Effect of interspecies quorum sensing on the formation of aerobic granular sludge

Sheng-Hua Zhang, Xin Yu, Feng Guo and Zhuo-ying Wu

ABSTRACT

Quorum sensing (QS) is a form of microbial communication that relies on small signal molecules to regulate group behaviors such as biofilm formation in response to population density. In this study, we attempted to apply the paradigm of bacterial QS to aerobic granular sludge (AGS) formation for wastewater treatment. An essential element of interspecies QS signals, boron, was added to a sequencing batch reactor (SBR) to stimulate AGS growth. Bioassays elaborated the activity of autoinducer-2 (AI-2). We found that boron accelerated AGS growth, resulting in improved settlement performance and increased biomass in the SBR. During continuous SBR operation, the AGS showed an obvious increase in AI-2 activity, which implies that interspecies QS was closely associated with AGS formation. Analysis of EPS showed that boron stimulated tryptophan production, and increased the hydrophobia of AGS. From these results, it was speculated that the addition of boron may have promoted the formation of boron complexed to (R)-4, 5-dihydroxy-2,3-pentanedione (DPD) as the precursor of AI-2, which resulted in accelerated interspecies QS. The results also suggested QS as a novel regulation target for the biogranulation process, such as AGS formation.

Key words | aerobic granular sludge (AGS), autoinducer-2 (AI-2), boron, auorum sensing (QS)

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INTRODUCTION

Aerobic granular sludge (AGS) is a promising technology for wastewater treatment. Granules are aggregates of microbes, and they are generally larger, denser, rounder, and more regular in appearance than other aggregates such as bioflocs (Mishima & Nakamura 1991). Aerobic granular sludge can prevent biomass loss significantly, thereby allowing reactors to operate at higher biomass concentrations. Compared to the anaerobic granulation process, AGS can overcome many drawbacks, including long start-up (normally 2–8 months), relatively high operation temperatures, and incapability of low-strength organic wastewater treatment (Moy *et al*. 2002; Tay *et al*. 2005).

However, the formation and maintenance of regularly shaped AGS is not easily implemented. Many previous studies have tried to reveal the intrinsic mechanism of this process. Many of them are focused on the impact of operation parameters. For example, shear force has been proven to play an important role in the cell-immobilization system and cell hydrophobicity. When bacteria become more hydrophobic, cell surface hydrophobicity can contribute to aggregation ability, such that increased cell-to-cell adhesion is observed (Tay *et al*. 2001; Liu *et al*. 2003). In addition, the property of the materials bound to the cell surfaces, mainly the extracellular polymers (EPS), is also the key factor for the formation of AGS. EPS are metabolic products accumulating on the surface of bacterial cells constituting proteins, polysaccharides, humic acids, and lipids, which could alter the physico-chemical characteristics of cellular surface such as charge, hydrophobicity and other properties.

EPS adsorbed on the surface of aerobic biogranules led to increased water content and loose-structure of biogranules (Nagaoka *et al*. 1996). Enrichment of certain EPS was helpful to initiate the aerobic granulation process, enhance granule stability, and resist shocks and toxins (Liu *et al*. 2004). Sheng & Yu (2006) and Adav *et al*. (2007) analyzed shifts in position or change in intensity of EEM peaks as evidence of chemical changes in the extracted EPS.

In this study, the concept of bacterial quorum sensing was applied to the formation of AGS in a sequencing batch reactor (SBR). Many bacteria synthesize and secrete small autoinducer (AIs) molecules into the surrounding environment, and as the population density increases,

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accumulation of these molecules eventually reaches a threshold concentration which, in turn, regulates gene expression. This process is called quorum sensing (QS). To date, several different AIs have been identified, but three systems have been more intensively studied, namely, oligopeptides, N-acylhomoserine lactones (AHLs), and autoinducer-2 (AI-2). While oligopeptides and AHLs are merely involved in cellular communication of gram-positive and gram-negative bacteria, respectively, AI-2 is produced by a large number of bacterial species and is universal for interspecies communication of both gram-positive and gram-negative bacteria. As indicator bacteria, *Vibrio harveyi* BB170, which lacks the LuxN receptor for AHLs but contains the sensor protein (LuxP) for AI-2 (Bassler *et al.* 1993) has been used extensively to investigate AI-2 production in bacteria. QS-controlled behaviors have been demonstrated to be involved in the development of bacteria biofilms, such as *Pseudomonas aeruginosa* (Davies *et al.* 1998), *Staphylococcus aureus* (Cuong *et al.* 2000) and *Streptococcus mutans* (Dilani & Dennis 2008). QS regulation of genes is directly involved in biosynthesis of the biofilm matrix (Yumiko & Roberto 2007). In view of its essential role in biofilm formation, strategies of disrupting QS systems have been discussed to control microbial attachment and membrane biofouling (Yeon *et al.* 2009).

