23S Ribosomal RNA mutations in halobacteria conferring resistance to the anti-80S ribosome targeted antibiotic anisomycin

Heidi Hummel and August Böck*

Lehrstuhl für Mikrobiologie der Universität München, Maria-Ward-Str. 1a, D-8000 München 19, FRG

Received January 28, 1987; Accepted February 20, 1987

ABSTRACT

Halobacterium (H.) halobium and H. cutirubrum mutants resistant to the anti-80S ribosome targeted inhibitor anisomycin were isolated. Three classes of mutants were obtained: Class I displayed a minimal inhibitory concentration (MIC) to anisomycin of 10 μg/ml, class II of 25 μg/ml and class III of at least 400 μg/ml. In vitro polyphenylalanine synthesis assays demonstrated that in those cases tested resistance was a property of the large ribosomal subunit. By primer extension analysis, each mutation class could be correlated with a distinct base change within the peptidyltransferase loop of 23S rRNA. In class I A2472 was changed to C, in class II G2466 was changed to C and in the high-level resistant class III C2471 was replaced by U. A double mutant - obtained by selection of a class I mutant for high-level anisomycin resistance - acquired the C2471 to U replacement of class III in addition to the class I mutation. The results provide information on the action of a eukaryotic protein synthesis inhibitor on archaebacterial ribosomes and demonstrate the suitability of organisms with a single rRNA transcriptional unit on the chromosome for direct selection of mutations in ribosomal RNA.

INTRODUCTION

Archaeobacteria display a heterogeneous response to protein synthesis inhibitors (1 - 3). One of the prominent features of the translational system of extreme halophilic and of methanogenic organisms is that it is susceptible to antibiotics which had been considered as classic eukaryotic 80S ribosome inhibitors (4, 5). Of most interest in this respect are compounds which interfere with the peptidyltransfer reaction. Examples are anisomycin, nardclasneme, bruceantin and some trichothecen antibiotics. They are potent inhibitors of in vitro polypeptide synthesis systems in these organisms and they display the same cross-resistance relationship to the

ribosome of anisomycin-resistant Methanobacterium mutants as they do to 80S ribosomes of resistant mutants from yeast (6). This, and the fact that inhibitors of the peptidyltransfer reaction of eubacterial ribosomes (chloramphenicol, erythromycin, tiamulin) are inactive against archaebacterial ribosomes, has lead to the conclusion that the peptidyltransfer domains of archaebacterial 70S and eukaryotic 80S ribosomes are, structurally, closely related (6).

Apart from this functional analogy there is, however, no information available which might indicate that the aforementioned eukaryotic and eubacterial inhibitors actually bind to homologous sites at the respective ribosomes. This is mainly due to a lack of knowledge with regard to the anisomycin binding site on the large subunit of 80S ribosomes; the interaction site of the eubacterial peptidyltransfer inhibitors with 70S ribosomes, on the other hand, is well characterized, mainly by the analysis of resistant mutants from mitochondria, chloroplasts and from plasmids carrying E. coli rRNA genes (for review see ref. 7).

In this communication we report on the analysis of mutants from Halobacterium (H.) resistant to the 80S ribosome inhibitor anisomycin. H. halobium and H. cutirubrum were chosen since they contain a single transcriptional unit for rRNA genes in their chromosome (8, 9). It is shown that this system enables the direct isolation of mutations in the gene for 23S rRNA and that three different base changes within the so-called peptidyltransferase loop of 23S rRNA determine different levels of resistance.

MATERIALS AND METHODS

Organisms and growth conditions

The extreme halophiles Halobacterium (H.) halobium DSM 670 and H. cutirubrum DSM 669 were cultivated in a medium containing 20 % NaCl; 2 % MgSO₄; 0.5 % KCl; 0.02 % CaCl₂; 0.5 % tryptone; 0.3 % yeast extract (H-medium). The pH was adjusted to 7 by the addition of 20 mM Tris/Cl pH 7.0. The cells were grown aerobically at 37°C; the generation time was about 6 h.
UV-mutagenesis

UV-mutagenesis was performed as described by Miller (10) with some modifications. Cells of *H. halobium* or *H. cutirubrum* were harvested at an *A*$_{420}$ of 0.6 - 0.8 by centrifugation at room temperature. The sedimented cells were suspended and diluted to about $10^7$ per ml in 20 % NaCl containing 2 % MgSO$_4$. After 15 min irradiation with UV-light of 254 nm wavelength about 99.9 % of the cells were killed under the conditions used (350 μW/cm$^2$). The cell suspension was concentrated by centrifugation and plated on H-medium solidified with 1.5 % agar to result in approximately 150 to 200 colonies per plate. 5,000 to 6,000 colonies of each strain were tested on plates containing 5 μg anisomycin/ml.

