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Stringent Factor Regulates Antibiotics Production and Morphological Differentiation of *Streptomyces clavuligerus*

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Abstract The involvement of the *relA* and *rsh* genes in the morphological and physiological differentiation of *Streptomyces clavuligerus* was evaluated with the *relA* and *rsh* genes mutants. The morphological differentiation of *S. clavuligerus* was greatly affected by the disruption of the *relA* gene, but not very much by the disruption of the *rsh* gene. The altered morphological characteristics were completely restored by the complementation of the corresponding disrupted genes. Thus, it was apparent that the mycelial morphology and clavulanic acid production were severely affected by the disruption of the *relA* gene. Production of clavulanic acid in the submerged batch culture and glycerol-limited chemostat showed that production was inversely related to the specific growth rate in the wild-type strain. However, the production of clavulanic acid in the Δ*relA* and Δ*rsh* null mutants was completely abolished. Therefore, it seems plausible that the stringent response of *S. clavuligerus* to starvation for amino acids is governed mainly by RelA, rather than Rsh, and that the (p)ppGpp synthesized immediately after the depletion of amino acids triggers the initiation of pathways for both morphological and physiological differentiation in this species.

Key words: Clavulanic acid, *relA*, *rsh*, ppGpp, chemostat

Stretomycetes are Gram-positive mycelial soil bacteria that produce a wide range of antibiotics and other secondary metabolites (physiological differentiation), whose production is very closely related to unique morphological differentiation characters to form substrate mycelia, aerial mycelia, and spores in solid culture. In liquid culture, physiological differentiation and morphological differentiation are generally confined to stationary phase cultures, when nutrients essential for growth are limiting [11, 12].

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It has widely been recognized that the accumulation of stringent factor ppGpp is one of the apparent responses of various bacteria to a nutritional shift-down [2]. The synthesis of ppGpp from ATP and GTP in *Escherichia coli* is accomplished by two enzymes, ppGpp synthetase (RelA) and ppGpp synthetase/hydrolase (SpoT) [11]. The RelA protein catalyzes ppGpp synthesis, under amino acid limitation, in association with ribosome [7]. The SpoT protein is a bifunctional enzyme which synthesizes ppGpp under carbon limitation in a ribosome-independent mode and also degrades (p)ppGpp by means of a manganese-dependent ppGpp pyrophosphohydrolase activity [6].

Although the roles of the stringent factor in *Streptomyces* spp. under nutrient limited condition are not well understood, there is physiological and genetic evidence to suggest that ppGpp plays an important role for initiation of antibiotic biosynthesis as an intracellular signaling molecule [2, 8, 15, 16, 22]. When the (p)ppGpp synthetase-encoding gene (*relA*) of *S. coelicolor* A3(2) is deleted, the *relA* null mutant fails to produce actinorhodin and undecylprodiosin under the conditions of nitrogen limitation [3, 4]. A second RelA/SpoT homologous protein, RshA, was also identified from the genome sequence of *S. coelicolor,* however, no experimental evidence is available to indicate if RshA, like RelA, can act as a bifunctional enzyme, both synthesizing and degrading (p)ppGpp [23].

The involvement of the unusual nucleotide (p)ppGpp during morphological and physiological differentiation of *Streptomyces clavuligerus* ATCC27064 has been elucidated. Two genes, *relA*, which encodes an 843 amino acid protein (RelA), and the *rsh* gene, which encodes a 738 amino acid protein (Rsh), were identified. The role of ppGpp on antibiotics production and morphological differentiation of *S. clavuligerus* were analyzed by comparing the phenotypes of the *relA* and *rsh* gene-disrupted mutants. It was found that the stringent response of *S. clavuligerus* to starvation for amino acids is governed mainly by RelA rather than

Rsh, and that the ppGpp synthesized immediately after the depletion of amino acids triggers the initiation of pathways for both morphological and physiological differentiation in *S. clavuligerus* [10]. Moreover, other study also shows that the stringent response is more apparently regulated by the limitation of phosphate [9].

In the current study, the profiles of morphological differentiation and production of clavulanic acid in various strains of *S. clavuligerus* were compared, and the effects of growth rate and specific nutrient uptake rate on antibiotics production are also discussed.

