Ecological insights into the underlying evolutionary patterns of biofilm formation from biological wastewater treatment systems: Red or Black Queen Hypothesis?

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Abstract
Interspecies interactions and phylogenetic distances were studied to reveal the underlying evolutionary adaptations of biofilms sourced from wastewater treatment processes. Based on 380 pairwise cocultures of 40 strains from two microbial aggregates (surface-attached and mobile aggregates [flocs]) at two substrate concentrations (LB broth and 0.1× LB broth), interspecies interactions were explored using biofilm classification schemes. There was a strong source-dependence of biofilm development formed by the monocultures, that is, a higher biofilm formation potential for strains from attached aggregates than for those from sludge flocs at both substrate concentrations. Interestingly, the results showed that total biofilm reduction was dominant in the dual-species biofilm sourced from flocs in both LB broth (67.37%) and 0.1× LB broth (64.21%), indicating high interspecific competition in mobile aggregates and the independence of substrate concentrations. However, biofilm reduction was higher (33.68%) than induction (19.37%) for the biofilms formed by surface-attached aggregates in LB broth, while the opposite trend was apparent in 0.1× LB broth, suggesting the occurrence of indeterministic processes for biofilm formation and important roles of substrate concentrations. In addition, the more closely related phylogenetic relationships of cocultures from mobile aggregates were consistent with higher competition compared with those from surface-attached aggregates. Overall, the underlying evolutionary patterns of biofilms formed from mobile aggregates consistently followed the essence of the “Red Queen Hypothesis,” while biofilms developed from surface-attached aggregates were not deterministic. This study advanced our understanding of biofilm-related treatment processes using the principles of microbial ecology.

KEYWORDS
biofilm, evolutionary process, interspecies interactions, microbial aggregates, substrate concentrations

1 | INTRODUCTION

Microorganisms preferably grow and live in aggregates, either in surface-attached forms (biofilms) adhering directly to the sub-stratum, or in mobile aggregates (flocs) that form without any sub-stratum (Costerton et al., 1987; Flemming et al., 2016). Based on growth preference, biological wastewater treatment takes full advantage of microbial aggregates in engineered ecosystems (Ahmed, Tian, & Delatolla, 2019; Bagheri & Mirbagheri, 2018; Mahendran, Lishman, & Liss, 2012). When coexisting with other species in the same environment, microorganisms could coevolve with others via interspecies interactions (Madsen et al., 2016). The principles of
microbial ecology suggest that the evolutionary processes of coexisting multispecies were critical for the resistance and functions of microbial communities (Burmolle, Ren, Bjarnsholt, & Sørensen, 2014; Hua, Joyce, Fong, & Palsson, 2006; Madsen et al., 2016). As one ubiquitous survival form, biofilms can be formed as microbial aggregates in biological treatment processes (moving bed biofilm reactor [MBBR] or floc-based membrane bioreactor [MBR]), causing either beneficial or detrimental effects on the reactor performance (Guo et al., 2019; Meng et al., 2009). Thus, understanding the underlying evolutionary patterns of biofilm formation is important for biofilm regulation in biological wastewater treatment systems.

To date, two evolutionary laws have been proposed to interpret the occurrence of natural phenomena (Castrodeza, 1979; Morris, Lenski, & Zinser, 2012). In the “Black Queen Hypothesis,” long-term coexistence could lead to interspecific cooperation and dependence of species (Burmolle et al., 2014; Morris et al., 2012). Substantial studies have found that cooperation dominates in microbial communities with robust coexistence (Elias & Banin, 2012; Schink, 2002). However, the “Red Queen Hypothesis” claims that competition and antagonistic interactions were predominant between coexisting bacteria (Burmolle et al., 2014; Liow, Van Valen, & Stenseth, 2011). This hypothesis emphasized that species must evolve constantly to survive for limited resources (Hibbing, Fuqua, Parsek, & Peterson, 2010; Liow et al., 2011). Foster and Bell (2012) also proposed that the typical result of adaptation was competition rather than cooperation among cocultures from a tree rainwater pool. These previous studies focusing on natural environments or pure bacteria provide fundamental clues for the understanding of biofilm formation in engineered systems (Foster & Bell, 2012; Madsen et al., 2016).

Wastewater concentrations often vary in engineered systems (Lagarde et al., 2005; Teerlink, Hering, Higgins, & Drewes, 2012). As previous studies demonstrated, biofilm formation is dependent on nutrient availability and facilitated by interspecies interactions (Burmolle et al., 2014; Freilich et al., 2011; Hibbing et al., 2010). Generally, a lower substrate concentration could cause more competition and reduced biofilm formation compared to higher substrate concentrations (Hibbing et al., 2010; Yan, Nadell, & Bassler, 2017). However, classic consumer-resource models reveal that when multiple species compete for a growth-limiting resource, the only possible outcome is competitive exclusion, unless specific circumstances are applied (Ghoul & Mitri, 2016; Goldford et al., 2018). For instance, a multispecies community, compared to a mono-species culture, can counter substrate limitation via cross-feeding (Goldford et al., 2018) and/or a time-sharing strategy (Liu et al., 2017). Based on these previous studies, we hypothesized that the evolutionary trajectories of biofilm formation might be influenced by variations in wastewater concentration, mainly due to its indispensable role in bacteria growth.

