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Outcompeting Presence of Acyl-Homoserine-Lactone (AHL)-Quenching Bacteria over AHL-Producing Bacteria in Aerobic Granules

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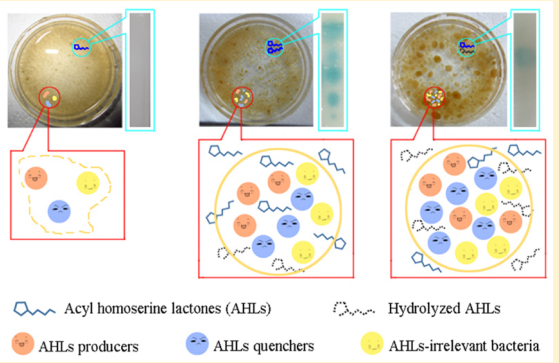
Supporting Information

ABSTRACT: The roles of acyl-homoserine-lactone (AHL)-based quorum sensing in biofilm formation for biological wastewater treatment have been widely recognized. In previous studies about the role of AHLs in biofilm formation, the focus is always on AHL-producing bacteria, while attention to AHL-quenching bacteria is not paid. In this work, 600 microbial isolates were screened from aerobic granules, a special type of biofilm, based on their AHL-producing or -quenching ability. The isolates with quenching activity were mainly *Rhodococcus* sp.; others included *Sphingopyxis* sp. and *Bacillus* sp. This result indicates the outcompeting presence of AHL-quenching bacteria over AHL-producing bacteria and a high complexity of AHL-based communication in the aerobic granules and also implies the possible overlooking of the role of AHLs in biofilm formation in previous studies.



INTRODUCTION

Quorum sensing (QS) is a phenomenon through which bacteria synchronize specific gene expression via the production, accumulation, and reorganization of signal molecules such as acyl-homoserine-lactones (AHLs), furanosyl borate diester, and oligopeptides.[1] These signal molecules, or autoinducers, are continuously synthesized and secreted and accumulate as the size of bacterial community increases. When the signal molecule concentration reaches a threshold value, some community-level behaviors are initiated.[2,3] Previous studies of single cultures or cocultures with pure species have revealed the vital role of AHLs in tuning group behaviors, such as biofilm formation. Thus, AHL-mediated QS has been introduced to tune biofilm formation in wastewater treatment systems.

However, it is worth noting that, compared with these pure cultures, activated sludge is much more complex. In biological wastewater treatment reactors, multiple bacterial species coexist, among which some can produce AHLs while others can quench them.[4−6] AHLs are known to be involved in biofilm formation. An aerobic granule is a specific form of biofilm formed by self-aggregation of bacteria and frequently found in wastewater treatment systems. Thus, it is reasonable to expect that, like in normal biofilm systems, AHL producers and quenchers also coexist in aerobic granules. However, so far in QS-related studies, focus is always on AHL-producing bacteria, while attention to AHL-quenching bacteria is not paid.[8−11] The AHLs detected in bioreactors are merely an overall result of the interactions between AHL producers and quenchers, and in some cases, the overall AHL concentration is too low to be detected despite the existence of local QS activity.[7] This may lead to misinterpretation of the microbial community behaviors in biological wastewater treatment reactors. Thus, the possible interactions between AHL producers and quenchers in aerobic granules deserve an in-depth investigation.

Because the AHL synthetase genes are heterogeneous,[8] it is impossible to directly detect the AHL-producing ability of a bacterium by using polymerase chain reaction (PCR) or rRNA probing technologies. By comparison, a bacterial screen could be used as an effective tool to distinguish the AHL producers and quenchers. By using this approach, various AHL-related bacteria have been screened from natural and engineered microbial systems,[8,9] and some of them have been applied for the fouling control of membrane bioreactors.[10] Therefore, in this work, both AHL producers and quenchers were screened

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from aerobic granules, and a total of 600 isolates were obtained and their AHL-producing and -quenching capabilities were evaluated. With the screening results, the coexistence of both bacterial groups in the aerobic granules was demonstrated and the species were identified, and their roles in the aerobic granulation of activated sludge were elucidated.

MATERIALS AND METHODS

Cultivation of Aerobic Granules. Aerobic granules were cultivated in a lab-scale sequencing batch reactor, fed with synthetic wastewater. Details about the reactor and the wastewater are provided in the Supporting Information.

AHL-Producing and -Quenching Activities of Sludge. To determine the AHL-producing activity of the sludge, mixed liquor was collected from the reactor and AHLs were extracted with ethyl acetate. To examine the AHL-quenching activity, mixed liquor was collected from the reactor, supplemented with a given amount of C8-HSL to a final concentration of 200 nM, and then incubated in a thermostat shaker. The remaining C8-HSL was extracted with ethyl acetate and assessed as described below. The quenching activity of the seed sludge was also determined. The detailed measurement procedures are given in the Supporting Information.