MATERIALS AND METHODS

Microorganisms and Preparation of Media

Streptomyces clavuligerus ATCC 27064 was used as the wild-type strain. Mutants obtained from the wild-type are listed in Table 1. The strains of *S. clavuligerus* were cultured on a solid inorganic salts starch medium (ISP4, Difco Laboratories, Detroit, MI, U.S.A.) at 30°C for 14 days. Spores of the wild-type strain and the complementary strain formed on the agar plates of the ISP4 medium were harvested and suspended in 20% (v/v) glycerol nutrient medium. Substrate mycelium grown on the agar plates of the ISP4 medium were harvested, fragmented aseptically, and then suspended in 20% (v/v) glycerol nutrient medium. The spores and fragmented mycelia suspension (about 10^{11} colony forming units per ml) were stored at -70°C.

Seed culture medium (TSB) for antibiotic production consisted of 1.0% (w/v) maltose, 0.5% glucose, 1.7% Bacto tryptone, 0.3% Bacto soytone, 0.25% NaCl, and 0.25% K_2HPO_4 (pH was adjusted to 7.0 before steam sterilization). Production medium for the batch culture consisted of 1.0% (w/v) glycerol, 0.6% arginine, 0.2% KH_2PO_4, 0.06% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$, 0.005% $MnCl_2 \cdot 4H_2O$, and 0.005% $ZnCl_2$. Small amount of medium for batch culture was sterilized by autoclaving, and glucose, NH_4Cl, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ were aseptically added to the autoclave after sterilization through membrane filtration (0.2 μm, Millipore). Medium for the glycerol-limited continuous culture contained (w/v): 0.4% glycerol, 0.1% NH_4Cl, 0.2% KH_2PO_4, 0.005% $MgSO_4 \cdot 7H_2O$, 0.005% $FeSO_4 \cdot 7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$, 0.005% $MnCl_2 \cdot 4H_2O$, and 0.005% $ZnCl_2$. Medium for continuous culture was prepared by membrane filtration (0.2 μm, Millipore). The pH of the media was adjusted to 7.0. The sterilized medium was collected in previously sterilized bottles (20 l) and kept at 4°C until use.

Solid Cultures for Observation of Morphological Differentiation

The stored spores or fragmented mycelia were inoculated on the ISP4 medium by streaking and cultured at 30°C. The growth phenotypic characteristics of the strains were observed after culturing for 14 days on ISP4 solid medium. Then, the changes in morphological characters through cross between wild-type and the disruption mutants were observed.

Antibiotics Production in Submerged Batch and Chemostat Cultures

Antibiotic production in batch culture by the wild-type and mutant strains of *S. clavuligerus* was assessed in a jar fermentor (KF-5l, Ko-Biotec). One ml of the spore suspension was inoculated to 100 ml of seed culture medium in 500-ml culture flasks and cultured at 30°C for 3 days on a rotary shaker (150 rpm). In batch culture, the seed culture (300 ml) was inoculated to 3 l of production medium in the jar fermentor. Glycerol-limited chemostat was followed by the batch culture, where the sterilized medium was fed into the culture vessel from the sterilized medium reservoir by using a peristaltic pump. A constant volume of culture (1.5 l) was maintained by overflowing the culture medium through a port installed at the side vessel of the fermentor. In order to prevent back contamination, glass traps were installed on the lines for medium supply line and culture overflow line, respectively. A positive pressure was maintained in the culture vessel to help outflow and also to prevent back contamination. The culture temperature was maintained at 30°C, and pH was adjusted to 7.0 by automatic addition of 1 N HCl or 1 N NaOH. Agitation was fixed at 300 rpm, and aeration was controlled to 1 volume of air per volume of medium per min.

Analytical Methods

To measure cell growth, triplicate samples (10 ml) of cultures were collected on preweighted filters (Whatman GF/C) by vacuum filtration. The filter cakes were washed twice with 10 ml of 0.85% NaCl solution and once

Table 1. Microorganisms and their characteristics used in this study.

