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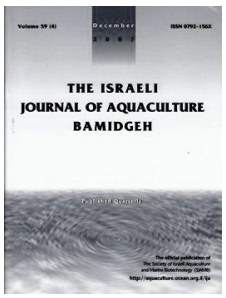

Detection of Acylated Homoserine Lactone (AHL) in the Heterotrophic Bacteria Z-TG01 and Its Ecological Action on the Algae, *Chlorella vulgaris*

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Key words: *Chlorella vulgaris*, bacteria Z-TG01, quorum sensing, acylated homoserine lactones (AHL), ecological action

Abstract

Heterotrophic bacteria Z-TG01 was isolated from the phycosphere of *Chlorella vulgaris* and identified as *Sphingomonas paucimobilis* by VITEK 2 Compact automated system with an excellent confidence level (99.0% probability). We detected acylated homoserine lactones (AHL) bioactivity in the growth process of Z-TG01 using *Agrobacterium tumefaciens* KYC55. The effects of *C. vulgaris* on AHL bioactivity, as well as the mutual influence of *C. vulgaris* and Z-TG01 in a co-culture system, were investigated. Results showed that AHL bioactivity of bacteria Z-TG01 varied with the bacteria density and was inhibited by supernatants of *C. vulgaris* culture in the early and middle exponential phases within 2 h ($p<0.01$), and in the late exponential phase within 1.5 h ($p<0.05$ or $p<0.01$). Within a certain cell density, bacteria Z-TG01 and *C. vulgaris* promote mutual growth in a co-culture system.

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Introduction

Many bacteria can synthesize and release chemical hormone-like signals called autoinducers that regulate physiological processes such as virulence, secondary metabolism, and bioluminescence (Whitchead et al., 2002). This phenomenon, named quorum sensing (QS), enables single-celled bacteria to collectively control gene expression and synchronize group behavior when the concentration of signal molecules reach a threshold level (Jayaraman and Wood, 2008). Different types of signal molecules are produced by bacteria, with acylated homoserine lactones (AHL) being the most common cell-to-cell communication signal in gram-negative bacteria (Horswill et al., 2007; Boye and Wisniewski-Dyé, 2009).

Bacteria coexist around algae cells, and the habitat of phytoplankton-associated bacteria is called a phycosphere (Sapp et al., 2007). Algae and the numerous heterotrophic bacteria in its phycosphere constitute a microcosm with close ecological interactions. Algae can release large amounts of organic compounds that can be assimilated by the bacteria that, in turn, may provide inorganic nutrients and growth factors to the algae. Such bacterium-alga interactions play an important role during biogeochemical cycling within the microbial loop (Azam, 1998).

Algae, as well as higher plants, can produce compounds that mimic QS signals (Bauer and Robinson, 2002). Bacteria from the phycosphere of *Alexandrium tamarense*, the algae that causes red tide, lead to lysis of the algae (Wang et al., 2010). The manipulation by algae of bacterial growth through inhibition or inactivation of QS offers a new opportunity to control bacteria. Heterotrophic bacteria Z-TG01 was isolated from the phycosphere of *Chlorella vulgaris* and the effects of this alga on the AHL bioactivity of Z-TG01 were investigated using *Agrobacterium tumefaciens* KYC55 (Zhu et al., 2003). In this study, the mutual effects of *C. vulgaris* and Z-TG01 were investigated.

Materials and Methods

Materials. Chlorella vulgaris was provided by the College of Fisheries, Ocean University of China, and grown in conical flasks containing f/2 medium under a 12L:12D photoperiod with a light density of 60 μmol/m^2/s at 20±0.5°C. *Agrobacterium tumefaciens* KYC55 (pJZ372/pJZ384/pJZ410) and *A. tumefaciens* R10 (pCF218), provided by Prof. Zhu Jun of Nanjing Agricultural University, were cultured in Luria-Bertani (LB) media containing 1 μg/ml tetracycline at 28°C with a stirring speed of 180 rpm.

