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Study on the Acyl-Homoserine Lactone Production of a Gram-Negative Bacterium *Sphingomonas xenophaga* by Co-Cultivation with Variovorax paradoxus

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ABSTRACT

Acyl-homoserine lactone is one of the signal molecules produced for the cell-to-cell communication in Gram-negative bacteria. When bacteria reach the high cell density at a threshold level, they produce acyl-homoserine lactone. Acyl-homoserine lactone can bind to the receptor and then induce the target gene expressions resulting in the decrease of the bacterial populations. From the co-cultivation of *Sphingomonas xenophaga* that can produce acyl-homoserine lactone and *Variovorax paradoxus* that can utilize acyl-homoserine lactone as an energy source, it was found that the optimum conditions for co-cultivation both bacteria were LB medium and incubation temperature at 20°C. In addition, *V. paradoxus* could degrade acyl-homoserine lactone produced from *S. xenophaga* as an energy source resulting in the increase of the 2-fold higher growth rate. The slower death of *S. xenophaga* by co-cultivation is due to the decrease of acyl-homoserine lactone level.

Key words: quorum sensing, acyl-homoserine lactone, co-cultivation

INTRODUCTION

From results decade ago it was shown that bacterial cells have a capability to communicate each other, coordinate their activities and finally form "community". Bacteria use chemical compounds as signal molecules for communication. Gram-positive bacteria produce and release signal molecules as small peptides (Dunny and Leonard, 1997) whereas Gram-negative bacteria produce and release signal molecules in various types of compounds. One well-studied chemical compound is acyl-homoserine lactone (AHL) (Greenberg, 2000). This molecule is produced and released from bacterial cells when cells reach the high cell density at a threshold level. AHL can bind to the receptor, subsequently regulate some gene expressions and finally control the bacterial populations (Fuqua *et al*., 1994). The system which bacteria can monitor own populations when their growth reaches the high cell density has been termed "Quorum sensing".

Acyl-homoserine lactone has a common structure as 3-oxo-C_6-HSL shown in Figure 1. It is composed of lactone ring and acyl side chain. The acyl side chain has normally 6 carbon atoms and can vary from 4 to 16 carbon atoms. Moreover, at the third carbon of acyl chain, it can be fully saturated or bound to hydroxyl group or carbonyl group. AHL has many types distinguishing from

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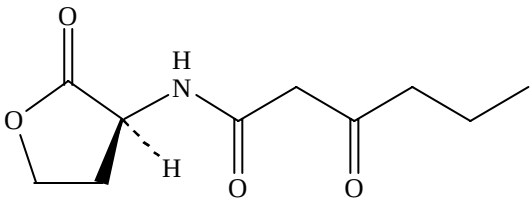

Figure 1 Structure of 3-oxo-C_6-homoserine lactone.

the length of carbon chain and the substitution at third carbon in acyl side chain. Each type shows the different specificity.

In this study, our attention was focused on a Gram-negative bacterium *Sphingomonas xenophaga*. It can produce AHL responding to the high cell density. Its shape is rod and its color is yellow. In addition, it can utilize phenanthrene, $C_{14}H_{10}$ aromatic hydrocarbon in oil, as a carbon source (Supaka *et al*., 2001; Baraniecki *et al*., 2002; Prak and Pritchard, 2002). In many bacteria, quorum sensing can modulate a variety of physiological processes, for example bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, plasmid conjugation transfer and the production of virulence determinants in animal, fish, and plant pathogens (Williams *et al*., 2000). One idea to control the amount of AHL is the use of AHL degrading enzymes that have a commercial value as modulators of cell-to-cell signaling. A recent report showed that *Variovorax paradoxus* was able to degrade and use AHL signals as the sole energy and nitrogen sources (Leadbetter and Greenberg, 2000). In this study, co-cultivation of both organisms was done to study the effect of AHL decrease because of *V. paradoxus* on growth of *S. xenophaga*. Firstly the optimum conditions for both growths were determined and subsequently the co-cultivation of both organisms was performed in one tube and in a dialyzing vessel tube.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Sphingomonas xenophaga and *Variovorax paradoxus* were isolated from seawater and grown in Lauria Bertani broth (LB) or Marine broth (MB). Reporter strain *Agrobacterium tumefaciens* A136 (Fuqua and Winans, 1996) was grown in LB medium supplemented with 4.5 µg/ml tetracycline and 50 µg/ml spectinomycin with shaking at 100 rpm, 30°C and overnight.