Determination of the minimal inhibitory concentration (MIC)

MIC values were determined as described (1). The antibiotics tested were anisomycin, erythromycin, tylosine, chloramphenicol, tiamulin and cycloheximide. Growth was monitored after 48 h at 420 nm.

Preparation of crude extracts, ribosomes, ribosomal subunits and S100

Cells of the halophilic bacteria were suspended in an equal volume of ribosome buffer (11) and broken by passage through a French pressure cell at 104 MPa. The crude extract was obtained after two consecutive low speed centrifugations (10 min at 10,000 rpm, 20 min at 15,000 rpm). The upper 2/3 of the supernatant was used. Ribosomes were isolated by centrifugation of the crude extract at 100,000 x g for 3 h. The preparation of the S100 was exactly as described (11).

For subunit preparation, the ribosomal pellet was suspended in dissociation buffer (3 M KCl; 0.5 M NH$_4$Cl; 10 mM MgSO$_4$; 20 mM Tris/Cl pH 8), incubated for 30 min at 40°C and layered on top of a 10 - 30 % sucrose density gradient prepared in the same buffer. The subunits were separated by centrifugation at 40,000 rpm for 90 min in a TV850 vertical rotor at 15°C.

In vitro polypeptide synthesis system

Poly(U) dependent polyphenylalanine synthesis was carried out as described (11). In some cases, S100 and ribosomes were
substituted by S30 crude extract. A_{260} of the crude extract was used in this case. The incubation time was 2 h at 40°C.

**Primer extension experiments**

Total ribosomal RNA was isolated from 70S ribosomes by the method described elsewhere (12). Oligonucleotide primers were synthesized with a DNA synthesizer (Applied Biosystems), cleaved from the support and the protective acetyl group removed as described in the user bulletin of the manufacturer. The oligonucleotides were purified by polyacrylamide gel electrophoresis (13, 14). The cDNA sequences were determined by hybridizing 0.1 μg of the oligonucleotide, which had been 5' end-labelled with [γ-^32P]ATP (5,000 C/mmol; Amersham) (15), to 10 μg of ribosomal RNA. The hybridization reaction was performed in 9 μl reaction mixtures containing 100 mM KCl, 50 mM Tris/Cl pH 8.3 by heating to 95°C for 1 min, then to 55°C for 20 min, and by cooling quickly (16).

Sequencing reactions were performed (17) using 1 U of reverse transcriptase (25,000 U/ml; Boehringer) for 1 h at 42°C. The concentration of the dNTP's (Boehringer) was 200 μM and that of the appropriate dideoxy NTP's (Boehringer) was 40 μM. The reaction mixture was chilled on ice and the reaction stopped as described (17). cDNA was fractionated on 8% polyacrylamide sequencing gels in the presence of 7 M urea.

**Antibiotics**

The antibiotics used were obtained from the following sources: anisomycin (Pfizer), erythromycin (Boehringer), tylosine and chloramphenicol (Serva); cycloheximide (Upjohn Co.), tiamulin (Sandoz).

**RESULTS**

**Mutant isolation**

The minimal inhibitory concentration (MIC) of anisomycin for H. halobium and H. cutirubrum in liquid medium is in the range of 0.1 to 0.5 μg/ml (Fig. 1). Mutants were isolated on plates containing the antibiotic at 5 μg/ml; after the UV mutagenesis procedure employed, they appeared with a frequency of one amongst about 1,000 cells plated. The mutants were tested for their resistance level; three classes could be
Figure 1: Minimal inhibitory concentrations for anisomycin. Tubes were inoculated with ~10^7 cells/ml and OD_{420} was measured after 48 h incubation at 37°C. The number of mutants showing identical phenotype is given in brackets. (○) wild-type H. halobium and H. cutirubrum, (△) low level resistant mutants, class I (3), (□) low level resistant mutants, class II (2), (■) high level resistant mutants, class III (9), (▲) double mutant (class I plus class III).

Differentiated: Class I was completely inhibited in its growth by 10 μg/ml of anisomycin, class II by 25 μg/ml and class III was only partially inhibited by 400 μg/ml, the highest concentration tested (Fig. 1). Three class I mutants (one from H. cutirubrum and two from H. halobium), two class II mutants from H. cutirubrum and nine class III mutants (six from H. cutirubrum and three from H. halobium) were chosen for further studies.

