

## ORIGINAL ARTICLE

# High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation

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**Biofilms that form on roots, litter and soil particles typically contain multiple bacterial species. Currently, little is known about multispecies biofilm interactions and few studies have been based on environmental isolates. Here, the prevalence of synergistic effects in biofilm formation among seven different soil isolates, cocultured in combinations of four species, was investigated. We observed greater biofilm biomass production in 63% of the four-species culture combinations tested than in biofilm formed by single-species cultures, demonstrating a high prevalence of synergism in multispecies biofilm formation. One four-species consortium, composed of *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans* and *Paenibacillus amylolyticus*, exhibited strong synergy in biofilm formation and was selected for further study. Of the four strains, *X. retroflexus* was the only one capable of forming abundant biofilm in isolation, under the *in vitro* conditions investigated. In accordance, strain-specific quantitative PCR revealed that *X. retroflexus* was predominant within the four-species consortium (>97% of total biofilm cell number). Despite low relative abundance of all the remaining strains, all were indispensable for the strong synergistic effect to occur within the four-species biofilm. Moreover, absolute individual strain cell numbers were significantly enhanced when compared with those of single-species biofilms, indicating that all the individual strains benefit from inclusion in the multispecies community. Our results show a high prevalence of synergy in biofilm formation in multispecies consortia isolated from a natural bacterial habitat and suggest that interspecific cooperation occurs.**

*The ISME Journal* (2015) 9, 81–89; doi:10.1038/ismej.2014.96; published online 17 June 2014

## Introduction

Environmental biofilm systems are comprised of many diverse cohabiting bacteria (Hall-Stoodley *et al.*, 2004). For example, soil is typically high in bacterial abundance and diversity and includes readily available surfaces suggesting it is a good setting for multispecies biofilm formation (Burmølle *et al.*, 2012). Members of multispecies biofilms may influence each other antagonistically; for example, through resource competition or production of inhibitory compounds (Rao *et al.*, 2005), or synergistically; via mechanisms such as syntrophy (cometabolism), biofilm induction or enhanced resistance (Burmølle *et al.*, 2014). We have previously observed strong synergy among four epiphytic isolates from a marine environment. When comparing single-species biofilms with four-species biofilms, enhanced biofilm biomass was observed in the latter, in addition to higher resistance to antimicrobial agents and better protection from

bacterial invasion (Burmølle *et al.*, 2006). Likewise, Diaz *et al.* (2012) revealed a synergistic partnership between *Candida albicans* and streptococci in which *C. albicans* improved the ability of streptococci to form biofilms on abiotic surfaces and on an oral mucosa analog (Diaz *et al.*, 2012). In addition, several studies have shown that some species that are unable to form biofilms in isolation can promote mixed-species biofilm biomass (Filoche *et al.*, 2004; Sharma *et al.*, 2005; Yamada *et al.*, 2005; Klayman *et al.*, 2009). In a recent study, Lee *et al.* (2014) addressed the protective effect of the resistant species in a three-species biofilm. These resistant species protected the more sensitive ones from inhibitory compounds and the overall species ratio remained constant, implying that resistance mechanisms may serve as public goods in multispecies biofilms (Lee *et al.*, 2014). These examples are all indicative of synergistic effects in multispecies biofilm formation, but the prevalence and impact of these effects among natural bacterial isolates has, to the best of our knowledge, not yet been addressed.

Multispecies biofilms have an essential role in maintaining the ecological balance in soil (Burmølle *et al.*, 2012) and, compared with single-species biofilm or planktonic counterparts, seem to confer

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Received 20 December 2013; revised 8 May 2014; accepted 10 May 2014; published online 17 June 2014

benefits such as increased resistance to antibacterial compounds, enhanced protection from desiccation and protozoan predation, and elevated rates of horizontal gene transfer (Davey and Ootole, 2000; Jefferson, 2004; Sørensen *et al.*, 2005). Therefore, a pressing need exists for research directed toward understanding social interactions and selective forces that drive bacterial biofilm communities. Specifically, the gains/losses in biofilm biomass production and the protection level afforded to each strain in a mixed community must be evaluated, enabling identification of the underlying interactions as cooperative or competitive. In the present study, we define a synergistic effect as an increase in the amount of biofilm biomass produced in cocultures in comparison with that produced by the best biofilm former in monoculture. When such synergistic biofilm enhancement leads to an increase in cell numbers of all strains present in the biofilm, we interpret this as a cooperative trait.

