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A Novel Two-Component System *amrB-amkB* Involved in the Regulation of Central Carbohydrate Metabolism in Rifamycin SV-Producing *Amycolatopsis mediterranei* U32

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Abstract. A novel two-component signal transduction system *amrB-amkB* was cloned from rifamycin SV-producing *Amycolatopsis mediterranei* U32, and their biochemical functions as a response regulator and a histidine protein kinase, respectively, were proven. The *amrB* disruption mutant was generated by insertional inactivation with the aparmycin resistance gene. The metabolic response to the absence of *amrB* gene was determined by a biochemical profiling technique in which the concentration changes of metabolic intermediates were measured by gas chromatography with time-of-flight mass spectrometry (GC/TOF-MS). Although the phenotype analyses of the *amrB* gene disruption mutant showed no significant change with respect to rifamycin SV production and morphological differentiation, the global metabolomic analyses found the concentration levels of some key intermediates in the TCA cycle and glycolysis pathway were affected by an *amrB* gene disruption event. The primary results suggested that *amrB-amkB* genes might be involved in the regulation of central carbohydrate metabolism in *A. mediterranei* U32.

Bacteria must modulate their gene expression repertoire in order to survive the hostile environments. This adaptive response is often mediated by two-component regulatory systems, generally consisting of a sensor-histidine protein kinase and a response regulator, which use reversible protein phosphorylation to regulate the adjustment to the new environment [10]. Two-component signal transduction systems are widely found in eubacteria, archea, and fungi. It has been estimated that most enteric bacteria harbor more than 40 different two-component systems [21]. Several model systems, including sporulation in *Bacillus subtilis* and chemotaxis in *Escherichia coli*, have been extensively studied [19].

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. Earlier studies showed that morphological differentiation and antibiotic production are controlled in part by a common mechanism [13]. Among these, several clearly involve two-component signal transduction systems, such as *afsQ1/afsQ2* [15] and *absA/absB* of *S. coelicolor* [1], *chiS/chiR* of *S. thermoviolaceus* [16], and *cutR/cutS* of *S. lividans* [4]. The *S. coelicolor* A3(2) genome project completed recently revealed 107 sensor and regulator homologous genes [3]. Although most of them are still functionally unknown, the existence of such large numbers of two-component systems in the streptomycetes genome clearly implies that they might play very important physiological roles. The elucidation of their functions will certainly enhance our understanding of the complex regulatory networks of secondary metabolite production and present intelligent guidance to our efforts to increase antibiotic titers.

The *Amycolatopsis mediterranei* U32 is a producer of rifamycin SV, an important antibiotic against *Mycobacterium tuberculosis* [5]. The polyketide synthase (PKS) responsible for rifamycin biosynthesis has been cloned from *A. mediterranei* recently [2]. On the other hand, presently the knowledge about gene regulation is very limiting. In this paper, we reported the molecular

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and biochemical characterization of a novel two-component system, *amrB-amkB* from *A. mediterranei* U32. The enzymatic functions of *amrB* and *amkB* as a response regulator and a histidine kinase, respectively, were demonstrated by biochemical studies. Furthermore, the gene replacement experiments and metabolomic analyses demonstrated that *amrB-amkB* was involved in the regulation of carbohydrate metabolism in *A. mediterranei* U32.

Materials and Methods

Materials and general molecular biology methods. (γ-^{32}P)ATP and (α-^{32}P)CTP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Restriction enzymes, *Taq* polymerase and T4 ligase, were from Shanghai Promega (Shanghai, China). The molecular biology manipulation was performed as described in Molecular Cloning [20].

Bacterial strains and growth conditions. *E. coli* strains were grown in LB medium [20] supplemented with 150 μg/mL ampicillin, 15 μg/mL tetracycline, or 30 μg/mL kanamycin when necessary. *A. mediterranei* strain was grown in Ben's medium at 28°C [26] supplemented with 30 μg/mL aparamycin or 34 μg/mL chloromycetin for mutants carrying the resistance gene [12].