Effect of medium types on growth

S. xenophaga and *V. paradoxus* were separately grown in tubes containing 15 ml of LB or MB. They were incubated in a temperature gradient incubator with shaking at 50 rpm, 30°C. The optical density at 660 nm of all cultures was measured.

Effect of temperature on growth

S. xenophaga and *V. paradoxus* were separately grown in tubes containing 15 ml of the appropriate medium. They were incubated in a temperature gradient incubator with shaking at 50 rpm from 20 to 30°C. The optical density at 660 nm was measured.

Co-cultivation of both cultures in one tube

S. xenophaga and *V. paradoxus* precultures were inoculated in tubes containing 15 ml of the selected medium with various ratios of both cell optical densities (1:1, 1:10, 10:1, 100:1). The cultures were incubated in the incubator with shaking at 50 rpm and the appropriate temperature. After 2 days of incubation, supernatant was collected by centrifugation at 13,000xg for 10 min and filtrated by using 0.22 µm millipore filter before adding to the reporter strain *A. tumefaciens* A136 culture for the determination of β-galactosidase activity expression.

Co-cultivation of both cultures in a dialyzing vessel tube

A dialyzing vessel tube (Figure 2) was invented from Prof. Teruhiko Beppu from Nihon University, Fujisawa-shi, Kanagawa, Japan. It consists of 2 partitions that are separated by a dialyzing membrane with a 0.1 μm filter. The vessel containing 50 ml of one-fifth concentration of LB was autoclaved and the pure cultures of *S. xenophaga* and *V. paradoxus* were inoculated into each partition, respectively. The cultures were incubated with shaking at 100 rpm, 20°C. The cultures were sampled for study the growth by measuring optical density at 660 nm and study AHL amount by measuring the β-galactosidase activity.

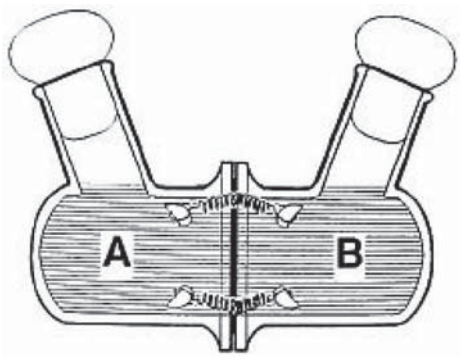

Figure 2 Dialyzing vessel tube (Ohno *et al.*, 1999).

Monitoring expression of AHL by measurement of β-galactosidase activity

AHL expression by measuring of β-galactosidase activity was done according to method of Sambrook *et al*. (1989).

RESULTS AND DISCUSSION

Growth of *S. xenophaga* and *V. paradoxus* in different kinds of media

The cultures were incubated separately in either LB or MB in the temperature gradient incubator with shaking at 50 rpm, 30°C. It was shown that at 30°C *V. paradoxus* was grown only in LB but was hardly grown in MB, whereas *S. xenophaga* was grown in both media but its growth was higher in LB (Figure 3). Interestingly, growth of *V. paradoxus* was faster than that of *S. xenophaga* in LB. In addition, in MB *S. xenophaga* was able

