Applied Microbiology and Biotechnology
https://doi.org/10.1007/s00253-018-8801-3

ENVIRONMENTAL BIOTECHNOLOGY

Detection and cell sorting of *Pseudonocardia* species by fluorescence in situ hybridization and flow cytometry using 16S rRNA-targeted oligonucleotide probes

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Received: 23 August 2017 / Revised: 19 December 2017 / Accepted: 21 January 2018

Abstract

Pseudonocardia spp. are receiving increasing attention due to their ability to biodegrade recalcitrant cyclic ether pollutants (e.g., 1,4-dioxane and tetrahydrofuran), as well as for their distinctive ecological niches (e.g., symbiosis with ants/plants and production of antibiotics). Isolating and characterizing *Pseudonocardia* spp. is thus important to discern their metabolic and physiological idiosyncrasies and advance their potential applications. However, slow growth, low cell yield, and dissimilar colony morphology hinder efficient isolation of *Pseudonocardia* using conventional plating methods. Here, we develop the first fluorescent probe (Pse631) targeting the 16S rRNA of *Pseudonocardia* members. In combination with flow cytometry and cell sorting, in situ hybridization with this probe enables sensitive and specific detection of *Pseudonocardia* cells in mixed cultures and enriched environmental samples without significant false positives, using *Escherichia coli*, *Bacillus subtilis*, and *Mycobacterium* spp. as negative controls. *Pseudonocardia dioxanivorans* CB1190 cells labeled with Pse631 as a positive control were detected when their relative abundance in the total bacterial community was as low as 0.1%. Effective separation of *Pseudonocardia* cells from the mixed consortium was confirmed by quantitative PCR analysis of sorted cells. This study provides a culture-independent high-throughput molecular approach enabling effective separation of *Pseudonocardia* populations from complex microbial communities. This approach will not only facilitate subsequent molecular analyses including species identification and quantification, but also advance understanding of their catabolic capacities and functional molecular diversity.

Keywords *Pseudonocardia* · Fluorescence in situ hybridization · Flow cytometry · 1,4-Dioxane · Tetrahydrofuran

Introduction

Pseudonocardia is a genus of *Actinobacteria* that has received significant attention due to its unique ecological roles and application in biotechnology, pharmaceutics, agriculture, and environmental remediation. Similar to other actinomycetes, members of *Pseudonocardia* produce bioactive secondary metabolites, including antibiotics (Carr et al. 2012; Dekker et al. 1998; Embley 1992; Oh et al. 2009) and immune-modulating agents (Li et al. 2012). Some *Pseudonocardia* species can form a commensal relationship with fungus-farming leaf-cutting ants that cause serious crop reduction (20~30%) in tropical and subtropical America (Currie et al. 1999; Zhang et al. 2007). Thus, detection and control of *Pseudonocardia* may enhance the ant management and promote crop yield (Della Lucia et al. 2014).

Pseudonocardia spp. are found in diverse environments, such as soils, water, activated sludge, and plant roots (Kohlweyer et al. 2000; Li et al. 2010a; Zhao et al. 2012), and are renowned for their metabolic versatility. Though some isolates exhibit autotrophic growth via fixation of atmospheric carbon dioxide/monoxide (Grostern and Alvarez-Cohen

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00253-018-8801-3) contains supplementary material, which is available to authorized users.

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Published online: 21 February 2018

2013; Park et al. 2008), most *Pseudonocardia* spp. live heterotrophically under aerobic or microaerophilic conditions. Members of *Pseudonocardia* can degrade various priority pollutants, including aromatic hydrocarbons (e.g., benzene and toluene) (Kohlweyer et al. 2000), methyl sulfides (Reichert et al. 1998), and halogenated compounds (e.g., chloroethene, 1,2,3,5-tetrachlorobenzene, and monofluorophenols) (Kämpfer and Kroppenstedt 2004; Kim et al. 2010; Lee et al. 2004).

Notably, *Pseudonocardia* species are predominantly associated with the biodegradation of recalcitrant cyclic ether pollutants, particularly 1,4-dioxane (dioxane) and tetrahydrofuran (THF), which are prevalent water contaminants (Malley et al. 2001; Mohr et al. 2010). To date, 34 bacterial strains have been isolated from various environments based on their capability to degrade dioxane and/or THF (Fig. 1 and Table S1). It is remarkable that more than one half (18 out of 34) of the isolates belong to the *Pseudonocardia* genus. A recent study randomly selected 13 *Pseudonocardia* strains from global cell culture resource facilities, and 11 of these strains (~ 85%) utilized dioxane and/or THF as the sole carbon and energy source (Inoue et al. 2016). Furthermore, two archetypic degraders of dioxane and THF (i.e., *Pseudonocardia dioxanivorans* CB1190 (Mahendra and Alvarez-Cohen 2005; Parales et al. 1994) and *Pseudonocardia tetrahydrofuranoxydans* K1 (Kohlweyer et al. 2000)) also belong to this genus. Hence, converging lines of evidence point to a strong link between *Pseudonocardia* spp. and their potential for bioremediation of cyclic ethers. Although not all *Pseudonocardia* spp. degrade dioxane or THF, it is very plausible that *Pseudonocardia* spp. that grow and thrive at dioxane/THF-contaminated sites are directly participating in the bioremediation process.

Given the significance of *Pseudonocardia* in industrial, agricultural, and bioremediation practices, expediting the identification and characterization of novel *Pseudonocardia* strains is important to advance understanding of their metabolic and physiological idiosyncrasies. However, traditional isolation techniques (e.g., enrichment and serial dilution for separating colonies on plates) are challenged by the fastidious nature of *Pseudonocardia* growth, low cell yields, dissimilar colony morphologies, and dormant stages of their life cycles (e.g., spore formation) (Embley 1992). For instance, the cell

