Characterizing Growth Promoters and Inhibitors of SAR11 *Pelagibacter* sp. HTCC7211

by

Connie Lee

A PROJECT

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Microbiology (Honors Scholar)

Presented May 29, 2013
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Connie Lee for the degree of Honors Baccalaureate of Science in Microbiology presented on May 29, 2013. Title: Characterizing Growth Promoters and Inhibitors of SAR11 *Pelagibacter* sp. HTCC7211.

Abstract approved:

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Stephen Giovannoni

SAR11 is a clade of marine bacteria that are the most abundant heterotrophs in ocean surface water. They play a significant role in marine carbon cycling. Although many culturing experiments have been done on SAR11, there was no research that focused on verifying the observations about inhibition of SAR11 growth by organic compounds. I compiled a growth nutrient profile for future reference. The experiment reported here used a prototype strain of SAR11, *Pelagibacter* sp. HTCC7211 (HTCC7211), to verify the effect of the 12 selected compounds at varying concentrations on the growth of HTCC7211. HTCC7211 was grown on a defined artificial seawater (ASW) containing inorganic and organic nutrients, vitamins, methionine, glycine, and pyruvate. In addition to the ASW medium, each selected compound was added to the ASW at varying concentrations. I compared the specific growth rates of each treatment and reported here the effects of each treatment on the growth of HTCC7211. The specific growth rates of HTCC7211 under organic acid and osmolyte treatments are highest at medium concentrations. The patterns of HTCC7211 growth rates under amino acid treatments are complicated. The limitations of this experiment include no duplicates or triplicates for control group and treatment groups, slow doubling time of HTCC7211, and low pH value of the cultures. The findings of this experiment require further investigations; however, the

results still serve as a foundation or reference for future culturing experiments and the emerging metabolomic research that will help understand SAR11's role in nutrient cycling in marine ecosystems.

Key Words: SAR11, *Pelagibacter* sp. HTCC7211, culturing, nutrient inhibition

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Chair, Department of Microbiology

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Connie Lee, Author

ACKNOLEDGMENT

I would like to acknowledge and extend my heartfelt gratitude to all those who gave me the possibility to complete this thesis:

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Dated _____

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Connie Lee

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DEDICATION

This thesis is lovingly dedicated to my parents, who have been my constant source of inspiration. My parents, Kane Lee and Grace Fang, have given me the drive and discipline to tackle any task with enthusiasm and determination. Without their love and support this project would not have been made possible.

Introduction

As oceans encompass a majority of the planet's surface, marine ecosystems play an important role in balancing other major ecosystems on Earth. Although marine environments are characterized as low-nutrient states, the oceans are still able to support the majority of metabolic energy and carbon required for oceanic organisms. The capability of supporting numerous lives under low nutrient states comes from the intense nutrient cycling between the microbial communities. Noticing the importance of the microbial world, the focus of biology oceanography has been shifted from the traditional macroorganism food-web dynamics to the interactions of microorganisms in maintaining nutrient cycling (Giovannoni and Stingl, 2005). Therefore, studying ecologically important marine bacteria and their metabolic behaviors is a major ongoing research field that helps better understand the mechanisms which maintain the cycling of essential substances.

Among all aquatic environments, ocean water contains the least amount of organic matter in the form of dissolved organic carbon (Fry, 1990). Challenged by the low-nutrient state of the ocean, many marine bacteria have adapted/evolved to be able to grow well in low concentrations of substrate. Although some oligotrophs can only grow in low concentrations of carbon compounds, others can grow at both low and high concentrations. Many marine oligotrophic bacteria have been found to have high affinity uptake systems and low saturation constants for growth, allowing them to grow effectively on low concentrations of nutrient. Moreover, oligotrophs can adapt their substrate affinity when growing on different substrates at different concentrations.

However, even though oligotrophs can adapt to different nutrient concentrations, their growth can still experience nutrient inhibition if the amount of nutrient exceeds their utilizing capacity (Fry, 1990).

