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Detection of an alkene monooxygenase in vinyl chloride-oxidizing bacteria with GeneFISH

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ABSTRACT

Fluorescence in situ hybridization (FISH) can provide information on the morphology, spatial arrangement, and local environment of individual cells enabling the investigation of intact microbial communities. GeneFISH uses polynucleotide probes and enzymatic signal amplification to detect genes that are present in low copy numbers. Previously, this technique has only been applied in a small number of closely related organisms. However, many important functional genes, such as those involved in xenobiotic degradation or pathogenesis, are present in diverse microbial strains.

Here, we present a geneFISH method for the detection of the functional gene *etnC*, which encodes the alpha subunit of an alkene monooxygenase used by aerobic ethene and vinyl chloride oxidizing bacteria (etheneotrophs). The probe concentration was optimized and found to be 100 pg/μl, similar to previous geneFISH reports. Permeabilization was necessary for successful geneFISH labeling of Mycobacteria; sequential treatment with lysozyme and achromopeptidase was the most effective treatment. This method was able to detect *etnC* in several organisms including Mycobacteria and *Nocardioides*, demonstrating for the first time that a single geneFISH probe can detect a variety of alleles (>80% sequence similarity) across multiple species.

Detection of *etnC* with geneFISH has practical applications for bioremediation. This method can be readily adapted for other functional genes and has broad applications for investigating microbial communities in natural and engineered systems.

1. Introduction

Molecular biological tools such as real-time quantitative polymerase chain reaction (qPCR) and next generation high-throughput DNA and RNA sequencing are frequently used microbial ecology techniques that provide quantitative and semi-quantitative information about the composition and activity of microbial communities in natural and engineered environments. Unlike these popular techniques, fluorescence in situ hybridization (FISH) can provide complementary information on the morphology, spatial arrangement, and local environment of individual cells (Moter and Göbel, 2000; Nielsen et al., 2009), allowing a unique opportunity to investigate how microorganisms interact with each other and their physical environments.

FISH typically uses probes complimentary to the target organism's ribosomal RNA (rRNA), which provides taxonomic information, but does not necessarily identify their ecological function (Moter and Göbel, 2000). Many important functions, such as nitrogen fixation, are distributed across genera (Brock et al., 2006), while others may only be present in a few strains within a single genus, such as certain dehalogenase genes important in xenobiotic degradation (Löffler et al., 2013). As interest has shifted from community composition to function, FISH has been applied to protein coding functional genes as well as rRNA (Kubota and Kawakami, 2016; Matturro and Rossetti, 2015; Moraru et al., 2010; Zwirglmaier et al., 2004). The detection of functional genes allows organisms to be identified based on their ecological role, rather than phylogeny. Direct detection of functional genes would improve understanding of microbial community structure and would be invaluable for the study of broadly distributed genes such as those involved in xenobiotic biodegradation pathways, antibiotic resistance, and pathogenicity.

GeneFISH is a method for the detection of functional genes using digoxigenin labeled polynucleotide probes in conjunction with enzymatic signal amplification (catalyzed reporter deposition (CARD)). This method has only been applied in a handful of studies and was used to detect functional genes in a single species or a group of closely related microorganisms (Kubota and Kawakami, 2016; Matturro and Rossetti,

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2015; Moraru et al., 2010). There are many potential challenges to using geneFISH in diverse bacteria. The primary challenges are finding probe sequences and hybridization conditions which enable the detection of many alleles of the target gene. Another challenge lies in developing an appropriate permeabilization method that enables large polynucleotide probes to access the target genes, particularly in bacteria with tough cell walls such as the Actinobacteria.

Here, we describe a geneFISH method for detecting and quantifying phylogenetically diverse aerobic ethene utilizing bacteria, known as etheneotrophs. Many etheneotrophs can also utilize vinyl chloride (VC), while others can cometabolize VC with ethene as a primary substrate. VC is common groundwater contaminant, and the detection of etheneotrophs has practical applications in bioremediation (Moran et al., 2007). Etheneotrophs include members of the Actinobacteria, (*Mycobacterium*, *Nocardioides*) and alpha, beta, and gamma proteobacteria (*Ochrobactrum*, *Ralsontia*, *Pseudomonas*) (Coleman et al., 2002; Danko et al., 2006; Elango et al., 2006; Verce et al., 2000).

The genes involved in aerobic VC degradation in etheneotrophs have been found on large, apparently linear, plasmids in several strains (Danko et al., 2004; Mattes et al., 2005). The first enzymatic step in aerobic ethene and VC biodegradation is to break the carbon double with the addition of oxygen to form an epoxide; either epoxyethane when ethene is the substrate or chlorooxirane when VC is the substrate (Mattes et al., 2010). Epoxidation is catalyzed by an alkene monooxygenase, a multi-component enzyme related to soluble methane monooxygenases and other soluble di-iron monooxygenases (SDIMOs) (Coleman et al., 2006; Hartmans et al., 1991). The epoxide is then conjugated to coenzyme M (CoM) by an epoxyalkane:CoM transferase and shuttled to central metabolism (Coleman and Spain, 2003b). The gene *etnC* encodes the alpha subunit of the alkene monooxygenase and *etnE* encodes the epoxyalkane:CoM transferase. Both *etnC* and *etnE* have been used as biomarkers for etheneotrophs in prior studies (Liang et al., 2017a; Liang et al., 2017b; Liu et al., 2018; Mattes et al., 2015; Richards et al., 2019). An increase in abundance of these genes has been associated with an increase in VC attenuation rates (Liang et al., 2017b).