Determination of AHLs. The AHLs were detected by thin layer chromatography (TLC) according to a previous report.[11] TLC plates were purchased from Merck KGaA (115683|TLC Silica gel 60 RP-18 F_{254}s, Merck KGaA). Reporter strain *Agrobacterium tumefaciens* JZA-1 was used to detect AHLs. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) was purchased from Sangon Biotechnology Co.

Isolation of Bacteria from Aerobic Granules. Aerobic granules were collected from the reactor on day 90 and washed three times with sterilized 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and ground. Then, the sludge samples were resuspended in HEPES buffer and shaken vigorously. By serial dilution in sterile HEPES, the suspension was plated out on R2A agar and incubated at 30 °C. To avoid missing the isolates with a slower growth rate, in the 10 day incubation, the single clones were picked on days 6, 8, and 10 and transferred to 96-well plates containing 100 μL of R2A medium per well. The picked colonies were marked to prevent them from being repicked. The AHL-producing and -quenching activities of these strains were then analyzed.

Screening and Identification of AHL-Producing and -Quenching Strains. To screen the AHL producers, the isolates were incubated for 48 h and then centrifuged. Twenty microliters of supernatant was transferred into another 96-well plate; each well contained an 80 μL overnight culture of reporter strain JZA-1. X-gal (20 mg/L) was also added as a color-developing agent. After overnight incubation, the wells that showed blue color were marked, and the corresponding strains were collected and stored in a −80 °C freezer. To screen the AHL-quenching strains, after a 48 h incubation, 50 μL of the culture was transferred to a new 96-well plate containing 50 μL of fresh R2A medium and 10 μM C8-HSL and incubated overnight. After centrifugation of the culture overnight after overnight incubation, 20 μL of supernatant was collected and transferred into another 96-well plate containing an 80 μL overnight culture of reporter strain JZA-1 and 20 mg X-gal/L in each well. After overnight incubation, the wells that showed no color were marked and the corresponding strains were collected and stored in a −80 °C freezer. Finally, the collected AHL-producing and -quenching candidates were cultured and purified again by repeating the procedures described above to obtain pure strains.

The 16S rDNA of isolated strains was amplified with forward primer 27F and reverse primer 1492R using a mix (Sangon Biotechnology Co.). DNA extraction was conducted using an Ezup bacterial DNA extraction kit (Sangon Biotechnology Co.). PCR amplification was performed using a 2720 thermal cycler (ThermoFisher Scientific Inc.). ClustalX version 2.0 (Science Foundation Ireland, Dublin, Ireland) was used for sequence alignment of the PCR product. Phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0.

RESULTS AND DISCUSSION

Production and Evolution of AHLs in the Reactor. The granulation process and the variation of AHLs in the granulation process of activated sludge are shown in Figure 1a. The seeding sludge exhibited no AHL-producing activity,

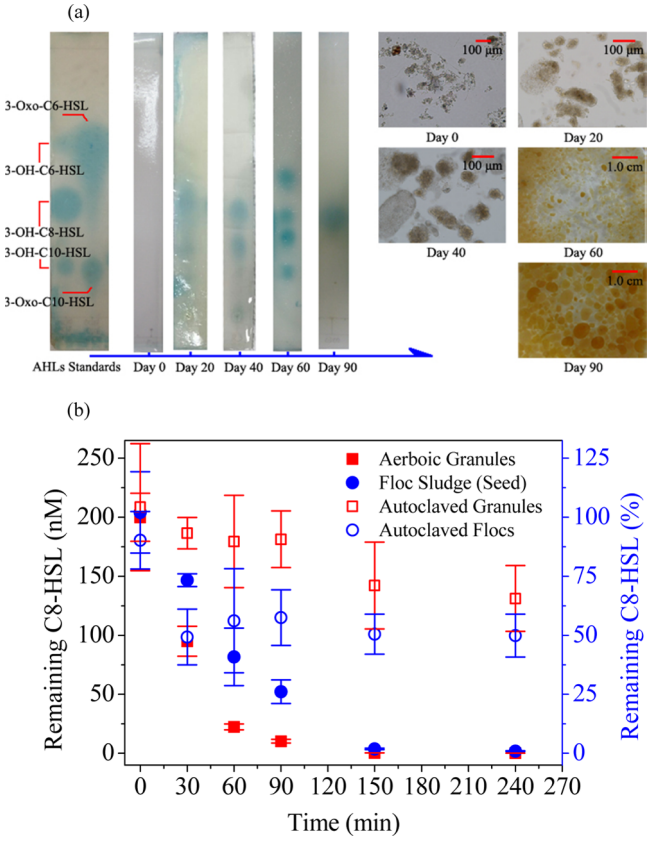

Figure 1. Evolution of sludge morphology and AHL-producing profiles during a 90 day operation of the reactor (a) and AHL-quenching activity of the mature aerobic granules added to C8-HSL (b).