From the above it is reasonable to regard QS as a driving factor to promote the formation of AGS since it has the same biological nature as microbial aggregates. We hypothesized that, in principle, AGS and biofilm have the same biological nature as microbial aggregates, and the formation of AGS could be promoted through QS, in which intercellular communication is strengthened to obtain AGS.

Boron is widely available in nature and is essential for vascular plants and several other organisms (Loomis & Durst 1992), but it has not been assigned a functional role in biological systems. Our results provide speculation for a biochemically defined function for boron in bacterial quorum sensing. Boron complexed to DPD is an essential requirement for the activity seen in AI-2 (Meijler *et al.* 2004). Compared with previously characterized autoinducers, AI-2 is a furanosyl borate diester formed from the reaction of ring-closed (R)-4, 5-dihydroxy-2,3-pentanedione (DPD) and boric acid (Chen *et al.* 2002), and AI-2 has an extremely uncommon structure with the presence of a boron atom (Meijler *et al.* 2004). Given its electron density and stability, boron was considered for the atom bridging the diester, and the borate diester is relatively stable (Chen *et al.* 2002). In our study, boron was added into the SBR to evaluate the AGS formation process and the activity of QS autoinducers. Addressing the QS characteristics of AGS will provide insight into microbial granules and may facilitate engineering and granulation as a promising technology in biological wastewater treatment.

MATERIALS AND METHODS

Cultivation of aerobic granules

Two columns (120 cm high, 50 mm in diameter) with a working volume of 1.96 l were used as SBRs during the study. The SBRs were seeded with freshly activated sludge obtained from a domestic wastewater treatment plant in Xiamen City, China, and operated at a cycle time of 4 h, composed of 5 min settling, 5 min discharging, 7 min filling, and 223 min aeration. The same volume exchange ratio of 40% and superficial air up-flow rate of 2.7 cm s^{-1} were applied. Reactor 1 (R1) was taken as the control, and Reactor 2 (R2) was the tested one. Synthetic wastewater with the following composition was used: Sodium acetate was the sole carbon source, 1.27 g L^{-1}; NH_4Cl, 191 mg L^{-1}; K_2HPO_4, 56 mg L^{-1}; $MgSO_4{\cdot}7H_2O$, 14 mg L^{-1}; $FeSO_4{\cdot}7H_2O$, 11 mg L^{-1}; $CaCl_2{\cdot}H_2O$, 17 mg L^{-1}, H_3BO_3 (only added in R2), 200 mg L^{-1}; trace solution 1 ml L^{-1}. This gave a total COD of 5 kg m^{-3} d^{-1}. The composition of the trace solution was: $FeCl_3{\cdot}6H_2O$, 1,500 mg L^{-1}; $CuSO_4{\cdot}5H_2O$ 30 mg L^{-1}, KI 30 mg L^{-1}, $MnCl_2{\cdot}4H_2O$ 120 mg L^{-1}, $Na_2MoO_4{\cdot}2H_2O$ 60 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 120 mg L^{-1}, $CoCl_2{\cdot}6H_2O$ 150 mg L^{-1}.

Assay of AI-2

In the last 1 min before settling, about 2 mL mixture in the SBR was collected and centrifuged at 12,000 rpm for 10 min. The supernatant was filtered through a 0.22 μm filter and then transferred to a clean tube as cell-free culture. The AI-2 activity in the aerobic granular sludge was measured using the *Vibrio harveyi* BB170 bioluminescence reporter assay, with *Vibrio harveyi* BB120 used as positive control. The AI-2 detection mechanism of *Vibrio harveyi* is described by Michiko (2005). The bioluminescence of *Vibrio harveyi* BB170 was measured by a multifunctional microplate reader (SpectraMax M5, America).