The geneFISH method presented here uses a probe targeting a region of *etnC* which is highly conserved amongst etheneotrophs, but not other SDIMOs, allowing sensitive detection of diverse etheneotrophs while limiting non-specific labeling of related SDIMO sequences which may coexist in the environment. This method could be readily adapted to other bacteria, and would be particularly useful in the study of Actinobacteria, which play an important role in many natural and engineered processes and have been challenging to study by FISH (Davenport et al., 2000; Sekar et al., 2003).

2. Materials and methods

2.1. Cultures

Pure cultures of *Mycobacterium rhodesiae* strain JS60 (ATCC BAA-494) and *Nocardioides* sp. JS614 (ATCC BAA-499) were grown on minimal salts media (MSM) with VC as a carbon source as previously described (Coleman et al., 2002). The wild-type *Mycobacterium* sp. JS623, which can only cometabolize VC, (Coleman and Spain, 2003a) was grown in MSM with ethene instead. *Methylocystis* sp. strain Rockwell (ATCC 49242), a methanotroph, was grown in MSM with methane as a carbon source. *E. coli* K-12 (ATCC 10798) was grown in lysogeny broth.

2.2. Probe synthesis

A 360 bp region of the *etnC* gene from *Mycobacterium* sp. strain JS60 flanked by the primer pair NVC105-NVC106 (Coleman et al., 2006) was selected as a candidate probe (NCBI Accession AY243034.1/754–1110). The primer and probe sequences are shown in Table 1. Specificity of the probe sequence was checked against the NCBI nr/nt database using a discontiguous/megaBLAST search.

DNA was extracted from *Mycobacterium* JS60 using a PowerWater Sterivex kit (Qiagen, Germantown, MD). The kit protocol was modified, omitting the filtration step, and adding liquid culture directly to the bead tubes. Samples were heated at 90 °C for 5 min prior to bead beating to improve cell lysis.

Digoxigenin-labeled polynucleotide probes were synthesized by PCR using a PCR-DIG Probe Synthesis kit (Roche, Basel, Switzerland), per the manufacturer's instructions. Unlabeled PCR products were also generated as a control. Approximately 10 ng of genomic DNA was used as a template for each 50 µl reaction. The primer concentration was 0.5 mM. The thermal cycler program was as follows: initial denaturation at 95^0 for 5 min; followed by 35 amplification cycles (95 °C for 30 s, 54 °C for 30 s, 72 °C for 40 s), and a final extension at 72 °C for 7 min. Product quality was checked by agarose gel electrophoresis. The DIG-labeled probe appeared larger than the unlabeled control (Fig. S1), indicating successful incorporation of digoxigenin. Unlabeled PCR products were cloned into One Shot™ TOP10 *E. coli* following the manufactures instructions (Invitrogen, Carlsbad, CA).

2.3. Melting temperature determination

Melting temperature (T_m) was determined for the DIG-labeled probe, unlabeled control products, and equal amounts of probe and control in a modified hybridization buffer and in two post hybridization wash buffers. The modified hybridization buffer consisted of 5× saline sodium citrate buffer (SSC), 20 mM EDTA, 0.1% (m/v) SDS, 10% (m/v) dextran sulfate, and formamide ranging from 0 to 50% (*v*/v). The formamide concentration was varied to modulate effective $T_{m.}$ Buffer compositions are detailed in Table S1. Probe and/or unlabeled control products (220 ± 20 ng) were added to hybridization buffer or wash buffer with the addition of SYBR Green I and ROX dyes, both at 1× final concentration. Melt curves were generated by denaturing the samples at 95 °C for 15 s, annealing for 1 min, then heating the samples 0.05 °C/s to 95 °C while measuring fluorescence using a Quant-studio 7 qPCR instrument.

2.4. GeneFISH

2.4.1. Fixation

Liquid samples (1.0 ml) were removed from the cultures described above during mid-exponential growth (OD_{600}: 0.1–0.2 cm^{-1}). Tween 80 was added to reduce cell clumping (final concentration 0.02%), then the samples were briefly vortexed and centrifuged at 10,000 x*g* for 1 min to pellet the cells. The supernatant was decanted, and the cells were resuspended in fixative (either 4% paraformaldehyde (PFA) in

Table 1
PCR primer and FISH probe sequences used in this study.

Probe/Primer	Sequence (5′-3′)	Reference
NVC105	5′-CAGGAGTCSCTKGACCGTCA-3′	Coleman et al., 2006
NVC106	5′-CARACCGCCGTAKGACTTTGT-3′	Coleman et al., 2006
JS60 *etnC*	5′-CAGGAGTCGCTTGACCGTCACTTCTGGCACCAGCATCAGTCGATGGACACGCTGGTCGGTGTTCTTTCGGAG-TACTTCGCCGTGGAACGCCCTTGGGCTTACAAGGATGTCTGGGAGGAGTGGGTCGTGGACGACTTCGTCGGC-TCTTACATGAGTCGACTGAGTCCGTTCGGGCTGAAGCCGCCGGCGAGGCTTGGTGATGTCGCCCGGTACGTC-AATGACATGCACCATTCAGTGGCGATCGCGCTTGCGGCTATGTGGCCGCTGAACTTCTGGCGGACCGACCCC-ATGGGTCCGGCAGATTACGAATGGTTTGAGAACCACTACCCTGGCTGGACCAAGTCCTACGGCGGTTTG-3′	This study

