Ribosomal Proteins Involved in the Suppression of Streptomycin Dependence in *Escherichia coli*

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Suppression of streptomycin dependence in *Escherichia coli* strain K-114, a spectinomycin-sensitive strain, is correlated with modification of 30S ribosomal protein P4, the component modified in spectinomycin-resistant mutants. The mutant is unusual in that reversion from dependence has previously been correlated only with modification in 30S protein P4a. Introduction into K-114 of another mutation conferring spectinomycin resistance results in a further alteration in protein P4.

The 30S ribosomal subunits from streptomycin-dependent mutants of *Escherichia coli* contain a specific altered protein, P10, which was shown by in vitro ribosome reconstitution experiments to be the determinant of the dependence phenotype (1). [Nomenclature of 30S ribosomal proteins is that of Nomura (14); corresponding designations used by other laboratories are found in references 7 and 9.]

Spontaneous revertants from streptomycin dependence to independence can be selected by plating dependent cells on medium lacking the drug. Each independently isolated strain exhibits a characteristic level of resistance to streptomycin but does not require the drug for growth. Genetic analysis of revertants demonstrated that many of them result from mutations near but not coincident with *strA*, the locus conferring streptomycin dependence (8). Using the techniques of ribosome reconstitution, two-dimensional gel electrophoresis, or phosphocellulose column chromatography, several laboratories have recently reported that a number of revertant strains contain an altered 30S ribosomal protein, P4a (2, 6, 10). The observation that ribosomes from revertants contain an altered protein other than P10 lends support to the genetic data of Hashimoto (8), which suggest that many streptomycin-independent mutants are not true revertants to the wild type, but result from mutation at a nearby site (i.e., the streptomycin-dependent phenotype is suppressed).

To determine whether specific modification of protein P4a is the only possible structural change associated with suppression of streptomycin dependence, we have compared ^{14}C -labeled 30S ribosomal proteins from several

independently isolated revertant strains with ^3H -labeled proteins from isogenic wild-type or dependent 30S subunits by gradient elution from phosphocellulose columns. Of nine revertant strains examined, five showed alterations in 30S proteins detectable on phosphocellulose; all but one involved shifts in the position of the P4a protein peak. However, one revertant, *E. coli* strain K-114, has a modification in protein P4, the protein previously reported as being the modified component in 30S ribosomes from spectinomycin-resistant mutants (3).

MATERIALS AND METHODS

Bacterial strains and media. The isogenic *E. coli* strains K-100 (streptomycin-sensitive) and the spontaneous streptomycin-dependent mutant derived from it (K-105) have been described previously (4). Strain K-114 is a spontaneous revertant of K-105 selected for ability to grow in the absence of streptomycin. K-114spc2 is a spontaneous mutant of K-114 selected on agar containing 100 μg of spectinomycin/ml. *E. coli* strain AB2834d-1 is a spontaneous streptomycin-dependent mutant of strain AB2834 (obtained from J. Davies).

For in vivo characterization of strains, cells were grown in Tryptose phosphate broth (TPB) and plated on agar containing 1.5% Difco agar in TPB. Isotopic labeling of cultures was done in tris(hydroxymethyl)aminomethane (Tris)-maleate medium supplemented with 0.2% glucose and 20 μg of methionine/ml as previously described (10). Streptomycin sulfate was obtained from Calbiochem; spectinomycin was a gift from the Upjohn Corp.

In vivo response of strains to spectinomycin and streptomycin. The response of K-100, K-105, K-114, and K-114spc2 to the antibiotics was analyzed in two ways. (i) Cells were grown to log phase in TPB without added antibiotic and then plated on

agar plates containing various concentrations of antibiotics (see Table 1). Growth of each strain on these plates was determined after 48 hr of incubation at 37 C. (ii) Log phase cells were grown to a concentration of 2×10^7 to 3×10^7 /ml at 37 C in TPB. At time zero the culture was split into three flasks containing either no drug, streptomycin (100 μ g/ml), or spectinomycin (100 μ g/ml). At $t = 0$ and at $t =$ one mass doubling, appropriately diluted samples from each flask were plated on Tryptose phosphate-agar plates which contained no drug. Colonies were counted after a 48-hr incubation at 37 C. Mass doubling time was determined in a Zeiss PMQ-II spectrophotometer.