As microorganisms typically live in dense multispecies communities (Flemming et al., 2016; Wingender & Flemming, 1999), investigating interspecies interactions is far from straightforward (Faust & Raes, 2012). Although the emerging multomics have been applied to decipher the cooccurrence networks of multiple species by calculating pairwise species correlations (Deng et al., 2019; Lima-Mendez et al., 2015; Zhang, Zhou, Li, & Meng, 2017), they were expensive and not necessarily suitable for the in-depth analysis of interspecies interactions (Ghoul & Mitri, 2016). To address these concerns, several studies have attempted to extrapolate from pure cultures to complex communities with elaborate experiments (Reisner, Holler, Molin, & Zechner, 2006; Vetsigian, Jajoo, & Kishony, 2011; Wintermute & Silver, 2010). However, most of these studies were not representative of wastewater treatment systems, as they merely focused on some special species or phenotypes (Foster & Bell, 2012). Because cooperation in multispecies communities could result in “emerging community properties,” such as enhanced biofilm biomass, biofilm phenotypes have been applied to reveal the underlying interspecies interactions (Liu et al., 2019; Ren, Madsen, Sorensen, & Burmolle, 2015). By evaluating the gains/losses of biofilm biomass (Burmølle et al., 2006; Ren et al., 2015), it is possible to construct a more nuanced and strict multiregime biofilm classification scheme, enabling the identification of the underlying interspecies interactions of cocultures from natural environment (Madsen et al., 2016). Based on the study of 1,235 cocultures, Madsen et al. (2016) showed the practicality of such a scheme for processing a large number of samples. Thus, this biofilm classification scheme could be promising for the unbiased and detailed investigation of interspecies interactions in wastewater treatment systems. In addition, it is assumed that the phylogenetic relationship is correlated with similarity in niches and resources (Liow et al., 2011). When occupying the same niche, a strain could influence the evolutionary trajectories of other species via resource competition (Liow et al., 2011). Hence, phylogenetic relationships and interspecies interactions can elucidate the underlying evolutionary processes (Liow et al., 2011).

In the current work, interspecies interactions of 40 strains from two microbial aggregates (surface-attached and mobile aggregates) at two substrate concentrations (LB broth and 0.1× LB broth) were studied with the help of the multiregime biofilm classification scheme. Phylogenetic relationships were also characterized by pairwise phylogenetic distances. The specific objective was to decipher the underlying evolutionary processes of biofilm formation by studying interspecies interactions and phylogenetic relationships. We expected to provide new insights into biofilm regulation in biological treatment processes from the principles of microbial ecology.

### 2 MATERIALS AND METHODS

#### 2.1 Strains isolation and identification

In this study, 40 strains were isolated from two microbial aggregates (Figure 1a, Step 1). Twenty strains were isolated from 8-day-old surface-attached aggregates (SA1-20) in a glass flow cell chamber (IBI Scientific) using activated sludge from a laboratory MBR as the source community; the other 20 strains were isolated from mobile aggregates (MA1-20) from a full-scale MBR plant (Guangzhou, China) with the sludge retention time of approximately 15–20 days (Maddela, Zhou, Yu, Zhao, & Meng, 2018). Herein, strains from each aggregate had previously coexisted and adapted to each other for several days: 8 days for SA1-20 and 15–20 days for MA1-20.
The isolation and identification of these strains followed the description of Maddela et al. (2018), and details are available in the supplementary material. For similarity analysis, the 16S Ribosomal RNA (rRNA) sequences were blasted online (http://www.ncbi.nlm.nih.gov/BLAST). Then, the sequence data of these strains were deposited in GenBank, and the detailed identification and accession numbers of these strains are listed in Table S1.

2.2 | Growth curves and growth rate of isolations

To measure growth rates, exponential phase cultures were first adjusted to an optical density at 595 nm (optical density [OD]595) of 0.02 in Luria-Bertani broth (LB broth) using a microplate reader (Multiskan FC; Thermo Fisher Scientific). Then, the diluted cultures were incubated in 96-well plates at 30°C and 120 rpm. During the entire cultivation, OD595 was regularly measured until the stationary stage. The measurement of growth curves in 0.1× LB broth followed the same protocol as those in LB broth. The specific growth rate ($\mu$) of each strain was calculated from five consecutive OD595 measurements during the exponential phase ($\mu = \frac{\Delta \ln [OD_{595}]}{\Delta t}$, where $t$ is time; Berney, Weilenmann, Ihssen, Bassin, & Egli, 2006).

2.3 | Biofilm formation of single and dual-species

To construct the multiregime classification scheme, biofilm growth of monocultures and pairwise cocultures was conducted at two substrate concentrations (Figure 1b, Step 2). For all isolates mentioned, 380 pairwise cocultures were obtained, including 190 cocultures from surface-attached aggregates (SA1-20) and 190 cocultures from mobile aggregates (MA1-20; Figure 1b, Step 2). The exponential phase cultures were adjusted to OD395 of 0.02 in LB broth and then inoculated in 96-well microtitre plates with four replicates. The inoculum volumes for biofilm formation were 100 µl for monocultures or 50 µl of each species for cocultures. The experiments conducted in 0.1× LB broth followed the same dilution time and protocol as those in LB broth. Negative controls were also conducted using sterilized LB broth and 0.1× LB broth for the two dilutions. After 24 hr of incubation (stationary phase as shown in Figure S1) at 30°C without shaking, biofilm formation in the stationary stage was quantified by crystal violet (CV) assay with minor modifications (Dai, Raskin, & Xi, 2017; Simoes, Simoes, & Vieira, 2007). The bacterial biofilms in plates were stained with 125 µl of 0.5% CV stains for 20 min and then washed three times with deionized water to remove the unbound stains. The plates were emptied and left to dry. Finally, 150 µl of 33% acetic acid was added for 15 min to release the bound stains, and biofilm biomass was measured at OD595 using the microplate reader. OD395 of negative controls was recorded as ODcontrol.

2.4 | The multiregime classification scheme for the assessment of interspecific biofilm formation

The multiregime classification scheme used for the assessment of interspecific biofilm formation is listed in Figure 1c (Step 3), as used in a previous study (Madsen et al., 2016). Briefly, five different classifications...
were used: synergistic induction, induction, unresolved regime, reduction, and antagonistic reduction (Madsen et al., 2016). As one bacterial phenotype, interspecific biofilm formation was regarded as a response to interspecific interactions (Burmølle et al., 2014; Madsen et al., 2016). It is likely that the level of niche overlap and the probability of competition were positively correlated (Liow et al., 2011). Without niche overlap, synergistic biofilm induction would occur as a result of strong interspecific cooperation when biofilm was enhanced in cocultures (>sum). Biofilm induction was designated if the biofilm biomass of cocultures was higher than that of the better individual biofilm producer (>maximum), indicating weak interspecific cooperation. With complete niche overlap, biofilm reduction was assumed if less biofilm formed in cocultures than the average of monocultures due to interspecific competition (<average). If the biofilm biomass formed by cocultures was less than that formed by the poorer biofilm producer, the antagonistic reduction was assigned (<minimum) to reveal strong competition or antagonism. To ensure stringency, the unresolved regime was included between the regime of biofilm induction and reduction, suggesting an overall weaker influence of interactions on biofilm formation (Madsen et al., 2016).

