Journal of Zhejiang University SCIENCE
ISSN 1009-3095
http://www.zju.edu.cn/jzus
E-mail: jzus@zju.edu.cn

A pair of two-component regulatory genes *ecr*A1/A2 in *S. coelicolor*[*]

LI Yong-quan (李永泉)[†1], CEN Pei-lin (岑沛霖)[2], CHEN Shi-fei (陈时飞)[1,3],
WU Dan (吴 丹)[1], ZHENG Jing (郑 静)[1]

([1]*College of Life Science,* [2]*Institute of Bioengineering, Zhejiang University, Hongzhou 310027, China*)
([3]*Drug Adiministration of Zhejiang Province, Hongzhou 310012, China*)
[†]E-mail: lyq@mail.hz.zj.cn

Received Jan. 24, 2003; revision accepted May 20, 2003

Abstract: Two-component genes are kinds of genetic elements involved in regulation of antibiotic production in *Streptomyces coelicolor*. DNA microarray analysis revealed that *ecr*A1/A2, which mapped at distant sites from *red* locus and encode respectively the kinase and regulator, expressed coordinately with genes of Red specific biosynthetic pathway. *ecr*A1 and *ecr*A2 gene-disruptive mutants were constructed using homogenotisation by reciprocal double crossover. Fermentation data showed that the undecylprodigiosin (Red) level of production was lower than that of wild-type strain. However, the change of the actinorhodin (Act) production level was not significant compared with wild type. Thus, these experiment results confirmed that the two-component system *ecr*A1/A2 was positive regulatory element for *red* gene cluster.

Keywords: *Streptomyces coelicolor,* Two-component system, Antibiotic gene cluster, *ecr*A1/A2
Document code: A **CLC number**: Q813.4; Q78; Q939.13

INTRODUCTION

S.coelicolor as a model system has been extensively studied with the aid of genetic and molecular techniques. Its ability to produce four antibiotics (Hopwood *et al*., 1995), namely actinorhodin (Act), undecylprodigiosin (Red), Calcium-Dependent Antibiotic (CDA) and methylenomycin (Mmy), enables studies of pathyway-specific and pleiotropic regulation of antibiotic production. In which, Red is a red-pigmented antibiotic and Act is coelicolor-pigmented antibiotic that enable the genetic study of coordinate regulation using a straightforward visual screen. Pathway-specific regulator genes, *act*II-*orf*4 (Fernandez-Moreno *et al*., 1994) and *redD* (Takano, *et al*., 1992), respectively control productions of Red and Act. In addition, the two-component system also controls expression of structural, regulatory and resistance genes in the actinorhodin and undecylprodigiosin biosynthesis pathways. DNA microarray analysis revealed that *ecr*A1/A2, which mapped at distant sites from *red* locus, encode respectively the kinase and regulator and expressed coordinately with genes of Red specific biosynthetic pathway (Huang *et al*., 2001). The location of *ecr*A1 /A2, *redD* and *act*II-*orf*4 in the chromosome DNA sequence of *S.coelicolor* (www.sanger.com) and their function are listed in Table 1. The works reported in this research are construction of *ecr*A1 and *ecr*A2 gene-disruptive mutants and confirmation of their function.

MATERIALS AND METHODS

Bacterial strains and culture conditions

[*] Project (No.20172046) supported by the National Natural Foundation of China

Table 1 The location and function of *ecr*A1/A2 and pathway-specific regulator

Gene name	Location	DNA length	Possible function
*ecr*A1 (SCC121.20c)	2712740−2713423	683 bp	Response regulator
*ecr*A1 (SCC121.21c)	2713432−2714775	1343 bp	Sensor kinase
RedD (SCO5877)	6432566−6433618	1052 bp	Patway-specific regulator
*act*II-*orf*4 (SCO5085)	5528094−5528861	767 bp	Patway-specific regulator

Escherichia coli DH5*α* was grown at 37 ºC in Luria-Bertin (LB) medium supplemented with kanamycin (50 µl/ml, Sigma Chemical Company, St. Louis, MO, USA) or apramycin (50 µl/ml, Sigma Chemical Company, St. Louis, MO, USA), if necessary. *E. coli* ET12567 (*dam*$^-$) was cultured at 37 ºC in LB medium supplemented with chloramphenicol (30 µl/ml, Sigma Chemical Company, St. Louis, MO, USA), kanamycin or apramycin was also added when necessary.

