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METHODS

High-Throughput Screening of Multispecies Biofilm Formation and Quantitative PCR-Based Assessment of Individual Species Proportions, Useful for Exploring Interspecific Bacterial Interactions

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Abstract Multispecies biofilms are predominant in almost all natural environments, where myriads of resident microorganisms interact with each other in both synergistic and antagonistic manners. The interspecies interactions among different bacteria are, despite the ubiquity of these communities, still poorly understood. Here, we report a rapid, reproducible and sensitive approach for quantitative screening of biofilm formation by bacteria when cultivated as mono- and multispecies biofilms, based on the Nunc-TSP lid system and crystal violet staining. The relative proportion of the individual species in a four-species biofilm was assessed using quantitative PCR based on SYBR Green I fluorescence with specific primers. The results indicated strong synergistic interactions in a four-species biofilm model community with a more than 3-fold increase in biofilm formation and demonstrated the strong dominance of two strains, *Xanthomonas retroflexus* and *Paenibacillus amylolyticus*. The developed approach can be used as a standard procedure for evaluating interspecies interactions in defined microbial communities. This will be of significant value in the quantitative study of the microbial composition of multispecies biofilms both in natural environments and infectious diseases to increase our understanding of the mechanisms that underlie cooperation, competition and fitness of individual species in mixed-species biofilms.

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Introduction

Biofilms are defined as polymeric matrix-enclosed bacterial communities associated with surfaces or interfaces [1]. They are considered the dominant lifestyle of bacteria both in environmental ecosystems and human hosts, and typically comprise a large number of different bacteria living together [2]. Soil is an example of an environment that contains a large number of very diverse bacteria and numerous available surfaces for multispecies biofilm formation [3].

Coresidence of diverse bacteria in multispecies biofilms is likely to catalyse complex interactions, resulting in increased or decreased biofilm biomass [4–7], which may in turn affect the overall function of the biofilm community. As example, *Pseudomonas aeruginosa* PAO1 and *Burkholderia sp*. NK8 showed enhanced biofilm formation in a dual-species biofilm, directly benefitting bioremediation potential, as chlorobenzoates were more efficiently degraded [8] and similar biofilm synergies were observed in drinking water systems [7]. Despite an increase in biofilm-related studies over the past decades, most of these have focused on monospecies biofilms or specific ecological niches such as mixed-species oral biofilms. Our current knowledge regarding the prevalence, physiology and complexity of multispecies biofilm is still incomplete, partly due to the lack of reproducible screening methods.

Two measurements are of particular importance when exploring interactions in biofilms; namely, the biomass (or

productivity) of the total biofilm and of the individual strains. Measurements of the overall biomass can, when compared with the amount that each of the residing species are able to produce as monospecies biofilms, be used to distinguish the effect of interactions on the extent of biofilm that is formed, as to whether this is synergistic, neutral or antagonistic. Being able to differentiate the success of each different bacterial member of the biofilm can furthermore resolve interactions as being mutual, commensal or parasitic, which is fundamental for exploring and understanding the selective forces operating in and shaping multispecies communities.

Several methods and protocols have been developed for studying biofilms. Flow cells combined with confocal scanning laser microscopy [9] is the most favoured tool and has the advantage of enabling one to obtain quantitative information on both the overall biomass and of the individual strains if combined with correct labelling techniques (e.g. fluorescence in situ hybridization, FISH) but is unfortunately not suited for high-throughput comparative screening studies. Furthermore, identification of specific strains in multispecies biofilms by this approach is highly labour-intensive and requires expert handling in order to avoid pitfalls such as uneven staining due to the limited probe penetration into biofilms, artefacts caused by hybridization and dehydration procedures in FISH [10, 11]. Microtiter plates are suitable for performing biofilm quantification, usually based on crystal violet retention, owing to their high-throughput screening capability and the simplicity of protocols [12]. However, the reproducibility of this assay is problematic, especially when multispecies consortia are analysed. In addition, molecular analysis of biofilm cells requires efficient and complete disruption of the cells followed by extraction of the target molecules, which still needs to be improved in multispecies biofilms.

In this study, we report an easily applicable and reproducible approach for consistently quantifying multispecies biofilm formation and to evaluate interactions, in regard to the overall biofilm formation and relative proportions of individual species. We present an optimized DNA extraction protocol and SYBR Green-based quantitative PCR (qPCR) assay for the selective, rapid and sensitive detection of four species in multispecies biofilm. The reproducibility and broad applicability of this specific detection procedure makes this method useful for most types of defined biofilms, not limited to the soil isolates used in this study.

