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Point mutations in the 3' minor domain of 16S rRNA of *E.coli*

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ABSTRACT

Point mutations were produced near the 3' end of E. coli 16S rRNA by bisulfite mutagenesis in a 121 base loop-out (1385 to 1505) in a heteroduplex of wild type (pKK3535) and deletion mutant plasmids. Two highly conserved, single stranded regions flank an irregular helix (1409-1491) in the area studied. Only a single mutation was isolated in the flanking regions, a transition at C1402, (normally methylated on the base and ribose in rRNA). Mutations occurred throughout the irregular helix. All mutant rRNAs were processed and assembled into 30S subunits capable of interacting with 50S subunits. Growth rates ranged from faster to significantly slower than cells with the wild type transcript. In particular, mutations at C1467 or C1469 cause slow growth. These two transitions (in a bulge region within the helix) reduced the bulge by additional base pairing.

INTRODUCTION

The 3' minor domain of 16S ribosomal RNA is involved in a number of ribosomal functions. The importance of this region is reflected by the sequence conservation of certain regions within this domain. For example, the sequence around position 1400 is highly conserved and thought to be in a single stranded conformation at a site close to the tRNA binding site. Evidence for this comes from cross linking studies in which the cytosine at position 1400 was shown to cross link to the 5' wobble base of tRNA (1). Sequence comparisons of this region and studies on the reactivity of G1405 toward the single strand specific agent kethoxal indicate that bases 1399 to 1409 are in a single stranded conformation (2). Another conserved single stranded region is located between positions 1492 and 1505. This region contains the site of action of the protein synthesis inhibitor colicin E3, which inactivates ribosomes by a single endoribonucleolytic cleavage between A1493 and G1494 (3).

Separating these two conserved, single stranded regions is a large irregular helical structure from position 1409 to 1491. This helix is a variable feature in secondary structure models from divergent organisms. In

general the sequence is not conserved and often deletions or extensions are required to describe its secondary structure (4). It is probable that the region extends into the base of the 30S subunit. In eukaryotic small subunits a pair of projections are apparent at the base of the subunit and one of these may be correlated with an extension in the secondary structure of the rRNA within the irregular helix between positions 1409 and 1491 (5).

In addition to this irregular helix, the 3' minor domain contains a second helix between positions 1506 and 1529 that is capped by a four base single stranded loop containing several bases modified by methyl groups. Two adjacent adenosine residues in this loop, normally doubly methylated, are associated with resistance to the antibiotic kasugamycin in organisms lacking the methylase required for this modification (6). Finally the 3' end terminates with a conserved single strand region known to interact with the 5' end of mRNA during initiation of protein synthesis (7).

In an earlier report we have characterized and described the role of the 3' end of 16S rRNA in ribosomal functions by producing and analyzing a collection of deletion mutations within the coding region for the 3'minor domain of 16S rRNA (8). Here we have used one of these mutations to construct a series of point mutations in the region covered by the deletion. The deletion we chose for this study covers the two highly conserved single stranded regions around positions 1400 and 1500 and the irregular helix separating these two regions. Point mutations were produced by bisulfite deamination of cytosine in the plasmid pKK3535. Because this is a high copy number plasmid containing two active ribosomal RNA promoters, mutations that give rise to lethal products will not survive. This approach allows us to sample the region for nonlethal mutations and also indicates regions of possibly lethal changes.

MATERIALS AND METHODS

Restriction enzymes were purchased from New England BIOLABS and were used as specified by the vendor. Large fragment of DNA polymerase 1 and T4 DNA ligase were from BRL. 32P orthophosphate , gamma labeled ATP and alpha thio(35S)dATP are products of New England Nuclear.

Cell Types and Plasmids

Strains MC1061 and CSR603 (9) and plasmids pKK3535 (10) and pHAE2-82 (8) have been described. BD817 (ung-) was a gift from B. Duncan.

Plasmid DNA Isolation

Large scale isolation of plasmid DNA was accomplished by standard

techniques employing cesium chloride density gradient centrifugation. Density gradient purified plasmid DNA was used in the bisulfite mutagenesis protocol. For restriction analysis plasmid DNA was isolated from cultures grown on agarose plates by a technique employing lysozyme and detergent for cell lysis.