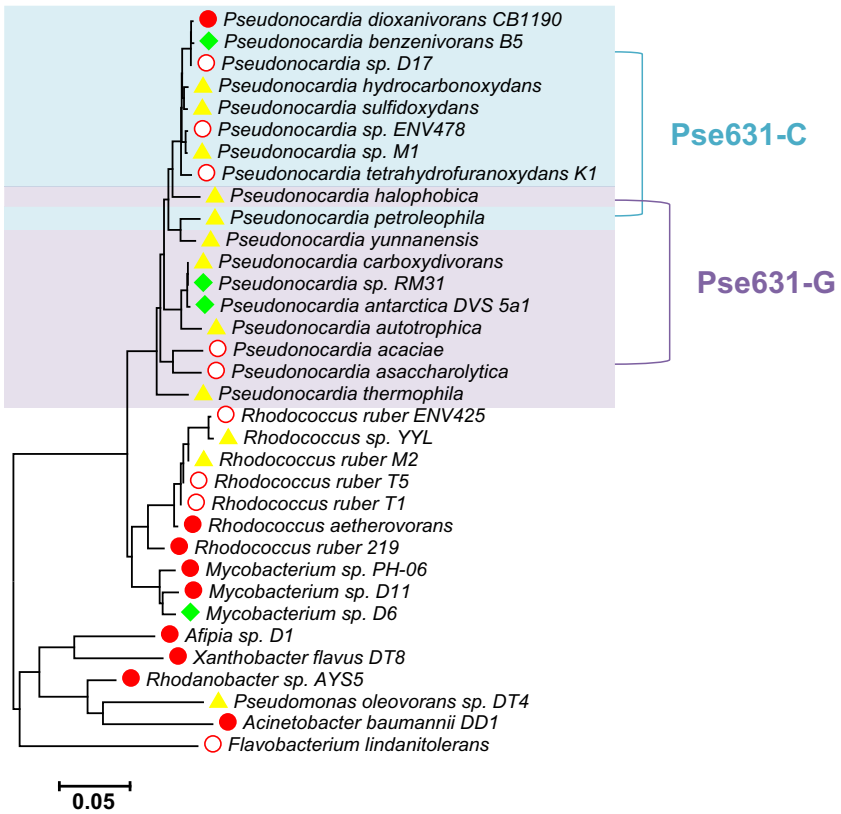

Fig. 1 Phylogenetic relationship of known dioxane and THF degraders and the coverage of designed probes (Pse631-C and Pse631-G) targeting the genus of *Pseudonocardia*. Bacterial strains capable of metabolizing both dioxane and THF are indicated with solid red circle. Strains that can metabolize THF and cometabolize dioxane with THF as the primary substrate are indicated by hallow red cycle. Dioxane degraders whose ability to degrade THF is unknown or absent are indicated with solid green diamond. THF degraders whose ability to degrade dioxane is unknown or absent are indicated by a solid yellow triangle. *Pseudonocardia* species targeted by the probes Pse631-C and Pse631-G are shadowed in blue and purple, respectively. The tree was generated from the alignment of the full 16S rRNA sequences (approximately 1400 nucleotides in length) retrieved from NCBI database using the maximum-likelihood algorithm by MEGA

yield of the dioxane-degrading CB1190 is lower than 0.1 mg protein/mg dioxane even under optimal growth conditions (Kelley et al. 2001; Mahendra and Alvarez-Cohen 2006; Parales et al. 1994). Though many *Pseudonocardia* strains have been isolated, recent studies using culture-independent techniques (e.g., 454-pyrosequencing) uncovered a discrepancy between the results from culture-dependent and culture-independent approaches, underlining possible biases when screening for *Pseudonocardia* by conventional isolation techniques (Mueller et al. 2010; Sen et al. 2009). For example, during agar plating, *Pseudonocardia* might be outcompeted by fast-growing bacteria (e.g., *Bacillus* and *Streptomyces*) as they can quickly deplete the available nutrients and become dominant by amensalistically generating antibiotics or acids (Morón et al. 1999).

In contrast, the use of hybridization probes in conjunction with fluorescence in situ hybridization (FISH) or modern flow cytometry (FCM) technology avoids these cultivation biases and enables in situ detection and identification of *Pseudonocardia* species (Amann et al. 1990; Ishii et al. 2010; Wang et al. 2010). These methods rely on fluorescent probes that bind to specific sequences of the chromosome and form probe-target hybrids with a high degree of sequence complementarity when conditions become favorable for hybridization. As depicted in Fig. S1, environmental samples are first fixed to stabilize and permeabilize the cells to facilitate penetration by the probes. Then, a favorable hybridization condition (e.g., temperature, salt, and denaturant concentrations) is provided to maximize the formation of the intracellular probe-target hybrids. After washing out the excess probes, the hybridized cells in the samples can be either visualized by epifluorescence microscopy or sorted and quantified by flow cytometry.

In this study, we present a novel fluorescently labeled rRNA-targeted oligonucleotide probe for imaging, separating, and enumerating *Pseudonocardia* cells. Coverage and specificity of this probe are evaluated based on empirical and computational assessment. In combination with high-throughput flow cytometry, this effort enables synchronized quantification and cell sorting of *Pseudonocardia* species from complex microbial mixtures recovered from target environments. This approach would facilitate molecular analysis of sorted cells to advance our understanding of their ecological traits and potentiate applications ranging from antibiotic synthesis to bioremediation of cyclic ether contaminants.

Materials and methods

Oligonucleotide probe design and synthesis

To design genus-specific probes targeting *Pseudonocardia* species, multiple sequence alignment by ClustalX 2.1 (Larkin et al. 2007) was employed using the 16S rRNA genes retrieved from the National Center for Biotechnology Information (NCBI) database for a wide phylogenetic variety of bacterial strains, including all cyclic ether-degrading bacteria indicated in Fig. 1 and Table S1. First, the nine hypervariable regions in their 16S rRNA genes were determined using the sequence of *Escherichia coli* K-12 as the reference (Brosius et al. 1978). Within each hypervariable region, sequence fragments that are only conserved among *Pseudonocardia* species but distinct from the other genera (e.g., *Mycobacterium* and *Rhodococcus*) were selected for further probe development. The nucleotide fragments that are shorter than 18 bp were eliminated to ensure a margin of specificity for probe design (Dieffenbach et al. 1993). To refine the probe-targeting nucleotide sequences, the starting and end positions of each probe candidate were manually adjusted to gratify the design criteria for oligonucleotide probes based on Keller and Manak (1989). Probes were then designed as the reverse complementary sequences of the target nucleotides.