Materials and Methods

Soil Isolates and Culture Conditions

The bacterial strains used in this study (Table 1) were obtained from agricultural soil as described previously [13]. From the total strain pool isolated by de la Cruz-Perera et al. [13], we selected seven strains based on growth compatibility (see below). Two of the selected strains (6 and 7) were not described by de la Cruz-Perera et al., but these were isolated and identified by procedures identical to those referred to above.

Optimization of Growth Media

To determine the optimal growth conditions and evaluate the biofilm-forming capabilities of the seven selected soil isolates, each strain was grown individually in Minimal Medium (basal medium 500 mL: $NaH_2PO_4 \cdot H2O$ 0.5 g, $K_2HPO_4 \cdot 3H_2O$ 2.125 g, NH_4Cl 1.0 g, pH 7.2; trace metals 500 mL: nitrilotriacetic acid 0.0615 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.006 g, $ZnSO_4 \cdot 7H_2O$ 0.0015 g, $MnSO_4 \cdot H_2O$ 0.0015 g, pH 7.0) supplemented with 0.2 % D(+)-Glucose, nutrient-low R2B medium (yeast extract 0.5 g, proteose peptone 0.5 g, casamino acids 0.5 g, glucose 0.5 g, soluble starch 0.5 g, sodium pyruvate 0.3 g, K_2HPO_4 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, in 1 L distilled water) and nutrient-rich TSB medium (tryptic soy broth, Merck KgaA, Germany). When solid medium was needed, 1.5 % (wt/vol) agar was added.

The strains were subcultured from frozen glycerol stocks onto tryptic soy agar (TSA) plates for 48 h at 24 °C, and then colonies were transferred onto Minimal Medium agar plates containing 0.2 % glucose, R2B agar plates or TSA plates. Colonies from solid type media were inoculated into 5 mL liquid media of the same type and incubated with shaking (250 rpm) at 24 °C overnight.

Cultivation of Biofilms

Both 96-well cell culture plates (cat. no. 655 180, Greiner Bio-One, Germany) and Nunc-TSP lid system (cat. no. 445497, Thermo Scientific, Denmark), which comprises a 96-well plate lid with pegs that extend into each well, were used to cultivate biofilms. The seven selected strains were screened

Table 1 Identification of the seven soil isolates by 16S rRNA analysis

Strain no.[a]	GenBank accession no.	Closest relative[b]
1	JQ890536	*Pseudomonas lutea*
2	JQ890538	*Stenotrophomonas rhizophila*
3	JQ890537	*Xanthomonas retroflexus*
4	JQ890542	*Ochrobactrum rhizosphaerae*
5	JQ890539	*Microbacterium oxydans*
6	JQ890541	*Arthrobacter nitroguajacolicus*
7	JQ890540	*Paenibacillus amylolyticus*

[a] The numbers 1 to 7 were designated to the seven strains for simplification

[b] The sequences had 98 to 100 % base identity to the closest relative in GenBank

for biofilm formation as single species and in four-species combinations as described below.

The colonies (grown on agar plates for 24 h) were inoculated into 5 mL of TSB medium and incubated overnight at 24 °C with shaking (250 rpm). One hundred to 600 μL of these stationary phase bacterial cultures were transferred to fresh TSB medium and grown until an optical density at 600 nm (OD600) of ~1.0 was reached. The cell suspensions were then adjusted to an OD600 of 0.15 by dilution in TSB medium. A total of 160 μL of monospecies or four mixed species (40 μL of each species) exponentially growing cultures were added to each well. To some wells, the same volume of fresh TSB medium was added to obtain a background value, which was subtracted from values obtained from the wells containing cells. The plates were sealed with Parafilm and incubated with shaking (200 rpm) at 24 °C for 24 h. The biofilm assays were performed three times on different days (biological replicates) with four technical replicates every time.

Quantification of Biofilm Formation by Use of CV and TTC

A modified version of the crystal violet (CV) method for detection of biofilms using 96-well cell culture plate [12] was applied as previously described. Additionally, the assay was further modified for quantifying biofilms formed on pegs of the Nunc-TSP lid system, previously referred to as the Calgary method [14]. After 24 h incubation, in order to wash off planktonic cells, the peg lid was transferred successively to three microtiter plates containing 200 μL phosphate buffered saline per well, followed by staining of the biofilms formed on the pegs with 180 μL of an aqueous 1 % (*w*/*v*) CV solution. After 20 min of staining, the lid was rinsed again three times and then placed into a new microtiter plate with 200 μL of 96 % ethanol in each well. After allowing 30 min for the stain to dissolve into the ethanol, the absorbance of CV at 590 nm was determined by using an EL 340 BioKinetics reader (BioTek Instruments, Winooski, Vt.). The CV-ethanol suspension was diluted with 96 % ethanol when the OD590 was above 1.1.