Bisulfite Mutagenesis

Bisulfite mutagenesis was carried out following a published procedure (11). The plasmids pHAE2-82 and pKK3535 were linearized by digestion with the restriction enzymes Hpa 1 and EcoR V respectively. The linearized plasmids were mixed, denatured, renatured and treated with bisulfite according to the published procedure (11). Samples of DNA treated for 30, 60, 120 and 240 min. with bisulfite were rapidly dialysed against 10 mM Tris-HCl, pH=8.0 and 1 mM EDTA for two hours to remove the sodium bisulfite. The DNA was precipitated by ethanol, resuspended and used to transform competent BD817 cells.

Cloning of Mutants

Bisulfite treated heteroduplex DNA was used to transform BD817 cells made competent by calcium chloride treatment (12). Cells were plated on agar plates with LB broth and 50 ug/ml ampicillin. For three consecutive days, each new colony that appeared on the plates was streaked on similar plates but with 200 ug/ml ampicillin to insure that the ampicillin resistance plasmid was present. From these ampicillin resistant cells single colonies were used to seed agarose plates with LB broth and 200 ug/ml ampicillin. Plasmid DNA was isolated and analyzed for wild type size by restriction mapping using EcoR 1. To detect point mutations by sequencing, an EcoR 1 , Xba 1 fragment containing the region covered by the deletion was cloned into the single strand producing phage M13. The DNA from clones with mutations was used to transform the UV-damage, repair deficient cell line CSR603.

In vivo Labeling of Plasmid Transcripts

Labeling of rRNA coded by the mutant plasmids was accomplished by use of the modified maxicell technique of Stark et al.(9) with minor changes. Cells scraped from plates were used to inoculate 25 ml liquid cultures of LB that were then grown at 30 C to an optical density of 0.55 at 600 nm. A portion of this culture (12.5 ml) was irradiated for 18 sec. Cells were allowed to recover for 2 hr. at 30 C, treated with cycloserine and allowed to recover an additional 6 hrs. At 8 hours after irradiation cells were centrifuged and resuspended in a defined medium with 32P orthophosphate and labeled for 12 hrs at 30 C. Cells were lysed as described and a portion of the lysate was

extracted with phenol in order to prepare RNA. The RNA was analyzed by electrophoresis on composite gels composed of 3% polyacrylamide and 0.5% agarose with Peacock's buffer of tris-borate and EDTA (13). Gels were electrophoresed at 250 volts for 6 hrs at 4 C. The gels were fixed and stained by Stains-all (14), dried and autoradiographed. The distribution of radiolabeled rRNA into ribosomal subunits was analyzed by two dimensional gel electrophoresis. Ribosomes separated in the first dimension were deproteinized by SDS and electrophoresed as rRNA in the second dimension as described (14).

Growth Curves

The doubling times for CSR603 cells harboring the mutant plasmids were measured at 37 C in LB broth with 200 ug/ml ampicillin. The cultures were inoculated with cells grown on plates to insure that fast growing revertant cells did not affect the doubling times.

Dideoxynucleotide Sequencing

Wild type size plasmid DNA isolated from clones transformed by bisulfite treated DNA was digested with the restriction enzymes EcoR 1 and Xba 1 and ligated to M13mp11 digested with the same two enzymes. The ligation mixture was screened for the insert containing the region subjected to bisulfite mutagenesis by dot blot hybridization using a 21 base oligonucleotide with a sequence corresponding to positions 1346 to 1366 in 16S rRNA. Positive clones were then sequenced by the dideoxynucleotide chain termination method (15) using the same oligonucleotide as a primer. Because bisulfite mutagenesis results in transitions of C to T(or U) and G to A only the C and G tracks were sequenced. Mutations are observed as an absent band from an otherwise normal pattern.