None of these mutants exhibited an altered phenotype relative to other inhibitors of the large ribosomal subunit, for example, erythromycin, tylosine, chloramphenicol, tiamulin or cycloheximide (data not shown). The mutations, therefore, appear to specifically affect anisomycin binding.

In view of the reported genetic instability of Halo-bacteria (18), the anisomycin resistance mutations were tested for their frequency of reversion to anisomycin sensitivity. Four of the high-level resistant mutants (two from H.
cutirubrum and two from *H. halobium*) were grown for 30 to 35 generations in the absence of anisomycin; no revertants to sensitivity could be detected amongst about 2,000 colonies tested.

The mutations affect the 50S subunit

The effect of anisomycin on in vitro polyphenylalanine synthesis was analysed to confirm that resistance is caused by ribosomal mutations and to test whether the different levels of in vivo resistance (see Fig. 1) show correlation with different in vitro sensitivities. Fig. 2 shows the results; in vitro polypeptide synthesis by the high-level resistant mutant, *H. halobium* Hh513A, was not at all affected by up to 200 µM of the antibiotic; extracts of the low-level class I mutant *H. halobium* Hh73A, on the other hand, were affected in their activity but to a lesser degree than the wild-type.

Ribosomal subunits were then prepared from wild-type *H. halobium* and from two high-level resistant mutants and were used in subunit exchange experiments. In each of the two mutants, resistance of in vitro polyphenylalanine synthesis to anisomycin was completely determined by the mutant 50S subunit.

![Figure 2: Poly(U) directed polyphenylalanine synthesis of crude extracts from *H. halobium* wild-type (○), a low level resistant mutant of class I (□) and a high level resistant mutant (△). The 100 % values are 3863 cpm for the wild-type, 4139 cpm for the low level resistant mutant Hh73A and 1831 cpm for the high level resistant strain Hh513A.](image-url)
(results not shown). These results indicate that anisomycin inhibits growth of halobacteria - like that of Methanobacterium formicicum (6) and of eukaryotes (19, 20) - by interference with the large ribosomal subunit.

**23S rRNA sequence analysis**

The biochemical basis of the resistance mutations was investigated. Although mutations in some protein of the large ribosomal subunit could not be precluded, there was some circumstantial evidence to suggest that 23S rRNA alterations might be involved. This assumption was based on the known action of anisomycin on the peptidyltransfer reaction of eukaryotic ribosomes (19), on the competition between anisomycin and other peptidyltransferase inhibitors for binding at the large ribosomal subunit of eukaryotic ribosomes (21, 22) and on the cross-resistance of mutants selected for anisomycin resistance with these compounds (6, 20). From studies with eubacterial-type ribosomes (eubacteria, mitochondria, chloroplasts) it was known that base changes in the so-called peptidyltransferase loop located in the 3' half of the 23S rRNA molecule can confer resistance to compounds interfering with the peptidyltransfer reaction (for review see ref. 7). We have, therefore, determined the sequence of this part of the 23S rRNA from the halobacterial mutants.

Since the complete primary structure of the H. halobium 23S rRNA gene was available (23) the "primer extension" method (16, 17) could be used for determination of any base alterations within the peptidyltransferase loop. Fig. 3 outlines the procedure. Three oligonucleotides were synthesized complementary to the 23S rRNA sequences boxed in Fig. 3. The 5' endlabelled oligonucleotides were used as primers for cDNA synthesis employing total rRNA (16S plus 23S) as template.

Initially, three high-level resistant mutants were sequenced and compared with the wild-type. cDNAs primed by oligonucleotides 1 and 2 (see Fig. 3) were identical for the wild-type and mutant sequences but that derived from oligonucleotide 3 differed from the wild-type showing the same single basepair change: G\textsubscript{471} was replaced by an A, indicating that in 23S rRNA C\textsubscript{2471} was replaced by U (Fig. 4d). The
residual mutants were therefore analysed employing oligonucleotide 3 only. The following results were obtained.

The other six high-level resistant mutants displayed the same base change as that depicted in Fig. 4d (not shown). Therefore, high-level resistance appears to be due to a C/G to T/A transition at position 2471 irrespective of whether the mutants were derived from H. halobium or H. cutirubrum. Fig. 4c shows that class I mutants possess a change in the adjacent
Figure 4: Sections of the cDNA sequences of the wild-type strains and the mutants from nucleotide 2461 (top) to 2476 (bottom). Base-changes are boxed and the position within the sequence is marked by an arrow. (a) H. halobium, H. cutirubrum (their sequences are identical), (b) low-level resistant mutants of class II, (c) low level resistant mutants of class I, (d) high level resistant mutants, (e) high level resistant double mutant.

