Abstract  Two-component and phosphorelay signal transduction systems are the major means by which bacteria recognize and respond to a variety of environmental stimuli. Although several model systems, including sporulation in *Bacillus subtilis* and chemotaxis in *Escherichia coli*, have been extensively studied, the two-component signal transduction systems in industrially important actinomycetes are not well studied. We report the molecular and biochemical characterization of a novel two-component signal system, *amrA-amkA*, from the rifamycin-SV-producing *Amycolatopsis mediterranei* U32. The deduced sequences of *amkA* and *amrA* contain all the structural features that are highly conserved in the typical bacterial histidine kinases and response regulators, respectively. BLAST analyses showed that AmrA and AmkA displayed high similarities to AfsQ1/AfsQ2 of *Streptomyces coelicolor* and MtrA/MtrB of *Mycobacterium tuberculosis*. The *amrA* and *amkA* genes were over-expressed and the gene products were purified from *E. coli*. Biochemical studies showed that AmkA is able to autophosphorylate, supporting its functional assignment as a histidine kinase. That AmrA functions as the cognate response regulator for histidine kinase AmkA was demonstrated by in vitro phosphotransfer from [γ-32P]ATP-labeled AmkA to AmrA. Rifamycin SV production was also decreased by 10–20% in *amrA* or *amkA* gene disruption mutants under the tested condition. Although the detailed regulatory mechanism is still unknown, this is the first report regarding the involvement of two-component signal systems in rifamycin biosynthesis in the genus *Amycolatopsis*.

Keywords  *Amycolatopsis mediterranei* U32 · Two-component regulatory system · Signal transduction · Rifamycin SV production

Abbreviations  *Am* Aparmycin · *Amp* Ampicillin · *Cm* Chloromycetin · *Kan* Kanamycin · *IPTG* Isopropyl-β-D-thiogalactopyranoside · *Tc* Tetracycline

Introduction  Bacteria must modulate their gene expression repertoire in order to survive hostile conditions or exploit their environment. This adaptive response is often mediated by two-component regulatory systems, consisting of a membrane sensor (histidine kinase) and a cytoplasmic response regulator. These regulatory systems enable bacteria to sense and respond to environmental conditions even when the stimulus does not penetrate the cytoplasm (Parkinson 1993; Hoch 2000). In response to an appropriate signal, autophosphorylation occurs at a conserved histidine residue in the cytoplasmic domain of the sensor (Goudreau and Stock 1998). Binding of environmental signals causes activation of the autokinase resulting in ATP hydrolysis and phosphorylation of a histidine on a phosphotransferase subdomain of the autokinase. The phosphotransferase subdomain functions together with a regulator domain of a response regulator to which its phosphoryl group is transferred resulting in an aspartyl-phosphate. Regulator domains normally inhibit the output domain of the response regulator and phosphorylation relieves this inhibition freeing the output domain to carry out its function, which is usually transcription activation (Parkinson and Kofoid 1992). Signal propagation in two-component systems requires precise interaction between phosphoryl donors and acceptors to ensure the correct response. Therefore, it is not surprising that bacteria such as *Escherichia coli*, *Bacillus subtilis*...
and *Pseudomonas aeruginosa* possess 30–40 different pairs of two-component systems, each dedicated to unique signals (Soncini et al. 1996; Mizuno 1997; Fabret et al. 1999).

The gram-positive bacterial actinomycetes are characterized by their complex morphological differentiation, resembling that of filamentous fungi (aerial mycelium), and the ability to produce a wide variety of secondary metabolites. This complex life cycle and differentiation undoubtedly requires various levels of regulation and signal transduction mechanisms (Chater 1993). Earlier studies showed that antibiotic production is controlled in part by two-component signal systems. The best-studied examples are *afsQ1afsQ2* (Horinouchi and Beppu 1992a; Ishizuka et al. 1992) and *absAabsB* (Brian et al. 1996; Aceti and Champness 1998; Anderson et al. 2001) signal systems controlling antibiotic production in *Streptomyces coelicolor*, and the *chiSchiR* signal transduction systems involved in the regulation of chitinase in *Streptomyces coelicolor*, *Streptomyces thermoviolaceus* and *Streptomyces lividans* (Tsuibo et al. 1999; Kormanec et al. 2000). Several two-component systems were also demonstrated to be involved in the regulation of morphological differentiation in streptomycetes, such as *cseBcsec* which regulates cell wall integrity in *S. coelicolor A3(2)* (Paget et al. 1999), and the response regulator homologue *ramR* which causes accelerated aerial mycelium formation in *S. lividans* (Ma and Kendall 1994). The *S. coelicolor A3(2)* genome sequencing project completed recently at the Sanger Center of University of Cambridge has revealed more than 100 genes homologous to previously identified sensors and regulators from streptomycetes and other bacteria (http://www.sanger.ac.uk). Although the function of most of these genes is still unknown, the existence of such a large number of two-component systems in the streptomycetes genome clearly implies that they play very important physiological roles. Thus, elucidation of their functions will certainly benefit our understanding of the complex regulatory networks of secondary metabolites production and aid in our efforts to increase antibiotic titers.

