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Codon usage as a reason for unsuccessful search for amber-suppressor mutants in *Streptomyces lividans*?

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Summary

An amber mutation was created in the CAT gene of plasmid vector pACYC184 and this modified plasmid was fused with the *Streptomyces* vector pIJ702 for use as an indicator for the identification of *Streptomyces* strains carrying nonsense suppressor tRNA mutations. The resulting hybrid plasmid pGM1109 was introduced into the chloramphenicol-sensitive mutant M252 of *Streptomyces lividans*. Chloramphenicol-resistant colonies were isolated and characterized. None of them was a nonsense suppressor mutant. The failure to obtain such mutants is discussed on the basis of codon usage in streptomycetes.

1. Introduction

Much of our information about the genetics and molecular biology of bacteriophages such as the coliphages lambda and T4 and *Salmonella* phage P22 has come from studies of nonsense mutants. These conditional lethal mutations, such as the amber mutation, have genetic defects which are lethal in wild-type hosts but may be overcome in suppressor strains in which the mutants can replicate successfully. Suppression of nonsense codons occurs generally by species of tRNA whose anticodon is altered, and which is therefore able to read the nonsense codon and to insert a specific aminoacid rather than terminate the polypeptide. Nonsense mutants are not only of great value for the analysis of phage replication but have a wider and general value in studying metabolic pathways in other organisms. Unfortunately, such mutants are not available for studies on actinophages and streptomycetes since nonsense suppressor strains have not been isolated from streptomycetes. In this paper we report an attempt to isolate such suppressor strains. After completion of our experiments a paper was published (Paradiso *et al.* 1987) which describes a technique similar to the one we have used and which also reports a negative result. We believe that this failure requires a reasonable explanation. We therefore discuss the possible causes of the inability to detect *Streptomyces* mutants which can suppress nonsense defects in a gene.

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2. Materials and Methods

Bacterial strains are listed in Table 1

Plasmids. E. coli plasmid pACYC184: *tet*^{r} *cam*^{r}; 4 kb (Chang & Cohen, 1978); *Streptomyces* plasmid pIJ702: *tsr*^{r} *mel*$^+$; 5·8 kb (Katz, Thompson & Hopwood, 1983)

Media. L Broth, YEME, R5 (modified R2YE) and SNA were prepared according to Hopwood *et al.* (1985). TSB: tryptic soy broth powder (Difco) 3% (w/v), yeast extract 1% (w/v), sucrose 10% (w/v); after autoclaving, pH was adjusted to pH 7·4, $CaCl_2 . 2H_2O$ was added to a final concentration of 0·14% (w/v), $MgCl_2 . 6H_2O$ to 1·0% (w/v). SMA: soy bean meal 2% (w/v), mannitol 2% (w/v) and Difco agar 2·2% (w/v) were dissolved in tap water; after autoclaving, $MgSO_4 . 6H_2O$ (final concentration 0·01 M) and $Ca(NO_3)_2 . 6H_2O$ (f.c. 8 mM) were added.

(i) *Preparation of plasmids*

E. coli plasmids were prepared as previously described (Ish-Horowicz & Burke, 1981). *Streptomyces* plasmids were prepared by the method of Kieser (1984) with the following modifications: bromocresol green was omitted from the lysozyme solution; the extract was treated with RNase after the first phenol extraction and after precipitation of the DNA by sodium acetate and isopropanol. This was followed by a second phenol extraction and precipitation with isopropanol. All subsequent stages described by Kieser were omitted.

Table 1. *Bacterial strains*

Escherichia coli	
490A *met leu thi rpsL recA sup*	G. Hobom
C600 *thr leu thi lacY tonA supE*	—
No.76 Hfr *thr thi ilv relA spc recA sup*$^-$	—
JM83 *ara* Δ (*lac-proAB*) *rpsL* ϕ*80 lacZ* Δ*M15 sup*$^-$	J. Messing
Salmonella typhimurium	
DB21 prototrophic *sup*$^-$	—
su70 $cys_{am}sup^+$	—
Streptomyces lividans	
TK24 prototrophic, *str*^{r}, naturally *cam*^{r}	—
M252 prototrophic, *cam*^{s}	Bibb & Cohen (1982)

(ii) *Transformation*

E. coli and *Salmonella* strains were made competent and transformed by the method of Lederberg & Cohen (1974). For *Streptomyces* transformation, mycelia were converted to protoplasts as described in Hopwood *et al.* (1985) except that treatment with lysozyme was at 37 °C. Transformation was performed according to Thompson *et al.* (1982) or by the 'Rapid small scale procedure' (Hopwood *et al.* 1985). Transformation mixtures were plated on R5 medium and incubated for about 24 h at 30 °C to permit regeneration and expression. Transformants were selected for thiostrepton resistance by overlaying the plates with 2 ml SNA + 500 μg/ml thiostrepton (gift from the Squibb Institute for Medical Research) and incubation was continued for a further 3 days to obtain sporulated transformed colonies.