All the obtained oligonucleotide probe candidates were then evaluated based on their coverage and specificity using two freely accessible web-based platforms, ProbeMatch (Cole et al. 2005) from RDP database (http://rdp.cme.msu.edu/probematch) and probeCheck (Loy et al. 2008) from the Silva database (http://www.microbial-ecology.net/probecheck). Using the two equations listed below, coverage and specificity of each designed probes were estimated based on the number of probe hits (perfect match and one-nucleotide mismatch) and the total number of available 16S rRNA sequences belonging to the targeted genus and the domain of Bacteria in the RDP database. To determine the binding stabilities of probe-target hybrids, probeCheck was also used to estimate their free energy (ΔG) of hybridization. Sequence, target site, and other properties of our designed probes are listed in Table 1. Probe Pse631 with the highest coverage, specificity, and binding energy were chosen for the genus of *Pseudonocardia*. Coverage and specificity were calculated as follows:

$$\text{Coverage} = \frac{\text{number of target strains detected}}{\text{total number of target strains tested}} \times 100\%$$

$$\text{Specificity} = \frac{\text{number of non-target strains undetected}}{\text{total number of non-target strains tested}} \times 100\%$$

For the optimization of hybridization and empirical assessment of coverage and specificity by epifluorescence microscopy, oligonucleotide probes Pse631-C, Pse631-G, and EUB338 with 5' TYE™ 563 label were synthesized and purified by Integrated DNA Technologies, Inc. (Coralville, IA). For the application of flow cytometry, Pse631 (mixture with

Table 1 Properties of the genus-specific probes targeting the 16S rRNA genes of *Pseudonocardia*

	Probe characteristics
Probe name	Pse631
Target genus	*Pseudonocardia*
Hypervariable region	V4
E. coli positions[a]	631–652
ΔG[b] (kcal/mol)	− 26.43
GC%	59.1
Length (bp)	22
Sequence	5′-AGTSATGCCCGTATCGACCGCA-3′ (5′-AGTCATGCCCGTATCGACCGCA-3′ for Pse631-C) (5′-AGTGATGCCCGTATCGACCGCA-3′ for Pse631-G)

[a] Position numbering was based on the *E. coli* system of nomenclature (Brosius et al. 1978)

[b] Free energy (ΔG) for intact matched probe-target hybrids was calculated using two-state hybridization algorithm by probeCheck

equal amount of Pse631-C and Pse631-G) was synthesized with Alexa Fluor®-488 dye at the 5′ end with endured brightness and photostability.

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 2. Most strains were obtained from either the Leibniz Institute-German Collection of Microorganisms and Cell Cultures (DSMZ) or the American Type Culture Collection (ATCC). *Mycobacterium* sp. PH-06, *Rhodococcus jostii* RHA1, and *Methylosinus trichosporium* OB3b were kindly provided by Dr. Yoon-Seok Chang (POSTECH, Pohang, South Korea), Dr. William Mohn (University of British Columbia, Vancouver, Canada), and Dr. Jeremy Semrau (University of Michigan, Ann Arbor, MI), respectively.

Pseudonocardia dioxanivorans CB1190 was cultured in ammonia mineral salt (AMS) medium amended with dioxane (100 mg/L) as the carbon and energy source (Li et al. 2010b). Other actinomycetes (*Pseudonocardia tetrahydrofuranoxydans* K1, *Pseudonocardia antarctica* DVS 5a1, *Mycobacterium* sp. PH-06, and *Rhodococcus jostii* RHA1) were grown in R2A medium. *Proteobacteria* (*Escherichia coli* K12, *Pseudomonas aeruginosa* PAO1, *Burkholderia cepacia* G4) and *Bacillus subtilis* were grown in Luria-Bertani (LB) broth. *Methylosinus trichosporium* OB3b was grown in nitrate mineral salt (NMS) medium amended with methane (20%, vol/vol) in the headspace as the sole carbon source (Whittenbury et al. 1970). *Desulfovibrio vulgaris* Hildenborough and *Halanaerobium congolense* were grown as recommended by DSMZ in the anaerobic chamber (Coy Lab, Grass Lake, MI) with sulfate and thiosulfate as the sole electron acceptors, respectively.

Cell fixation

All bacterial cells were harvested during logarithmic growth by centrifugation of an aliquot (ca. 2 mL) in microcentrifuge (10,000×*g*, 10 min). The cell pellets were washed three times

Table 2 Specificity and coverage tests for the designed phylogenetic probes

Microorganism strain	Strain number	Taxonomy (family/phylum)	Gram stain	Phylogenetic probe hybridization[a]	
				EUB338[b]	Pse631
Pseudonocardia dioxanivorans CB1190	DSM-44775	*Pseudonocardineae/Actinobacteria*	+	+	+
Pseudonocardia tetrahydrofuranoxydans K1	DSM-44239	*Pseudonocardineae/Actinobacteria*	+	+	+
Pseudonocardia antarctica DVS 5a1	DSM-44749	*Pseudonocardineae/Actinobacteria*	+	+	+
Mycobacterium sp. PH-06	NA[c]	*Mycobacteriaceae/Actinobacteria*	+	+	−
Escherichia coli K12	ATCC-10798	*Enterobacteriaceae/Gamma-Proteobacteria*	−	+	−
Pseudomonas aeruginosa PAO1	ATCC-15692	*Pseudomonadaceae/Gamma-Proteobacteria*	−	+	−
Burkholderia cepacia G4	DSM-11737	*Burkholderiaceae/Beta-Proteobacteria*	−	+	−
Methylosinus trichosporium OB3b	ATCC-35070	*Methylocystaceae/Alpha-Proteobacteria*	−	+	−
Desulfovibrio vulgaris Hildenborough	DSM-644	*Desulfovibrionaceae/Delta-Proteobacteria*	−	+	−
Rhodococcus jostii RHA1	NA	*Nocardiaceae/Actinobacteria*	+	+	−
Bacillus subtilis	DSM-10	*Bacillaceae/Firmicutes*	+	+	−
Halanaerobium congolense	DSM-11287	*Halanaerobiaceae/Firmicutes*	−	+	−

[a] + indicates a positive visualization was obtained after hybridization with the indicated phylogenetic probe; − indicates no positive visualization was obtained after hybridization with the indicated phylogenetic probe

[b] EUB338 is an universal probe targeting the prokaryotic domain

[c] Strain source is indicated in "Materials and Methods"

with 1 × phosphate-buffered saline (PBS, Thermo Scientific, Waltham, MA). As fixation is the primary step that permeabilizes cells for hybridization, different strategies are used to enhance the fixation efficiency for pure or mixed cultures based on well-established methods. For Gram-positive bacteria, washed cells were fixed in 1 mL of ethanol/PBS (1:1, *v*/*v*) and incubated at room temperature for 2 h (Roller et al. 1994). For Gram-negative bacteria, cells were resuspended thoroughly in 1 mL of 4% paraformaldehyde fixative at 4 °C for 3 h (Amann et al. 1990). After fixation, all cells were washed twice with 500 μL 1 × PBS and stored at − 20 °C prior to further analysis.