Co-chromatography of 30S ribosomal proteins on phosphocellulose. Techniques for labeling, extraction, and fractionation of ribosomal proteins have been previously described (10). Briefly, the technique is as follows: 30S ribosomal subunits derived from two strains, one grown in 3 H-labeled amino acids and the other in 14 C-labeled amino acids, were exposed together to 67% acetic acid containing 0.03 M $MgCl_2$ for 45 min with stirring. After removal of the precipitated ribonucleic acid (RNA) by low-speed centrifugation, proteins were dialyzed extensively against standard urea buffer (6 M urea, 50 mM NaH_2PO_4 , 12 mM methylamine, 10 mM 2-mercaptoethanol) and applied to a 0.9 by 35 cm phosphocellulose (high-capacity Mannex-P) column equilibrated with standard buffer (pH 6.5). Proteins were eluted with a linear gradient of NaCl (0.0 to 0.55 M) in a total of 575 ml of standard urea buffer at a flow rate of 0.15 ml/min. Protein in each 2-ml fraction was precipitated with cold 10% trichloroacetic acid, collected on a glass fiber filter, and counted.

The identity of the P4 protein peak was determined by detection of the position of an altered protein in a number of independently isolated spectinomycin-resistant mutants of *E. coli*. Since spectinomycin resistance is determined by modification of P4 (3), detection of an altered peak in the spectinomycin-resistant 30S proteins versus wild-type 30S proteins confirmed the position of P4 and allowed numbering of the other peaks.

TABLE 1. Antibiotic sensitivity of wild-type and mutant strains

Strain	Growth ^a			
	No drug	Streptomycin (100 μ g/ml)	Streptomycin (100 μ g/ml)	Spectinomycin (100 μ g/ml)
K-100	+++	—	—	—
K-105	—	—	+++	—
K-114	+++	++	—	—
K-114spc2	+++	++	—	++

^a Relative growth of strains on agar plates containing the indicated drug concentration is expressed as: (+++) heavy growth; (++) moderate growth; (—) no growth.

RESULTS

Analysis of 30S ribosomal proteins. Figure 1 shows the elution profile of 3 H-labeled wild-type (K-100) 30S proteins co-chromatographed with 14 C-labeled mutant (K-114) proteins. Whereas no detectable change in the position of the P4a peak (noted by arrow in Fig. 1) is evident in the two strains, protein P4 from K-114 ribosomes elutes earlier than the corresponding component from K-100 ribosomes. As previously reported, when 30S proteins from wild-type and streptomycin-dependent (K-105) isogenic strains are compared, no such alteration appears (10). This observation demonstrates that reversion from streptomycin dependence to independence can involve a change in a 30S protein other than P4a.

Genetic analysis of K-114 suppressor and spectinomycin-resistant mutations. Preliminary genetic experiments support the contention that the mutation in K-114 suppressing streptomycin dependence and the spectinomycin-resistance mutation affect the same gene product. Spontaneous mutants showing high-level resistance to spectinomycin were isolated from strain K-114. Phage P1kc produced in one of these strains, K-114spc2 (*aroE*⁺, *spc*^r, *su114*, *str*^d), was used to transduce streptomycin independence into a streptomycin-dependent recipient, AB2834d-1 (*aroE*⁺, *spc*^r, *su*⁺, *str*^d). Techniques for transduction were those of Lennox (12). Streptomycin-independent transductants (resulting from the transfer of the K-114 suppressor, *su114*) were selected on agar plates without any antibiotic. Of 143 streptomycin-independent transductants analyzed, the per cent contrasduction of *su114* with the unselected markers *spc*^r and *aroE*⁺ was 100 and 70% respectively. All of the transductants had the same response to streptomycin (low-level resistance) as the donor strain, K-114. Although precise mapping of the K-114 suppressor requires analysis of many more transductants, our inability to separate the suppression of dependence from spectinomycin resistance shows that *su114* and *spc* are in the same or very closely linked genes.

We compared the elution profiles of 30S proteins from K-114 and K-114spc2, a mutant of K-114 selected for ability to grow on agar containing 100 μ g of spectinomycin/ml, by co-chromatography on phosphocellulose. As shown in Fig. 2, the profiles are identical except at the P4 protein position where the two peaks do not coincide. The introduction of the spectinomycin-resistant mutation into strain K-114 is expressed as a further alteration in protein P4, which causes it to elute earlier

FIG. 1. Co-chromatography of 30S ribosomal proteins derived from K-100 (^3H -labeled) and K-114 (^{14}C -labeled) on phosphocellulose at pH 6.5. ^3H counts/minute ($\bullet$); ^{14}C counts/minute ($\times$). Arrows indicate the position of two wild-type proteins (P4 and P4a) referred to in the text.