Parallel with the CV-based biofilm assays described above, an alternative method, based on 2, 3, 5-triphenyltetreazolium chloride (TTC) reduction, was also implemented and evaluated. After 24-h incubation, the pegs with attached cells were rinsed three times as described above. Thereafter, the peg lid was mounted on a new microtiter plate with 200 μL fresh medium containing 0.01 % TTC. The plate was then sealed with Parafilm, wrapped in foil and incubated with shaking (200 rpm) for another 24 h. In order to resuspend the formed formazan, the peg lid was transferred to a new microtiter plate containing 200 μL of 96 % ethanol per well. Finally, the absorbance was measured at 490 nm.

The statistical analyses were conducted using ANOVA test (SPSS version 17.0 for Windows). A p value of ≤0.05 was regarded as a statistically significant difference.

DNA Extraction from Four-Species Biofilms

The 24-h biofilms formed on peg lids were rinsed three times with PBS to remove non-adherent bacteria as described above. Then pegs were broken from the lid from the back (i.e. without direct contact to the biofilm-covered part of the peg) using sterile forceps and transferred into Lysing Matrix E tubes (provided by the FastDNA™ SPIN Kit for soil). Each peg was placed in one tube. Aliquots of 882 μl of sodium phosphate buffer was added to each tube and biofilms were disrupted from pegs by bead beating using the Savant FastPrep FP120 for 40s at setting 6.0. The pegs were removed and stained with crystal violet to verify that after bead beating, all the cells were detached from the pegs. Ninety-eight microlitres of lysozyme solution (20 mg/ml), dissolved in sodium phosphate buffer was added, and the samples were incubated for 1 h at 37 °C. Next, 122 μL of MT buffer was added to each sample, followed by bead beating twice for 40s at setting 6.0 with cooling on ice during the short time interval. The complete cell lysis was visually confirmed with an optical microscope. Genomic DNA was then extracted using FastDNA™ SPIN Kit for soil (Qbiogene, Illkirch, France) according to the manufacturer's instructions and quantified with a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

DNA Sequence Analysis and Design of the Species-Specific Primers

Multiple alignment of sequences from the four strains 2, 3, 5 and 7 was done using the DNAMAN software (version 7, Lynnon corporation). The species-specific primer pairs for SYBR Green qPCR were designed manually based on the variable regions of 16S rRNA genes (Table 2) according to the guidelines set by Primer Express (version 3.0) from Applied Biosystems with an approximate maximum amplicon size of 300 bp. The obtained best fitting primers were checked by DNAMAN to avoid hairpins or primer–dimer formations. The specificity of each pair of primers was checked against the other three, non-target strains using conventional PCRs as follows. Reaction mixtures of 25 μL contained 16 μL H_2O, 1 μL genomic DNA, 5 μL 5× Phusion HF Buffer, 0.5 μL 10 mM dNTPs, 1 μL 10 μM of each primer, and 0.5 μL Phusion DNA polymerase (Phusion high-fidelity PCR kit; Finnzymes, Espoo, Finland). Amplifications were performed with the following cycling protocol: 5 min at 95 °C, followed by 30 cycles of 30s at 94 °C, 30s at 61 °C/62 °C, 20s at 72 °C and a final elongation step of 5 min at 72 °C in DNA Engine Dyad Peltier Thermal Cycler (MJ Research Inc.). The

Table 2 Species-specific primers based on 16S rRNA gene sequences

Strain no.	Identity	Primer position	Sequence (5′–3′)	Length (bp)	Product size (bp)
2	*S. rhizophila*	FP 159	GCCTTGCGCGGATAGATG	18	240
		RP 399	CGGGTATTAGCCGACTGCTT	20	
3	*X. retroflexus*	FP 135	GCCTTGCGCGATTGAATG	18	252
		RP 387	CCGTCATCCCAACCAGGTATT	21	
5	*M. oxydans*	FP 935	TCAACTCTTTGGACACTCGTAAACA	25	213
		RP 1148	CATGCGTGAAGCCCAAGAC	19	
7	*P. amylolyticus*	FP 800	GATACCCTTGGTGCCGAAGTT	21	145
		RP 945	CGGTCAGAGGGATGTCAAGAC	21	

The numbers in the primer position show the positions of the target sites of each species

FP Forward primer, *RP* Reverse primer

amplified products were separated by 1.0 % agarose gel electrophoresis, stained with ethidium bromide and photographed under UV illumination.