Previous studies have primarily focused on describing synergistic biofilm interactions based on defined, pairwise interactions. Here we assess the prevalence of synergism in four-species biofilm formation in order to determine whether synergistic effects are frequent or rare among the strain collection tested. Different bacterial soil isolates were obtained from one specific soil environment simultaneously. These isolates are therefore presumed to have coexisted. One selected four-species biofilm community was investigated for the presence and progression dynamics of the individual strains at different stages of biofilm development by use of strain-specific quantitative PCR (qPCR). We demonstrate a high prevalence of synergistic effects in multispecies biofilm formation, and enhancement in cell numbers of all four species in the selected multispecies biofilm, indicating that cooperative forces shape these communities.

## Materials and methods

### *Bacterial strains used in this study*

Seven agricultural, bacterial isolates, previously identified and characterized, were used in this study: (1) *Pseudomonas lutea*, (2) *Stenotrophomonas rhizophila*, (3) *Xanthomonas retroflexus*, (4) *Ochrobactrum rhizosphaerae*, (5) *Microbacterium oxydans*, (6) *Arthrobacter nitroguajacolicus* and (7) *Paenibacillus amylolyticus* (de la Cruz Perera *et al.*, 2013; Ren *et al.*, 2013).

### *Biofilm quantification by use of crystal violet assay*

Biofilm formation was assayed and quantified as previously described (Ren *et al.*, 2013). Briefly, exponential phase cultures of the seven selected strains were adjusted to an optical density at 590 nm (OD<sub>590</sub>) of 0.15 in tryptic soy broth (Merck KgaA, Darmstadt, Germany) medium and then inoculated

into Nunc-TSP plate (Cat. No. 445497, Thermo Scientific, Hvidovre, Denmark). The inoculum volumes were 160 µl for monospecies biofilms and 40 µl for each species in four-species biofilms. After 24-h incubation at 24 °C with shaking (200 r.p.m.), biofilm formation was quantified by a modified crystal violet (CV) assay based on the Calgary device (Ceri *et al.*, 1999; Ren *et al.*, 2013).

The four-species consortium composed of strains 2, 3, 5 and 7 was examined for synergy in all possible combinations of two-, three- and four-species biofilm. The inoculum volumes of each strain were equivalent and added up to a total of 160 µl. Biofilm assays were performed as described above.

The biofilm experiments were repeated three times on three independent days with four replicates each time. The statistical analyses were conducted using analysis of variance test (SPSS version 17.0 for Windows; SPSS Inc., Chicago, IL, USA). *P*-values <0.05 were regarded as statistically significant.

### *Effects of spent biofilm culture supernatants on biofilm formation*

Single-species biofilms of strains 2, 3, 5 and 7 were prepared as described above. Supernatants were generated by filtering planktonic fractions through 0.2-µm pore-size filters. No viable bacteria were detected in the supernatants. Cultures of strains 2, 3, 5 and 7 were grown to exponential phase and then adjusted to an OD<sub>590</sub> of 0.3 in 2 × tryptic soy broth media. The four strains were then mixed in equal volumes in all possible combinations of three. Subsequently, 80 µl of each of the mixtures was spiked with 80 µl supernatant derived from the fourth species. Comparative controls were done by adding 80 µl of sterile water instead of supernatant to the three member cocultures. Next, the cocultures were inoculated in wells of microtiter plates in four replicates and incubated at 24 °C for 24 h. Biofilms formed on the pegs were quantified by using the CV assay as described above.

### *Strain-specific qPCR on biofilm and planktonic fractions*

Cell numbers of monocultures and cocultures of strain 2, 3, 5 and 7 were assessed by qPCR as follows: the four-species biofilms attached to the pegs and planktonic cells in wells were collected at six time points (4 h, 8 h, 12 h, 16 h, 20 h and 24 h after coinoculation). In addition, both single-species biofilms and associated planktonic fractions were sampled at 24 h. Three replicates were prepared at each time point. Cells were lysed by lysozyme digestion and bead beating, followed by DNA extraction using FastDNA SPIN Kit for soil (Qbiogene, Illkirch, France) and species-specific qPCRs as previously reported (Ren *et al.*, 2013).