RESULTS

Bisulfite Mutagenesis

Plasmids pKK3535 and pHAE2-82 were used with bisulfite mutagenesis to create point mutations within a defined region near the 3' end of 16S rRNA. The plasmid HAE2-82 contains a 121 base deletion within the coding region for 16S rRNA extending from positions 1385 to 1505 (8). A mixture of the linearized plasmids was denatured and renatured to produce heteroduplexes containing a single stranded region for bisulfite mutagenesis as described in Methods and Materials. Cytosine residues were deaminated to uracil and transformation of this uracil-containing DNA resulted in transitions of C to T and G to A. Transformants were isolated from a strain (BD817) deficient

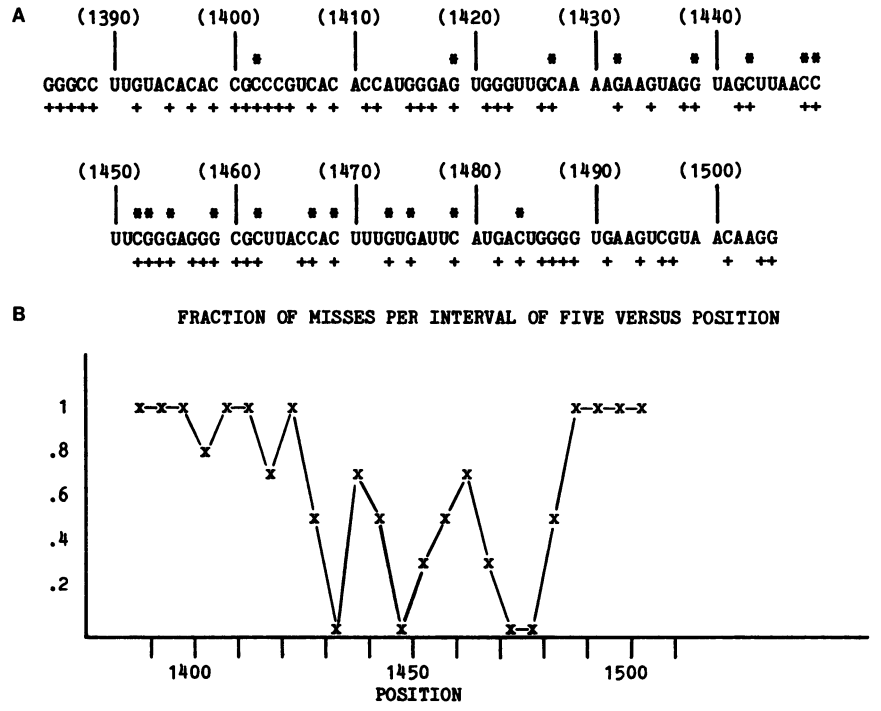

FIGURE 1: In "A" the sequence of the region in pKK3535 subjected to bisulfite mutagenesis is shown with potential target positions underscored (+) (G indicates C on opposite strand). Locations of mutations recovered are labeled (*).
In "B" the fraction of misses per interval of five positions is plotted against position. The date is calculated by partitioning the sequence into groups of five and dividing the number of target sites not recovered as mutations (misses) by the total number of target site in the interval. (As an example, in the interval from 1460 to 1464 two of the three target sites were not recovered as mutations. Thus a value of 0.67 is plotted for the interval starting at position 1460.)

in the DNA repair enzyme uracil glycosylase. Plasmids were screened by restriction mapping and those of wild type size were then sequenced for cytosine and guanosine transitions.

Distribution of Mutations

The sequence of the region of the 16S rRNA subjected to mutagenesis is presented in Figure 1. Within the 121 base target site 65 positions (labeled +) were potentially reactive with bisulfite, representing positions of C or G. Of these 65 sites available, transitions were isolated in only 19 positions (labeled *). The distribution of potentially reactive sites not