(iii) *Plasmid curing*

Spores of *Streptomyces lividans* were grown in TSB by shaking at 30 °C for about 8 h. Acridine orange was added to a final concentration of 20 μg/ml, and incubation was continued for 30–35 h. This treatment usually resulted in 70–90% of the surviving cells losing the plasmid (detected by replica plating the colonies of the survivors onto thiostrepton medium).

Enzymes: Restriction endonucleases and T4-ligase were purchased from Boehringer and Gibco BRL and were used according to the recommendations of the manufacturers.

3. Results

(i) *Isolation of an amber-mutant in the CAT gene of pACYC184*

The CAT gene of plasmid pACYC184, which is derived from transposon Tn9, confers resistance to chloramphenicol and is expressed in both *E. coli* and *Streptomyces lividans* under the control of the same (*E. coli*) promoter (Bibb & Cohen, 1982). An amber mutation was induced within this gene which could be suppressed in well characterized suppressor strains of Enterobacteriaceae. To produce this mutation *Salmonella typhimurium* strain DB21 carrying pACYC-184 was grown overnight in LB with 40 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, appropriate dilutions of the surviving cells were plated on LB-agar + 20 μg/ml tetracycline and the resulting colonies were replica plated onto LB-agar with 100 μg/ml chloramphenicol. From 8000 tetracycline-resistant colonies two appeared to be chloramphenicol sensitive. Their plasmids were isolated, transferred into the *Salmonella* nonsense suppressor strain su70, and transformants were selected for tetracycline resistance. By replica plating these transformants could be shown to be also chloramphenicol-resistant. To reconfirm that the CAT gene of both plasmids carried nonsense mutations, the plasmids were reisolated and transformed into *E. coli* strains 76 (*sup*$^-$) and 490A and C600, both of which are *sup*$^+$ strains. Transformants were selected on tetracycline and also on chloramphenicol medium. Chloramphenicol resistant transformants were obtained only from strains 490A and C600, but not from *sup*$^-$ strain 76. Tetracycline resistant transformants could be selected from all recipient strains, but, as shown by replica plating, only the transformants of 490A and C600 were also resistant to chloramphenicol. Since C600 carries the suppressor *supE* which suppresses only amber mutations, but none of the other nonsense mutations, it was concluded that both mutants from pACYC184 carried an amber mutation (TAG) in the CAT gene. One of them was named pGM1107 and used for further experiments.

(ii) *Construction of a shuttle vector*

In order to transfer pGM1107 into *Streptomyces lividans*, this plasmid was fused with the *Streptomyces* vector pIJ702. pGM1107 was linearized by cutting the unique *Bam*HI site in the Tc gene and pIJ702 was opened at the unique *Bgl*II site which is located in the tyrosinase gene controlling synthesis of melanin. After ligation the plasmids were introduced into *E. coli* strain 490A, and chloramphenicol resistant transformants were selected. By digestion with *Eco*RI it was shown that plasmids from such transformants

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Table 2. *Chloramphenicol-resistant revertants of M252 carrying pGM1109*

Selective concentration	Revertants assayed	Chromosomal revertants	Plasmid revertants	Suppressor mutants
10 μg/ml	24	24	0	0
7 μg/ml	6	6	0	0
5 μg/ml	38	15	23	0
Total	68	45	23	0

had a length of 9·8 kb which corresponds to the sum of both components, pIJ702 and pGM1107. One of these plasmids, pGM1109, was used to transform the chloramphenicol sensitive mutant strain M252 of *Streptomyces lividans* to thiostrepton resistance, and white (*mel*$^-$) colonies were selected. To demonstrate that the relevant marker (CAT_{am}) was still present in pGM1109, *E. coli* strains JM83 (*sup*$^-$) and 490A (*sup*$^+$) were transformed with the bivalent plasmid extracted from M252. Cam^r transformants could be obtained only from strain 490A. Since the Tc gene was inactivated by the fusion, a positive control by selecting tetracycline-resistant transformants was not possible. However, the quality of competent cells was proven by parallel transformation with pGM1107, pACYC184 and with pCRI (a plasmid isogenic to the fused plasmid pGM1109, with the exception that it carries the intact CAT gene).