Extraction and assay of EPS from mixed liquid

About 15 mL of mixed activated sludge or aerobic granular sludge liquid was collected once a week from the SBR. The

extraction of extracellular polymers (EPS) from the mixed liquid is described by Zhang *et al.* (1999). The three-dimensional excitation-emission matrix (EEM) of the EPS samples were also determined as mentioned in previous literature (Aryal *et al.* 2009).

Other analytical methods

Mixed liquid suspended solids (MLSS) and sludge volume index (SVI) were determined by standard methods (Eaton *et al.* 1995). The size of the granular sludge was measured by a laser particle size analysis system (Mastersize 2000, Malvern, UK). The morphology of the aerobic granular sludge was observed with an Olympus SZX9 microscope.

RESULTS AND DISCUSSION

The effect of boron addition on quorum sensing and subsequently on granule formation was investigated. This study was conducted over 35 days as AGS could be well formed and remain stable, and obvious differences between the control and tested reactors occurred in this duration. The granular sludge properties and the quorum sensing performances are described for the two reactors.

Granular sludge properties

In both reactors, the size of the granule increased with culture time (see Figure 1(g)). The first granules were observed one week after the running of R1 and R2, a significant

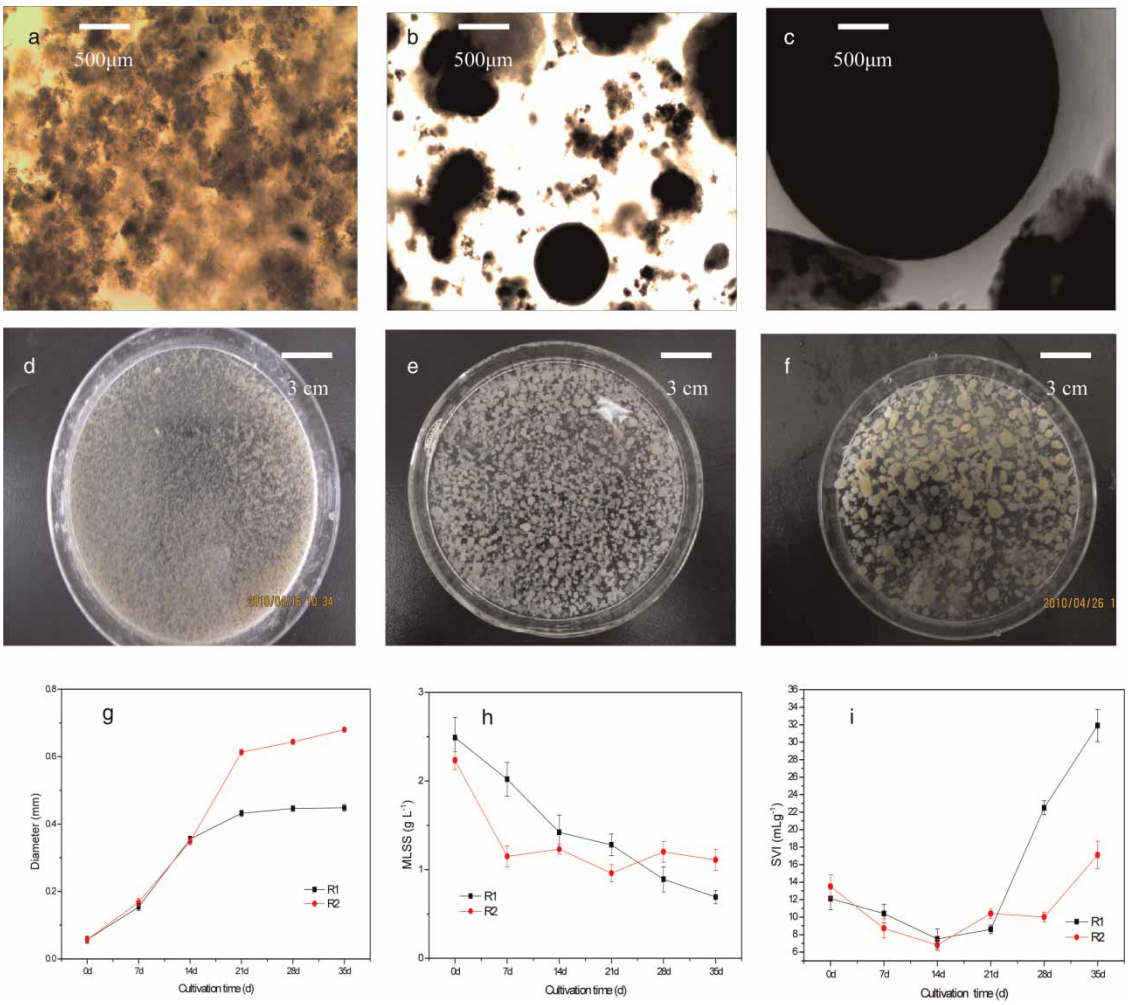

Figure 1 | The growth of aerobic granular sludge during 35 d cultivation in R1 and R2. Morphologies of aerobic granular sludge sampled at 0 d (a and d); 21 d (b: R1, c: R2); 35 d (e: R1, f: R2). Average granular diameter change is shown in (g). MLSS versus cultivation time is shown in (h). SVI versus culture time is shown in (i).

amount of granules were observed and granular size increased faster in R2 than R1 after day 14. Biomass was a mixture of intact granules in the two reactors, and the color was not uniform. After 21 days' cultivation, the average diameter of the granules was 0.43 mm in R1 and 0.61 mm in R2. At the end of cultivation (35 days), the average diameter of granules in R1 and R2 were 0.45 mm and 0.68 mm, respectively, and AGS in R2 was tighter and more regular than in R1 (see Figure 1(a–f)).