Plasmid Standards Used for Absolute Quantification

A plasmid standard containing the target region was prepared for each primer pair as follows. The 16S rRNA gene fragments were amplified by conventional PCR using the corresponding primers as mentioned above. The products were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Plasmids were isolated with QIAprep Spin Miniprep Kit (Qiagen Gmbh, Hilden, Germany). The qualities were evaluated by agarose gel electrophoresis and concentrations were measured by Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA). 16S rRNA gene copy numbers were calculated assuming that the average molecular mass of a double-stranded DNA molecule is 660 g/mol. Four standard curves were generated using triplicate 5-fold dilutions of plasmid DNAs, and the corresponding slope was used to calculate PCR amplification efficiency (E) according to the equation of $E = 10^{(-1/\text{slope})}$. 16S rRNA gene copy numbers in unknown samples were then determined by interpolation from the standard curve using their respective threshold cycle (Ct) values. The Ct value represents the number of PCR amplification cycles needed to produce fluorescence intensity above a pre-defined threshold.

Quantitative PCR Based on SYBR Green I Fluorescence

For each sample, four separate qPCR reactions were performed with the Mx3000P™ Real-time PCR System (Stratagene, USA) using each pair of species-specific primers. Each of the reaction components per 20 μL were 7 μL H_2O, 10 μL 2× Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies), 1 μL 10 μM of each primer and 1 μL genomic DNA (either standard or sample). The PCR programmes were carried out as follows: 95 °C for 10 min, 40 cycles of 95 °C for 30s, 59–62 °C for 30s and 72 °C for 20s/30s, followed by a standard melting/dissociation curve segment. Each sample was run in triplicates and a negative control was included in every run.

Results

Isolates and Media Optimization

The seven selected strains, representing common soil bacteria, were all able to grow on Minimal Medium supplemented with 0.2 % D(+)-Glucose (data not shown). These strains were selected for further media optimization and multispecies biofilm formation. The strain identities and the accession numbers of the 16S rRNA gene sequences, used in this study for designing specific primers (strain 2, 3, 5 and 7), are shown in Table 1.

All seven strains showed slower growth on Minimal Medium+Glucose (0.2 %) and R2A plates than on TSA plates and took much more time to reach exponential growth phase in liquid media at 24 °C (data not shown). Since all seven strains grew well on TSA/TSB, this medium was chosen for further cultivation of mono- and four-species biofilms.

Assay for Biofilm Formation

Monospecies biofilm formation was assayed both with and without the peg lid system using both the CV retention and the TTC reduction assays (Fig. 1a–c). To evaluate the reproducibility within technical replicates and between individual experimental series (biological replicates), all biofilm assays were performed in four replicates and conducted three individual times on different days under the same conditions.

Compared with the 96-well cell culture plate, the Nunc-TSP lid system gave less variation between replicates and between individual days, which were directly reflected in the biofilm formation of strains 1, 2 and 4. These three strains, which were identified as poor biofilm formers in the Nunc-TSP lid system (Fig. 1b), showed significantly different