S.coelicolor strain lyqA2001 (Km^r, Apr^s), lyqA 2002 (Km^r, Apr^s) and M145 (wt) were manipulated and their spores were stored at −20 ºC in 20% glycerol as described previously (Kieser *et al*., 2000). For fermentation, the spores were pre-germinated as described (Kieser *et al*., 2000), and then inoculated into modified liquid medium (R_5^-), pH 7.2, lacking additional KH_2PO_4, $CaCl_2$ and L-proline, supplemented with kanamycin (20 µl/ml). 6% PEG8000 was added to enhance cell dispersal and antifoam 289 was added (0.05% v/v) before use. R_5 medium was also used for the regeneration of *S.coelicolor* protoplast.

DNA manipulation and transformation

Recombinant DNA technique was implemented and *E.coli* plasmid DNA was prepared according to the standard procedures described by Sambrook *et al.*(1989). Competent *E.coli* cells were prepared and transformed by standard methods (Sambrook *et al.*, 1989). Lysozyme and some restriction endonucleases used were from Boehringer Mannheim (Amersham biosciences, USA). Purification of DNA fragments from PCR or agarose was carried out with QIAquick Purification Kit (QIAGEN Companies, Valencia, CA, USA). *Streptomyces* genomic DNA was prepared as described by Kieser *et al.*(2000). The alkaline lysis method was used to prepare plasmid from *S.coelicolor*, and its plotoplasts were prepared and transformed also according to Kieser *et al.*(2000).

Construction of plasmid and gene-disruption

pKC1139, a high copy number and temperature-sensitive *E.coli-Streptomyces* shuttle vector, digested with *BamHI/HindIII*, was used as a backbone to construct both pKCA2001 and pKCA2002. The strategy of constructing plasmid is illustrated in Fig.1. For construction of these two plasmids, every left and right fragment DNA was amplified by PCR using genomic DNA of wild-type *S.coelicolor* M145's genomic DNA as template. The central fragment DNA Km was also amplified by PCR using pUC119 as template, which was Kanamycin resistance gene. The pairs of primers for every PCR are listed in Table 2.

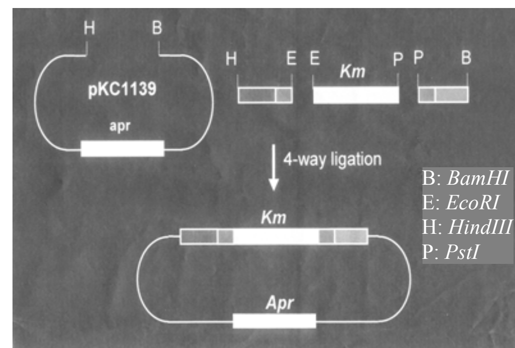


Fig.1 Construction of gene disrupted vectors

All PCR reactions were carried out using *pfu* DNA polymerase with hot start, and the PCR products were purified using QIAquick PCR Purification Kit (QIAGEN Company, USA) after separation by agarose gel electrophoresis. The PCR amplified DNA fragments were digested with the corresponding restriction endonucleases and cloned into the *HindIII/BamHI* digested pKC1139 by four-way ligation to create pKCA2001 and pKCA2002 (Fig.1). The gene-disruption was carried out using