phosphate buffered saline (PBS) (ThermoFisher, Waltham, MA), or in 50% ethanol in phosphate buffered saline (PBS)) for 1 h at room temperature. Initial experiments showed that ethanol fixation was inadequate and PFA fixation was used in the remaining experiments (Fig. S2). Fixed cells (50–200 μl) were diluted in 5 ml PBS and filtered onto 0.2 μm pore-size GTTP polycarbonate filters (Millipore) backed by a 0.45 μm nitrocellulose support filter, under gentle vacuum (25 in. Hg). The filters were rinsed with 5 ml PBS, briefly air dried, then dipped in molten 0.1% low melting temperature agarose to reduce cell detachment during subsequent steps. Agarose-coated filters were dried face-up on parafilm for 10 min and divided into 6–8 pieces using dissecting scissors.

2.4.2. Permeabilization and peroxidase inactivation

Several chemical and enzymatic permeabilization methods were evaluated as shown in Table 2. For each treatment, the filters were placed face-down in a petri dish containing the permeabilization solution and incubated as shown. Enzyme solutions were prepared immediately before use. Lysozyme from hen egg white (Sigma Aldrich, St. Louis, MO) was prepared in 100 mM Tris-HCl and 50 mM EDTA. Achromopeptidase (Kodak Chemical, Japan) was prepared in 0.01 10 mM Tris-HCl and 0.01 10 mM NaCl. A previously reported permeabilization method using lysozyme followed by Triton-X 100 (Cimino et al., 2006) was tested as reported and also with the with the detergent and lysozyme steps reversed. This was done to determine if the detergent would disrupt the outer leaflet and improve peptidoglycan access for lysozyme. After permeabilization, endogenous peroxidases were inactivated by placing the filters in a petri dish containing 0.01 M HCl and incubating for 10 min at 37 °C. Filters were then rinsed in PBS.

2.4.3. Hybridization

Hybridization buffer consisted of 5× SSC, 20 mM EDTA, 0.1% (m/v) SDS, 10% (m/v) dextran sulfate, and 40% (v/v) formamide (Table S1). Everything except the formamide was combined and heated at 60 °C with occasional vortexing until the dextran sulfate was fully dissolved. The solution was cooled to room temperature before adding the formamide. Probe was added to the hybridization buffer immediately before use; concentrations ranged from 10 to 1000 pg/μl. Sheared salmon sperm DNA (sssDNA)(Invitrogen, Carlsbad, CA), was also added (100 μg/ml) as blocking agent to minimize non-specific binding of the probe.

Hybridization was performed in 0.2 ml PCR tubes to minimize evaporation. Filters were carefully placed into the tubes (1–2 pieces/tube) and hybridization buffer (150 μl) was added. Samples were denatured at 80 °C for 20 min, then hybridized at 46 °C for 18 ± 1 h in a thermal cycler. At the end of hybridization, excess buffer was pipetted off and the filters were washed to remove unbound probe. The samples were first washed 2 × 5 min with wash buffer 1 (WB1)(2× SSC, 0.1% SDS), then 1 × 5 min and 1 × 30 min with wash buffer 2 (WB2)(0.1× SSC, 0.1% SDS). These wash buffers are routinely used for DNA hybridization, including previous use with geneFISH (Moraru et al., 2010; Sambrook and Russell, 2006). Wash buffer compositions are detailed in Table S1. All washes were performed at 50 °C with pre-warmed buffers.

2.4.4. Immunolabeling

Samples were blocked using 2% bovine serum albumin (BSA) (Amresco, Solon, OH) in PBS for 30 min at 37 °C. Excess blocking solution was pipetted off, the immunolabeling solution was added and the samples were incubated at 37 °C for 1 h. The immunolabeling solution contained 1 U/ml Anti-DIG HPR (Roche, Basel, Switzerland) with 2% BSA in PBS.

2.4.5. Catalyzed reporter deposition (CARD)

CARD buffer consisted of 20% (*w/v*) dextran sulfate and 2 M sodium chloride in PBS. Hydrogen peroxide (final concentration 0.0015% *v*/v) and Alexa Fluor 488 tyramide reagent (10 μl/ml) (Invitrogen, Waltham, MA) were added to the buffer immediately before use. CARD buffer (150 μl) was added to each sample and incubated for 10 min at 37 °C in the dark. The samples were immediately washed in PBS 2 × 5 min, then dehydrated by immersing the filter in 96% ethanol for 1 min. Excess moisture was removed by touching the filters to a piece filter paper.

2.4.6. Mounting and counter stain

Filters were mounted onto glass slides using Prolong Diamond antifade media with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Samples were cured overnight at room temperature prior to imaging.

2.4.7. Microscopy and image processing

Imaging was performed at the University of Iowa Central Microscopy Research Facility using a Leica SP8 inverted confocal laser scanning microscope using a 63× objective lens (numerical aperture 1.4). Excitation and emission wavelengths were: DAPI: 405/430–480 nm, Alexa Fluor 488: 490/510–530 nm. A minimum of 5 fields of view and 300 individual cells were imaged for each sample. DAPI stained cells represented the entire bacteria population, while those showing Alexa Fluor 488 fluorescence were labeled by geneFISH. Images were processed in ImageJ using an automatic cell counting macro. DAPI and Alexa Fluor 488 channels were processed independently. Manual cell counts were periodically compared with the automatic counts for quality control.

