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Inducible Amber Suppressor for *Bacillus subtilis*

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An amber suppressor variant of *Bacillus subtilis* tyrosyl-tRNA was constructed and placed under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter. Addition of IPTG resulted in a 50-fold increase in the expression of an *rpsD-lacZ* fusion containing a UAG amber codon. This system permitted isolation of a conditional lethal mutant which required IPTG for growth.

Conditional mutants such as temperature-sensitive and nonsense mutants are extremely useful in the analysis of essential genes. Isolation of nonsense mutants requires nonsense suppressor strains, which are widely available in *Escherichia coli* (1). For *Bacillus subtilis*, few nonsense mutants have been identified because of the lack of convenient, well-characterized nonsense suppressors. A few suppressor mutants have been described (3, 12). The *sup-3* and *sup-44* alleles were both mapped to a position near 36° on the 360° *B. subtilis* map (7). The *sup-3* allele was shown to be an ochre (UAA) suppressor variant of a lysyl-tRNA (2, 9, 11), while *sup-44* is an ochre suppressor variant of a leucyl-tRNA (9); ochre suppressors suppress both ochre (UAA) and amber (UAG) mutations because of wobble. No specific amber (UAG) or opal (UGA) suppressor mutants have been reported, although natural suppression of UGA occurs at a low frequency (9). In our analysis of the *tyrS* gene, which is regulated by transcription antitermination directed by uncharged tyrosyl-tRNA ($tRNA^{Tyr}$)(6), we required a variant of $tRNA^{Tyr}$ capable of interaction with UAG amber codons. We therefore constructed the *B. subtilis* equivalent of the *E. coli supF* allele (4) and placed the tRNA gene under control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{spac} promoter on a multicopy plasmid. This construct permits IPTG-inducible amber suppression in *B. subtilis* and should be useful in a number of gram-positive systems for the isolation and characterization of new classes of conditional mutants.

Construction of an amber suppressor variant of $tRNA^{Tyr}$. The gene for $tRNA^{Tyr}$ is located in the *trnD* cluster (15). This region was isolated by PCR and inserted into bacteriophage M13. The $tRNA^{Tyr}$ gene was subjected to oligonucleotide-directed mutagenesis to generate a variant with an altered anticodon, and PCR was used to generate a DNA fragment containing the mutated tRNA gene, with a *Hin*dIII site upstream and a *Sal*I site downstream of the gene (Fig. 1). The 170-bp tRNA fragment was then inserted into plasmid pDG148 (14), digested with *Hin*dIII and *Sal*I, so that its expression would now be under the control of the IPTG-inducible P_{spac} promoter; this plasmid also contains the *E. coli lacI* gene under control of the *penP* promoter and ribosome binding site (Fig. 1), giving constitutive synthesis of LacI protein. The resulting plasmid was designated pFG-SUP; an identical construct in which the wild-type $tRNA^{Tyr}$ was placed under P_{spac} control was designated pFG-WT.

Construction of *rpsD*(Am)-*lacZ*. Since no well-characterized amber mutations were available for *B. subtilis*, an amber mutant variant of an *rpsD-lacZ* translational fusion (5), which is highly expressed in *B. subtilis*, was constructed. A 0.9-kb *Hin*dIII fragment containing the promoter and amino-terminal coding region of *rpsD* was generated by PCR with a mutagenic oligonucleotide which changed amino acid 54 from lysine (AAG) to a UAG amber codon in the amplified product. This fragment was inserted into a *lacZ* fusion vector, as previously described (5), and the resulting plasmid conferred β-galactosidase production in *E. coli* strain DH5α (*supE44*) but not in strain MC1000 (suppressor minus). The *rpsD*(Am)-*lacZ* fusion was crossed onto specialized transducing phage SPβ and was introduced into the *B. subtilis* chromosome.

IPTG-inducible amber suppression. Plasmid pFG-SUP or pFG-WT was introduced into strains carrying either the wild-type or amber mutant *rpsD-lacZ* fusions, and β-galactosidase production was monitored during growth in the presence or absence of IPTG (Table 1). Addition of IPTG (0.2 mM) resulted in a 50-fold increase in β-galactosidase production from the amber mutant fusion in cells containing the suppressor plasmid but had no effect on expression of the amber mutant fusion in cells expressing the wild-type tRNA. Background expression of the amber mutant fusion in cells containing the suppressor plasmid in the absence of IPTG was very low, indicating that the P_{spac} system is tightly controlled. Expression of the wild-type fusion in cells containing the amber suppressor plasmid in the presence of IPTG was slightly reduced, indicating that expression of the suppressor tRNA has a modest inhibitory effect. If the lysine-to-tyrosine substitution upstream of the β-galactosidase coding region did not affect the function or stability of the fusion protein, then the suppression level under these conditions was approximately 18%, within the range for efficient nonsense suppressors in *E. coli* (8).