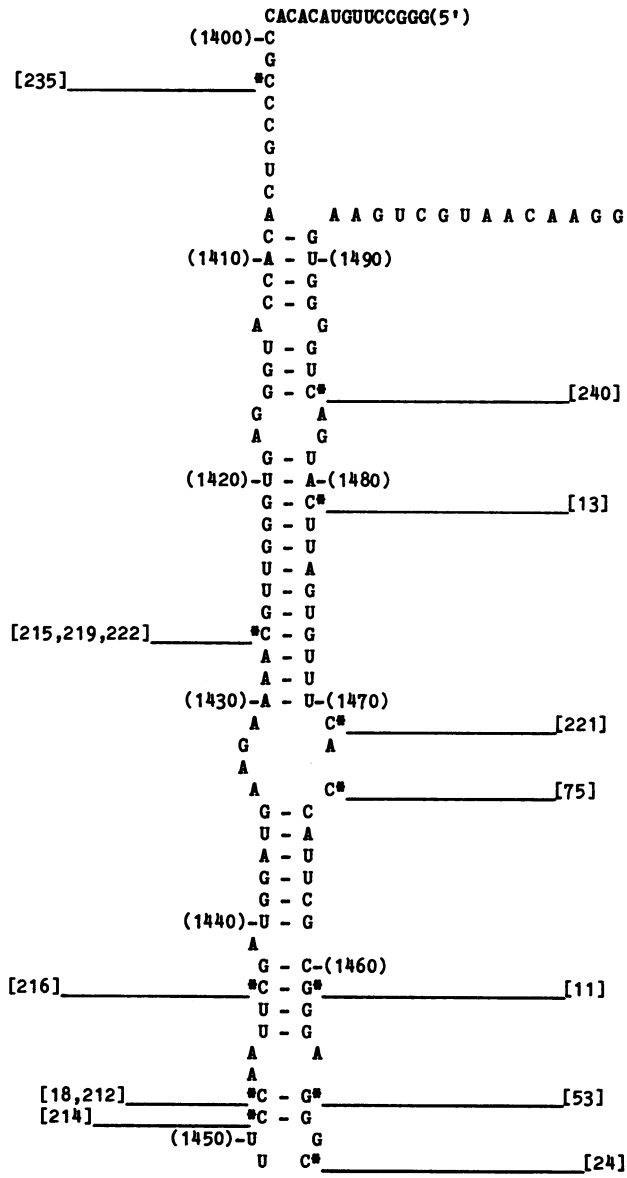

FIGURE 2: SINGLE POINT MUTATIONS. The secondary structure model for the region of 16S rRNA covered by the deletion mutation used for bisulfite mutagenesis is shown. Bracketed numbers indicate plasmids with point mutations at the position labeled with (*). Mutations are transitions of the wild type sequence shown here. For example, 240 (top, right) is a C to U transition at position 1484.