Table 2 The pairs of primers for every PCR

	Sequence	Site
Left Arm of pKCA2001 (0.88 kb, upstream of *ecr*A1)		
Primer 1:	5’ TATTAAGCTTCCACCAGGCGCTCGAACTGATCCG 3’	(contained in *HindIII* site)
Primer 2:	5’ TTTTGAATTCGTTCTGGTCGTCCGCCAGCAGCAC 3’	(contained in *EcoRI* site)
Right Arm of pKCA2001 (0.87 kb, downstream of *ecr*A1)		
Primer 1:	5’ AAAACTGCAGAGCCCGCTCACCGCGAAGACCCAC 3’	(contained in *PstI* site)
Primer 2:	5’ AATTGGATCCCGAGGACACCTTCCCGCACTTCGC 3’	(contained in *BamHI* site)
Left Arm of pCKA2002 (0.94 kb, upstream of *ecr*A2)		
Primer 1:	5’ ATAAAAGCTTCCGATCAGCGTCATCAGCCCCACC 3’	(contained in *HindIII* site)
Primer 2:	5’ TATAGAATTCGGGTTCGTCGCTGTCGTGGTTGCC 3’	(contained in *EcoRI* site)
Right Arm of pKCA2002 (0.92 kb, downstream of *ecr*A2)		
Primer 1:	5’ AAAACTGCAGGGAGAGAACCGCATGACGATACGC 3’	(contained in *PstI* site)
Primer 2:	5’ AAATGGATCCGCAGAACGAGCGGGACGTCAACAC 3’	(contained in *BamHI* site)
Cental Fragment DNA (km, 1.0 kb)		
Primer 1:	5’ ATTTGAATTCTAGAGGATCCCCTGATACCGCTCGCCG3’	(contained in *EcoRI* site)
Primer 2:	5’ TATTCTGCAGCCAGAGTCCCGCTCAGAAGAACTCGTC3’	(contained in *PstI* site)

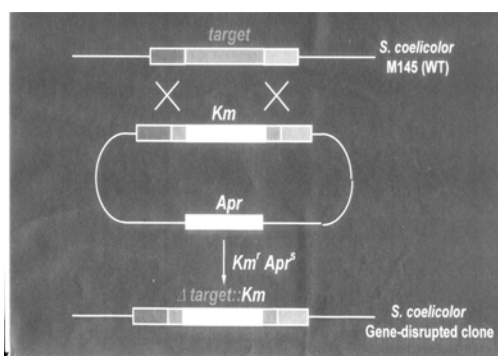


Fig.2 Homogenotisation by reciprocal double crossover

homogenotisation by reciprocal double crossover, as illustrated in Fig.2. Confirmation, selection and screening of mutants were carried out according to Kieser *et al*.(2000).

Confirmed PCR

Confirmed PCR reactions were carried out by using lyqA2001 genomic DNA as template with the forward primer (5’TATTAAGCTTCCACCAGGCG CTCGAACTGATCCG3) and the reverse primer (5’AATTGGATCCCGAGGACACCTTCCCGCACT TCGC3’), and using lyqA2002 genomic DNA as template with the forward primer (5’ATAAAAGCTT CCGATCAGCGTCATCAGCCCCACC3’) and the reverse primer (5’AAATGGATCCGCAGAACGA GCGGGACGTCAACAC3), respectively.

Southern hybridization

Genomic DNA of lyqA2001, lyqA2002 and WT M145 were digested with restriction endonucleases *BamHI* and *KpnI* and the fragments, separated by agarose gel electrophoresis, were transferred to nylon membranes (Southern, 1979). The probes were prepared by PCR using pKCA2001 plasmid DNA as template with pair of primers as the same as that of confirmed PCR, and using pKCA 2002 plasmid DNA as template with pair of primers as the same as that of confirmed PCR, respectively. The Digoxigenin (Dig)-dUTP was used to label DNA probes by the random priming method. Hybridization was carried out at 65 ºC for 16 h and then stringency washes. The labeled DNA was detected by the chemiluminescence procedure (Amersham Biosciences, Boehringer Mannheim, USA).

Antibiotic assays for *S.coelicolor*

Actinorhodin (Act): Sample was treated with 1N KOH to achieve pH 12, and then the culture was filtered through a 0.2 μ filter unit. Absorbance of the filtrate was measured at 640 nm spectrophotometrically.