but after a short period of acclimation (~20 days), AHLs were detected. Accompanied with the granulation of sludge, both the activity and type of the produced AHLs in the reactor increased initially (day 40 to day 60). However, when the granule size further increased and became mature after 90 days, only one type of AHL was detected. One possible explanation is that the development of AHL-producing and -quenching groups in the granules was not synchronous. Also, the decreased type of AHLs might be related to the stratified structure of the aerobic

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granules and/or the growing population size of the AHL-degrading bacteria.

The quenching activity of the mature aerobic granules was also determined by the addition of C8-HSL (Figure 1b). The added C8-HSL was rapidly degraded by the aerobic granules, and the degradation was completed within 150 min, exhibiting a great quenching activity of the matured granules. This result indicates the net production of AHLs was greater and the possibility of producing more types of AHLs could not be excluded. This is consistent with a previous work,[7] and the screening for AHL-producing and -quenching strains should be conducted to explore this phenomenon.

AHL-Producing and -Quenching Strains. A total of 600 clones were picked and screened for their AHL-producing and -quenching activity. As shown in Figure 2, most isolates with

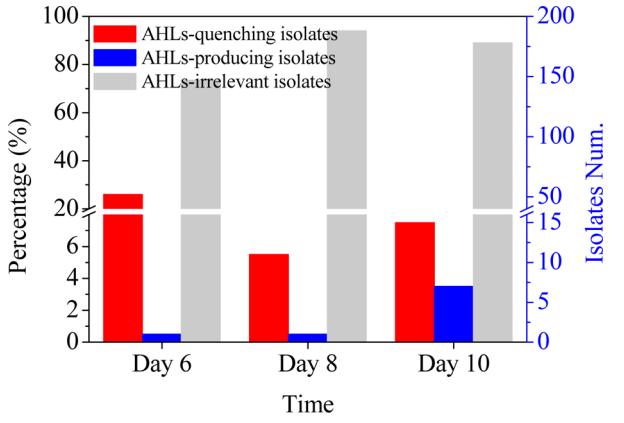

Figure 2. Percentage of the AHL-producing and -quenching isolates collected on days 6, 8, and 10.

AHL-producing activity were picked on day 10, and those with quenching activity were mostly picked on day 6. This indicates a growth rate of the AHLs producers in the complex microbial consortium relatively lower than that of the AHL quenchers. AHL-producing groups with a lower growing rate could reach a large population size, which could be partially used to explain why AHLs were more likely to be detected in a sludge system with a longer solid retention time and/or higher sludge concentration. This result shows that, although AHL producers and quenchers coexisted in aerobic granules, most isolates exhibited neither AHL-producing nor AHL-quenching activity and the number of AHL-producing isolates was smaller than that of the AHL-quenching isolates. This is agreement with a previous report,[7] in which both the quenching ability of activated sludge and local AHL production were detected.

Identification of AHL-Producing and -Quenching Isolates. Among the 600 isolates, 87 showed AHL-related activities, including nine AHL-producing and 78 AHL-quenching strains. On the basis of the colony morphology and restriction endonuclease analysis, 12 quenching isolates and three producing isolates were chosen for further identification. The 16S rDNA analysis shows that the AHL-producing strains belong to genus *Shinella* and *Sphingomonas*, and quenching strains include *Rhodococcus*, *Bacillus*, and *Sphingopyxis*. A phylogenetic tree was constructed (Figure 3). The production of AHLs by *Shinella* sp. has been reported previously.[12] In the work presented here, *Shinella* sp. showed the highest level of AHL production among all the AHL-producing isolates. Because this group of bacterium can also degrade pyridine,[13]

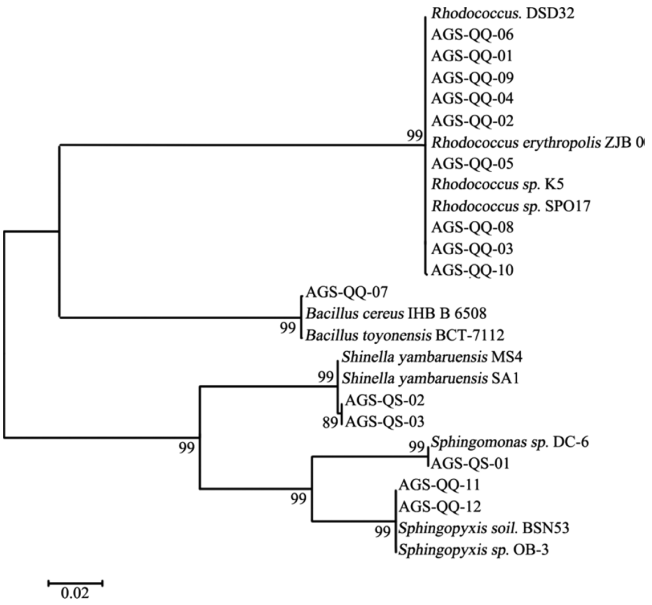

Figure 3. Phylogenetic tree constructed on the basis of 16S rDNA analysis.

