A new mutation in 16S rRNA of *Escherichia coli* conferring spectinomycin resistance

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ABSTRACT

We report a novel mutation, C1066U in 16S rRNA which was selected for resistance to spectinomycin, an antibiotic which inhibits ribosomal translocation. The minimal inhibitory concentration (MIC) of spectinomycin determined for this mutant (15 µg/ml) is greater than with the wild-type plasmid (5 µg/ml) but lower than with the well known C1192U mutation (>80 µg/ml). The C1066U mutation also increases the cells sensitivity to fusidic acid, another antibiotic which inhibits translation at the translocation stage, whereas C1192U is unchanged relative to the wild type. We discuss why the acquisition of resistance to one of these drugs is often associated with hypersensitivity to the other.

INTRODUCTION

The C1192U transition was the first identified mutation in 16S rRNA leading to spectinomycin resistance (Spc^R) (1). Since then several other mutations in helix 34 (2) have been found to give rise to Spc^R (Fig. 1) and a binding site for spectinomycin in helix 34, in the vicinity of C1063 and G1064, has been suggested from protection studies (3). It has been known for a long time that Spc^R mutations can also be isolated in the ribosomal protein S5 (4). However recent *in vitro* reconstitution experiments show that the binding of spectinomycin is independent of S5 (5) indicating rRNA as the major determinant in the binding site.

The major effect of spectinomycin *in vitro* is on the interaction of elongation factor G (EF-G) with the ribosome (6). This suggests a role for helix 34 in ribosomal translocation. Helix 34 has been proposed to melt during the elongation cycle and spectinomycin may interfere with translation by stabilizing the helix (7). Until recently all known mutations in 16S rRNA causing Spc^R disrupted a basepair. It has now been shown that the disruption is not a prerequisite for Spc^R since the replacement of the G1064–C1192 with any other base pair also gives a Spc^R phenotype (7). This indicates that the G1064–C1192 base pair is directly involved in the binding of spectinomycin.

The position of helix 34 in the ribosome has recently been re-evaluated. Cross linking of U1052 in the lower part of helix 34 to the 3' nucleotide of the codon in the A site suggests that helix 34 has a position in the vicinity of the decoding site, at least transiently (8). Furthermore, an interaction between C1192U and a complex mutation close to the decoding site (C1409A,

Figure 1. Secondary structure of the upper part of helix 34 of 16S rRNA in *E. coli* (24) with the novel C1066U mutation indicated by an open U. In addition, all other known Spc^R mutations are shown. The mutations are A1191G,C, G1193A in *C. reinhardtii* chloroplast (25); G1064A (20), A1191C, C1192U (26) in *N. tabacum* chloroplast; G1193A (20) in *N. undulata* chloroplast and C1192U (1), C1192U,G,A (19), G1064U,C,A, G1064U–C1192A, G1064C–C1192G, G1064A–C1192U (7) in *E. coli*. All numbers are converted into *E. coli* numbering.

C1411G)–(G1489C, G1491U) has been observed where the C1192U mutation restores the protein synthesis activity of this mutant (9). It is interesting to note that several mutations in the 1409–1491 region cause resistance to kanamycin (10), since kanamycin resistance is also observed for some mutations in EF-G (11,12).

We have previously shown that mutations in EF-G selected as resistant to fusidic acid, an antibiotic which locks EF-G-GDP on the ribosome after translocation, often increase the cells sensitivity to spectinomycin (11). Here we report a new Spc^R mutation in helix 34 of 16S rRNA, C1066U. We have characterized this mutant in terms of growth rate and level of resistance to spectinomycin and fusidic acid. The connection between the resistance to spectinomycin and fusidic acid is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids

The XAC (=XA90) (13) derivatives US463 and US634, were kindly provided by Dr M. Rydén Aulin, Department of Microbiology, Stockholm University. The genotype of US463 is *ara*, *argE*(UGA), Δ (*lac proB*), *nalA*, *thi*, *metB* and of US634 is *ara*, *argE*(UGA), Δ (*lac proB*), *nalA*, *thi*, *recA*, *srl*. MV1190 Δ (*lac proAB*), *thi*, *supE*, Δ (*srl-recA*)306::Tn10/F':*traD*36, *proAB*, *lacI*^q ZAM15 from Bio-Rad, was used to propagate M13mp19 clones and to prepare single stranded DNA for sequencing. The plasmid pUB34 is identical with pKK3535 (14) except for the *HindIII* site

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in the pBR322 part which has been removed by filling in with Klenow fragment and subsequent blunt end ligation.