A consortium was obtained from the enrichment of a groundwater sample collected at the source zone of a historically contaminated site in San Antonio, TX. 16S rRNA metagenomic sequencing analysis discovered the two dominant genera of the consortium are *Mycobacterium* (Gram positive) and *Sediminibacterium* (Gram negative) (data not shown). Thus, we adapted the fixation method for sediment samples (Llobet-Brossa et al. 1998). Briefly, collected cells were fixed with 4% paraformaldehyde fixative at 4 °C for up to 24 h. After washed twice with 500 μL 1 × PBS, cells were then resuspended in 1 mL of ethanol/PBS (1:1, *v*/*v*) for storage.

FISH and microscopic imaging

An efficient in-solution hybridization approach was developed based on previously developed standard protocols (Manti et al. 2011; Pernthaler and Amann 2004). Briefly, the cell pellets were washed with 1 × PBS and resuspended by adding 90 μL hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl [pH 8], 0.01% [wt/vol] sodium dodecyl sulfate [SDS], 20 or 25% [wt/vol] formamide depending on the probes) and 10 μL 50 ng/μL fluorescent probe stock (final concentration of ~ 500 nM) in 2-mL centrifuge tubes. After incubation for 2 h at 46 °C, the suspended cell hybridization solution was diluted with 1.5 mL of pre-warmed (48 °C) washing buffer (225 or 159 mM NaCl depending on the formamide concentration in the hybridization buffer, 5 mM EDTA, 20 mM Tris–HCl [pH 8], 0.01% [wt/vol] SDS) prior to the centrifugation (10,000×*g*, 2 min). The cell pellets were rinsed again to remove unbounded probes with 0.5 mL of washing buffer and incubated at 48 °C for 15 min. Finally, the cell suspension was kept on ice and filtered with a 0.22-μm polycarbonate membrane for microscopic observation of staining. The filtered membrane was carefully cut into two 1 cm × 1 cm membrane squares, which were further stained with 50 μL of 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI, Thermo Scientific, Waltham, MA) and then gently rinsed with sterilized 1 × PBS to remove unbounded stains. Samples were then mounted on glass slides and examined using an Olympus IX71 inverted fluorescence microscope (Olympus, Center Valley, PA) equipped with a × 100 oil immersion objective. DAPI- and probe-labeled images were obtained separately from the same field of view (exposure time for 100 ms) using the accordant filter sets. Probe/dye-conferred fluorescence of each imaged cell was quantified as the mean pixel intensity by ImageJ (Schindelin et al. 2015).

The optimal formamide concentrations for probes Pse631-C and Pse631-G were determined by changing its concentration in the hybridization buffer with an increment of 5 or 10% from 0 to 60% at one fixed incubation temperature (i.e., 46 °C). Concentrations of NaCl in washing buffer were changed accordingly to eliminate undesirable mismatched probe-target hybrids. Two *Pseudonocardia* strains, *P. dioxanivorans* CB1190 and *P. antarctica* DVS 5a1, were used as model strains to empirically evaluate probe-target dissociation under the varying hybridization and washing conditions. Two web-based tools, ProbeMelt from DECIPHER (Wright et al. 2014) (http://decipher.cee.wisc.edu/ProbeMelt.html) and Formamide Curve Generator from mathFISH (Yilmaz et al. 2011) (http://mathfish.cee.wisc.edu/formamide.html), were employed to simulate designed probe hybridization efficiencies with the tested strains at different concentrations of the denaturant formamide.

Flow cytometry and cell sorting

Flow cytometry was performed using an FACS AriaII Flow Cytometer (BD Biosciences, San Jose, CA) equipped with a red laser (wavelength of 640 nm), a violet laser (wavelength of 405 nm), yellow-green laser (wavelength of 561 nm), a blue laser (488 nm wavelength), and an UV laser (wavelength of 355 nm) controlled by a computer equipped with the software BD FACS Diva 8.0 (BD Bioscience, San Jose, CA). Sheath pressure was set to 70 psi, and nozzle size was 70 μm. Forward scatter (FSC), side scatter (SSC), and blue (450 ± 25 nm, DAPI) and green fluorescence (530 ± 15 nm, Alexa Fluor 488) signals were measured and analyzed in logarithmic amplification mode. The bacteria exhibiting fluorescence signals above the cutoff of DAPI were gated using the scattergrams of SSC versus DAPI, and Pse631-labeled cells (gated by AF488+) were set similarly in the scattergrams of SSC versus Alexa Fluor 488. Plots displayed 10,000–50,000 events, and target gates were defined according to all negative control samples (e.g., non-Pse631-labeled *Pseudonocardia* cells and Pse631-labeled cells that are not *Pseudonocardia*). Cell sorting was performed on the same flow cytometer with the drop drive frequency set at 88.7 kHz/s. Each event falling within the gate AF488+ was sorted in a sort envelope of 1.2 drops at a rate of 1×10^2 to 1×10^3 events/s. In order to achieve high purity and recovery, the purity precision mode was used. Sorted cells were collected in sterile microcentrifuge tubes (Eppendorf, Hamburg, Germany).

To further assess the sensitivity of the cell-sorting method, fixed CB1190 cells were mixed with consortium cells to

achieve a serial dilution of percentage abundance (10, 3, 1, 0.3, and 0.1%), and then in-solution hybridization was employed to the cell mixtures with probe Pse631. The total bacteria in the mixture were enumerated as the positive DAPI-stained cells detected by flow cytometry. The relative abundance of the hybridized cells was estimated as the ratio of Pse631-stained cells detected by flow cytometry (i.e., the events gated by AF488+) to the total bacteria. The detection efficiency of the method was calculated as the ratio of the relative abundance of the detected CB1190 cells to the amendment percentage prior to the hybridization and flow cytometric analysis.