SAR11 is a clade of marine bacteria that is the most abundant hetero-oligotrophs in ocean surface water with an estimated global population size of 10^{28} cells (Morris *et al*., 2002). Due to their abundance in the marine environments, SAR11 bacteria have a significant role in marine carbon cycling (Malmstrom *et al*., 2005; Morris *et al*., 2005). Thus, many laboratory studies are being performed to investigate the metabolic mechanisms and evolution of SAR11 in order to gain better knowledge of its ecological role in marine, low nutrient state environments. Since the identification of the SAR11 clade, many studies have shown signs that SAR11 cannot tolerate high concentration of nutrients. There are many reported and unreported observations on potential growth promoters and inhibitors of the growth of SAR11 (Malmstrom *et al*., 2004; Schwalbach *et al*., 2009; Tripp *et al*., 2009; Carini *et al*., 2012). However, there was no research which definitively demonstrated the inhibition of SAR11 growth by organic compounds. Therefore, I decided to characterize the previously-observed effects of twelve selected compounds on the growth of SAR11. I hope to compile a growth nutrient profile of SAR11 for future metabolomic experiments which will help define the adaptability to SAR11 of high nutrient concentrations.

The experiment reported here uses *Pelagibacter* sp. HTCC7211 (HTCC7211), a member of the SAR11 clade, to characterize the effect of the selected compounds at varying concentrations on the growth of HTCC7211. Based on the previous culture media used for HTCC7211, a defined medium was developed to study the nutrient

requirements of HTCC7211. Each selected compound was added at varying concentrations, and cell growth of each culture was monitored by flow cytometer. Based on previous reported and unreported observations on the growth of HTCC7211, I hypothesized that HTCC7211 would have a preference for specific concentrations of each compound in order to reach optimal growth. I report here that the growth of HTCC7211 can be promoted or inhibited by the selected compounds at varying concentrations, indicating that HTCC7211 has a preference for specific concentration of compounds for its growth.

Materials and Methods

Organism source

HTCC7211 was originally isolated from the Sargasso Sea (Stingl *et al*., 2007).

Media preparation

The basic medium used in this experiment is artificial seawater medium (ASW), which contains base salts, macronutrients, trace metals, vitamins, low nutrient heterotrophic media (LNHM) components, two amino acids, and pyruvate (Table 1). The base salts, macronutrients, and trace metals were added to nanopure water at the specified concentrations, and the medium was autoclaved to remove any contaminants. To establish that the bicarbonate-based buffer system mimicking the ocean surface water, the autoclaved nanopure water was sparged with 0.1 μM-filtered CO_2 for at least 6 hours, followed by sparging with O_2 for overnight (Connon and Giovannoni, 2002; Carini *et al*., 2012). The vitamins, LNHM components, amino acids, and pyruvate were added under sterile condition after autoclaving and sparging. All liquid media were tested for sterility before use by checking for contaminants with Guava Technologies flow cytometer (Millipore). The pH value of the final media was 7.5. Test compounds were then respectively added to flasks containing the basic medium prior to bacterial inoculation.

Table 1. Components of the Basic Medium

Compound	Final concentration
Base salts	
NaCl	481 mM
$MgCl_2•6H_2O$	27 mM
$CaCl2•2H_2O$	10 mM
KCl	9 mM
$NaHCO_3$	6 mM
$MgSO_4•7H_2O$	2.8 mM
Macronutrients	
$(NH_4)_2SO_4$	400 μM
NaH_2PO_4 (pH 7.5)	50 μM
Trace minerals	
$FeCl_3•6H_2O$	117 nM
$MnCl_2•4H_2O$	9 nM
$ZnSO_4•7H_2O$	800 pM
$CoCl_2•6H_2O$	500 pM
$Na_2MoO_4•2H_2O$	300 pM
Na_2SeO_3	1 nM
$NiCl_2•6H_2O$	1 nM
Vitamins	
B_1	6 μM
B_3	800 nM
B_5	425 nM
B_6	500 nM
B_7	4 nM
B_9	4 nM
B_{12}	700 pM
Myo-inositol	6 μM
4-Aminobenzoic acid	60 nM
LNHM components	
NH_4Cl	10 μM
KH_2PO_4	1 μM
FeCl	10 nM
Amino acids	
L-glycine	50 μM
L-methionine	10 μM
Carbon source	
Pyruvate	100 μM

*The table is derived from Carini *et al*., 2012

Compound preparation

Each of the twelve test compounds were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) or other commercial vendors and were of reagent grade quality (Carini *et al*., 2012). Stock solutions of the twelve test compounds were prepared with nanopure water, and the stock solutions were 0.1 mM and 0.1M. The concentrations being tested in this experiment were then prepared from the stock solutions. Each stock solution was filtered through 0.2 μM-filter under sterile conditions before use.

