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Bacillus subtilis Mutant Allele *sup-3* Causes Lysine Insertion at Ochre Codons: Use of *sup-3* in Studies of Translational Attenuation

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The mutation *sup-3* in *Bacillus subtilis* suppresses ochre (TAA) mutations at each of three codons in the 5′ end of the *cat-86* coding sequence. The suppressor is shown to insert lysine at ochre codons. The efficiency of suppression by *sup-3* is about 15%, as determined by changing a *cat-86* Lys codon (codon 12) to an ochre codon and measuring the level of CAT in the suppressor-containing strain. The results obtained are discussed in light of previous observations that ochre mutations at *cat* leader codons 2 and 3 can be phenotypically suppressed by *sup-3*, whereas ochre mutations at leader codons 4 and 5 cannot. Translation of the *cat* leader is essential to inducible expression of *cat*. Our data support the interpretation that the nature of amino acids 2 through 5 of the leader peptide contributes to determining whether chloramphenicol can stall a ribosome in the leader, which in turn leads to induction of *cat* expression.

Inducible resistance to chloramphenicol and erythromycin in gram-positive bacteria is due to a regulatory mechanism termed translational attenuation (1, 5, 9, 22). In this form of gene control, the ribosomally targeted antibiotic inducer stalls a ribosome in a regulatory leader sequence. This stalled ribosome causes the destabilization of a downstream region of RNA secondary structure that normally sequesters the ribosome-binding site for the coding sequence of the drug resistance gene. Thus, induction of *cat* genes by chloramphenicol or *erm* genes by erythromycin activates the translation of the corresponding mRNA (see, e.g., reference 8).

Ribosome stalling that leads to *cat* induction requires that the aminoacyl site of the stalled ribosome be placed at leader codon 6 (1, 4, 7). Chloramphenicol causes a ribosome to stall at this leader site because *cat* leaders contain a chloramphenicol stall sequence (E. J. Rogers, U. J. Kim, and P. S. Lovett, submitted for publication). This sequence of four codons (codons 2 through 5), or the corresponding sequence of four amino acids in the leader peptide, apparently promotes the stalling of a chloramphenicol-sensitized ribosome (Rogers et al., submitted).

Replacement of *cat* leader codons 2 through 5 with ochre codons blocks chloramphenicol induction (1, 4). Such mutations prevent a ribosome from access to both the stall sequence and leader codon 6. The loss of inducibility resulting from replacement of leader codons 2 and 3 with the ochre codon can be partially restored if the mutant genes are placed in strains of *Bacillus subtilis* containing the nonsense suppressor *sup-3* (4; U. J. Kim and P. S. Lovett, unpublished results). Unexpectedly, ochre mutations at leader codons 4 and 5 could not be phenotypically suppressed by *sup-3* (4; Kim and Lovett, unpublished). Since the amino acid sequence of the leader may be a crucial factor in drug-mediated ribosome stalling, we were interested in determining the nature of the amino acid that is inserted at ochre codons by *sup-3*. In the present study we have found that the suppressor mutation causes lysine to be inserted at ochre codons and have determined the efficiency of suppression.

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MATERIALS AND METHODS

Bacteria and plasmids. *B. subtilis* BR151 (*trpC2 metB lys-3*) and PY22 (*trpC2*) are nonsuppressing, wild-type cells. CB313 (*sup-3*), obtained from Charles Stewart (Rice University, Houston, Tex.), was used to suppress ochre mutations made in the *cat-86* coding sequence. The version of *cat-86* used throughout this study is the constitutively expressed gene in pPL703-C2 (14). This gene was under the control of the P4 promoter (19). Growth conditions and all plasmid manipulations were as previously described (7).

Assay for CAT. Chloramphenicol acetyltransferase (CAT) was assayed at 25°C by the colorimetric method of Shaw (20). Protein was determined by the Bradford procedure (2). CAT specific activity is reported as the number of micromoles of chloramphenicol acetylated per minute per milligram of protein at 25°C.

Site-directed mutagenesis. Conversion of *cat-86* codons 7, 9, and 12 to ochre codons (TAA) was performed by oligonucleotide-directed mutagenesis as previously described (7, 21, 23).

CAT purification and amino acid sequencing. CAT was purified as previously described (14). Amino acid sequencing was performed by automated Edman degradation.

RESULTS

***sup-3* causes lysine to be inserted at ochre codons.** The N-terminal 13 amino acids of *cat-86*-specified CAT were determined from the sequence of the gene and by Edman degradation of the corresponding protein (Fig. 1) (13, 16). Codon 7 specifies Glu, and codon 9 specifies Tyr. Both of these codons were separately changed to the ochre codon, TAA, generating two mutants, which we have designated M7-TAA and M9-TAA. Neither mutant specified detectable levels of CAT in nonsuppressor strains of *B. subtilis*, whereas both specified measurable CAT levels in a suppressor strain carrying the *sup-3* mutant allele (Table 1). CAT protein specified by M7-TAA and M9-TAA in the *sup-3*-containing host cells was purified and subjected to automated Edman degradation. The protein specified by M7-TAA in the suppressor mutant strain contained Lys as amino acid 7, rather than the wild-type amino acid Glu. Each of the other N-terminal amino acids though amino acid 10 was the

