Bacterial Type I Glutamine Synthetase of the Rifamycin SV Producing Actinomycete, Amycolatopsis mediterranei U32, is the Only Enzyme Responsible for Glutamine Synthesis under Physiological Conditions

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Abstract The structural gene for glutamine synthetase, glnA, from Amycolatopsis mediterranei U32 was cloned via screening a genomic library using the analog gene from Streptomyces coelicolor. The clone was functionally verified by complementing for glutamine requirement of an Escherichia coli glnA null mutant under the control of a lac promoter. Sequence analysis showed an open reading frame encoding a protein of 466 amino acid residues. The deduced amino acid sequence bears significant homologies to other bacterial type I glutamine synthetases, specifically, 71% and 72% identical to the enzymes of S. coelicolor and Mycobacterium tuberculosis, respectively. Disruption of this glnA gene in A. mediterranei U32 led to glutamine auxotrophy with no detectable glutamine synthetase activity in vivo. In contrast, the cloned glnA+ gene can complement for both phenotypes in trans. It thus suggested that in A. mediterranei U32, the glnA gene encoding glutamine synthetase is uniquely responsible for in vivo glutamine synthesis under our laboratory defined physiological conditions.

Key words glnA; glutamine synthetase; Amycolatopsis mediterranei; rifamycin SV

Rifamycin is a clinically useful macrolide antibiotic produced by Gram-positive actinomycete Amycolatopsis mediterranei. As a result of the importance of rifamycin in treatment of infectious diseases caused by Mycobacterium, A. mediterranei has been the focus of research in several laboratories worldwide [1].

As in most other bacteria, ammonium is assimilated in A. mediterranei mainly through the sequential action of glutamine synthetase (GS) and glutamate synthase while the ammonium supply is limited [2]. The origin of nitrogen in the key intermediate for rifamycin SV biosynthesis, 3-amino-5-hydroxybenzoic acid, was suggested from the amine group of glutamine on the basis of the incorporation experiment using the 15N-labeled nitrate, glutamate or glutamine [3,4]. It was further postulated that GS of A. mediterranei might be involved in the synthesis of 3-amino-5-hydroxybenzoic acid [2]. Therefore, the regulation of this enzyme has been studied physiologically with respect to the synthesis of rifamycin SV and the results suggested that the GS activity of A. mediterranei U32 could be the rate limiting step in rifamycin SV biosynthesis [5,6].

In vitro studies suggested that the GS of A. mediterranei U32 is composed of 12 identical subunits, each with molecular mass approximately 55 kDa, arranged in two superimposed hexagonal rings [7]. This arrangement is similar to the structure of a typical prokaryotic type I GS (GI). Although the regulation of GS in the Gram-negative enteric bacteria, such as that in Escherichia coli, is well documented [8], comparable information about the regulation of their counterparts in Gram-positive bacteria has been limited and the mechanism seems to be quite

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different [8]. In vivo studies indicated that the GS of *A. mediterranei* U32 did not respond significantly either to ammonium shock or to the treatment with snake venom phosphodiesterase [9]. In addition, no difference was observed in the catalytic properties of GS isolated from cultures grown with various kinds of nitrogen sources. Therefore, it was postulated that the activity of this enzyme was not regulated by reversible adenyllylation-deadenyllylation mechanism like most GSI enzymes, including some very closely related Gram-positive bacteria, such as *Streptomyces coelicolor* [10] and *Corynebacterium glutamicum* [11].

To elucidate the molecular mechanism of GS regulation, we cloned and characterized the GS structural gene *glnA* from *A. mediterranei* U32. We successfully used the cloned gene to produce a *glnA* null mutant, which clearly indicated that it is the only gene in U32 encoding a functional GS protein under normal physiological conditions.