Cloning and sequencing of *amrB-amkB* genes. Two degenerate oligonucleotides corresponding to the conversed domains in the response regulator genes were designed as PCR amplification primers, Prime 1: 5′-CCGGAATTCTNGACCTNATGCTRCCTGG-3′ Primer 2: 5′-GGAATTCGTAACCWASACCGCGSACMGT-3′. Both PCR primers have an *Eco*RI site at their 5′ end (underlined). PCR was performed (30 cycles, 94°C 30 s, 55°C 30 s, 72°C 60 s) with *A. mediterranei* U32 genomic DNA as a template. The PCR product with expected size (460 bp) was gel excised and cloned into pGMET (Shanghai Promega, Shanghai), and confirmed by sequencing (ABI Matrix 241, Model 377, Takara Biotech. Dalian Co., Ltd., Dalian). The PCR fragment was then labeled with α-^{32}P-dCTP (Primer-A-Gene, Promega, Madison, WI) and used to probe the *A. mediterranei* U32 cosmid library. The library was constructed in the cosmid vector pLAFR3 as described before [26]. A 6.0-kb *Kpn*I fragment from the positive cosmid clones was subcloned into pBluescript II/KS (+) (Promega, Madison, WI), generating pHKK for further studies. The sequences were analyzed by FramePlot 2.3.2* software (available online http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl). The putative hydrophobicity profile analysis was performed by 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 *amrB* gene, a 1.7-kb *Nar*I-*Bgl*II fragment from pHKK was cloned into expression vector pET28a (Novagen, Madison, WI) at *Nhe*I-*Bam*HI sites to yield the plasmid pETRB. The pETRB was transferred into *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) for over-expression. Expressed AmrB protein was purified with Ni-NTA agarose chromatography, as described in the product manual (Novagen, Madison, WI). The purified AmrB was refolded according to the method described by Inoue et al. [15]. Refolded AmrB was concentrated with the ultrafilter column (Millipore 10,000, Bedford, MA), then kept in TESG buffer containing 50% glycerol at −70°C for further studies. The AmrB activity was stable up to 1 month.

Phosphorylation assay. The purified histidine protein kinase HupT of *Rhodobacter sphaeroides* was used as a phosphoryl donor to perform in vitro protein phosphorylation reaction, according to the methods described by Dischert et al. [6]. (The purified HupT was kindly provided by Y. Wu, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.) The HupT was incubated with 10 μCi [μ-^{32}P] ATP (3000 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ) in a total volume of 10 μL of TKME buffer (50 mM Tris-HCl, 50 mM KCl, 10 mM MgCl, 0.5 mM EDTA, pH 8.0) for 15 min at 25°C; then the purified AmrB as a recipient was added. The reaction was stopped by the addition of 4× Laemmli sample buffer after 10 min. The reaction products were heated at 56°C for 10 min and analyzed by SDS-PAGE (5% stacking and 15% separating gel). The gel was exposed to Hyperfilmβmax X-ray film (FUJI Photo Film Co. Ltd., Japan) at −80°C for 1 day.

Disruption of the response regulator *amrB* gene and phenotype analysis. The *Nar*I-*Bgl*II fragment containing *amrB* gene was cloned into *Cla*I and *Bam*HI double-digested pBluescript II KS(+) (Promega), generating pKSRB. The 1.6-kb *Eco*RV-*Bam*HI apramycin resistance gene fragment from pULVK2A [17] was cloned into *Bam*HI-*Sma*I sites of pKSRB to generate pKSRBA. The 3.1-kb fragment of pKSRBA, released by *Not*I (end-filled with the Klenow fragment I) and *Kpn*I, was then cloned into *Sma*I-*Kpn*I sites of pSP2 to generate pSPRBA (*amr*B::Am^r). 1.5 μg pSPRBA plasmid DNA was electrophorated into *A. mediterranei* U32-competent cells with GENE PULSER II System (Bio-Rad, Hercules, CA) as described before [26]. Following several rounds of propagation of the single crossover mutants in the absence of any antibiotics, colonies resistant to aparmycin but sensitive to chloromycetin were obtained; two of them were designated as *A. mediterranei amr*B RMB1 and RMB2 mutants and were used for phenotype analyses. The double crossover events were confirmed by Southern hybridization.