The PCR programs were adjusted as follows: 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 64 °C for 20 s and 72 °C for 20 s, followed by a standard melting/dissociation curve segment. Each sample was assayed in duplicate wells and a no-template control was included in every run. As the exact copy numbers of the 16 S rRNA gene per cell in the four species are currently unknown, the cell numbers were calculated based on other species in the same genus as previously described (Ren *et al.*, 2013).

## Results

### *Biofilm formation by single-species and four-species consortia*

The prevalence of synergistic effects in biofilm formation among the seven soil isolates was examined by coculturing all possible combinations of four strains. A total of 35 different four-species consortia was screened for biofilm formation by using the Nunc-TSP lid system and quantified by the modified CV-staining method, previously demonstrated to be suitable and reproducible (Ren *et al.*, 2013).

For assessment of whether synergistic effects dominated in the individual four-species biofilm, the measured absorbance of the multispecies biofilm ( $Abs_{590}$  MS) was related to that of the best single-species biofilm former present in the relevant combination ( $Abs_{590}$  BS) as follows: ( $Abs_{590}$  MS – standard deviation, s.d.) > ( $Abs_{590}$  BS + s.d.) = synergism.

This is based on the assumption that in the absence of interactions: (1) cell density of single-species biofilms and multispecies biofilms is equal, so no additional biofilm is formed by multiple species cultures than by single-species cultures when similar nutrients are available unless interactions causing synergistic effects occur, and (2) the best biofilm former dominates the biofilm. Figure 1 shows data from a representative data set. Data from the three biological replicates are presented as Supplementary material (Supplementary Table S1).

A total of 22 four-species-combined cultures, accounting for 63% of all combined cultures (35 in total), showed synergy in biofilm formation, as indicated by enhanced biomass production when compared with that of single-species cultures (Figure 1). In total, 10 of the 22 combined cultures showing synergy were composed of only poor biofilm-forming strains. The synergistic effect was especially strong in combinations 1-2-5-7 and 1-2-6-7, in which biofilm biomass had increased by more than fivefold ( $P < 0.05$ ) (Figure 1b, Supplementary Table S1). This may indicate that interspecific interactions had led to cooperative biofilm formation by these strains despite an inability to form biofilms individually.

The majority of the 13 combinations that were not identified as interacting synergistically with respect

to biofilm formation produced amounts of biofilm that were equal to the best biofilm former (Figures 1a and b) with two exceptions. Combinations 1-2-3-4 and 2-3-4-5 showed four-species biofilm formation plus standard deviation ( $Abs_{590}$  MS + s.d.), which was lower than monospecies biofilm formation (best strain) minus standard deviation ( $Abs_{590}$  BS – s.d.). In these two cocultures, competitive interactions may dominate.

### *Synergy in biofilm formation among *S. rhizophila*, *X. retroflexus*, *M. oxydans* and *P. amylolyticus**

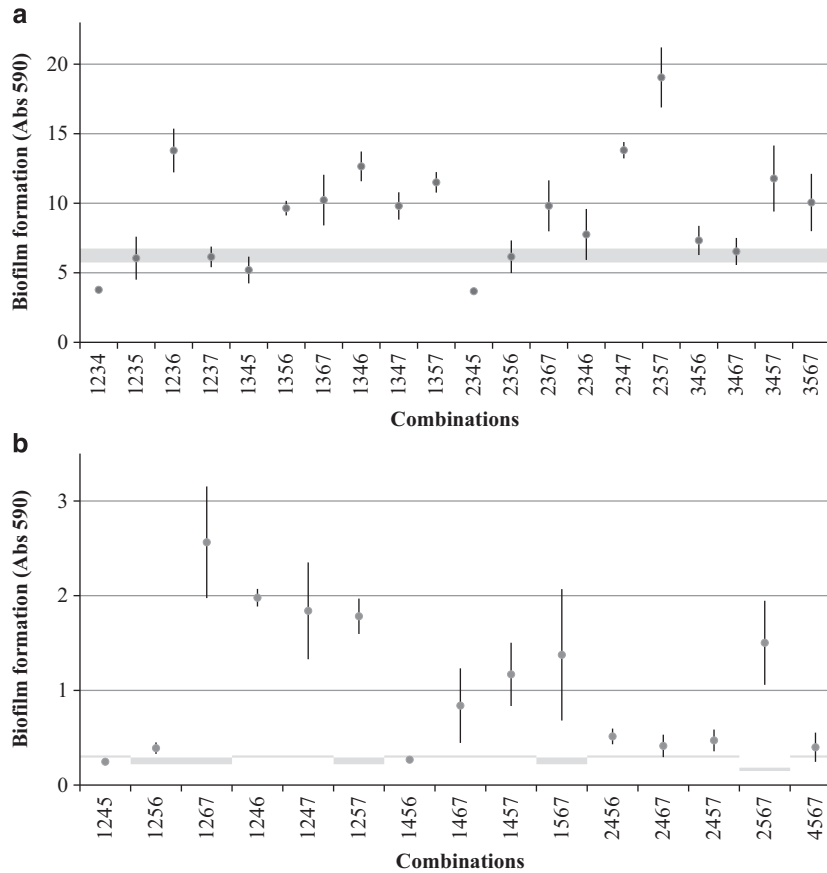
An example of strong synergy was found in strain combination 2-3-5-7 (*S. rhizophila*, *X. retroflexus*, *M. oxydans* and *P. amylolyticus*, respectively, Figure 1 and Supplementary Table S1). To determine whether all four strains contributed to the enhanced biomass, each strain was grown as a single-species biofilm and in all possible combinations as two, three and four-species biofilms and analyzed for synergy in biofilm formation as described above (Figure 2).

