Rapid detection of rRNA group I pseudomonads in contaminated metalworking fluids and biofilm formation by fluorescent in situ hybridization

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Metalworking fluids (MWFs), used in different machining operations, are highly prone to microbial degradation. Microbial communities present in MWFs lead to biofilm formation in the MWF systems, which act as a continuous source of contamination. Species of rRNA group I Pseudomonas dominate in contaminated MWFs. However, their actual distribution is typically underesti-mated when using standard culturing techniques as most fail to grow on the commonly used Pseudomonas Isolation Agar. To overcome this, fluorescent in situ hybridization (FISH) was used to study their abundance along with biofilm formation by two species recovered from MWFs, Pseudomonas fluorescens MWF-1 and the newly described Pseudomonas oleovorans subsp. lubricantis. Based on 16S rRNA sequences, a unique fluorescent molecular probe (Pseudo120) was designed targeting a conserved signature sequence common to all rRNA group I Pseudomonas. The specificity of the probe was evaluated using hybridization experiments with whole cells of different Pseudomonas species. The probe's sensitivity was determined to be

10^3 cells/ml. It successfully detected and enumerated the abundance and distribution of Pseudomonas indicating levels between 3.2 $(\pm 1.1) \times 10^6$ and 5.0 $(\pm 2.3) \times 10^6$ cells/ml in four different industrial MWF samples collected from three different locations. Biofilm formation was visualized under stagnant conditions using high and low concentra-tions of cells for both P. fluorescens MWF-1 and P.oleovorans subsp. lubricantis stained with methylene blue and Pseudo120. On the basis of these observations, this molecular probe can be successfully be used in the management of MWF systems to monitor the levels and biofilm formation of rRNA group I pseudomonads.

Keywords: Metalworking fluid · rRNA group I Pseudomonas · FISH · 16S rRNA · Biofilms

Introduction

Metalworking fluids (MWFs) are used in different indus-tries to reduce heat and friction between the machining system and the workpiece. MWFs are formulated to improve the longevity of the equipment by preventing metal corrosion (Gilbert et al. 2010a) but are highly prone to physical, chemical, and microbial contamination result-ing in the deterioration of the lubricants. In addition, several health hazards are associated with microbial contamination of MWFs (Cyprowski et al. 2007; Dilger et al. 2005) as they support the growth of wide varieties of microorgan-isms, including (opportunistic) pathogens such as Myco-bacterium sp., Escherichia coli, Legionella sp., and bacteria belonging to the genus Pseudomonas. The pseudomonads are the primary colonizers and predominant bacteria found in contaminated MWFs mainly acting as deteriorgens (Mattsby-Blatzer et al. 1989; Khan and Yadav 2004; van

der Gast et al. 2003). Some are also associated with health and safety risks such as skin dermatitis, endotoxin exposure, asthma, and other respiratory illnesses (Gilbert et al. 2010b; Selvaraju et al. 2005). The commonly found pseudomonads in MWFs are *Pseudomonas oleovorans* subsp. *oleovorans*, *Pseudomonas oleovorans* subsp. *pseudoalcaligenes*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and the newly described *Pseudomonas oleovorans* subsp. *lubricantis* belonging to rRNA group I (Saha et al. 2011; Gilbert et al. 2010a; Saha 2009; Khan and Yadav 2004; van der Gast et al. 2003; Virji et al. 2000).

Bacteria colonizing MWFs may also be capable of forming biofilms (Mattsby-Blatzer et al. 1989). It can be composed of single or multiple species of organisms including Gram-positive or Gram-negative bacteria along with yeast and protozoa (Vu et al. 2009). The development of a biofilm depends on the availability of nutrients and surface for attachment (Stoodley et al. 2002; Costerton et al. 1999). Biofilm formation mainly occurs in the sump, which holds the in-use MWFs and is recognized in the industry as scum or slime (Fishwick et al. 2005; Veillette et al. 2004). Microorganisms growing within the biofilm are protected from the surrounding environment and are highly tolerant to different disinfection methods. Once established within the system, the biofilms also serve as continuous sources of contamination of MWFs (Mariscal et al. 2007).

Previous studies have reported that rigorous cleaning and the use of biocide were not completely successful in eliminating the rRNA group I pseudomonads but only in reducing their concentration in MWFs (Marchand et al. 2010; Dilger et al. 2005). Therefore, early detection and identification of pseudomonads in MWFs is important for the development of better fluid management regime such as control of microbial load (e.g., by UV disinfection or ozone treatment) and protection of the workers from exposure to harmful contaminants (Selvaraju et al. 2005; Johnson and Phillips 2002). Since these pseudomonads are primary colonizers (Mattsby-Blatzer et al. 1989) of MWFs, it is also important to investigate their biofilm forming capability in the sumps, which provide excellent habitat for biofilm formation.

