Research in Microbiology 165 (2014) 2–13

www.elsevier.com/locate/resmic

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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Received 5 June 2013; accepted 3 September 2013
Available online 17 October 2013

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 ($MLVA_{13}$-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 $MLVA_{13}$-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.

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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http://dx.doi.org/10.1016/j.resmic.2013.10.004

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 $MLVA_9$-London [45] and $MLVA_9$-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 $MLVA_{13}$-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 Versailleux, 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