Quantitative PCR

To evaluate the effectiveness of our cell-sorting method, quantitative PCR (qPCR) using TaqMan chemistry was employed to quantify the CB1190 cells (enumerated as the abundance of *thmA* genes coding for THF/dioxane monooxygenases) (Li et al. 2013) and the total bacteria (enumerated by 16S rRNA genes) (Nadkarni et al. 2002) in the sorted cells. The primer and probe sequences are provided in Table S2. The sorted cells were spun down at 10,000 rpm for 10 min and washed three times with autoclaved distilled water. The cells were resuspended in 20 μL DNA-free water, and then subject to three boiling-freeze cycles (95 °C for 10 min and − 70 °C for 10 min) to release the genetic materials. The qPCR reaction mixture (a total volume of 20 μL) consisted of 1 μL of the obtained DNA, 300 nM forward and reverse primers, 150 nM fluorogenic probe, 10 μL of TaqMan universal master mix II (Applied Biosystems, Foster City, CA), and DNA-free water. qPCR was performed with the CFX 96™ Real-Time System (Bio-Rad, Hercules, CA) using the following temperature program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions (10^{-4} to 100 ng of DNA/μL) of the extracted genomic DNA of CB1190 were utilized to prepare the calibration curves for both *thmA* (one copy per genome) and 16S rRNA (three copies per genome) genes. The calculation of copy numbers and relative abundance of CB1190 cells has been previously described (Li et al. 2017; Li et al. 2013). Student *t* test was used to determine whether differences in the gene ratio between cells before and after the cell sorting were significant at the 95% confidence level.

Results

16S rRNA-targeted probe design

Pse631 is a degenerate probe consisting of two oligomers (Pse631-C and Pse631-G). The singular degenerate site on the 16S rRNA is located at the 649th base from the 5′ end (G or C) based on the *E. coli* system of nomenclature. As shown in Fig. 1, Pse631-C targets the two archetype cyclic ether degraders (CB1190 and K1) and a number of *Pseudonocardia* species (e.g., *P. benzenivorans* B5, *P. hydrocarbonoxydans*, *P. sulfidoxydans*, and *P. petroleophila*) that are known for their capability to degrade a wide range of priority organic pollutants. For instance, THF-grown ENV478 can degrade a variety of ethers, including dioxane and its related solvent 1,3-dioxolane, the plasticizer bis-2-chloroethyl ether (BCEE), and the gasoline additive methyl *tert*-butyl ether (MTBE) (Vainberg et al. 2006). However, probe Pse631-C only covers approximately 12% of the *Pseudonocardia* species with a perfect match based on the 1873 16S rRNA gene sequences retrieved from RDP (Table 3). The other probe, Pse631-G, is complementary to 16S rRNA genes from the majority of the rest of the species (~ 74%) belonging to the genus of *Pseudonocardia*. Among these species, there are a few strains that thrive under harsh conditions (e.g., cold temperature and high salinity), including *P. halophobica*, *P. antarctica*, and *P. thermophila*, suggesting the potential to target, separate, and characterize extremophilic strains.

A theoretical estimation using ProbeMatch (Table 3) shows that Pse631 can effectively hybridize with the majority (> 86%) of the *Pseudonocardia* species. Such high coverage of probes did not sacrifice their ability to preclude false positives. As indicated in Table 3, specificity of our designed probes was greater than 99.9%. Formation of mismatched probe-target hybrids can be greatly eliminated but not completely precluded, via optimizing the hybridization condition. By enabling a matching distance of 1 (i.e., one mismatch, insert, or delete is allowable when matching a probe to a sequence), our probe design exhibited an improved coverage (> 91%) and maintained high specificity (> 99.9%), suggesting the high resilience of our probe to occasional mismatching during in situ hybridization. Compared to our probe, oligonucleotide probes targeting other genera from the order of *Actinomycetales* offer comparable or less coverage and specificity based on the ProbeMatch analysis (Table 3).

Optimization of hybridization (laboratory and computation tests)

To optimize the hybridization system, probe-conferred fluorescence was examined under varying hybridization stringencies. Pure cultures of CB1190 and DVS 5a1 were used as the perfect targets for probes Pse631-C and Pse631-G, respectively. Figure 2 shows that the average signals per cell remained at comparable levels until the added formamide concentration reached 25% for both probes Pse631-C and Pse631-G.

In addition to the empirical tests, mathematical models DECIPHER and mathFISH were used to provide theoretical guidance on the thermodynamic feasibility and mismatch

Table 3 Theoretical coverage and specificity estimation of phylogenetic oligonucleotide probes targeting *Pseudonocardia* and other genera in the order of Actinomycetales

Probe name	Target genus	Coverage		Specificity		Reference
		No mismatch (%)	1 mismatch (%)	No mismatch (%)	1 mismatch (%)	
Pse631	*Pseudonocardia*	86.01	91.40	99.99	99.95	This study
Pse631-C	*Pseudonocardia* (partial)	12.22	87.56	< 100[b]	99.96	This study
Pse631-G	*Pseudonocardia* (partial)	73.79	90.12	99.99	99.98	This study
Myb736b	*Mycobacterium*	75.85	79.05	98.86	97.44	(de los Reyes et al. 1997)
Myc657	*Mycobacterium*	75.65	84.78	99.61	99.38	(Davenport et al. 2000)
Gor596	*Gordonia*	87.57	92.61	< 100	< 100	(de los Reyes et al. 1997)
Modesto	*Modestobacter*	62.26	70.35	< 100	99.98	(Urzi et al. 2004)
Geo/Blasto	*Geodermatophilus*	38.46	53.58	< 100	< 100	(Urzi et al. 2004)
APYO183	*Trueperella*	63.39	72.68	100	< 100	(Werckenthin et al. 2012)
MNP1[a]	*Corynebacterineae*	75.06	92.47	99.30	98.94	(Schuppler et al. 1998)

Calculation of probe coverage and specificity was based on the 16S rRNA sequence collection retrieved from the RDP database updated in March 2017

[a] MNP1 was designed to probe nocardioform actinomycetes. Thus, sequences obtained in the suborder *Corynebacterineae* were used for coverage and specificity estimation

[b] < 100% indicates a specificity between 99.99 and 100%

discrimination for in situ hybridization experiments. Results from both models corroborated favorable equilibrium thermodynamics for both designed probes with sufficient hybridization affinity but low risk to produce false positives or false negatives. In general, our experimental data fit better with the hybridization curves simulated by DECIPHER, which recommended formamide concentrations ranging from 20 to 30% (Fig. 2). Therefore, considering results from both empirical tests and theoretical computation, 25% formamide was chosen in the later hybridization for Pse631, a mixture of Pse631-C and Pse631-G.

