JOURNAL OF BACTERIOLOGY, July 1968, p. 215-220

Vol. 96, No. 1
Printed in U.S.A.

Procedure for Identifying Nonsense Mutations

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Received for publication 9 April 1968

A method has been devised for the rapid identification of nonsense mutations (UAG, UAA, UGA codons) in *Salmonella*. The mutations to be tested are reverted, and the revertants are replica-printed onto lactose plates spread with lawns of tester strains. These tester strains contain F′ *lac* episomes with nonsense mutations in the episomal *Z* gene. The revertants are infected with the episome from the tester strain lawn. Because *S. typhimurium* is unable to ferment lactose, only those revertants which have nonsense suppressors are able to grow on lactose. If colonies appear on the lactose plate, it may be concluded that the original strain carries a nonsense mutation, since nonsense suppressors suppress the mutant phenotype.

We have designed a method for screening mutants of *Salmonella typhimurium* to determine which are of the nonsense type (4–6, 18, 21). The method is based on the expectation that nonsense suppressor-carrying strains are found frequently among the revertants of nonsense mutants, but are not found among the revertants of missense or frameshift mutants (19). The test has been designed so that nonsense suppressor-carrying strains can be detected by directly printing revertants of the mutant to be tested onto lawns of tester strains. This is simpler than tests using nonsense mutants of a phage (10, 14), since it is not necessary to pick and grow individual revertants.

The tester strains each carry an F′*lac* episome with a nonsense mutation in the β-galactosidase (*lacZ*) gene. Revertants of a mutation are replica-plated onto a lawn of a tester strain on minimal medium containing lactose as a sole carbon source. Bacteria in each revertant clone receive the F′*lac* episome, but, since *Salmonella* is unable to ferment lactose (*Bergey's Manual of Determinative Bacteriology*), only those revertants which have nonsense suppressors able to suppress both the original mutation and the nonsense mutation on the introduced F′*lac* episome are able to grow on the lactose plates. When a large proportion of the revertants of a given mutant strain are found by this test to grow on lactose plates spread with a tester strain, it can be concluded that the strain is a nonsense mutant.

This paper describes the use of five tester strains for the identification of nonsense mutations. Mutations in the *hisC* gene and the *hisG* gene have been classified by this method.

MATERIALS AND METHODS

Media. The minimal medium used was medium E of Vogel and Bonner (17). Glucose (1%) was used as a carbon source unless otherwise indicated. For solid media, agar (Difco) was added at a final concentration of 1.5%. Nutrient Broth or Nutrient Agar (Difco) was used as maximal medium. MacConkey agar was used as a fermentation indicator medium.

For lactose plates, minimal medium was modified to eliminate the citrate (the ratio of monobasic to dibasic phosphate being changed to bring the *p*H back to neutrality), and lactose (1%) was used as the carbon source. In 200 ml of 50× medium, the following are contained: KH_2PO_4, 39.4 g; K_2HPO_4 (anhydrous), 49.7 g; and $NaH(NH_4)PO_4 \cdot 4H_2O$, 35.0 g. The sterile 50× salts, a 1,000× solution of sterile $MgSO_4 \cdot 7H_2O$ (2 g/10 ml) and the filter-sterilized lactose (Difco) were added to previously autoclaved 1.5% agar before the plates were poured.

Chemicals. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Diethylsulfate was obtained from Fisher Scientific Co., Pittsburgh, Pa. The acridine half

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mustard, ICR-191, was a gift from Hugh H. Creech (7). Lactose was purchased from Difco Laboratories.

Bacterial strains. The *S. typhimurium hisC* and *hisG* gene mutants were supplied by P. E. Hartman. The *hisC* mutants were previously classified as amber, ochre, missense, or frameshift mutants (19). We are grateful to W. A. Newton for *Escherichia coli* strains X82,U281, and YA596, and to D. Zipser for *E. coli* strains NG219 and NG813. The genotypes and sources of all strains used are given in Table 1.

Phage strains. Stocks of phage P22 were obtained from P. E. Hartman. T4 phage was obtained from M. Gellert and P. Ross. SP6 phage was obtained from N. Zinder (20).

Construction of tester strains. The mutant F'*lac* episomes were transferred from *E. coli* to *S. typhimurium* strains carrying suppressors which could suppress the mutation in the introduced F'*lac* episome, thus enabling the *Salmonella* recipient to grow on lactose. Donors and recipients were mixed on lactose agar, and lactose-positive colonies were selected.