(iii) *Selection for* cam^r *revertants*

From M252 carrying pGM1109 we selected spontaneous revertants at a frequency of about 10^{-7} which were resistant to 10 μg/ml chloramphenicol. These could be either (1) backmutants in the nonsense codon of the plasmid borne Cm gene; or (2) chromosomal revertants of M252 to its original natural resistance; or (3) nonsense suppressor mutations producing a tRNA species able to read the amber codon. To distinguish between these different kinds of cam^r revertants, plasmids were recovered and used to transform *E. coli* strains JM83 and 490A and *Streptomyces* strains M252. In case (1) *sup*$^-$ strains JM83 and M252 should yield chloramphenicol resistant transformants. If only the *sup*$^+$ strain 490A yielded such transformants, the reversion could be a chromosomal backmutation of M252 (case 2) or caused by a nonsense suppressor mutation (case 3). Therefore, chloramphenicol resistant revertants were cured of the plasmid pGM1109 by treatment with acridine orange and assayed for sensitivity or resistance ot thiostrepton and chloramphenicol. Table 2 shows that only chromosomal revertants could be detected. The selective stress was therefore lowered to 7 and 5 μg/ml chloramphenicol resp. This allowed chromosomal as well as plasmid revertants to be detected (Table 2). Lowering the drug concentration to 3 μg/ml caused a significant background growth of sensitive M252 so that weakly resistant colonies could not be distinguished.

(iv) *Selection for highly reverting auxotrophs*

Since these attempts to select suppressor mutants failed, we tried a method which was used successfully for selecting the *Salmonella* suppressor strain su70 (not published): Auxotrophic mutants which were not further analysed for their growth requirements were induced by hydroxylamine (Tessman, 1968) and those which reverted easily to prototrophy were isolated. A series of such revertants were transformed with pGM1109 to thiostrepton resistance and assayed for chloramphenicol resistance by replica plating; however, no amber suppressor mutant could be detected.

4. Discussion

We have attempted to isolate spontaneous mutants of *S. lividans* which are able to suppress an amber defect in the CAT gene of the *E. coli* plasmid pACYC184. Three events could lead to chloramphenicol resistant cells: (1) restoration of the natural resistance of *S. lividans* which has been lost in strain M252; (2) backmutation of the amber defect of the plasmid gene, and (3) suppression due to a change in the anticodon of a particular tRNA. Only classes (1) and (2) could be found; their frequencies depended significantly on the concentration of chloramphenicol used for selection. At higher levels only chromosomal revertants could be found. This may indicate that the expression of the *E. coli* gene under its original promoter even in a multicopy plasmid does not confer levels of resistance as high as the chromosomal gene of *S. lividans*. True suppressor mutants were not observed, which confirms the recent report by Paradiso *et al.* (1987). The nucleotide sequences of 12 genes of 10 different *Streptomyces* species (Table 3) show that all three nonsense codons are used to terminate translation. Therefore, tRNAs able to read these nonsense codons cannot exist, and an earlier suggestion that in streptomycetes a natural suppressor would exist, cannot be valid (Lomovskaya *et al.* 1980).

Streptomycetes have an unusually high G+C content (Enquist & Bradley, 1971). We determined an

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Table 3. *Genes the nucleotide sequences of which have been evaluated in the text*