It has long been known that classical culturing techniques are not only tedious to perform but also lack specificity and speed along with accuracy (van der Gast et al. 2001). With MWFs, it has been reported that culture-based techniques not only take several days to provide results but only <5% of bacteria can be recovered on the available culture media, thus underestimating the total bacterial population present at any given time in the contaminated MWF samples (Saha et al. 2010b; Selvaraju et al. 2005; van der Gast et al. 2003). Due to the limitations associated with these techniques, there was a need for the development of molecular-based methodologies for the detection and enumeration of bacteria present in MWFs (Saha et al. 2010b; Rhodes et al. 2008; Khan and Yadav 2004). Culture-independent molecular techniques such as denaturing gradient gel electrophoresis (DGGE) and real-time PCR (qPCR) were developed for the detection and enumeration of bacteria present in MWFs (Saha et al. 2010b; Rhodes et al. 2008; Yadav et al. 2006; van der Gast et al. 2001).

Fluorescent in situ hybridization (FISH) is commonly used for the detection of microorganisms from different environmental samples and offers a cost-effective alternative detection solution. The microbial diversity of a wide variety of natural environments ranging from river water systems (Kenzaka et al. 1998), sea-water (Tang et al. 2005), corals (Ainsworth et al. 2006), and sulfidic and acid mines (Kock and Schippers 2008) have been successfully studied using FISH. Recently, the utilization of FISH has rapidly increased in the medical diagnostics for detection and enumeration of pathogens associated with different infections (Hogardt et al. 2000; Waar et al. 2005; Oosterhof et al. 2006). FISH of whole bacterial cells involving the use of an oligonucleotide probe targeting 16S rRNA molecules universally present in all bacteria has several advantages over culture-based methods (Amann et al. 1990; Kenzaka et al. 1998). FISH permits direct visualization of viable cells has reduced time of detection and is highly specific (Tang et al. 2005). Due to these advantages, earlier studies successfully used FISH for the investigation of biofilm in different environments (Thurnheer et al. 2003; Dige et al. 2007). However, it has some limitations such as autofluorescence, photobleaching, insufficient permeability, and low rRNA content due to the physiological state of the cells (DeLong et al. 1989; Hogardt et al. 2000).

The overall objective of this study was to design a unique fluorescent-labeled oligonucleotide probe targeting a specific region of the 16S rRNA molecules of the rRNA group I pseudomonads to develop FISH assays. Using this probe, the specific objectives of this study were (a) rapid detection and enumeration of rRNA group I *Pseudomonas* in contaminated MWF samples, and (b) study of biofilm development by *P. fluorescens* and *P. oleovorans* subsp. *lubricantis* recovered from used MWFs using different levels of cells.

Materials and methods

Bacterial cultures

Cultures of *P. oleovorans* subsp. *oleovorans* (ATCC 8062), *P. oleovorans* subsp. *pseudoalcaligenes* (ATCC 17440), *Pseudomonas alcaliphila* (ATCC BAA 571), *Pseudomonas mendocina* (ATCC 2541), along with *P. oleovorans* subsp.

lubricantis (ATCC BAA 1494) and *P. fluorescens* MWF-1 recovered in an earlier study (Saha et al. 2010a) were selected for the study as they belong to the *Pseudomonas* rRNA group I. *Escherichia coli* (ATCC 23848) was selected as the negative control. All strains were grown and maintained on Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) (BD, Franklin Lakes, NJ). All strains were grown for 24–48 h at 37°C except for *P. fluorescens* MWF-1, which was grown at 30°C.

Metalworking fluid samples

Four samples of used MWFs were used for the study. The samples were obtained from three different industries located in Michigan and were of different ages and collected from different machining systems (Table 1).

Probe design and specificity

An oligonucleotide probe based on 16S rRNA sequences of the sixteen species of *Pseudomonas* belonging to the rRNA group I was designed for the study. The sequences of *P. oleovorans* subsp. *oleovorans*, *P. oleovroans* subsp. *pseudoalcaligenes*, *P. alcaliphila*, *P. mendocina*, *P. oleovorans* subsp. *lubricantis*, *P. stutzeri*, *P. alcaligenes*, *P. resinovorans*, *P. aeruginosa*, *P. putida*, *P. cichorii*, *P. syringae*, *P. chlororaphis*, *P. fragi*, *P. agarici*, and *P. fluorescens* were obtained from the Ribosomal Database Project (RDP) (Cole et al. 2008) and from GeneBank (Benson et al. 2008) for analysis and design of the probe using different software. The sequences were initially aligned with Clustal X (Thompson et al. 1997) and were manually screened for the signature sequence for the rRNA group I. Three different probe design software, i.e., ARB probe tool at www.arb-home.de; Allele ID at www.premierbiosoft.com; and Integrated DNA Technologies (IDT) at www.idtdna.com were evaluated for analysis of the sequences for designing of the unique probe targeting the 16S rRNA molecules of the rRNA group I pseudomonads.

