*STREPTOMYCIN-INDUCED OVERSUPPRESSION IN E. COLI**

By Luigi Gorini and Eva Kataja

DEPARTMENT OF BACTERIOLOGY AND IMMUNOLOGY, HARVARD MEDICAL SCHOOL, BOSTON

Communicated by Herman M. Kalckar, April 6, 1964

Any model for the action of extracistronic suppressors[1-3] that involves the mistaken reading of a codon appearing often in the genome, predicts that if the efficiency of suppression is high, the suppressor would be lethal. We have found strains in which this "oversuppression" can be brought about by an external agent, streptomycin (Sm).

This Sm-induced oversuppression appears to be the combined result of two suppressors of the same defect. These strains result from a second suppressor mutation in a Sm-suppressible (conditional Sm-dependent, CSD) parent. The CSD strains require either a growth factor or Sm for growth and have been demonstrated[4] to contain a competent Sm^r mutation and a defect in a structural gene, whose suppression is Sm-dependent. *In vitro* experiments[5] have provided support for the suggestion that Sm acts in the CSD strains to cause specific mistakes in the genetic code.

We shall describe here the strains that contain the two suppressor functions, and show that in the presence of Sm their combined effect, although increasing the level of the suppressible defective enzyme, decreases the levels of other enzymes in the cell and inhibits growth. We propose to call this phenomenon "oversuppression."

Isolation and Characterization of the Mutants.—B4S-7^{SD} is a CSD-arginine mutant containing a suppressible defect in the ornithine transcarbamylase structural gene (OTC^{SD}) and a streptomycin-activated suppressor.[4] Spontaneous revertants to arg^+ were isolated (frequency 10^{-7}) from a histidine-requiring derivative of this strain by spreading the cells (about 10^8 cells/plate) on solid minimal medium A[6] + his + glucose. It was expected that the revertants would be Sm^r like the original Sm^r parent of these strains. However, 4 out of 38, when tested with the standard concentration of Sm (500 μg/ml) appeared sensitive. One of these mutants is B4S-71.

The sensitivity of these mutants is different from the wild-type sensitivity. In minimal medium + histidine, 75 per cent of a growing culture of B4S-71 survives a one-hr exposure to 500 μg/ml Sm at 37°. In rich medium the survival is 10 per cent, whereas that of the standard Sm^s strain is less than 0.001 per cent in both media. B4S-71 is slow-growing in minimal medium + histidine as compared with the original prototrophic parent, B4S-7 (OTC^+ Sm^r) (Table 1). Addition of arginine to the medium restores wild-type growth rate. When small amounts of Sm are added, the growth rate decreases sharply. The upper limit of Sm concentration permitting a measurable growth depends on the composition of the medium: in minimal medium, with or without arginine, this limit is 12 μg/ml; in enriched medium, 50 μg/ml (Table 1).

Mutants of B4S-71 resistant to high levels of Sm were isolated (approximate frequency 10^{-6}) by spreading the cells on solid enriched medium + Sm (500 μg/ml). All 358 mutants examined were CSD mutants indistinguishable from the original B4S-7^{SD} his^-. This suggests that the revertant phenotype had been the result of a suppressor mutation affecting both the OTC^{SD} and Sm^r characteristics.

TABLE 1

INFLUENCE OF SM ON GROWTH RATE OF B4S-71

	Doubling Time (min)							
	B4S-71 Sm μg/ml						B4S-7 Sm μg/ml	
Medium	0	5	8	12	25	50	0	50
Minimal	87	90	106	140	No growth	No growth	55	55
" + arginine	65	89	109	153	" "	" "	55	55
Enriched	34	34	—	48	85	Linear growth	30	30

Media: Minimal: Medium A + 0.1% glucose + 28 μg/ml histidine. Minimal + arginine: Medium A + 0.1% glucose + 100 μg/ml arginine + 28 μg/ml histidine. Enriched: Medium A + 0.2% glucose + 0.2% casamino acids + 0.2% yeast extract.

Doubling time was determined from Lumetron O.D. measurements at 490 mμ of cultures growing at 37° with shaking. Growth rates of B4S-7, the original prototrophic Sm^r parent of B4S-71, are given here for comparison.

