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Selection of Ribosomal Mutants by Antibiotic Suppression in Yeast

Abstract. *Wild-type* Saccharomyces cerevisiae *is highly resistant to streptomycin. A histidine auxotroph was found which could grow without histidine in the presence of high concentrations of streptomycin. Selection for derivatives of this strain which could be suppressed by much lower concentrations of streptomycin yielded streptomycin-sensitive mutants which are cold-sensitive and have altered ribosomal profiles.*

Mutants selected for resistance to such antibiotics as streptomycin and neomycin have been useful in the analysis of ribosome structure and function in bacteria (*1*). The selection of these mutants is positive, and the fact that resistance results in altered ribosomes can be demonstrated by comparing the sensitivity to the antibiotic of protein-synthesizing systems in vitro with ribosomes from the mutant or wild-type strain (*2*). The general method can be used to determine which of the two subunits is altered (*3*). The study of streptomycin-resistant mutations in bacteria has established the effects of streptomycin and of the state of the genetic locus of streptomycin resistance on the fidelity of translation of messenger RNA, and on the efficiency of genetic suppression of nonsense mutations (*4, 5*). Similar effects occur with neomycin (*6, 7*).

One approach to a genetic analysis of ribosome function in yeast would involve isolation of the same sorts of antibiotic resistance mutations that have already been demonstrated to be ribosomal in bacteria. Two types of ribosomes are normally present in yeast: mitochondrial ribosomes, similar to those of bacteria in their inhibition by antibiotics, and cytoplasmic ribosomes, insensitive to antibiotics that are effective against bacterial ribosomes, but sensitive to cycloheximide (*3*). An inhibitor of mitochondrial ribosomes will prevent growth on a nonfermentable carbon source, such as glycerol, the utilization of which requires oxidative phosphorylation, and hence mitochondrial function (*9*), whereas an inhibitor of cytoplasmic ribosomes or of both cytoplasmic and mitochondrial ribosomes will inhibit growth on glucose as well (*10*). *Saccharomyces cerevisiae*, strain S288C (from R. K. Mortimer, University of California at Berkeley), proved to be highly resistant to streptomycin (*11*) and to neomycin. It grows in the presence of more than 40 mg of streptomycin per milliliter or 4 mg of neomycin per milliliter in YEPD (1 percent yeast extract, 2 percent Bacto-peptone, and 2 percent glucose) or petite medium (3 percent glycerol, 1 percent yeast extract, 2 percent Bacto-peptone, and 0.025 percent glucose). An in vitro protein-synthesizing system (*12*) of cytoplasmic ribosomes from this resistant strain proved resistant to 10 mg of streptomycin per milliliter, thus suggesting that exclusion of streptomycin from the cell was not the basis of streptomycin resistance (*13*). Two possible explanations remained: (i) that in yeast cytoplasmic ribosomes there is no protein component comparable to the streptomycin protein of bacterial ribosomes, and (ii) that the wild-type ribosomes in our yeast strain is equivalent to a streptomycin-resistant bacterial ribosome. In the latter case, it should be possible to isolate streptomycin-sensitive mutants. Reports that *Endomyces magnusii* (a yeastlike fungus) is sensitive to streptomycin supported this possibility (*14*). One hundred and twenty strains of *Saccharomyces cerevisiae* (collection of H. J. Phaff, University of California, Davis) were screened in an attempt to find a naturally occurring streptomycin-sensitive strain of *Saccharomyces*. One strain, KB-140, was inhibited by 3 mg of streptomycin per milliliter and was found to be sensitive in an in vitro protein-synthesizing system (*13*); but this strain could not be mated or sporulated, and hence was useless for genetic studies.

Attempts by us and by S. R. Snow to obtain a streptomycin-sensitive derivative of S288C by mutagenesis and counterselection via nystatin (*15*) in the presence of streptomycin (10 mg/ml) in YEPD medium failed repeatedly.

Gorini, working with *Escherichia coli*, was able to select positively for transduction of streptomycin sensitivity into a streptomycin-resistant strain (*5*). His resistant strain contained a conditional streptomycin-dependent lesion in ornithine transcarbamylase; that is, it required either arginine or streptomycin for growth (*7*). When present in a streptomycin-sensitive strain, the same mutation in ornithine transcarbamylase was suppressed by far lower concentrations of streptomycin. Streptomycin-sensitive transductants of the resistant strain could be selected by plating on medium containing only low concentrations of streptomycin and no arginine. This suggested that, if we could find yeast strains with nutritional requirements suppressible by high concentrations of streptomycin, streptomycin-sensitive strains might be found by selecting for strains suppressible by low concentrations of streptomycin.