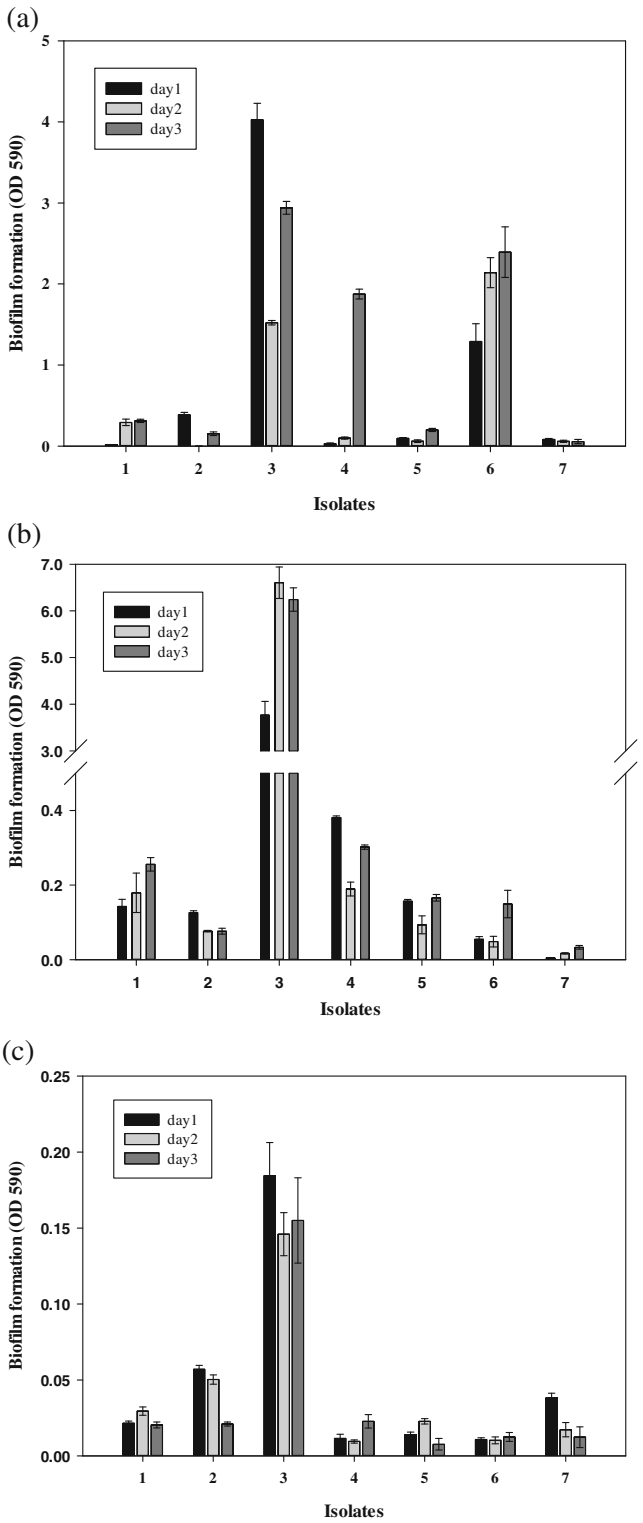

Fig. 1 Biofilm formation in 96-well cell culture plate (**a**) and Nunc-TSP ▶ lid system (**b**, **c**) by the seven isolates. After 24 h of incubation, the biofilm formation was quantified by staining with crystal violet followed by absorbance measurements at 590 nm (**a**, **b**) or the presence of reduced TTC was quantified by measuring the absorbance at 490 (**c**). *Three parallel bars* represent means ± standard error for four replicates on three different days representing three biological replicates (day 1, day 2 and day 3). *1*, *Pseudomonas lutea*; *2*, *Stenotrophomonas rhizophila*; *3*, *X. retroflexus*; *4*, *Ochrobactrum rhizosphaerae*; *5*, *Microbacterium oxydans*; *6*, *Arthrobacter nitroguajacolicus*; *7*, *P. amylolyticus*

abilities for biofilm formation from day to day ($P < 0.05$) when using the 96-well cell culture plate (Fig. 1a). Another difference between the assays conduced in the two types of microtiter plates was the amount of biofilm formed by strain 6, which displayed biofilm formation in the 96-well cell culture plate, but was incapable of attaching to the pegs. The Nunc-TSP lid system could not only provide more reproducible results, but it also removed the possibility that aggregation may be linked to sedimentation of the microorganisms in test wells [15], which might have been the case for strain 6 (subpanels a vs. b of Fig. 1). Based on these characteristics, the Nunc-TSP lid system was chosen as a better device for high-throughput screening for synergistic interactions in multispecies biofilm formation.

In addition to the CV method, which quantifies total attached material, we also used the TTC method, which evaluates cell activity. Respiring cells reduce the colourless TTC solution to the red insoluble formazan, which can be dissolved in 96 % ethanol and measured spectrophotometrically. The data shown in Fig. 1c represents the TTC absorbance at 490 nm. Low day-to-day variation was observed, but the low output signals resulted in less distinct difference between biofilm formers and non-formers. In addition, some absorbance measurements were close to the resolution limit of the Bioreader. Therefore, the TTC reduction assay was not applied for the following mixed-species biofilm formation.