Mutants of the same phenotype, i.e., slow-growing prototrophs inhibited by Sm in a similar manner have been isolated so far from the following CSD strains: $meth^{SD}$, $leuc^{SD}$, $val\text{-}isol^{SD}$, lys^{SD}. They constitute 2–50 per cent of the revertants.

Genetic Recombination Experiments.—That the parental OTC^{SD} marker has remained unaltered in B4S-71 was shown by a genetic recombination experiment using K 10 try^- OTC^+ Sm^s as a donor. This cross reveals the number of genetic determinants of the arg^+ Sm-inhibited phenotype. Parental types were eliminated by the omission of histidine and tryptophane from the selection plates. Four types of selection plates were used: minimal medium for selection of all arg^+ recombinants; Sm (500 μg/ml) for selection of arg^+ Sm^r and CSD; arginine + Sm for selection of arg^+ Sm^r, arg^- Sm^r, and CSD; and arginine for scoring of all possible recombinants. Table 2 lists the classes and numbers of recombinants obtained.

TABLE 2

TYPES OF RECOMBINANTS OBTAINED IN CROSSES K10 TRY^- × B4S-71 HIS^-

	Plates on Which Recombinants Were Selected			
Phenotypes found	Minimal	Sm	Sm + arg	Arg
Arg^+ Sm^s	9	—	—	10
Arg^+ $Sm^{inhib.}$	184	—	—	155
Arg^+ Sm^r	2	16	8	4
Arg^- Sm^s	—	—	—	23
CSD	—	26	80	13
Total scored	195	41	88	205
No. recomb./10^6 Hfr	130	1.5	2	160

Recombination experiment: parental cultures were grown to log phase (1×10^8 to 5×10^8 cells/ml) in enriched medium (Table 1) at 37°. Equal volumes were mixed and incubated at 37° without shaking for $2^1/_2$ hr. Washed samples were plated on the selection media.

Screening of recombinants which appear Sm^s on Sm (500 μg/ml) plates: cell suspensions (about $10^6 - 10^7$ cells/ml) were pregrown at 37° for 2 hr in minimal medium + glucose + arg; exposed to Sm (50 μg/ml) for 15 min; and tested for viability in the absence of Sm.

CSD recombinants were scored as those showing no growth on minimal medium, but growing on minimal + Sm and on minimal + arg. The ability of the CSD recombinants to grow in Sm varied widely (see text).

The appearance of Sm^r recombinants indicates that although both parents are phenotypically Sm^s, the recipient harbors the Sm^r marker. The appearance of the CSD phenotype among the recombinants shows that the OTC^{SD} marker has remained unaltered in the mutational step from $B4S\text{-}7^{SD}$ to B4S-71. These observations confirm the occurrence in B4S-71 of a suppressor outside the OTC locus which simultaneously suppresses both the OTC^{SD} mutation and the Sm^r mutation, producing an intermediate (bacteriostatic) type of Sm-sensitivity. Given a Su^+ having these properties, one can predict the phenotypes of recombinants obtainable from a cross Hfr: OTC^+ Su^-Sm^s × F^-: OTC^{SD} Su^+Sm^r as listed in Table 3. The pre-

TABLE 3

PREDICTED PHENOTYPES OF RECOMBINANTS EXPECTED FROM THE CROSS: Hfr: OTC$^+$ Su$^-$ Sm^s × F$^-$: OTD^{SD} Su$^+$ Sm^r

Expected recombinants	Phenotype
(1) Arg^{SD} Su$^+$ Sm^r	Arg$^+$ Sm$^{inhib.}$
(2) Arg$^+$ Su$^+$ Sm^r	" "
(3) Arg$^+$ Su$^-$ Sm^s	Arg$^+$ Sm^s
(4) Arg$^+$ Su$^+$ Sm^s	" "
(5) Arg^{SD} Su$^+$ Sm^s	" "
(6) Arg^{SD} Su$^-$ Sm^s	Arg$^-$ Sm^s
(7) Arg^{SD} Su$^-$ Sm^r	CSD
(8) Arg$^+$ Su$^-$ Sm^r	Arg$^+$ Sm^r

The recombinant genotypes are those predicted on the assumption that the one-gene change from B4S-7^{SD} to B4S-71 is a change from Su$^-$ to Su$^+$ and that the K10 donor is Su$^-$.

dicted recombinant phenotypes are all found in the cross K 10 × B4S-71 (Table 2). The appearance of the CSD recombinant class also suggests that the Su$^+$ is external to the Sm locus as well as to the OTC locus. If the Su$^+$ had occurred at the Sm locus (Sm$^{r'}$), the phenotype of the OTC^{SD} Sm$^{r'}$ recombinant would be arg$^+$ Sm-inhibited, and the CSD phenotype would not appear at all among the recombinants.