### Materials and Methods

#### Bacteria strains, plasmids and culture conditions

The bacterial strains and plasmids used in the present study are listed in Table 1. *E. coli* strains were grown in

<table>
<thead>
<tr>
<th>Strain and plasmid</th>
<th>Characteristics</th>
<th>Source and Strain</th>
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<tbody>
<tr>
<td><strong>Amycolatopsis mediterranei</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U32</td>
<td>A high yield producer of rifamycin SV</td>
<td>This laboratory</td>
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<tr>
<td>P32</td>
<td><em>glnA::apr</em> mutant of U32</td>
<td>This study</td>
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<tr>
<td>P32’</td>
<td>P32 (pVK2.6AEr)</td>
<td>This study</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
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<tr>
<td>DH5α</td>
<td><em>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA) U169q80d lacZ ΔM15</em></td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>XL1-blue</td>
<td><em>hsdR17 supE44 recA1 endA1 gyrA46 thi relA1 lac F' [proAB’ lacI’ lacZ ΔM15::Tn10 (Tc</em>)]*</td>
<td>Stratagenes</td>
</tr>
<tr>
<td>JM110</td>
<td><em>dam dcm supE44 endA1 hsdR17 thi leu rpsL lacY galK galT ara tonA thr tsx Δ(lac-proAB) F’ [traD36 proAB’ lacI’ lacZ ΔM15]</em></td>
<td>Stratagenes</td>
</tr>
<tr>
<td>YMC11</td>
<td><em>thi endA hsr Δlac169 htr C klebs Δ(glnG-glnA)</em></td>
<td>Magasanik</td>
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<tr>
<td><strong>Plasmid</strong></td>
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<tr>
<td>pLAFR3</td>
<td>cos, Mob’, Trα’, IncP, Tc&lt;sup&gt;k&lt;/sup&gt;</td>
<td>[16]</td>
</tr>
<tr>
<td>pBluescript II KS+</td>
<td>Cloning vector</td>
<td>Stratagenes</td>
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<tr>
<td>pBluescript II SK+</td>
<td>Cloning vector</td>
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<tr>
<td>pLEW3</td>
<td><em>Streptomyces coelicolor glnA</em> gene in pIC19H</td>
<td>[22]</td>
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<tr>
<td>pULVK2A</td>
<td>6.8 kb, am km/neo</td>
<td>[19]</td>
</tr>
<tr>
<td>pUC19E</td>
<td>A 1.7kb <em>KpnI</em> Er’ cassette in pUC19</td>
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<tr>
<td>pWT2</td>
<td>PLAFLR3 cosmide clone containing <em>A. mediterranei glnA</em></td>
<td>This study</td>
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<tr>
<td>pWT2-6.0B1</td>
<td>A 6.0 kb <em>BamHI</em> fragment of pWT2 containing <em>glnA</em> gene, expression orientation in pBluescript II KS+</td>
<td>This study</td>
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<tr>
<td>pWT2-6.0B2</td>
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<td>PKSAM</td>
<td>A 1.5 kb <em>EcoRV-PstI</em> Am&lt;sup&gt;k&lt;/sup&gt; cassette in pBluescript II KS+</td>
<td>This study</td>
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<td>pPeng2</td>
<td>A 1.5 kb <em>PstI</em> fragment cloned into pKSAM</td>
<td>This study</td>
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<tr>
<td>pPeng3</td>
<td>A 1.5 kb <em>KpnI-EcoRV</em> fragment cloned into pPeng2, Δ<em>glnA::Am</em></td>
<td>This study</td>
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<tr>
<td>pKSFP1</td>
<td>A 1.1 kb <em>BamHI-PstI</em> fragment of pWT2-6.0B1 in pBluescript II KS+</td>
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<td>pKSFP2</td>
<td>A 1.5 kb <em>PstI</em> fragment of pWT2-6.0B1 in pKSFP1</td>
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<td>pVK2.6A</td>
<td>A 2.6 kb <em>BamHI-EcoRI</em> fragment of pKSFP2 in pULVK2A</td>
<td>This study</td>
</tr>
<tr>
<td>pVK2.6AEr</td>
<td>A 1.7 kb erythromycin resistance cassette inserted into pVK2.6A</td>
<td>This study</td>
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</table>
either LB medium or M9 minimal medium supplemented with biotin (0.01 mg/ml) and thiamine (0.1 mg/ml) [12]. *A. mediterranei* U32 was grown in Bennet medium [13] or in S medium [14] for total chromosomal DNA extraction. A modified YEME medium [15], with supplements of MgCl$_2$ (5 mM), CaCl$_2$ (5 mM), glycine (1%, W/V) and KNO$_3$ (0.6%, W/V) was used to grow cultures for electrocompetent cell preparation. For the selection of *glnA* disruption mutants, 0.4% glutamine was added to the Bennet medium and the nutritional requirements of the mutants were tested on the minimal medium (0.5 g L-Asn, 0.5 g K$_2$HPO$_4$, 0.2 g MgSO$_4$$\cdot$7H$_2$O, 0.01 g FeSO$_4$$\cdot$7H$_2$O, 10 g glucose per liter) [13]. When appropriate, antibiotics were added at the following final concentrations: ampicillin (100 μg/ml) and tetracycline (15 μg/ml) for *E. coli*; apramycin (30 μg/ml) and erythromycin (200 μg/ml) for *A. mediterranei*.