The kinetics of suppression of the *rpsD*(Am)-*lacZ* fusion with different concentrations of IPTG was monitored in an attempt to maximize suppression (Fig. 2). Addition of 0.04 mM IPTG resulted in 18-fold induction after 2 h, and 0.2 mM IPTG resulted in 56-fold induction, whereas a further 5-fold increase in IPTG level to 1 mM had only a small additional stimulatory effect and resulted in a slight inhibition of growth. Expression of the fusion was maximal after 2 h of incubation; the drop in β-galactosidase activity after 3 h could be due to reduced expression of *rpsD* as the cells entered stationary phase.

Isolation of conditional amber mutants. The utility of the amber suppression system for identification of amber muta-

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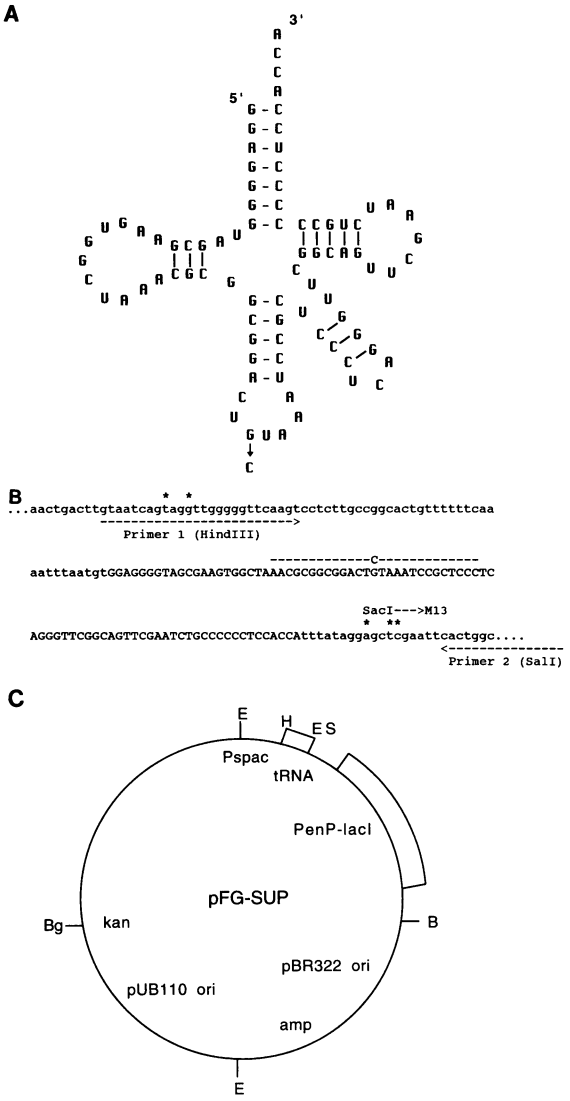

FIG. 1. Amber suppressor variant of *B. subtilis* $tRNA^{Tyr}$. (A) Structure of *B. subtilis* $tRNA^{Tyr}$. Arrow indicates the G-to-C substitution in the anticodon. (B) Construction of amber suppressor tRNA. The sequence of the $tRNA^{Tyr}$ gene in the *trnD* cluster is shown in uppercase. A 1.05-kb region of *trnD* was initially isolated by PCR with primers hybridizing upstream and downstream of the $tRNA^{Tyr}$ gene; the primers contained sites for cleavage by *Bam*HI and *Sac*I restriction enzymes, permitting insertion into M13 mp19. Oligonucleotide-directed mutagenesis was used to alter the G in the anticodon to a C; the position of the mutagenic oligonucleotide is shown above the tRNA gene sequence. The mutated gene was isolated by PCR with oligonucleotides hybridizing upstream (primer 1) and downstream (primer 2); the positions of the PCR primers are shown below the gene sequence. Primers 1 and 2 contained *Hin*dIII and *Sal*I sites, respectively, so that the PCR product could be digested with these enzymes, and the resulting 170-bp DNA fragment was inserted into plasmid pDG148 digested with *Hin*dIII and *Sal*I. Asterisks indicate positions where the *trnD* sequence was altered to introduce restriction sites. (C) Structure of plasmid pFG-SUP, in which the amber suppressor variant of $tRNA^{Tyr}$ is under control of P_{spac}. Restriction enzyme cleavage sites are as follows: E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; and S, *Sal*I. Plasmid pFG-WT is identical to pFG-SUP except that it contains the wild-type $tRNA^{Tyr}$ gene. kan, kanamycin; amp, ampicillin; ori, origin of replication.