Selection

Mutagenesis was employed on US463/F Δ 14 (15) harbouring pUB34 using ethyl methanesulphonate, in essence the same method which generated C1192U originally (16). Of the resulting overnight culture, 0.2 ml was spread on plates containing 30 μ g/ml spectinomycin and 50 μ g/ml ampicillin. After two days incubation at 37°C, all the colonies from each plate were pooled in LB with 20 μ g/ml spectinomycin and grown overnight. Plasmids were prepared from each pool and transformed into US634 selecting for ampicillin resistance. The transformants were purified on plates with 200 μ g/ml ampicillin and thereafter screened for Spc^R on plates with 50 μ g/ml spectinomycin. About half of the transformants were Spc^R at this level.

Mapping, sequencing and quantification

Plasmid DNA was prepared from 21 mutants and cut with *Aat*II to see if they were mutated at, or close to, the classical 1192 site, as a mutation here destroys one of the two *Aat*II sites in pUB34. Only three of the plasmids retained both *Aat*II sites. One of these three was mapped by exchanging individually the overlapping *Apa*I-*Xba*I and *Sma*I-*Sma*I fragments purified by agarose gel electrophoresis, between the mutated plasmid and pUB34 (the wild type plasmid) and subsequently screening for Spc^R. The correct orientation of the *Sma*I fragment was confirmed by cutting with *Eco*RI. Both fragments conferred Spc^R showing that the mutation lies in the region of overlap. The complete 453 base pair *Apa*I-*Sma*I fragment was sequenced in M13mp19 with an universal primer, identifying the new mutation C1066U on both strands, using the T7 sequencing kit from Pharmacia, Uppsala. Sequencing showed that the two other plasmids retaining both *Aat*II sites also had the C1066U mutation. Three of the plasmids which had lost one of the *Aat*II sites were similarly mapped by fragment exchange and sequenced and in each case carried the previously identified Spc^R mutation C1192U. One of these plasmids carrying C1192U was used as a control in our subsequent experiments.

30S, 70S and disomes were isolated from US634/p1066U and US634/p1192U according to (17) and the relative amounts of mutant 16S rRNA were determined as described in (18) except that 10 U of AMV reverse transcriptase (Promega) were used and the reaction time extended to 45 min. For the C1192U and the C1066U mutants the oligomers 1194 (5'-GGGCCATGAT-GACTTGA-3') respectively 1068 (5'-CATTTTACAACAC-GAGC-3') were used as primers in the quantification. The resulting oligomers were separated on a 20% polyacrylamide/urea gel and the bands in the autoradiogram quantified using a Bio-Rad Model 620 Video Densitometer.

Minimal inhibitory concentration and growth rate

Minimal inhibitory concentration (MIC) was measured as described in (11). The levels of spectinomycin used were 0, 5, 10, 15, 20, 30, 40, 80 μ g/ml (spectinomycin sulphate 60% active) with 50 μ g/ml ampicillin. For fusidic acid 0, 10, 15, 20, 25, 30, 50, 60 μ g/ml of the sodium salt were used in the presence of 1 mM EDTA and 50 μ g/ml ampicillin. The MIC was defined as the

Figure 2. Relative amounts of mutant 16S rRNA in different fractions from polysome profiles of strains containing the C1066U and C1192U mutations. Lane 1, disomes from the C1192U mutant using primer 1194, indicated on the left of the autoradiogram the 17mer oligonucleotide represents excess of primer, the 19mer the chromosomal encoded wild type 16S rRNA, and the 39mer the plasmid encoded 16S rRNA with the C1192U mutation. In lanes 2-6 primer 1068 is used together with: no template, lane 2; disomes from the C1192U mutant, lane 3; disomes from the C1066U mutant, lane 4; 70S ribosomes from the C1066U mutant, lane 5; 30S subunits from the C1066U mutant, lane 6. In lanes 2-6 the position of the 17mer representing excess primer is indicated on the right side of the autoradiogram, the 19mer represents the wild type 16S rRNA and the 22mer the plasmid encoded 16S rRNA with the C1066U mutation.

concentration where no growth was visible after 20 h incubation in LB without agitation at 37°C. Three independent measurements of MIC were made, with double samples of each strain, giving the same result each time. Growth rates were measured in LB supplemented with 0.2% glucose and 200 μ g/ml ampicillin.

