292	57	10	13	7	3	2.5	9	2	99	3	4	3	2.5	1	1	4	2	
EML1293, EML1294, EML1295	58	10	7	7	3	2.5	9	2	99	3	4	3	2.5	1	1	4	2	

(continued on next page)

Table 3 (continued)

Sample	MT MLVA ₁₃ -Lyon	MC	VNTR														
			61	207	209	77	127	142	172	211	213	214	215	216	217	222	223
EML1296	59	10	14	7	3	2.5	9	2	12	3	4	3	2.5	1	1	4	2
EML1297	60	10	7	7	3	2.5	9	2	99	3	4.5	3	2.5	1	1	4	2
EML1316, EML1319, EML1320, EML1321, EML1324, EML1325	61	9	11	11	3	2	8	1	12	2	1	2	2	1	2	3	4
EML1317	62	9	11	11	3	2	8	1	12	2	1	2	2	1	2	4	2
EML1318, EML1330	63	17	11	7	3	1.5	8	6	11	2	3	5	4	2	2	4	2
EML1322, EML1323	64	13	14	4	5	2.5	8	4	9	6	3	4	6	3	5	4	2
EML1326, EML1327	65	17	11	7	3	1.5	8	6	11	2	3	5	99	2	2	4	2
EML1328	66	17	11	7	3	1.5	8	6	11	2	3	5	2	2	2	4	2
EML1329	67	17	11	7	3	1.5	8	6	11	2	3	5	3	2	2	4	2
EML1331	68		11	13	9	5	9	1	10	3	6	3	2	2	6	5.5	3
EML1332	69		11	9	3	2.5	9	2	8.5	2	5	6	2.5	3	1	5	2
EML1333	70		10	9	4	2.5	9	4	12	6	1	3	6	2	4	4	4
EML1334	71	10	11	7	3	2.5	9	2	99	3	4	3	2.5	1	1	2	2
EML1335	72	11	8	11	3	2.5	8	4	10	7	5	3	4	2	1	2	2
EML1336	73	11	7	11	3	2.5	8	4	10	7	5	3	4	2	1	2	2

Based on the observations mentioned above, ms172 data and ms127 data were removed from clustering analysis. A new MLVA scheme called MLVA₁₃-Lyon, showing high discriminatory power and typability of 90% was then defined (Table 4). MLVA typing of the 105 isolates using the MLVA₁₃-Lyon scheme led to differentiating 73 MTs (Table 3). These isolates were clustered into 17 MCs. As a comparison, the MLVA₉-London and MLVA₉-Utrecht schemes segregated the isolates into 70 MTs and 62 MTs, respectively.

The typability and HGDI of the three MLVA schemes were compared (Table 4). The lower typability of the MLVA₉-London scheme was partly due to the numerous absences of amplification observed with ms172. The HGDI of the different MLVA schemes were superior to 0.982 (Table 4), while the MLVA₉-Utrecht scheme had significantly lower discriminatory power ($p = 0.028$).

The three typing schemes were highly congruent at the MT level, with the lowest adjusted Rand coefficients of 0.772 and 0.833 for MLVA₉-Utrecht vs MLVA₉-London, and MLVA₁₃-Lyon respectively. The adjusted Rand coefficient between MLVA₉-London and MLVA₁₃-Lyon was 0.916. In addition, two isolates with the same MT in one of the MLVA schemes had a high probability of being classified in the same MT with

another of the two MLVA schemes, as indicated by the adjusted Wallace coefficient (Table 5).

Representative snake isolates from feces and breeding facilities previously characterized using PFGE [5] were selected to validate the typing achieved by the MLVA₁₃-Lyon scheme on a restricted number of isolates of known diversity. Results showed that this scheme enabled discrimination of isolates in a manner similar to that obtained by PFGE (Fig. 2). In some instances, the MLVA₁₃-Lyon scheme showed more discriminatory power than PFGE, as isolates belonging to the same PFGE type clustered in different MTs. For example, isolates EML64, EML100 and EML109 of the MC16 clustered into two distinct MTs differing at ms61 and ms213 markers (MT19 and MT29), while they were grouped into a single PFGE type (clone PFGE IX). Likewise, MLVA typing of isolates EML91, EML138 and EML145 belonging to PFGE CC I composed of two PFGE types segregated them into three MTs clustered in MC7 (MT25, MT30 and MT31 respectively), with EML91 and EML145 showing different genotypes of one repeat unit at the ms61 marker.