We looked for streptomycin suppression of approximately 50 missense mutations in His_1 (obtained from C. Korch). The mutants were plated on minimal medium (Difco yeast–nitrogen base, without amino acids) with and without streptomycin (5 mg/ml) and incubated at 30°C for 1 to 2 weeks. One mutant grew only on the plate containing the streptomycin. This streptomycin-suppressible strain, 66A4-295 (His_{1-34}, α), was treated with ethylmethanesulfonate in phosphate buffer, *p*H 7, for 1 hour, washed twice with sterile water, suspended in 4 percent thiosulfate for 10 minutes, washed twice with sterile water, and then plated on minimal medium plates containing streptomycin at 1 μg/ml, 10 μg/ml, or 50 μg/ml and incubated at 30°C. After 36 hours, colonies were selected and placed on the following mediums: minimal, YEPD, and YEPD plus streptomycin (10 mg/ml). A streptomycin-sensitive mutant was obtained after we

Table 1. Antibiotic-sensitive mutants.

Strain	Gene	Isolation procedure	Sensitivity to neomycin (μg/ml)*		Sensitivity	Growth at 15°C‡
			Haploid	Diploid†		
AA-1	*str 1*	Suppression§	100	Resist.‖	Recessive	+
AA-79	*Neo 5*	Nys, c.s.¶	700	700	Dominant	—
AA-80	*Neo 6*	Nys, c.s.¶	500	1000	Semidominant	+
AA-81	*neo 7*	Nys, c.s.¶	100	Resist.‖	Recessive	—
AA-89	*Neo 11*	Suppression§	900	2500	Semidominant	—
AA-91	*Str 13*	Suppression§	100#	100#	Dominant	—

* In YEPD medium. † Diploid formed by crossing mutant with haploid wild-type XC500A. ‡ Unable to grow at 15°C is indicated by (—); the wild type grows at 4°C and is indicated by (+). § Suppression of nutritional requirement by low concentrations of streptomycin in minimal media. ‖ Resistant to concentrations of neomycin sulfate greater than 5 mg/ml. ¶ Nystatin counterselection (Nys, c.s.) in presence of 1 mg of neomycin sulfate per milliliter. # Also sensitive to the same concentrations of streptomycin.

had screened approximately 500 colonies. This mutant, AA-1, has the genotype (*str1*, His_{1-34} α). A concentration of 500 μg of streptomycin per milliliter in YEPD inhibits growth of this mutant on agar plates, whereas a diploid, AA-1d, formed by crossing AA-1 with a wild-type haploid, XC500A, is insensitive to 20 mg of streptomycin per milliliter on the same agar plates, an indication that sensitivity is recessive. The doubling time for AA-1 in the absence of streptomycin was essentially the same as that of the wild type, whereas no growth occurred in the presence of 10 mg of streptomycin per milliliter. The heterozygous diploid grows at the rate of the wild type both in the presence and absence of this concentration of antibiotic.

The mutant, AA-1, was tested for cross-sensitivity to a series of other antibiotic and chemical inhibitors. We found that it was also sensitive to neomycin at 100 μg/ml. The parent strain, 66AA-295, is resistant to 5 mg of neomycin per milliliter and at least 20 mg of streptomycin per milliliter. Sensitivity to both these antibiotics maps at the same locus or at immediately adjacent loci, suggesting that the mutation conferring streptomycin sensitivity also conferred neomycin sensitivity.

Mapping studies of AA-1 indicate that the streptomycin character segregates as a nuclear gene. The gene is not linked to a centromere nor is it linked to any of some 40 widely distributed loci tested for linkage (*13*).