S. clavuligerus strains	Characteristics	Reference
ATCC27064	Wild-type strain: clavulanic acid producer	[10]
SMF387	The *relA* gene in the wild-type strain was disrupted	
SMF389	The disrupted *relA* gene in SMF387 was complementary restored	
SMF3815	The *rsh* gene in the wild-type strain was disrupted	
SMF3816	The disrupted *rsh* gene in SMF3815 was complementary restored	

Table 2. Fermentation kinetic parameters of the wild-type, *ΔrelA* mutant, and *Δrsh* mutant of *S. clavuligerus*, calculated from the glycerol-limited chemostat cultures.

Kinetic parameters	Wild-type (ATCC 27064)	*ΔrelA* mutant (SMF387)	*Δrsh* mutant (SMF3815)
$Y_{x/glu}$ (g g^{-1})	1.67	0.85	1.56
q_{gly} (g g^{-1} h^{-1})	0.09	0.04	0.16
q_{amm} (g g^{-1} h^{-1})	0.004	0.004	0.011
q_{cla} (g g^{-1} h^{-1})	0.90	0.01	0.55

with 10 ml of distilled water and then dried at 80°C for 24 h and reweighed. The concentration of glycerol and phosphate in the supernatant was determined by the methods of Quesenberry and Lee [20] and Pierpoint [18]. The concentration of ammonium ion was measured immediately after the sampling by using a specific ion analyzer (Model EA940, Orion Research), and the concentration of clavulanic acid was quantified by HPLC after modification with imidazole [5, 17].

Fermentation Kinetic Parameters

Data from continuous cultures were analyzed for specific growth rate (μ), and specific rate of cla production (q_{cla}). Kinetic parameters in continuous cultures were calculated as follows as suggested by Pirt [18]. $\mu=D$, where D is the dilution rate (h^{-1}); $q_{cla}=D$*cla/x, where cla is the steady state production of clavulanic acid.

Chemicals, Reagents, and Reproducibility

All reagents were purchased from Sigma (Steinheim, Germany). Experiments were carried out twice and mean values are given.

RESULTS

Disruption of the *relA* and *rsh* Genes and the Growth Characteristics on Solid Medium

The growth and morphological characteristics of the wild-type (ATCC27064), *ΔrelA* (SMF387), *Δrsh* (SMF3815), and the complementary restored mutants (SMF389 and SMF3816) of *S. clavuligerus* were examined after growing for 14 days on ISP4 medium (Fig. 1). In comparison to the wild-type strain which formed abundant aerial mycelia and spores, the mutants showed very different characteristics. The *ΔrelA* mutant showed a bald appearance, resulting from a lack of aerial mycelia and spore formation (Fig. 1a). However, the growth characteristics of the *Δrsh* mutant was not significantly changed in growth characters compared to the wild-type (Fig. 1b). Both complementary strains restored the growth phenotype characters of the wild-type strain. Microsopic observation revealed that the *ΔrelA* mutant grew to form short, branched, and clumped mycelia, whereas