Cultivation details

250 mL polycarbonate flasks were used to culture HTCC7211. The flasks were acid-washed and autoclaved before use. Each flask contained 50 mL ASW medium with the addition of one of the twelve test compounds selected for characterization. Each compound being tested was added in varying concentrations (0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, and 1000 μM) into individual flasks. One control group containing 50 mL ASW medium without any additional compounds was also prepared, resulting in a total of 73 flasks. The stock culture of HTCC7211 was maintained in ASW prior to this experiment. HTCC7211 was then added to each of the 73 flasks (1×10^4 cells per ml or 5×10^5 cells per flask). All cultures were grown in dark at 20°C. The pH of the cultures was within the range of 7.0-7.2.

Measurement of growth

Cellular proliferation was measured by harvesting (200 µl) of bacterial culture from each flask and aliquoted into a 96-well plate. Each sample was then stained with the DNA stain, SYBR Green I (Invitrogen), for at least one hour in the dark. The cells were then counted using a Guava Technologies flow cytometer (Millipore) at 48- or 72- hour intervals (Stingl *et al*., 2007; Tripp *et al*., 2008; Carini *et al*., 2012). Growth of each culture was monitored until all cultures had reached stationary phase.

Results

To better correlate and compare compounds with similar properties, I separated the compounds into two groups during analysis: (1) organic acids and osmolytes, and (2) amino acids. Using the cell density of each culture obtained from the flow cytometer, I generated the growth curve of HTCC7211 under each treatment. I then selected two points that make up the steepest slope in the exponential phase of the growth curve to calculate the specific growth rates of HTCC7211 under the specific treatment. I compared the specific growth rates by creating a bar graph of the specific growth rate of each culture under each treatment. Although the doubling time and maximum cell density of each culture were not used for comparison in this report, they were also calculated (See Appendix). We defined the low concentrations to be 0.01μM, 0.1 μM; medium concentrations were 1 μM, and 10 μM; high concentrations were 10 μM and 100 μM.

In the first group, cells grown with taurine, dimethylsulfoniopropionate (DMSP), and betaine all had higher specific growth rates when the compounds were added in medium concentrations (Figure 1). Cells grown with oxaloacetate had an increasing specific growth rate as concentration of the compound increased (Figure 1). Cells grown with γ-aminobutyric acid (GABA) had a decreasing specific growth rate as concentration of the compound increased (Figure 1).