Given these results, the MLVA₁₃-Lyon scheme seemed to be consistent with PFGE typing, unlike the MLVA₉-Utrecht scheme that showed a weaker discriminatory power and did

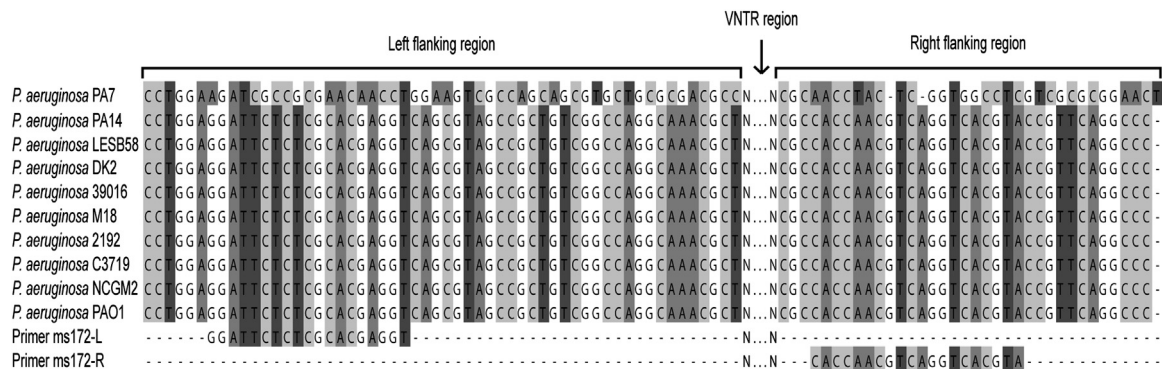


Fig. 1. Alignment of the genomic DNA sequences of type strains of *P. aeruginosa* bordering the ms172 marker, and primers used for amplification.

not segregate isolates according to PFGE. In contrast, the MLVA₉-London scheme that showed high discriminatory power with a threshold of one difference between two distinct MCs led to segregation of isolates belonging to PFGE CC I and clone IX in different MCs (data not shown).

3.2. Diversity of soil and manure isolates

The 62 isolates from soil ($n = 23$) and manure ($n = 39$) typed using the MLVA₁₃-Lyon scheme were resolved into 41 MTs, with 26 MTs clustered into 10 MCs (Fig. 2). High diversity was observed with an HGDI of 0.971 and 16 isolates (61.5%) showing a single MT.

Twelve out of 23 soil isolates showed seven MTs and clustered into three MCs (MC9, MC13 and MC17). Eleven isolates showed a single MT and did not cluster with any of the defined MCs. *P. aeruginosa* MT diversity was observed both within and between samples. For instance the eight isolates originating from soil sampled at Ouagadougou, Burkina Faso, had a different MT, and none grouped within an MC. Similarly, the three isolates from hydrocarbon-impacted soil in Paris showed three single MTs. Finally, three MTs clustered into two MCs (MC9 and MC13) were observed among the nine isolates from Neuves-Maison, and two MTs clustered into one MC (MC17) were observed among the isolates from Chinon soil. It should be noted that MTs and MCs identified among the soil isolates never clustered with isolates of different geographic origins.

Among manure isolates, 34 out of 39 isolates were resolved into 20 MTs clustered into eight MCs. Five isolates showed a unique MT and did not cluster within MC. MT diversity was also observed between and within manure samples. For instance, the five isolates from cattle manure from Versailles clustered into two MCs (MC6 and MC14) of two isolates and one isolate (EML1268) with a unique MT. Similarly, isolates of horse manure from St Olive showed three MTs, with two being clustered in the same MC (MC12). However, we were able to cluster isolates from different animal manures and from different farms into the same MC. For instance, MC3 (consisting of a single MT, MT49) clustered one isolate from cattle manure sampled in Feucherolles with seven isolates of composted horse manure from two farms in Versailles. Similarly, MC10 included isolates from the same animal manure i.e. that of horses, but of different origin: indeed, eight isolates were from manure sampled at Versailles and one isolate was from manure sampled at Marcy l'Etoile, yet these isolates showed seven different MTs.