Base at position 2472. In the cDNA sequence a T is replaced by a G which corresponds to the A\textsubscript{2472}T replacement by a C in the 23S rRNA; remarkably, all three class I mutants analysed had the same alteration.

Finally, both representatives of class II mutants had an altered position 2466 within the 23S rRNA (Fig. 4b): Due to a C/G to G/C transversion in the gene, G\textsubscript{2466} was replaced by C in the rRNA. Fig. 5 summarizes these alterations at the RNA level.

A low-level resistant class I mutant with the defined alteration at position 2472 of the 23S rRNA was used to select high-level resistant derivatives. The cDNA of one of the presumptive double mutants obtained was sequenced. Fig. 4e shows that the 23S rRNA had acquired the alteration at position 2471 determining high-level resistance in addition to the mutation at position 2472 of the ancestor. The respective double mutant was analysed for anisomycin resistance in vivo (Fig. 1). It
Figure 5: Base changes of 23S rRNA responsible for anisomycin resistance. The altered nucleotides are circled.

appeared to exhibit an even higher level of resistance than that characteristic of class III mutants.

DISCUSSION

Mutations causing anisomycin resistance have been correlated with base changes at three closely linked positions within the peptidyltransferase loop of 23S rRNA from halobacteria. Due to the lack of a gene transfer system for these organisms there is no direct proof that these alterations are indeed responsible for the resistance phenotype. There are, however, a number of arguments which make it highly likely that the 23S rRNA sequence changes described are conferring resistance: (i) identical mutations were identified in two (closely related) organisms, *H. cutirubrum* and *H. halobium*; (ii) independently isolated mutants of each class possess the same base change and at identical positions; (iii) each type of 23S rRNA sequence alteration is correlated with a distinct resistance phenotype; (iv) each of the mutants analysed had a single base alteration within the cDNA primed by oligonucleotide 3 but not in those primed by oligonucleotides 1 and 2.

Corroborating evidence is also provided by the phenotype of the double mutant described. It possesses the 23S rRNA
sequence changes of both class I and class III mutants which act additively in increasing resistance above the level conferred by class III mutations alone. In a strict sense, however, we cannot preclude the possibility that some other mutation in 23S rRNA or in some ribosomal protein may contribute to the anisomycin resistance phenotype.

Alterations within the same region of 23S rRNA involved in anisomycin resistance in halobacteria have been shown to cause resistance to chloramphenicol in eubacterial-type ribosomes (for review see ref. 7). Interestingly, the same exchange of C\textsuperscript{2471} for U - as present in class III mutants - has been shown to be responsible for chloramphenicol resistance in murine mitochondrial mutants (24). In chloramphenicol resistant human mitochondria C\textsuperscript{2471} is substituted by an A (25). The identical structural basis of mutations to chloramphenicol resistance in eubacterial-type ribosomes and to anisomycin resistance in archaeabacterial ribosomes strongly supports the notion that the two compounds bind to homologous sites and that the respective functional domain of archaeabacterial ribosomes exhibits a close topological relationship with that present in eukaryotic 80S ribosomes (6).

The results described extend the examples of antibiotics for which target-site resistance can be brought about by single base changes of ribosomal RNA (for review see ref. 7, 26). It is plausible, but by no means proven, that the respective sequence positions serve as contact sites; the different resistance levels caused by closely linked base changes provide some argument for this assumption in the case of anisomycin. It can be definitely stated, however, that the ribosomal protein moiety must contribute to the specificity of the binding site since the same type of sequence alteration in a strongly conserved region of rRNA causes chloramphenicol resistance on eubacterial and anisomycin resistance on archaeabacterial ribosomes, respectively.

Lastly, we would like to stress the usefulness of organisms with a single rRNA transcriptional unit for the simple and rapid isolation of rRNA mutants. Many of the archaeabacteria contain just a single copy of these genes (27).
and since they display a unique pattern of susceptibility, they may be valuable tools for studies on antibiotic target sites and on their mechanism of action.

ACKNOWLEDGMENT

We are greatly indebted to G. Wich for many technical suggestions, to G. Sawers for reading the manuscript and to M. Geier for editorial help. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

*To whom correspondence should be addressed

REFERENCES

17. Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin,