The results obtained by the Nunc-TSP lid system showed that only strain 3 was able to establish biofilm on its own; CV values obtained for this strain were more than 10-fold higher than for any of the other strains (Fig. 1b). Based on these results, strain 3 was identified as being a good biofilm former, whereas the remaining six strains were characterized as poor biofilm formers.

Biofilm Formation by Single-Species and Four-Species Consortia

The seven selected strains were screened for biofilm formation, by using the Nunc-TSP lid system, as single species and in all possible combinations of four, in order to identify a four-species model consortium, suitable for studies of interspecies interactions. As shown in Fig. 2, the combination of strains 2, 3, 5 and 7 gave no significant variation between replicates and between individual days, indicating the high reproducibility of the assay. Obviously, with strain 3 as exception, the strains showed weak ability to form biofilm. However, when the four isolates coexisted in the biofilm, the biofilm biomass increased by >300 % compared to the single-species biofilms,

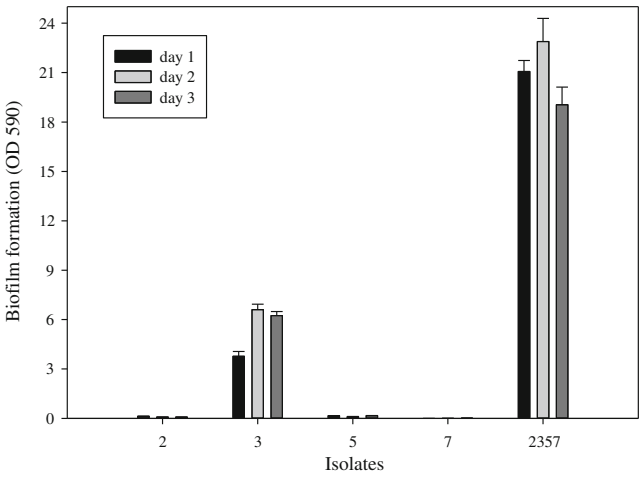

Fig. 2 Biofilms formed by single-species and four-species communities (*2*, *Stenotrophomonas rhizophila*; *3*, *X. retroflexus*; *5*, *M. oxydans*; and *7*, *P. amylolyticus*) in Nunc-TSP lid system. After 24 h of incubation, the biofilm formation was quantified by staining with crystal violet. *Three parallel bars* represent the average absorbance at OD 590 nm for four replicates on three different days representing three biological replicates (day 1, day 2 and day 3). The *bars* represent means ± standard error for four replicates

indicating a strong synergy in this multispecies biofilm. This four-species consortium was therefore selected as a multispecies biofilm model for further studies of the species dynamics.

Quantification of Four Strains by Quantitative PCR in Multispecies Biofilm

DNA was successfully extracted from the biofilms by excision of entire pegs followed by cell disruption. The biofilms were completely removed from the pegs (confirmed by CV staining, data not shown), and complete cell lysis was confirmed by microscopy. The amount of DNA extracted from multispecies biofilms composed of strains 2, 3, 5 and 7 was 1.9–2.1 μg/peg.

Based on the variable regions of 16S rRNA genes, four pairs of specific primers were designed for SYBR Green qPCR (Table 2). For simplification and to enable quantification based on SYBR Green, the primers were designed for application in four separate reactions for each sample. The specificity of the primers was confirmed by conventional PCRs, verifying that primers were strictly species specific. The high linearity of the Ct values plotted in the standard curves was verified by the correlation coefficient (RSq) values of >0.99. The amplification efficiencies (E) ranged from 80 to 90 % (Supplementary Fig. 1). No detectable peaks that were associated with primer–dimer or other non-specific PCR products were observed in the melting curves (data not shown), and only the single bands of the expected size amplicon in each qPCR assay were detectable by agarose gel electrophoresis (data not shown). The standard plasmid DNA used for the standard curves ranged from 3×10^6 to 3×10^7 copies/μL and were used in 5-fold dilution series. The DNA extracted from biofilms was diluted appropriately to ensure that all the unknown samples were within the range of the standard curves. 16S rRNA gene copy number of each species was calculated and is shown in Table 3. As the exact copy numbers of the 16S rRNA gene per cell in the four species are currently unknown, the cell numbers were estimated based on other species in the same genus [16–19].

