Novel Antibiotics, Amythiamicins IV. A Mutation in the Elongation Factor Tu Gene in a Resistant Mutant of *B. subtilis*

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Since the multiple drug resistance of MRSA is still expanding, new anti-MRSA drugs with different structures and activities have to be sought. Amythiamicin A, B, C and D, new members of the cyclic thiazolyl peptide family, were isolated for their effectiveness against MRSA[1~3]. Looking for possible molecular targets for future anti-MRSA drugs, we attempted to elucidate the mechanism of action of amythiamicins. Cyclic thiazolyl peptide antibiotics, such as thiostrepton, siomicin, thiopeptin, and sporangiomycin are all known to act on 50S ribosomal subunit, resulting in inhibition of protein synthesis[4,5]. The target of thiostrepton was studied in more detail using reconstituted 50S ribosomal subunits from *B. megaterium*, and was found to be located on a ribosomal protein that was equivalent to *E. coli* L-11[4]. In contrast, GE2270, a newcomer to this family, has recently been reported to act on elongation factor Tu, rather than the 50S ribosomal subunit[6,7]. Amythiamicin A closely resembles GE2270 in structure (Fig. 1), hence we asked whether amythiamicins would act on Tu, on the 50S ribosomal subunit, or on something else, including its inhibition of protein synthesis as a whole. We approached this problem by selecting a mutant(s) that was resistant to amythiamicin, analyzing the Tu gene of the mutant for a possible mutation, and testing whether the mutant remained sensitive to thiostrepton, an inhibitor of the 50S ribosomal subunit. The Tu genes of several bacteria have been sequenced[8],

Fig. 1. Structures of amythiamicin A and GE2270A.

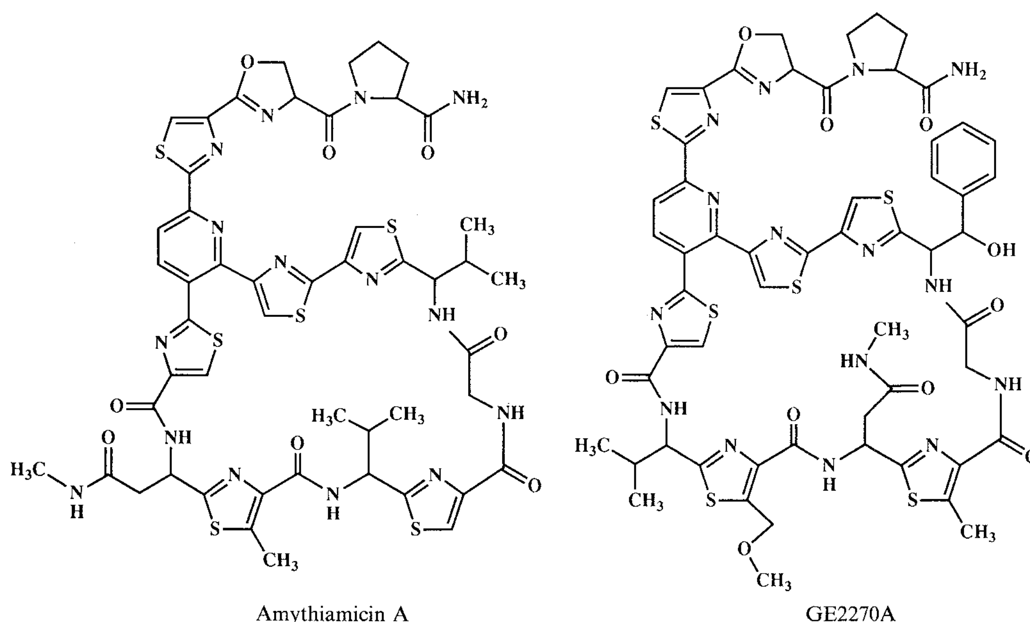
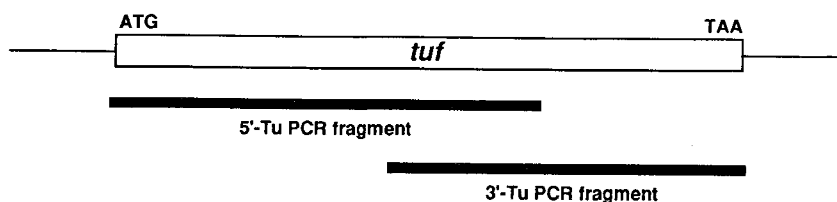