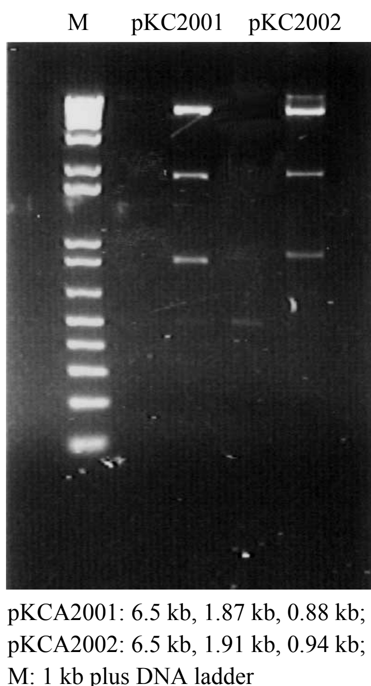
Undecylprodigiosin (Red): Sample was first extracted with 1 N KOH to solubilize actinorhodin, then centrifuged at 3000×g for 5 min.The mycelial pellet was washed and dried by vacuum, and then extracted with pH=2 methanol acidified with 1N HCl overnight at room temperature. The filtrate was detected at 540 nm spectrophotometrically after passing through a 0.2 μ filter unit.

EXPERIMENT RESULTS AND ANALYSIS

Construction of plasmid

The key factor affecting four-way ligation reaction was the mole ratio of the fragment DNA and Vector pKC1139, because their concentrations decided the collision ratio in the solution. The lower the concentration, the less the collision ratio. However, too high concentration would reduce the free space in the reaction solution and reduce the collision ratio. The experiment results of four-way ligation confirmed that the suitable ratio of fragment DNA and vector pKC1139 was 3:1−5:1 after digestion with the corresponding restriction endonucleases, and fragment DNA suitable concentrations were about 10−25 ng/μl (1 kb DNA as the criterion).

The electrophoresis of constructed plasmid pKCA2001 and pKCA2002 is shown in Fig.3 after amplification, purification and digestion with *HindIII/EcoRI.* In which and all of other electrophoresis experiments, the 1 kb plus DNA ladder was used as DNA marker (M) (Fig.4). Since both central fragment Km as the inserted DNA and pKCA1139 contained *EcoRI* site; every plasmid produced three bands after digestion. The sites of pKCA2001 should be 6.5 kb, 1.87 kb and 0.88 kb, and of pKCA2002 should be 6.5 kb, 1.91 kb and 0.94 kb. Every band site shown in Fig.3 is right.



pKCA2001: 6.5 kb, 1.87 kb, 0.88 kb;
pKCA2002: 6.5 kb, 1.91 kb, 0.94 kb;
M: 1 kb plus DNA ladder

Fig.3 Electrophrosis of constructed vectors after digesting with *HindIII* and *EcoRI*

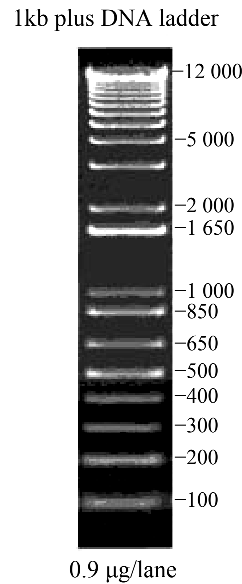
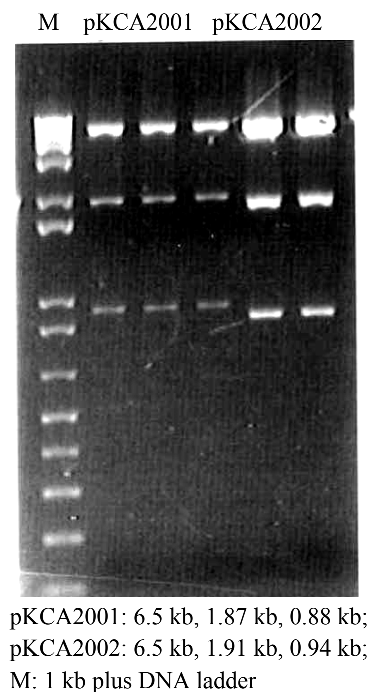


Fig.4 The size of DNA marker

Construction of gene-disruptive mutants

Both pKCA2001 and pKCA2002 were first transformed into *E.coli* DH5*α* followed by transformation into *E.coli* ET12567 to obtain the demethylated plasmids before transformation into *S.coelicolor* M145 protoplast. The electrophoresis of digested plasmids from transformants are shown in Fig.5.