2.5 | Pairwise phylogenetic distances

Based on 16S rRNA gene sequences, pairwise phylogenetic distances were calculated in MEGA7 (Kumar, Stecher, & Tamura, 2016). All sequences of isolates were first aligned by ClustalW, and then pairwise phylogenetic distances were calculated using the Maximum Composite Likelihood model (Mardia, Kent, Hughes, & Taylor, 2009; Tamura et al., 2011). The phylogenetic tree was also constructed using the Maximum Likelihood method in MEGA7 (Mardia et al., 2009; Tamura et al., 2011).

3 | RESULTS

3.1 | Biofilm formation and growth rate of monocultures

For easier interpretation of the results, some previous studies classified the biofilm formation potential based on the biofilm biomass (Liebana et al., 2016; Simes et al., 2007; Stepanovic et al., 2007). Due to the higher biomass here, we modified the classification criteria according to those previous studies: weak biofilm potential (ODcontrol < OD ≤ 5 × ODcontrol), moderate biofilm formation potential (5 × ODcontrol < OD ≤ 10 × ODcontrol), and strong biofilm formation potential (OD > 10 × ODcontrol). The ODcontrol value of negative controls in LB broth was the same as that in 0.1× LB broth, and thus we used the same classification criteria for biofilm formation in both substrates.

At both substrate concentrations, biofilm formation potentials of monocultures from surface-attached aggregates were higher than those from mobile ones (Figure 2). Among monocultures from mobile aggregates, only five strains (MA3, 4, 12, 15, and 20) presented strong biofilm formation potentials in LB broth (OD595 > 0.8), while all strains presented moderate or weak biofilm formation potentials in 0.1× LB broth (OD595 < 0.8). However, a total of 11 strains from surface-attached aggregates presented strong biofilm formation potentials (OD595 > 0.8) in LB broth, and seven strains maintained strong biofilm formation potentials (OD595 > 0.8) in 0.1× LB broth. Overall, the results suggested that the fitness of strains from surface-attached aggregates was higher than that from mobile aggregates in the biofilm-forming environment, and this fitness was independent of substrate concentration.

Growth rates and attachment ability are two indispensable factors influencing biofilm formation (Komlos, Cunningham, Camper, & Sharp, 2005; Moons, Michiels, & Aertsen, 2009). In the present study, the growth rates of strains isolated from mobile aggregates were comparable to those from surface-attached aggregates for both substrate concentrations, despite the evident restriction in 0.1× LB broth (Figure 2 and Figure S1). Thus, the higher biofilm formation potentials of strains from surface-attached aggregates might not be attributed to growth rates. The varying biofilm potentials of these isolated strains could be associated with the phenotypes of the strains, such as extracellular polymeric substances (EPS) production (Karunakaran & Biggs, 2011; Maddela et al., 2018; Vanysacker, Boerjan, Declerck, & Vankelecom, 2014) and mobility (Xia, Chen, Chen, Qian, & Liu, 2016). We recently found that the functional groups of Amide II and α-1,4-glycosidic linkage largely determined the biofouling process of the bacterial strains (Maddela et al., 2018).
3.2 Interspecific biofilm formation of cocultures from mobile aggregates

Interspecific biofilm formation of cocultures from mobile aggregates is depicted in the color-coded scatterplots (Figure 3a,b), as a function of the average amount of biofilm produced by monocultures. The observations showed comparable trends of biofilm classifications among cocultures from mobile aggregates at two substrate concentrations (Figure 3c,d). Total biofilm induction, including synergistic induction and induction, was not prominent among cocultures in LB broth (15.79%) or 0.1× LB broth (20.53%; Figure 3c,d). In particular, synergy was negligible for both substrate concentrations (0% and 2.11% in LB broth and 0.1× LB broth, respectively; Figure 3c,d). The low biofilm induction among cocultures from mobile aggregates indicated that the prior coexistence could not facilitate interspecific biofilm induction sourced from mobile aggregates, which was not consistent with the result of Madsen et al. (2016). However, biofilm reduction was pronounced for both substrate concentrations, that is, 67.37% in LB broth and 64.21% in 0.1× LB broth. Note that this method was based on the comparison of biofilm biomass for cocultures and monocultures (Madsen et al., 2016). Thus, biofilm reduction was not attributed to the inability of biofilm formation (Ren et al., 2015), but rather interspecies interactions that resulted in the phenotypic response of biofilms. With the observation of pronounced biofilm reduction, cocultures from the mobile aggregates were dominated by competition rather than cooperation (Madsen et al., 2016). At both substrate concentrations, the observations of cocultures from mobile aggregates showed comparable trends for both total biofilm induction and reduction, suggesting that interspecific biofilm formation of strains from mobile aggregates was consistent and independent of substrate concentrations.

3.3 Interspecific biofilm formation of cocultures from surface-attached aggregates

According to the color-coded scatterplots (Figure 4a,b), the distribution of interspecific biofilm classifications of cocultures from surface-attached aggregates is depicted in Figure 4c,d. Among cocultures from surface-attached aggregates, the unresolved regime was the most commonly observed outcome in LB broth (46.85%) and 0.1× LB broth (43.68%), suggesting the weak influence of social interactions on biofilm formation (Madsen et al., 2016). The weak social interactions might be due to the weak adaption of strains in short-term prior coexistence (8 days). Nevertheless, the distinctive trends of total biofilm induction or reduction were observed at two substrate concentrations. In LB broth, the total proportion of antagonistic biofilm reduction and biofilm reduction was higher (33.68%) than total biofilm induction (19.47%), implying interspecific competition was also nonnegligible in the surface-attached aggregates. However, the total proportion of biofilm reduction was lower (22.63%) than biofilm induction (33.68%) in 0.1× LB broth. The distinctive trends for biofilm classifications at both substrate concentrations indicated that substrate concentration could affect interspecific biofilm formation of cocultures from surface-attached aggregates.