The MLVA also showed that isolates from different regions and different types of samples (soil or manure) could belong to

Table 4
Typability and Hunter-Gaston discriminatory Index (HGDI) with 95% confidence Interval of each compared MLVA scheme.

MLVA scheme	Typability (%)	HGDI index	Confidence interval
MLVA ₁₃ -Lyon	90	0.987	0.979 – 0.995
MLVA ₉ -London	70	0.984	0.976 – 0.993
MLVA ₉ -Utrecht	93	0.982	0.974 – 0.993

Table 5
Adjusted Wallace coefficient indicating congruence between the different MLVA schemes.

	MLVA ₁₃ -Lyon	MLVA ₉ -London	MLVA ₉ -Utrecht
MLVA ₁₃ -Lyon		1 (1.000–1.000)	0.986 (0.972–1.000)
MLVA ₉ -London	0.847 (0.801–0.893)		0.835 (0.788–0.882)
MLVA ₉ -Utrecht	0.724 (0.652–0.797)	0.724 (0.652–0.797)	

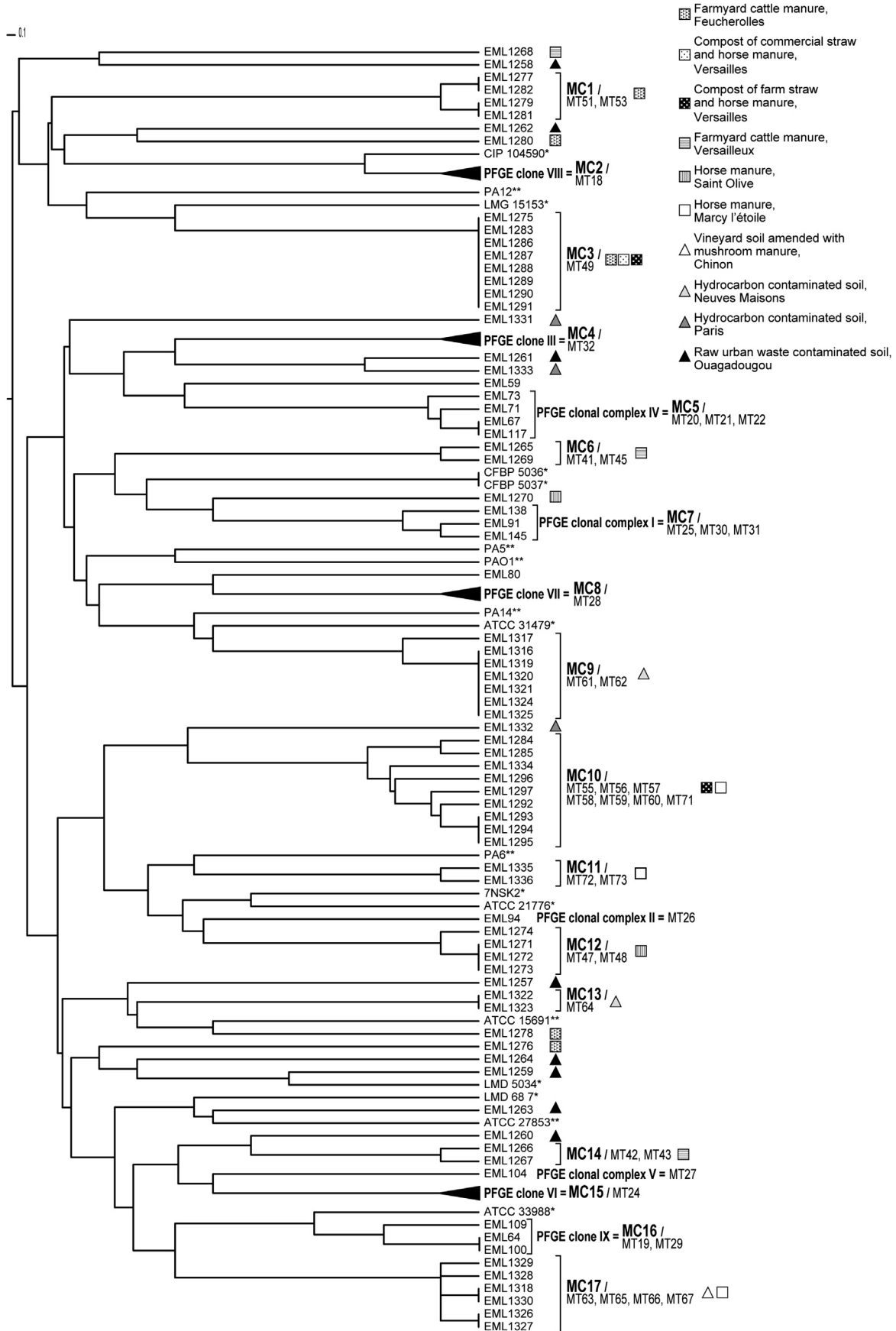
Numbers in brackets are 95% confidence intervals.