Figure 1(h) shows the biomass concentration in R1 and R2. From day 0 to day 35, as a result of hydraulic selection pressure (Qin, *et al*. 2004), a part of the floc sludge was washed out of the reactor, and the biomass concentration decreased slowly in both R1 and R2. From day 21 to day 35, R2 gradually reached a steady state, and the biomass concentration in R2 was bigger than R1, indicated by the stable biomass concentrations of 1.11 gMLSS L^{-1}. Sludge volume index (SVI) is commonly used to indicate settleability and compactness of activated sludge in environmental engineering. Figure 1(i) shows the SVI values in R1 and R2, which clearly demonstrates that SVI in R1 and R2 were normal and show no sludge bulking phenomenon.

Characterizations of AI-2

It has been suggested that AI-2 can serve as a universal interspecies quorum sensing signaling molecule (Bassler *et al*. 1993). Figure 2 shows the AI-2 activity in R1 and R2, which indicates that aerobic sludge granulation and boron addition improved AI-2 activity of aerobic sludge cells significantly. From day 0 to day 14, AI-2 activity increased quickly from 0.83 to 2.33 in R1 and from 0.83 to 8.37 in R2. During day 14 to day 35, AI-2 activity reached a steady state in both reactors, indicated by stable AI-2 activity of 1.2 in R1 and 1.8 in R2. In all experimental stages, AI-2 activity was higher in R2 than R1, which demonstrated that better cell communication occurred in R2.

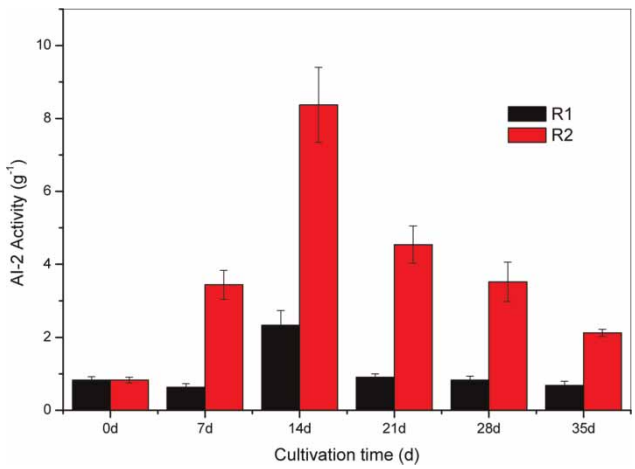

Figure 2 | Variation of AI-2 activities of AGS sampled at different culture time. The AI-2 activity was represented by the highest luminescence value ratio between samples and AB medium control sample of per gram sludge (g^{-1}). The luminescence was measured using the *V. harveyi* BB170 AI-2 bioassay.

