ESCHERICHIA COLI MUTANTS DEFICIENT IN EXORIBONUCLEASES

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SUMMARY

Strain S296, isolated by screening 2000 colonies after nitrosoguanidine mutagenesis, yields extracts with less than 1% of wild-type RNase activity against (^{3}H) poly(U). Unlike other E. coli strains, S296 grows with a doubling time of about 2 hr., both in nutrient broth and in minimal medium, and at 30°, 37° and 42°. The strain retains 10 to 20% of wild-type exonuclease activity against (^{3}H) rRNA or T4 phage-specific mRNA; but two further mutants, made by screening mutagenized colonies of strain S296, are reduced to 3% of wild-type activity against those substrates as well.

INTRODUCTION

Many proteins are present in cells in large excess over the level required for normal growth. As a result, cells can tolerate some severe mutational lesions in vital genes (reviewed in ref. 1). The growth rate of the mutant cells may be affected little if at all, though in some instances the mutated element will be at a limiting level, producing a selective retardation of a particular process.

Such mutants have been isolated for E. coli DNA polymerase 1 (E.C.2.7.7.7; ref. 2); for tRNA nucleotidyl transferase (2.7.7.25; ref. 3); and for RNase III (4-6). In each case, screening of colonies from a mutagenized culture turned up mutants low enough in specific enzyme levels to permit critical studies in whole cells and extracts. The inference that this simple method might provide a generally useful alternative to temperature-sensitive mutants for the study of many enzymes (1) led us to ask whether a similar analysis would be possible for exoribonucleases - and in particular, for the K^+-activated RNase II which accounts for much of the exonuclease activity in E. coli cell extracts (7-9).

MATERIALS AND METHODS

For mutant screening, 10 ml overnight cultures of E. coli strain C3 (ref. 10) and of colonies from mutagenized cultures (11) were centrifuged, resuspended in 1 ml of RNase II assay buffer (10 mM Tris-Cl, pH 7.5; 150 mM NH_4Cl; 1 mM

$MgCl_2$; ref. 7) and lysed in an ice-water bath by three 15-sec bursts of cavitation with a Biosonik probe. 1 µl of C3 lysate was sufficient to degrade essentially all added (^3H) poly(U) to alcohol-soluble nucleotides in a standard incubation for 30 min (Fig. 1). Ten µl of each lysate was assayed to identify candidates with low activity, and confirmatory assays were carried out on alumina-ground crude extracts or soluble proteins cleared of ribosomes by centrifugation (ref. 8).

Ribonuclease activity was measured at 35° in 50 µl of assay buffer along with enzyme and 2 µg of rRNA (77,000 cpm), 2 µg of poly(U) (41,000 cpm), or 0.8 µg of T4 mRNA (70,000 cpm). Twenty µl (200 µg) of carrier ribosomal RNA and 0.2 ml of chilled absolute ethanol were then added. After 15 to 20 min on ice, the mixture was centrifuged at 8000 x g for 10 min to remove the precipitate, and 150 µl of the supernatant was counted in 5 ml of toluene scintillation fluid containing 10% protosol (New England Nuclear).

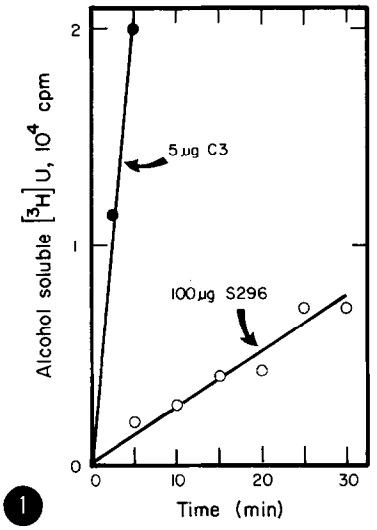
To prepare T4 mRNA as a substrate, E. coli B was labeled from 8 to 13 min after infection at 37° (12), with 50 µCi of (^3H) uridine/ml. RNA was phenol-extracted and fractionated in a sucrose gradient (13). Fractions sedimenting faster than 10S, excluding the absorbance peaks of 16S and 23S rRNA, were pooled, brought to 0.1 M NaCl, and precipitated with ethanol. The (^3H) T4 mRNA, or rRNA similarly prepared from growing cells labeled for 90 min, was then stored frozen in water solution.

RESULTS AND DISCUSSION

In each of two sets of screening trials, 1000 slow-growing colonies were tested after nitrosoguanidine mutagenesis of strain C3. Two mutants were found, extracts of which satisfied the phenotypic requirement: one of the first set, with about 10% of wild-type enzyme activity; and strain S296 (the 296th colony tested in the second lot), with less than 1% of wild-type activity against poly(U) (Figure 1). The mutant extract gave no inhibition of enzyme activity when mixed with wild-type extracts, so that it was truly deficient.

The physiology of strain S296 has not been analyzed in detail, but we have noted that the strain grows at a slow rate compared to the parental; and that unlike other reported bacterial strains, the growth rate is comparable in minimal or fortified media (Fig. 2), and at 30°, 37°, and 42° (data not shown).

Whereas extracts of strain S296 assayed against poly(U) were reduced to less than 1% of the wild-type activity, they retained 10 to 20% of the parental strain activity against (^3H) T4-specific mRNA or (^3H) ribosomal RNA (e.g., Fig. 3). The specific deficiency in RNase II in the mutant has since been proven, and the bulk of the remaining activity has been found to reside in a new exonuclease which can be more easily detected in extracts of the mutant (Gupta, R.S., Kasai, T., and Schlessinger, D., ms. submitted to J. Biol. Chem.).



