Systematic and Applied Microbiology xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Polyphasic characterization of four soil-derived phenanthrene-degrading *Acidovorax* strains and proposal of *Acidovorax carolinensis* sp. nov.

David R. Singleton[a,*], Janice Lee[b], Allison N. Dickey[c], Aaron Stroud[b], Elizabeth H. Scholl[c], Fred A. Wright[c], Michael D. Aitken[a]

[a] *Department of Environmental Sciences and Engineering, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC 27599-7431, USA*
[b] *Department of Biology, University of North Carolina,Chapel Hill, NC, 27599-3280, USA*
[c] *Bioinformatics Research Center, North Carolina State University, Raleigh, NC, USA*

ARTICLE INFO

Article history:
Received 30 March 2018
Received in revised form 30 May 2018
Accepted 1 June 2018

Keywords:
Acidovorax
Phenanthrene
Polycyclic aromatic hydrocarbons

ABSTRACT

Four bacterial strains identified as members of the *Acidovorax* genus were isolated from two geographically distinct but similarly contaminated soils in North Carolina, USA, characterized, and their genomes sequenced. Their 16S rRNA genes were highly similar to those previously recovered during stable-isotope probing (SIP) of one of the soils with the polycyclic aromatic hydrocarbon (PAH) phenanthrene. Heterotrophic growth of all strains occurred with a number of organic acids, as well as phenanthrene, but no other tested PAHs. Optimal growth occurred aerobically under mesophilic temperature, neutral pH, and low salinity conditions. Predominant fatty acids were $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{16:0}$, and $C_{18:1}\omega7c$, and were consistent with the genus. Genomic G+C contents ranged from 63.6 to 64.2%. A combination of whole genome comparisons and physiological analyses indicated that these four strains likely represent a single species within the *Acidovorax* genus. Chromosomal genes for phenanthrene degradation to phthalate were nearly identical to highly conserved regions in phenanthrene-degrading *Delftia*, *Burkholderia*, *Alcaligenes*, and *Massilia* species in regions flanked by transposable or extrachromosomal elements. The lower degradation pathway for phenanthrene metabolism was inferred by comparisons to described genes and proteins. The novel species *Acidovorax carolinensis* sp. nov. is proposed, comprising the four strains described in this study with strain NA3^T as the type strain (=LMG 30136, =DSM 105008).

Introduction

Bacteria within the *Acidovorax* genus are found in a diverse range of habitats and have varied functions within those environments. Strains now classified as *Acidovorax* have been associated with soils [11,45], wastewater treatment plants [23,46], plants [19,20,35,63], and clinical samples [59]. Mirroring the diversity inherent in this widespread distribution, isolates within this diverse genus have been linked to a variety of phenotypes ranging from plant pathogenicity, to denitrification, to the biodegradation of contaminants.

Environmentally derived 16S rRNA gene sequences from the *Acidovorax* genus have frequently been associated with samples impacted by petroleum contamination which typically contain high concentrations of polycyclic aromatic hydrocarbons (PAHs) [18,26,41,58]. Of particular interest for PAH bioremediation, the isolates *Acidovorax delafieldii* strains P4-1 [44] and TNA921 [47], *Acidovorax temperans* strain GTI-19 [6], and several uncharacterized *Acidovorax* strains [40] have been demonstrated to grow on the three-ring, low-molecular-weight (LMW) PAH phenanthrene. In a prior study of weathered PAH-contaminated soils treated via biostimulation in ex situ, lab-scale, slurry-phase bioreactors, we directly linked the degradation of phenanthrene to uncultivated members of the *Acidovorax* genus through stable-isotope probing (SIP) [25,49]. Additional SIP experiments of bacterial PAH degraders in a similarly contaminated soil from France also directly linked *Acidovorax* organisms to the disappearance of phenanthrene in those samples [38]. We subsequently isolated several strains of

* Corresponding author. Present address: Pratt School of Engineering, Department of Civil & Environmental Engineering, 121 Hudson Hall, Box 90287, Duke University, Durham, NC 27708, USA.
E-mail address: david.r.singleton@duke.edu (D.R. Singleton).

https://doi.org/10.1016/j.syapm.2018.06.001
0723-2020/

phenanthrene-degrading bacteria, including one (designated *Acidovorax* strain NA3) with high 16S rRNA gene sequence identity to SIP-detected *Acidovorax* sequences that could grow on the PAH phenanthrene [50].