Isolation and identification of Z-TG01. The *C. vulgaris* culture was filtered through a 0.45-μm-pore-size filter membrane, and the supernatants obtained with continuous gradient dilution were plated onto 2216E media (5 g tryptone, 1 g yeast extract, 0.01 g $FePO_4$, 1 l aged sea water) supplemented with 1.0% agar. After 7 days of culture, one of the dominant bacteria was isolated, purified, and identified by VITEK 2 Compact Automatic Bacteria Identification System (BioMérieux, France) using GN cards. The identified bacteria was named Z-TG01 and cultured in 2216E medium at 28°C with a stirring speed of 180 rpm.

Detection of AHL bioactivity in Z-TG01. The AHL bioactivity of bacteria Z-TG01 was tested every 3 h in a continuous culture of 100 ml 2216E medium. The axenic 2216E medium was used as a negative control and the stationary phase of *A. tumefaciens* R10 as the positive control. AHL bioactivity was determined by measuring ß-galactosidase bioactivity according to the method of Zhang et al. (2009) with some modifications. *Agrobacterium tumefaciens* KYC55 (5×10^7) were inoculated into 10 ml *A. tumefaciens* (AT) culture medium (Fuqua and Winans, 1994) containing 1 ml supernatant of Z-TG01. For each sample, absorbance at OD_{600} was recorded after about 10 h, and then 200 μl supernatant of the AT culture medium was removed and brought up to 1 ml by addition of 800 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, pH 7.0). Three drops of chloroform and 1 drop of 0.1% SDS were added, and the solution was vortexed for 10 s and placed into a 30°C water bath for 5 min. Reactions were initiated by the addition of 200 μl of 4 mg/ml ortho-nitrophenyl-β-D-galactopyranoside (ONPG), and stopped by adding 600 μl of 1 M Na_2CO_3 when the reaction mixture became yellow. After centrifugation at 10,000 *g*, absorbance at OD_{420}

was read from the supernatant of the reaction mixture. LB medium containing 1 µl/ml tetracycline was used as a control under the same conditions. ß-galactosidase units were calculated as: Miller units = $(1000 \times OD_{420})/(OD_{600} \times T \times V)$, where OD_{420} was read from the supernatant of the reaction mixture, OD_{600} reflected the cell density of *A. tumefaciens* KYC55 cultured about 10 h in AT medium, T = the time of the reaction in minutes, and V = the volume of culture used in the assay in ml.

Effects of C. vulgaris on the AHL bioactivity of Z-TG01. Supernatants of the algae culture in the lag, early exponential, middle exponential, late exponential, stationary, and degradation phases were mixed with an equal volume of supernatant of Z-TG01 culture in the stationary phase; the supernatant of Z-TG01 without any supernatant of the algae culture was used as the control. AHL bioactivity was assayed every 30 min for 2 h.

Mutual influence of C. vulgaris and Z-TG01. Suspensions of fresh Z-TG01 culture were added to axenic *C. vulgaris* (10^6 cells/ml) in the exponential phase to final Z-TG01 concentrations in the algae cultures of 10^4, 10^5, 10^6, 10^7, and 10^8 cells/ml and cultured under a 12L:12D photoperiod with a light density of 60 µmol/m^2/s at 20±0.5°C. Algae cell density, chlorophyll *a* (Chl-*a*) content, AHL bioactivity, and bacteria density of the co-culture system were measured every day. The number of algae was determined by microscopy in a blood counting chamber and the number of bacteria was counted using the plate count method. The relative growth rates of the algae and the bacteria were calculated as $K = (\lg N_t - \lg N_0)/t$, where N_t = cell density just before assay, N_0 = the initial cell density, and t = culture time in days.

About 20 ml of the culture media was centrifuged at 4,500 rpm for 10 min. The resulting pellet was washed twice with Na-phosphate buffer (50 mM, pH 7.2), ground in 10 ml chilled 80% acetone, and laid at 4°C under a dim light for 24 h. The homogenate was centrifuged at 12,000 rpm for 10 min at 4°C. Chl-*a* concentration in the supernatant was determined by spectrophotometry following the method of Lorenzen (1967): Chl-*a* = $V \times [11.64(OD_{663} - OD_{750}) - 2.16(OD_{645} - OD_{750}) + 0.1(OD_{630} - OD_{750})]$, where V = extraction volume and OD_λ = optical density at wavelength λ (nm).