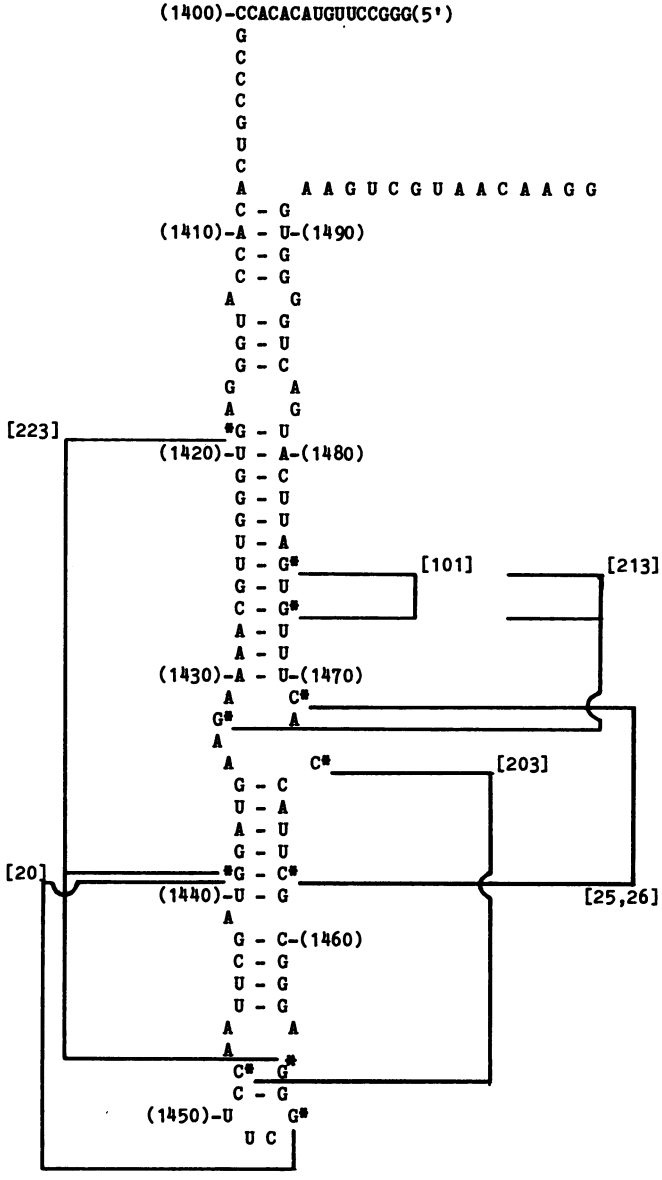

FIGURE 3: MULTIPLE POINT MUTATIONS. The secondary structure model for the region of 16S rRNA covered by the deletion mutation used for bisulfite mutagenesis is shown. Bracketed numbers indicate plasmids with multiple point mutations. Solid lines connecting positions labeled by (*) correspond to point mutations found in the plasmid listed in brackets. Mutations are transitions of the wild type sequence shown here. For example, 223 is a plasmid with three transitions (of G to A) at G1419, G1439 and G1455.

cloned as transitions is depicted in the graph in the lower half of Figure 1. It is evident from the figure that mutations are clustered in the center of the sequence corresponding to that portion of the sequence coding for the irregular helix from position 1409 to 1491 in 16S rRNA. Conversely there is a striking lack of mutations in the regions coding for the two highly conserved single stranded regions centered around positions 1400 and 1500 respectively. For the region around position 1400 only one mutation, located at position 1402, was recovered while within the region around position 1500 no mutations were recovered. Because mutagenesis was carried out in the plasmid pKK3535 which has two active ribosomal promoters, mutations giving rise to products seriously affecting cell growth may have been selected against.

Location of Mutations

The location of mutations is shown in Figures 2 and 3. Point mutations involving only single sites on a plasmid are shown in Figure 2 while plasmids with multiple point mutations are shown in Figure 3.

Transcription and Incorporation into 30S Subunits

Mutant plasmids were cloned into the UV damage repair deficient cell line CSR603 in order to determine the fate of transcripts by the modified maxicell technique (9). In all cases transcripts from mutant plasmids were observed. In addition the transcripts appeared to be processed normally to mature 16S rRNA. The processed transcripts were found in 30S subunits capable of interacting with 50S subunits in 10 mM $MgCl_2$ as analyzed by two dimensional gels (data not shown).

Effects of Mutations on Growth Rates

CSR603 cells harboring mutant plasmids were grown in liquid culture with 200 ug/ml ampicillin to determine the effects of mutant plasmids on growth rate. The data, presented as cell doubling times, are shown in Table 1. The location of the mutations and the clone designations are also recorded. We observed a wide range of growth rates. For example, cells harboring mutations at positions C1467 or C1469 grew with doubling times nearly twice as long as cells harboring the plasmid pBR322. In contrast, the mutation involving C1452 grew slightly faster than cells harboring the parent plasmids pKK3535. In addition to variations in growth rates, a variation in colony morphology was also observed. Cells containing plasmids with mutations at C1402, and the double mutation at C1462 and C1469, grew on plates with uniform sized colonies. In contrast, cells with other mutant plasmids such as C1452 gave rise to colonies with a variety of sizes. Colony purification did not correct this phenotype.

TABLE 1

LOCATION	MUTATION	CLONE	DOUBLING TIME(min.)
G1459	G to A	11	63
C1479	C to U	13	55
C1448	C to U	18, 212	61, 70
G1439, G1453	G to A	20	61
C1452	C to U	24	48
C1462, C1469	C to U	25, 26	87, 87
G1455	G to A	53	62
C1467	C to U	75	70
G1473, G1475	G to A	101	61
C1448, C1467	C to U	203	84
G1432, G1473, G1475	G to A	213	61
C1449	C to U	214	68
C1427	C to U	215, 219, 222	60, 56, 58
C1443	C to U	216	54
C1469	C to U	221	78
G1419, G1439, G1455	G to A	223	57
C1402	C to U	235	63
C1484	C to U	240	58
wild type		pKK3535	51
		pBr322	42
no plasmid			41