TABLE 1. IPTG-inducible suppression of *rpsD*(Am)-*lacZ* expression

Fusion	Plasmid	IPTG (0.2 mM)[a]	β-Galactosidase activity (Miller units)[b]	Induction (+IPTG/−IPTG)
rpsD(Am)-*lacZ*	pFG-WT	−	1.0	
		+	0.88	0.88
	pFG-SUP	−	4.5	
		+	220	49
rpsD-lacZ	pFG-WT	−	1,700	
		+	1,700	1.0
	pFG-SUP	−	1,500	
		+	1,200	0.80

[a] Cell cultures were grown in Luria-Bertani medium to early logarithmic growth phase, split, grown in the presence (+) or absence (−) of IPTG (0.2 mM) for 2 h, and then harvested and assayed for β-galactosidase activity.
[b] β-Galactosidase activity was determined as described by Miller (10).

tions in genes essential for growth or sporulation was tested by random mutagenesis of cells containing the suppressor plasmid and screening for isolates which were dependent on IPTG for growth or sporulation. Exponentially growing cells were treated with ethyl methanesulfonate (1%) for 2.5 h, washed, allowed to grow for several hours, and then plated on media containing neomycin (5 μg/ml) and IPTG (0.2 mM). After overnight growth, colonies were patched onto nutrient sporulation plates (13) with or without IPTG. Of 1,000 colonies tested, one isolate, designated AMB2, was able to grow only on media containing IPTG, while a second isolate, designated AMB1, grew slowly on both media but sporulated only in the presence of IPTG. When the AMB2 strain was transferred from tryptose blood agar base plates containing 0.2 mM IPTG to Luria-Bertani broth without IPTG, growth was normal for approximately three generations and then slowed (Fig. 3); the initial growth was presumably due to residual IPTG, suppressor tRNA, and full-length gene product present in the cells, all

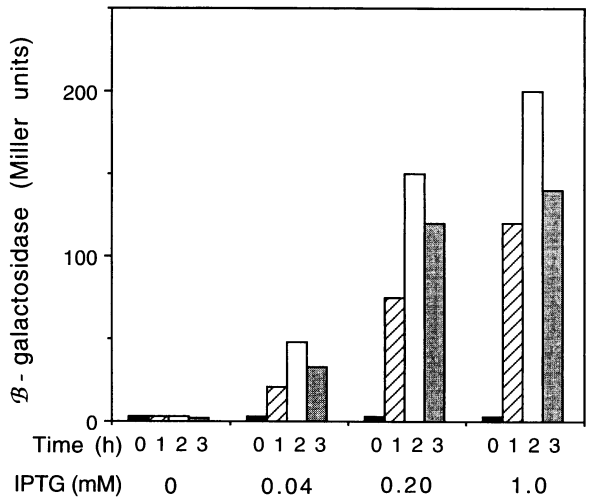

FIG. 2. IPTG induction of *rpsD*(Am)-*lacZ* expression. A strain containing the *rpsD*(Am)-*lacZ* fusion carried on specialized transducing phage SPβ and plasmid pFG-SUP was grown in Luria-Bertani medium (10) to early exponential growth phase. The culture was split, IPTG (0, 0.04, 0.2, or 1.0 mM) was added, and samples were harvested at 1-h intervals. β-Galactosidase activity was determined as described by Miller (10).

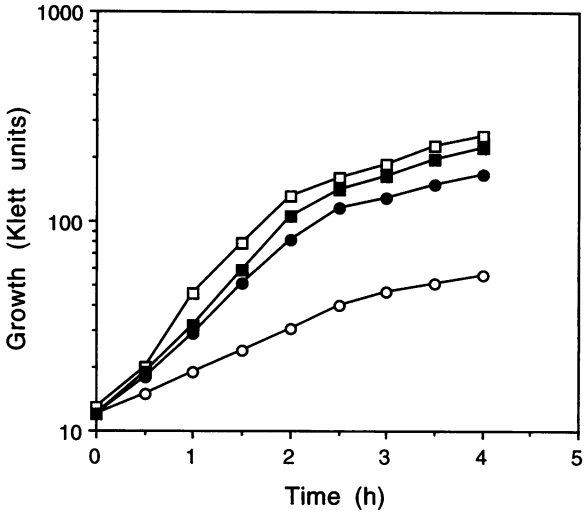

FIG. 3. IPTG-dependent growth of the AMB2 mutant. Wild-type (□, ■) and AMB2 mutant (○, ●) cells containing plasmid pFG-SUP were grown on tryptose blood agar base (Difco) plates containing 0.2 mM IPTG and were inoculated into Luria-Bertani medium with (■, ●) or without (□, ○) 0.2 mM IPTG. Cells were grown for three doublings and then were diluted into fresh medium, and growth was monitored with a Klett-Summerson colorimeter.

of which were eventually diluted by growth and cell division. These results demonstrate that the suppressor system can be used to isolate conditional amber mutants. The time course of the effect of IPTG removal is likely to be variable for different amber mutants, depending on the levels of the product required.

This is the first report of an inducible nonsense suppression system for *B. subtilis*. This system permits a simple on/off switch for expression of a target gene containing a UAG amber mutation by addition or removal of IPTG. The suppressor tRNA is carried on a plasmid containing a pUB110 replicon and should be useful in any system in which this type of plasmid can be propagated and the P_{spac} promoter is active.

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