**DNA manipulation**

Total chromosomal DNA was isolated from *A. mediterranei* U32 as described [15]. Restriction enzyme digestions, subcloning procedures, plasmid isolation, gel electrophoresis, Southern hybridization and dot hybridization were carried out by standard methods [12]. DNA probes used for hybridization were labeled with $^{32}$P dCTP by either random priming using the Prime-a-Gene Labeling System (Promega, Madison, USA) or nick translation using a kit from Pharmacia (Uppsala, Sweden).

**Construction of a genomic library of *A. mediterranei* U32**

Chromosomal DNA isolated from *A. mediterranei* U32 was partially digested with Sau3A and size fractionated on a 10%–40% sucrose gradient. The fractions containing fragments of approximately 20–30 kb long were pooled, precipitated with ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na$_2$EDTA, pH 8.0). The cosmid vector pLAFR3 [16] was digested with BamHI, and diphosphorylated by calf thymus alkaline phosphatase. The vector (1 μg) and the chromosomal DNA (2 μg) were ligated, packaged in *vitro* using the λ DNA packaging kit (Boehringer Mannheim Biochemicals, Mannheim, Germany) as recommended by the manufacturer. The packaged phage was used to infect DH5α. Colonies were selected on LB agar plates containing tetracycline and X-gal (40 μg/ml). Approximately 5000 independent clones were obtained, with an average insert size of approximately 25 kb.

**DNA sequencing and analysis**

Restriction fragments were subcloned into pBluescript II KS+ or pBluescript II SK+. Overlapping deletion fragments from the segments lacking an appropriate restriction enzyme site for subcloning were generated with the Erase-a-Base system (Promega). Single-stranded DNA for sequencing was obtained by the modified single-strand rescue protocol [17]. Sequencing was carried out on both strands by the chain termination method [18]. Sequence data were analyzed using the DNAsis software package (Hitachi Software Engineering Co., Hitachi, Japan) and deposited in GenBank (accession No. AF050112).

**Construction of plasmids for *glnA* disruption and complementation**

The construct used for *glnA* disruption was made in three steps as shown in Fig. 1(A). First, the 1.5-kb *EcoRV*-*PstI* fragment of pULVK2A [19], which contains an apramycin resistance cassette, was cloned into pBluescript II KS+ to form pKSAM. The 1.5-kb *PstI* fragment of pWT2-6.0B1 containing the 3‘ end of *glnA* gene was cloned into pKSAM so that the 3′ region of the *glnA* gene was right after the apramycin resistance cassette, resulting in pPeng2. Second, the 1.5-kb *KpnI*-*EcoRV* fragment of pWT2-2.7X containing the upstream region of *glnA* was cloned into pPeng2 digested with *KpnI* and *EcoRV*. The resulting plasmid, pPeng3, contained a deleted version of *glnA* gene (a 0.83-kb *EcoRV*-*PstI* fragment containing the 5′ region of the *glnA* gene was deleted and replaced by a 1.5-kb apramycin resistance cassette). With this construct, the apramycin resistance cassette flanked by the *glnA* 1.5-kb DNA on either the 5′ side or the 3′ side was used for homologous recombination.

The construction of plasmids for *glnA* complementation experiment is shown in Fig. 1(B). First, the 1.1-kb *BamHI*-*PstI* fragment of pWT2-6.0B1 containing the 5′ end of *glnA* gene was cloned into pBluescript II KS+ to form pKSBP1. The 1.5-kb *PstI* fragment of pWT2-6.0B1 containing the 3′ end of *glnA* gene was cloned into pKSBP1 to form pKSBP2. Second, the 2.6-kb *BamHI*-*EcoRI* fragment of pKSBP2 containing a complete fragment of *glnA* was cloned into pULVK2A [19] digested with *BamHI* and *EcoRI* to form pVK2.6A. Finally, a 1.7-kb *KpnI* fragment of pUC19E containing an erythromycin resistance cassette was inserted into the *KpnI* site of pVK2.6A to form pVK2.6AEr, which was used to complement for the *glnA* null mutant.