Two and three-species biofilms did not differ significantly ( $P > 0.05$ ), with the exception of combinations 3-5 ( $P = 0.026$ ) and 3-5-7 ( $P = 0.001$ ), when compared with the amount of biofilm produced by single-species cultures. Strain 2, *S. rhizophila* and strain 7, *P. amylolyticus* were very weak biofilm-formers when grown alone, but both stimulated biofilm formation of the other three species. When the four isolates were cocultured, the biomass increased by more than fourfold compared with that of single-species biofilms and two–fivefold compared with that of three-species biofilms. This illustrates that each of these four strains were essential for strong synergy induction. Interestingly, this applied both to the good biofilm former, strain 3, *X. retroflexus*, as well as the three other strains that only produce very small amounts of biofilm in monocultures.

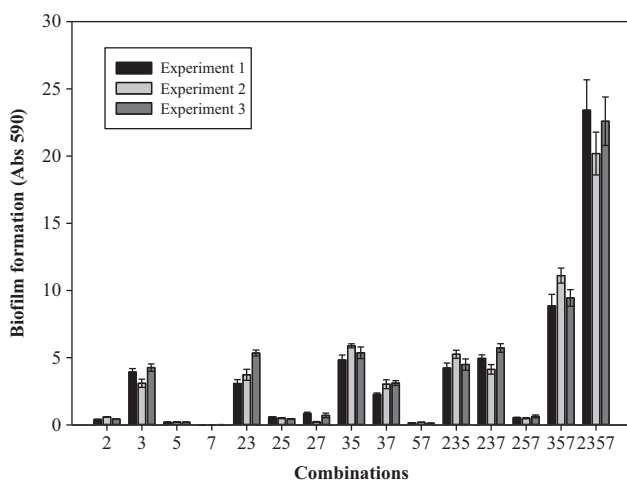
No significant differences were observed ( $P > 0.05$ ) between three-species biofilms with and without spent, cell-free supernatant. This indicates that the presence of the fourth species was required in all combinations for the synergistic effect to occur, or alternatively that compounds in the supernatant were only transiently effective during the 24 h of biofilm formation.

### *Cell numbers in monocultures and cocultures*

Specific qPCRs were performed in order to evaluate the total cell numbers of each strain in both biofilms and planktonic communities at different stages of multispecies biofilm development. Additionally, the cell numbers in single-species biofilms and planktonic cells at 24 h were measured. The DNA samples from different time points were diluted appropriately in order to yield results within the dynamic range of standard curves generated using



**Figure 1** Four-species biofilm formation of seven soil isolates. The observed data points (dark-gray dots) were collected by quantifying multispecies biofilm formation of all possible four-species cocultures using the CV assay. Error bars represent standard deviations of four replicates. Gray bars indicate the amount of monospecies biofilm produced by the best biofilm former present in the specific combination. Data points (including standard deviations) above gray areas indicate synergistic effects in four-species biofilms (see text for further details). The data obtained from combinations containing strain 3 are shown in **a**, whereas other combinations (without strain 3) are shown in **b** in order to visualize the data points at the lower end of the range. Strains: 1: *P. lutea*, 2: *S. rhizophila*, 3: *X. retroflexus*, 4: *O. rhizosphaerae*, 5: *M. oxydans*, 6: *A. nitroguajacolicus*, 7: *P. amylolyticus*.



