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| 보 문 |

Effects of Cell-free Culture Fluids for the Expression of Putative Acyltransferase in *Corynebacterium glutamicum*

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코리네형 균주의 Acyltransferase 발현에 미치는 세균배양액의 효과

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Autoinduction is mediated by signaling molecules known as autoinducers (AIs) that are produced, released and detected by bacterium itself. We recently reported that *Corynebacterium glutamicum* possesses an autoinduction system which secretes autoinducers during the stationary-phase of growth, triggering the expression of acyltransferase gene. However, it is still not clear what may act as autoinducers for the autoinduction in *C. glutamicum*. In this study, we compared the inducing effects of cell-free culture fluids obtained from a number of microbes including *Agrobacterium tumefaciens*, *Vibrio harveyi*, and *Escherichia coli*. Fluids from *A. tumefaciens* did not increase the expression of acyltransferase, whereas fluids from *V. harveyi* BB120 (AI-1$^+$, AI-2$^+$) did. Interestingly, the expression was increased by the fluids obtained from the early exponential-phase culture of BB120. Furthermore, this induction was not observed by the fluids from autoinducer mutants of *V. harveyi* MM77 (AI-1$^-$, AI-2$^-$) and BB152 (AI-1$^-$, AI-2$^+$). Unlike the effect shown by BB152, fluids from *E. coli* (AI-1$^-$, AI-2$^+$) still induced the acyltransferase expression. Taken together, these results suggest that *C. glutamicum* autoinducers seem to be unidentified molecules which do not belong to AI-1 or AI-2.

***Keywords*:** *Corynebacterium glutamicum*, acyltransferase, cell-free culture fluid

The autoinduction is mediated by a range of autoinducers, which are produced, released extracellularly and detected by bacterium itself (Miller and Bassler, 2001). When bacterial cultures reach a sufficient cell-population density, the concentration of autoinducers reaches a minimal threshold level, allowing physiological modulation through altering expression of a number of genes including virulence factors (Waters and Bassler, 2005). So far, diverse Gram-nagative and positive bacteria have been reported to have autoinduction by using lipid-based molecules, termed acylhomoserine lactones (AHLs) or peptide-based molecules, termed oligopeptide autoinducers to communicate with each other (Waters and Bassler, 2005).

Corynebacterium glutamicum is well known for its biotechnological importance in the industrial production of amino acids, such as glutamate, lysine and threonine (Malumbres and Martin, 1996; Hermann, 2003). Due to its industrial importance, *C. glutamicum* has been intensively subjected to study the metabolic pathways of amino acids biosynthesis since it is first discovered in 1957. However, information on the regulatory mechanisms at the level of gene expression including autoinduction still remains limited (Brinkrolf *et al.*, 2007). Recently, we first reported the presence of autoinduction in *C. glutamicum* by identifying a putative acyltransferase (NCgl0350), whose expression is highly induced by autoinducers present in the cell-free culture fluids from the stationary-phase culture of *C. glutamicum* (Shin *et al.*, 2011). The potent autoinducers in the fluids seemed to be small molecules because they are resistant to heat-treatment and proteinase K treatment. Interestingly, the expression of acyltransferase was also increased by fluids obtained from the culture of *C. ammoniagenes*, a closely related strain to the *C. glutamicum*, as well as of *Pseudomonas aeruginosa*, a bacterium well known to mediate autoinduction through acylhomoserine lactones.

The autoinducers for acyltransferase induction seem to commonly present in a number of bacteria and may potentially

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confer interspecies communication. In this study, we were interested in further exploring the effects of fluids obtained from the culture of different bacteria including *Agrobacterium tumefaciens*, *Vibrio harveyi* and *Escherichia coli*.

Materials and Methods

Bacterial strains and culture conditions

A. tumefaciens (KCTC22158) and *E. coli* DH5α were grown in LB (yeast extract, 0.5%; tryptone, 1%; and NaCl, 1%; all w/v) at 37℃, while *C. glutamicum* was grown in MB (yeast extract, 0.4%; tryptone, 1%; Soytone, 0.4%; and NaCl, 0.5%; all w/v) at 30℃. *V. harveyi* was grown in LB containing 3% NaCl at 37℃.

Preparation of cell-free culture fluids

All bacterial strains tested were grown in proper broth with shaking for 36 h or as indicated in the text. To obtain the supernatant, the culture broth was centrifuged for 10 min at 10,000×g at 4℃. The cell-free culture fluids were prepared by passing the supernatant through a cellulose acetate filter with a 0.2 μm pore size. The fluids were stored at -20℃.