It should be noted that the CSD recombinant class indicated in Table 2 is not a homogeneous one. Although some clones were clearly CSD showing rapid growth on Sm medium, others showed visible growth only after several days' incubation, and still others showed almost no growth after prolonged incubation on Sm. Since, however, many of the latter type had appeared originally on the Sm selection plates in the absence of arginine, they could not be excluded with certainty from the CSD class and are hereafter referred to as "weak CSD." We shall show that these have acquired the $R_{arg}$$^+$ gene and are repressed in the arginine pathway.

In order to map the markers involved, interrupted mating experiments were done between a K 10 Sm^s donor and B4S-71 and several other B derivatives. The entry of the Su$^-$ marker into B4S-71 was detected by the early appearance of Sm^r recombinants (Tables 4 and 5). Since the low efficiency of recombination in crosses

TABLE 4

INTERRUPTED MATING EXPERIMENTS: TIME OF ENTRY OF K MARKERS INTO B RECIPIENTS

Recipient		Selective medium	Time of Entry	
Strain	Relevant markers		K10 markers	Min (±4)
B90	OTC$^-$ Lac$^-$	(Minimal-glucose) + arg + lactose	Lac$^+$	15
"	" "	Minimal	OTC$^+$	18
B4S-71	OTC^{SD} Su$^+$ R$^-$ his$^-$	" + Sm + his	Su$^-$	20
B meth^{SD}-1-BM	Meth^{SD} Su$^+$	" + Sm	Su$^-$	20
B meth^{SD}-1	Meth^{SD}	"	Meth$^+$	23
B4S-71	OTC^{SD} Su$^+$ R$^-$ his$^-$	" + Sm + his	$R_{arg}$$^+$	48
"	" " " "	" " arg	His$^+$	75

Donor: K10, injects markers in the order: O-Lac-OTC-val^s-meth-R_{arg}-Sm-his. Mating: As described in Table 2 except that aliquots were withdrawn at 5-min intervals. Mating pairs were separated by vigorous mixing (Vortex mixer), washed twice, and spread on selective plates. Counterselectors: Valine (10^{-3} *M*) was used in the selection plates to prevent growth of K10. Where Sm was used, two sets of plates were spread: one containing Sm (500 μg/ml) and another to which Sm was added after 5-hr incubation at 37°.

of K and B necessitates a high cell density on the selective plates decreasing the precision of the method, markers transferred less than 8 min apart cannot be distinguished with certainty. Nevertheless, both in crosses with B4S-71 and with the corresponding suppressed derivative of a CSD-methionine strain, the frequency of Sm^r recombinants increases abruptly at about 20 min, placing the Su in both crosses between the arg and meth markers, but well before the Sm locus which enters closely

TABLE 5

SCORING OF Sm^r RECOMBINANTS FROM INTERRUPTED MATING EXPERIMENTS BETWEEN K 10 × B4S-71 his^-

Time after start of mating (min)	Total found	Number scored	CSD		Arg+
			"Strong"	"Weak"	
0	4	4	4	0	0
10	7	7	7	0	0
20	43	43	41	1	1
30	78	20	20	0	0
40	94	20	17	1	2
50	259	20	13	3	4
60	500	30	21	8	1

The level of argininosuccinase was determined in 4 of the "weak" CSD clones appearing at 40, 50, and 60 min. The enzyme is repressed by arginine as in standard R_{arg}^+ strains (i.e., the ratio of enzyme under arginine-limitation to enzyme in arginine-excess is 1:10).

linked to R_{arg} at about 50 min under similar conditions.[7] This early entry of Su is detected only when Sm is added to the selection plates after a 5-hr preincubation at 37°. If, on the contrary, samples are plated directly on Sm selection plates, a delay of about 30 min is observed in the appearance of Sm^r recombinants, indicating that this expression of Sm^r, in a cell which is Sm-inhibited because of the additional Su^+ gene, has a phenotypic lag of about one generation.