The specificity and the coverage of the probe were evaluated against other bacterial (target and non-target) 16S rRNA sequences using different software and databases such as probeBase, and probeCheck at www.microbial.ecology.net along with RDP's ProbeMatch at www.rdp.cme.msu.edu and NCBI's BLAST database (Altschul et al. 1990). The secondary structure of the 16S rRNA molecule was also evaluated to confirm the potential binding site of the probe to the 16S rRNA molecule (Behrens et al. 2003). The probe was synthesized by Integrated DNA Technologies (IDT) (Coralville, IA). The unique probe, Pseudo120, was labeled with a fluorescent dye, Cy3 on the 5' end; the universal bacterial probe EUB338 (Waar et al. 2005) was labeled with FAM on the 3' end of the oligonucleotide.

Fluorescent in situ hybridization of pure cultures

The FISH experiments were performed according to the procedure of Pernthaler et al. (2001) and Hugenholtz et al. (2000) with the modifications described below. Pure culture of six different species of *Pseudomonas* and *E. coli* were used as reference strains to test the specificity of the probe. Following hybridization and washing each slide was covered with coverslip and the edges were sealed with clear nail polish. The prepared slides were immediately processed for image analysis and documentation.

Microscopy and quantification

Imaging was performed using a Zeiss Axiovert 200 M inverted microscope with an AxioCam fluorescence camera (Zeiss, Hamburg, Germany). The images were processed using image documentation software CELL C (Selinummi et al. 2005) and DAIME (Daims et al. 2005). The CELL C software was used to count the number of cells per field.

Table 1 Enumeration of rRNA group I pseudomonads in used MWFs using FISH (values of cell count per milliliter represents mean of ten replicates (n=10))

Used MWF samples[a]	Age of Sample (months)	HPC[b]	PIA[c]	Cell count (per milliliter)[d]	Pseudomonads (%)[e]
1	4	$<1.0\times10^1$	$<1\times10^1$	$3.8\ (\pm2.0)\times10^6$	85.9
2	6	9.2×10^4	$<1\times10^1$	$5.0\ (\pm2.3)\times10^6$	84.5
3	6	$<1.0\times10^1$	$<1\times10^1$	$3.2\ (\pm1.1)\times10^6$	84.6
4	24	1.0×10^5	3.3×10^4	$3.4\ (\pm1.8)\times10^6$	72.4

[a] The four samples were collected from three different locations

[b] Heterotrophic plate count in colony forming units (CFU) per milliliter

[c] *Pseudomonas* isolation agar (PIA) was used to recover culturable *Pseudomonas* species

[d] CELLC image analysis software was used to determine the cell count per milliliter of used MWF samples using the Pseudo120 probe

[e] Percentages were calculated by analysis of all bacterial cells stained with EUB338 and pseudomonads with Pseudo120 probe

Bacterial cells per milliliter of the sample were determined using the formula modified from Hogardt et al. (2000): $N_s = (N_f/V) \times M_f \times 10^3$, where N_s=cell count per milliliter of the sample; N_f=mean cell count per field for ten randomly selected field of view; V=volume of sample in each well; and M_f=number of field per square centimeter of the well.

FISH of used MWF samples

The protocol used for the fixation, hybridization, and microscopic analysis of the used MWF samples was similar to that used with pure cultures (Pernthaler et al. 2001) with the following modifications. A 10-ml volume of used MWF sample was fixed using 30 ml volume of 4% paraformaldehyde (PFA) solution for 12 h at 4°C. The MWF sample was then centrifuged at 10,000×*g* for 20 mins, the pellet was washed twice with 1× phosphate buffered saline (PBS) (pH 7.2), resuspended in a mixture of 100% ethanol and PBS (1:1 *v*/*v*) and stored at −20°C. The fixed cells (diluted if necessary with 1× PBS) were hybridized for 2 h at 46°C using a hybridization buffer containing 30% formamide and a mixture of the Pseudo120 and the EUB338 probes.

Preparation of biofilm

Biofilms were prepared according to a modified version of the method described in Niemira and Solomon (2005). Pure cultures of *P. fluorescens* MWF-1 and *P. oleovorans* subsp. *lubricantis* were each grown in a 50 ml of sterile centrifuge tube containing 10 ml of TSB for 24–48 h at optimum temperature (30°C for *P. fluorescens* and 37°C for *P. oleovorans* subsp. *lubricantis*) to obtain a cell concentration of 10^8 colony forming units (CFU)/ml. Precleaned multi-well microscopic slides (Thermo Fisher Scientific Inc., Waltham, MA) were sterilized by autoclaving at 121°C for 15 min. These slides were suspended in 50 ml sterile polypropylene tubes containing 25 ml of TSB. Individual tubes were inoculated with 2.5 or 250 μl of each culture to obtain a starting concentration of 10^4 CFU/ml or 10^6 CFU/ml, respectively. The tubes were incubated under static conditions at the optimum temperature of each bacterium and examined at 48, 72, and 96 h. Each test was performed in duplicate. For both *P. fluorescens* MWF-1 and *P. oleovorans* subsp. *lubricantis*, preparation of biofilm was carried out separately using the dye methylene blue (Kreth et al. 2004) and the molecular probe Pseudo120.

