Soil Biology & Biochemistry 58 (2013) 313–322

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Application of *mxaF* functional gene sequence to determine genetic relatedness among environmental *Methylobacterium* strains (PPFMs)

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ARTICLE INFO

Article history:
Received 11 July 2012
Received in revised form
9 December 2012
Accepted 12 December 2012
Available online 7 January 2013

Keywords:
Methylobacterium
PPFM
mxaF
Soil

ABSTRACT

Pink-pigmented, facultatively methylotrophic bacteria (PPFMs) of the *Methylobacterium* genus utilize plant-derived methanol as a carbon and energy source when growing on plant roots. To accomplish this, they employ the methanol dehydrogenase (MDH) enzyme, for which *mxaF* is the structural gene. Changes in *mxaF* sequence can result in decreased enzyme efficiency. Environmental conditions such as soil pH can alter MDH efficiency and impact bacterial proliferation in soil by affecting the solubility and chemical speciation of metals and nutrients. Soil pH can be influenced by location, soil type, ecosystem type and plant cover. Phenotypic analyses as well as 16S rRNA and *mxaF* functional gene phylogeny and denaturing gradient gel electrophoresis (DGGE) were performed on nine New Jersey agricultural soil PPFM isolates to compare gene characteristics and to determine the feasibility of using *mxaF* as a genetic tool to distinguish between PPFM species. 16S rRNA and *mxaF* functional gene phylogeny of 114 New Jersey, South Carolina, and California forest and agricultural soil *Methylobacterium* isolates were examined in order to apply *mxaF* sequence as a tool for describing genetic relatedness among newly acquired soil *Methylobacterium* strains. The pH of soil sample source was considered as a potential influencing factor for PPFM species distribution and thus *mxaF* sequence in soil environments. Nucleotide sequences of PPFM 16S rRNA gene fragments were too similar to be useful for species characterization. DGGE separation of *mxaF* fragments revealed species-specific banding patterns for PPFM strains whereas 16S rRNA patterns were nearly identical for multiple strains. pH, location and plant type were found not to be a selecting factor for *mxaF* sequence and PPFM species distribution in soil, suggesting the robust and versatile nature of the *mxaF* gene in *Methylobacterium*.

1. Introduction

Methylobacterium species, pink-pigmented, facultatively methylotrophic (PPFM) bacteria, are ubiquitous in the environment, occupying several important niches such as plant leaves, stems, roots, and fruit, as well as fresh and salt water, air, dust, and soil (Omer et al., 2004; Van Aken et al., 2004; Madhaiyan et al., 2005). PPFMs specialize in the use of C_1 compounds, most notably methanol, as carbon and energy sources (Van Aken et al., 2004; Madhaiyan et al., 2005). Methanol is a byproduct of pectin metabolism during cell wall synthesis in actively growing areas of plants (Nemecek-Marshall et al., 1995; Galbally and Kirstine, 2002). It is also produced when aerobic bacteria in surface soils oxidize methane as it diffuses up from subsurface soils where methane is microbially produced. As methylotrophs, PPFMs take advantage of this metabolic and ecological niche when colonizing both above- and below-ground plant surfaces. Genes that control the production and conformation of the methanol dehydrogenase (MDH) enzyme are therefore essential for PPFMs living in both phyllosphere and rhizosphere environments.

mxaF is the structural gene for MDH, therefore controlling its conformation (McDonald and Murrell, 1997). It is ubiquitous in the *Methylobacterium* genus (Kasprzak and Steenkamp, 1983). *mxaF* contains several regions in which mutations can effect enzyme configuration and efficiency (McDonald and Murrell, 1997). Thus, *mxaF* sequence directly affects the ability of PPFMs to grow on methanol. Some PPFM MxaF proteins vary in their conformation due to differences in highly conserved regions of the *mxaF* gene nucleotide sequence (McDonald and Murrell, 1997). Differences may be exploitable as a tool for isolate characterization, although previous research has suggested that *mxaF* sequence is too highly conserved to distinguish at the species or even genus level for some bacteria (McDonald and Murrell, 1997).

We hypothesize that *mxaF* sequences are sufficiently conserved within the genus to be a reliable tool for determining genetic

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0038-0717/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.soilbio.2012.12.009

relatedness while reflecting adaptation to selective pressures from the environment. These adaptations could reflect species-level distinction. Because *mxaF* is a nonessential functional gene as compared to 16S rRNA, changes in *mxaF* nucleotide sequence affect methylotrophic metabolic efficiency but do not result in bacterial mortality, allowing for more variations in gene structure to persist in the environment (McDonald and Murrell, 1997; Jordan et al., 2002; Dötsch et al., 2010). Thus, we anticipated a greater level of nucleotide variation in *mxaF* gene fragments than occurs in 16S rRNA gene fragments. The occurrence of nucleotide variation in *mxaF* may potentially provide improved species resolution that will be useful for determining genetic relatedness of environmental strains. Therefore, the specific objectives of this research were to investigate the validity of using *mxaF* sequence as a tool by which *Methylobacterium* species can be characterized and to compare its usefulness to that of 16S rRNA gene sequence. To accomplish this, we applied *mxaF* sequence as a potential genetic characterization tool for *Methylobacterium* strains obtained from both agricultural and forest soils. Strains isolated from a blueberry skin biofilm were included for comparison to soil isolate sequences. Additionally, effects of ecosystem type and properties on *mxaF* sequence phylogeny were examined. Environmental pH can act as selective factors for bacterial enzymes, particularly those found in the periplasm as is MDH, influencing their function and transcription (Kasprzak and Steenkamp, 1983; Stancik et al., 2002). To justify the use of *mxaF* sequence as a genetic tool for determining the relatedness of PPFM species, it is necessary to demonstrate species specificity and robustness of *mxaF* sequence, regardless of environmental pH or transient phenotypes that may arise within strain variants.