Statistical analysis. All experiments were conducted in triplicate. Data, expressed as means±SD, were subjected to one-way analysis of variance (ANOVA, SPSS version 13.0) to determine significant differences between groups. Significant differences between treatments were determined by least significant difference multiple-range test.

Results

The Z-TG01 colony was round, 1.5-2.5 mm in diameter, semi-transparent, yellow-pigmented, convex with an entire margin, smooth, and wet in appearance. Using VITEK 2 Compact automated system, bacteria Z-TG01 was identified as *Sphingomonas paucimobilis* with an excellent confidence level of 99.0% probability (Table 1). Z-TG01 secreted AHL, the bioactivity of which varied with the bacterial density (Fig. 1). AHL bioactivity increased with the increasing bacteria density from the lag phase to the exponential phase, then decreased from the stationary phase to the degradation phase as the bacteria density decreased. The maximum AHL bioactivity reached 28.76 Miller units at 24 h.

Table 1. Biochemical characteristics of bacteria Z-TG01.

Test	*Result*	*Test*	*Result*	*Test*	*Result*	*Test*	*Result*	*Test*	*Result*
APPA	-	GGT	-	PLE	-	ILATk	-	CMT	-
ADO	-	OFF	-	TyrA	-	AGLU	+	BGUR	-
PyrA	-	BGLU	+	URE	-	SUCT	-	O129R	-
IARL	-	dMAL	+	dSOR	-	NAGA	-	GGAA	-
dCEL	+	dMAN	-	SAC	-	AGAL	+	IMLTa	-
BGAL	-	dMNE	-	dTAG	-	PHOS	-	ELLM	+
H2S	-	BXYL	-	dTRE	-	GlyA	-	ILATa	-
BNAG	-	BAIap	-	CIT	-	ODC	-	/	-
AGLTp	-	ProA	-	MNT	-	LDC	-	/	-
dGLU	-	LIP	-	5KG	-	IHISa	-	/	-

Supernatants of the *C. vulgaris* culture in the early and middle exponential phases significantly inhibited ($p<0.01$) the AHL bioactivity of bacteria Z-TG01 (Table 2). In the late exponential phase, inhibitory effects were exhibited at 1.5 h ($p<0.05$ or $p<0.01$). Supernatants of other multiplication phases had no significant ($p>0.05$) inhibitory or stimulatory effect on AHL bioactivity.

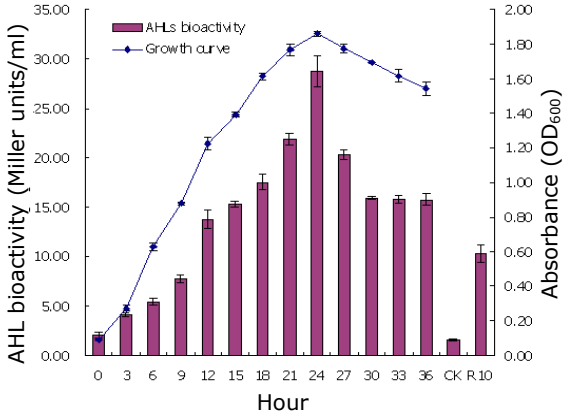

Fig. 1. Growth and acylated homoserine lactones (AHL) bioactivity in the supernatant of bacteria Z-TG01 culture.

Table 2. Effect of *Chlorella vulgaris* in different growth periods on the relative acylated homoserine lactones (AHL) bioactivity of bacteria Z-TG01 (%).

Growth phase	*Time*			
	0.5 h	*1 h*	*1.5 h*	*2 h*
Control	100±3	100±4	100±3	100±6
Lag	97±4	93±4	96±4	93±3
Early exponential	**74±4	**78±3	**83±5	**85±4
Middle exponential	**76±1	**75±1	**77±3	**68±5
Late exponential	*90±3	**89±3	*91±5	92±4
Stationary	94±7	95±3	98±6	100±6
Degradation	100±5	102±7	102±4	106±6

* significantly different from control (p<0.05)
** extremely significantly different from control (p<0.01)