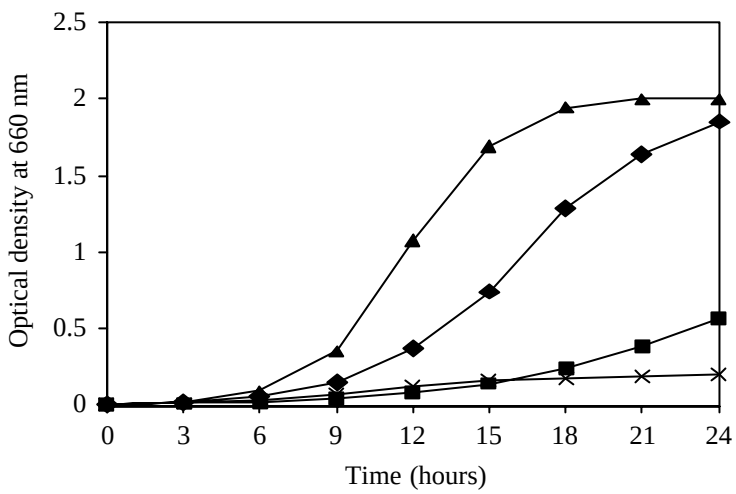

Figure 3 Growth of *S. xenophaga* and *V. paradoxus* in LB and MB (*S. xenophaga* in LB (◆) and MB (■) and *V. paradoxus* in LB (▲) and MB (X)).

to grow slowly and the maximum optical density was reached within 2 days (data not shown), whereas *V. paradoxus* was rarely grown. It was indicated that MB is not suitable for growing *V. paradoxus*. It might due to the less macroelements in MB. The medium which was suggested to grow both cultures in this case should be LB, however, *S. xenophaga* and *V. paradoxus* still have the different growth rates in this medium.

Growth of *S. xenophaga* and *V. paradoxus* from 20 to 30°C

S. xenophaga and *V. paradoxus* precultures were inoculated in 15 ml of LB. The cultures were incubated separately in the temperature gradient incubator from 20 to 30°C. The optical density at wavelength 660 nm of all cultures was shown in Figure 4. It was shown that the growth rates of *V. paradoxus* were higher than those of *S. xenophaga* at incubation temperature more than 22°C, however, the growth of *V. paradoxus* was decreased when incubated at 20°C. Moreover, at incubation temperature of 20°C growth pattern of *V. paradoxus* was quite similar to the growth of *S. xenophaga* at 22 and 20°C. Therefore, the incubation temperature at 20°C might be suggested for co-cultivation of *S. xenophaga* and *V. paradoxus*, although at this temperature both cultures were grown very slowly.

Co-cultivation of *S. xenophaga* and *V. paradoxus* together in one tube with the different ratios of cell density

S. xenophaga and *V. paradoxus* precultures were inoculated in LB with various ratios of both cell optical densities (*S. xenophaga* : *V. paradoxus* = 1:1, 1:10, 10:1, 100:1). The cultures were incubated in the temperature gradient incubator with shaking at 50 rpm at 20°C. After 2 days of incubation, supernatants were collected and filtrated before adding into *A. tumefaciens* A136 culture for determining the expression of β-galactosidase activity. *A. tumefaciens* A136 genetically modified by deleting *luxI* gene (for AHL synthesis) but still containing *luxR* (for AHL receptor) connected with gene encoding β-galactosidase producing enzyme was used as a reporter strain (Fuqua and Winans, 1996). From Figure 5, it was shown that the higher growth rates were found in the cultures containing higher ratios of *V. paradoxus* or the culture of *V. paradoxus*

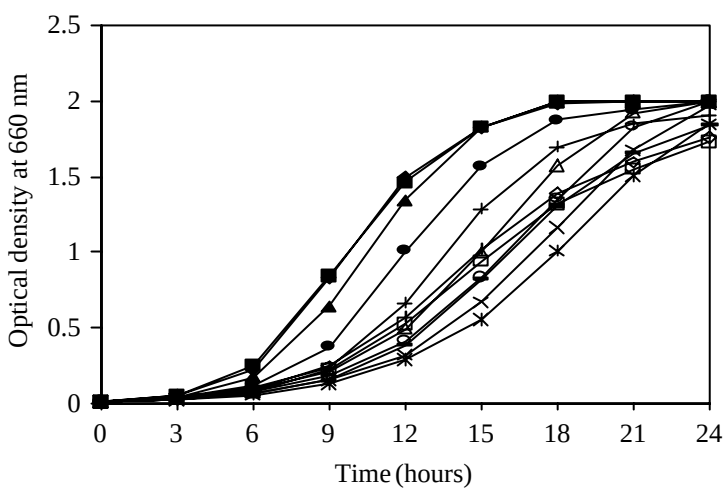

Figure 4 Growth of *S. xenophaga* and *V. paradoxus* at the different temperatures (*S. xenophaga* at 30° C (□) 28°C (◇) 26°C (△) 24°C (○) 22°C (X) 20°C (*) and *V. paradoxus* at 30°C (■) 28°C (◆) 26°C (▲) 24°C (●) 22°C (+) and 20°C (–)).