Gene	Source	No. of codons	Termination codon	Reference
aph	*S. fradiae*	269	UAG	Thompson & Gray (1983)
endoH	*S. plicatus*	314	UAG	Robbins *et al.* (1984)
ermE	*S. erythraeus*	371	UAG	Uchiyama & Weisblum (1985)
vph	*S. vinaceus*	287	UAG	Bibb *et al.* (1985)
Streptavidin	*S. avidinii*	184	UAG	Agarana *et al.* (1986)
amy	*S. hygroscopicus*	479	UAG	Hoshiko *et al.* (1987)
tsr	*S. glaucescens*	275	UGA	Huber *et al.* (1985)
mel	*S. antibioticus*	272	UGA	Bernan *et al.* (1985)
ORF 438	*S antibioticus*	146	UGA	Bernan *et al.* (1985)
afsB	*S. coelicolor*	244	UGA	Horinouchi *et al.* (1986)
hygB	*S. hygroscopicus*	333	UGA	Zalacain *et al.* (1986)
tsr	*S. azureus*	269	UAA	Bibb *et al.* (1985)

average ratio of 70·8 % for the 12 different genes given in Table 3 (i.e. 10164 bp). In Table 4 we have listed the usage of those codons whose corresponding tRNAs could be converted to an amber suppressor by a single base exchange in their anticodons. But then the original codon would no longer be recognized, unless it could be read by an isoaccepting tRNA molecule according to the wobble hypothesis (Crick, 1966). For most of the amino acids given in Table 4 there exist only the two synonymous codons listed. Only for Ser and Leu are six different codons available. Codons corresponding to wobble tRNAs ('wobble codons') are indicated in the table. The unique codon for Trp of course cannot be compensated. We see that codons read by wobble tRNAs are more frequent in streptomycetes than wobble codons. For *E. coli* and its phages a significant correlation between codon usage and the abundance of the corresponding tRNA has been shown (Ikemura, 1981). If this holds true also for streptomycetes, a mutation in the anticodon of the major tRNA to give a suppressor tRNA would require very efficient compensation. This is basically possible by the presumably minor tRNAs which would normally read the respective codon by wobble (Table 4). (An exception is the codon pair for Tyr where the major tRNA is able to read also the minor codon; but the minor tRNA may possibly not exist). Because of the low concentrations of minor tRNAs and since wobble will never give an optimal codon–anticodon association, mutation of a major tRNA to a suppressor tRNA would impose severe disadvantages on the cell. Additionally, suppression of nonsense codons can never restore the function of a mutated gene to 100% (in *E. coli* between 5% and 55% of the wild-type level was measured; Garen, 1968). So it is not surprising that suppressor mutants in streptomycetes appear extremely rare. In *E. coli*, however, where suppressor mutants can easily be obtained, the codon usages are mostly more balanced (Table 4; Maruyama *et al.* 1986), and for Lys and Glu the ratios are even reversed. The situation in *Streptomyces*, however, would immediately change if two copies of one of the potential suppressor tRNA genes were present in the cell (as for instance *supF* suppressor in *E. coli*). However, nothing is known about the frequencies of tRNA genes in this genus, and the unsuccessful search for suppressor mutations suggests

Table 4. *Usage for codons with cognate tRNAs able to mutate to amber suppressor tRNAs*

Amino acid	Codon	Number	Percentage in streptomycetes†	Percentage in *E. coli*‡
Leu	T*T*A*	1	0·5	8·5
	T*T*G	14	6·6	10·1
Ser	T*C*A*	6	3	8·9
	T*C*G	62	30·5	12·9
Gln	*C*AA*	10	11·2	28·0
	*C*AG	86	89·6	72·0
Lys	*A*AA*	1	1·3	76·0
	*A*AG	74	98·7	24·0
Glu	*G*AA*	35	17·6	71·4
	*G*AG	164	82·4	28·6
Tyr	TA*T*	3	3·4	47·7
	TA*C**	85	96·6	52·3
Trp	T*G*G	71	100	100

Nucleotide positions are underlined whose change in the cognate tRNA would lead to a nonsense suppressor

* The tRNAs corresponding to these triplets are able to read the synonymous triplet given in the Table due to an U (in case of Tyr G) at the wobble position

† Values from 12 different genes accounting for 3388 codons (Table 2)

‡ Values from 199 different genes accounting for 71,736 codons

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that they are usually unique in the genome of *Streptomyces lividans*. Furthermore, in streptomycetes UAG is used much more frequently as termination codon (Table 3) than in *E. coli*. Therefore, overreading of such signals in suppressor strains possibly would damage too many proteins, if suppression occurs efficiently. In *E. coli* the most common stop codon seems to be UAA (ochre); but ochre suppressors have a low efficiency (6–16%). Possible 5′-inosine bearing tRNAs which would accept C, U and A at the 3′-position of a codon, cannot account for the suppression of amber codons.

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