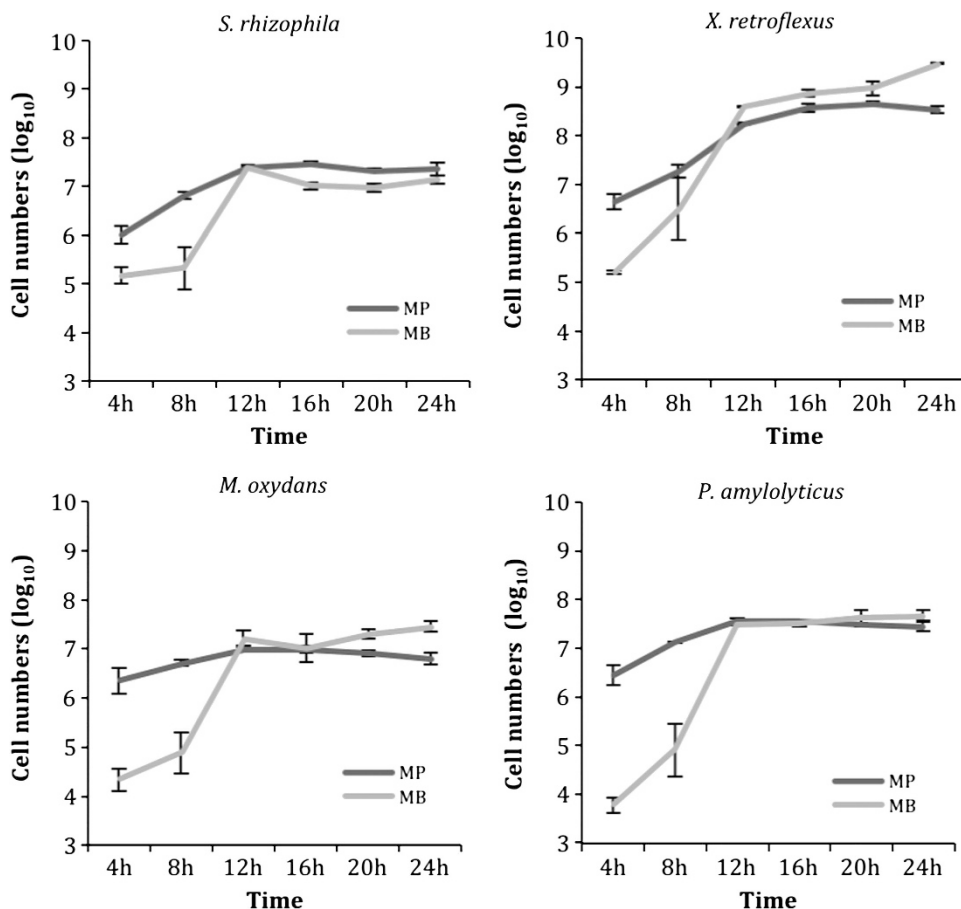
**Figure 2** Biofilm formation of four selected strains in monocultures and cocultures. Biofilms formed by four isolates 2: *S. rhizophila*, 3: *X. retroflexus*, 5: *M. oxydans* and 7: *P. amylolyticus* when equal aliquots of the diluted cultures were incubated in cocultures of two, three and four isolates. Assays for the detection of synergistic effects were performed three times (experiments 1, 2 and 3) with four replicates each time. Error bars represent  $\pm$  s.e.m. of four replicates.

duplicate 10-fold dilutions of purified plasmid DNA (Ren *et al.*, 2013). R-square values, based on threshold cycles of the standard curves, ranged from 0.988 to 1 and amplification efficiencies ( $E$ ) ranged from 84.7–98.0% (Supplementary Table S2).

The cell numbers of each species in the multispecies biofilm (microtiter peg) and in the associated planktonic fraction (microtiter well) are shown in Figure 3. In general, a marked increase in cell numbers was observed from 4–12 h for all of the four strains in the biofilm fraction. After 12 h, only the cell numbers of strain 3 increased continuously, whereas those of the other three species in the multispecies biofilm remained constant or decreased. Cell numbers of all four species in the planktonic fraction increased during the first 12–16 h, after which planktonic growth leveled off, indicating nutrient depletion and transition to a steady-state condition.