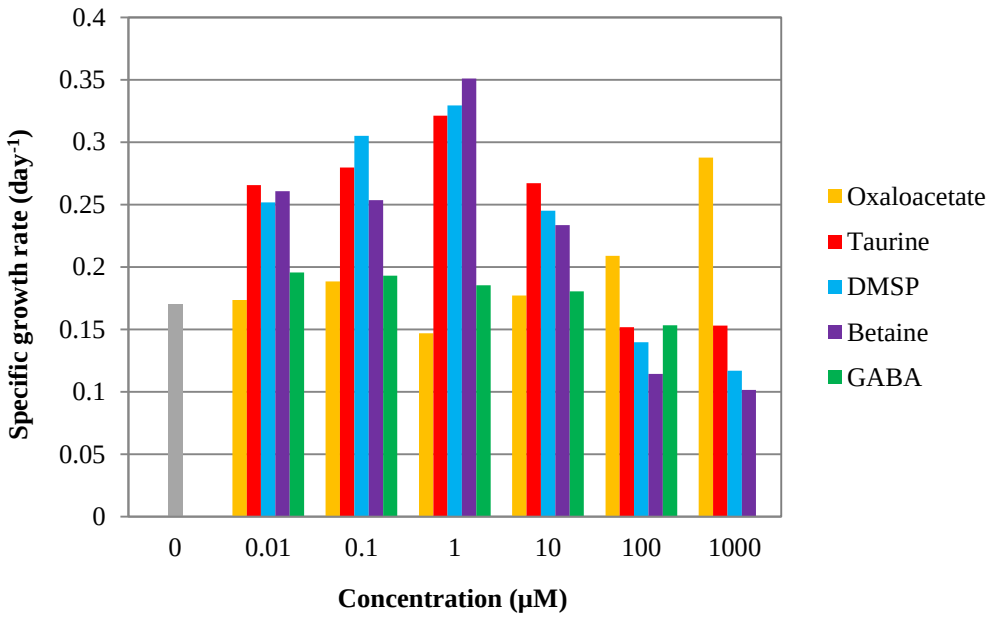

Figure 1. Comparison of the specific growth rate of HTCC7211 cultured in ASW containing organic acid and osmolyte at varying concentrations. Each bar represents one culture grown with the particular compound at the specific concentration.

In the second group of compounds, cells grown with L-cysteine had higher specific growth rates when the compound was added in low and medium concentrations. Comparing different concentrations of L-alanine added into the cultures, HTCC7211 also had higher specific growth rates when L-alanine was added in medium concentrations; however, comparing to the control group, cells grown with L-alanine did not reach a higher specific growth rate than the control group (Figure 2). Cells grown with L-histidine had a high specific growth rate at 0.01 µM, and a sudden decrease of specific growth rate at high concentration of the compound (Figure 2). Cells grown with L-threonine did not show significant preference for a specific concentration of the compound but was slightly higher than the control group (Figure 2). Cells grown with L-phenylalanine had a higher specific growth rate at 1000 µM, and the culture had no

obvious preference for low and medium concentrations of the compound (Figure 2). Cells grown with D-alanine had unusual, alternating promoting and inhibiting effects over varying concentrations on the cell growth (Figure 2). Cells grown with L-proline could tolerate medium and high concentrations, and the cells had the highest specific growth rate at 1 μM and 1000 μM; the culture had lower specific growth rates at low concentrations of the compound (Figure 2).

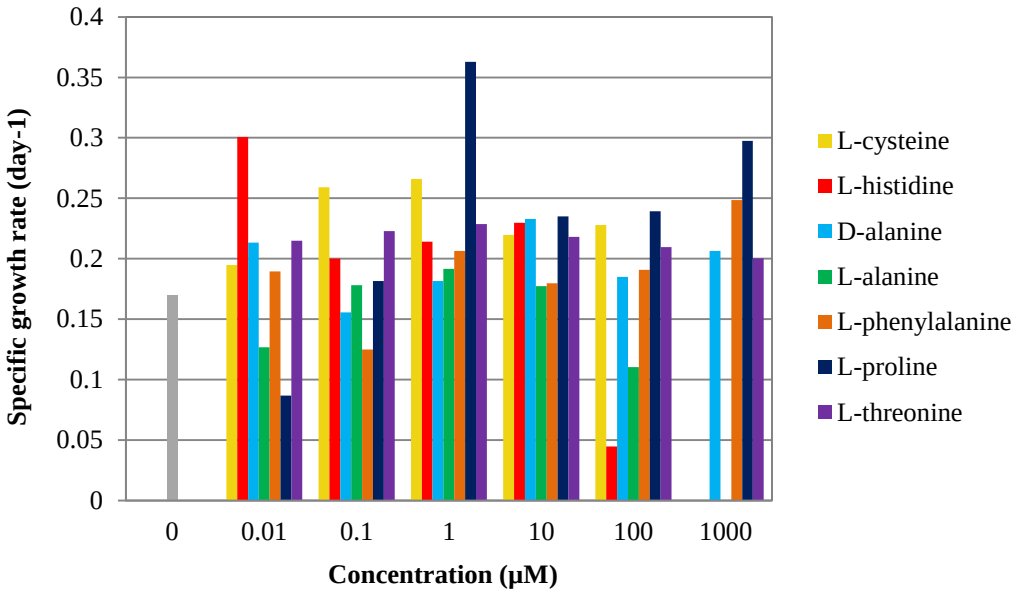

Figure 2. Comparison of the specific growth rate of HTCC7211 cultured in ASW containing amino acid at varying concentrations. Each bar represents one culture grown with the particular compound at the specific concentration.