**FIGURE 3** Distribution of interspecific biofilm classifications of cocultures from mobile aggregates at two substrate concentrations. Overview of the classification distribution of cocultures from mobile aggregates in (a) LB broth and (b) 0.1× LB broth, as a function of the average of biofilm formed by monocultures. Percentage distribution of interspecific biofilm classifications among cocultures from mobile aggregates in (c) LB broth and (d) 0.1× LB broth [Color figure can be viewed at wileyonlinelibrary.com]
In addition, a lower substrate concentration facilitated interspecific biofilm formation of cocultures from surface-attached aggregates in the present study (Figure 4). This was inconsistent with previous studies, which proposed that a lower substrate supply could cause more competition and reduced biofilm formation (Hibbing et al., 2010; Liu et al., 2017). The distinct biofilm responses of cocultures from two microbial aggregates were likely associated with the different strains from two source communities.

### 3.4 Phylogenetic distances of pairwise cocultures

Phylogenetic relationship was proposed to correlate with the niche overlap of cocultures (Philippot et al., 2010; Plata, Henry, & Vitkup, 2015), and niche differences increased with phylogenetic distances (Godoy, Kraft, & Levine, 2014). Thus, it was supposed that the phylogenetic distance of cocultures might be an underlying determinant of interspecific biofilm formation (Madsen et al., 2016). Lower pairwise phylogenetic distances implied that cocultures were phylogenetically more closely related (Liow et al., 2011). As observed in previous studies, species with phylogenetic distances <0.5 and those >1 showed distinct trends of resource difference and competitive exclusion (Godoy et al., 2014; Violle, Nemergut, Pu, & Jiang, 2011). As observed in Figure 5 and Figure S2, strains from mobile aggregates were more closely related due to the lower pairwise phylogenetic distances (<0.5), indicating higher niche overlap of cocultures from mobile aggregates compared to those from surface-attached ones (Emerson & Gillespie, 2008; Foster & Bell, 2012).

However, pairwise phylogenetic distances of cocultures from surface-attached aggregates were more distantly related (33.68% of those was >1), suggesting lower niche overlap than those from mobile aggregates (Foster & Bell, 2012). When strains from the two microbial aggregates were combined, 80% of pairwise phylogenetic distances were >1, resulting in the most distant pairwise phylogenetic distances compared to those from the single aggregate form. Our results indicated that strains from the same environment with prior coexistence could be more closely related than those without prior coexistence. Furthermore, phylogenetic relationships in mobile aggregates were more closely related than those from surface-attached ones.

**FIGURE 4** Distribution of interspecific biofilm classifications of cocultures from surface-attached aggregates at two substrate concentrations. Overview of the classification distribution of cocultures from surface-attached aggregates in (a) LB broth and (b) 0.1× LB broth, as a function of the average of biofilm formed by monocultures. Percentage distribution of interspecific biofilm classifications among cocultures from surface-attached aggregates in (c) LB broth and (d) 0.1× LB broth [Color figure can be viewed at wileyonlinelibrary.com]

**FIGURE 5** Distribution of pairwise phylogenetic distances among cocultures within mobile aggregates (MA & MA), surface-attached aggregates (SA & SA) or across two aggregates (MA & SA) [Color figure can be viewed at wileyonlinelibrary.com]
4 | DISCUSSION

From the classical principles of microbial ecology, the evolutionary process was critical to the resistance and function of microbial communities (Burmølle et al., 2014; Hua et al., 2006). However, much remains unknown about the evolutionary process of biofilm formation in biological wastewater treatment systems. Herein, we investigated the biofilm formation of strains from two common microbial aggregates in biological wastewater treatment systems for better biofilm regulation.

4.1 | The distinctive evolutionary patterns of biofilm formation with different sources

Following the biofilm classification scheme, our results exhibited high biofilm reduction among cocultures from mobile aggregates (Figure 3). Based on the “Black Queen Hypothesis,” bacteria deleted vital functions or pathways that could be provided by the surrounding bacteria to enhance their own fitness (Burmølle et al., 2014; Klasson, 2004). This hypothesis was invoked to explain the cooperation and dependence of species as a result of adaptation during long-term coexistence (Burmølle et al., 2014). In this study, strains from mobile or surface-attached aggregates had previously coexisted and adapted to each other for several days. However, the strains sourced from the mobile aggregates could not adapt to each other during the prior coexistence period, as indicated by the low biofilm induction (Figure 3). In contrast, high levels of competition were reflected by the pronounced biofilm reduction among these cocultures (Figure 3), indicating a high niche overlap of mobile aggregates (Foster & Bell, 2012; Lawrence et al., 2012). For instance, floc-based reactors used monomers as carbon sources for denitrification in biological wastewater treatment (Deng et al., 2019; Lu, Chandran, & Stensel, 2014). Due to the comparably high competition at two substrate concentrations, the underlying evolutionary pattern of biofilm formation from mobile aggregates might follow the essence of the “Red Queen Hypothesis” consistently, and be independent of substrate concentrations.

Among cocultures from surface-attached aggregates, lower biofilm reduction was observed compared to those from mobile aggregates, indicating they could adapt to each other during the prior coexistence. As reflected by the pronounced unresolved regime in Figure 4, it was inferred that the prior adaptation was weak. In LB broth, the proportion of total biofilm reduction was higher than that of biofilm induction (Figure 4), suggesting competition was higher than cooperation during biofilm formation. Generally, it was believed that there was more competition and reduced biofilm formation at lower substrate concentrations (Ghoul & Mitri, 2016; Hibbing et al., 2010). However, our results showed higher cooperation at lower substrate concentrations, analogous to the finding of Freilich et al. (2011) and Liu et al. (2017). Herein, the underlying evolutionary pattern of biofilm formation from surface-attached aggregates was not deterministic and was affected by substrate concentration.