FIG. 2. Co-chromatography of 30S ribosomal proteins derived from K-114 (^3H -labeled) and K-114spc2 (^{14}C -labeled) on phosphocellulose at pH 5.8. ^3H counts/minute ($\times$); ^{14}C counts/minute ($\bullet$). Arrows 1 and 2 indicate the position of elution of protein P4 from strains K-114spc2 and K-114, respectively. Arrow 3 indicates the expected position of elution of protein P4 from strain K-100 at pH 5.8 as determined from another column run. Minor differences in the profiles in Fig. 1 and 2 are due to the different pH at which each column was run.

than P4 from K-114 (Fig. 2, arrow 2). Protein P4 from the wild type, although not shown in this figure, would elute at the position marked by arrow 3.

In vivo response of mutants to spectinomycin and streptomycin. The effect of the modified protein P4 on reversing the streptomycin-dependent phenotype in K-114 is distinct from its previously reported role in determining the response of cells to spectinomycin. This conclusion is based on data presented in Tables 1 and 2 in which the responses of K-100, K-105, K-114, and the spontaneous spectinomycin-resistant mutant of K-114 (K-114spc2) to the drugs are compared. K-114 is phenotypically spectinomycin-sensitive, as

evidenced by (i) the inability to form colonies on agar plates containing spectinomycin (Table 1), and (ii) the inhibition of cell division in broth cultures containing the drug (Table 2). This pattern is identical to that of the isogenic wild-type strain, K-100. K-114 is also resistant to low levels of streptomycin (10 $\mu\text{g}/\text{ml}$) on plates; cells in liquid culture exposed for one generation to 100 μg of streptomycin per ml are more resistant to its killing action than the wild-type strain K-100. K-114spc2 retains the characteristic phenotypic response of K-114 to streptomycin even though it has acquired the additional mutation to spectinomycin resistance. This characteristic is true of all spectinomycin-resistant mutants of K-114

TABLE 2. *Effect of streptomycin and spectinomycin on cells in broth culture^a*

Strain	Mass doubling time (min)	Survival after one generation (%)		
		No drug	Streptomycin (100 µg/ml)	Spectinomycin (100 µg/ml)
K-100	24	200	< 1	82
K-114	31	200	17	94
K-114spc2	38	172	10	172

^a Results are presented as the per cent of cells surviving after a period of time equal to one mass doubling of the "no drug" control culture. Broth cultures contained the indicated concentrations of streptomycin or spectinomycin before plating on agar plates without drug as indicated in the text.

analyzed to date.

DISCUSSION

Although total reconstitution of ribosomes containing wild-type and mutant P4 and P4a will be needed as final proof of the function of these proteins in the suppression of streptomycin dependence, it seems clear that the streptomycin-dependent phenotype can be reversed by changes in either of two ribosomal proteins other than P10. This implies that the effect of streptomycin on the ribosome may involve the cooperative interaction of several structural proteins. Recently Chang and Flaks (5) have shown that, in addition to P10, the presence of two other 30S proteins (P8 and P11) is critical for binding of radioactive dihydrostreptomycin to 30S ribonucleoprotein particles derived from streptomycin-sensitive cells. Thus, although a mutational change in a single protein can be correlated with a specific mutant phenotype, the overall contribution of other ribosomal protein species cannot be excluded. Interaction of a modified protein P4 with the product of the *strA* gene (P10) is also implied in the ability of spectinomycin resistance to relieve restriction of the Su II amber suppressor function by certain streptomycin-resistant mutants (11). A similar type of interaction is also suggested for a modified protein P4a, identified as the ribosomal alteration conferred by the *ram* mutation of Rosset and Gorini (15, 16). The *ram* mutation also relieves restriction of suppression imposed by some streptomycin-resistant mutations.

It is of particular interest to note that streptomycin dependence may be suppressed by the alteration of either P4a, an "internal" pro-

tein (which is one of the first to interact with 16S ribosomal RNA in the in vitro assembly process), or P4, an "external" protein added late in the assembly sequence of Mizushima and Nomura (13).

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