The doubling times for CSR603 cells harboring point mutation plasmids are listed in the last column. Growth was at 37 C in LB media. Doubling times ranged within approximately 8% of the values listed. In addition, growth of colonies on plates apppeared to reflect growth rate differences observed in liquid culture. The first column indicates the location of the mutation(s). As indicated in the second column, all mutations are transitions, and the clone number designation is listed in the third column. Under the columns headed "Clone" and "Doubling Time" multiple entries within a row indicate isolates with the same mutation(s) and the corresponding doubling times for the isolates. (For example, clones 25 and 26 both have transitions at C1462 and C1469 and show doubling times of 87min. respectively.) pBR322 is included because pKK3535 is a derivative of this plasmid.

DISCUSSION

We have constructed a series of point mutations in a rDNA operon in a region coding for the 3' minor domain of E. coli 16S RNA using the mutagenic agent sodium bisulfite. The mutations were produced using a deletion mutant, lacking bases G1385 to G1505 in the 16S rRNA coding region in the cloned rrnB operon of E. coli on the plasmid pKK3535.

Heteroduplex plasmids formed between wild type pKK3535 and the deletion plasmid pHAE2-82 were treated with bisulfite and cloned in a cell line lacking the uracil glycosylase repair system. Bisulfite deamination of cytosine to produce uracil was propagated as C to T and G to A transitions. All of the point mutations cloned were within the region delimited by the original deletion mutation. This deletion covers two single stranded, highly

conserved sequences in the RNA, one around position 1400 and the other at position 1500 near the colicin E3 cleavage site. Separating these two regions is a large irregular helix between residues 1409 and 1491.

The distribution of the mutants obtained is not random. We are unaware of any reason for bias in the mutagenesis technique that would produce this distribution. For example, it is unlikely that secondary structure within the DNA is responsible. In fact most of the mutations (see below) occur within a region known to be involved in secondary structure within the rRNA. Most likely mutations in some regions are lethal or affect growth so severely that they cannot be isolated. All but one of the mutations studied were in the helix covering positions 1409 to 1491. No mutations were recovered in the 1500 region around the colicin E3 cleavage site. Within the region centered at residue 1400 only one mutation was recovered. This mutation is a C to U transition at position 1402. In wild type rRNA, position 1402 is modified by methylation of the base and of the 2' position of the ribose. We do not know the state of methylation of the uracil residue at position 1402 in the mutant. Cells carrying a plasmid with this mutation grow with a doubling time of approximately 60 min. in contrast to 50 min. for cells carrying wild type pKK3535. In addition, colonies of cells harboring the 1402 mutant plasmid exhibit scalloped edges although they are uniform in size and otherwise round in shape. These observations indicate that the mutation is being expressed. This is confirmed by observing transcription and processing of rRNA coded by the mutant plasmid in a modified maxicell system. The mutant rRNA is not only incorporated into 30S ribosomal subunits but these subunits can associate with 50S subunits to form 70S ribosomes *in vitro*. Similar results were obtained for all of the bisulfite mutations in this report.

All of the other point mutations isolated in this study were in the rRNA helix covering positions 1409 to 1491. Several mutations were recovered independently more than once. For example a C to U transition at position 1427 was represented by three clones and transitions at positions C1462 and C1469 in the same plasmid were represented by two clones. Although many single point mutations were recovered, several examples of multiple mutations were also found (an example of which is mentioned above). Additional examples of double and triple mutations include transitions of G1473,G1475 ;G1439,G1453; G1432,G1473,G1475,; and G1419,G1439,G1455. In addition to the example above, a double mutation involving C residues occurred at C1448, C1467.

Several point mutations give rise to the formation of GU base pairs in the RNA. At position C1484 a transition to U is expected to replace a GC base pair with a GU pair. This is also the situation for point mutations at C1479, C1427, C1443, C1448 and C1449. The irregular helix contains many examples of GU base pairs in the wild type sequence. In fact two GU pairs are adjacent to four of the point mutations in the helix at C1484, C1479, C1427 and C1443. Doubling times for these mutations range from 54 min. to 60 min. and do not represent particularly slow growing cells. (At C1448 and C1449 near the end of the helix, mutations result in slower growth.) The effect of converting a GC pair to a potential GU pair (C1443 to U1443) was not as severe (as measured by growth rate) for the cell as disruption of the GC pair by replacing it with an AC pair (G1459 to A1459). Growth rates for mutants at 1443 and 1459 are 53 and 63 minutes respectively.