The amber tester strains were then constructed by the nonselective transfer of the X82 and U281 episomes from the *Salmonella* suppressor strains into the *Salmonella* histidine deletion *hisFAHBCDG644*.

The ochre tester strain was constructed by introducing amino acid requirements into the *Salmonella* suppressor strain carrying episome YA596.

UGA tester strains were constructed by the transfer of episomes NG219 and NG813 from *E. coli* to the *Salmonella* histidine deletion mutant *hisCD129* carrying a UGA suppressor. A detailed description of the construction of each of the tester strains is given below.

(i) SB391 (containing F'lac with UAG mutation). The episome from strain X82 was transferred to *Salmonella* strain TA131 which carries an amber suppressor. The F'-duction was done by mixing 0.1 ml of log-phase Nutrient Broth cultures of both strains and plating the mixture on lactose plates. A lactose-positive colony was picked, and a single colony was isolated. The resulting strain, TA105, was sensitive to *Salmonella* phage P22 and grew on minimal lactose agar.

To transfer the episome from TA105 to the deletion mutant *hisFAHBCDG644*, the strains were mated by the method of Sanderson and Demerec (16). The desired strain, SB391 (Table 1) is Lac$^-$, but, unlike the parent deletion strain, can be mutated to Lac$^+$ by nitrosoguanidine. Strain SB391 was selected by plating portions of the mating mixture on MacConkey lactose plates, picking Lac$^-$ (white) colonies, and testing these for revertability to Lac$^+$ with nitrosoguanidine. As expected, episome transfer between the *S. typhimurium* strains was efficient; 30% of the Lac$^-$ strains tested carried the episome. One such episome-carrying strain was further tested and found to be sensitive to *Salmonella* phage P22 and resistant to coliphage T4. By episome transfer on histidine-lactose plates, the strain could enable *hisC527 sup-501* (TA131) to grow on lactose, but could not enable *hisC527*, the corresponding strain without the amber suppressor, to do so. This tester strain was given the strain number SB391 (Table 1).

(ii) SB392 (containing F'lac with UAG mutation). The episome from U281 was transferred to strain TA131 as described for TA105 above. The resulting strain, TA106, is TA131 (F'*lacU281*). The transfer of the episome from TA106 to *hisFAHBCDG644* was again done by the method of Sanderson and Demerec

TABLE 1. *Genotypes and sources of bacterial strains*

Strain or mutation no.	Genotype	Source
E. coli strains		
X82 [UAG in episome]	*lac* deletion (F'*lacX82*)	W. A. Newton
U281 [UAG in episome]	*lac* deletion (F'*lacU281*)	W. A. Newton
YA596 [UAA in episome]	*lac* deletion (F'*lacYA596*)	W. A. Newton
NG219 [UGA in episome]	*lacNG219 trp-8*(F'*lacNG219*)	D. Zipser
NG813 [UGA in episome]	*lacNG813 trp-8*(F'*lacNG813*)	D. Zipser
Salmonella strains		
hisFAHBCDG644	*his* deletion *hisFAHBCDG644*	P. E. Hartman
hisFAHBCD152	*his* deletion *hisFAHBCD152*	P. E. Hartman
TA131 (*hisC527su527-1*)	*hisC527 sup-501*	H. Whitfield
TA105	*hisC527 sup-501*(F'*lacX82*)	Constructed
SB391	*his644*(F'*lacX82*)	Constructed
TA106	*hisC527 sup-501*(F'*lacU281*)	Constructed
SB392	*his644*(F'*lacU281*)	Constructed
TA144 (*hisC117su117-7*)	*hisC117 sup-514*	H. Whitfield
TA107	*hisC117 sup-514* (F'*lacYA596*)	Constructed
TA108	*hisC117 sup-514 arg*$^-$ (F'*lacYA596*)	Constructed
SB393	*hisC117 sup-514 arg*$^-$ *ile*$^-$ (F'*lacYA596*)	Constructed
hisCD129	*his* deletion *hisCD129*	P. E. Hartman
TA216	*hisCD129 sup-533* (F'*lacNG219*)	Constructed
TA109	*hisCD129 sup-533* (from TA216)	Constructed
TA217	*hisCD129 sup-533* (F'*lacNG813*)	Constructed

(16), and *hisFAHBCDG644* (F'*lacU281*) was selected in the same way. The resulting strain is SB392.