In this study, the growth medium (LB broth) used may have acted as a selective force for the strains with similar resource preferences, which may have caused high competition. However, Madsen et al. (2016) observed a high degree of fitness interaction (i.e., cooperation) between cocultures in tryptic soy broth (Ren et al., 2015). In this work, we assumed the prior coexisting environment was the main force in shaping the evolutionary process, not the medium used for the experiment. The difference between our study and Madsen et al. (2016) could be due to the different bacterial sources. In nutrient-rich soil, bacteria could stabilize with tightly associated structures and collective metabolic activities (Mathieu & Sonea, 1995; Ren et al., 2015), leading to high cooperation of cocultures from this environment. However, in wastewater treatment systems, strains aggressively compete for the limited resources due to the high biomass (Ping Chu & Li, 2005; Sririviyarat & Randall, 2005; Wells et al., 2017). Thus, the ecological discoveries from the natural system cannot simply be extrapolated to the engineered systems.

4.2 | Potential clues for understanding the distinctive evolutionary patterns of biofilm formation from two microbial aggregates

As proposed in previous studies, phylogenetic relationships are correlated with the similarity of resources and niche (Liow et al., 2011; Madsen et al., 2016), and thus could be one potential clue for understanding the evolutionary pattern. Based on Darwin’s hypothesis, niche overlap would hamper the coexistence of species with more closely related phylogenetic relationships (Mason, de Bello, Doležal, & Lepš, 2011). This hypothesis supposed that closely related strains would compete for similar resources, and thus exclude each other (Vamosi, Heard, Vamosi, & Webb, 2009). However, Liow et al. (2011) proposed that strains with the closely related phylogenetic relationships would share similar adaptive traits, and thus they could coexist in a similar abiotic environment. In this study, the prior coexisting strains within the single aggregate form presented more closely related phylogenetic relationships than those from the different ones (Figure 5). The observation of phylogenetic relationships in this study was consistent with Liow et al. (2011), which was analogous to the selection process. The selection process was mainly attributed to the effects of the abiotic environment, in which the fitness differences determine the community structure (Xu, Yu, Zhang, & Meng, 2019; Zhou & Ning, 2017). In floc-based reactors, nutrient concentrations were more homogeneous (Deng et al., 2019), contributing to the high selection forces of abiotic environments in shaping the communities of mobile aggregates. As such, the more closely related bacteria with similar adaptive traits were selected in flocs, resulting in pronounced biofilm reduction (Figure 3). The pronounced biofilm reduction was maintained at different substrate concentrations (Figure 3), indicating that strong competition was consistent and independent of substrate concentrations. Thus, the evolutionary pattern of biofilm formation from mobile aggregates followed the essence of the “Red Queen Hypothesis,” and this process was consistent and independent of substrate concentration due to the more closely related phylogenetic relationships.
However, the less closely related phylogenetic relationships of cocultures from surface-attached aggregates were similar to the results of Godoy et al. (2014), which suggested that coexistence had no direct correlation with phylogeny. In the well-structured surface-attached aggregates (biofilm), the passive diffusion of nutrients could lead to nutrient gradients and different microenvironments due to the mass transfer process (Fux, Costerton, Stewart, & Stoddley, 2005; Stewart, 2012; Stewart & Franklin, 2008). This phenomenon might contribute to the larger niche and higher microbial diversity in the biofilm reactors than in floc reactors (Deng et al., 2019), and thus more distantly related bacteria could coexist in the surface-attached aggregates (Figure 5). Although previous studies suggested that phylogenetic relationships were correlated with resource or niche similarity (Liow et al., 2011; Madsen et al., 2016), Freilich et al. (2011) further proposed that a moderate level of resource similarity could maximize the potential for cooperation (Freilich et al., 2011). Thus, the less closely related phylogenetic relationship between cocultures from surface-attached aggregates (Figure 5) might maximize the potential for cooperation, leading to higher biofilm induction compared to mobile aggregates (Figure 3 and 4). However, biofilm induction or reduction presented opposite trends at two different substrate concentrations (Figure 4), suggesting this process was influenced by substrate concentrations. Thus, the evolutionary process of biofilm formation from surface-attached aggregates was not consistent due to the less closely related phylogenetic relationships.

A second potential clue for the distinctive evolutionary pattern might be the fitness difference in the biofilm-forming environment. Given that fitness can differ, strains specialized in one environment or another (Yan et al., 2017; Yawata et al., 2014). In this study, strains from surface-attached aggregates specialized in biofilm formation on the substratum, while those from mobile aggregates had lower biofilm formation potential on the substratum (Figure 2). Based on competition-dispersal tradeoffs, superior competitors could dominate within patches by colonization, whereas those with an inferior competition ability must migrate for the new resources or patches (Yawata et al., 2014). During the resource competition, this “fugitive” strategy endowed mobile aggregates with high migration abilities instead of colonization, while strains from surface-attached aggregates were inclined to colonize with inferior migration abilities. Thus, the distinct fitness of strains from two microbial aggregates in the biofilm-forming environment might also introduce distinctive biofilm responses to substrate concentrations due to the potential competition-dispersal tradeoffs.

4.3 | Implications for biological wastewater treatment

In engineered ecosystems, many processes are mainly based on a single aggregate form, such as sludge-based process (e.g., MBRs) or biofilm-based process (e.g., MBBRs; Ahmed et al., 2019; Meng et al., 2009; Ping Chu & Li, 2005; Regmi et al., 2016). In these processes, bacteria must struggle for limited resources due to high niche overlap (Deng et al., 2019; Vanyascker et al., 2014), leading to constant competition (Hibbing et al., 2010). This phenomenon is beneficial for wastewater treatment as the competition for pollutants contributes to high removal efficiency. However, this phenomenon is also a selection process, which could cause biodiversity loss (Zhou & Ning, 2017). For survival, bacteria must continually evolve to compete for limited resources, so they can survive in the ecosystem (Liow et al., 2011). As niches differ between mobile and surface-attached aggregates, microbial communities based on these two aggregate forms were distinct in wastewater treatment processes (Mahendran et al., 2012). For instance, the abundances of Zoogloea, ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in the attached forms were higher than those in mobile forms (Guo et al., 2019; Mahendran et al., 2012). The distinct communities of these two types of microbial aggregates could make very distinctive contributions to overall performance (Ahmed et al., 2019; Mahendran et al., 2012; Wingender & Flemming, 1999). By improving the niche differentiation, the engineering ecosystem could be more stable and efficient (Deng et al., 2019). Thus, integrating these two microbial aggregates (e.g., integrated fixed-film activated sludge) is a promising process for the enhancement of microbial diversity and wastewater treatment efficiency (Sriwiriyarat & Randall, 2005).