3. Results and discussion

3.1. Probe design and synthesis

A high degree of specificity is required for FISH probes to hybridize with target sequences while minimizing non-specific binding. The probe used in this study was prepared by PCR using the NVC 105–106 primer set (Table 1), which was designed to amplify a highly conserved region of *etnC* (Coleman et al., 2006). Unlike PCR primers, which only need short binding sites, a geneFISH probe must have a high degree of complementarity across its entire sequence to hybridize efficiently (Moraru et al., 2010). Since most known etheneotrophs are Mycobacteria, we selected the *Mycobacterium* JS60 gene sequence as a candidate probe and evaluated its specificity by performing a discontinuous/MEGA-BLAST search against the NCBI non-redundant database. This

Table 2
Permeabilization Treatments.

Treatment	Concentration	Temperature	Time (minutes)	Reference
1 M HCl	1 M	RT	30	(Nielsen et al., 2009)
Lysozyme	10 mg/ml	37 °C	60	(Nielsen et al., 2009)
Achromopeptidase	60 U/ml	37 °C	30	(Sekar et al., 2003)
Lysozyme, then Achromopeptidase	10 mg/ml	37 °C	60	(Sekar et al., 2003)
	60 U/ml		30	
Lysozyme, then Triton X-100	10 mg/ml	37 °C	60	(Cimino et al., 2006)
	0.1% (v/v)	RT	5	
Triton X-100, then lysozyme	0.1% (v/v),	RT	5	This study
	10 mg/ml	37 °C	60	

RT: Room temperature.

search retrieved sequences from several strains of etheneotrophs (Table S2). These sequences share a high degree of homology with the probe (>82% identity, > 94% query coverage, E value $<4 \times 10^{-88}$). No other significant matches were found, despite using MEGA-BLAST, which is intended to detect more distantly related sequences. This suggests that the candidate probe sequence is highly specific for *etnC* and is unlikely to hybridize with other sequences, including other SDIMOs.

In addition to specificity, probe size is a major consideration in geneFISH. A larger probe would allow for more fluorophores, and thus provide more sensitivity, but would reduce diffusion of the probe into the cells. The minimum number of fluorophores necessary to detect an individual cell are estimated at 370–1400 (Hoshino et al., 2008). We calculated that the probe used in this study would contain 50 digoxigenin labels, based on a length of 360 nt and 60% GC content. Assuming that each label can accommodate a single HRP, and each HRP can deposit 26–41 tyramides (Hoshino et al., 2008), hybridization of a single probe will catalyze the deposition of 1300–2100 fluorophores, more than enough to detect individual cells. The candidate *etnC* probe met the criteria for specificity and sensitivity and was advanced for further method development.

3.2. Hybridization conditions

The rate and specificity of hybridization are a function of the probe's melting temperature (T_m), the buffer composition, and the temperature at which the samples are hybridized. The high temperatures and long incubation times required for FISH would be detrimental to sample quality, so formamide is typically added to hybridization buffer to reduce the effective T_m. The probe T_m in hybridization buffer without formamide was higher than tested (>95 °C). The effective T_m of the DIG-labeled probe was 90 °C with 10% formamide and decreased linearly as the formamide concentration increased (Fig. 1). DIG-labeled probes had a slightly lower Tm (3.2–3.8 °C lower), than the unlabeled control PCR products at any given formamide concentration. Combinations of probe and unlabeled control PCR products also had a T_m lower than the unlabeled products (0.1—2.7 °C lower) but not a low as the probe alone. This demonstrates that the DIG-labels depress the T_m, but not enough to significantly interfere with hybridization. However, this may be an important finding for future studies considering longer probes or probes with a greater degree of DIG labeling.

It has been suggested that geneFISH cannot capture every allele of a gene, and that probes should have ≤5% mismatch with their target sequences (Moraru et al., 2010; Moraru et al., 2011). However, ex situ DNA:DNA hybridizations (i.e., Southern blot) are routinely done with greater degrees of mismatch (Sambrook and Russell, 2006). A common rule of thumb is that for every 1 °C below the $T_{m,\ effective}$, DNA:DNA hybridizations will tolerate approximately 1% sequence mismatch (Sambrook and Russell, 2006). We targeted hybridization temperatures at $T_{m,\ effective}$-25 °C, to balance hybridization rates (Eisel et al., 2008) with stringency requirements (i.e., tolerating ≤18% mismatch between probe and target sequences). Either the hybridization temperature or the formamide concentration can be selected, and the other adjusted accordingly to achieve the desired $T_{m,\ effective}$. For this study, we selected 46 °C as the hybridization temperature, resulting in a $T_{m,\ effective}$ of 71 °C in hybridization buffer with 40% formamide.

T_m was also measured in the post hybridization wash buffers, WB1 and WB2 to evaluate the stringency of the washes. The T_m of probe: unlabeled control hybrids in the low-stringency WB1 was >95 °C, indicating that only very poorly hybridized sequences would be removed in the initial wash. The T_m in the high-stringency WB 2 was 58 °C.

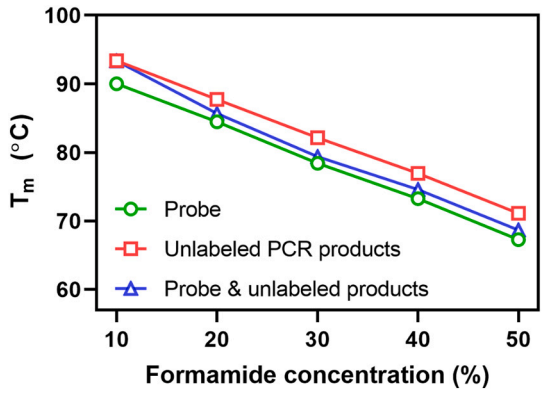

Fig. 1. Probe melting temperature (T_m) determination. The melting temperature of DIG-labeled probes, unlabeled control products, and combinations thereof were determined in hybridization buffer with variable formamide concentrations. Samples were analyzed in duplicate; error bars are omitted because they are smaller than the graph symbols.