(*iii*) *SB393* (*containing F'lac with UAA mutation*). The episome from *E. coli* YA596 was transferred to *Salmonella* TA144 (*hisC117 sup-514*), a strain carrying an ochre suppressor. The strains were mated for 2 hr in Nutrient Broth. T4 phage was added to kill the donors, and samples were plated on histidine lactose plates. Lac$^+$ colonies were picked. As expected, the resulting strain, TA107 (Table 1), was P22-sensitive. Attempts to transfer the episome to *hisFAHBCDG644* and to another histidine deletion, *hisFAHBCD152*, were not successful. Counterselection markers were then introduced into TA107 so that it could be used directly as a tester strain. To minimize the chance of producing a nonsense mutation, the first outside mutation was induced by ICR-191, an agent known to produce frameshift mutations (2, 3). An arginine auxotrophic marker was induced by ICR191, and an additional auxotrophic marker (Ile$^-$) was induced by diethylsulfate (12). Penicillin selections (8, 11) were employed in both cases. The resulting strain SB393 has the phenotype expected for the genotype *hisC117 sup-514 Arg$^-$ Ile$^-$* (F'*lacYA596*).

(*iv*) *TA216* (*containing F'lac with UGA mutation*). No Salmonella strains known to carry UGA suppressors were available. To induce a UGA suppressor, histidine deletion mutant, *hisCD129*, was mutagenized with diethylsulfate (12). The mutagenized culture of *hisCD129* was then mated with *E. coli* strain NG219. After mating, the cells were plated on histidine lactose plates. The *E. coli* donor was unable to grow because it is Lac$^-$ and because of its tryptophan requirement (Table 1). The recipient was unable to grow because it, too, was Lac$^-$. The only colonies expected to grow on the histidine lactose plates were those resulting from the transfer of a reverted F'*lac* episome, or from the introduction of a NG219 episome into a *hisCD129* cell carrying a UGA suppressor. Of 11 Lac$^+$ strains picked, six were identified as *hisCD129* carrying a UGA suppressor and the F'*lac* episome from NG219. The identification was based on the following properties. (i) The strains were sensitive to the *Salmonella*-specific phage P22. (ii) The strains were not sensitive to SP6, a female specific phage (9). (iii) The strains were Lac$^+$ His$^-$ and segregated out Lac$^-$ His$^-$ colonies on a lactose-MacConkey plate. One of these segregants, TA109, on reinfection with the NG219 or NG813 episomes from *E. coli* was again Lac$^+$ His$^-$. (iv) The strains could not transfer the ability to grow on lactose to wild-type *Salmonella*, and thus they do not contain F'*lac*$^+$ episomes. One of these strains was called TA216 and is of the genotype *hisCD129 sup-533* (F'*lacNG219*) (Table 1).

(*v*) *TA 217* (*containing F'lac with UGA mutation*). No lactose-positive colonies resulted when NG813 was mated with mutagenized *hisCD129* and plated on histidine lactose plates. Therefore, TA109 (a Lac$^-$ segregant of TA216) was used as a recipient of the episome from *E. coli* NG813. TA109 and NG813 were mated, and colonies were picked, isolated, and characterized as outlined for TA216. One which had the desired properties was given the strain number *TA217*, was used as a tester strain, and had the genotype *hisCD129 sup-533* (F'*lac NG813*) (Table 1).

Method for classifying mutants. When an auxotrophic mutant containing a nonsense mutation is reverted on a minimal plate, some of the revertant colonies appearing are not true back mutations but are due to a secondary mutation to a nonsense suppressor. By replica-plating the revertant colonies onto lawns of tester strains, those colonies containing a suppressor are identified.

Each of the histidine-requiring mutants to be tested for the presence of a nonsense mutation was grown overnight in Nutrient Broth. Each culture (0.1 ml) was then added to 2 ml of liquid 0.6% agar at 45 C containing 0.2 μmole of L-histidine. The suspension was mixed and poured onto the surface of a minimal glucose plate. After the surface agar had hardened, a crystal of nitrosoguanidine was placed in the center of each plate, and the plates were incubated at 37 C for 2 days. The trace of histidine made it possible to see the zone of nitrosoguanidine inhibition and resulted in more mutations per plate. Missense and nonsense mutants gave rise to 50 to 500 colonies just outside the zone of inhibition. Frameshift and deletion mutations are not induced to revert by nitrosoguanidine (19). Reversions of frameshift mutations were obtained spontaneously or with ICR-191 (2).