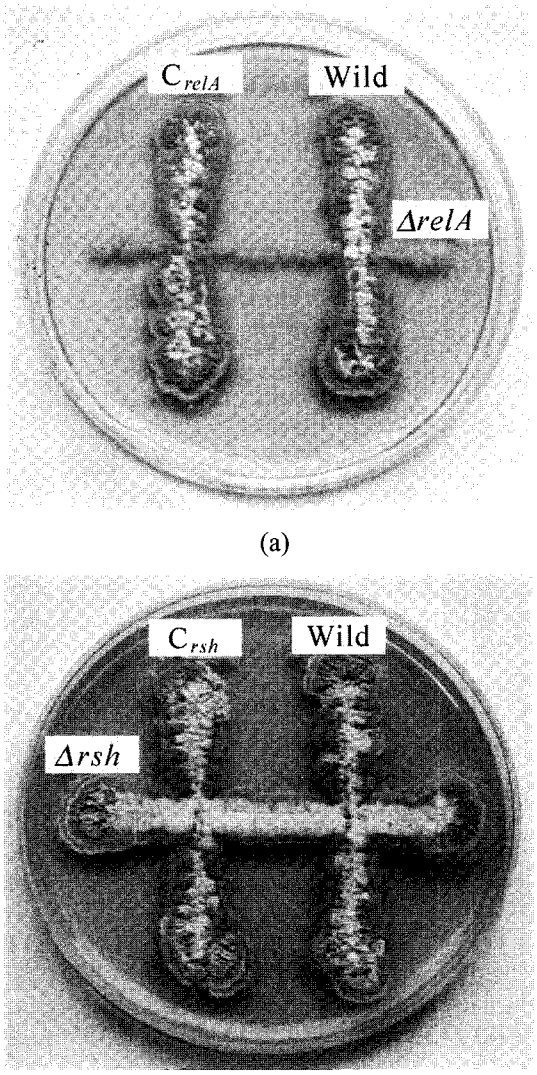

Fig. 1. The effects of *relA* and *rsh* genes on morphological differentiation of *S. clavuligerus*. *S. clavuligerus* ATCC 27064 (wild-type), *relA* mutant (*ΔrelA*), and *rsh* mutant (*Δrsh*) were cultured on ISP4 medium for 14 days.

the *Δrsh* mutant grew in a manner similar to the wild-type, but with slightly straighter hyphae and less branching (Fig. 2). Moreover, it was clear that the growth characters of the strains did not interfere with the strains that were cross-cultured on ISP4 agar medium. The mycelial growth of the *ΔrelA* mutant in submerged culture was retarded, compared to the wild-type (Figs. 3a and 3b), whereas the growth of the *Δrsh* mutant was not greatly altered (Fig. 3c).

Production of Clavulanic Acid in Batch and Chemostat Cultures

Mycelium growth, glycerol uptake, and clavulanic acid production in submerged batch cultures of wild-type, *ΔrelA* mutant, and *Δrsh* mutant of *S. clavuligerus* were compared.

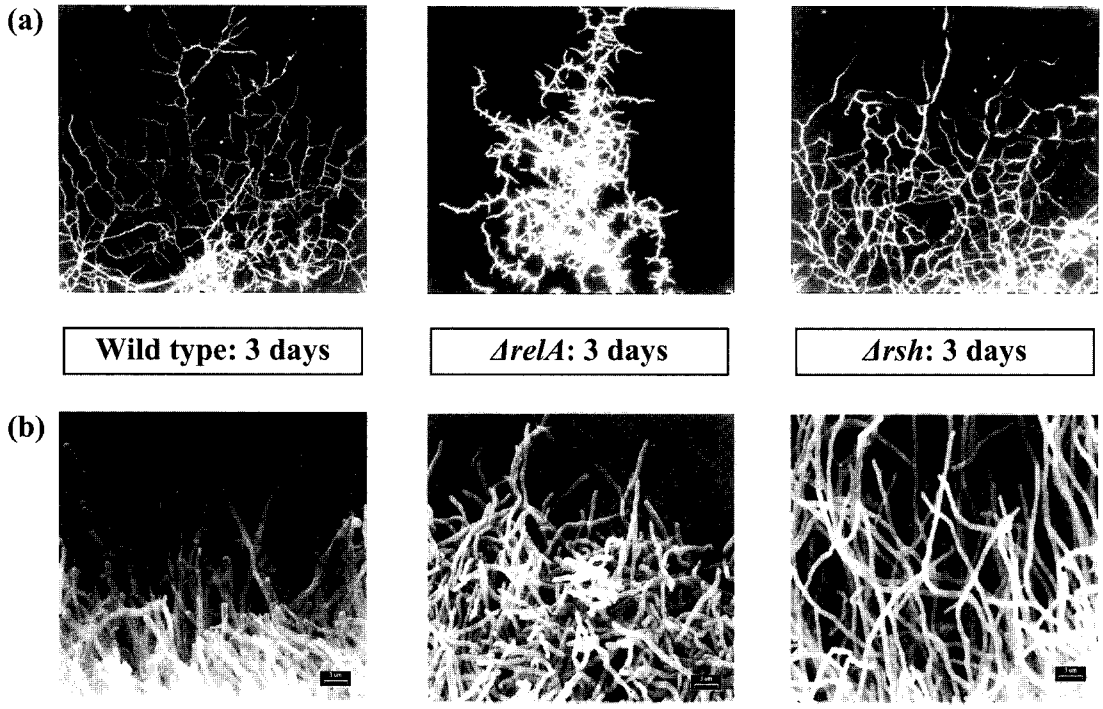

Fig. 2. Mycelial micrographs of wild-type, *ΔrelA* mutant, and *Δrsh* mutant strains of *S. clavuligerus*. The strains were grown on ISP4 medium for 3 days.