the same MC. MC17 comprised three horse manure isolates from Marcy l'Etoile and three isolates from a vineyard soil amended with mushroom manure in Chinon.

No isolates from soil and manure clustered with strains from international collections whatever their origin, nor with snake-related isolates. None of the MTs obtained for the 62 isolates from soil and manure and the 17 isolates from the international collection matched with well-known lineage from a database of 454 clinical isolates of *P. aeruginosa* MLVA types previously described [24,39,46,48] and publicly available at <http://mlva.u-psud.fr/mlvav4/genotyping/index.php> (data not shown). However, isolates EML91, EML138 and EML145 from snakes belonging to MC16 showed MTs having only two alleles different from an MLVA clonal complex that includes isolates from urine, pus and pleural exudate studied in [24]. Thus, the present work led to highlighting new MTs as well as new MCs that only include environmental isolates.

3.3. Antimicrobial resistance among soil and manure isolates of *P. aeruginosa*

Antimicrobial resistance of *P. aeruginosa* isolates was tested. Like the wild-type, all soil and manure isolates (100%) were resistant to minocycline and trimethoprim/sulfamethoxazole and sensitive to colistin, ciprofloxacin, isepamicin, amikacin, meropenem, aztreonam, ceftazidim (data not shown). Resistance to ticarcillin and ticarcillin/clavulanic acid was sporadically observed among isolates from manure: an isolate from cattle manure sampled at Feucherolles (EML1280) with a single MT and an isolate from horse manure sampled at St Olive (EML1274). The latter, belonging to MC12, showed this resistance profile, while all other isolates belonging to this MC had a wild-type phenotype. It is noteworthy that EML1274 differed from other isolates of MC12 in the number of copies at the ms61 marker. This resistance to ticarcillin and ticarcillin/clavulanic acid was also observed among soil isolates: EML1321 (MC49) and EML1322 (MC13) isolates from hydrocarbon-impacted soil in Lorraine, with the latter also being resistant to imipenem (MIC \geq 16 mg/ μ l). Others isolates included in these two MCs did not show an extended spectrum of antibiotic resistance. Resistance to ticarcillin and ticarcillin/clavulanic acid was also encountered in strains from international collections isolated from rhizospheres (7NSK2), hydrocarbon-contaminated soils



(CIP104590) and bulk soil (LMD5034, which was also resistant to pefloxacin, but not to ticarcillin alone). As a reminder, isolates EML88, EML138 and EML145 (PFGE clone VI) isolated from snake feces in the study of [5] were resistant to gentamicin and tobramycin, but this phenotype was not observed in isolates from soil and manure. Only strain PA6 of clinical origin from an international collection showed a resistance phenotype with additional resistance to ticarcillin, ticarcillin/clavulanic acid, piperacillin and piperacillin/tazobactam. No other isolate besides EML1322 showed resistance to imipenem.

4. Discussion

The extent of diversity of environmental *P. aeruginosa* isolates from non-aquatic environments and their similarity with clinical ones has been poorly investigated, partly due to the low number of available isolates from non-aquatic origin in international collections. We sought to fill this gap by investigating the genetic diversity of 62 isolates from soil and manure using the MLVA typing method.