Discussion

Multispecies biofilms are prevalent in almost all environments. A pressing need, therefore, exists to better the understanding of the social interactions and selective forces that drive bacterial communities in multispecies biofilm. During the past few decades, simultaneous staining of multiple species by FISH has been widely used in combination with confocal laser scanning microscopy (CLSM) for species differentiation in oral biofilms [20–22]. This approach is, however, not easily applicable to many different isolates from various environments and it is only partly quantitative. In this study, we have developed an approach to consistently quantify biofilm formation, which enables high-throughput screening of the prevalence of synergistic interactions and assessment of the proportions of individual bacterial species in biofilms. The general procedure of this approach is illustrated in Fig. 3 which can be used as a standard procedure for evaluating

Table 3 The copy numbers of 16S rRNA gene and the estimated cell numbers derived from four separate qPCRs

Strain no.	Identity	16S rRNA gene copies (per μL)	Estimated copy number of the 16S rRNA gene (per cell)	Estimated number of cells (per μL)
2	*S. rhizophila*	7.91E+006	4[a]	1.98E+006
3	*X. retroflexus*	1.98E+009	2[b]	9.90E+008
5	*M. oxydans*	8.89E+006	2[c]	4.45E+006
7	*P. amylolyticus*	9.95E+008	12[d]	8.29E+007

[a] The copy number was estimated to be 4, as *Stenotrophomonas maltophilia* is known to have four copies [17]

[b] The copy number of the 16S rRNA gene in *X. retroflexus* is estimated to be 2, as *Xanthomonas axonopodis*, *Xanthomonas campestris*, *Xanthomonas oryzae*, *Xanthomonas citri*, *Xanthomonas albilineans* are known to have two copies, respectively [18]

[c] The copy number of the 16S rRNA gene in *M. oxydans* is estimated to be 2, as *Microbacterium testaceum* is known to have two copies [19]

[d] The copy number of the 16S rRNA gene in *P. amylolyticus* is estimated to be 12, as other *Paenibacillus* species are known to have an average 12 copies [16]

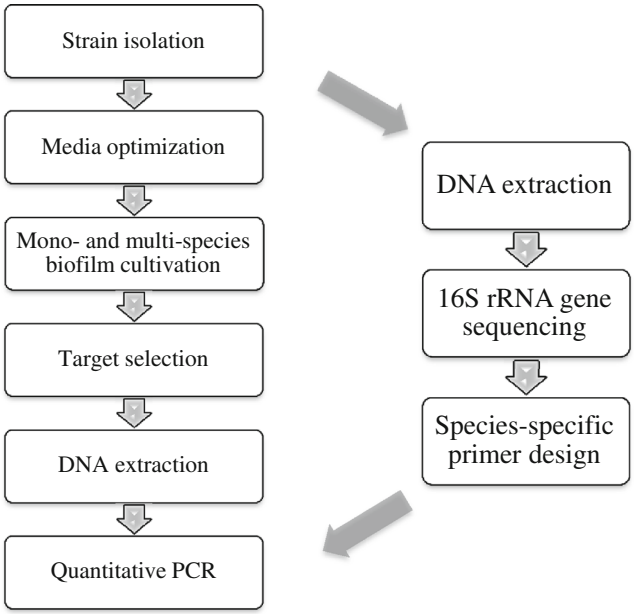

Fig. 3 General procedure for high-throughput screening and evaluation of interactions in multispecies biofilms. The *right-hand part* illustrates the parallel strain identification and primer design for use in qPCR

interspecies interaction in multispecies biofilms. The right-hand part of this figure illustrates the parallel strain identification and primer design. Clearly, this protocol is applicable for multispecies biofilms composed of a defined number of known species. Other approaches, including metagenomic analysis, are more suitable when interactions in more complex communities are explored. However, the standard method presented in this study could be much useful in some natural settings where species diversity is more restricted e.g. chronic infections or when limited key species are the research focus.

Multispecies Biofilm Assay

Mixed species may facilitate synergistic or antagonistic interactions between consortia members in biofilms. The co-culturing of four strains in this study provided an easy way to detect changes in biofilm formation from mono- to multi-species biofilms and allowed high-throughput screening of many isolates with high reproducibility. Biofilms have been proposed to exist in a fine balance between competition and cooperation [23], which can be tipped by various types of influences such as surrounding environmental factors and quorum sensing-dependent gene regulation. Inconsistent results in biofilm formation assays have previous been reported [24–27] due to different substrates, media, inoculum size and culture conditions, and the inconsistency is most likely to be worsened by the highly heterogeneous nature of multispecies biofilms. In this study, the three independent repetitive runs and four technical replicates per run were performed to assess the reproducibility of the quantitative analysis.