A number of *Acidovorax* strains have publicly available draft or finished genomes. While some of these represent *Acidovorax* strains capable of the metabolism of aromatic compounds (e.g., 2-nitrotoluene by *Acidovorax* sp. JS42 [22] or biphenyl and polychlorinated biphenyls by *Acidovorax* sp. KKS102 [27]), no public *Acidovorax* genomes are available from organisms capable of degrading any PAH. In fact, there is little genetic information available for the strains previously shown to grown on phenanthrene, with only a partial 16S rRNA gene sequence for *A. delafieldii* P4-1 available (GenBank accession number DQ282184). The only published work on the genetics of PAH metabolism within the genus was performed on *Acidovorax* strain NA3^T using partial sequences recovered from a fosmid library [50]. In that work, the genes likely to be associated with the first three steps of the "upper pathway" of PAH degradation in NA3^T (comprising a ring-hydroxylating dioxygenase [RHD], dihydrodiol dehydrogenase, and ring-cleavage dioxygenase) were shown to be highly similar to homologous genes in phenanthrene-degrading members of the *Alcaligenes*, *Delftia*, and *Burkholderia* genera within the Betaproteobacterial order *Bulkholderiales*. Although the genes in strain NA3^T were not directly linked to the transformation of phenanthrene, RNA transcripts derived from those genes were induced by the presence of either of the LMW PAHs phenanthrene or naphthalene added to the cell culture. A recent publication analyzing the genome of *Delftia* strain Cs1-4, a strain with putative phenanthrene-degradation genes nearly identical to those in *Acidovorax* NA3^T, revealed a genomic "phn island," a 232 kb region of the chromosome flanked by transposable elements containing the *phn* genes thought to be responsible for phenanthrene degradation [24]. It was suggested in that work that the genomes of organisms harboring highly similar genes, such as phenanthrene-degrading *Acidovorax* spp., may demonstrate a similar gene organization.

In this manuscript, we describe four *Acidovorax* strains isolated from two geographically distinct, weathered PAH-contaminated soils from former manufactured gas plant (MGP) sites in North Carolina, USA. Each isolate was characterized using a variety of physiological tests and the complete genome of each strain was determined. The 16S rRNA genes from all of the strains were highly similar to sequences derived from phenanthrene SIP [25,49], and all were capable of growth on phenanthrene as a sole source of carbon and energy. The genome of each isolate was examined for genes and genomic islands potentially associated with phenanthrene metabolism. Based on genotypic and physiological differences to characterized *Acidovorax* species, *Acidovorax carolinensis* sp. nov. is proposed to encompass all four strains described herein, with strain NA3^T as the designated type strain.

Methods and materials

Strain isolation

Acidovorax sp. strains P3 and P4 were isolated from crystalline phenanthrene-enriched, PAH-contaminated soil collected from the site of a former MGP site in Salisbury, NC, USA that had previously undergone treatment in a lab-scale, slurry-phase aerobic bioreactor [51]. Pure cultures of strains P3 and P4 were obtained by serial dilution of phenanthrene-enriched bioreactor slurry on nutrient agar (NA) plates (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Well-separated colonies were screened by PCR using *Acidovorax*-selective primers and conditions as previously described [48]. Strains P3 and P4 specifically were selected for analysis based on genomic differences among 10 PCR-verified *Acidovorax* isolates (see below). Strains NA2 and NA3^T were previously isolated in a similar manner, except that the source soil for treatment in the bioreactor was obtained from a former MGP site in Charlotte, NC, USA, and treated under slightly different conditions [50].