As Figure 2 shows, AI-2 activity in this study significantly improved with the addition of boron. In this case, LuxS catalyzed *S*-ribosylhomocysteine conversion to DPD and homocysteine. As pro-AI-2, DPD has a relatively compact five-carbon framework. The skeletal backbone of DPD is densely functionalized in a continuous distribution containing two hydroxy and two keto moieties, and which suggests that DPD is a highly reactive molecule and can exist in normal physiological conditions (Kennedy *et al*. 1995). In this study, AI-2 activity in R2 was substantially higher than in R1, which suggests that the addition of boron stimulated the formation of boron complexed to DPD, as well as the LuxP-AI-2 complex. The higher AI-2 activity observed in this study contributed to intercellular communication to promote AGS formation.

It is worth mentioning that AI-2 molecular containing boron seems only to be associated with environmentally isolated bacteria, such as bacteria in AGS. Miller *et al*. (2004) presented the crystal structure of a second AI-2 signal binding protein, LsrB from *Salmonella typhimurium*. They found that LsrB binds a chemically distinct form of the AI-2 signal, (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF), that lacked boron, and demonstrated that both autoinducer signals derived from 4,5-dihydroxy-2,3-pentanedione (DPD).

Analysis of EPS

The EEM spectra of the EPS in R1 and R2 at various operation times are shown in Figure 3. Three main peaks were readily identified from the EEM fluorescence spectra. The first main peak was identified at the excitation/emission wavelengths (Ex/Em) of 370/455 nm (Peak A), while the second (Peak B) and third (Peak C) main peaks were observed at the Ex/Em of 280/335 and 275/455 nm. Peak A was reported as humic acid-like, in which the fluorescence is associated with hydrophobic acids (Baker & Inverarity 2004), and Peak B was described as a protein-like peak, in which the fluorescence is associated with aromatic amino acid tryptophan (Coble 1996). Peak C was also regarded as humic acid-like. Tryptophan (protein-like substances) was dominant among organic matter with