only because *V. paradoxus* had slightly the higher growth rate than *S. xenophaga*. After 2 days of cultivation all growth curves approached to the stationary phase. The cell supernatants were sampled and β-galactosidase activity was measured and shown in Table 1.

Expectedly, the highest amount of β-galactosidase activity or AHL was found in the supernatant of *S. xenophaga* and *S. xenophaga* : *V. paradoxus* (100:1). Because *S. xenophaga* is a Gram-negative bacterium and was reported to produce AHL (Guan and Kamino, 2001), it is reasonable to detect the highest amount of AHL in supernatant of *S. xenophaga* only. In case of using supernatant of *S. xenophaga* : *V. paradoxus* in ratio of 100:1, the high amount of AHL or β-galactosidase activity was also found. It can be explained that the very low ratio of *V. paradoxus* inoculum size was not enough to show any effects on AHL even though *V. paradoxus* showed the

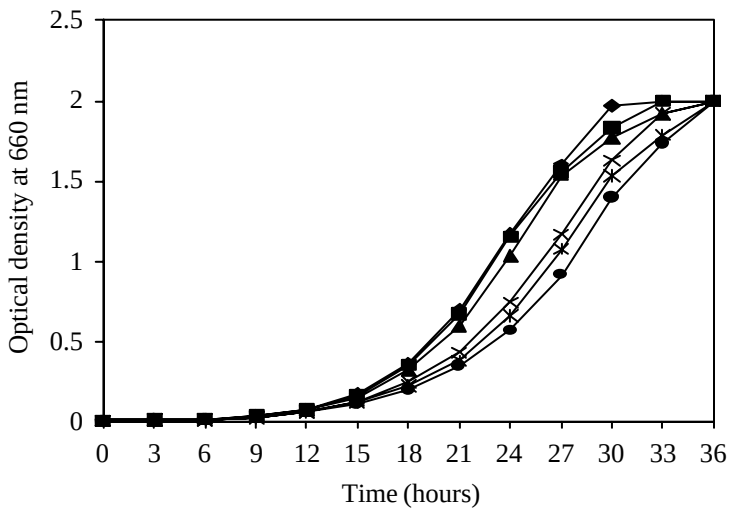

Figure 5 Growth of *S. xenophaga* and *V. paradoxus* in various ratios of the initial cell densities (*S. xenophaga* only (●), *V. paradoxus* only (■), *S. xenophaga* : *V. paradoxus* (1:1) (▲), *S. xenophaga* : *V. paradoxus* (10:1) (X), *S. xenophaga* : *V. paradoxus* (1:10) (◆) and *S. xenophaga* : *V. paradoxus* (100:1) (*)).

Table 1 β-galactosidase activity expressed by *Agrobacterium tumefaciens* A136 from various kinds of the cell supernatants.

Types of cell supernatants	Average β-galactosidase activity (units)
S. xenophaga	2782
V. paradoxus	5
S. xenophaga : *V. paradoxus* (1:1)	22
S. xenophaga : *V. paradoxus* (10:1)	1297
S. xenophaga : *V. paradoxus* (1:10)	10
S. xenophaga : *V. paradoxus* (100:1)	2757

AHL-degrading enzyme activity, the higher growth rate and the utilization of AHL as an energy source.

In this experiment, AHL could not be produced by *V. paradoxus* because no β-galactosidase activity could be determined. In addition, it was shown that AHL was utilized by *V. paradoxus* because the higher ratio of *V. paradoxus* resulted in the less AHL amount (from the comparison of the ratio of *S. xenophaga* : *V. paradoxus*). It could be concluded that AHL produced by *S. xenophaga* was utilized by *V. paradoxus* as an energy source via the AHL-degrading enzyme and implied that the AHL degrading enzyme might degrade all types of AHL produced in all Gram-negative bacteria by degrading either the acyl side chain or the lactone ring.