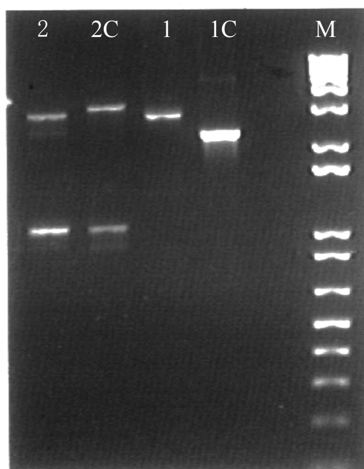
pKCA2001: 6.5 kb, 1.87 kb, 0.88 kb;
pKCA2002: 6.5 kb, 1.91 kb, 0.94 kb;
M: 1 kb plus DNA ladder

Fig.5 Electrophrosis of plasmid from *S.coelicolor* after digesting with *HindIII* and *EcoRI*

After transformation, propagation of transformants on non-selection R_5 medium at 30 ºC, and on R_5 medium supplemented with kanamycin at 37 ºC promoted double crossover recombination between the homologous sequence of pKCA2001 or pKCA2002 and *S.colicolor* M145 chromosome, resulting in replacement of *ecr*A1 or *ecr*A2 with kanamycin resistant gene. Then, sporulation of the transformants on R_5 plate supplemented with kana- mycin and with apramycin, respectively showing the desired phonotype (Km^r and Apr^s) was isolated. These recombinant clones were named lyqA2001 (*ecr*A1 disrupted mutant), lyqA2002 (*ecr*A2 disrupted mutant).

Confirmed PCR reaction and southern blotting

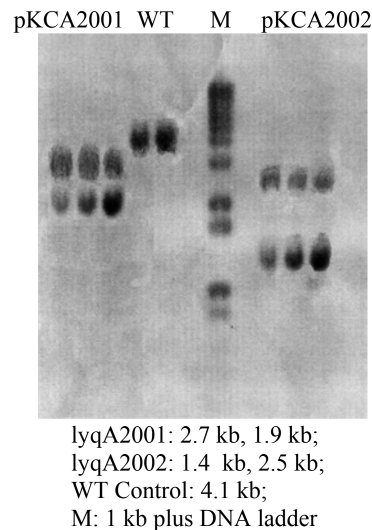
The two gene-disrupted mutants were confirmed by PCR (Fig.6) and Southern Blot hybridization analysis (Fig.7). Confirmed PCR reactions were carried out using the genomic DNA of gene-disruptive mutants lyqA2001 and lyqA2002 as template, respectively with the genomic DNA of WT strain M145 as control template. The size of PCR products should be 2.65 kb for lyqA2001 and 2.86 kb for lyqA2002; the control size should be 2.25 kb for lyqA2001 and 3.07 kb for lyqA2002. The electrophoresis of confirmed PCR products in Fig.6 demonstrated that every band was right.



1: lyqA2001(2.65 kb);
1C: Control(2.25 kb);
2: lyqA2002(2.86 kb);
2C: Control(3.07 kb);
M: 1 kb plus DNA ladder

Fig.6 Confirmed PCR from mutant genomic DNA

pKCA2001 WT M pKCA2002



lyqA2001: 2.7 kb, 1.9 kb;
lyqA2002: 1.4 kb, 2.5 kb;
WT Control: 4.1 kb;
M: 1 kb plus DNA ladder

Fig.7 Result of Southern Blotting for mutants and M145 (control)

Chromosomal DNA samples from lyqA2001, lyqA2002 and M145 were digested with either *BamHI* or *KpnI*, and probed with Digoxigenin (Dig) -dUTP labelled fragment DNA. lyqA2001 gave rise to 2.7 kb and 1.9 kb bands and lyqA2002 gave rise to 1.4 kb and 2.5 kb bands while WT M145 yielded 4.1 kb band, the size change from lyqA2001 and lyqA 2002 confirmed that both *ecr*A1 and *ecr*A2 were replaced by Km resistant gene.