**Disruption of *glnA* in *A. mediterranei* U32**

Electrocompetent cell preparations were made from mycelia of *A. mediterranei* U32 pre-grown in 30 ml of YEME with shaking at 200 rpm (28 ºC) for 48 h. Then
2.5 ml of the culture was used to inoculate 50 ml of modified YEME medium as described above and cultured under the same conditions. After 21–24 h incubation, the culture was harvested by centrifugation at 1600 g (4 °C) for 10 min and washed three times with 30 ml of ice-cold salt-free water. The cells were resuspended in 3 ml of electroporation solution (0.3 M sucrose, 15% glycerol). Aliquots of the suspension were stored in small plastic tubes at −70 °C.

Electroporation of *A. mediterranei* U32 was carried out with a Gene Pulser apparatus connected to a Pulser Controller (Bio-Rad Laboratories, Hercules, USA). Plasmid DNA for electroporation was prepared from the *E. coli* strain JM110, a *dam dcm* mutant [12] to reduce the possibility of cleavage in the recipient U32 strain. A 3.5-kb *ApaI-Stul* fragment from pPeng3, in which the 0.83-kb *EcoRV-PstI* fragment containing the 5’ region of the *glnA* gene [Fig. 1(B)] was replaced by an apramycin resistance cassette, was isolated, and then denatured by heating in 95°C for 5 min and chilling on ice immediately. To 60 μl U32 cell suspension, 0.1 μg of the denatured 3.5-kb *ApaI-Stul* DNA fragment was added, mixed and incubated on
ice. The mixture was transferred to a chilled electroporation cuvette (2 mm, Bio-Rad Laboratories), exposed to a single pulse at a field strength of 9 KV/cm.

Transformed competent cells were immediately mixed with 1 ml Bennet liquid medium and the suspension was incubated at 28 °C for 2–3 h to allow the expression of resistance genes. Then, cells were plated on Bennet agar medium containing 30 μg/ml apramycin. Candidate \( glnA \) null mutants were verified by colony polymerase chain reaction (PCR).

**Complementation of the \( glnA \) null mutant P32**

Electrocompetent cell preparations and electroporation of P32 (\( glnA \) null mutant) were made as described above, except that 0.4% glutamine was added in the modified YEME medium. Transformed competent cells were mixed with Bennet medium and incubated as described above. Then, cells were plated on Bennet agar medium containing 200 μg/ml erythromycin. The selected transformants harboring the complementary plasmids were further transferred to the minimal medium containing (NH₄)₂SO₄ as the sole nitrogen source to check the elimination of glutamine requirement of P32.

**Glutamine synthetase assay**

Glutamine synthetase (GS) was assayed by the \( \gamma \)-glutamyltransferase method described by Chiao et al. [3] including the culture conditions and extract preparation. Protein concentrations were determined by the Bradford method [20]. The GS activity was defined as the increase of absorbance at 540 nm per min per mg of total protein. The GS activities of P32 and P32' were measured in the same way as U32, except that 200 μg/ml erythromycin was added when P32' was cultured.

**Results and Discussion**

**Cloning the \( glnA \) gene from \( A. mediterranei \) U32**

To clone the \( glnA \) gene from \( A. mediterranei \) U32, a genomic library was constructed using cosmid pLAFR3 as the vector. Because the host strain for library construction, DH5α, contains a complete \( E. coli \) \( glnA \) gene, a modified screening method similar to that developed by Hosted et al. [21] was adopted. Dots of extracted DNA from the library were blotted to nylon filters so that each dot corresponded to a combination of 20 cosmid clones contained in the master library. Cosmids likely to contain the \( glnA \) gene were identified by probing the nylon filters with a \(^{32}\)P-labeled, 1.2 kb BamHI fragment corresponding to the N-terminal portion of the \( S. coelicolor \) \( glnA \) gene, isolated from pLEW3 [22] (Table 1). Two dots out of 192 combined clone groups, corresponding to 3840 individual clones, showed strong hybridization to the probe. One cosmid, designated pWT2 was finally identified and chosen for further analysis.