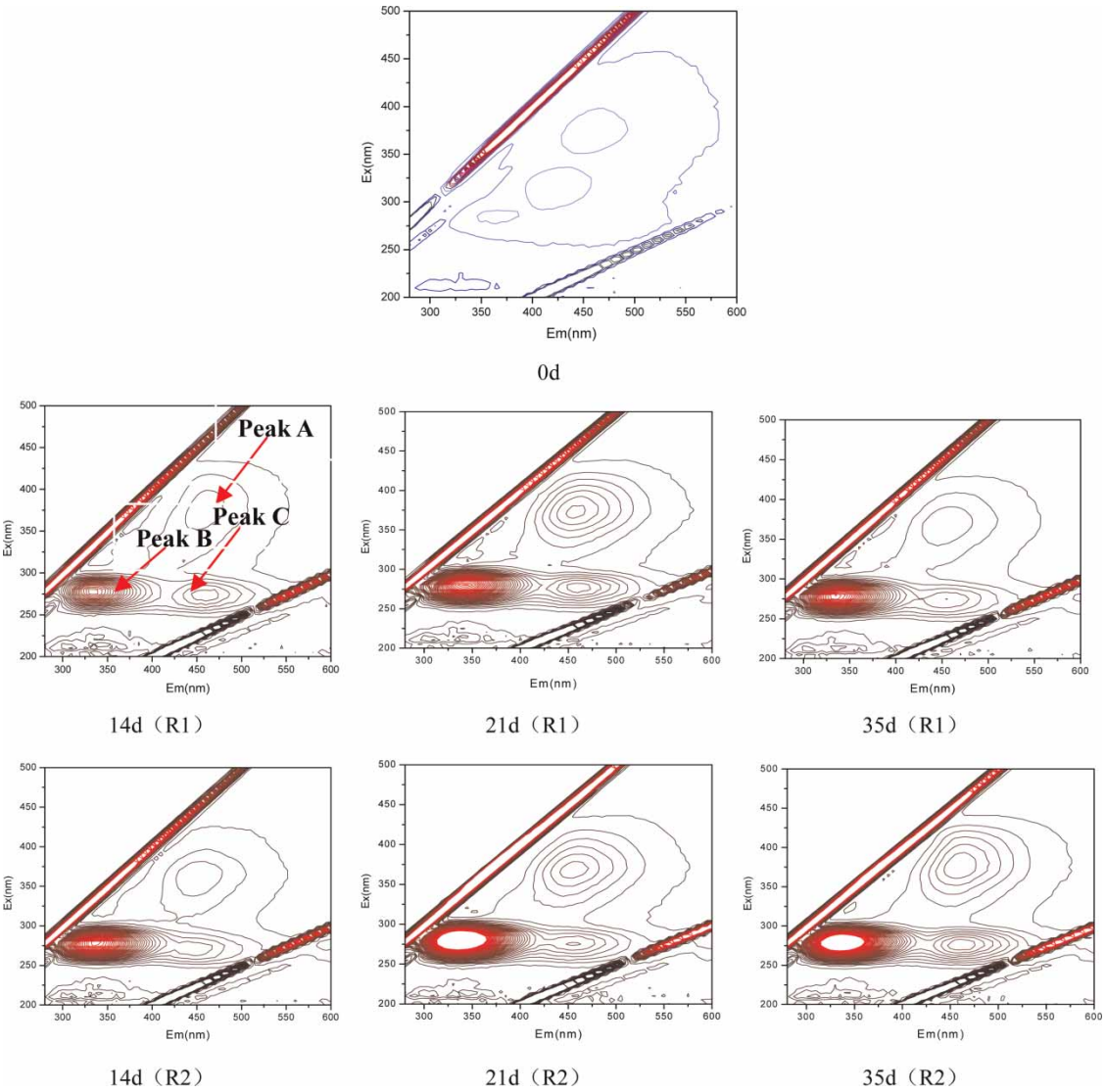

Figure 3 | EEM spectra of the EPS samples collected at different time in R1 and R2.

fluorescence characteristics in the EPS, with fluorescence intensity of tryptophan in R2 typically higher than in R1 (Table 1).

Previous studies have demonstrated that EPS production is controlled by quorum sensing in a population density dependent manner, e.g., the expression of EPS genes in *V. cholerae* (Bodman *et al*. 1998; Hammer & Bassler 2003). LasR is part of the quorum sensing system in *Pseudomonas aeruginosa* as opposed to *V. cholerae*. It has also been speculated that EPS-associated genes are likely controlled by LasR-3-oxo-C12-HSL on *Pseudomonas aeruginosa* biofilm formation (Shih & Huang 2002). As shown in Figure 3, while fluorescence peak locations did not change significantly during the AGS cultivation process, fluorescence peak intensities did. The EEM results reveal that the relative concentrations of humic acid-like substances were relatively steady during the AGS formation process in both reactors. Tryptophan fluorescence was dominant and had a maximum fluorescence at 280/335 nm (Table 1). Tryptophan fluorescence intensity was constantly higher in R2 than in R1. Importantly, tryptophan is a hydrophobic amino acid, and previous studies have shown that hydrophobicity is the main driving force for biogranulation (Liu *et al*. 2004). To our knowledge, therefore, the faster growth of AGS in R2 may be attributed to their hydrophobicity from hydrophobic substances represented by trytophan.

Table 1 | Fluorescence spectral parameters of the EPS sample

		Intensity of Peak A (Ex/Em: 370/455)	Intensity of Peak B (Ex/Em: 280/335)	Intensity of Peak C (Ex/Em: 275/455)
0 d		515	473	629
R1	14 d	367	2,795	743
	21 d	271	5,201	504
	35 d	411	4,619	365
R2	14 d	402	4,807	623
	21 d	275	7,153	522
	35 d	757	7,377	528

CONCLUSIONS

Boron was added to an SBR to cultivate AGS. Granular sludge properties, characterization of AI-2 activity, and analysis of EPS were used to elucidate the strengthening effect of boron on AGS formation. The addition of boron promoted the formation of AGS. We concluded that the faster growth of AGS in R2 may be related to boron stimulating the formation of boron complexed to DPD, which promoted AI-2 activity and intercellular communication. EEM fluorescence spectroscopy showed that the hydrophobicity that resulted from hydrophobic substances represented by trytophan might contribute to faster AGS formation. Our results provide new insight into the relationship between quorum sensing and the biogranulation process.

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