Discussion and Conclusions

Although many researchers have observed the effects of several compounds on the growth of SAR11 in other culturing experiments, they have not systematically characterized these observations. To help build a foundation in culturing HTCC7211, I selected twelve compounds that have previously-reported effects on growth of SAR11, and I tested their effects on the growth of HTCC7211. The scope of this experiment is limited to the initial screening and characterization of the selected compounds. Also, due to my limited knowledge of SAR11 metabolism, I will only attempted to give a general conclusions for the results observed from the specific growth rates of the selected compounds.

In the group of organic acids and osmolytes additions, taurine, DMSP, and betain have the highest specific growth rate at 1 μM. The specific growth rate graph of cells grown with these three compounds shows a concave-down curve. Adding these compounds can promote growth of HTCC7211 only up until 1 μM; the higher concentrations decrease the growth rate of the culture. This pattern suggests that HTCC7211 has an optimal growth rate at specific concentrations. This may be due to nutrient inhibition, which can come from the lack of necessary enzymes or the saturation of enzymes or transporters that process the nutrient (Schwalbach 2009). Therefore, when adding a concentration of compound that is higher than the enzymes' capacities, the nutrient may overwhelm the cell and cause cell death. Moreover, since the cultures were monitored for a period of time, the toxic waste excreted from the cells could build up and might have caused an inhibiting effect on the cells. Lastly, this phenomenon can also be

explained by osmolarity issues. Thus, taurine, DMSP, and betain can only promote HTCC7211's growth up to medium concentrations. Oxaloacetate shows a true, promoting effect on HTCC7211 as higher concentrations of oxaloacetate were consistently correlated with increased specific growth rates. HTCC7211 prefers high concentrations of oxaloacetate, indicating that oxaloacetate is a growth promoter of HTCC7211. I also noted that GABA inhibits HTCC7211's growth as the compound's concentration increases and even stops bacterial growth at 1000 μM.

In the group of amino acids compounds, the patterns of growth rates in each treatment are more complicated. L-cysteine also shows the nutrient inhibiting effect on HTCC7211's growth. L-cysteine only promotes bacterial growth when L-cysteine is added at the medium concentrations, and then L-cysteine inhibits growth at high concentrations. Thus, L-cysteine has a growth promoting effect on HTCC7211 only at the medium concentrations. Although L-alanine also shows nutrient inhibiting effect, adding L-alanine does not provide a growth-promoting effect comparing to the control. HTCC7211 prefers low concentrations of L-histidine, but it can also grow moderately well at medium concentrations of L-histidine. However, higher concentrations of L-histidine decrease or even inhibit the growth of the bacteria. HTCC7211 does not show a preference for L-threonine over varying concentration. Adding L-threonine seems to have no impact on HTCC7211's growth. HTCC7211 shows a high tolerance or nutrient preference to high concentrations of L-phenylalanine. Thus, L-phenylalanine is potentially a growth promoter for HTCC7211. I also noted that HTCC7211 cultures grown with D-alanine and L-proline show no visible patterns or trends of growth rates.

Therefore, the effects of D-alanine and L-proline on HTCC7211's growth are inconclusive, and they require further investigation.

Due to multiple limitations, this report only presents the results of the initial characterization of the selected compounds on HTCC7211's growth. Due to limited laboratory equipment and time available for the experiment, there were no duplicates or triplicates for any of the samples. Thus, the results presented here represent preliminary findings for the inhibitory or promoter effect of each compound. Moreover, the generation of HTCC7211 used in the experiment was later found to have a slower growth than normal doubling time (normal is 48 hours) of HTCC7211 cells growing under ASW medium. The pH of all samples, except the control group, was lower than the average pH of ASW medium (pH 8.0-8.2) due to the addition of the acidic test compounds. Future research repeating this experiment should perform triplicates and continuously monitor pH to provide more confidence in the data collected.