Biochemical profiling procedures. *A. mediterranei* strains were grown in Ben's medium at 28°C [26]. The cells were collected at three time points (24, 72, 144 h) by centrifugation at 6000 rpm for 10 min at 4°C. Pellets were washed twice with ice-cold water, then were lyophilized overnight at 4°C. Approximately 20 mg of dried ground cells was analyzed by gas chromatography with time-of-flight mass spectrometry (GC/TOF-MS) [9]. Commercially available reference compounds were obtained from Sigma-Aldrich (St. Louis, MO). The biochemical profiling data from wild-type *A. mediterranei* U32 strain were statistically analyzed, and the standard deviation (SD) for each individual intermediate was calculated and used as a baseline. The profiling data collected from the *amrB* mutant were compared with the baseline. Any compound with less than or equal to 1 standard deviation change (SDC) was considered unresponsive.

Results and Discussion

Cloning and sequence analyses of *amrB-amkB* signal system from *A. mediterranei* U32. Numerous antibiotic biosynthetic clusters have been cloned during the past decades, and the biosynthetic mechanisms elucidated have been applied successfully to produce novel antibiotics [11]. On the other hand, despite many years of research driven by the commercial importance, the knowledge about metabolic regulation in antibiotic-producing actinomycetes is very limited. In previous papers [26, 28], we have reported the characterization of a few eukaryotic-type serine/threonine protein kinases in-

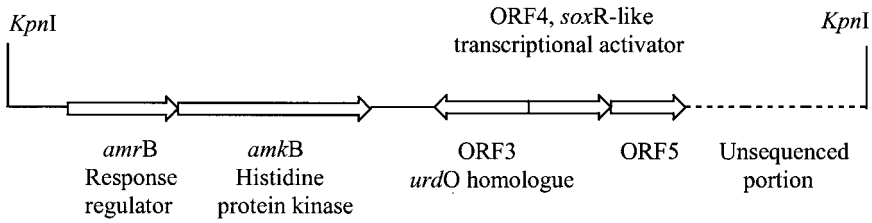

Fig. 1. Physical map of pHKK.

volved in the secondary metabolism in rifamycin SV-producing *A. mediterranei* U32. In this paper, we extended our investigation to the physiological role of two-component signal systems in *A. mediterranei* U32.

Amino acid sequence analysis of bacterial two-component response regulator genes showed two well-conserved regions: LDLMLPG at their N-terminus that is involved in the binding of phosphoryl group, and TVRGFGY at the C-terminus [19]. Two degenerate primers were designed corresponding to these two sequences to obtain a specific probe. The degree of degeneracy was decreased based on the infrequency of codon usage in *M. tuberculosis* [24] and *S. coelicolor* [25]. A 4.8-kb fragment from a positive clone was sequenced and was found to contain five complete open reading frames (ORF) with typical streptomycete codon bias. The sequences were submitted to GenBank under the accession number AJ318385, and their genetic organization is shown in Fig. 1. The *amrB* ORF starts at nt 626 (GTG) and stops at nt 1315 (TGA) with G + C% of 72.9%, encoding a protein of 229 aa. The downstream *amkB* gene is overlapped with *amrB* by 4 bp, a device that is thought to lead to translational coupling [23]. The *amkB* ORF starts at nt 1312 (GTG) and stops at nt 2721, with G + C% of 74.1%, and encodes a protein of 469 aa. The upstream region of the *amrB-amkB* operon shows no sequence corresponding to *E. coli* σ^{70}-like −10 and −35 hexamers [23]. ORF3, which is transcribed in the opposite direction, encodes a protein of 196 aa with 77% similarity to *urdO* (a putative short-chain alcohol dehydrogenase) of *S. fradiae* Tu2717 [8]. ORF4 encodes a protein of 159 aa with 60–70% similarity to some putative *soxR*-like transcriptional activators from *S. violaceoruber* or *S. coelicolor* A3(2) [14]. ORF5 encodes a putative hypothetical protein of 163 aa. The *Blast* of the deduced amino acid sequence of *amrB-amkB* displayed high identity (50–66%) to some known response regulators and histidine protein kinase genes, such as AfsQ1/AfsQ2 involved in actinorhodin production in *S. coelicolor* [14, 15], MtrA/MtrB [27] and PhoP/PhoR [7] systems from *M. tuberculosis.*