All five initial bacteria densities significantly increased the relative growth rate and Chl-*a* content of *C. vulgaris* (Fig. 2). The best stimulatory effects on *C. vulgaris* growth were obtained with 10^6 cells/ml, followed by 10^7, 10^5, 10^4, and 10^8. With time, the relative growth rate of the bacteria with 10^4 cells/ml initial density increased while the growth rate with 10^7 and 10^8 cells/ml initial density decreased (Fig. 3). The growth rate with 10^5 and 10^6 cells/ml initial density first increased, but then decreased. AHL activity increased rapidly in a bacteria-density-dependent manner before the exponential phase of the algae cells. However, once the algae entered the exponential phase, AHL activity increased slowly in the 10^4, 10^5, and 10^6 cells/ml treatments, although the number of bacteria increased rapidly, and then decreased rapidly as the bacteria decreased in the 10^7 and 10^8 cells/ml treatments.

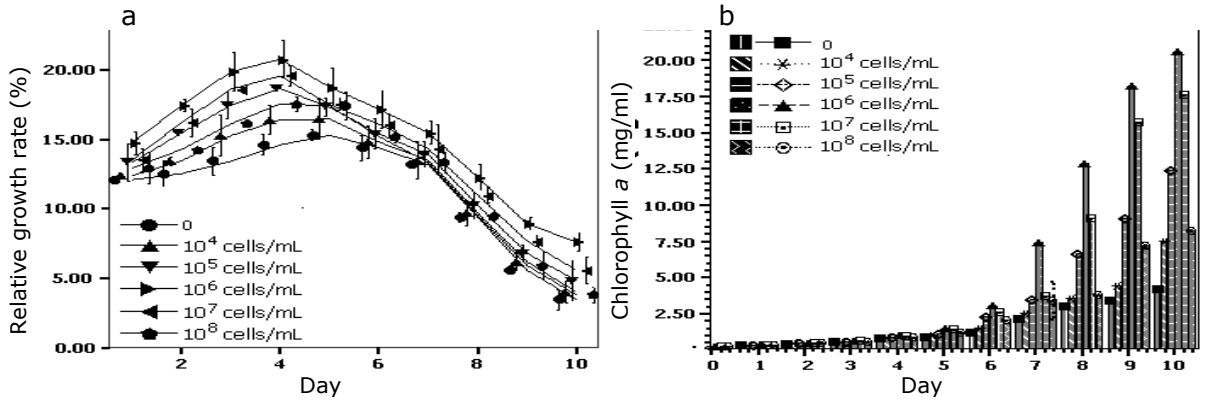

Fig. 2. Effect of density of bacteria Z-TG01 on (a) the relative growth rate and (b) chlorophyll *a* of *Chlorella vulgaris.*

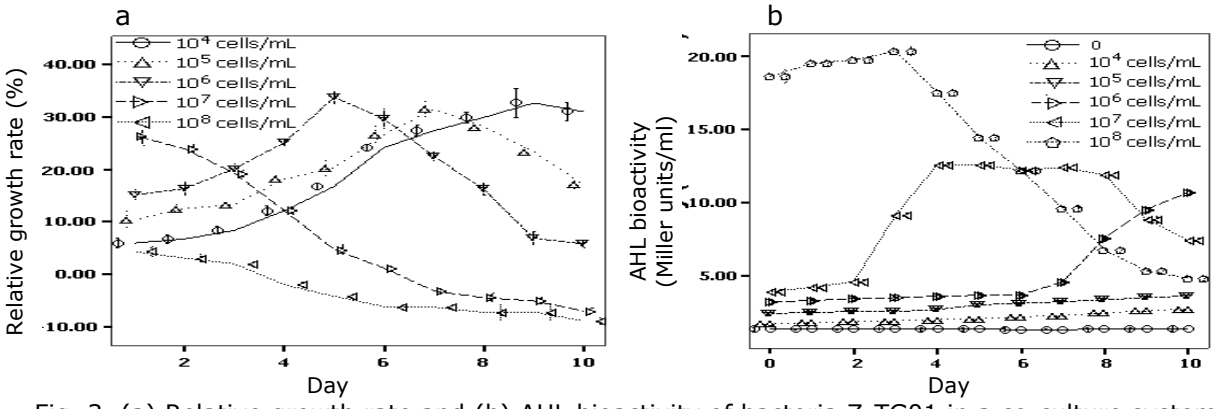

Fig. 3. (a) Relative growth rate and (b) AHL bioactivity of bacteria Z-TG01 in a co-culture system with *Chlorella vulgaris*.