The low AHL amount of cell supernatant of *S. xenophaga* : *V. paradoxus* in the ratio of 1:1 (Table 1) indicated that AHL degradation rate in *V. paradoxus* was higher than the AHL biosynthesis rate in *S. xenophaga*. AHL produced by *S. xenophaga* was supposed to be the nutrient source for *V. paradoxus* during the late log phase after the declination of nutrient, however, it might be the other interactive relationships between both organisms. Therefore, the dialyzing culture vessel was used in the next experiment to study each growth rate and the relationships between the AHL production by *S. xenophaga* and the AHL degradation by *V. paradoxus*.

Co-cultivation of *S. xenophaga* and *V. paradoxus* in the dialyzing culture vessel

S. xenophaga and *V. paradoxus* were inoculated separately in each partition of the dialyzing culture vessel containing 50 ml of 1/5 LB. For control, either *S. xenophaga* or *V. paradoxus* was inoculated to both partitions of each medium. All cultures were incubated with shaking at 100 rpm, 20°C and sampled for determining growth by mean of optical density at 660 nm and measuring AHL in the supernatants. In case of cultivation one microorganism, the results showed that AHL was found in the *S. xenophaga* supernatant during the late log phase period whereas AHL was not found in *V. paradoxus* supernatant. For the co-cultivation results, β-galactosidase activity in *V. paradoxus* side was found higher than usual because AHL produced by *S. xenophaga* was penetrated through filter membrane to *V. paradoxus* side. (Figure 6B) and β-galactosidase activity showed the highest peak at hour 36. Looking at the growth characteristics, *V. paradoxus* was grown faster than *S. xenophaga* during the late log phase period in 1/5 LB medium (Figure 6A). By co-cultivation method, AHL produced by *S. xenophaga* during the late log phase was utilized by *V. paradoxus* as an energy source, resulting in an increase of the 2-fold higher growth rate. The increase of growth rate was found in the limited medium like 1/5 of LB medium but not found in the enriched medium like LB medium (data not shown). Moreover, by co-cultivation method the growth pattern of *S. xenophaga* was slightly changed. The retarding of cell death in *S. xenophaga* during the stationary phase was shown. It might be explained that AHL produced by *S. xenophaga* was utilized by *V. paradoxus*, therefore AHL level was lower and not enough to bind the receptor for the induction of AHL-responding target genes resulting in no physiological changes and the slower death.

From co-cultivation results, it could be concluded that *V. paradoxus* could utilize AHL produced by *S. xenophaga* as an energy source, promoting its higher growth. However, there might be either some interaction of both organisms. In natural environment, bacteria can not live alone but form community with their own or different organisms, therefore, it is quite complicated to study the effect of AHL only by co-cultivation method. We are on progress to partially purify the AHL degrading enzyme and study the action of enzyme in order to control the bacterial populations.