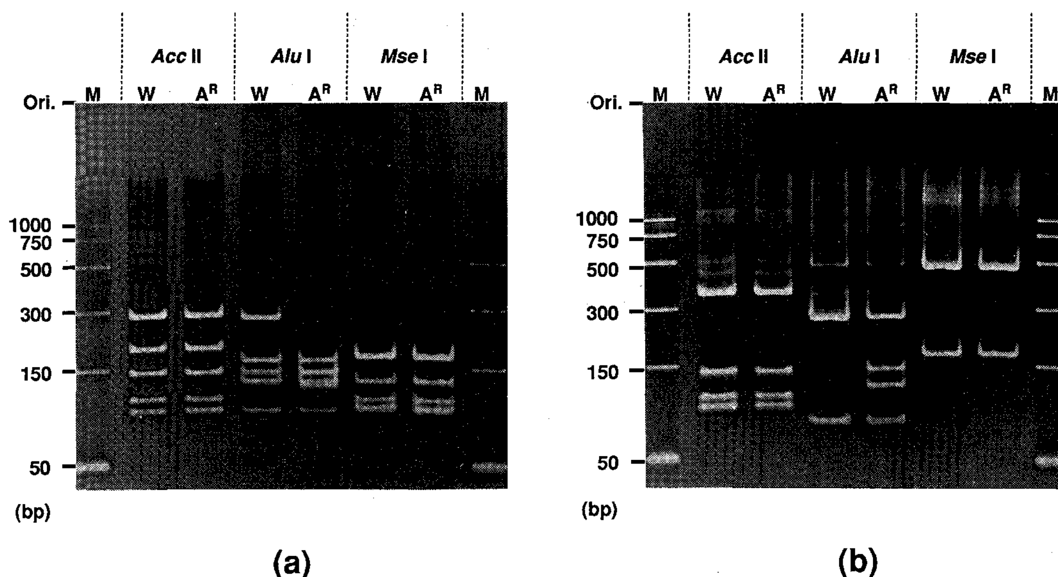
Fig. 2. Amplification of EF-Tu gene, *tuf*, from *B. subtilis* NRRL B-558 by PCR.



5′-Tu PCR fragment (811 bp) was produced by using 5′-Tu primer (5′-GGGAATTC/ATG[1]/GCT[2]/AAA[3]/GAA[4]-3′) and m3′-Tu primer 5′-GGAAGCTT/ACG[265]/GAA[264]/CAT[263]/TTC[262]-3′), while 3′-Tu PCR fragment (682 bp) by using m5′-Tu primer (5′-GGGGATCC/GCT[176]/CTT[177]/AAA[178]/G-3′) and 3′-Tu primer (5′-GGGGATCC/TTA[397]/CTC[396]/AGT[395]/GA-3′). The numerals on the primer sequences indicate codon numbers of *tuf*.

Fig. 3. Restriction fragment length polymorphism of PCR fragments for *tuf*.

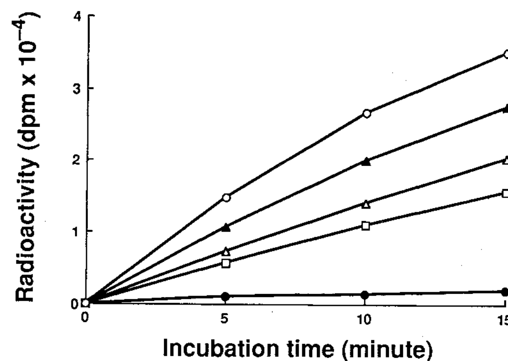
(a) for 5'-Tu PCR fragment and (b) for 3'-Tu PCR fragment. M indicates PCR markers (Novagen). W and A^R correspond, respectively, to the wild type of *B. subtilis* NRRL B-558 and a mutant therefrom resistant to amythiamicin A.