RESULTS AND DISCUSSION

Following selection for Spc^R a novel mutation, C1066U, in helix 34 of 16S rRNA was identified (see Materials and Methods). We also isolated the previously described Spc^R mutation C1192U. The growth rates of bacteria harbouring either of the mutant plasmids or the wild type plasmid are identical (Table 1). This suggests that expression of a 16S rRNA gene with C1066U, as is the situation with the C1192U mutation (1,19), is not detrimental to the cell. The relative amount of mutant 16S rRNA is ~80% in the 30S, 70S and disome fractions from the C1066U mutant (Fig. 2) which is the same level as found for the C1192U mutant in the disome fraction. The MICs show that the C1066U mutant has a low level of Spc^R compared to the C1192U mutant but it is clearly more resistant than the wild type. Since we have detected spectinomycin hypersensitive as well as Spc^R phenotypes in EF-G mutants selected as fusidic acid resistant (11), we decided to check the MIC of fusidic acid on the Spc^R mutants. The wild type and the C1192U mutant have a MIC of 50 μ g/ml whereas the C1066U mutant has a MIC of only 25 μ g/ml.

Table 1. Doubling time and MIC for the wild type and two Spc^R mutant plasmids

Strain	Doubling time (min)	MIC (µg/ml)	
		Spectinomycin	Fusidic acid
US634/pUB34	42	5	50
US634/p1066U	43	15	25
US634/p1192U	42	>80	50

It has been proposed that the base pairing in the upper part of helix 34 is necessary for the binding of spectinomycin (20) and that spectinomycin inhibits translation by binding to and stabilizing the helix (7). Recent experiments disrupting the basepairing in helix 34, by changing G1064 and then restoring the helix by a compensatory mutation at C1192, show that all changes at this base pair are Spc^R (7). Even the detrimental G1064C–C1192G mutant, which is predicted to have a more stable helix 34 than the wild-type, is Spc^R (7). The simplest explanation is that the binding site of spectinomycin is affected by all changes at this base pair. Previously only changes at the base pair G1064–C1192 have been shown to confer Spc^R in *E.coli* (1,19,7). In other species several other positions are known to give this phenotype when mutated (Fig. 1) and these mutations all disrupt base pairing in helix 34. It is conceivable that the specific G1064–C1192 base pair is necessary for the binding of spectinomycin. We speculate that disruptive mutations at other positions in the helix might affect the binding of spectinomycin indirectly by destabilizing the helix and thereby preventing the formation of the crucial G1064–C1192 base pair.

In *E.coli* the new mutation C1066U is the only isolated mutation conferring spectinomycin resistance which lies outside the G1064–C1192 basepair. The mutation C1066U disrupts a base pair near the top of helix 34. Although this basepair disruption fits the pattern seen with other Spc^R mutations, we note that basepairing at this position is unsupported by phylogenetic comparisons (21).

There are two additional observations which make this mutation unique. Firstly, the level of resistance is low compared with the mutations at position C1192. Secondly, in contrast to C1192U, it makes the cell more sensitive to fusidic acid (Table 1). Both the low level of resistance and the distance of C1066U from the protection site [C1063, G1064 (3)] compared with the relatively small size of spectinomycin (MW 334) argue that the resistance may not be the result of an impaired direct interaction of the mutated nucleotide and spectinomycin. Together with the fusidic acid hypersensitive phenotype, it suggests a more general perturbation in the interaction between EF-G and the ribosome.

The main effect of spectinomycin on translocation is to decrease the k_{cat} of the EF-G cycle on the ribosome (6). Fusidic acid, on the other hand, locks EF-G on the ribosome after completed translocation (22). We speculate that spectinomycin might inhibit the formation of a transition state between the pre- and post-translocation states of the ribosome. Thus, a low level of spectinomycin resistance could be achieved by improving the interaction of EF-G with the ribosome in the transition state. However, this might also strengthen the binding in the post-translocated complex leading to fusidic acid hypersensitivity. In line with this hypothesis is the observation that a fusidic acid resistant mutant (*fusA20*), causing an

impaired interaction between EF-G and the ribosome (23), also shows an increased sensitivity to spectinomycin (11).

Increased understanding of the spectinomycin and fusidic acid phenotypes will hopefully provide more insight into the mechanism of translocation. The possibility to combine characterised mutants in EF-G with mutants in the ribosome could be another way to approach the goal. From this perspective the new mutation at C1066 could be a useful tool in dissecting the mechanism of translocation.

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