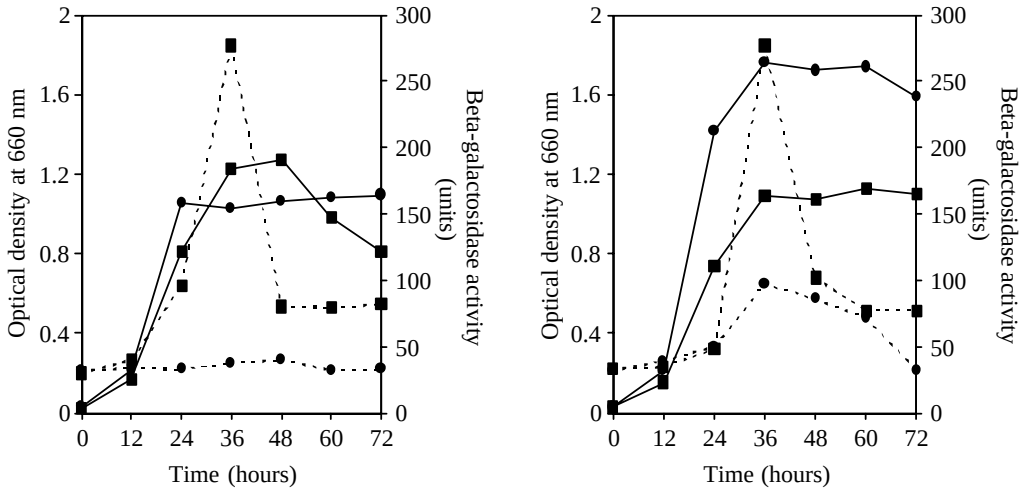

Figure 6 Growth and β-galactosidase activity of *S. xenophaga* and *V. paradoxus* in the dialyzing vessel tube when (A) both sides contain *S. xenophaga* only (■), or *V. paradoxus* only (●) (B) determined from *S. xenophaga* side of co-cultivation (■), and from *V. paradoxus* side of co-cultivation (●). Stragiht line (——) shows OD value whereas dash line (------) indicates the β-galactosidase activity.

CONCLUSION

In order to control the amount of a cell-to-cell signal molecule AHL, co-cultivation of *S. xenophaga* that produced AHL and *V. paradoxus* that was able to degrade AHL was performed. The optimum medium and incubation temperature used for co-cultivation of both bacteria were LB medium and 20°C, respectively. *V. paradoxus* could utilize AHL produced from *S. xenophaga* as an energy source, promoting the 2-fold higher growth rate of *V. paradoxus* and the slower death of *S. xenophaga.*

LITERATURE CITED

Baraniecki, C.A., J. Aislabie and J.M. Foght. 2002. Characterization of *Sphingomonas* sp. Ant17, an aromatic hydrocarbon-degrading bacterium isolated from antarctic soil. **Microb. Ecol.** 43(1) : 44-54.

Dunny, G.M. and B.A. Leonard. 1997. Cell-cell communication in Gram positive bacteria. **Annu. Rev. Microbiol.** 51 : 527-64.

Fuqua, C., S.C. Winans and E.P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. **J. Bacteriol.** 176(2) : 269-75.

Fuqua, C. and S.C. Winans. 1996. Conserved *cis*-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. **J. Bacteriol.** 178(2) : 435-440.

Greenberg, E.P. 2000. Acyl-homoserine lactone quorum sensing in bacteria. **The Journal of Microbiology** 38(3) : 117-21.

Guan, L.L. and K. Kamino. 2001. Bacterial response to siderophore and quorum-sensing chemical signals in the seawater microbial community. **BMC Microbiology.** 1 : 27-37.

Leadbetter, J.R. and E.P. Greenberg. 2000. Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus.* **J. Bacteriol.** 182(24) : 6921-6926.

Ohno, M., I. Okano, T. Watsuji, T. Kakinuma, K. Ueda and T. Beppu. 1999. Establishing the independent culture of a strictly symbiotic bacterium *Symbiobacterium thermophilum* from its supporting *Bacillus* strain. **Biosci. Biotechnol. Biochem.** 63(6) : 1083-1090.

Prak, D.J. and P.H. Prtichard. 2002. Degradation of polycyclic aromatic hydrocarbons dissolved in Tween 80 surfactant solutions by *Sphingomonas paucimobilis* EPA505. **Can. J. Microbiol.** 48(2) : 151-158.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. **Molecular Cloning : a Laboratory Manual I-III.** 2nd ed. Cold spring harbor laboratory press. New York. 1544 p.

Supaka, N., P. Pinphanichakan, K. Pattaragulwanit, S. Thaniyavarn, T. Omori and K. Juntongjin. 2001. Isolation and characterization of a phenanthrene-degrading *Sphingomonas* sp. strain P2 and its ability to degrade fluoranthene and pyrene via cometabolism. **ScienceAsia** 27 : 21-28.

Williams, P., M. Camara, A. Hardman, S. Swift, D. Milton, V.J. Hope, K. Winzer, B. Middleton, D.I. Pritchard and B.W. Bycroft. 2000. Quorum sensing and the population-dependent control of virulence. **Phil. Trans. R. Soc. Lond.** 355 : 667-680.