The reversion plates were replica-plated onto lawns of the tester strains on minimal lactose plates. The replicates were incubated at 37 C and read after 2, 4, and 6 days. Typical reversion plates and the corresponding tester strain plates are shown in Fig. 1.

The five tester strains were grown overnight in Nutrient Broth. The cultures were centrifuged, and the cells were suspended in half of the original volume of T2 buffer (9). These suspensions could be stored at 4 C for at least 1 week. The tester lawns were made by spreading 0.1 ml of the suspension on the surfaces of minimal lactose plates. (Alternately, lawns of the tester strains on Nutrient Agar or histidine-glucose plates were replica-plated onto minimal lactose plates. An overnight lawn could be printed onto at least 10 plates.)

RESULTS

The *hisC* mutants were screened with the five tester strains. The results are shown in Table 2. The *hisC* mutants were used as a test of the method, since these mutants had been classified previously by Whitfield et al. (19). All 22 of the *hisC* amber mutants gave a positive response. All six of the *hisC* ochre mutants also gave a positive response. However, fewer colonies were seen on the lactose plates with ochre mutants than with amber mutants. Amber mutants responded in 2 days, whereas most ochre mutants required 4 to 6 days for a positive response. None of the 23 *hisC* missense mutants, and none of the frameshift mutants responded in the test. None of the *hisC* mutations was a UGA mutation.

Twenty-one *hisG* mutants were also screened by this procedure. The revertants of three polar

mutants, *hisG200*, *hisG205*, and *hisG611*, did not show a positive response on lawns of SB391 (UAG), SB392 (UAG), or SB393 (UAA), but did show a positive response on lawns of the UGA strains TA216 and TA217 (Fig. 1). UGA suppressors are not able to suppress amber or ochre mutations (15, 21), allowing these mutations to be classified unambiguously as UGA mutations.

Tests for amber and ochre mutations were also applied to the *hisA* mutants by Margolies and Goldberger (13). None of the *A* gene mutants classified as nonsense by this test produced cross-reacting material (CRM), whereas at least 70% of the missense mutants were CRM-positive.

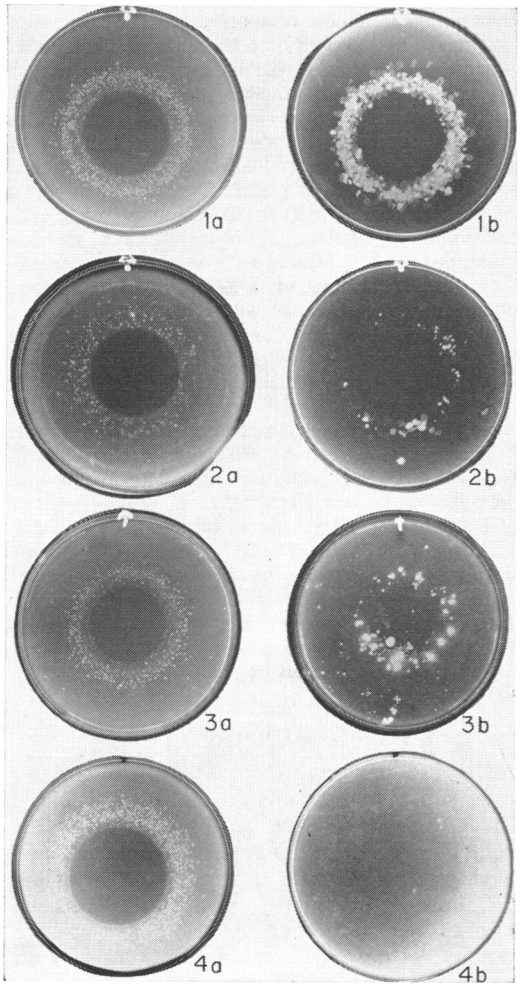

FIG. 1. *Mutants (1) hisC527 (amber), (2) hisC342 (ochre), (3) hisG200 (UGA), and (4) hisC464 (missense) were reverted to His+ as described in the text. The C mutant revertants were printed onto lawns of SB391 on lactose plates, and the hisG200 revertants were printed onto TA216 on lactose plates. Column a shows the reversion plates and column b shows the lactose replicates.*

TABLE 2. *Responses of tester strains to revertants of hisC mutants*[a]