3.3. Probe concentration

Probe concentrations were optimized in *E. coli* clones carrying plasmids with the same *Mycobacterium* JS60 *etnC* sequence that was used to generate the probes. This was done because *E. coli* are easily permeabilized with established methods. Permeabilization of etheneotrophs had not yet been done and was expected to be challenging.

Detection of microorganisms by either rRNA FISH or geneFISH is limited by the number of fluorophores deposited in a cell (Nielsen et al., 2009). A greater number of fluorophores creates a brighter signal that can be more readily detected and distinguished from background fluorescence (Hoshino et al., 2008). Increasing the probe concentration can increase the number of fluorophores deposited in the cell (Hoshino et al., 2008). However, it also increases the likelihood of non-specific binding (Moraru et al., 2010). The optimum probe concentration provides the highest labeling efficiency (i.e., number of probe-labeled target sequences divided by the total number of target sequence) while limiting non-specific probe binding and background fluorescence. The few existing geneFISH methods report a wide range in probe concentrations (25–1000 pg/ul), so we evaluated several probe concentrations between 2.5 and 1000 pg/ul (Matturro and Rossetti, 2015; Moraru et al., 2010).

Labeling efficiency was determined by normalizing the number of cells exhibiting Alexa Fluor 488 signal to those stained with DAPI. While the highest average labeling efficiency was seen with 10 pg/ul probe, there were no significant differences between 10 and 500 pg/ul (Fig. 2), with an average labeling efficiency of 31%. The negative controls had no

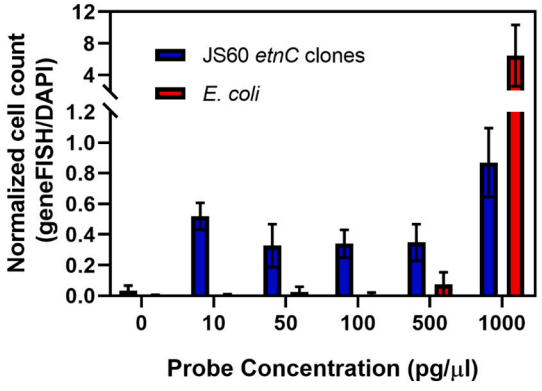

Fig. 2. Probe concentration optimization. *E. coli* clones carrying the partial *Mycobacterium* JS60 *etnC* sequence and regular *E. coli* (negative control) were hybridized with probe concentration between 0 and 1000 pg/μl. Alexa Fluor 488 cell counts were normalized to total (DAPI) cell counts to determine geneFISH labeling efficiency. Samples were prepared in triplicate and cells were counted in ≥5 fields of view per sample. Error bars represent the standard deviation between fields of view.

Alexa Fluor 488 signal when the probe concentrations were less than 500 pg/ul. When the probe concentration was 500 pg/µl, Alexa Fluor 488 fluorescence could be seen in the negative controls, indicating non-specific binding when the probe was present in excess. Non-specific binding was drastically worse with 1000 pg/ul probe, causing difficulties with the automatic cell counts and resulting in apparent labeling efficiencies $>>1$. A probe concentration of 100 pg/µl was selected for the final protocol since this was the highest probe concentration without appreciable non-specific signal. FISH probes are typically applied in excess to increases the opportunity of hybridization with a target sequence.

Routine use of clones in geneFISH is not necessary but was valuable for the development of this method. A successful geneFISH reaction requires many consecutive successful steps to generate a fluorescence signal, making it difficult to troubleshoot unsuccessful reactions. For this method, the low gene copy number and relative impermeability of Actinobacteria were both thought to be major challenges. The use of clones allowed the decoupling of hybridization from permeabilization, so that hybridization efficiency could be determined independently.

3.4. Permeabilization

FISH protocols often include a permeabilization step, designed to facilitate the diffusion of probes into fixed cells. Actinobacteria have an unusual cell envelope structure including a thin layer of peptidoglycan cross-linked arabinogalactan and an outer leaflet, analogous to the outer membrane of gram negatives In certain Actinobacteria, including the Mycobacteria, the outer leaflet includes hydrophobic mycolic acids, which form a significant barrier to diffusion (Carr et al., 2005; Davenport et al., 2000; Rahlwes et al., 2019). Several chemical and enzymatic permeabilization methods have been used for FISH with Actinobacteria (Carr et al., 2005; Davenport et al., 2000; Sekar et al., 2003). These permeabilization methods have found varying levels of success, which are often strain dependent. We hypothesized that the permeabilization method must disrupt the outer leaflet as a well as the cell wall. To this end, we evaluated several of the reported methods, as well as a novel method including Triton-X100 and lysozyme and designed to target the Actinobacterial cell envelope. *Mycobacterium* JS60 was chosen for the permeabilization experiments because it exhibits significant hydrophobic character (i.e., clumping, floating, adhering to containers) and was expected to be the most difficult of our experimental organisms to permeabilize.