Specificity and coverage of Pse631 with pure and mixed bacterial strains

To validate the coverage and specificity of the designed probes, FISH analysis (Table 2 and Fig. 3) indicated that our designed fluorescence probe Pse631 was capable of efficiently hybridizing with various strains in the genus of *Pseudonocardia*, including *P. dioxanivorans* CB1190, *P. tetrahydrofuranoxydans* K1, and *P. antarctica* DVS 5a1. Negative controls using pure and mixed bacterial strains from other genera that are phylogenetically close (e.g., *Rhodococcus jostii* RHA1, *Mycobacterium* sp. PH-06) or distant (e.g., *Burkholderia cepacia* G4, *Halanaerobium congolense*, and *Pseudomonas aeruginosa* PAO1) to *Pseudonocardia* were used to evaluate the potential for false-positive hybridization. These strains were selected because they cover a broad taxonomic diversity of bacteria (Table 2) and are present in a wide variety of environment niches. Our FISH results (Table 2 and Fig. 3) showed that none of these negative controls yielded false positive (i.e., none was visualized under the same hybridization and exposure condition by this Pse631 probe). Our method can effectively exclude non-specific hybridization with negative control strains that share very high 16S rRNA sequence identities with *Pseudonocardia* species (e.g., 93% as identities for both RHA1 and PH-06 compared to CB1190). These results confirmed the specificity of our newly developed probe, implying its usefulness to target the *Pseudonocardia* species that are "non-cultivable" or difficult for culturing with the help of fluorescence-assisted cell sorting.

Flow cytometry for efficient cell sorting

As illustrated in Fig. 4, 35.6 ± 4.2% of CB1190 cells (enumerated as the events in the defined gate AF488+) were labeled by probe Pse631 and quantified by flow cytometry using our phylogenetic probe. Similar labeling and detection efficiencies (30 to 55%) are commonly reported using fluorescence-conjugated oligonucleotide probes targeting *actinomycetes* and other bacteria after in situ hybridization (Hoshino and Schramm 2010; Manti et al. 2011; Sekar et al. 2003). The specificity of probe Pse631 was also verified with non-labeled CB1190 cells. Minimal false negatives were observed in Gram-positive and Gram-negative bacterial cells hybridized with Pse631, including *Escherichia coli* K12 (0.0%), *Bacillus subtilis* (0.0%), and *Mycobacterium* sp. PH-06 (0.1%). Staining with DAPI was used to estimate the total bacterial count.

The performance of our cell-sorting method was verified using a consortium enriched from a field sample. The 16S rRNA metagenomic sequencing data showed that *Pseudonocardia* spp. were absent in the consortium (data

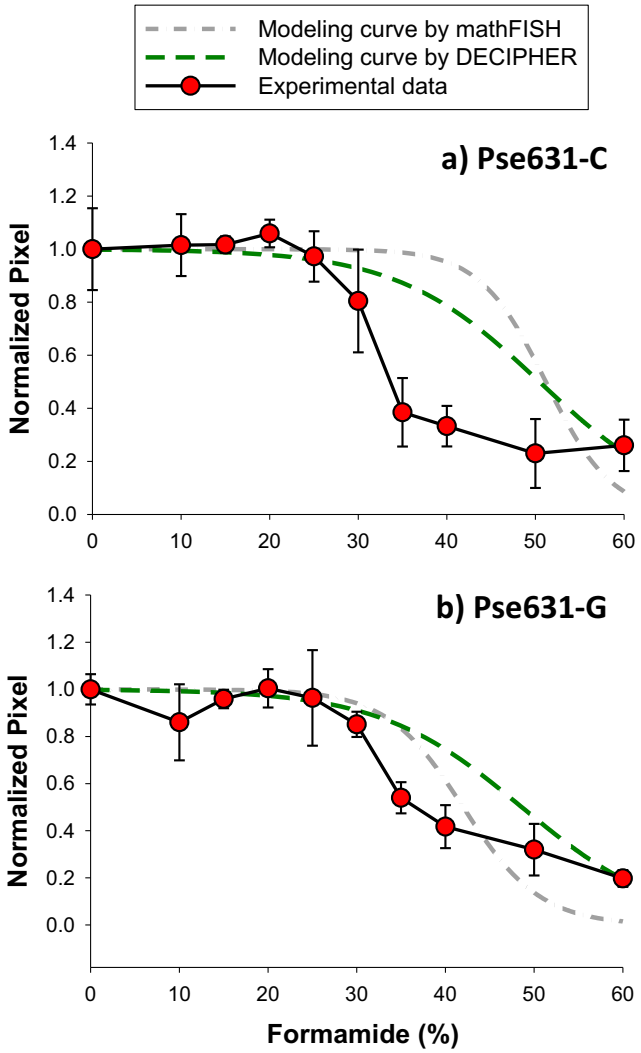

Fig. 2 Probe dissociation curve under various formamide concentrations for in situ hybridization of **a** Pse631-C with *Pseudonocardia dioxanivorans* CB1190 and **b** Pse631-G with *Pseudonocardia antarctica* DVS 5a1. Each experimental data point represents the average value of fluorescence intensity of approximately 50 cells normalized with the one obtained without the presence of formamide in the hybridization solution. Error bars indicate the standard deviations. Hybridization efficiency curves simulated by two theoretical models were based on two web tools, ProbeMelt from DECIPHER (Wright et al. 2014) and Formamide Curve Generator from mathFISH (Yilmaz et al. 2011)

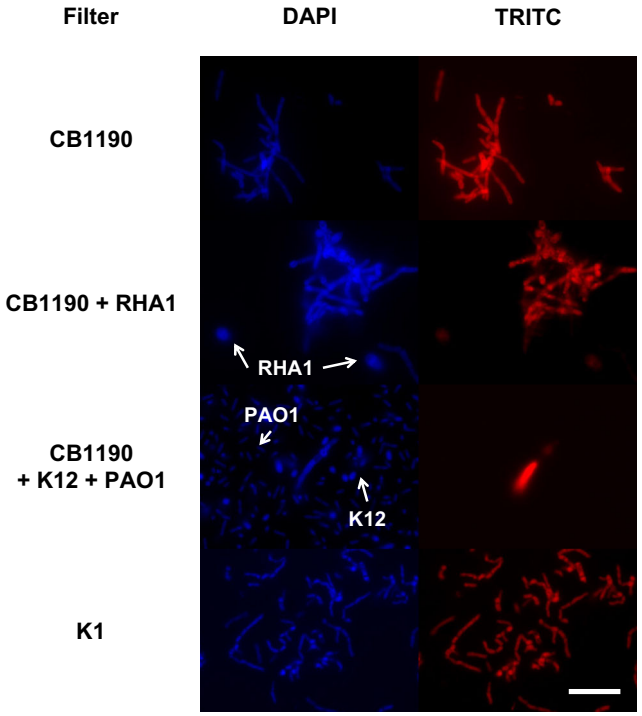

Fig. 3 Reference bacterial strains visualized by epifluorescence microscopy after hybridization with probe Pse631 (visualized with the TRITC filter), as well as DAPI, a fluorescent dye used to stain both live and fixed cells. The scale bar at the bottom right indicates a length of 10 μm