Discussion

Chlorella vulgaris is commonly used in aquaculture and can be a model organism for studying the QS interference of microalgae. The supernatant of *C. vulgaris* CCAP211/12 in the exponential phase strongly inhibited AHL-regulated pigment production in the reporter bacteria, *Chromobacterium violaceum* CV026, but stimulated green fluorescent protein (GFP) production in the reporter strain, *Escherichia coli* JB523 (Natrah et al., 2011a). AHL bioactivity can be impacted by environment factors such as pH, temperature, and light (Horswill et al., 2007). In addition to lactone hydrolysis, the 3-oxo-C12 AHL of *Pseudomonas aeruginosa* can spontaneously undergo a Claisen-like condensation reaction in an aqueous environment, forming a tetramic acid product, 3-hydroxydecyli-dene 5-(2-hydroxyethyl) pyrrolidine-2,4-dione (Kaufmann et al., 2005). Therefore, different methods for detecting AHL bioactivity can lead to different results. In the present study, a more precise quantitative manner using *A. tumefaciens* KYC55 was used to detect AHL and investigate the QS interfering effects of *C. vulgaris* on bacteria Z-TG01. Under the experimental conditions, supernatants of *C. vulgaris* in the exponential phase significantly ($p<0.01$) inhibited the AHL bioactivity of bacteria Z-TG01.

Eukaryotes such as algae, protozoa, and fungi have developed different defense mechanisms to interact with bacteria in the aquatic environment by producing secondary metabolites impacting QS (Natrah et al., 2011b). The marine red macroalga, *Delisea pulchra*, interferes with bacterial QS by producing compounds that mimic bacterial N-AHL QS signals (Givskov et al., 1996). Microalgae can secret reactive compounds that mimic bacterial signals and interfere with QS regulation in bacteria. *Chlamydomonas reinhardtii* can produce and secrete at least a dozen chromatographically separable substances that specifically stimulate LasR and CepR-mediated QS functions in the *Pseudomonas putida* CepRI'::GFP reporter strain (Teplitski et al., 2004). We speculate that *C. vulgaris* also can secrete reactive compounds that interfere with QS regulation and could be a promising micro-algae for controlling pathogenic bacteria in aquaculture.

Sphingomonas paucimobilis can grow and survive in hostile conditions such as low temperature, low nutrient concentrations, and toxicity. Widely distributed in nature, it has been isolated from plants, soils, and subsurface sediments (Wolf et al., 2003; Vanbroekhoven et al., 2004; Wang et al., 2009). The ability to secrete polysaccharide might play an important role in simulating the effects of *S. paucimobilis* on the growth of *C. vulgaris* (Gusman et al., 2010). In addition, AHL degradation products could stimulate plant growth by triggering certain physiological bioactivities of plant-associated bacteria. Stomatal conductance and transpiration of the bean, *Phaseolus vulgaris* L., increased significantly (from 20% to 30%, $p\leq0.05$) 42 h after 10 nM homoserine lactone was supplied to the roots (Joseph and Phillips, 2003). *Sphingomonas paucimobilis* can enhance the disease-resistance of plants and exhibits antagonism against the phytopathogenic fungus *Verticillium dahliae* (Berg and Ballin, 1994). In the present study, *S. paucimobilis* significantly simulated the growth of *C. vulgaris* within a certain cell density in a co-culture system. Therefore, we believe *S. paucimobilis* could be used as a probiotic against algae proliferation in aquaculture.

In conclusion, heterotrophic bacteria Z-TG01 was isolated from the phycosphere of *C. vulgaris* and identified as *S. paucimobilis* with an excellent confidence level (99.0% probability). The AHL bioactivity of bacteria Z-TG01 varied with its density and can be inhibited by supernatants of *C. vulgaris* in the early and middle exponential phases within 2 h ($p<0.01$), and in the late exponential phase within 1.5 h ($p<0.05$ or $p<0.01$). Within a certain cell density, bacteria Z-TG01 and *C. vulgaris* promote mutual growth in a co-culture system.

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