All the permeabilization methods tested resulted in an increase in the Alexa Fluor 488 labeled cell fraction compared to the control (Fig. 3), indicating that permeabilization allows for increased diffusion of probe and anti-DIG antibodies into the cells. All the permeabilization methods showed variable performance and were not significantly different from each other with an average labeling efficiency of 35%. However, permeabilization with lysozyme followed by achromopeptidase resulted in the greatest average labeling efficiency (42%) and the most reproducible results. These findings agree with a previous study where permeabilization with lysozyme followed by achromopeptidase was the most effective method for detecting fresh water actinobacteria by FISH (Sekar et al., 2003). Overall labeling efficiency remains relatively low, but on par with other geneFISH reports (Moraru et al., 2010).

During the permeabilization tests, it became clear that imaging with a CLSM offers important advantages compared with a standard epifluorescent microscope for geneFISH. GeneFISH signals from *Mycobacterium* JS60 were much less bright than was seen with the *E. coli* clones, regardless of permeabilization method. This is likely the result of fewer copies of the target gene in *Mycobacterium* JS60 than in the *E. coli* clones. However, the microscope could be easily adjusted to produce a clear fluorescence signal in the JS60 samples. The polycarbonate filters used to mount the cells exhibited weak autofluorescence across a wide range of wavelengths, including the range used to detect probes. The confocal aperture and the shallow depth of field was able to exclude most of this

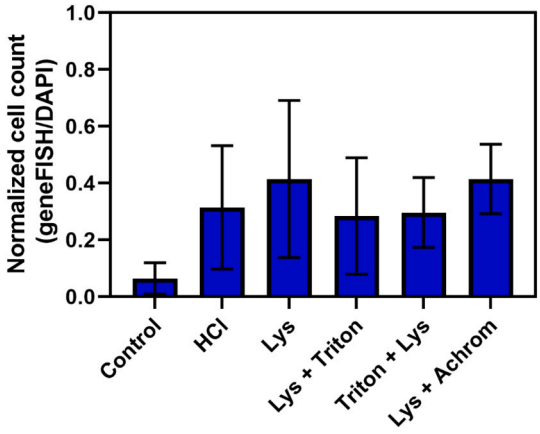

Fig. 3. Normalized cell counts of *Mycobacterium* JS60 cells permeabilized by various methods. *Mycobacterium* JS60 cultures were treated as described for *etnC* clones, with an additional permeabilization step prior to hybridization. The control samples were not permeabilized. The number cells labeled by geneFISH is normalized to the total (DAPI) cell count. Lys: Lysozyme, Triton: Triton-X 100, Achrom: Achromopeptidase. Samples were prepared in triplicate and cells were counted in ≥5 fields of view per sample. Error bars represent the standard deviation between fields of view.

background, which allowed the relatively weak geneFISH signals in *Mycobacterium* JS60 to be counted despite this potential interference. Use of a CLSM would be particularly useful for examining environmental samples, were autofluorescent minerals and organic compounds are common sources of background.

3.5. GeneFISH in etheneotrophs

The geneFISH protocol for *etnC* was evaluated in several organisms, including the etheneotrophs *Mycobacterium* JS60, *Mycobacterium* JS623, and *Nocardioides* JS614, as well as *E. coli* clones carrying the partial JS60 *etnC* sequence. *Methylocystis* strain Rockwell and *E. coli* K-12 were included as negative controls. *Mycobacterium* sp. JS623 is closely related to *Mycobacterium* JS60, while *Nocardioides* JS614 is more distantly related, but still an Actinobacteria. No etheneotrophs belonging to the proteobacteria could be obtained for this study, so the *E. coli* clones carrying the *Mycobacterium* JS60 *etnC* sequence were used as a proxy. The *E. coli* clones will have a cell envelope characteristic of the proteobacteria. The methanotroph *Methylocystis* sp. strain Rockwell was included as a negative control because it also possesses a methane monooxygenase gene related to *etnC*. Similar methanotrophs commonly coexist with etheneotrophs and can cometabolize VC (Liang et al., 2017b; Richards et al., 2019), and may generate false positive results for this method.

All the etheneotrophs and the *E. coli* clones carrying the *Mycobacterium* JS60 *etnC* sequence were successfully labeled by geneFISH (Fig. 4). The clones had the highest labeling efficiency at 47% (Fig. 5) and produced the brightest signal of all the strains tested. The signal was so bright that the detector gain had to be reduced about 30% compared to the other strains to avoid over saturation. This was expected, since *E. coli* are relatively easy to permeabilize and the clones carry several copies of the target sequence, providing many opportunities for the probe to bind. All the etheneotrophs had a labeling efficiency between 9 and 27%, demonstrating that this geneFISH method can detect several alleles of *etnC* in etheneotrophs with a variety of cell envelope characteristics.