A Southern analysis of both pWT2 and \( A. mediterranei \) U32 chromosomal DNA digested with BamHI or BglII, probed with a \( S. coelicolor \) \( glnA \) fragment showed the same hybridization pattern (Fig. 2). This result confirmed that the cloned fragment was originated from \( A. mediterranei \) U32. However, although there were no other detectable minor bands seen in this Southern analysis, the possibility of presence of the second GS-encoding gene, \( glnII \), cannot be excluded because of the high heterogeneity between the sequences of \( glnA \) and \( glnII \) [23].

Restriction analysis indicated that pWT2 contained an insert of approximately 25 kb. Localization of the probable \( glnA \) gene in pWT2 was achieved by Southern hybridization studies of various restriction enzyme digestions of pWT2 probed with the \( S. coelicolor \) \( glnA \) probe (data not shown).

![Fig. 2](http://www.abbs.info; www.blackwellpublishing.com/abbs)
The cloned *glnA* of U32, expressed by the *lac* promoter in an *E. coli* *glnA* null mutant, was able to complement for its nutritional requirement of glutamine

An *E. coli* glutamine auxotroph, YMC11, was transformed with the pWT2-6.0B1, pWT2-6.0B2 (Fig. 3), pWT2 or pBluescript II KS+ (as a negative control) individually. Transformants selected on LB medium containing ampicillin were transferred to M9 minimal medium containing 0.1% NH₄Cl as the sole nitrogen source. After 48 h incubation at 37 °C, only transformants harboring pWT2-6.0B1 were able to grow on minimal medium. Under identical conditions, no growth was observed for transformants harboring pWT2, pWT2-6.0B2 or pBluescript II KS+. Because pWT2-6.0B2 is a *glnA* subclone identical to pWT2-6.0B1 except for opposite insertion orientation, it is likely that the cloned *A. mediterranei* U32 *glnA* gene in pWT2-6.0B1 was expressed through the *lac* promoter rather than its own promoter. Thus, this complementation experiment not only functionally verified the cloned U32 *glnA* but also identified the expression orientation of the *glnA* protein-coding sequence within the cloned fragment.

The cloned *A. mediterranei* U32 *glnA* gene encodes a 466 amino acid residue-long protein highly homologous to the bacterial type 1 glutamine synthetase

The nucleotide sequence of the 2.6 kb *BamHI-PstI* DNA fragment was determined [Fig. 4(A)]. The *glnA* structural gene was identified as an open reading frame beginning with a GTG codon at coordinate 392 and ending with a TGA stop codon at coordinate 1792. This open reading frame encoded a protein of 466 amino acids with a calculated molecular mass of 52 kDa. It was in good agreement with the size of the GS subunit of approximately 55,000 estimated from SDS-polyacrylamide gel electrophoresis [7]. The 15 N-terminal amino acid residues (excluding the translational start amino acid residue, N-formylmethionine) of the purified GS, determined by N-terminal sequencing (unpublished data), were in good agreement with the amino acid sequence deduced from the *glnA* nucleotide sequence starting at the GTG codon. There was a discrepancy of three residues after the 9th codon, i.e., Arg10, Asp14 and Asp16, deduced from the DNA sequence versus the Ala10, Ala14 and Cys16 determined by the N-terminal peptide sequencing. Besides the high possibility of experimental errors from protein sequencing, we tend to believe that Arg10, Asp14 and Asp16 are more reliable residues because the deduced amino acid sequence, 10-Arg-Leu-Ile-Ala-Asp-Glu-Asp-16, is well conserved in all the GlnA proteins of streptomycetes and other actinomycetes (data not shown).

The G+C content of *glnA* DNA was 66.5%, close to the overall G+C content of *A. mediterranei* strains (66%–69%) [24]. As in many other genes of the actinomycetes, the codon usage was extremely biased at the third position, i.e., those with a G or C at the third position accounted for 96.8% of the codons used in *A. mediterranei* U32 *glnA*.