Determination of optimal growth conditions

Strains were generally maintained on either NA or R2A plates (Difco), or in nutrient broth (NB; Difco). For determining optimal growth conditions for each strain, cultures in triplicate 5-mL tubes of NB were tested at 25 °C, 27.5 °C, 30 °C, 32.5 °C, and 35 °C with shaking at 250 rpm. Optimal exponential growth rates were determined by measuring turbidity at OD_{600} using a DR/3000 Spectrophotometer (Hach, Loveland, CO, USA). Growth at 4 °C, 15 °C, 37 °C, and 40 °C was tested by examining NA plates for growth after 2 weeks. Growth under varying pH was determined by buffering triplicate 5-mL NB tubes with 50 mM of (2-(*N*-morpholino)ethanesulfonic acid) monohydrate (St. Louis, MO; pH 5.5, 6.0, and 6.5), (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) (Acros Organics, NJ; pH 7.0, 7.5 and 8.0), or Tris·HCl (pH 8.9 and 9.0), and incubating at 32.5 °C and 250 rpm for 7 h. Growth in NB (pH 7.0, 32.5 °C) amended with 0%, 1%, 2%, 3%, 4%, and 5% NaCl was also tested. Production of indigo was examined by amending NA plates with 1 mM indole (Aldrich Chemical Co., Milwaukee, WI, USA). Growth under low oxygen conditions was tested by incubating NA plates in a sealed vessel in the presence of a GasPak™ EZ anaerobe sachet (BD, Franklin Lakes, NJ) for two weeks at room temperature (~23 °C). Unless otherwise stated, strains were routinely grown aerobically in NB buffered to pH 7.5 with no additional salt, and incubated 32.5 °C.

Cellular morphology

The morphology of overnight cultures was determined by scanning electron microscopy. Weak pellets were obtained by centrifugation for 2 min at ~1000 × *g* and the pellets washed twice with phosphate buffered saline (PBS). Specimens were observed and images acquired as previously described [13]. Motility of the strains was further examined using tubes of nutrient broth containing 0.3% agar (motility agar). Stabs of separate single colonies grown on NA plates were inoculated into triplicate tubes and incubated statically at 32.5 °C. Cell suspensions in PBS derived from motility agar tube cultures were stained with Remel™ Flagella Stain (Thermo Scientific, Waltham, MA) according to manufacturer's directions and examined under oil-immersion light microscopy.

Chemotaxonomic and physiological characteristics

Catalase activity was tested by adding 3% hydrogen peroxide (v/v) solution to cells freshly scraped from the surface of an NA plate. Oxidase activity was determined by adding a few drops of freshly-prepared 1% *N*′,*N*′,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (Acros Organics, NJ) to cells scraped from a plate onto filter paper [30]. DNase activity of the strains was tested by streaking each isolate on DNase agar plates with toluidine blue (Remel, Lenaxa, KA, USA). Starch hydrolysis was examined using starch agar plates stained with Gram's iodine solution after 48 h of growth [32]. Gelatin hydrolysis was tested with a nutrient gelatin medium and examining for liquefaction of the media [15]. Protease activity was analyzed by streaking each of the strains on nutrient agar plates amended with 0.1% casein, as well as skim milk agar plates [2]. Urease activity was examined using slants of Christensen's Urea Agar [12]. Nitrate reduction was determined by growing cells in triplicate tubes of nitrate broth (0.5% pep-

Please cite this article in press as: D.R. Singleton, et al., Polyphasic characterization of four soil-derived phenanthrene-degrading *Acidovorax* strains and proposal of *Acidovorax carolinensis* sp. nov. Syst. Appl. Microbiol. (2018), https://doi.org/10.1016/j.syapm.2018.06.001