The cell numbers of each strain in the multispecies biofilm and in the single-species biofilms at 24 h are shown in Figure 4. For all of the

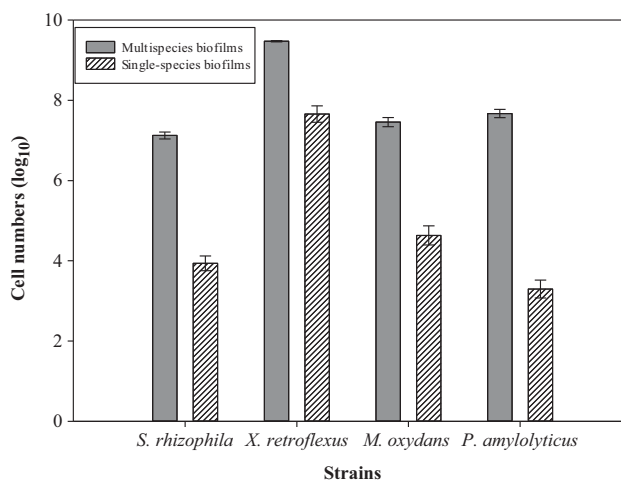




**Figure 3** Cell numbers of biofilm (light gray lines) and planktonic (dark gray lines) fraction of each strain in four-species cocultures. The estimated cell numbers per peg or well, respectively, of each strain in four-species biofilms and associated planktonic fractions of *S. rhizophila*, *X. retroflexus*, *M. oxydans* and *P. amylolyticus* at six time points (4 h, 8 h, 12 h, 16 h, 20 h and 24 h after coincubation) based on qPCR. MP, multispecies planktonic cells; MB, multispecies biofilms. Each point represents the mean of three replicates, with vertical lines representing  $\pm$  s.d.

four species, cell numbers were significantly higher ( $P < 0.05$ ) in four-species biofilms in comparison to single-species biofilms, indicative of individual fitness gains from joining the multispecies biofilm.

The ratio of total cell numbers at 24 h of the four strains (*S. rhizophila*, *X. retroflexus*, *M. oxydans* and *P. amylolyticus*) was 4:900:9:15 in multispecies biofilms (Table 1). Thus, there was a strong dominance of *X. retroflexus* in the four-species biofilm, as this strain constituted more than 97% of the total cell number. It is noteworthy that, in contrast to biofilms (Figure 4), planktonic cell numbers of the four species were within equivalent ranges or lower in the four-species coculture than in single-species planktonic biomass (Table 1). The latter was especially pronounced for *M. oxydans*, where a log<sub>2</sub>-fold reduction was observed when comparing planktonic cell numbers of this strain in coculture with that in monoculture ( $6.42 \times 10^6$ – $1.58 \times 10^8$ ). The summarized cell numbers from the biofilm and the planktonic fraction from a specific microtiter well are not representative of the total cell number of that well, because there are other surfaces



**Figure 4** Cell numbers of each strain in single and multi species biofilms. The estimated cell numbers of each strain (*S. rhizophila*, *X. retroflexus*, *M. oxydans* and *P. amylolyticus*) in multispecies (gray bars) and single-species (striped bars) biofilms at 24 h. Bars represent means  $\pm$  s.d. for three replicates.

**Table 1** Absolute cell numbers per peg/well measured by quantitative PCRs at 24 h in multispecies planktonic cells, single-species planktonic cells, multispecies biofilms and single-species biofilms, and each value is the mean of three replicates

Strains	Multispecies planktonic cells	Single-species planktonic cells	Multispecies biofilms	Single-species biofilms
<i>S. rhizophila</i>	2.33E+07	5.79E+07	1.35E+07	9.14E+03
<i>X. retroflexus</i>	3.38E+08	5.46E+08	2.97E+09	4.92E+07
<i>M. oxydans</i>	6.42E+06	1.58E+08	2.93E+07	4.73E+04
<i>P. amylolyticus</i>	2.81E+07	1.33E+07	4.78E+07	2.10E+03

available for bacterial attachment in these wells besides the pegs.