Mutant	No. in class	SB391 (UAG)	SB392 (UAG)	SB393 (UAA)	TA216 (UGA)	TA217 (UGA)
hisC amber						
Class I.....	17	+	+	−	−	−
Class II.....	5	+	+	+	−	−
hisC ochre						
Class I.....	1	+	+	−	−	−
Class II.....	1	+	+	+	−	−
Class III....	1	+	−	+	−	−
Class IV.....	2	−	−	+	−	−
Class V.....	1	−	+	−	−	−

[a] Various histidine mutants were reverted and printed onto lawns of tester strains as described in the text. The members of each class were as follows: *hisC* amber; class I, *31*, *43*, *50*, *340*, *341*, *364*, *426*, *434*, *439*, *446*, *491*, *501*, *507*, *508*, *525*, *527*, and *544*; class II, *121*, *487*, *869*, *879*, *881*; *hisC* ochre; class I, *514*; class II, *342*; class III, *117*; class IV, *151*, *354*; class V, *502*.

In some cases, the result required additional experiments to define clearly the mutational events involved. Revertants of some of the *hisC* ochre mutants produced only a small number of colonies (one to five) on the tester lawns on lactose. In such cases, the Lac+ colonies must be isolated. The strains must be checked by transduction to be sure that the original mutation is still present. Several isolated colonies which appeared on tester plates of some *hisC* missense mutants were characterized in this way. Phage grown on Lac+ revertants were used to transduce deletion *his-FAHBCDG644* to growth on minimal medium. Since transductants appeared, it could be concluded that the prototrophic phenotype was the result of an event within the *C* gene, and was not due to a suppressor. Such colonies may have resulted from a secondary suppressor mutation or from infection of a back mutant by a reverted *F'lac*+ episome.

DISCUSSION

The episome test identifies UGA mutations unambiguously, but does not definitely distinguish between amber and ochre mutations. UGA suppressors do not suppress amber and ochre mutations (15, 21). Strains which respond to the UGA episomes are therefore inferred to carry a UGA mutation. Since ochre suppressors suppress either amber or ochre mutations (5), strains which

respond to amber or ochre episomes cannot be uniquely classified as either amber or ochre. Ochre suppressors may be expected to occur among the revertants of amber mutants (*hisC* class II). In general, however, amber suppressor strains are observed in 2 days and ochre only in 4 to 6 days on the lactose plates.

All the nonsense suppressors capable of suppressing a particular mutation are not necessarily detected. The test requires that a suppressor suppress both the nonsense mutation on the episome and the chromosomal nonsense mutation. Suppressors which are not able to suppress both mutations would not be detected.

The test was designed for *Salmonella*, which is unable to ferment lactose (*Bergey's Manual*). The method can be applied to any reasonably stable Lac$^-$ strains which can act as recipients of the F'*lac* episomes. Deletions would be better than stable point mutants, because recombination between the epismal and chromosomal *lacZ* genes would be eliminated.

Nonsense mutants have been identified by showing that some of their revertants permit the growth of nonsense phage mutants (5, 10, 14). The episome test described here is easier to use, since individual revertants need not be isolated. Osborn et al. (14) have been able to distinguish suppressor loci by patterns of nonsense phage growth on suppressor-carrying strains. This can also be done by testing the ablity of a suppressor-carrying strain to suppress a large number of nonsense F'*lac* episomes.

The tester strains have also been used to map nonsense suppressors in *Salmonella* (*manuscript in preparation*). Auxotrophic strains with markers near suppressor loci can be transduced to prototrophy with phage grown on a suppressor-carrying strain. The transductants are then printed onto lawns of tester strains on lactose plates to score for the percentage of cotransduction of the suppressor. Transductants carrying suppressors can be selected directly by mixing phage and the recipient, and spreading this mixture on lactose plates with an episome donor which is phage resistant (D. B. Fankhauser, *personal communication*). A counterselecting marker in the phage-resistant strain prevented the growth of cells carrying episomes with reverted *lac* mutations.

UGA mutations appear to be rare in our system, though they are common in the *lac* operon (21). None was found in the *hisC* gene, whereas three were found in the *hisG* gene. The three *hisG* UGA mutants are all polar and map at the operator end of the first structural gene of the histidine operon. They may be allelic, as they do not recombine with one another (1).

ACKNOWLEDGMENTS

We are grateful to Gerald R. Fink and Robert G. Martin for many helpful suggestions.

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