E. coli, one of the negative controls, had only trace geneFISH signal, showing that the geneFISH reaction is specific to *etnC* (Fig. 5). Normalized cell counts could not be obtained for *Methylocystis* cultures because only a small number of DAPI stained *Methylocystis* cells were observed on the filters that had undergone the full geneFISH protocol. This was initially thought to be the result of cell lysis or detachment

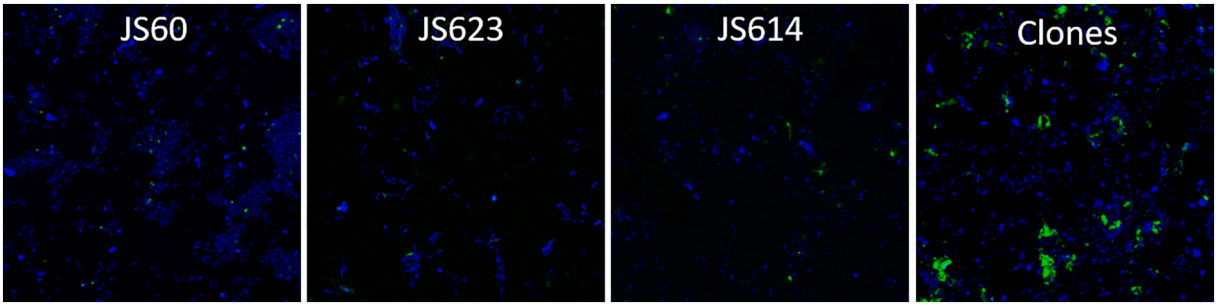

Fig. 4. Micrographs of GeneFish-labeled etheneotrophs and *etnC* clones. All cells stained by DAPI are blue. Cells that were successfully labeled by GeneFISH are green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

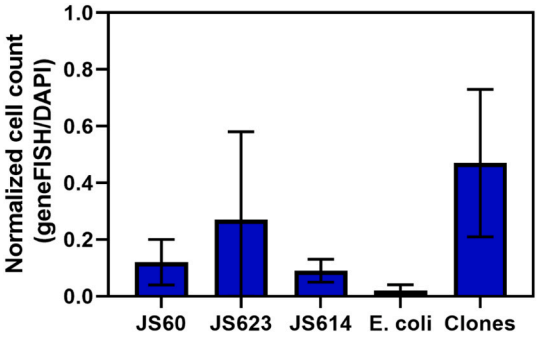

Fig. 5. Normalized cell counts of etheneotrophs and control strains. The number cells labeled by *etnC* GeneFISH is normalized to the total (DAPI) cell count. Samples were prepared in triplicate and cells were counted in ≥5 fields of view per sample. Error bars represent the standard deviation between fields of view.

during the permeabilization steps. In a separate experiment, geneFISH was performed on *Methylocystis* samples without permeabilization, with only lysozyme treatment, and with both lysozyme and achromopeptidase treatment as done in the samples described above. Only trace geneFISH signal was seen in any of the *Methylocystis* samples, indicating that the *etnC* probe did not bind to methane monooxygenase sequences present in this strain (Fig. 6). There was an obvious decrease in the number of DAPI stained cells with lysozyme permeabilization compared to the unpermeabilized samples (Fig. 6). This is an important finding as it shows that the aggressive permeabilization necessary for geneFISH with Actinobacteria will result in the loss of some more sensitive cells.

4. Conclusions

The geneFISH method described here enables the detection of *etnC* in diverse etheneotrophs. There are many alleles of *etnC*, some of which may have relatively low sequence similarity to one another and are closely related to other monooxygenase genes that are found in similar niches, such as methane monooxygenases (Coleman et al., 2006; Liang et al., 2017b; Richards et al., 2019). This necessitates the careful selection of probe sequence and hybridization conditions to capture the greatest number of *etnC* sequences while limiting non-specific binding. The exact number of *etnC* gene copies present in an individual cell (i.e., the plasmid copy number) is not known but is thought to be low. Detection of such low abundance targets likely require a large probe, in addition to signal amplification, to generate a measurable fluorescence signal. Most known etheneotrophs are Actinobacteria, which often have tough and impermeable cell envelopes, requiring aggressive permeabilization for large geneFISH probes to enter the cells.

This geneFISH method was optimized, and the final protocol is detailed in Table 3. This is an important advancement in FISH methodology for several reasons. First, this method demonstrates that geneFISH can detect a single functional gene in diverse bacterial species,

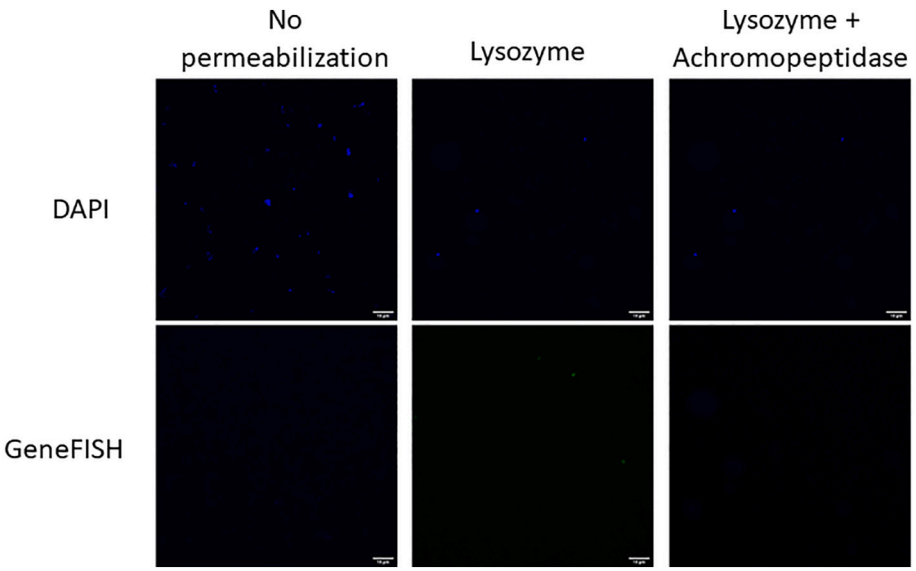

Fig. 6. Micrograph of *Methylocystis* cells permeabilized by various methods. The culture stained with DAPI (blue) to assess the relative level of cell lysis between treatments and labeled with geneFISH (green) to assess potential non-specific binding of the *etnC* probe to methane monooxygenase sequences in this strain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Final CARD-FISH Protocol for the detection of etheneotrophs.