Staining and imaging of biofilms

Following incubation, the slides were carefully removed from the centrifuge tubes gently rinsed for 10 s with sterile distilled water to remove unattached cells and stained with methylene blue for 30 s. Separate sets of slides were used for staining with the Pseudo120 probe. For FISH, the slides were gently rinsed with sterile PBS (pH 7.2) to remove unattached cells and immediately fixed in 4% PFA solution for 3 h at 4°C. After fixation the slides were rinsed with PBS and stored in a mixture of PBS and 100% ethanol (1:1 *v*/*v*) at 4°C until used for hybridization. The slides were used within 3 days after fixation for hybridization. FISH was performed using 30% formamide at 46°C as described previously. Image analysis of both methylene blue and fluorescent-labeled probe-stained biofilm was performed using the Zeiss Axiovert 200 M inverted microscope with AxioCam fluorescence camera. Bright field and epifluorescence microscopy was used for methylene blue and FISH, respectively. To determine the thickness and 3D image analysis of the biofilm, the 6D platform of the AxioCam software was used.

Results

Probe design and FISH of pure cultures

The ARB probe design software was successful in designing a 23-bp probe, 5'-ACT ACC AGG CAG ATT CCT AGG CA-3', specific to the rRNA group I pseudomonads based on alignment of the 16S rRNA sequences available for all the sixteen rRNA group I pseudomonads. After determining the theoretical specificity of the probe using different software and databases, the probe was tested for its specificity under varying stringent conditions with pure cultures of different pseudomonads (Fig. 1). The stringency condition of the hybridization reaction depends on the optimum fluorescent intensity of a given probe. The fluorescent intensity depends on several factors such as the rRNA content of the cells (dependent upon the physiological state of the cells), fluorescence label used, probe and formamide concentration along with the temperature of hybridization (Thurnheer et al. 2003). The probe labeled with the Cy3 dye was successful in penetrating the bacterial cells and the optimum stringent conditions of binding to the complimentary 16S rRNA sequence was determined to be between 30% and 40% formamide at 46°C using a final probe concentration of 0.5 ng/μl. There were no observable differences in the fluorescence intensity between 30% and 40% formamide. Also, no fluorescence signal was detected with *E. coli* cells using the same stringent conditions indicating the specificity of the probe.

A similar experiment was carried out to optimize the hybridization conditions of the universal bacterial probe EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') labeled with the fluorescent dye FAM. Here, the optimum condition was determined to be 30% formamide at 46°C with a final probe concentration of 0.5 ng/μl. Due to the loss of signal

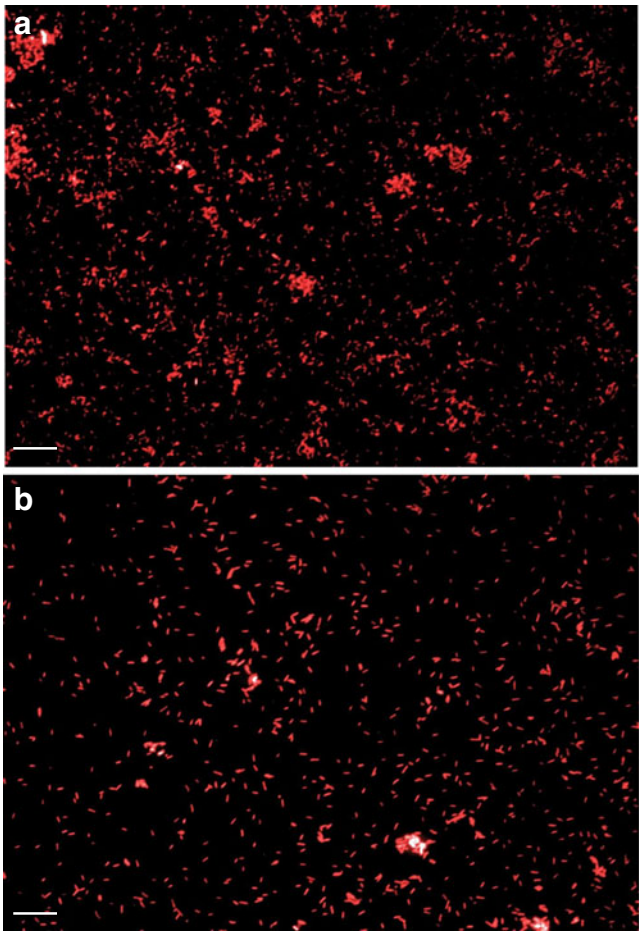

Fig. 1 Epifluorescent micrographs of the 16S rRNA FISH study with pure cultures using the Pseudo120 probe. **a** *P. oleovorans* subsp. *pseudoalcaligenes* ATCC 17440^T; **b** *P. alcaliphila* ATCC BAA 571^T. Both the cultures were grown in TSB for 24 h at 35°C. *Bar*, 5 μm

of the FAM dye at 40% formamide, all further hybridization reactions for the analysis of contaminated MWF samples were carried out using 30% formamide for both Pseudo120 (Cy3) and EUB338 (FAM) probes.