2. Materials and methods

2.1. Strain isolation

Multiple soil samples were collected from New Jersey (NJ), California (CA), and South Carolina (SC) locations. Information about the soil sample locations and properties are provided in Table 1. NJ samples were collected from the top 15 cm of soil using a hand trowel. Surface soil was collected to ensure that rhizosphere bacteria would be present. Only plant tissues such as large roots, stems and leaves were removed from the samples. Small roots and other debris were included in the samples. Approximately 500 g of soil was collected per sample in plastic Ziploc bags and placed immediately into coolers with ice. Upon arrival at the laboratory, each sample was mixed inside the bag and then split in half, with some soil refrigerated for the purpose of obtaining isolates. The remainder was frozen at −20 °C until the DNA was extracted and analyzed. California and South Carolina soil samples were also taken from the top 15 cm of the soil horizon. California samples were provided via mail by Dr. Sabine Goldberg of the U.S. Salinity Laboratory (Riverside, CA, 92507) and South Carolina samples were

Table 1
Soil sample characteristics (as of December, 2009). Soil samples ranged from active agricultural soils with current crops and fallow agricultural fields to forest soil. CFU/g measurements were conducted in the laboratory and pH measurements were performed using an electronic pH electrode.

Sample ID(s)	Sample location	Soil type & texture	Land use history	Avg. GWC (%)	pH	CFU of PPFM per gram of soil
SC-1	Clemson University's Pee Dee Research & Education Center, Clemson, SC	Noboco loamy sand (fine-loamy, siliceous, subactive, thermic Oxyaquic Paleudults)	Agricultural	12.41	6.35	6.40E+01
SC-2	Clemson University's Pee Dee Research & Education Center, Clemson, SC	Noboco loamy sand (fine-loamy, siliceous, subactive, thermic Oxyaquic Paleudults)	Agricultural	12.94	7.25	5.20E+01
SC-3	Clemson University's Pee Dee Research & Education Center, Clemson, SC	Lynchburg sandy loam (fine-loamy, siliceous, semiactive, thermic Aeric Paleudults)	Agricultural	13.53	6.80	1.46E+02
SC-4	Clemson University's Pee Dee Research & Education Center, Clemson, SC	Lynchburg sandy loam (fine-loamy, siliceous, semiactive, thermic Aeric Paleudults)	Agricultural	12.44	6.80	1.48E+02
SC-5	Coastal Plains Soil, Water & Plant Research Center, Florence, SC	Coxville sandy loam (Fine, kaolinitic, thermic Typic Paleaquults)	Agricultural	19.92	5.25	4.00E+01
SC-6	Coastal Plains Soil, Water & Plant Research Center, Florence, SC	Norfolk sandy loam (Fine, kaolinitic, thermic Typic Kandiudults)	Agricultural	18.67	5.40	1.54E+03
SC-7	Coastal Plains Soil, Water & Plant Research Center, Florence, SC	Norfolk sandy loam (Fine, kaolinitic, thermic Typic Kandiudults)	Agricultural	16.94	5.55	4.40E+01
SC-8	Coastal Plains Soil, Water & Plant Research Center, Florence, SC	Norfolk sandy loam (Fine, kaolinitic, thermic Typic Kandiudults)	Agricultural	16.60	5.10	2.38E+02
SC-9	Next to Coastal Plains Soil, Water & Plant Research Center, Florence, SC	Norfolk sandy loam (Fine, kaolinitic, thermic Typic Kandiudults)	Forest	13.98	5.20	3.00E+01
CA-1	University of California, Riverside Farm, Riverside, CA	Arlington Fine Sandy Loam	Agricultural	0.48	7.35	2.00E+00
CA-2	University of California, Riverside Farm, Riverside, CA	Buren fine sandy loam	Agricultural	0.52	7.00	5.00E+01
CA-3	University of California, Riverside Farm, Riverside, CA	Hanford fine sandy loam	Agricultural	1.13	7.40	6.00E+00
CA-4	University of California, Riverside Farm, Riverside, CA	Ramona sandy loam	Agricultural	0.67	7.85	3.60E+01
CA-5	Twisselman Rd. Kern County, CA	Twisselman clay	Agricultural	2.79	7.80	1.00E+02
NJ-1	Rutgers Adelphia Research Farm in Freehold, NJ	Holmdel sandy loam	Agricultural	6.60	6.30	2.30E+02
NJ-2	Horticulture Farm II, Rutgers, New Brunswick, NJ	Sandy loam	Agricultural	9.79	5.75	1.67E+02
NJ-3	Pine Barrens in Ocean County, NJ	Downer loamy sand	Forest	1.11	4.20–4.65	1.8E+04
NJ-4	Pine Barrens in Ocean County, NJ	Downer loamy sand	Forest	1.32	3.90–4.00	1.9E+04
NJ-5	Pine Barrens in Ocean County, NJ	Downer loamy sand	Forest	1.24	3.50–3.65	3.0E+03