In order to optimize the method, we sought to develop an MLVA scheme based on a low number of markers and enabling good typability and high discriminatory power. As no data on the diversity and typing ability of environmental isolates were available, 15 markers previously used in the 9-marker MLVA schemes developed by [45] and [46] were selected. Among the 15 markers, two of them did not fulfil the criteria of typability and discriminatory power. The ms172 marker, which often led to non-amplification, and the ms127 marker, which provided little discrimination, were thus removed from the analysis. Despite the congruence between the MLVA13-Lyon scheme and the two simplified MLVA9-Utrecht and MLVA9-London schemes, typability and discriminatory indexes as well as comparison to the clustering of snake isolates with PFGE revealed that our scheme was the most efficient for the typing and clustering of environmental isolates. As suggested by [39], the typing of a larger number of VNTRs increases the probability of subtyping some clones. Thus, the MLVA₁₃-Lyon scheme might be accurate in epidemiological studies and analysis of the population structure of large sets of clinical and environmental *P. aeruginosa* isolates.

As previously reported for epidemiological studies [46], strong diversity reflected by the identification of 41 MTs was observed among our 62 soil and manure isolates. Such diversity has also been observed in studies on aquatic, animal and other non-clinical isolates [32,33]. Ten MLVA clusters were clearly identified. Most of these clusters were composed of isolates of the same geographic origin and from the same source of sample (i.e. soil or manure), with a few exceptions (MC3, MC10 and MC17). These findings are similar to those of a recent study by [43] that compared cattle, aquatic and

clinical strains of *P. aeruginosa* and observed that genotypes of cattle strains differed from those of human clinical strains. However, our results contrasted with those obtained by [33], who observed no clear correlation between the clonal complexes and geographic origin or habitat. Unlike studies on *P. aeruginosa* population structure [50], genotypic divergence was observed between isolates from soil and manure and isolates from an international collection and from snake feces and breeding facilities. Furthermore, comparison of soil and manure MTs with the *P. aeruginosa* MLVA database showed that none of the MTs from soil or manure corresponded to already defined lineages. Such divergence was also observed by [43] with regard to the clonality of cattle and human strains. It is noteworthy that isolates from snakes belonging to MC16 showed MTs with only 2 different alleles, with an MLVA clonal complex that includes isolates from urine, pus and pleural exudate studied in [24]. However the absence of relationships between our terrestrial genotypes and clinical ones could be due to our sampling strategy, since a limited number of different geographic origins as well as a low number of isolates, especially from soil, were tested. As discussed in [23], this might have biased our evaluation of the diversity of *P. aeruginosa* within each habitat, i.e. soil and manure, and consequently, the potential correlation between clones or clonal complexes and habitat.

No common MTs were found among the soil isolates having different geographic origins. Although the sample size was limited, because of the low abundance of *P. aeruginosa* populations in terrestrial environments [1,6,12], it was observed that soil isolates exhibited differing levels of diversity according to their geographic origin. For instance, each of the 8 isolates from Ouagadougou showed a unique MT. Conversely, low diversity was observed within the soil contaminated with hydrocarbons sampled at Neuves-Maison where the 9 isolates clustered within 2 MCs. These differences in isolate diversity could be related to the differing severity of selective pressure of hydrocarbon and raw urban waste [30,42]. However, 3 MTs were observed among the 4 isolates obtained from the hydrocarbon-contaminated soil collected in Paris. It would thus be interesting to analyze larger sets of isolates from a more diverse soil type set under various anthropic constraints and geographic origins to obtain a more accurate measurement of the selection impact of human activities on the diversity and structure of the soil population of *P. aeruginosa*.

Isolates from manure also exhibited diversity between and within samples. Nevertheless, some isolates of differing geographic and animal origin were clustered. The clustering of isolates from the same host, i.e. the horse, but from different farms located in different regions of France, like those in the MC10, suggests the existence of widespread clones adapted to a host. The spread of major cattle clones in different

Fig. 2. Dendrogram deduced from clustering analysis of the 105 isolates. MLVA types (MT) and MLVA clonal complexes (MC) are indicated for clusters of two isolates or more. Pictograms refer to animal and geographic origins of isolates. Isolates marked with an “**” are from international collections of environmental origin, “***” from international collections of clinical origin. Branches with isolates characterized by a similar MLVA type and a similar PFGE type (i.e. PFGE clone) were collapsed.