Stage	Step	Description
1. Fixation and immobilization	1.1	Transfer 1 ml of culture to a centrifuge tube. Add 10 µl 5% Tween-80 and mix. Centrifuge at 10,000 ×*g* for 1 min. Decant the supernatant.
	1.2	Add 1 ml 4% PFA and mix. Incubate 1 h at RT. Combine 50–200 µl fixed cells with 5 ml PBS.
	1.3	Label the edge of a 0.2 µm GTTP filter with a soft graphite pencil. Place a 0.45 µm support filter onto the glass support. Place the GTTP filter over it and attach the filtration tower. Filter the fixed cells under gentle vacuum (ca. 30 in Hg). Rinse the tower with 5 ml of PBS.
	1.4	Dip the filters into molten 0.1% low melting temp agarose, coating both sides. Place face up on a piece of parafilm and dry at 37 °C for 10 min.
2. Permeabilization and peroxidase inactivation	2.1	Place the agarose-embedded filters face down in a petri dish containing 0.1% Triton X-100. Incubate for 10 min at RT. Rinse the filter in PBS.
	2.2	Place the filters face down in a petri dish containing 10 mg/ml lysozyme solution. Incubate for 60 min at 37 °C. Rinse filter in PBS.
	2.3	Place the agarose-embedded filters face down in a petri dish containing 0.01 M HCl for 10 min at RT. Rinse filter in PBS. Briefly air dry.
3. Hybridization	3.1	Cut the filters into 6–8 pieces. Place the filter pieces into 0.2 ml PCR tubes (1–2 sections per tube).
	3.2	Add probe and sheared salmon sperm DNA blocking agent to hybridization buffer. Mix, and aliquot 150 µl into each 0.2 ml tube and cap tightly.
	3.3	Heat the samples to 80 °C for 15 min. to denature the DNA. Hybridize the sample at 46 °C for 18 h. After hybridization, remove solution via pipette.
	3.4	Keep the filter sections in the tubes. Wash 2 × 5 min with WB1. Then wash 1 × 5 min then 1 × 30 min with WB2. All washes are done at 50 °C. Remove excess wash buffer and continue to without drying the filters.
4. Immunolabeling	4.1	Add 150 µl blocking solution to each tube. Incubate for 30 min at 37 °C.
	4.2	Prepare fresh 1 U/ml Anti-DIG HRP solution. Add 150 µl anti-DIG solution to each and incubate 60 min at 37 °C.
	4.3	Pipette off excess solution, rinse 2 × 5 min with PBS at RT.
5. CARD	5.1	Prepare fresh CARD solution. Add 150 µl CARD solution to each tube. Incubate for 10 min at 37 °C.
	5.2	Pipette off excess, rinse 2 × 5 min with PBS at RT.
	5.3	Pipette off PBS. Add 150 µl absolute ethanol and incubate for 1 min at RT
6. Mounting/DAPI	6.1	Remove the filters from the tubes and place face up onto glass slides. Allow to air dry in the dark for ~10 min.
	6.2	Add 1 drop mounting media to the filter. Carefully lower #1.5 cover slip over the sample. Firmly press the cover slip to distribute the antifade and remove bubbles.
	6.3	Cure overnight in the dark at RT prior to imaging. Keep samples at 4 °C for extended storage.

rather than only closely related species, thus separating function and phylogeny. Second, this method demonstrates that, with the appropriate choice of target sequences and hybridization conditions a single geneFISH probe can detect a wide range of gene alleles (≥82% similarity), simplifying the investigation of mixed communities without a priori knowledge of their composition. Third, we have developed a permeabilization method which is suitable for the introduction of large polynucleotide probes into diverse bacteria, particularly the Actinobacteria which play a significant role in many natural and engineered systems including antibiotic production, xenobiotic degradation, wastewater treatment, and even human disease (Barka et al., 2016; Davenport et al., 2000; Mattes et al., 2010; Sharma et al., 2014).

We have also noted the advantaged of using a CLSM with geneFISH and identified potential applications for studying microbial communities including biofilms. This detailed method could be readily adapted to the investigation of other functional genes and bacteria and may be combined with other techniques such as rRNA FISH and microautoradiography enabling study of the interplay between taxonomy, functional genes, and ecological function (Moter and Göbel, 2000). Although only relative quantification was used in this study, geneFISH can be easily used for quantitative studies as well. If the area of a microscopic field of view is known, cell counts can be extrapolated to the total filter area and volume of filtrate.

Relatively low detection efficiency is an ongoing challenge in geneFISH methodology. The large probe, low gene copy number, and tough cell walls of the organisms used in this study all likely contribute to the low efficiency. Even when permeabilization is not thought to be problematic (i.e. in *E. coli* clones), labeling efficiencies are below 50%. However, quantitative analysis of functional genes in the environment are often concerned with large (several orders of magnitude) differences between samples (Liang et al., 2017b; Richards et al., 2019). Even the relatively low efficiencies seen in this study can provide useful quantitative estimates of gene abundance. Future geneFISH studies may improve labeling efficiency by further optimizing probe size, hybridization conditions, and permeabilization techniques.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2021.106147.

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