The sequence AAGGAG located 14–9 nucleotides upstream from the translational start codon GTG was likely to function as the ribosomal binding site *in vivo* [25]. Immediately downstream from the *glnA* coding region at nucleotide 1816–1859 was an inverted repeat that could potentially form a hairpin loop in the mRNA, suggesting the possibility of rho-independent transcription termination.

Comparison of the inferred GS sequences from *A. mediterranei* U32 to those from other bacteria showed that the five well defined regions related to the active centers of these enzymes were all conserved in *A. mediterranei* U32 GS [Fig. 4(B)]. Of the primary sequences of GSI determined so far, *A. mediterranei* U32 GS was most closely related to that of *Mycobacterium tuberculosis* (Protein ID AAB70038, 72% identity). It was also closely related to the enzyme of *S. coelicolor* (Protein ID CAB90845, 71% identity).

The Tyr403 of the *A. mediterranei* U32 GS was of particular interest because the amino acid sequence flanking it (18 amino acids) made it a potential site of adenyllylation as characterized in *E. coli* GS [26]. This region was highly conserved in all GS enzymes whose activities were regulated by adenyllylation [Fig. 4(B)] [27], suggesting that

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**Fig. 3**  Restriction map of the DNA fragment containing the *Amycolatopsis mediterranei* U32 *glnA* gene and subclones used in complementation tests

The solid bar represents the coding region of *glnA* with the arrow indicating the direction of transcription. Subclones used for complementation tests were constructed by inserting the *BamHI* fragment of the perspective *glnA* primary clone into pBluescript with opposite directions as shown above the restriction map with the *lac* promoter of the vector indicated. B, *BamHI*; Ev, *EcoRV*; H, *HincII*; P, *PstI*; Sa, *SacI*; Sm, *SmaI*; X, *XhoI*.
Glutamine Synthetase in Amycolatopsis mediterranei U32

The U32 enzyme might have the potential to be similarly modulated. However, previous studies showed that A. mediterranei U32 GS was not regulated by adenylylation [9]. Although many scenarios might explain this discrepancy, it was possible that repeated mutagenesis of the industry strains had already inactivated the post-translational modification of the key enzyme in the biosynthesis of rifamycin SV.

As in other Gram-positive bacteria, the tryptophan residue of region I, which was thought to complete the active site formed between adjacent subunits of the Gram-negative bacteria [28], was substituted by phenylalanine in the
A. mediterranei U32 as well as other Gram-positive eubacteria and archaea GS [Fig. 4(B)]. Although this variation is a rather conservative one, which might not alter the functionality of the enzymes, it is likely significant with respect to the evolution of the molecule.

**Gene knock-out and complementation experiments together with the assay of in vivo GS activities proved that the cloned glnA gene encodes the primary functional GS in A. mediterranei U32**

Quite a few actinomycetes, such as certain species of the genera Streptomyces [29,30] and Frankia [31], had two distinct GS enzymes encoded by glnA (or glnI) and glnII. On the basis of phylogeny, it is possible that A. mediterranei might have a second gene (glnII) encoding another GS.

To investigate this possibility, the cloned glnA gene was disrupted from A. mediterranei U32 chromosome via double-crossover homologous recombination (see “Materials and Methods”). Approximately four apramycin resistant colonies were obtained for per microgram of plasmid transformed, on Bennet agar supplemented with 0.4% glutamine. The disruption of the glnA gene in the A. mediterranei U32 chromosome was verified by PCR (Fig. 5), indicating that the glnA null mutant, P32, was derived from the designed double-crossover recombination [Fig. 5(A)]. Phenotypically, strain P32 failed to grow on minimal medium [Fig. 6(A,D)], unless high concentration of glutamine was added [Fig. 6(B)]. However, if plasmid pVK2.6Aer, constructed for in vivo glnA expression from its original promoter, was transformed into P32, the resulting strain, P32', was able to grow on minimal medium without glutamine [Fig. 6(C)].

Because P32 was unable to grow in minimal medium without glutamine supplemented, we assayed the GS activities in the crude cell extracts of A. mediterranei U32, P32, and P32' grown in Bennet liquid medium (see “Materials and Methods”). Under the experimental growth and assay conditions, no GS activity was detectable in P32 although high GS activities were detected in U32 and P32' (Table 2).