Hungarian regions was also reported by [43]. However, isolates from horse and cattle manure sampled at 3 farms located close to one another in the Ile-de-France were clustered into MC3. This observation undermines the hypothesis of host-adapted strains and suggests, like other studies, that there is no correlation between the *P. aeruginosa* clone and the habitat such as the host [31,36]. As these isolates originated from neighboring farms, the spread of a clone in different hosts could occur over a restricted area. The clustering of horse manure isolates and mushroom manure amended soil isolates in MC17 support the notion of environmental and host adaptability. But since French mushroom manure used for field amendment is partly composed of horse manure, all these isolates might have an animal origin. [43] highlighted the possibility of natural exchange between environmental and animal strains. Therefore, the survival of *P. aeruginosa* after amendment should be assessed to elucidate the origin of the isolates from amended soils.

Antibiotic resistance profiles were performed to determine a potential link between resistance properties and the selective pressure that might be exerted in the various habitats. Almost all isolates from soil and manure showed wild-type antibiotic resistance profiles. Two isolates from hydrocarbon-impacted soils from Neuves-Maisons were found to be resistant to ticarcillin, coupled or not with clavulanic acid. One of them was also resistant to imipenem. In their study, [19] found resistance to at least 2 antibiotic classes, including extended spectrum penicillins and carbapenem, in 33% of isolates from hydrocarbon-contaminated sites. This suggested that hydrocarbon contamination could lead to a selection of resistant phenotypes. No resistance was observed among isolates from Burkina Faso soils. As antibiotic use for human or animal medicine is low in that country, the presence of antibiotics in such amended soils, and consequently, antibiotic selective pressure, is unlikely. However, as Zn, Pb and Hg contamination was reported in these soils (Hien, personal communication) metal contamination could act as a selective pressure that might co-select antibiotic resistance and metal resistance [2].

Considering manure isolates, only 2 out of 39 isolates displayed resistance to ticarcillin and ticarcillin/clavulanic acid. These results contrasted with a previous work wherein antibiotic resistance was detected among isolates from manure containing compost, especially against carbapenem and third-generation cephalosporin [20]. This low resistance could be explained by low selective pressure, i.e. low amounts of antibiotics in manure and low exposure in animals. Detailed information on the different types and intensity of antimicrobial selective pressure exerted on our isolate collection could not be obtained. However, in these livestock farms that are not intensive, antibiotics are not delivered at sub-therapeutic levels and are used only occasionally to treat infections (mainly oxytetracycline in bovine farms). Consequently antibiotics might have been present rarely, both in animals and manure, and/or at a low level and for a short period of time. Details on the eventual doses and frequencies of antibiotic administration to the animals and measurement of antibiotic amounts in manure would thus be needed to reach a conclusion concerning the exact role of

antibiotic treatment and environmental dispersion on resistance selection. One of the manure-resistant isolates was clustered with others that displayed a wild-type phenotype, suggesting that the genetic content and organization of a strain is not correlated with its resistance phenotype [21].

In summary, this study demonstrates that the MLVA method is suitable for the typing of *P. aeruginosa* isolates from terrestrial environments. It can be concluded that the population structure of *P. aeruginosa* from manure and soil was highly diverse, with strains diverging from those in international strain collections of environmental and clinical origin. Whether a correlation between genotype and habitat exists could not be inferred based on our data due to the insufficient richness of our set of isolates. No significant antibiotic resistance was observed among these isolates, suggesting that environmental conditions encountered in soil and manure did not favor antibiotic resistance selection. Further studies on a set of unrelated and more diverse isolates are thus needed: i) to elucidate relationships between strains capable of colonizing animals and soil, and the broader set of *P. aeruginosa* isolates found in aquatic environments and in hospitals; and ii) to evaluate the distribution of terrestrial genotypes and their ability to colonize various niches and evolve towards resistant epidemic clones.

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