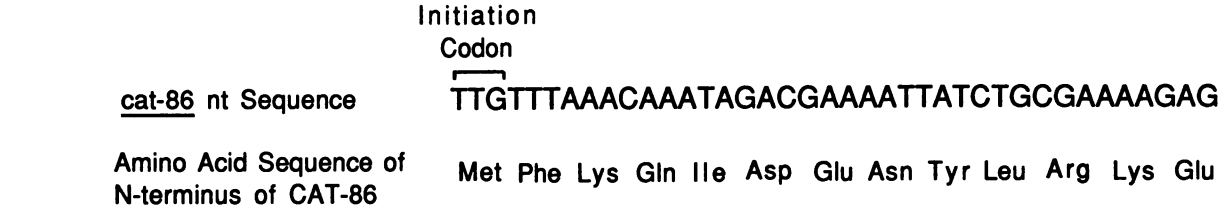

FIG. 1. Sequence of the first 13 codons of the *cat-86* coding region and the corresponding amino acid sequence. The DNA sequence was reported by Harwood et al. (13), and the amino acid sequence was determined by direct sequencing of the CAT protein (16). The total coding sequence of *cat-86* consists of 220 codons (13).

same as specified by the wild-type gene. Comparable results were obtained with the suppressed protein encoded by M9-TAA, except that Lys was present as amino acid 9, replacing the Tyr found at that position in the wild-type protein. All of the remaining N-terminal amino acids through amino acid 10 were the same as specified by the wild-type *cat-86* gene. We conclude that *sup-3* causes insertion of Lys at ochre codons.

Efficiency of suppression. The observed efficiency of suppression by *sup* alleles as measured by in vivo assays is likely to be a function of primarily two factors: the efficiency of insertion of the suppressing amino acid at a particular nonsense codon, and the efficiency with which the resulting protein performs a catalytic function. To more accurately determine the efficiency of suppression by *sup-3*, we changed a Lys codon in *cat-86* (codon 12) to an ochre codon and then measured the return to function of CAT activity in the suppressor-containing mutant. The return to function of CAT would then be a measure of the insertion efficiency of Lys by the *sup* allele. Our results indicate that the efficiency of suppression by *sup-3* was 15% by this method.

DISCUSSION

Previous studies indicate that the ability of chloramphenicol to stall a ribosome in the *cat-86* leader depends on the nature of leader codons 2 through 5 (Rogers et al., submitted). These codons, and/or the corresponding amino acids of the leader peptide, act to stall a chloramphenicol-sensitized ribosome such that the aminoacyl site of the ribosome occupies leader codon 6. Replacement of leader codon 3, 4, or 5 with the ochre codon (TAA) prevents chloramphenicol from inducing the gene (1). It has been observed that the loss of inducibility due to replacement of *cat-86* leader codon 3 with the ochre codon could be partially restored if the mutant gene was inserted into *B. subtilis* strains containing the *sup-3* mutation (Kim and Lovett, unpublished). The level of restoration of inducibility was approximately 8% of the inducibility of the wild-type gene. The loss of inducibility due to ochre mutations at leader codon 4 or 5, however, could not be suppressed under the same conditions.

cat-86 leader codon 3 specifies Lys (1). Hence, suppression of the ochre mutation at codon 3 by *sup-3* caused insertion of the amino acid that is normally found at this position in the wild-type leader. By contrast, leader codons 4 and 5 encode Thr and Asp, respectively (1). The inability of the suppressor mutation to phenotypically suppress ochre mutations at codons 4 and 5 is probably due, at least in part, to a reduction in function of the stall sequence when a Lys residue is inserted at position 4 or 5 of the leader.

Dick and Matzura (4) have demonstrated that replacement of leader codon 2 (Lys) of the pUB112 *cat* gene by an ochre codon blocks induction and that this loss of inducibility can be partially suppressed by *sup-3*. However, the loss of inducibility due to ochre mutations at leader codons 4 and 5 (Ser and Glu, respectively) could not be suppressed by *sup-3*. Collectively, the results with both *cat-86* and the pUB112 *cat* gene support the notion that the sequence of the *cat* leaders from amino acids 2 through 5 is essential for the successful stalling of a ribosome by chloramphenicol.

sup-3 is one of several nonsense suppressor mutations identified in *B. subtilis* (3, 6, 12, 14, 15, 17, 18). *sup-3* was originally isolated in a study by Georgopoulos (12), and it has been shown to cause insertion of an amino acid that is more basic than that inserted as a result of another nonsense suppressor, *sup-1* (10, 11). Our data directly demonstrate that *sup-3* results in the insertion of Lys residues at ochre codons. *sup-3* is therefore the first nonsense suppressor mutation in *B. subtilis* to be correlated with the insertion of a specific amino acid.

TABLE 1. Level of expression of *cat-86* and the mutant derivatives M7-TAA and M9-TAA in wild-type and suppressor hosts

Gene	CAT sp act in the following host[a]:	
	Wild type	*sup-3*
cat-86	15.05	13.6
M7-TAA	<0.01	0.20
M9-TAA	<0.01	0.21

[a] The wild-type host was PY22; the *sup-3* host was CB313. CAT specific activity is reported as micromoles of chloramphenicol acetylated per minute per milligram of protein.

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