Morphological study

For morphological study, two mutants were propagated on R_5 medium plate supplemented with Kanamycin for 9 d at 30 ºC, which showed there were no morphological and physiological differentiation between mutants and wild type. However, the color of strains indicated that undecylprodigionsin (Red) production was reduced greatly.

Growth kinetics and antibiotic production of mutants

Both growth kinetics and antibiotic production of lyqA2001, lyqA2002 and wild-type grown in R_5^- liquid medium were measured and compared (Figs.8–10), which revealed that there was less difference between the two mutants and M145 in cell growth and Act production. However, compared with wild-type strain, the Red production of mutants decreased about 60%. In addition, the experiment

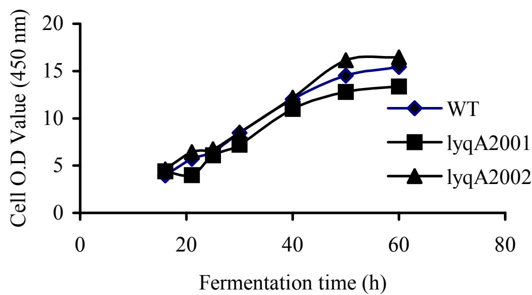


Fig.8 Cell growth kinetics of mutants and wild-type

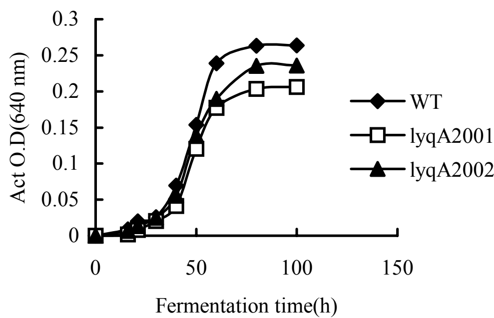


Fig.9 *Actinorhodin* (Act) production of mutants and wild-type

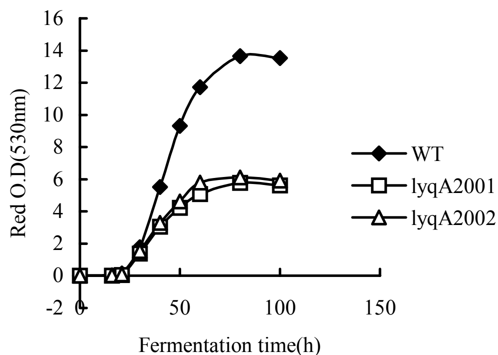


Fig.10 *Undecylprodigiosin* (Red) production of mutants and wild-type

results suggested the *ecr*A1/*ecr*A2 only affected the expression of *red* gene cluster, which could led to the conclusion that the *ecr*A1/*ecr*A2 two-component genes exerted positive control over expression of red gene cluster.

DISCUSSION AND CONCLUSION

In some studies, if the transcription levels of two pathway-specific regulatory genes, *act*II-*orf*4 and *redD* were affected by expression or disruption of some specific gene, the correspond production of antibiotics Act and Red were changed in the some way. However, some genes, whose disruption did not affect the transcription of pathway-specific regulatory genes, could change the antibiotic production (Floriano and Bibb, 1996), which suggested the *act*II-*orf*4 and *redD* are not the only factors that directly control the expression of the Act and Red biosynthesis gene cluster. In this case, *ecr*A1/*ecr*A2 genes did affect Red production.

Two-component system is a subtle way to regulate the antibiotic biosynthesis gene cluster. Most of these regulation elements such as *abs*A1/A2, *afs*Q1/*afs*Q2 and *cutR/cutS* have negative function (Aceti and Champness, 1998; Ishizuka *et al*., 1992; Chang *et al*., 1996). Some of them can yield morphological differentiation by gene-disruption or increasing copy number. However, *ecr*A1/*ecr*A2 genes have positive regulation over the Red biosynthesis gene cluster and their gene-disruption did not produce morphological change. More interesting is that the *ecr*A1/*ecr*A2 only affect Red production but not Act production.