The weak CSD phenotype appeared among the Sm^r recombinants at about 50 min (Table 5). Since the arginine repressibility locus enters at about this time,[7] the enzyme argininosuccinase of these recombinants was tested for its repressibility by arginine. Four recombinants were tested, and all were found to be repressible, although the parent B4S-71 is a derepressed strain. That the CSD character can appear arg^- rather than CSD-arg when enzyme repression is superimposed on the OTC^{SD} genotype, is understandable in terms of the low level of the Sm-induced correction.[4] One expects the CSD phenotype to arise only in cells having the potential to produce enzyme in great excess of the minimum required to support growth. Since recombinants occurring in crosses of K × B exhibit intermediate levels of repression of the arginine pathway,[7] we suggest that the inhomogeneity of the CSD recombinant class in these crosses is due to the segregation of the multigenic determinants of repression of the arginine enzymes.

Enzyme Levels.—The Su^+ gene in B4S-71 acts to restore a low level of OTC activity. When this strain is grown in the presence of Sm, the Sm-activated suppressor acts to increase the level of OTC activity 3–4-fold (Fig. 1). Sm also acts to increase the OTC activity of the parent, $B4S\text{-}7^{SD}$. It can be seen, however, that the combined action of the two suppressors in the presence of Sm results in an OTC level more than twice as high as the sum of the levels produced by either suppressor acting alone. In contrast, the two suppressors acting together reduce drastically the level of other enzymes in B4S-71. Figure 1 shows that the levels of the three enzymes studied (argininosuccinase (AS) of the arginine biosynthetic pathway, aspartate transcarbamylase (ATC) of the uridylate pathway, and β-galactosidase) were all decreased in the presence of Sm. In the parent strain $B4S\text{-}7^{SD}$, streptomycin does not affect these levels, nor is there an effect on the original strain (B4S-7 (Sm^r OTC^+)).

For three of the enzymes studied, OTC, AS, and β-gal, similar enzyme levels and streptomycin effects were found whether the cells were grown in minimal or in enriched media. The behavior of ATC, however, was dependent on the growth medium. When B4S-71 was grown in enriched medium, ATC levels were high,

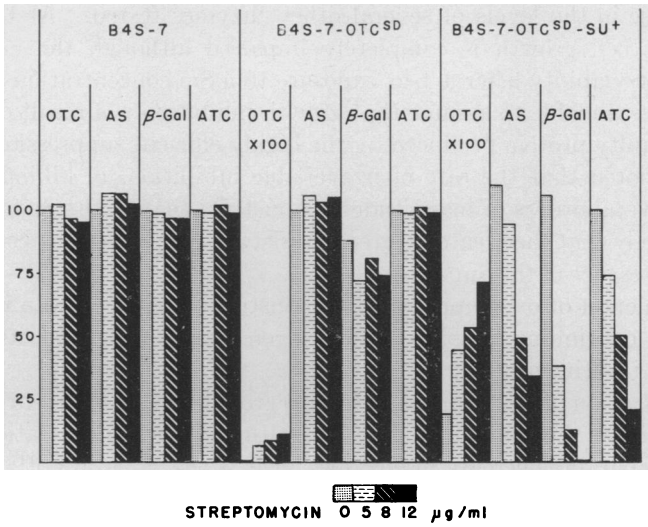

FIG. 1.—Influence of Sm on the level of OTC and other enzymes in suppressed and nonsuppressed strains. OTC: ornithine transcarbamylase; AS: argininosuccinase; β-gal: β-galactosidase; ATC: aspartate transcarbamylase. Growth medium: the three strains were grown in enriched medium + 0.2% glucose. Thiomethyl-galactoside ($5 \times 10^{-4}M$) was added to the growth medium of cultures assayed for β-galactosidase. Enzyme preparations: cell extracts obtained by sonication were used in the case of OTC, AS, and ATC, and suspensions of toluenized cells in the case of β-gal. Enzyme determinations: OTC was assayed by citrulline formation;[9] ATC by ureidosuccinic acid formation;[10] AS by determining arginine formed, as urea;[11] and β-gal by orthonitrophenylgalactoside hydrolysis.[12] Enzyme levels are expressed as per cent of the B4S-7 level in the absence of Sm. Control experiments established that in all 3 strains, Sm added to the enzyme assay mixture does not interfere with activity of the enzymes studied.