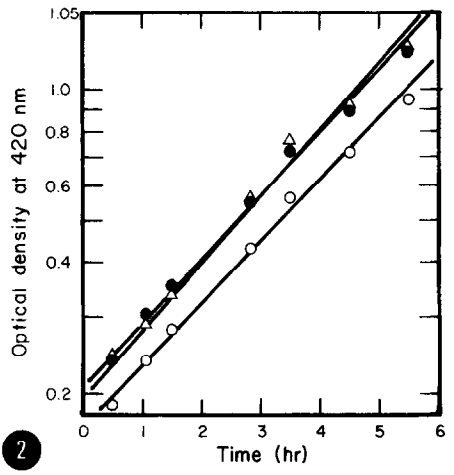
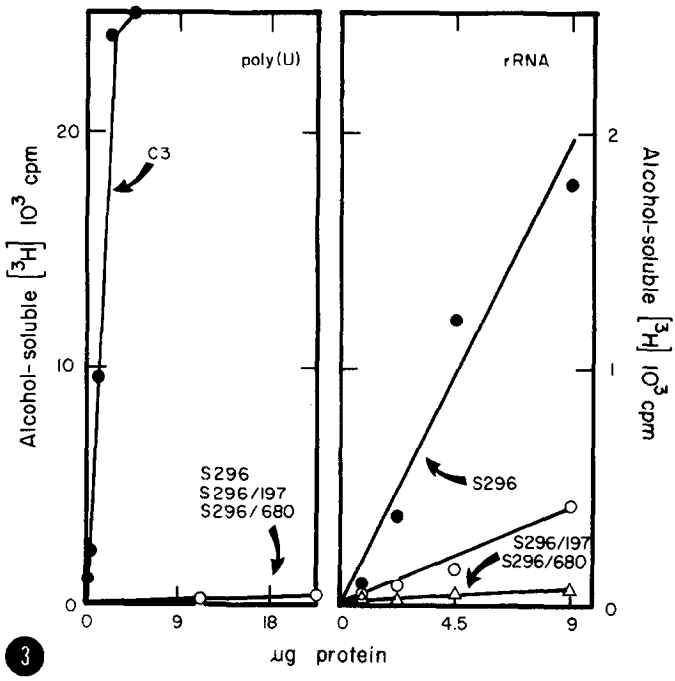


Fig. 1. Degradation of (^{3}H) poly(U) by crude extracts of strains C3 and S296. Assay as in MATERIALS AND METHODS, using samples from alumina-ground cells extracted in assay buffer. Note that from strain S296, 100 µg of protein is used, as compared with 5 µg from strain C3.

Fig. 2. Growth of strain S296 in different media. The growth rate was followed by the optical density of the culture at 420 nm. Cultures were grown from single colonies in minimal salts containing glucose, tryptophan and methionine (ref. 10; —o—); in the same medium fortified with 0.4% technical grade casein hydrolysate (—Δ—) or in L broth (1% tryptone - 0.5% yeast extract - 0.5% NaCl (—•—).

Strains with even lower exonuclease levels than S296 have been isolated by repeating the screening procedure with (^{3}H) ribosomal RNA as a substrate. From two mutagenized cultures of strain S296, 520 and 515 colonies, respectively, were tested. Two colonies, one from each culture, showed low levels of activity; they were purified as strains S296/197 and S296/680. Cell extracts from these mutant strains showed activity much lower than their parental strain S296 (Fig. 3). Tested against (^{3}H) T4 mRNA they showed a comparable reduction in activity (Fig. 4).

One might expect that the mutant strains would show complex lesions induced by nitrosoguanidine. However, the mutants reported here very likely result from one or a very few lesions, because their frequency is comparable to that of mutants scored as lac$^-$ or RNase I$^-$ in control screenings. Also,



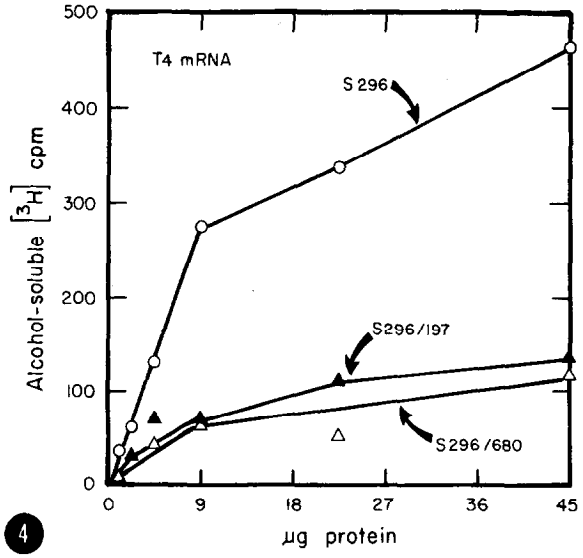


Fig. 3. Degradation of (^{3}H) poly(U) (left) and (^{3}H) rRNA (right) by soluble protein of strains C3, S296, S296/197, and S296/680. Assay as in MATERIALS AND METHODS, with portions of soluble protein from alumina-ground cells.

Fig. 4. Degradation of (^{3}H) T4 mRNA by soluble proteins of strains S296, S296/197, and S296/680. Assay as in MATERIALS AND METHODS, with soluble protein from alumina-ground cells.

all the mutant strains grow at 30° on minimal medium containing methionine and tryptophan - i.e., with the same nutritional requirements as their parental strain C3.

While the genetic analysis of these mutants remains to be pursued, they may already be useful in many studies of RNA function and metabolism, where low levels of endogenous nuclease activity are frequently an advantage.

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