Membrane biofouling is one of the main obstacles impeding the wide application of MBRs (Meng et al., 2009). From an engineering standpoint, the initial adhesion is critical for biofouling formation (Vanyascker et al., 2014). One primary biofouling source is bacteria with high biofilm formation potential, known as the pioneer (Vanyascker et al., 2014), which could stimulate the initiation of biofouling (Lu et al., 2014). In this study, strains from surface-attached aggregates were specialized in biofilm formation, while those from mobile aggregates had lower biofilm formation potentials (Figure 2). This observation also indicated that initial biofouling is attributed to the sludge communities with high biofilm formation abilities. Previous studies reported that biofilm communities were distinct from sludge communities (Huang, De Wever, & Diels, 2008; Matar, Bagchi, Zhang, Oerther, & Saikaly, 2017; Zhang, Choi, Dionysiou, Sorial, & Oerther, 2006). Thus, targeting the pioneers with high biofilm formation potentials rather than the total sludge microbial community could facilitate biofouling control (Vanyascker et al., 2014; Zhang et al., 2006).

Biofilm-based processes, such as MBBR, are promising and emerging due to their high robustness and resilience (Regmi et al., 2016; Seetha, Bhargava, & Kumar, 2010; Young, Delatolla, Kennedy, LaFlamme, & Stintzi, 2017). In the present study, the robustness and resilience of biofilm were also demonstrated by the higher biofilm formation potentials of monocultures (Figure 2) and higher cooperation of cocultures from surface-attached aggregates at lower substrate concentrations (Figure 4). In a well-structured biofilm, passive diffusion of nutrients could lead to the nutrient gradients and different microenvironments due to mass transfer limitations (Fux et al., 2005; Stewart, 2012; Stewart & Franklin, 2008). This could further contribute to the larger niche difference and higher microbial diversity.
diversity in the biofilm reactors than those in floc reactors (Deng et al., 2019), which could also be indicated by the distantly related phylogenetic relationships of strains from surface-attached aggregates (Figure 5).

Apart from the pairwise biofilm formation, biofilm classifications could also be applied to investigate higher-order biofilm formation only if bacteria were dissecible (Freilich et al., 2011; Madsen et al., 2016). In a previous study, culturability was regarded as one limitation in the investigation of complex microbial communities using this method (Madsen et al., 2016). However, Martiny (2019) showed that approximately 97.3 ± 2.3% of cells could be cultured with substantially advanced cultivation techniques. Therefore, this method could be further verified with these advanced techniques in the future. In addition, this study was mainly based on short-term evolutionary patterns of the prior coexisting bacteria from two microbial aggregates, and understanding of long-term coadaptation processes is needed for more valuable information in the future. To our knowledge, only a few large-scale cultivation-independent studies have evaluated biofilm formation and interspecies interactions in situ (Foster & Bell, 2012; Madsen et al., 2016). In this study, the underlying evolutionary patterns of biofilm formation in biological wastewater treatment systems were studied by investigating large numbers of cocultures from microbial aggregates, which would provide new insight into biofilm regulation in engineered ecosystems.

5 | CONCLUSIONS

The main conclusions of this work can be summarized as follows:

(1) Biofilm formation potentials of monocultures from surface-attached aggregates were higher than those from mobile aggregates independent of substrate concentrations.

(2) Biofilm reduction was pronounced among cocultures from mobile aggregates at two substrate concentrations, indicating high interspecific competition independent of substrate concentration.

(3) Interspecific biofilm reduction or induction of cocultures from surface-attached biofilms presented opposite trends at both substrate concentrations, suggesting this behavior was influenced by substrate concentrations.

(4) The phylogenetic relationships of cocultures from mobile aggregates were more closely related than those from the surface-attached ones, implying higher niche overlap of cocultures from mobile aggregates than those from surface-attached ones.

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REFERENCES

Ahmed, W., Tian, X., & Delatolla, R. (2019). Nitrifying moving bed biofilm reactor: Performance at low temperatures and response to cold-shock. Chemosphere, 229, 295–302. https://doi.org/10.1016/j.chemosphere.2019.04.176

Bagheri, M., & Mirbagheri, S. A. (2018). Critical review of fouling mitigation strategies in membrane bioreactors treating water and wastewater. Bioresource Technology, 258, 318–334. https://doi.org/10.1016/j.biortech.2018.03.026

Berney, M., Weillemann, H. U., Ihsen, J., Bassin, C., & Egli, T. (2006). Specific growth rate determines the sensitivity of Escherichia coli to thermal, UVA, and solar disinfection. Applied and Environmental Microbiology, 72(4), 2586–2593. https://doi.org/10.1128/aem.72.4.2586-2593.2006

Burmalle, M., Ren, D., Bjarnsholt, T., & Sørensen, S. J. (2014). Interactions in multispecies biofilms: Do they actually matter? Trends in Microbiology, 22(2), 84–91. https://doi.org/10.1016/j.tim.2013.12.004

Burmolle, M., Webb, J. S., Rao, D., Hansen, L. H., Sørensen, S. J., & Kjelleberg, S. (2006). Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Applied and Environmental Microbiology, 72(6), 3916–3923. https://doi.org/10.1128/aem.02022-05

Castrodaza, C. (1979). Nonprogressive evolution, the Red Queen hypothesis, and the balance of nature. Acta Biotheoretica, 28(1), 11–18. https://doi.org/10.1007/bf00546777

Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. Annual Review of Microbiology, 41, 435–464. https://doi.org/10.1146/annurev.mi.41.100187.002251