As the marine microbial world gains attentions in the oceanography and marine ecology, scientists start to investigate their role in balancing and cycling scarce nutrients in the marine environments. Although genomics and metagenomics can help researchers quickly get a glimpse of the metabolism of marine oligotrophs, culturing is still important because cultures can be used to characterize and evaluate hypotheses made from genomic data (Giovannoni and Stingl, 2007). The culturing experiments described here provide evidence that supports previous culturing research, showing that some compounds are inhibitory to SAR11's growth. Even though the experiment only provides preliminary results and the problem requires further investigation, the results reported here should still be able to serve as a reference for future experiments. Having a SAR11 growth

profile will improve and optimize the efficiency and consistency of future culturing research. In addition, this research gives a foundation to design a metabolomics project that studies the chemical process of bacterial metabolism. The knowledge obtained from this research can help understand SAR11's role in nutrient cycling in the marine communities, which may further our knowledge of marine ecosystems.

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APPENDIX

HTCC7211 growth inhibitors and promoters

Compound	Concentration (μM)	Specific growth rate (day^{-1})	Doubling time (days per division)	Maximum cell density (10^6 cells/mL)
Negative control		0.170	4.08	30.3
Oxaloacetate	0.01	0.173	4.00	32.4
	0.1	0.188	3.68	53.0
	1	0.147	4.72	12.8
	10	0.177	3.91	29.0
	100	0.209	3.32	121
	1000	0.288	2.41	236
Taurine	0.01	0.266	2.61	47.7
	0.1	0.280	2.48	29.8
	1	0.321	2.16	42.9
	10	0.267	2.59	50.7
	100	0.152	4.57	14.7
	1000	0.153	4.53	0.303
L-cysteine	0.01	0.195	3.56	52.0
	0.1	0.259	2.68	53.1
	1	0.266	2.61	53.9
	10	0.220	3.16	6.97
	100	0.228	3.04	0.988
	1000	0	N/A	0.0321
DMSP	0.01	0.252	2.75	59.6
	0.1	0.305	2.27	42.5
	1	0.329	2.10	42.5
	10	0.245	2.83	27.4
	100	0.140	4.96	11.0
	1000	0.117	5.93	10.2
Betaine	0.01	0.261	2.66	83.9
	0.1	0.253	2.73	60.6
	1	0.351	1.98	61.2
	10	0.234	2.97	12.4
	100	0.114	6.07	1.29
	1000	0.102	6.83	0.705
L-histidine	0.01	0.301	2.30	45.3
	0.1	0.200	3.46	0.934
	1	0.214	3.24	0.784
	10	0.230	3.02	86.4
	100	0.045	15.5	0.854
	1000	0	N/A	0.0676

Compound	Concentration (μM)	Specific growth rate (day^{-1})	Doubling time (days per division)	Maximum cell density (10^6 cells/mL)
Negative control		0.170	4.08	30.3
D-alanine	0.01	0.213	3.25	62.8
	0.1	0.156	4.45	7.05
	1	0.182	3.82	14.6
	10	0.233	2.98	85.9
	100	0.185	3.75	93.1
	1000	0.206	3.36	16.7
L-alanine	0.01	0.127	5.47	10.4
	0.1	0.178	3.89	26.7
	1	0.191	3.62	25.7
	10	0.177	3.91	26.0
	100	0.110	6.29	0.255
	1000	0	N/A	0.0332
L-phenylalanine	0.01	0.189	3.66	27.8
	0.1	0.125	5.56	42.7
	1	0.206	3.36	52.3
	10	0.180	3.86	24.5
	100	0.191	3.64	49.2
	1000	0.249	2.79	176
L-proline	0.01	0.0868	7.98	11.0
	0.1	0.181	3.82	29.1
	1	0.363	1.91	59.7
	10	0.235	2.95	69.8
	100	0.239	2.90	168
	1000	0.298	2.33	176
L-threonine	0.01	0.215	3.22	41.3
	0.1	0.223	3.11	13.0
	1	0.229	3.03	82.7
	10	0.218	3.18	40.5
	100	0.209	3.31	74.0
	1000	0.200	3.46	6.03
γ-aminobutyric acid (GABA)	0.01	0.196	3.54	29.2
	0.1	0.193	3.59	50.5
	1	0.185	3.74	57.1
	10	0.181	3.84	4.36
	100	0.153	4.52	0.696
	1000	0	N/A	0.0688

*All culture contains basic medium (artificial seawater, 50μM glycine, 10μM methionine, and 100μM pyruvate). Negative control contains only the basic medium. All flasks are incubated under 20°C. Each treatment only has one flask.

**A growth rate of 0 cell/mL/day represents that the culture had negative growth.