*Amycolatopsis mediterranei* U32 is a producer of rifamycin SV, an important antibiotic against *Mycobacterium tuberculosis* (August et al. 1998). The biosynthetic cluster of rifamycin has recently been cloned and sequenced from *A. mediterranei* (August et al. 1998; Tang et al. 1998). A detailed analyses of this cluster will provide more information about the genetic structure of rifamycin polyketide synthase, and the genes characterized could be modified to produce novel forms of rifamycins that may be effective against rifamycin-resistant microorganisms. Present knowledge about the regulation of antibiotic production and morphological differentiation in *A. mediterranei* U32 is very limited, and no signal transduction system similar to that in *S. coelicolor A3(2)* has ever been described. In previous papers (Zhang et al. 2000; Yang et al. 2001), we reported the characterization of a few eukaryotic-type serine/threonine protein kinases which might be involved in the secondary metabolism of rifamycin-SV-producing *A. mediterranei* U32. The results strongly implied that the signal transduction mechanisms involved in the transfer of a phosphoryl group might play very important roles in the metabolic regulation of *Amycolatopsis*. In this paper, we report the molecular and biochemical characterization of a novel two-component system, *amrA-amkA*, from *A. mediterranei* U32. The enzymatic functions of *amrA* and *amkA* as a response regulator and histidine kinase, respectively, were demonstrated by direct phosphotransfer between them. Furthermore, gene replacement experiments demonstrated that *amrA-amkA* might play a role in rifamycin SV production by *A. mediterranei* U32.

### Materials and methods

**Bacterial strains and growth conditions**

Strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in LB medium (Sambrook et al. 1989) supplemented with 150 μg ampicillin/ml, 15 μg tetracycline/ml or 30 μg kanamycin/ml when necessary. *A. mediterranei* strains were grown in Ben’s medium (glucose 0.1%, tryptone 0.02%, yeast extract 0.4%, beef extract 0.01%, glycerol 0.1%, nitrate potassium 0.7%, w/v, pH 7.2) at 28 °C (Zhang et al. 2000). The medium was supplemented with 30 μg aminoglycoside/ml or 34 μg chloromycetin/ml for mutants carrying the resistance gene (Hopwood et al. 1985).

**Cloning and sequencing of *amrA-amkA* genes**

Two degenerate oligonucleotides corresponding to the conserved domain in the response regulator gene were designed as PCR amplification primers:

- Primer 1: 5′-CCGGATTATCTGACCTNATGCTRCCTGG-3′
- Primer 2: 5′-GGAATTCCGAACWASACCGACGACMGMT-3′

Both PCR primers have an EcoRI site at their 5′ end (underlined). PCR (30 cycles, 94 °C 30 s, 55 °C 30 s, 72 °C 60 s) was performed using *A. mediterranei* U32 genomic DNA as a template. The PCR product with the expected size (460 bp) was excised from the gel (Gel Isolation Kit, Shanghai Waston, Shanghai), cloned into pGMET-easy (Shanghai Promega, Shanghai) and confirmed by sequencing (ABI Matrix, model 377, Takara Biotech, Dalian Co., Dalian). The fragment was then labeled with [α-32P]-dCTP (Promer-A-Gene, Promega, Madison, Wis.) and used to probe an *A. mediterranei* U32 cosmid library. The library was constructed in the cosmid vector pLAFR3 as described before (Staskawicz et al. 1987; Zhang et al. 2000). A 7.0-kb EcoRI fragment from the positive cosmid clones was subcloned into pBluescript II/KS (+) (Promega) and used to probe an *A. mediterranei* U32 cosmid library. The library was constructed in the cosmid vector pAPAF3 as described before (Staskawicz et al. 1987; Zhang et al. 2000). A 7.0-kb EcoRI fragment from the positive cosmids clones was subcloned into pBluescript II/KS (+) (Promega), generating pWJ1 for further studies. The sequences were analyzed by FramePlot 2.3* software (available online http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl), BioEdit software (Hall 1999), BLAST2 (Tatusova and Madden 1999), or the GCG software package (Genetics Computer Group, University of Wisconsin, Madison, Wis.). The putative hydrophobicity profile was analyzed using TMPred software available online (http://www.ch.embnet.org/software/TMPRED_form.html).

**Expression in *E. coli*, gene product purification, and refolding**

To over-express *amrA* gene in *E. coli* BL21(DH5), plasmid pETRA was constructed by PCR with two synthetic oligonucleotides as primers, with the positive cosmid DNA as template:

- Primer 3: 5′CATATGACTGACGGGTTGGCGGC 3′
- Primer 4: 5′AAGCTTACGAAACCGGACGAGCAG3′

Primer 3 contains a Ndel site and primer 4 an HindIII site (underlined). The PCR product was purified directly from the gels, di-