## Discussion

### *High prevalence of synergism in biofilm formation*

Single-species biofilms are rare in natural environments; especially in agricultural soil where microcommunities exposed to organic matter have the potential to develop into multispecies biofilms with high bacterial density and diversity (Rodríguez and Bishop, 2007; Narisawa *et al.*, 2008; Burmølle *et al.*, 2010). Such conditions in bacterial habitats are likely to facilitate the development of intricate relationships between different species. While many previous studies have focused on interspecies interactions within oral microbial communities (Palmer *et al.*, 2001; Sharma *et al.*, 2005; Wang and Kuramitsu, 2005; Kuramitsu *et al.*, 2007; Saito *et al.*, 2008), research on multispecies biofilms composed of soil bacteria is still in its infancy. In this study we assess, for the first time, the prevalence of interactions leading to synergism in biofilm formation. This was done by screening seven soil bacteria, isolated from one microhabitat, for biofilm formation in four-species cocultures.

In total, 63% of four-species biofilms showed synergistic effects in biofilm formation. This is in agreement with the assumption that the driving force in bacterial community development is self-organization and cooperation rather than competition of individual microorganisms (Davies *et al.*, 1998; Daniels *et al.*, 2004; Parsek and Greenberg, 2005). The recently presented 'Black Queen Hypothesis' provides an explanation as to how multispecies cooperation can evolve (Morris *et al.*, 2012). This hypothesis considers cooperation in complex bacterial communities as being a consequence of species adapting to the presence of each other. In order to enhance individual fitness, species delete vital functions or pathways that are provided by the surrounding bacteria. This leads to a communal dependency and is, therefore, an irreversible commitment to living in close association with other species, which may often require development of more complex systems to ensure that the co-existence is

maintained. When applying the Black Queen Hypothesis on the results of the present study, the strains may reside within the multispecies biofilm in order to keep vital partners in close contact, or alternatively, the ability of biofilm formation could be the function that is lost by some of the species.

Synergistic interactions were also observed when four poor biofilm-formers were cocultured (Figure 1b), verifying previous findings in which individual biofilm-forming ability is not necessarily an indicator of potential in multispecies biofilms (Burmølle *et al.*, 2006; Bharathi *et al.*, 2011). This shifting of biofilm pattern from weak to moderate or strong can be the result of metabolic interactions (Møller *et al.*, 1998), enhanced coaggregation (Rickard *et al.*, 2003), organized spatial distribution (Skillman *et al.*, 1998) and/or facilitated initial surface attachment (Simões *et al.*, 2008; Klayman *et al.*, 2009), for example, bridging bacteria may facilitate the association of other species that do not coaggregate directly with each other. Thus, species that do not form biofilms as single strains may benefit from the advantages associated with biofilm formation, including enhanced protection from external stress and expanded niche availability, through engagement with multispecies communities.

Only two of the four-species cocultures tested showed a reduction in biofilm biomass in comparison with the best monospecies biofilm former present, indicating a low prevalence of strong competition. In contrast to our results, Foster and Bell, (2012) recently showed that the great majority of interactions in pairwise species combinations of bacterial isolates from tree-hole rainwater pools were net negative and very few strong higher-order positive effects arose from combinations composed of more than two species (Foster and Bell, 2012). The two studies differ with respect to bacterial habitats targeted for isolation (soil vs tree holes), productivity parameters assessed (biofilm formation vs CO<sub>2</sub> production) and alternate definitions of synergism/cooperation. Such factors may explain the observed differences. The tree-hole species tend to use similar resources, which may be the key factor that leads to competition among microbes (Lawrence *et al.*, 2012). While, in nutrient-rich agricultural soil, bacteria are likely to be tightly associated and individual bacterial consortia may stabilize their environment by their collective metabolic activities (Mathieu and Sonea, 1995). The key to whether bacterial species compete or cooperate may lie in their potential for long-term coadaptation and degree of niche overlap. We are currently investigating the significance of these parameters for the net interactions of bacterial communities.