Bacterial growth-permissive assay

C. glutamicum strain C3 carrying vector control (CON) or C6 carrying pHS09004 (P_{0350}:CAT) were used for this assay as previously described (Shin *et al.*, 2011). Brifely, pHS09004 was constructed as a fusion plasmid containng a promoterless chloramphenicol acetyltransferase (*cat*) gene under the control of acyltransferase (NCgl0350) promoter. The strains were grown overnight at 30℃ in MB medium containing 30 μg/ml of kanamycin. The culture broth was diluted by 1:2,000 (v/v) into an assay medium. The assay medium comprised a MB medium mixed with an equal volume of cell-free culture fluids in the absence or presence of 40 μg/ml of chloramphenicol. In control experiments, fresh MB medium alone was used. The strains were grown in the assay medium with shaking at 30℃ for 24 h. The culture growth was quantified by measuring the cell density at OD_{600} and used as an indication for *cat* expression

Results and Discussion

Acyltransferase expression was induced by cell-free culture fludis obtained from the stationary-phase culture of *P. aeruginosa*, indicating that the fluids could have similar autoinducers present in *C. glutamicum*. To further explore the effects of fluids obtained from other bacteria, we examined the effect of cell-free culture fluids obtained from *A. tumefaciens* culture grown for the indicated time as shown in Fig. 1. Both C3 carrying vector control (CON) or C6 carrying pHS09004 (P_{0350}:CAT) was not grown in MB mixed with fluids in the presence of chloramphenicol, indicating that the fluids did not induce the expression of *cat* under the control of the acyltransferase

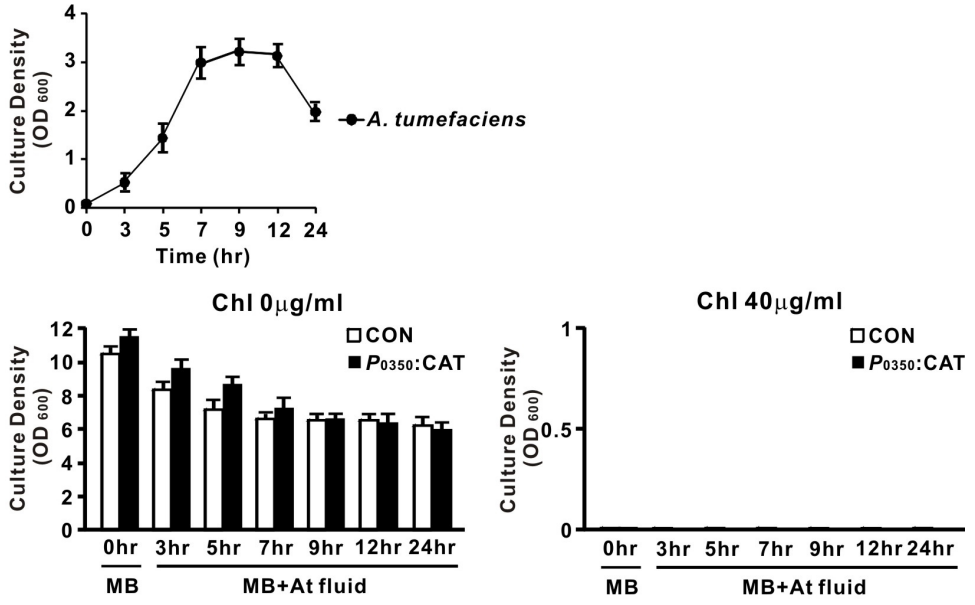

Fig. 1. Acyltransferase expression by cell-free culture fluids from *A. tumefaciens*. The growth of *A. tumefaciens* wild-type (upper) was measured at 3, 5, 7, 9, 12, and 24 h of culture. Culture density at OD_{600} was measured to quantify the growth of *C. glutamicum* carrying either vector control (CON) or pHS09004 (P_{0350}:CAT) in response to the cell-free culture fluids (At fluid) obtained at the indicated culture time in the absence or presence of 40 μg/ml chloramphenicol (Chl). The data represent three independent experiments.

(NCgl0350) promoter, rendering resistance to chloramphenicol. In the absence of chloramphenicol, all strains grew, although the cell density in the MB mixed with fluids was reduced little compared to that in MB. This is likely due to a reduced amount of nutrients present in the MB mixed with fluid compared to fresh MB medium alone. Although *A. tumefaciens* is known to have the LuxR homologue TraR whose stability is increased upon AI-1 binding, the autoinduction is activated at the plant-bacteria interface and requires both plant- and bacteria-produced signals (Waters and Bassler, 2005). This could be a reason why we did not detect the effect of *A. tumefaciens* fluids for the induction.