Additional experiments for the selection and isolation of mutants, with low concentrations of streptomycin or neomycin being used for suppression, have yielded many sensitive mutants as well as one that is apparently streptomycin-dependent. The phenotypes of a number of these are summarized in Table 1. Dominance studies with diploids formed by crossing our mutants with a haploid wild-type, XC500A, yielded three classes of mutants with respect to their dominance characteristics: dominant sensitivity, semidominant sensitivity, and recessive sensitivity. Cooper *et al.* (*10*) have isolated a mutant of *Saccharomyces cerevisiae* with a recessive resistance gene *cyh8* (ac^r_8) that is ribosomal. Dominant sensitivity (that is, recessive resistance) is also a property of merodiploids formed in *Escherichia coli* with mutations known to reside in the ribosome (*16*).

Growth studies with AA-13 (a segregant from a cross of AA-1 and S1-16A) with genotype (*str1*, α) show an inhibitory effect within 4 hours when exposed to neomycin at 600 and 1000 μg/ml. Total cell counts and viable counts as well as optical density were determined for each sample. The viable count remained constant for the period 4 to 24 hours at a neomycin concentration of 1 mg/ml. The viable count dropped after 24 hours, an indication that the antibiotic was killing the cells rather than causing stasis at this time. An explanation for the failure of the first attempts with the nystatin counterselection could now be attributed to the fact that the original levels used were too high to be static and therefore caused killing.

These data indicated that the nystatin counterselection should work if YEPD plus 600 to 1000 μg of neomycin per milliliter were used. We performed such an experiment and found that our prediction was correct; that is, approximately 3 percent of surviving organisms were sensitive mutants. The only required modification of the nystatin counterselection procedure was to grow the cells, after they had been subjected to nitrogen starvation and before the nystatin was added on YEPD plus 1 mg of neomycin per milliliter for 7 hours. This procedure allowed adequate time for the drug to exert its static effect and, at the same time, protected the sensitive mutants from nystatin killing.

Selection for mutations that are likely to result in altered cytoplasmic ribosomes has thus been successful. Isolation of mitochondrial mutants sensitive to neomycin or streptomycin was

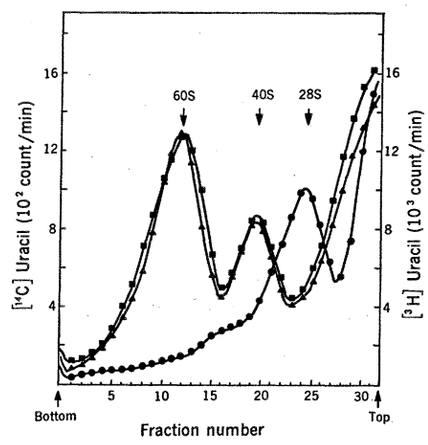

Fig. 1. Sucrose gradient sedimentation analysis of ribosomal subunits from the parent (S288C) and the cold-sensitive mutant (AA-89) grown in YEPD at 30°C and 15°C. (■) AA-89 growing exponentially at 30°C with 4.0 μc of [^{3}H]uracil (6.85 c/mmole) per millimeter at 30°C for two doublings. (●) AA-89 growing exponentially at 30°C was shifted to 15°C for 30 minutes and then [^{14}C]uracil (0.2 μc/ml; 52 mc/mmole) was added for 12 hours (0.7 doubling), at which time growth was stopped. (▲) S288C growing exponentially at 30°C was shifted to 15°C for 30 minutes, then [^{3}H]uracil (4.0 μc/ml; 6.85 c/mmole) was added, and growth was allowed to continue for two doublings. Cells were broken in an Eaton press (*19*), and equal amounts of S288C (15°C) plus AA-89 (15°C) and AA-89 (30°C) plus AA-89 (15°C) were mixed. A 0.1-ml sample of the two mixtures was layered on separate 5-ml portions of sucrose gradients (5 to 20 percent) in low Mg^{2+} buffer [tris(hydroxymethyl)aminomethane (tris), 0.01*M*; 0.1*M* NaCl, 0.1 m*M* $MgCl_2$, *p*H 7.5]. The gradients were centrifuged for 185 minutes at 37,000 rev/min (SW39 rotor, 4°C, Beckman model L2-50 ultracentrifuge). Fractions (ten drops were collected into vials via a multispeed transmission pump (Harvard Apparatus), and 6 ml of Bray's solution (*20*) was added before counting in a Nuclear-Chicago Mark II liquid scintillation spectrometer. The counts of ^{3}H and ^{14}C were corrected for channel overlap and plotted by computer.