Clavulanic acid production in the wild-type coincided with the decline of mycelium concentration and with the accumulation of ammonium ions (Fig. 3a). However, the production of clavulanic acid in the batch cultures of the *ΔrelA* mutant and *Δrsh* mutant was almost abolished, although the mycelium growth rate of the strains was not significantly altered by the disruption of the *relA* and *rsh* genes (Figs. 3b and 3c).

In order to evaluate the relationship between the growth rate and the production of clavulanic acid, glycerol-limited chemostats were carried out with the wild-type strain and the mutants (Fig. 4a and 4b). Steady-state data in the

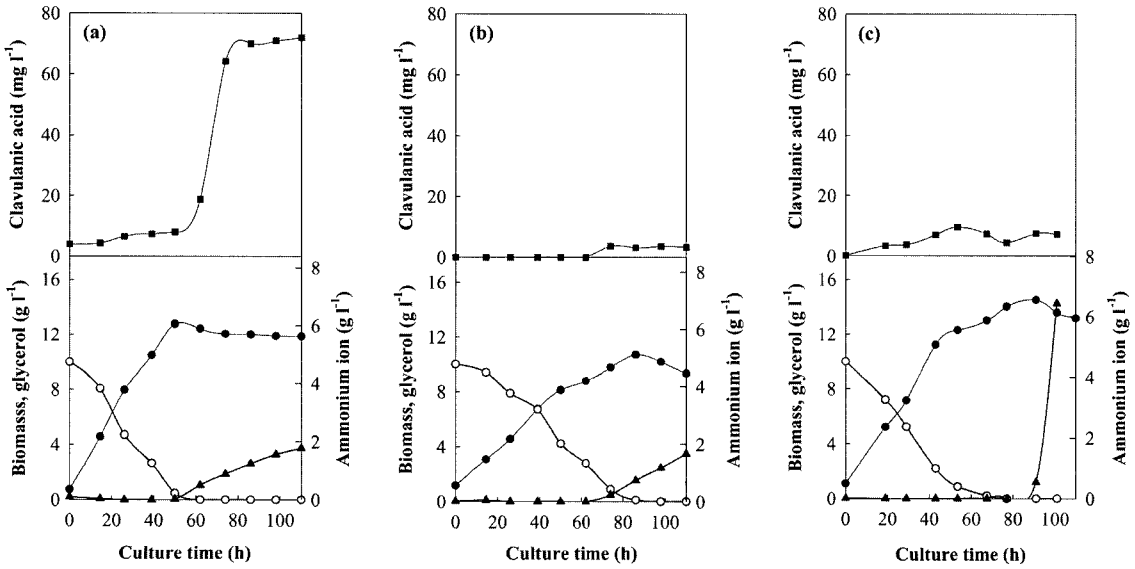

Fig. 3. The changes of biomass (●), glycerol (○), ammonium ion (▲), and clavulanic acid production (■) of wild-type, *ΔrelA* mutant, and *Δrsh* mutant of *S. clavuligerus* during batch cultures.

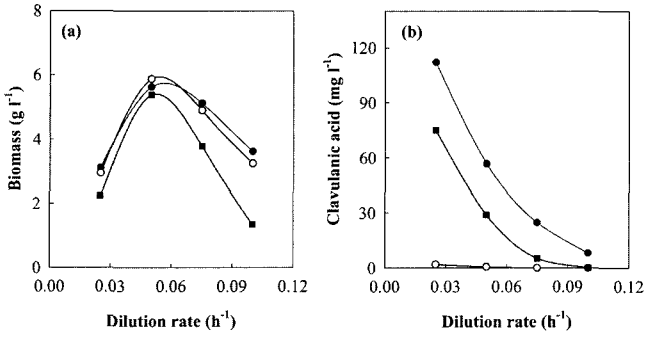

Fig. 4. Relationship between stringent factors and clavulanic acid production of wild-type (●), *ΔrelA* mutant (○), and *Δrsh* mutant (■) of *S. clavuligerus* grown in continuous cultures.

chemostats showed that the production of clavulanic acid in the wild-type was inversely related to the growth rate. However, clavulanic acid production in the *ΔrelA* mutant was completely abolished at all the growth rates, and the production in the *Δrsh* mutant was significantly lower, but still sustained.