### *Enhanced biofilm formation in cocultures*

When exploring the strain dynamics of the selected four-species community, the strongest synergy was only induced when all the four strains

(*S. rhizophila*, *X. retroflexus*, *M. oxydans* and *P. amylolyticus*) were cocultivated (Figure 2). This demonstrates that each strain had an important role in the enhanced biofilm formation irrespective of single-species biofilm formation ability. Divergence in resources use may be one factor leading to the increased productivity of the entire community. By differential resource consumption and metabolism of waste products produced by other species, the consortium collectively decomposes medium substrates more efficiently. In the present study, the synergistic enhancement of biomass of the four cocultured species was only observed when grown as a biofilm; planktonic coculturing did not lead to changes in overall biomass (data not shown) and we did not observe this synergistic effect when adding spent, cell-free supernatant to the three-species biofilms. This indicates that a structured environment and the physical presence of the interacting cells are highly important in maintaining synergy. Thus, syntrophy may explain the observed synergy, but only when coupled with a structured environment that enables tight cell–cell associations, optimizing product/substrate availability. Similar observations have previously been reported (Stewart *et al.*, 1997; Hansen *et al.*, 2007).

qPCR results showed a strong dominance (>97%) of *X. retroflexus* in the four-species biofilm, which is consistent with the results from the CV assay where *X. retroflexus* was the only good biofilm former out of the four strains. Despite its monospecies biofilm-forming abilities, cell numbers of strain *X. retroflexus* were enhanced approximately 60-fold when cocultured in biofilms with the other strains. The remaining three strains constituted <3% of the total cell number of the four-species biofilms; however, they all showed enhanced cell numbers in the multispecies biofilm by over three orders of magnitude when compared with cell numbers in monospecies biofilms. Thus, although *S. rhizophila*, *M. oxydans* and *P. amylolyticus* are present in low abundances and show poor capability of monospecies biofilm formation, they do stimulate biofilm formation of *X. retroflexus* and their cell numbers increase when present in the multispecies biofilm. This significant change in capacity of biofilm formation may be explained by the fact that species evolving in communities may have higher growth rates when assayed in the presence of other species (Lawrence *et al.*, 2012), especially in structured communities (Stewart *et al.*, 1997; Hansen *et al.*, 2007). *P. amylolyticus*, the strain for which cell numbers received the greatest enhancement, has previously been reported to produce antibacterial agents with broad-spectrum activity against both Gram-negative and Gram-positive bacteria (DeCrescenzo Henriksen *et al.*, 2007). However, in this study, this strain appeared to stimulate rather than inhibit growth of the other three strains and no biofilm-stimulating or -inhibitory effects of spent *P. amylolyticus* supernatants were observed. Further

studies to identify the changes in gene expression patterns in this four-species biofilm will lead to a deeper understanding of the underlying molecular mechanisms of synergy in bacterial communities.

#### *Synergy in biofilm formation indicate collective cooperation*

Several observations in this study indicate that bacteria increase fitness from joining multispecies biofilms. If this fitness advantage applies to all of the species present, the underlying interaction is categorized as being cooperative (West *et al.*, 2007). Four such observations are discussed here. (i) We observed a high prevalence of biofilm synergy in four-species biofilms. This implies selection for living in multispecies communities, indirectly indicating that bacteria may benefit from joining multispecies biofilms. The obtained fitness advantage may be caused by growth promotion that enhances bacterial biomass and thereby the direct fitness, but also the advantages gained from the biofilm-associated bacterial protection may be of major significance in natural high-stress environments. (ii) Each of the four species in the selected community was indispensable for the synergistic effect observed on biofilm biomass. These vital interdependencies may evolve over many years under continuous selective pressures, whereby only fitness-enhancing relationships are favored. (iii) The cell numbers of all four species were higher in multispecies biofilms than in their respective single-species biofilms. Thus, when focusing on biofilm-associated growth, there is a direct fitness advantage for all strains from joining the four-species biofilm. (iv) The observed synergistic effect only applies to the four-species biofilm; cell numbers in the planktonic fraction decreased for three out of the four strains. Based on this, there seem to be selection for, or forces that drive, bacteria to form multispecies biofilms. Alternatively, syntrophy occurs in the biofilm, whereby the growth rates of biofilm-resident strains are enhanced.

In conclusion, the observations presented in this study suggest multispecies biofilms as being a favorable bacterial habitat, in which protection and potential for engagement in mutually beneficial cooperative interactions prevail.

#### **Conflict of Interest**

The authors declare no conflict of interest.

#### **Acknowledgements**

We thank Timothy Evison and Urvish Trivedi for text edition and Karin Vestberg and Anette Hørdum Løth for technical assistance. This study was partly funded by grants to SJS and MB from the Danish Innovation Consortium SiB, ref no: 11804520, and The Danish Council for Independent Research; ref no: DFF-1335-00071 and ref no: DFF-1323-00235 (SIMICOM).



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