A successful hybridization reaction was observed with a mixed culture of *E. coli* and *P. fluorescens* when hybridized simultaneously with both the probes under optimized conditions indicating the efficacy of the probes and the assay (Fig. 2). Between the two dyes, the Cy3 conjugate expressed the brightest signals. Since photobleaching of both the dyes was observed during experimentation, all the steps were performed under dark conditions along with immediate visualization and imaging of the slides limiting the exposure of the dyes to light, thus reducing the loss of signal due to photobleaching.

Probe sensitivity

Following optimization of the hybridization reaction, the sensitivity of the Pseudo120 probe was determined using different concentrations (10^1–10^4 cells/ml) of different *Pseudomonas* species. The probe's sensitivity was found to be 10^3 cells/ml, using the previously described hybridization conditions. The absence of signal from 10^2 to 10^1 cells/ml could be attributed to the loss of cells from the microscopic slide during sample preparation as very few cells were observed on the slides when viewed under bright field microscopy (to confirm the attachment of cells).

FISH of used MWF samples

The oligonucleotide-FISH protocol was evaluated to test the detection and quantification efficiency of the probes in four different used MWF samples collected from different industries (Table 1). Both the probes (specific and non-specific) were successful in co-detection of pseudomonads along with other bacteria present in used MWF samples (Fig. 3) The FAM-conjugated universal probe was successful in detecting all the bacteria present in the samples (the green fluorescence). The Cy3 conjugated (Pseudo 120) probe emitted red fluorescence when bound to pseudomonads and appeared orange-yellow after superimposing the images (using *daime* image analysis software) as the pseudomonads bound both the FAM and Cy3 conjugates. The fluorescence intensity of both the probes was different from the pure cultures (more active) due to the difference in the physiological state of the cells, which is directly correlated to the ribosomal content of the cells (Thurnheer et al. 2003). The difference in physiological state could be attributed to (a) bacteria present in used MWFs being in a complex chemical environment as compared to the pure cultures and/or (b) due to the age of the used MWF samples there could be a possibility of nutrient depletion.

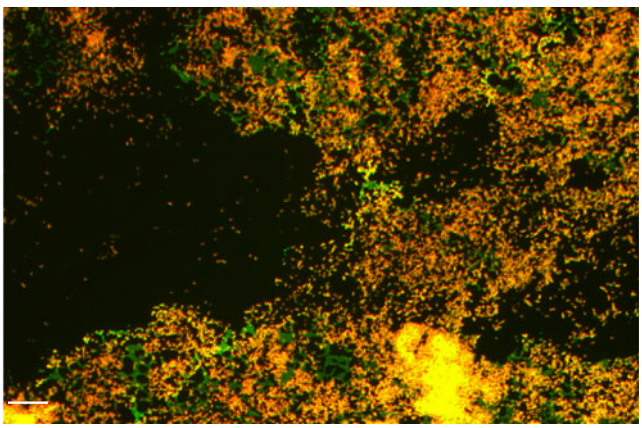

Fig. 2 Epifluorescent micrographs of the 16S rRNA FISH study using the Pseudo120 and the EUB338 probe. Mixed culture of *E. coli* and *P. fluorescens*. *E. coli* grown in TSB for 24 h at 35°C and *P. fluorescens* grown in TSB at 30°C for 24 h. *E. coli* labeled with universal EUB338 probe tagged with FAM (*green*) and *P. fluorescens* labeled with both Pseudo120 probe tagged with Cy3 and EUB 338 tagged with FAM (*orange-yellow*). *Bar*, 5 μm

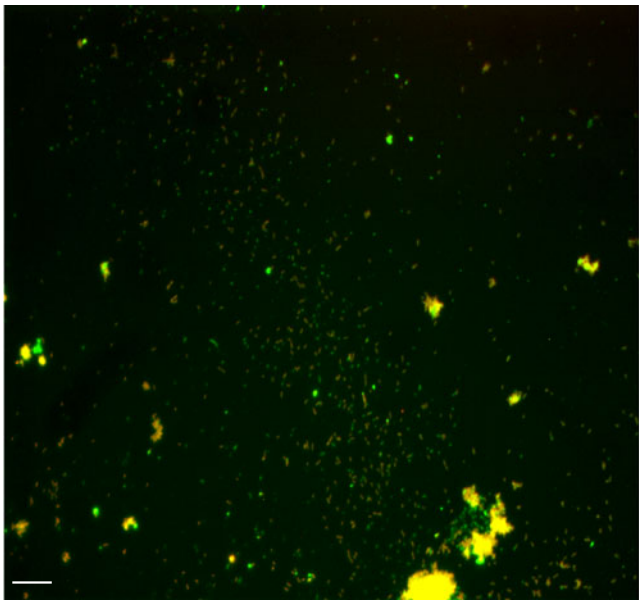

Fig. 3 Epifluorescent micrograph of the 16S rRNA FISH study for the analysis of contaminated MWF sample using Pseudo120 and the EUB338. Contaminated MWF sample hybridized simultaneously with Pseudo120 probe tagged with Cy3 dye and EUB338 probe tagged with FAM dye (*green*). Bacteria other than *Pseudomonas* appearing green as hybridized with EUB338. *Pseudomonas* appearing orange-yellow as hybridized with both Pseudo120 tagged with Cy3 (*red*) and EUB338 tagged with FAM (*green*). *Bar*, 5 μm