DISCUSSION

This paper deals with the involvement of two *relA/spoT* homologue genes, *relA* and *rsh*, in morphological differentiation and antibiotic production (physiological differentiation) in *S. clavuligerus*. The morphological differentiation of *S. clavuligerus* was greatly affected by the disruption of the *relA* gene, but not much by the disruption of the *rsh* gene. The altered morphological characteristics were completely restored by complementation of the corresponding disrupted genes. The minor changes in mycelial morphology of the *Δrsh* gene was presumably associated with the consequent accumulation of ppGpp and pppGpp after amino acid starvation [10].

Clavulanic acid production in the wild-type coincided with the decline of mycelium concentration and with the accumulation of ammonium ions, suggesting that clavulanic acid production is closely related to the metabolism of amino acids. Kinetic data for growth and antibiotic production were more accurately determined with glycerol-limited chemostat, which showed that the production of clavulanic acid was inversely related to the growth rate and that the productions of clavulanic acid and cephamycin were optimum at a dilution rate of 0.025 h^{-1}. The results presented in this paper indicate that the growth rate plays an important role in determining the onset of antibiotic production in *S. clavuligerus*, and that the initiation of biosynthesis can be triggered by the level of ppGpp. It is believed that the products of *rel*A play an essential role in the regulation of the level of (p)ppGpp, and in turn, in the initiation of clavulanic acid biosynthesis in *S. clavuligerus*.

In a previous report, it was found that the production of ppGpp and pppGpp in *S clavuligerus* was completely abolished in the *ΔrelA* mutant, and that the levels of ppGpp and pppGpp were significantly lowered in the *Δrsh* mutant. The data indicated that the *relA* gene has a more dramatic effect on the production of (p)ppGpp than does *rsh* [10]. Mycelial morphology on the agar medium was severely affected by the disruption of the *relA* gene, and both of these phenotypes are probably associated with inability of the mutants to accumulate (p)ppGpp after amino acid starvation. However, the mutation of the *rsh* gene resulted in only minor changes in ppGpp and pppGpp accumulation, which was more directly regulated by phosphate rather than amino acids or carbon sources.

Therefore, it seems plausible that the stringent response of *S. clavuligerus* to amino acids starvation is governed mainly by RelA, rather than Rsh, and that the (p)ppGpp synthesized immediately after the depletion of amino acids triggers the initiation of pathways for both morphological and physiological differentiation in this species. The changes in clavulanic acid production upon the deletion of the gene most likely result from the changes in (p)ppGpp concentration, an essential factor regulating the production of clavulanic acid in *S. clavuligerus*.

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REFERENCES

1. Bascaran, V., L. Sanchez, C. Hardisson, and A. F. Brana. 1991. Stringent response and initiation of secondary metabolism in *Streptomyces clavuligerus*. *J. Gen. Microbiol.* **137:** 1625-1634.
2. Cashel, M., D. R. Gentry, V. J. Hernandez, and D. Vinella. 1996. The stringent response, pp. 1458-1496. *In*: Neidhardt F. C. *et al.* (eds.). *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, vol. 2, Chapter 92. American Society for Microbiology, Washington, DC, U.S.A.
3. Chakraburtty, R. and M. J. Bibb. 1997. The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. *J. Bacteriol.* **179:** 5854-5861.
4. Chakraburtty, R., J. White, E. Takano, and M. J. Bibb. 1996. Cloning, characterization and disruption of a (p)ppGpp

synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **19:** 357-368.
5. Foulstone, M. and C. Reading. 1982. Assay of amoxycillin and clavulanic acid, the components augmentin, in biological fluids with high performance liquid chromatography. *Antimicrob. Agents Chemother.* **22:** 753-762.
6. Gentry, D. R. and M. Cashel. 1996. Mutational analysis of the *Escherichia coli spoT* gene identifies distinct but overlapping regions involved in ppGpp synthesis and degradation. *Mol. Microbiol.* **19:** 1373-1384.
7. Haseltine, W. A. and R. Block. 1973. Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. *Proc. Natl. Acad. Sci. USA* **70:** 1564-1568.
8. Hoyt, S. and G. H. Jones. 1999. *relA* is required for actinomycin production in *Streptomyces antibioticus*. *J. Bacteriol.* **181:** 3824-3829.
9. Jin, W., H. K. Kim, J. Y. Kim, S. G. Kang, S. H. Lee, and K. J. Lee. 2004. Cephamycin C production is regulated by *relA* and *rsh* genes in *Streptomyces clavuligerus* ATCC 27064. *J. Biotechnol.* **114:** 81-87.
10. Jin, W., Y. G. Ryu, S. G. Kang, S. K. Kim, N. Saito, K. Ochi, S. H. Lee, and K. J. Lee. 2004. Two *relA/spoT* homologous genes are involved in the morphological and physiological differentiation of *Streptomyces clavuligerus*. *Microbiology* **150:** 1485-1493
11. Kang, S. G., W. Jin, M. J. Bibb, and K. J. Lee. 1998. Actinorhodin and undecylprodigiosin production in wild type and *relA* mutant strains of *Streptomyces coelicolor* A3(2) grown in continuous culture. *FEMS Microbiol. Lett.* **168:** 221-226.
12. Kim, C. J., Y. K. Chang, G.-T. Chun, Y.-H. Jeong, and S. J. Lee. 2002. Physiological characteristics of immobilized *Streptomyces* cells in continuous cultures at different dilution rates. *J. Microbiol. Biotechnol.* **12:** 557-562.
13. Lee, C.-H., B.-J. Kim, G. J. Choi, K. Y. Cho, H. Yang, C. Shin, S. Min, and Y. Lim. 2002. *Streptomyces* with antifungal activity against rice blast causing fungus, *Magnaporthe grisea*. *J. Microbiol. Biotechnol.* **12:** 1026-1028.
14. Metzger, S., E. Sarubbi, G. Glaser, and M. Cashel. 1989. Protein sequences encoded by the *relA* and *spoT* genes of *Escherichia coli* are interrelated. *J. Biol. Chem.* **264:** 9122-9125.
15. Ochi, K. 1986. Occurrence of the stringent response in *Streptomyces* spp. and its significance for the initiation of morphological and physiological differentiation. *J. Gen. Microbiol.* **132:** 2621-2631.
16. Ochi, K. 1987. Changes in nucleotide pools during sporulation of *Streptomyces griseus* in submerged culture. *J. Gen. Microbiol.* **133:** 2787-2795.
17. Paradkar, A. S. and S. E. Jensen. 1995. Functional analysis of the gene encoding the clavaminate synthase 2 isoenzyme involved in clavulanic acid biosynthesis in *Streptomyces clavuligerus*. *J. Bacteriol.* **177:** 1307-1314.
18. Pierpoint, W. S. 1957. The phosphatase and metaphosphatase activities of pea extract. *Biochem. J.* **65:** 67-76.
19. Pirt, S. J. 1975. Parameters of growth and analysis of growth data, pp. 4-14 *In*: *Principles of Microbe and Cell Cultivation.* Blackwell, Oxford, U.K.
20. Quesenberry, M. S. and Y. C. Lee. 1996. A rapid formaldehyde assay using purpald reagent: Application under periodation conditions. *Anal. Biochem.* **234:** 50-55.
21. Sohng, J. K., H. C. Lee, K. Liou, E. B. Lee, S.-Y. Kang, and J.-S. Woo. 2003. Cystocin, a novel antibiotic, produced by *Streptomyces* sp. GCA0001: production and characterization of cystocin. *J. Microbiol. Biotechnol.* **13:** 483-486.
22. Strauch, E., E. Takano, H. A. Baylis, and M. J. Bibb. 1991. The stringent response in *Streptomyces coelicolor* A3(2). *Mol. Microbiol.* **5:** 289-298.
23. Sun, J., A. Hesketh, and M. J. Bibb. 2001. Functional analysis of *relA* and *rshA*, two *relA/spoT* homologues of *Streptomyces coelicolor* A3(2). *J Bacteriol* **183:** 3488-3498.