According to the sequence similarity, the response regulators may be divided into several subfamilies [22]. The deduced amino acid sequence of AmrB has the highly conserved N-terminal domain (Asp^{9}, Asp^{10}, Asp^{52}, and Lys^{101}) [10] and a characteristic conserved C-terminal effector domain for DNA binding activity, which suggested that AmrB might belong to the OmpR-PhoP family [22]. The deduced amino acid sequence of AmkB contains all the features characteristic of the histidine protein kinase family. It has the conserved histidine residue (position 261), presumably the site of phosphorylation, and other four highly conserved sequences termed N, G1, F, and G2 boxes [7]. The AmkB contains two putative hydrophobic regions sufficiently long to span the membrane, one from aa 17 to 40, the other from aa 171 to 190; this suggests that it might be membrane-located as a signal receptor (data not shown).

Over-expression of *amrB* gene and biochemical characterization of the gene products. To characterize the putative two-component system AmrB-AmkB, the response regulator *amrB* gene was over-expressed as a histidine-tagged fusion protein as described in Materials and Methods. pETRB was introduced into *E. coli* BL21 (DE3) cells, the culture was first incubated at 37°C until the OD_{600} reached 0.4, and the cells were collected 2 h after induction by 0.5 mM of IPTG. SDS-PAGE analyses of the crude extract showed a clear extra protein band at approx. 31 kDa, which is the correct size of the sum of AmrB and its his-tag (Fig. 2). The protein was purified by Ni^{2+}-affinity column and refolded in vitro [15]. To test whether response regulator AmrB can be phosphorylated in vitro by histidine protein kinase, we used a heterologous histidine protein kinase HupT as a phosphoryl donor. The [γ-^{32}P] ATP-phosphorylated HupT was incubated with purified AmrB protein for 10 min; the mixture was then loaded onto SDS-PAGE. Autoradiography of SDS-PAGE gel showed a clear 31-kDa labeled band corresponding to AmrB protein (Fig. 3). The results suggested that AmrB is able to function as a response regulator and to participate in the two-component signal transduction reaction.

Disruption of *amrB* gene in *A. mediterranei* U32 and phenotype analysis of the mutants. To elucidate the physiological role of *amrB-amkB* in *A. mediterranei* U32, a few *amrB* gene knockout mutants were constructed by inserting an aparamycin-resistance gene into