V. harveyi is a bacterium well studied about autoinduction, which depends on the production and detection of two different autoinducers, AI-1 (3OH C_4-HSL) synthesized by LuxLM and AI-2 synthesized by LuxS (furanosyl borate diester) (Cao and Meighen, 1989; Bassler *et al.*, 1994). To determine whether these molecules are involved in the induction of acyltransferase in *C. glutamicum*, we examined the effect of cell-free culture fluids obtained from the culture of *V. harveyi* wild-type BB120 grown for 3, 5, 12, and 24 h. As shown in Fig. 2, the growth of the BB120 strain reached mid-exponential phase after 5 h of culture and entered the stationary phase after 12 h. In the presence of chloramphenicol, the C6 strain did not grow in MB alone or MB+BB120 fluid from 12 and 24 h cultures. However, growth was clearly observed in the MB+BB120 fluid from 3 and 5 h cultures, especially by 3 h. These results suggest that potent autoinducers were produced in *V. harveyi* during the early-exponential growth phase. Although quorum activity is generally dependent on the increase of bacterial population, it is also influenced by diverse biochemical and physiological conditions such as the presence of preferred carbon source, temperature, pH and osmolarity. It has been reported that *E. coli* and *Salmonella typhimurium* increase AI-2 production during exponential phase whereas the production is reduced by entering stationary phase (Surette and Bassler, 1998).

Next, we examined the effects of cell-free culture fluids obtained from either *V. harveyi* MM77 or BB152 culture grown for 3, 5, and 36 h. MM77 is a double mutant strain incapable of producing both AI-1 and AI-2 (Mok *et al.*, 2003) whereas BB152 is a single mutant incapable of producing only AI-1 (Bassler *et al.*, 1994). The growth of both MM77 and BB152 was similar to that of the BB120 (data not shown). However, in the presence of chloramphenicol, the C6 strain did not grow in MB+MM77 fluid or MB+BB152 fluid from 3 and 5 h cultures (data not shown). These results suggest that AI-1 might play a role as a potent autoinducer. However, it was reported that the production of AI-1 is highly limited to *V. harveyi* and *V. parahaemolyticus*, the closely related *Vibrio* species, indicating that the AI-1 is used for intraspecies communication. Whereas, AI-2 production is widespread in Gram-negative and Gram-positive bacteria and used for interspecies communication

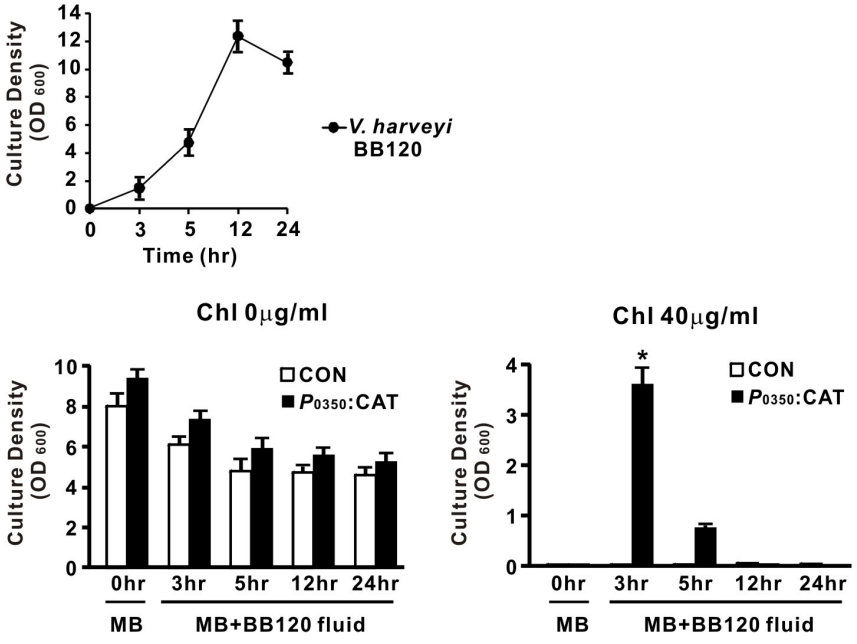

Fig. 2. Acyltransferase expression by cell-free culture fluids from *V. harveyi* BB120. The growth of *V. harveyi* wild-type BB120 (upper) was measured at 3, 5, 12, and 24 h of culture. Acyltransferase expression was measured by the same procedures described in the legend of Fig. 1. The data represent three independent experiments.