Dai, D., Raskin, L., & Xi, C. (2017). The effect of interactions between a bacterial strain isolated from drinking water and a pathogen surrogate on biofilms formation diverged under static vs flow conditions. Journal of Applied Microbiology, 123(6), 1614–1627. https://doi.org/10.1111/jam.13596

Deng, Y., Ruan, Y., Ma, B., Timmons, M. B., Lu, H., Xu, X., ... Yin, X. (2019). Multimics analysis reveals niche and fitness differences in typical denitrification microbial aggregations. Environment International, 132, 1–9. https://doi.org/10.1016/j.envint.2019.105085

Elias, S., & Banin, E. (2012). Multispecies biofilms: Living with friendly neighbors. FEMS Microbiology Reviews, 36(5), 990–1004. https://doi.org/10.1111/j.1574-6976.2012.00325.x

Emerson, B. C., & Gillespie, R. G. (2008). Phylogenetic analysis of community assembly and structure over space and time. Trends in Ecology & Evolution, 23(11), 619–630. https://doi.org/10.1016/j.tree.2008.07.005

Faust, K., & Raes, J. (2012). Microbial interactions: From networks to models. Nature Reviews Microbiology, 10(8), 538–550. https://doi.org/10.1038/nrmicro2832

Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., & Kjelleberg, S. (2016). Biofilms: An emergent form of bacterial life. Nature Reviews Microbiology, 14(9), 563–575. https://doi.org/10.1038/nrmicro.2016.94

Foster, K. R., & Bell, T. (2012). Competition, not cooperation, dominates interactions among culturable microbial species. Current Biology, 22(19), 1845–1850. https://doi.org/10.1016/j.cub.2012.08.005

Freilich, S., Zarecki, R., Eilam, O., Segal, E. S., Henry, C. S., Kupiec, M., ... Ruppin, E. (2011). Competitive and cooperative metabolic interactions in bacterial communities. Nature Communications, 2(1), 589. https://doi.org/10.1038/ncomms1597
Lawrence, D., Fiegna, F., Behrends, V., Bundy, J. G., Phillimore, A. B., Lagarde, F., Tusseau-Klasson, L. (2004). Evolution of minimal H. pylori genomes. *Microbiology, 150*(9), 2507-2515. https://doi.org/10.1099/00221087-227-9-2507

G o l d f o r d , J. E., Lu, N., Bajic, D., Estrada, S., Tikhomirov, M., Sanchez, N., McAvoy, M., et al. (2015). Biodegradation of phenolic compounds by a novel bacterial strain. *Journal of Industrial Microbiology and Biotechnology, 42*(6), 641-651. https://doi.org/10.1007/s10295-015-1790-9

Huang, L., Yuan, S., Meng, F., & Sorensen, S. J. (2016). Emergent simplicity in microbial community organization in multispecies biofilms. *The ISME Journal, 10*(3), 517-530. https://doi.org/10.1038/s41396-019-0494-9

Madsen, J. S., Røder, H. L., Sorensen, H., Burmølle, M., & Sørensen, S. J. (2016). Coexistence facilitates interspecific biofilm formation in complex microbial communities. *Environmental Microbiology, 18*(8), 2565-2574. https://doi.org/10.1111/1462-2920.13335

Martini, A. C. (2019). High proportions of bacteria are culturable across major biomes. *The ISME Journal, 13*(8), 2125-2128. https://doi.org/10.1038/s41396-019-0410-3

Mathieu, L. G., & Sonea, S. (1995). A powerful bacterial world. *Science, 269*(5226), 1489-1491. https://doi.org/10.1126/science.7882185

Matar, G. K., Bagchi, S., Zhang, K., Oerther, D. B., & Saikaly, P. E. (2017). Functional diversity of biofilm communities in full-scale membrane bioreactors (MBR): Sludge cake formation and fouling characteristics. *Water Research, 113*, 212-221. https://doi.org/10.1016/j.watres.2016.07.049

Moons, P., Michiels, C. W., & Aertsen, A. (2009). Bacterial interactions in biofilms. *Critical Reviews in Microbiology, 35*(3), 157-168. https://doi.org/10.1080/10408410902808943

Morris, J. J., Lenski, R. E., & Zinser, E. R. (2012). The Black Queen Hypothesis: Evolution of dependencies through adaptive gene loss. *mBio, 3*(2), e00036-00012. https://doi.org/10.1128/mBio.00036-12

Philippot, L., Andersson, S. G., Battin, T. J., Prosser, J. I., Schimel, J. P., Whitman, W. B., & Hallin, S. (2010). The ecological coherence of high bacterial taxonomic ranks. *Nature Reviews Microbiology, 8*(7), 523-529. https://doi.org/10.1038/nrmicro2367

Ping Chu, H., & Li, X.-Y. (2005). Membrane fouling in a membrane bioreactor (MBR): Sludge cake formation and fouling characteristics. *Biotechnology and Bioengineering, 90*(3), 323-331. https://doi.org/10.1002/bit.20409

Plata, G., Henry, C. S., & Vitkup, D. (2015). Long-term phenotypic evolution of bacteria. *Nature, 517*(7534), 369-372. https://doi.org/10.1038/nature13827

Regmi, P., Holgate, B., Miller, M. W., Park, H., Chandran, K., Wett, B., & Bott, C. B. (2016). Nitrogen polishing in a fully anoxic anaerobic MBBR treating mainstream nitritation-denitrification effluent. *Biotechnology and Bioengineering, 113*(3), 635-642. https://doi.org/10.1002/bit.25826
Reischer, A., Holler, B. M., Molin, S., & Zechner, E. L. (2006). Synergistic effects in mixed Escherichia coli biofilms: Conjugative plasmid transfer drives biofilm expansion. Journal of Bacteriology, 188(10), 3582–3588. https://doi.org/10.1128/jb.188.10.3582-3588.2006

Ren, D., Madsen, J. S., Sorensen, S. J., & Burmølle, M. (2015). High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. The ISME Journal, 9(11), 81–89. https://doi.org/10.1038/ismej.2014.96