In contrast to examples of a loss of a base pairing,(G1459 to A1459, above) the point mutant at C1467 involves a position within a nonpaired region in the secondary structure. Transition of C to U at this position gives rise to the potential for creating a base pairing with A1434 thus forming a new AU base pair. Creation of potential base pairing arises with the transition at C1469 as well. We note that there is significant reduction in growth rate with both of these mutations. Double mutations, such as clones 203, 25 and 26 which involve transitions at C1467 or C1469, also produce significantly prolonged doubling times. These results indicate that this nonpaired loop region in the large irregular helix of the 3' minor domain of 16S rRNA is very important for proper ribosome function.

We have also isolated mutations that change GU pairs to AU pairs but examples of these involve clones with multiple mutations. At position G1419 transition to A now allows for an AU pairing. The plasmid with this mutation carries two additional changes at G1439 and G1455. G1439 and G1455 are paired with C1462 and C1448 respectively in the wild type sequence and the transitions at G1439 and G1455 convert two GC pairs to AC pairs. Thus the triple mutation results in the exchange of a GU for an AU pair and the replacement of two GC pairs with two AC pairs. Only a marginal decrease in growth rate is evident for this triple mutation (57 min.), which is surprisingly less than for G1455 to A1455 alone (62 min.). Another example of a mutation that changes a GU pair to an AU pair occurred at position G1475. This alteration is accompanied by a second change at G1473 that results in the formation of an AC pair. This particular two-position mutation also occurred in a plasmid with a third mutation at position G1432,

which is within a nonpaired region of the secondary structure. The transition of G1432 to A1432 should not result in additional base pairing in this region. The double and triple mutant plasmids have the same 61 min. doubling time. Thus we have several examples of AC pairs created with only marginal effects on growth.

Two independent clones were isolated harboring a plasmid with a transition at position C1448 (mutants 18 and 212). Both clones transcribe and process rRNA from the plasmid and the 16S rRNA is incorporated into 30S subunits that associate with 50S subunits. The clones also grow at slower rates than that observed for cells with wild type plasmids. However the colony morphology from the two clones is quite different. One clone (18) gives rise to slow growing colonies with uniform size and shape. In contrast the other clone (212) produces colonies with a wide variation in colony size. Both large and small colonies are observed in clone 212. Upon replating several times, selecting the same size colony each time, either large or small colonies, variation in colony size is still maintained. The plates derived from large colonies have a predominance of larger colonies and the plates derived from small colonies have a predominance of small colonies. We do not understand this observation and are exploring the possibility that one of the plasmids contains a second mutation. While it is possible that second site mutations are present in other clones we think this is unlikely for two reasons. First, no mutations were found in regions adjacent to the sites covered by the deletion. Second, in two other cases where the same mutation was isolated more than once, the phenotypes were the same (compare clones 24 and 25 or 215, 219 and 222). This indicates either the absence of other mutations, or the unlikely possibility that the plasmids have obtained phenotypically identical second site mutations.

An interesting point mutation was isolated in the terminal loop structure. Plasmid 24 with C1452 mutated to U gives rise to fast growing, irregularly shaped colonies. The doubling time in liquid culture of cells with this plasmid is 48 min., slightly faster than cells with the wild type plasmid pKK3535. We have no explanation for the accelerated growth rate.

In summary, we have isolated a variety of point mutants in a defined region of 16S rRNA. We have measured the effects of these mutants <u>in vivo</u> (growth rate alterations, rRNA processing) and can see that certain areas are very important. The most notable examples are alterations in an open loop by mutations at positions 1467 and 1469, which greatly effect the growth rate. With the development of techniques to isolate pure mutant rRNA we anticipate

that we will be able to conduct more precise in vitro functional studies on these mutants.

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