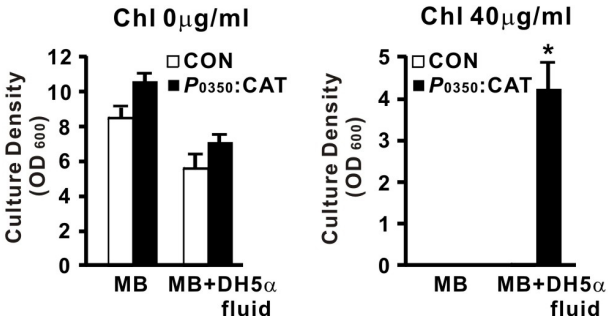

Fig. 3. Acyltransferase expression by cell-free culture fluids from *E. coli* DH5α. Cell-free culture fluids were obtained from the stationary-phase culture of *E. coli* DH5α. Acyltransferase expression was measured by the same procedures described in the legend of Fig. 1. The data represent three independent experiments.

(Bassler *et al.*, 1997; Surette and Bassler, 1999; Miller and Bassler, 2001).

To confirm the effect of AI-1, we obtained and examined the effect of fluids from *E. coli*, which is known to produce AI-2 like molecules but not AI-1 like molecules because they do not have a *luxI* gene (Surette and Bassler, 1998; Walters and Sperandio, 2006). As shown in Fig. 3, C6 strain was grown in MB mixed with the fluids in the presence of chloramphenicol, indicating that AI-1 might not be required for acyltransferase expression and that there might be new-type of molecules which do not belong to AI-1 or AI-2. Interestingly, acyltransferase expression was induced by fluids from not *V. harveyi* mutants but wild-type, suggesting that the new-type of molecules might work in hierarchical system. In the other hand, as the structure of AI-1 in *E. coli* and *V. harveyi* is not identical, we can not rule out the possibility that *V. harveyi* AI-1 may play a role inducing acyltransferase expression.

Further investigation is currently ongoing to determine autoinducers and to identify regulators responsible for the autoinduction in *C. glutamicum*. *C. glutamicum* belongs to the high GC Gram-positive actinomycetes including *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, and *Streptomyces coelicolor* (Bentley *et al.*, 2002). Possible presence of autoinduction in *M. tuberculosis* has been proposed, and the speculated signaling molecule was low molecular weight molecules (American Society for Microbiology. General Meeting 1999). However, the phenomenon of autoinduction is relatively unexplored in these microbes. The identification of autoinduction in *C. glutamicum* will contribute to better understanding autoinduction not only in *C. glutamicum* for the purpose of industrial benefit but also in these important human pathogens.

적 요

이 연구의 목적은 코리네형 균주(*Corynebacterium glutamicum*)에 존재하는 acyltransferase 유전인자의 발현에 관여하는 autoinducer에 대한 정보를 얻기 위해 다양한 종류의 균주에서 얻은 세포배양액(cell-free culture fluids)을 처리하여 발현에 미치는 효과를 조사하는 것이다. 다양한 배양시간동안 얻은 *Agrobacterium tumefaciens*의 세포배양액은 acyltransferase의 발현을 전혀 증가시키지 못하는 것으로 관찰되었다. 반면 AI-1과 AI-2를 분비하는 것으로 알려진 *Vibrio harveyi* BB120 (AI-1$^+$, AI-2$^+$)을 지수성장기까지 배양시켜 얻은 세포배양액은 acyltransferase의 발현을 증가시키는 것으로 관찰되었다. 이들 autoinducer가 미치는 효과를 조사하기 위하여 돌연변이 균주인 MM77 (AI-1$^-$, AI-2$^-$)과 BB152 (AI-1$^-$, AI-2$^+$)를 사용하여 조사한 결과, 이들 균주에서 얻은 세포배양액은 BB120와는 달리 acyltransferase의 발현을 증가시키지 않는 것으로 나타났다. 이러한 결과로 보아 acyltransferase의 발현은 AI-1에 의한 효과로 보이며, 이를 확인하기 위해 *V. harveyi* BB152와 같이 AI-2만을 분비하는 것으로 알려진 *Escherichia coli* (AI-1$^-$, AI-2$^+$)를 사용하였다. 하지만 *E. coli* 세포배양액은 acyltransferase의 발현을 증가시키는 것으로 관찰되었다. 이들 결과는 코리네형 균주에 존재하는 autoinducer는 기존에 알려진 형태의 신호분자와 차이가 있을 것이며, 코리네형 균주외에 *V. harveyi*와 *E. coli*에도 존재하여 종간 상호작용(interspecies communication)에 관여할 것으로 보인다.

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