Schink, B. (2002). Synergistic interactions in the microbial world. Antonie Van Leeuwenhoek, 81(1), 257–261. https://doi.org/10.1023/A:1020579004534

Seetha, N., Bhargava, R., & Kumar, P. (2010). Effect of organic shock loads on a two-stage activated sludge-biofilm reactor. Bioresource Technology, 101(9), 3060–3066. https://doi.org/10.1016/j.biortech.2009.12.055

Simoes, L. C., Simoes, M., & Vieira, M. J. (2007). Biofilm interactions between distinct bacterial genera isolated from drinking water. Applied and Environmental Microbiology, 73(19), 6192–6200. https://doi.org/10.1128/AEM.00837-07

Sriviriyarat, T., & Randall, C. W. (2005). Performance of IFAS wastewater treatment processes for biological phosphorus removal. Water Research, 39, 3873–3884. https://doi.org/10.1016/j.watres.2005.07.025

Stepanovic, S., Vukovic, D., Hola, V., Di Bonaventura, G., Djukic, S., Cirkovic, I., & Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMAIS, 115(8), 891–899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

Stewart, P. S. (2012). Mini-review: Convection around biofilms. Biofouling, 28(2), 187–198. https://doi.org/10.1080/08927014.2012.662641

Stewart, P. S., & Franklin, M. J. (2008). Physiological heterogeneity in biofilms. Nature Reviews Microbiology, 6(3), 199–210. https://doi.org/10.1038/nrmicro1838

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGAS: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28(10), 2731–2739. https://doi.org/10.1093/molbev/msr121

Teerlink, J., Hering, A. S., Higgins, C. P., & Drewes, J. E. (2012). Variability of trace organic chemical concentrations in raw wastewater at three distinct sewersheds scales. Water Research, 46(10), 3261–3271. https://doi.org/10.1016/j.watres.2012.03.018

Vamosi, S. M., Heard, S. B., Vamosi, J. C., & Webb, C. O. (2009). Emerging patterns in the comparative analysis of phylogenetic community structure. Molecular Ecology, 18(4), 572–592. https://doi.org/10.1111/j.1365-294X.2009.04001.x

Vanysek, L., Boerjan, B., Declerck, P., & Vankelecom, I. F. (2014). Biofouling ecology as a means to better understand membrane biofouling. Applied Microbiology and Biotechnology, 98(19), 8047–8072. https://doi.org/10.1007/s00253-014-5921-2

Vetsigian, K., Jajo, R., & Kishony, R. (2011). Structure and evolution of Streptomyces interaction networks in soil and in silico. PLoS Biology, 9(10), e1001184. https://doi.org/10.1371/journal.pbio.1001184

Violle, C., Nemergut, D. R., Pu, Z., & Jiang, L. (2011). Phylogenetic limiting similarity and competitive exclusion. Ecology Letters, 14(8), 782–787. https://doi.org/10.1111/j.1461-0248.2011.01644.x

Wells, G. F., Shi, Y., Laurenzi, M., Rosenthal, A., Szivak, I., Weissbrodt, D. G., ... Morgenroth, E. (2017). Comparing the resistance, resilience, and stability of replicate moving bed biofilm and suspended growth combined nitrification-anammox reactors. Environmental Science & Technology, 51(9), 5108–5117. https://doi.org/10.1021/acs.est.6b05878

Wingender, J., & Fleming, H. C. (1999). Autoggregation of microorganisms: Flocs and biofilms (1, Biotechnology Second, Completely Revised Edition.). Weinheim, Germany: Wiley-Blackwell Verlag GmbH.

Wintermute, E. H., & Silver, P. A. (2010). Emergent cooperation in microbial metabolism. Molecular Systems Biology, 6(1), 407. https://doi.org/10.1038/msb.2010.66

Xia, J., Chen, J., Chen, Y., Qian, G., & Liu, F. (2018). Type IV pili biogenesis genes and their roles in biofilm formation in the biological control agent Lysobacter enzymogenes OH11. Applied Microbiology and Biotechnology, 102(2), 833–846. https://doi.org/10.1007/s00253-017-8619-4

Xu, R., Yu, Z., Zhang, S., & Meng, F. (2019). Bacterial assembly in the bio-cake of membrane bioreactors: Stochastic vs. deterministic processes. Water Research, 157, 535–545. https://doi.org/10.1016/j.watres.2019.03.093

Yan, J., Nadell, C. D., & Bassler, B. L. (2017). Environmental fluctuation governs selection for plasticity in biofilm production. The ISME Journal, 11(7), 1569–1577. https://doi.org/10.1038/ismej.2017.33

Yawata, Y., Cordero, O. X., Menolascina, F., Hehemann, J.-H., Polz, M. F., & Stocker, R. (2014). Competition-dispersal tradeoff ecologically differentiates recently speciated marine bacterioplankton populations. Proceedings of the National Academy of Sciences of the United States of America, 111(15), 5622–5627. https://doi.org/10.1073/pnas.1318943111

Young, B., Delatolla, R., Kennedy, K., LaFlamme, E., & Stintzi, A. (2017). Post carbon removal nitrifying MBBR operation at high loading and exposure to starvation conditions. Bioresource Technology, 239, 318–325. https://doi.org/10.1016/j.biortech.2017.05.024

Zhang, K., Choi, H., Dionysiou, D. D., Sorial, G. A., & Oeterher, D. B. (2006). Identifying pioneer bacterial species responsible for biofouling membrane bioreactors. Environmental Microbiology, 8(3), 433–440. https://doi.org/10.1111/j.1462-2980.2005.00909.x

Zhang, S., Zhou, Z., Li, Y., & Meng, F. (2017). Deciphering the core fouling-causing microbiota in a membrane bioreactor: Low abundance but important roles. Chemosphere, 195, 108–118. https://doi.org/10.1016/j.chemosphere.2017.12.067

Zhou, J., & Ning, D. (2017). Stochastic community assembly: Does it matter in microbial ecology? Microbiology and Molecular Biology Reviews, 81(4), e00002-e00017. https://doi.org/10.1128/mmbrr.00002-17

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