In Streptomyces, glnA disruption did not induce a glutamine auxotrophic phenotype [10,32] and it was proven that the presence of a second functional GS, encoded by glnII was responsible for this phenotype. Although for A. mediterranei U32, the glnA null mutant, P32, was glutamine auxotrophic on minimal medium and no GS activity was detectable in vivo when P32 was grown in rich medium.

![Fig. 5](image-url)  
**Fig. 5**  
Schematic illustration of the design (A) and the results (B) of the colony polymerase chain reaction experiment for verifying the structure of the glnA null mutant (P32) of Amycolatopsis mediterranei U32

Genomic DNA of U32 and P32 was amplified with primer CF/CA2 (lanes 1 and 2) or with primer CF/CA1 (lanes 3 and 4), respectively. The 1-kb molecular size marker was from Gibco-BRL. Primer CF, 5'-CTGCTGCTCACACCGACTCT-3'; primer CA1, 5'-AAGTGGAGCTCGATGACTTC-3'; primer CA2, 5'-GGCGAGTTCCTCGACACGTA-3'. Template for PCR was picked from a single colony, and polymerase chain reaction (PCR) was carried out for 30 cycles in a common reaction system except that DMSO of 10% final concentration was added. As expected, the 4.2-kb ΔglnA::Am PCR product in lane 2 was 800 bp larger than the 3.4-kb glnA product in lane 1. In lane 3, there was a 1.5-kb PCR product corresponding to the 5'-half of the glnA gene but no product was found in lane 4. U32, strain U32 chromosome; P32, strain P32 chromosome. Amr, Apramycin resistance gene.
GS enzymes, probably encoded by glnII-like genes, can become active in *A. mediterranei* U32.

It has been known that addition of certain nitrogen compounds to the fermentation medium is essential for high yield in antibiotic production because they possibly stimulate antibiotic biosynthesis either as precursors or as stimulants [33]. In the case of *A. mediterranei* U32, it was tempting to speculate that these nitrogen compounds exerted their roles by increasing glnA gene transcription and thus the GS levels. Particularly, the global effect of addition of certain nitrogen compounds (e.g., nitrates) to the fermentation culture was attributed to the positive correlation between the increase of GS activity and the yield of rifamycin SV production [2,5,6,9]. With the only copy of gene encoding the functional GS cloned, it will be feasible to further investigate both the regulation of primary metabolism responding to the availability of nitrogen sources and the mechanism of stimulating secondary biosynthesis of rifamycin SV exerted by the external addition of nitrogen.

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**Table 2** Glutamine synthetase specific activities of *Amycolatopsis mediterranei* U32, P32, and P32' grown in Bennet liquid medium

<table>
<thead>
<tr>
<th>Strain with relevant genotype</th>
<th>GS activity After 24 h</th>
<th>GS activity After 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>U32 (glnA')</td>
<td>3.539±0.202</td>
<td>3.627±0.031</td>
</tr>
<tr>
<td>P32 (glnA')</td>
<td>0.000±0.031</td>
<td>−0.058±0.034</td>
</tr>
<tr>
<td>P32' (glnA', pVK2.6AErglnA')</td>
<td>2.443±0.252</td>
<td>3.884±1.246</td>
</tr>
</tbody>
</table>

Glutamine synthetase activities were measured as shown in “Materials and Methods” using crude cell extracts of corresponding strains. Erythromycin was added to the medium when P32' was cultured. The enzymatic units expressed in the table are [Δ540 min−1·(mg protein)−1]. Data shown in the table were the average of three independent measurements.

Fig. 6 Glutamine auxotrophy of the *Amycolatopsis mediterranei* glnA null mutant, P32

The strains of *A. mediterranei* were used: the prototype U32, the glnA null mutant P32, and the glnA complementation strain P32', i.e., P32 (pVK2.6AEr). MM media without amino acid supplements (plate A, C and D) or supplemented with 0.4% glutamine (plate B) were used to test the nutritional requirements of the strains.

(Bennet). However, transformation of a plasmid carrying the expressed glnA' gene into P32 could simultaneously complement for these two phenotypes in *trans*. Therefore, one might conclude that at least under these experimental physiological conditions, the cloned glnA encoded GS is the only functional enzyme responsible for glutamine synthesis in *A. mediterranei* U32. Of course, one can not exclude the possibility that under certain conditions other
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