Biofilm formation under static condition

Both *P. fluorescens* and *P. oleovorans* subsp. *lubricantis* were capable of forming biofilms after 72 h of incubation under static condition when inoculated with 10^6 CFU/ml. This study for the first time reports the biofilm formation by *P. oleovorans* subsp. *lubricantis*. Single cell attachment was observed on the surface of the glass slide within 48 h of incubation for both species (Figs. 4a and 5a). Formation of microcolonies was observed within 72 h of incubation (Figs. 4b and 5b). Increase in colonization and thickness of the biofilm was observed with brightfield microscopy within 96 h of incubation (Figs. 4c and 5c). Similarly, biofilm formation was observed for both the bacteria when inoculated with low (10^4 CFU/ml) concentration of cells. However, in contrast to the higher cell density, the formation of microcolonies with 10^4 CFU/ml was observed after 96 h of incubation, and the increase in colonization and the thickness was observed within 120 h of incubation (data not presented). The probe Pseudo120 was also successful in staining the cells present in the biofilm. It led to the observation of the spatial architecture of the biofilms and differences in microcolony formation between *P. fluorescens* and *P. oleovorans* subsp. *lubricantis* (Fig. 6).

The thickness of the biofilm formed by *P. fluorescens* was approximately 40 μm when stained with methylene blue and 25 μm when stained with Pseudo120 (Fig. 6a). The difference in thickness could be due to the fact that there was water loss during the hybridization process. In contrast, the thickness of the biofilm formed by *P. oleovorans* subsp. *lubricantis* was 35–70 μm when stained with Pseudo120 probe (Fig. 6b) and 80–120 μm when stained with methylene blue. The observed patterns of colonization were different between *P. oleovorans* subsp.

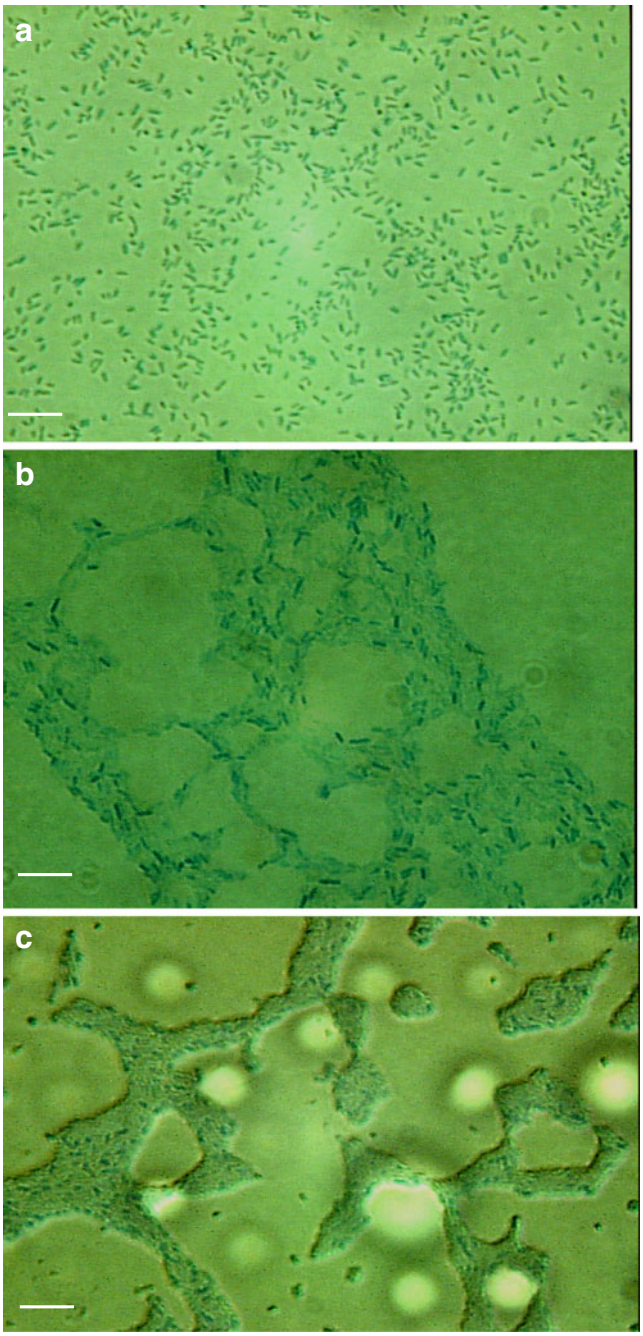

Fig. 4 Biofilm formation by *P. fluorescens* on glass surface using 10^6 CFU/ml after **a** 48 h, **b** 72 h, and **c** 96 h of incubation. Biofilms were stained with methylene blue and images were taken by bright field microscopy. *Bar*, 5 μm