polycyclic aromatic hydrocarbon,[12] and 3-methyl-sulfolane[14] and reduce nitrate,[15] it is hypothesized that some of the metabolic activities might be regulated by its QS system, as reported for other bacterial species such as *Pseudomonas*.[16] Another AHL-producing isolate, AGS-QS-02, belongs to genus *Sphingomonas*, which also possess a wide range of substrate degradation ability.[17] Although its AHL-producing ability has been reported previously,[18,19] it is unclear which roles the QS system plays in the degradation of refractory organics.

Most of the AHL-quenching isolates belong to genus *Rhodococcus*, which has been successively applied to membrane fouling control.[10,20] The presence of this group in the aerobic granules indicates its high AHL-quenching potential. The other two AHL-quenching isolates belong to genus *Sphingopyxis* and *Bacillus*. This finding is consistent with previous studies, in which some members of these species were found to possess strong AHL-quenching ability.[21−24]

More AHL-quenching bacteria over AHL-producing bacteria were found in the aerobic granules in this work. Because the detected AHL signal was a result of the balance between AHL-producing and -quenching communities, the increased AHL level in the sludge granulation process suggests that the aerobic granules possessed an AHL-producing activity higher than the AHL-quenching activity. Taking together the relative smaller group size of AHL-producing strains, roles of AHLs in biofilm formation, and the fade-out of AHLs with growing granule size, we concluded that the ratio of the AHL producers and quenchers was another important factor influencing the granulation of activated sludge.

TLC Assay of the AHLs Produced by the AHL-Producing Isolates. AHLs from the AHL-producing isolates were extracted and assayed by the TLC method (Figure S1). The isolates from genus *Shinella* produced three different types of AHLs, and the isolates from genus *Sphingomonas* produced two. Information about the AHL-mediated metabolisms in these two strains is lacking, especially for *Shinella* sp. As mentioned above, these two strains can metabolize a wide spectrum of substrates, including some toxic contaminants. QS circuits in bacteria are often related to the expression of certain

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genes that are significant for the thriving and survival of bacteria.[25] In recent studies of AHL-related wastewater treatment, the quenching activity of some bacteria to mitigate membrane fouling is a focus. However, biofilm formation in other biological wastewater treatment, such as aerobic granules and reactors with biofilm carriers, is desirable and could be enhanced by AHL-based QS. Thus, further studies to explore the relationship between QS and the degrading ability of bacteria could be helpful in optimizing biological wastewater treatment and bioremediation processes.

Coexistence of AHL-Producing and -Quenching Bacteria. The experimental results clearly demonstrate the coexistence of the AHL-producing and -quenching bacteria as well as other irrelevant species in the aerobic granules. Most isolates exhibited no AHL activity; i.e., they neither produced nor quenched AHLs. The number of AHL-quenching isolates (78) was far greater than the number of AHL-producing isolates (9), although the exact ratios of these three bacterial groups need to be investigated further.

The findings in this work will give rise to four questions to be answered in future studies. First, AHLs are known to improve multispecies biofilm formation, but how much AHLs could be viewed to be "sufficient" to take effect and whether AHLs at an overly high level are detrimental to biofilm formation could not be determined. Second, are AHLs indispensable for biofilm formation, and could a multispecies biofilm be formed in the absence of AHL-producing bacteria or even rich in AHL-quenching bacteria? Third, which roles do the AHL-quenching bacteria and other bacteria play in a multispecies biofilm? AHL-quenching bacteria can quench and provide extracellular polymeric substances to the biofilm matrix, while other bacteria without a response to AHLs are the majority of microbial strains in a biofilm. Finally, compared with other factors influencing biofilm formation, what is the contribution of AHLs to multispecies biofilm formation? The coexistence of AHL-producing and -quenching bacteria in the aerobic granules suggests a high complexity of AHL-based communication in multispecies biofilm formation, which has been overlooked previously. This warrants further in-depth investigations.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00332.

Additional figures and a detailed description of methods (PDF)

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Notes

The authors declare no competing financial interest.

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