Poor reproducibility of the microtiter plate assay (reflected by higher standard errors (Fig. 1a)) can be caused by pipetting uncertainties, pieces of biofilm detaching, etc. In the Nunc-TSP lid system, the number of pipetting steps is dramatically reduced, which decreases the handling-induced variability. This was best reflected by the lower standard errors, indicative of relatively uniform biofilms throughout the four replicate wells (Fig.1b, c and Fig. 2).

TTC reduction, as a simple colorimetric method, has been widely used to evaluate the cell activity in plant tissue [28], fungal spores, yeast and bacteria cultures [29]. The low TTC values observed in this study were probably due to the relatively low metabolic activities of cells and the enhanced levels of non-biological material within mature biofilm compared to the CV assay where the extracellular matrix and all bacterial cells are stained. The combined use of CV and the respiratory indicator CTC (5-cyano-2,3-ditolyl tetrazolium chloride) in high-throughput biofilm assays was previously reported by Pitts et al., who also found cells that had lost metabolic activity could still contribute to the total amount of biomass [30]. Therefore, in this study, TTC was less suitable for quantitative biofilm measurements of microbial biomass.

DNA Extraction and qPCR Analysis of Species Distribution

Comparative studies of gene and protein expression of biofilm-associated cells have proven that these are significantly different from planktonically grown cells [31, 32]. The protocol optimized in the present study successfully detached and lysed both the Gram-negative and Gram-positive cells and is likely to become useful in many applications of DNA-based biofilm research. With the appropriate modification, the protocol is additionally suitable for RNA and protein extractions.

qPCR has been successfully applied for quantifying bacterial abundance in plaque biofilm [33], faucet biofilm [34] and biofilms in wounds [35] due to its speed, sensitivity and specificity. The targets of qPCR can be species-specific genes or 16S ribosome RNA genes [36, 37]. 16S rRNA gene proved to be an excellent target for both quantitative broad-range PCR [38] and group-/species-specific PCR [39, 40] in complex communities. The qPCR assay offers several advantages over FISH: low risk of contamination by amplified products, the simplicity and rapidity of data analysis and low detection limit. Using the real-time TaqMan assay, Price et al. [41] were able to examine specific bacterial populations in biofilms grown from human saliva and their susceptibility to chlorhexidine. Ren et al. reported a consistent function-related bacterial distribution in anode biofilms using both FISH and qPCR analyses [42].

In the present study, we describe a specific qPCR assay to examine the population dynamics in multispecies biofilms.

The significant synergistic interaction observed in a biofilm consisting of four soil bacteria make this consortium a powerful model to study development and interactions in multispecies biofilms. For this work, 16S rRNA gene was targeted for SYBR Green assay profiling of the four strains run in separate reactions. The hypervariable regions interspersed with the conserved regions make 16S rRNA gene an attractive target for both universal and specific primers. In addition, the public databases of 16S rRNA gene sequences are easily accessible, including GenBank, Greengenes and Ribosomal Database Project, which are valuable for bacterial identification and investigation in microbial ecology and evolution. TaqMan and SYBR Green qPCR are two frequently used assays. Despite of the significantly high specificity of the detection with TaqMan probe, SYBR Green qPCR is widely used due to the low cost and the ease in designing primers and optimizing assays. Maeda et al. [43] have reported that there are no significant differences between the TaqMan and SYBR Green chemistry in their specificity and sensitivity. However, the effect of SYBR Green qPCR is also limited in terms of the number of species it is practical to analyse. In such cases, the use of TaqMan would be preferred in a complex community to enable more species to be analysed, as it opens up for multiplex qPCR, which only requires one specific probe per target (instead of two specific primers) and reduces the amount of samples being run.

Concluding Remarks

Overall, we present a sensitive and reliable high-throughput method to investigate the interspecific interactions between bacterial isolates with the static co-culture assay followed by a qPCR assay that uses species-specific primers to measure the 16S rRNA gene numbers of each species in multispecies biofilms. By quantification and comparisons of the biomass of each species when grown alone and in the multispecies biofilm, understanding of the interspecific interactions (cooperative, mutualistic, competitive) that operate in the multispecies biofilm, is obtainable. To our knowledge, this presented approach applied in screenings for overall synergism and antagonism within multispecies biofilms composed of soil isolates is firstly reported in this study. As the ubiquity of biofilm formation is receiving increasingly more attention among scientists, this developed method will be a valuable tool for studying the social interactions and selective forces that drive complex bacterial communities in natural environments as well as infectious diseases.

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