6 units, and decreased in Sm to 1 unit. For cells grown in minimal medium (±arginine) the ATC level was low, 1.5 units, and was reduced only slightly, to 1 unit, by Sm. Since Sm reduces the growth rate of B4S-71 in minimal medium (Table 1), increased repression of ATC may occur in minimal medium + Sm, obscuring any other Sm effect on the enzyme level. We do not think that the effect of Sm on the β-galactosidase level is due to catabolite repression[8] since the magnitude of the effect is the same in minimal or enriched medium, and the dramatic reduction in enzyme level in enriched medium appears with 5 μg/ml of Sm where the growth rate is not measurably changed (Table 1). The results with OTC and AS could not be affected by any control mechanism, since these two enzymes in the same pathway are always controlled in parallel, but behave oppositely under conditions of oversuppression.

Discussion.—The results of the genetic studies presented here demonstrate that in addition to a Sm-activated suppression mechanism, strain B4S-71 contains a suppressor of the OTC^{SD} mutation which functions even in the absence of Sm, and which maps in the region between arginine and methionine as does the suppressor of a meth^{SD} mutation in another CSD strain. In addition it has been shown that in the presence of Sm the suppression of the OTC^{SD} mutation is considerably greater than the sum of suppression effects produced by either the Su^{+} marker alone or Sm alone (as in B4S-7^{SD}). Moreover, in the presence of increasing Sm concentrations up to 12 μg/ml, the increased suppression level in the cell is also observable as a

drastic decrease in the levels of several other enzymes tested. At the higher Sm concentrations cell growth is completely impaired although the culture retains 10–75 per cent viability after a 1-hr exposure to a Sm concentration as high as 500 μg/ml. It is reasonable to assume that growth inhibition is a result of overloading the cell with faulty protein produced by the highly efficient suppression mechanism. It should be noted that the rate of irreversible inhibition, or killing, of standard Sm^s cells is several orders of magnitude greater than that of the Su^+ strains examined. It is likely that the greater degree of resistance shown by the Su^+ strains is a result of the presence of the Sm^r gene.

The phenomenon of oversuppression demonstrates that a cell can tolerate a high level of error, continuing to grow, slowly, even when burdened with as much as 50–80 per cent inactive protein.

The concentration of Sm required to inhibit growth of B4S-71 in enriched medium is 2–3 times higher than in minimal medium although the concentration required to produce a comparable decrease in enzyme levels is the same in both media. This is consistent with the idea that to some extent the cell can be compensated for a high level of faulty enzymes by supplying many of their end products in the medium.

The interaction between Sm and the Su^+ gene is not understood. They may act via completely independent mechanisms or via the same mechanism, and in either case they may produce the same or different amino acid shifts. Since there is evidence[5] that the Sm-activated suppressor may act at the level of the ribosome-messenger RNA-transfer RNA complex, if the two suppressors operate via the same mechanism, then the action of Su^+ should also be at the ribosomal level. The fact that some degree of synergism is observed between the two suppressors in the suppression of the OTC^{SD} defect, does not permit a choice between the possibilities of their having independent or similar mechanisms, since one can visualize models which predict synergism, in both cases. If two separate mechanisms exist producing different amino acid shifts, then it sould be possible to demonstrate that the protein (OTC) formed by the Sm-activated suppression (i.e., in B4S-7^{SD}: OTC^{SD} Su^- Sm^r) is different from that formed by the Su^+ suppression (i.e., in B4S-71: OTC^{SD} Su^+ Sm^r, grown without Sm).

Summary.—The growth-inhibitory loading of a cell with faulty protein resulting from a highly efficient suppression mechanism has been demonstrated in a strain of *E. coli* in which the suppression level can be controlled by an external agent, Sm.