and therefore our strategy seemed feasible. *B. subtilis* NRRL B-558 was shake-cultured in nutrient broth supplemented with 0.1% (w/v) yeast extract (NBY medium), in which 0.12 μg/ml of amythiamicin A (the most active member of amythiamicins) had been dissolved. Selection of amythiamicin-resistant mutants was successful upon only one overnight shake-culture. The resistant colonies were isolated on agar plates containing the antibiotic at the same concentration as used for the selection. Maximum drug concentrations that allowed the mutants to grow were determined using the broth dilution method. A mutant that gained 100-fold or higher resistance to amythiamicin (abbreviated as A^R) was arbitrarily chosen and submitted to analysis of Tu gene for a possible mutation. The structure gene for *B. subtilis* Tu consists of 396 codons[8]. Two PCR fragments spanning respectively the 5' neighboring region and the 3' neighboring region of the structure gene, partially overlapping with each other, were produced (Fig. 2) and analyzed for RFLP using restriction endonucleases Acc II, Alu I and Mse I (Fig. 3). Both of the two PCR fragments showed RFLP with Alu I as shown in Figs. 3a and 3b; an Alu I band (*ca.* 270 bp; in 3b, two different fragments coincide here as a broad band) of Wild type disappeared in A^R, while smaller bands (120 ~ 150 bp) appeared or became denser instead. The result indicated that Tu gene of A^R had mutated to yield an additional Alu recognition site (AGCT) within the overlapped region of the two PCR fragmetns. The 5' neighboring region was again amplified by PCR under conditions where either primer for PCR carried a 5'-end phosphate, so that the products might carry a 5'-end phosphate either on the template strand or on the coding strand. Each product was digested with λ exonuclease to destroy a strand carrying the 5'-end phosphate, and the remaining strand was submitted as a template to the base sequence analysis using Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochem. Co. 70777). The result indicated that the Tu gene of A^R underwent a missense mutation at 228th codon, from GTT(Val) to GCT(Ala), that yielded a new Alu I site AGCT in which the mutant codon was included. The amino acid sequence of *E. coli* Tu has been

Fig. 4. Effect of amythiamicins on poly(U)-directed poly-(Phe) synthesis.

○: Without antibiotic, ●: amythiamicin A, △: amythiamicin B, ▲: amythiamicin C, □: amythiamicin D.



Cell-free protein synthesis directed by poly(U) was conducted as reported previously[11] with minor modifications as follows: The reaction mixture contained in 300 μl, 29 units of pyruvate kinase, 10 μM [^{14}C]Phe (1.85 MBq/ml, 1.77×10^3 MBq/mmol), 150 μg of *E. coli* tRNA, 300 μg of *E. coli* K12Q13 S30 and 60 μg of poly(U).The assay mixtures were incubated at 37°C with or without 10^{-5} M antibiotic. At the times indicated, 90 μl samples were withdrawn and transferred to paper discs (Whatman 3 MM, 2.4 cm diameter).

functionally divided into 3 domains; Domain I (1~200) is responsible for binding to GTP/GDP and aminoacyl tRNA, while Domain II (210~295) and Domain III (301~393) to EF-Ts[9]. Although the present mutation is localized in Domain II, the replacement of Val^{228} for Ala will not affect the affinity of Tu to Ts but only to amythiamicin.

Amythiamicins inhibited poly U-directed poly Phe synthesis in a cell free protein synthesizing system including S30 from *E. coli* K12Q13, in the decreasing order of activities of amythiamicin A, D, B, and C, on the molar concentration basis (Fig. 4). Since this order paralleled that of the strength of activities in inhibiting growth of various Gram-positive bacteria including MRSA, there is no doubt that amythiamicins are inhibitors of protein synthesis. Cyclic thiazolyl peptides are promising candidates for anti-MRSA drugs, since no resistance has been established in MRSA to thiostrepton (this study, data not shown), amythiamicins[1] and GE2270[10].

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