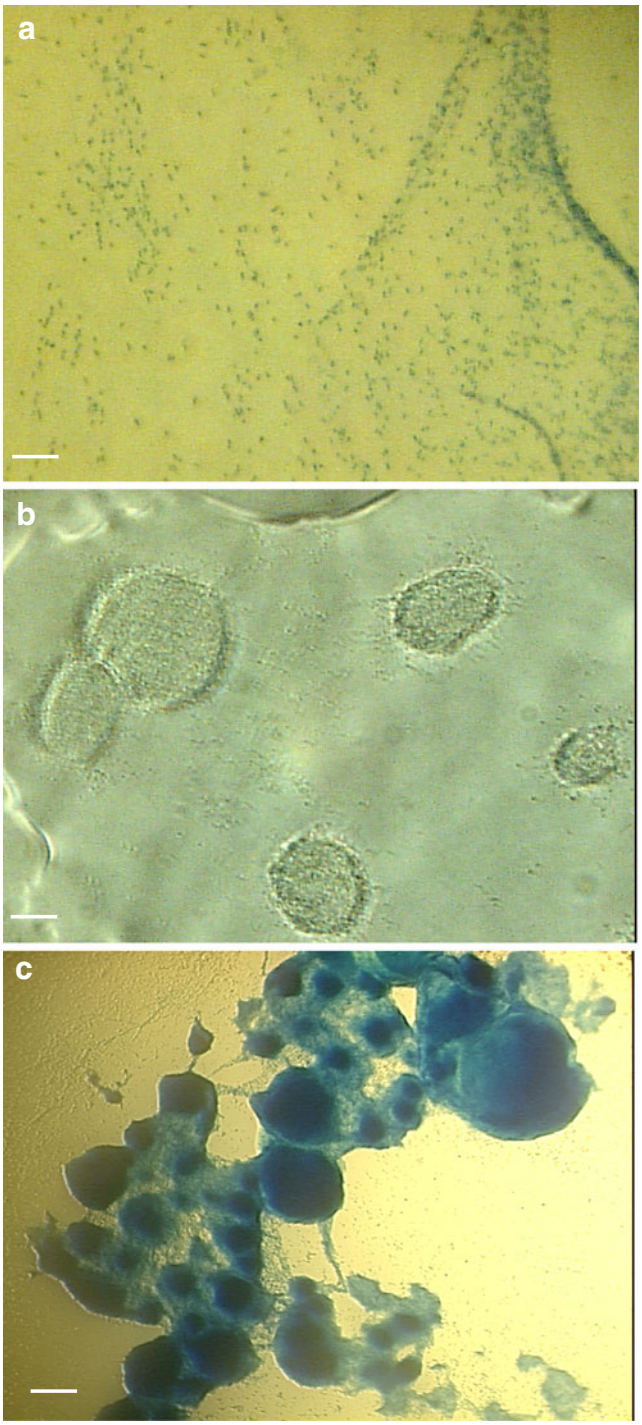

Fig. 5 Brightfield micrographs of biofilm study of *P. oleovorans* subsp. *lubricantis* on glass surface after **a** 48 h, **b** 72 h, and **c** 96 h of incubation. Biofilms were stained with methylene blue. *Bar*, 5 μm

lubricantis and *P. fluorescens*. After 96 h of incubation, the cells of *P. oleovorans* subsp. *lubricantis* colonized as tight circular clusters on the surface of the glass slide and the clusters were connected with layers of cells, whereas *P. fluorescens* did not exhibit any circular clustering pattern of the cells.

Discussion

In this study, the Pseudo120 probe was designed to target the ribosomal RNA molecules, which are ubiquitously present in high numbers in viable cells (Amann et al. 1995) to estimate the abundance of the rRNA group I pseudomonads and study the biofilms. The specificity of the probe depends on several factors such as sequence of the probe, size of the probe, hybridization conditions, and labeling techniques (Bouvier and del Giorgio 2003). The specificity of Pseudo 120 was not affected by the size or sequence of the probe; however, labeling of the probes (EUB338 and Pseudo120) with different fluorescent dyes (FAM, CY3) affected the stringency condition of the hybridization reaction.