The skillful technical assistance of Mrs. Frayda Oston and Mrs. Regine Weil are gratefully acknowledged.

* This work was supported by a USPHS grant (AI-02011-06) from the National Institute of Allergy and Infectious Diseases, an American Cancer Society grant (E-226-B), and USPHS fellowship (5-F1-GM-19,085-02) from the National Institute of General Medical Sciences.

[1] Garen, A., and O. Siddiqi, these PROCEEDINGS, **48**, 1121 (1962).

[2] Brody, S., and C. Yanofsky, these PROCEEDINGS, **50**, 9 (1963).

[3] Benzer, S., and S. P. Champe, these PROCEEDINGS, **48**, 1114 (1962).

[4] Gorini, L., and E. Kataja, these PROCEEDINGS, **51**, 487 (1964).

[5] Davies, J., W. Gilbert, and L. Gorini, these PROCEEDINGS, **51**, 883 (1964).

[6] Davis, B. D., and E. S. Mingioli, *J. Bacteriol.*, **60**, 17 (1950).

[7] Gorini, L., W. Gundersen, and M. Burger, in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 173.

[8] Magasanik, B., in *Cellular Regulatory Mechanisms*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 26 (1961), p. 249.

[9] Jones, M., L. Spector, and F. Lipmann, *J. Am. Chem. Soc.*, **77**, 819 (1955).

[10] Gerhart, J. C., and A. B. Pardee, *J. Biol. Chem.*, **237**, 891 (1962).

[11] Ratner, S., W. P. Anslow, and B. Petrack, *J. Biol. Chem.*, **204**, 115 (1953).

[12] In *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, 1962), vol. 5, pp. 212–213.

---

*CELL-FREE PROTEIN SYNTHESIS: MESSENGER COMPETITION FOR RIBOSOMES**

BY R. HASELKORN AND V. A. FRIED

DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF CHICAGO

Communicated by Raymond E. Zirkle, April 27, 1964

In a previous communication,[1] it was shown that the over-all kinetics of incorporation of amino acids into polypeptides in a cell-free system from *E. coli* could be accounted for by the following scheme:

$$M + nR \underset{k_2}{\overset{k_1}{\rightleftharpoons}} MR_n \underset{k_4}{\overset{aa\ k_3}{\rightleftharpoons}} MR_n' \underset{aa}{\overset{k_5}{\rightarrow}} MR_n'P$$

in which M = polynucleotide messenger, R = single ribosome, aa = amino acids or their activated intermediates, and P = polypeptide product. When poly U and TYMV-RNA were compared as messengers, the rate of association of poly U with ribosomes was found to be greater than that of TYMV-RNA.[2] These observations have been extended, and in this communication we present results indicating that the association equilibria, as well as forward rates, differ for different polynucleotides. Association of messenger with ribosomes can, under suitable conditions, be the rate-limiting step of amino acid incorporation *in vitro*. An *in vivo* system in which secondary structure of entire messenger RNA molecules is equilibrated, could therefore utilize this feature for controlling the rate of synthesis of particular proteins.

Materials and Methods.—Amino acid incorporation into TCA-precipitable material was measured exactly as described previously.[1] C^{14}-labeled proline and phenylalanine were obtained from New England Nuclear Co., and had specific activities of 205 and 360 mC/mmole, respectively. Poly U and poly C were generously provided by Dr. Stanley Yachnin, who synthesized them from nucleoside diphosphates with *M. lysodeikticus* polynucleotide phosphorylase. These polymers had $S_{20,w}$ = 4.3 S and 4.0 S, respectively, in 0.15 *M* NaCl, 0.01 *M* Tris pH 7.6. Poly C labeled with P^{32} was prepared by Dr. F. Fox in S. B. Weiss' laboratory by using heat-denatured *P. fluorescens* DNA as primer in a reaction catalyzed by *M. lysodeikticus* RNA polymerase. When CTP^{32} is the only nucleotide present, P^{32}-poly C is the product.[3] The product was completely sensitive to pancreatic ribonuclease, formed a helical complex with poly G, and after separation from the DNA primer by centrifugation in Cs_2SO_4, had an ultraviolet absorption spectrum identical with that of poly C made with polynucleotide phosphorylase.[4] Attach-