not shown). Thus, a series of concentrations of CB1190 cells was diluted with the consortium cells to assess probe sensitivity and efficiency of our cell-sorting method. As shown in Fig. 5 and S2, the detection efficiency was 36.7 ± 4.1% for mixed samples with 10% of CB1190, which is comparable to that of pure CB1190 cells (i.e., 35.6 ± 4.2%). The detection efficiency of our approach increased with decreasing relative abundance of CB1190 (Fig. 5) probably due to the greater ratio of our probes to the target cells. Though probe design is of the primary significance for effective hybridization, probe-to-target ratio also affects the success of hybridization and detection especially in complex environmental matrices mimicked by our experiment. The optimum concentration of *Pseudonocardia* cells for our method was 0.3% given that its overall efficiency was as high as 89% (Fig. 5). Thus, for enriched samples with relatively high abundance of *Pseudonocardia* cells, appropriate dilution prior to staining is recommended to improve the overall detection efficiency for cell-counting or cell-sorting purposes. Our approach also exhibited reliable sensitivity when the percentage of CB1190 cells in the mixed consortium was as low as 0.1%, and no significant false positives occurred for the control containing consortium cells without CB1190 (Figs. 5, S2e, and S2f).

To further verify the specificity of our method, the relative abundance of CB1190 cells in the sorted cells and original consortium samples mixed with 10% of CB1190 was quantified and compared using TaqMan qPCR assays. Theoretically, in the genome of CB1190, the ratio of *thmA* to 16S rRNA gene is 0.33 (Sales et al. 2011). Comparably, this *thmA*/16S rRNA ratio was determined by qPCR analysis to be 0.27 ± 0.05 in the genomic DNA of pure CB1190 cultures (Table 4). After cell sorting, the *thmA*/16S rRNA ratio in sorted cells remained 0.20 ± 0.06, which is statistically indiscernible ($p < 0.05$) from the ratio in pure CB1190 cells. This demonstrates that most of the sorted cells were CB1190. The *thmA*/16S RNA ratio in sorted cells was more than 10 times greater than that detected in the original cell mixture before the sorting (0.02 ± 0.01), further corroborating that our method can specifically sort

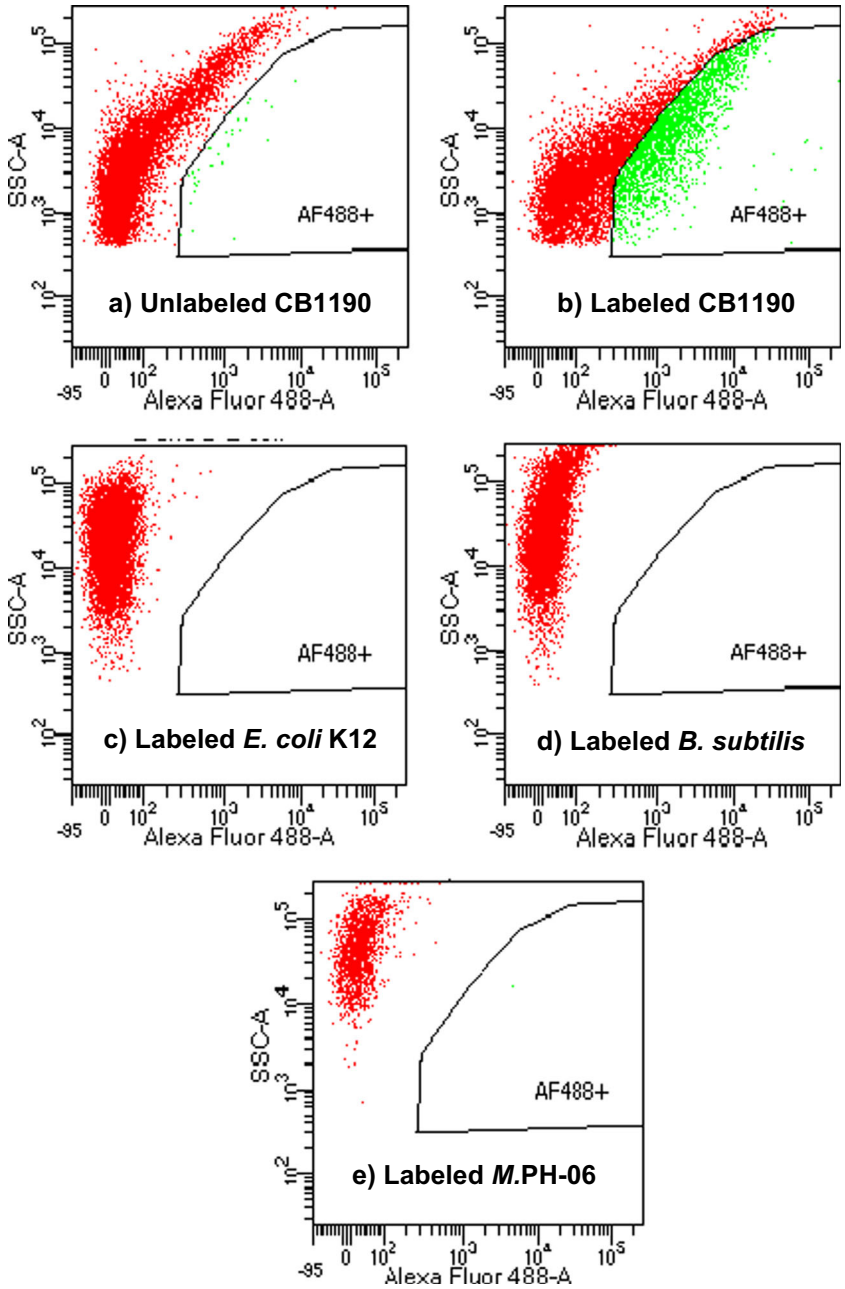

Fig. 4 Performance of probe Pse631 verified by flow cytometry in scattergrams of SSC (90° sidelight scatter) vs. Alexa Fluor 488 (green fluorescence). Red dots indicate the total bacteria labeled by DAPI (the events with high DAPI fluorescence) and green dots indicate bacteria labeled by the Pse631 probe (the events with high Alexa Fluor-488 signals in AF488+ gate). The strains *M.* PH-06, *E. coli* K12, *B. subtilis*, and unlabeled CB1190 were considered as negative control here

Pseudonocardia cells from a mixed consortium. Overall, our results suggest high sensitivity and reliable specificity of our Pse631 probe in complex environmental samples.