Based on the FISH analysis using the Pseudo120 probe, the average distribution of the rRNA group I pseudomonads in different samples was about 4.2 (± 1.0)$\times 10^6$ cells/ml, which was consistent with the findings of Selvaraju et al. (2005). However, the viable count levels of *Pseudomonas* recovered on a commonly used recovery medium,

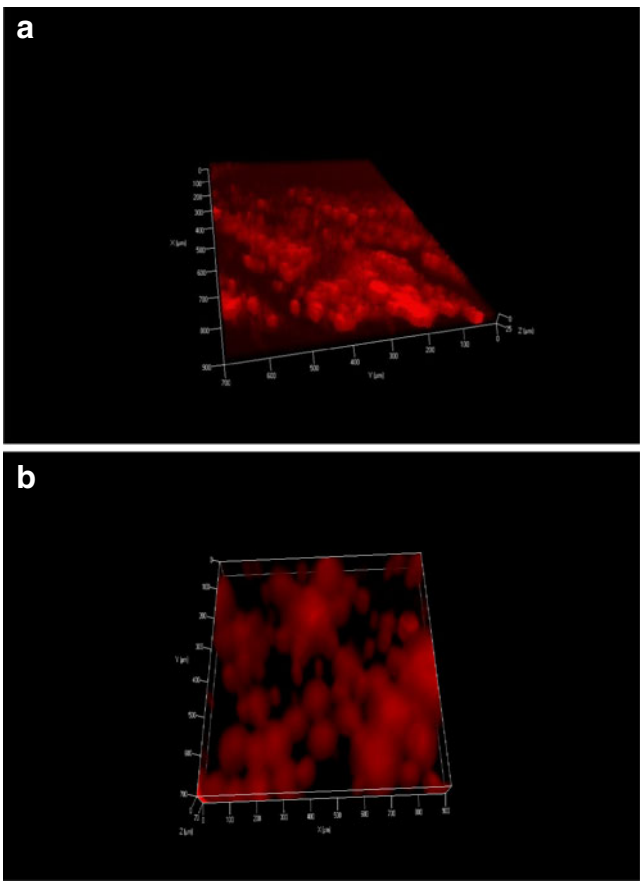

Fig. 6 Epifluorescent micrographs of biofilm study on glass surface after 96 h of incubation for **a** *P. fluorescens* and **b** *P. oleovorans* subsp. *lubricantis*. Three-dimensional view showing approximate thickness; biofilm stained with Pseudo120 probe conjugated with Cy3 dye

Pseudomonas Isolation Agar, with these same samples resulted in recoveries of only in the range of $<1\times10^1$ to 3.3×10^4 CFU/ml (Saha et al. 2010b), indicating the disadvantage of using traditional culturing techniques. The overall percentage distribution of planktonic pseudomonads was found to be in the range of 72.4–85.9%, indicating that the pseudomonads are the dominant group of microorganisms found in used MWFs. Apart from FISH, other methods such as gas chromatographic analysis of 3-hydroxylauric acid as a chemical determinant of *Pseudomonas* species (Mattsby-Blatzer et al. 1989), fatty acid methyl ester analysis (van der Gast et al. 2003) and DGGE (Rabenstein et al. 2009) reported similar results. Using 16S gene survey, Perkins and Angenent (2010) reported species belonging to the genus *Alcaligenes* to be predominant (>40%), but the study also detected *Pseudomonas* species (2%) in MWFs. In contrast to FISH, these techniques involved indirect quantification of bacterial cells and required more specialized instrumentation and skills.

Using a low concentration of inoculums delayed biofilm formation by both the bacterial strains, indicating that lowering the concentration of cells via disinfection could delay the biofilm formation by at least 24 h in the sump of the MWF systems. Architectural differences were observed in the biofilms of *P. fluorescens* and *P. oleovorans* subsp. *lubricantis*. Similar species-specific patterns in the spatial architecture of biofilms formed by pure cultures of *P. aeruginosa, P. fluorescens*, and *Vibrio parahaemolyticus* have earlier been reported by Lawrence et al. (1991). The difference in spatial architecture of biofilms might lead to physiological heterogeneity that contributes to reduced susceptibilities to different antimicrobial agents (Xu et al. 1998; Folkesson et al. 2008). Thus, based on the differences in the colonization pattern and thickness, *P. fluorescens* and *P. oleovorans* subsp. *lubricantis* might respond differentially to disinfection methods. The microscopic analysis showed that the newly described subspecies, *P. oleovorans* subsp. *lubricantis* is a better biofilm former than *P. fluorescens* under similar conditions.

In conclusion, the unique probe (Pseudo120) was found to be highly specific for the *Pseudomonas* belonging to the rRNA group I. The probe was also successful in studying the distribution and quantification of the pseudomonads in used MWFs. This work demonstrated that the Pseudo120 probe can be used for the rapid detection of these bacteria from complex environments such as MWFs. Also, based on the levels of *Pseudomonas* detected in the contaminated samples using the Pseudo120 probe, these appear to be the dominant bacteria found in MWFs. Both *P. fluorescens* and *P. oleovorans* subsp. *lubricantis* were capable of forming biofilms under high and low concentration of cells under static condition. Since biofilm formation was observed to be delayed by 24 h under low concentration of cells, implementation of suitable disinfection methods along with proper fluid monitoring system (routine analysis of MWFs using FISH) could prolong the life of in-use MWFs. Due to the difference in the spatial architecture and thickness of biofilms formed by the two bacterial strains, further research is recommended to investigate the efficacy of different disinfectants on the biofilms formed in the sump of the MWF systems.

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