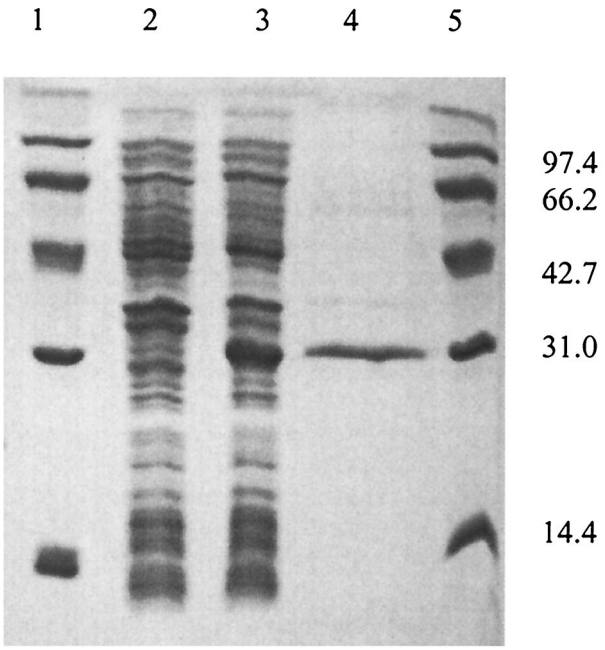

Fig. 2. SDS-PAGE analyses of *amrB* gene expression. Lane 1: molecular mass standards; Lane 2: *E. coli* BL21 (DE3); Lane 3: *E. coli* BL21 (DE3) with the pETRB induced with 0.5 mM IPTG; Lane 4: the purified AmrB; Lane 5: molecular mass standards (kDa).

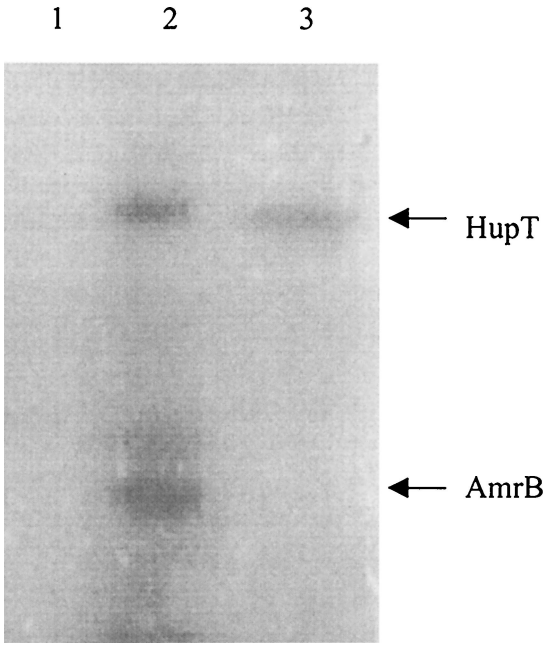

Fig. 3. Phosphorylation of AmrB in vitro. Lane 1: AmrB protein only; Lane 2: phosphorylated HupT and AmrB; Lane 3: phosphorylated HupT only.

its ORF. The *amrB* mutant RMB1 and RMB2 and the wild-type strain *A. mediterranei* U32 were cultivated in various media (media with NO_3^-, $NH4^+$, or glutamic acid as the sole nitrogen source, and with glucose, glycerol, or sucrose as the sole carbon source), and their phenotypes, including the time courses of cell growth and rifamycin SV production, colony morphology, and colony pigments formation, were compared. However, no significant difference could be detected, suggesting that the *amrB-amkB* operon might have negligible effect on the cell differentiation and antibiotic biosynthesis.

Central carbohydrate metabolism was affected by the *armB* gene disruption. To further investigate the regulatory roles of the *amkB-amrB* system, the biochemical profiling technique was applied to detect the concentration change of the major cellular intermediates associated with the *amrB* disruption. The wild-type U32 strain and the *amrB* mutants were cultivated under identical conditions, and the cells were collected and analyzed by GC-MS. To minimize the variation during the sample preparation, all wild-type and mutant samples were prepared in triplicate. Compound identification was performed by comparison of mass spectra and retention times with those obtained with commercially available reference compounds. Relative amounts of the various compounds were obtained by normalizing the intensity of individual ion traces to the response of internal reference compounds.