Antibiotic production in *streptomyces* species generally is dependent on the growth phase and involves the expression of physically clustered regulatory and biosynthetic genes. Our data showed that antibiotic pathway genes are coordinately regulated at the level of transcription during *S.coelicolor* growth; and that this model of regulation has helped to define the physical boundaries of biosynthetic loci.

The regulatory network is very complicated, so a through analysis of the regulatory network structure is essential for a complete understanding of gene regulatory pattern and morphology including physiological development in *streptomyces* and other complex microorganisms. Ultimately, it will provide new strategies for manipulating secondary metabolism and for finding novel compounds as well as to increase production of valuable biologically active natural products.

ACKNOWLEDGEMENT

We are grateful to the Sanger Center for access to

the *Streptomyces coelicore* genome sequence; and express our heartfelt thank to the Chinese Scholarship Council's sponsor who helped the author finish part of this research work smoothly and for Prof. Wei-Shou Hu and Prof. David H. Sherman's guidance during the author's stay in the University of Minnesota, USA.

References

Aceti, D.J., Champness, W., 1998. Transcriptional regulation of *Streptomyces coelicolor* pathway-specific antibiotic regulators by the *absA* and *absA* loci. *Journal of Bacteriology*, **180**(12):3100-3106.

Chang, H.M., Chen, M.Y., Shieh, Y.T., Bibb, M.J., Chen, C. W., 1996. The *cutRS* signal transduction system of *Streptomyces* repress the biosynthesis of polyketide antibiotic actinorhodin. *Molecular Microbiology*, **21**(5): 1075-1085.

Fernandez-Moreno, M.A., Martinez, E., Caballero, J.L., Ichinose, K., Hopwood, D.A., Malpartida, F., 1994. DNA sequence and functions of the *act*VI region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor* A3(2). *J. Biol Chem.*, **269**(40):24854-24863.

Floriano, B., Bibb, M., 1996. afsR is a pleiotropic but conditionally regulatory gene for antibiotic production in in *Streptomyces coelicolor* A3(2). *Mol. Microbiol*, **21** (2):385-396.

Hopwood, D.A., Chater, K.F., Bibb, M.J., 1995. Genetics of antibiotic production in *Streptomyces coelicolor* A3(2), a model streptomyces, *Biotechnology*, **28**:65-102.

Huang, J.Q., Lih, C.J., Pan, K.H., Cohen, S.N., 2001. Stanley N. Global anylysis of growth phase responsive gene expression and regulation of antibiotic biosynthetic pathways in *Streptomyces coelicolor* using DNA microarrays. *Genes and development*, **15**(23):3183-3192.

Ishizuka, H., Horinouchi, S., Kieser, H.M., Hopwood, D.A., Beppu, T., 1992. A putative two-component regulatory system involved in secondary metabolism in *Streptomyces spp*. *Journal of Bacteriology*, **174**(23):7585-7594.

Kieser, T., Bibb, M.J., Chater, K.F., Hopwood, D.A., 2000. *Practical Streptomyces genetics*. John Innes Foundation, Norwich,United Kingdom.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory.

Southern, E.M., 1979. Measurement of DNA length by gel electrophoresis. *Anal. Biochem.*, **100**:319-323.

Takano, E., Gramajo, H.C., Strauch, E., Andres, N., White, J., Bibb, M.J., 1992. Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). *Mol. Microbiol*, **6**(19):2797-2804.

Welcome visiting our journal website: ***http://www.zju.edu.cn/jzus***
Welcome contributions & subscription from all over the world
The editor would welcome your view or comments on any item in the journal, or related matters
Please write to: Helen Zhang, Managing Editor of JZUS
E-mail: **jzus@zju.edu.cn** Tel/Fax: 86-571-87952276