Discussion

Isolating and separating difficult-to-cultivate *Pseudonocardia* populations from complex communities facilitate subsequent molecular analyses to discover new species and understand their metabolic and physiological characteristics and functional niches. Here, we designed a 16S rRNA-based probe and used (culture-independent) FISH-flow cytometry to efficiently detect and separate microbial populations of interest that potentiate molecular analysis to align their genetic identities with metabolic potentials. Note that Pse631, similar to many other well-designed probes (e.g., Myc657 and Gor596 in Table 3), targets the nucleotide sequences within the 16S rRNA hypervariable region V4 (i.e., *E. coli* 16S rRNA positions 576–682) (Table 1). As highlighted in Fig. 1, all cyclic ether-degrading *Pseudonocardia* can be accurately targeted by either Pse631-C or Pse631-G except *P. asaccharolytica*. The 16S rRNA gene of this strain has one mismatch with Pse631-G at the position of 631 with G rather than T, and this mismatch is located at the 3′ end of the probe. Hybridization probably occurs between the probe and

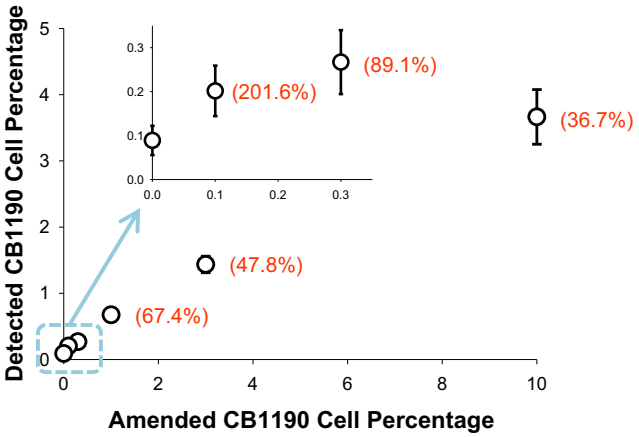

Fig. 5 Relative abundance of the hybridized CB1190 cells detected by flow cytometry in relation to the amendment percentage of CB1190 cells when mixed with the enriched consortium. Detection efficiency is indicated as the percentage in the parentheses next to the respective data symbol

this single mismatched target, since defects towards either end of the probes usually lead to minor signal reduction (10–20%) (Naiser et al. 2008; Pozhitkov et al. 2006; Wick et al. 2006). Therefore, all known cyclic ether-degrading *Pseudonocardia* isolates can be efficiently targeted by Pse631 (Fig. 1).

With this designed phylogenetic probe, a high-throughput culture-independent approach was developed and optimized to integrate flow cytometry with fluorescence-activated cell sorting (FACS). 16S rRNA is an ideal target for FACS to isolate the dominant bacteria from environmental samples or enriched consortia. The ubiquity of 16S rRNA and its relatively high copy numbers (10^4–10^5 per cell) in prokaryotic cells are conducive to strong signals (Giovannoni et al. 1988), while targeting functional genes can be more technically challenging and requires signal amplification. Our approach eliminates the use of potentially biased cultivation and DNA extraction methods, both of which are time-consuming and laborious. Additionally, sorted cells remain accessible for molecular analysis, since fluorescent probe-stained and sorted cells have been successfully used as templates for PCR amplicon sequencing (Sekar et al. 2004).

Despite the hybridization limitation caused by cell permeabilization and molecular binding between probes and targets, the detection efficiency of FACS is tightly associated with the heterogeneity of fluorescent signals from labeled cells and the stringency to preclude false positives. Our hybridized CB1190 cells possessed a wide range of fluorescence intensities and sizes (Fig. 4b), suggesting the applicability of our method to target cells with different morphological properties and at different growth stages. To ensure the accuracy and exclude false positives, (1) the gate AF488+ was set at high stringency that excludes background signal from autofluorescence and (2) negative and positive controls, such as pure culture or spiked environmental samples, are prerequisites to achieve correct gate for accurate analysis.

The high sensitivity and specificity of our method — demonstrated with pure and mixed cultures — corroborate its feasibility to detect and sort *Pseudonocardia* cells from various environments. This could help identify novel cyclic ether degraders and associated genes from field samples collected from contaminated sites. Since our method can reliably sort out CB1190 cells from a mixed culture when CB1190 makes up as few as 0.1% of the total population, it should be able to target other *Pseudonocardia* cells that would be enriched in systems exhibiting cyclic ether degradation activity. Our method may also facilitate monitoring and enumeration of CB1190 and other *Pseudonocardia* strains when used for in situ bioaugmentation of dioxane contaminated sites.

In summary, this study presents a cultivation-free strategy to separate and concentrate genus-specific subpopulations by using our fluorescently labeled oligonucleotide probes and flow cytometry. This will expedite discovering novel *Pseudonocardia* strains and discerning the molecular basis for specific metabolic traits of interest (e.g., antibiotic production, biodegradation of cyclic ethers, and C_1 autotrophic fixation). The obtained genetic sequences and metabolic information will be valuable to expand the current database of reference genes or genomes for the development of closed-format molecular approaches (e.g., qPCR and microarray) and reroute the open-format next-generation sequencing resources to increase the portion of informative reads (e.g., bypassing community-focused metagenomics to single-cell analysis) (Zhou et al. 2015).

Table 4 Quantification of *thmA*/16S rRNA ratios by qPCR

Sample	*thmA*/16S rRNA ratio	Relative abundance of CB1190 cells[a]
CB1190	0.27 ± 0.05	81 ± 15%
Sorted cells	0.20 ± 0.06	60 ± 18%
Enriched consortium	NA[b]	0%
Enriched consortium with 10% of CB1190	0.02 ± 0.01*	8.5 ± 4.2%*

*Significant decreases compared to CB1190 ($p < 0.05$)

[a] Each CB1190 genome contains three copies of 16S rRNA genes. An average of 4.2 copies per genome was assumed for the microbial mixtures (Větrovský and Baldrian 2013)

[b] The abundance of *thmA* genes in the enriched consortium was below our method detection limit (approximately 7000 copies per gram of biomass)

Acknowledgements The authors thank Joel M. Sederstrom (Baylor College of Medicine) for the assistance with flow cytometry.

Funding This study was funded by SERDP (Grant # ER-2301).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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