The cells from three time points (24, 72, and 144 h, corresponding to lag phase, log growth phase, and stationary phase respectively) were collected for biochemical profiling analyses. Eighty-six compounds detected were chemically identified, including most of the amino acids, monosaccharides, disaccharides, fatty acids, and some intermediates. With a 1.0 time of standard deviation change (SDC) as a cutoff value, 28 compounds were found responsive to *amrB* disruption with the concentration changes of 1.2–5.0 times of standard deviation (SDC) when compared with the wild-type baseline (Table 1). The analyses showed that only four metabolomes—fumarate, glyceraldehyde-3-P, aspartic acid, and folic acid—showed moderate changes of about 1.2–1.3 times of SDC in the lag phase. However, significant changes were found at the log phase when the cells were subjected to fast growth and antibiotic biosynthesis. Twenty-eight metabolomes were found responsive to *amrB* disruption at log phase, the majority involved in central carbohydrate metabolism. By pathway analyses, we found that the cellular concentration of most of the intermediates in the tricarboxylic acid (TCA) cycle and glycolysis pathways were increased in the *amrB* mutant, including five intermediates in the TCA cycle (α-ketoglutarate, citrate, fumarate, malate, and oxaloacetate) and four intermediates in the glycolysis pathway (fructose-6-P, glucose-5-P, glyceraldehyde-3-P, and pyruvate).

Table 1. Metabolome changes associated with *amrB* gene disruption[a]

Compound name	SDC		
	Lag phase (24h)	Log phase (72h)	Stationary phase (144h)
Carbohydrate metabolism			
α-Ketoglutarate	N	5.2	2.3
Citrate	N	2.7	1.4
Fructose-6-P	N	2.3	N
Fumarate	1.2	2.9	1.5
Glucose-5-P	N	3.9	1.7
Glyceraldehyde-3-P	−1.2	1.7	−1.4
Malate	N	1.9	2.9
Ornithine	N	1.8	N
Oxaloactetate	N	3.3	1.7
Phosphoenolpyruvate	N	−1.3	2.1
Pyruvate	N	1.7	N
Ribose	N	2.7	N
Succinate	N	−1.7	N
Succose	N	−1.4	2.9
Amino acid metabolism			
Aspartic acid	−1.3	−1.9	N
Glycine	N	−2.7	N
Histidine	N	3.2	2.2
Lysine	N	−1.7	−1.4
Threonine	N	−1.2	N
Tryptophan	N	3.5	N
Other			
Aminobenzoic acid	N	1.9	1.4
Aminobutyric acid	N	1.5	1.4
Adenosine	N	1.8	1.9
Folic acid	−1.2	−1.9	−1.4
Palmitic acid	N	−2.9	−1.8
Pipecolic acid	N	1.8	2.9
Pyridoxine	N	−1.9	1.5
Riboflavin	N	2.7	1.9

[a] The cellular concentration changes of metabolomes were indicated as times of the standard deviation (SDC) compared with the wild-type strain. N: no change. One time of standard deviation was used as the cutoff value.

The cellular concentration of α-ketoglutarate was affected the most, 5.2 times of SDC increased in *amrB* mutant, followed by 3.9 times of SDC change of glucose-6-P and 3.3 times of SDC change of oxaloacetate (Table 1). The results strongly suggested that the *amrB-amkB* signal system might play a role in the regulation of central carbohydrate metabolism. Twenty-two metabolomes were responsive at the stationary phase in the *amrB* mutant. However, most responsive metabolomes showed less change at the stationary phase than at the log phase, suggestive that the regulation by the *amrB* gene could be growth phase dependent.

In *B. subtilis*, it has previously been found that the 3-phosphoglycerate kinase gene of the glycolysis pathway was regulated by a *resD-resE* two-component signal transduction system that is essential for aerobic and anaerobic respiration [18]. Pyruvate dehydrogenase of the TCA cycle in *Arabidopsis thaliana* was also regulated by a putative histidine protein kinase [29]. So far, no evidence was found that central carbohydrate metabolism was controlled by a two-component system in antibiotic-producing Actinomycetes. Our primary result that the cellular concentration of major intermediates in the TCA cycle and the glycolysis pathway was significantly increased in the *amrB* mutant suggested that certain genes of central carbohydrate metabolism might be under the control of the *amrB-amkB* signal network in *A. mediterranei* U32. Although further biochemical work needs to be done to explain its molecular mechanism, this is the first report regarding the possible regulatory role of a two-component system on central carbohydrate metabolism in Actinomycetes.

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