also possible with the use of nystatin counterselection and by substituting a nonfermentable carbon source (glycerol) for glucose, along with a few modifications as follows. (i) Cells were grown for 48 hours on yeast extract and peptone (YEP) plus 5 percent glucose before EMS treatment in order to reduce the number of mitochondria per cell. These glucose-repressed conditions result in cytological changes similar to those seen with anaerobic growth, namely, a reduction from 15 or 20 mitochondria in a respiring cell to two or three mitochondria per repressed cell. (ii) The cells were returned to YEP plus 5 percent glucose after treatment with ethylmethanesulfonate to enhance the segregation of a single mitochondrial population of a sensitive mutant. (iii) After nitrogen starvation, the cells were grown on petite medium plus 1 mg of neomycin per milliliter before addition of nystatin. (iv) Finally the cells were grown on petite medium for 24 hours after the nystatin was washed away. One of the ten mutants isolated is unable to grow on petite medium containing 800 μg of neomycin per milliliter, but grows well on petite medium without neomycin and on YEPD plus 3 mg of neomycin per milliliter. This indicates that some mitochondrial function is impaired by the addition of neomycin. We have been unable to analyze this mutant genetically because the ascospores do not germinate.

Strain AA-89, which was selected for streptomycin sensitivity by suppression with low concentrations of streptomycin and which is also cold-sensitive (allelic with streptomycin sensitivity), has an altered ribosomal profile (Fig. 1) when grown at the nonpermissive temperature (15°C); thus it shows the cold-sensitive phenotype defective in subunit assembly similar to those reported for bacteria (*17*). The cytoplasmic ribosomes isolated from this mutant when grown at the permissive temperature (30°C) are sensitive to neomycin and streptomycin in an in vitro protein-synthesizing system (*13*). Our results indicate that AA-89 is a ribosomal mutant in the sense of Apirion (*18*), and we conclude that the streptomycin and neomycin sensitivity of the strain results from this alteration in ribosome structure.

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Environmental Nitroso Compounds: Reaction of Nitrite with Creatine and Creatinine

Abstract. *Creatine reacts with nitrite under acid conditions to produce first sarcosine and then* N*-nitrososarcosine, which is a weak carcinogen in the rat. Creatinine reacts with acidified nitrite to produce either creatinine-5-oxime or 1-methylhydantoin-5-oxime, depending on reaction conditions. The toxicity and environmental significance of these compounds is not yet known.*

The carcinogenicity of large numbers of nitrosamines has been demonstrated in various tissues of many animal species (*1*). It is possible that nitrosamines will form in the environment whenever nitrite and secondary or tertiary amines occur together. Certain foodstuffs have already been shown to contain simple alkyl nitrosamines (*2*), and reaction of nitrite and various secondary amines in human gastric juice at *p*H 1.5 in vitro also yielded nitrosamines (*3*). These compounds might represent a health hazard (*4*), and their possible relevance to the etiology of human cancer has been reviewed (*1, 5, 6*).

Nitrates and nitrites are frequently used as preservatives and for color fixation in foods. Extensive use of nitrates as fertilizers can lead to high accumulated levels in water supplies and plant tissue. Microorganisms, particularly the coliform group or *Clostridia*, reduce nitrate to nitrite; thus nitrite can accumulate during the storage of fodder crops and during the storage and processing of vegetables and food to which nitrate has been added.

Creatine, present in muscular tissue of many vertebrates, is a normal constituent of meat. A large portion of creatine in muscle is combined with phosphoric acid as phosphocreatine, but this compound is readily hydrolyzed to creatine and creatinine in acid solution (*7*). Creatinine, the end product of creatine metabolism, is found together with creatine in muscle tissues, milk, and blood. Other significant sources of creatinine include grain seeds and other vegetable matter, certain fish, and crab meat extract (*8*).

In view of the abundance of nitrite, creatine, and creatinine in our environment, and because these compounds are frequently ingested simultaneously in a normal diet, we thought that it was important to investigate their mutual reactivity.

We first demonstrated the formation of *N*-nitrososarcosine by reaction of creatine with nitrite: *N*-nitrososarcosine has been shown to induce cancer of the esophagus in the rat (*6*). To a stirred solution of 5.2 g of creatine (Nutritional Biochemicals) dissolved in 15 ml