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Isolation of Nonsense Suppressor Mutants in *Pseudomonas*

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A strain of *Escherichia coli* harboring the drug resistance plasmid RP1 was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and mutants were isolated in which ampicillin resistance had been lost due to an amber mutation in the plasmid. One of these mutants was again treated, and a strain was isolated in which tetracycline resistance was also lost due to an amber mutation in the plasmid. The plasmid containing amber mutations in the genes *amp* and *tet* was named pLM2. This plasmid could be transferred to strains of *Pseudomonas aeruginosa, P. phaseolicola,* and *P. pseudoalcaligenes*. Mutants resistant to ampicillin and tetracycline could not be obtained from *P. phaseolicola* carrying pLM2. However, strains of *E. coli, P. aeruginosa,* and *P. pseudoalcaligenes* carrying the plasmid did produce mutants simultaneously resistant to both antibiotics. All of the mutants of *E. coli* had developed nonsense suppressors since they became phenotypically *lac*$^+$, although harboring a *lac* amber mutation, and formed plaques with amber mutants of phages PRR1 and PRD1 that attack organisms carrying RP1. Approximately 20% of the resistant mutants of *P. aeruginosa* and *P. pseudoalcaligenes* were sensitive to the amber mutant of PRD1. These mutants were of variable stability and grew somewhat more slowly than their parent strains. One of the suppressor mutants of *P. pseudoalcaligenes*, designated ERA(pLM2)S4, was used for the isolation of nonsense mutants of bacteriophage ϕ6, a virus having a segmented genome of double-stranded ribonucleic acid and an envelope of lipids and proteins.

Genetic suppression of nonsense mutations was first described in *Escherichia coli* (4, 6). Nonsense suppressors have been subsequently demonstrated in other microorganisms such as *Bacillus subtilis* (12) and *Saccharomyces cerevisiae* (6). These mutations have been of great value in the elucidation of viral structure and morphogenesis since they allow the isolation of nonsense mutants of bacteriophages, which can be analyzed in a manner that can identify the product of the gene in which each nonsense mutation occurs. Using these mutants that produce only fragments of particular proteins, it has been possible to work out morphogenetic pathways in phage development and to determine the roles of particular proteins in phage development (10, 19, 20, 24). We have been studying the development of bacteriophage ϕ6, which is of special interest because it has a genome composed of three pieces of double-stranded ribonucleic acid (RNA) and an envelope structure composed of an icosahedral core with a lipid-containing, membrane-like outer layer (16, 17, 22). To facilitate this study, we endeavored to isolate suppressor mutants of *Pseudomonas phaseolicola,* the natural host of this bacteriophage. We found that such mutants could not be obtained in this strain, but we did succeed in isolating a new host for ϕ6, called *P. pseudoalcaligenes* ERA. Suppressors were obtained in this strain, as well as in *P. aeruginosa,* using plasmid pLM2, which was prepared in this study.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1. *P. phaseolicola* HB1OY and bacteriophage ϕ6 were obtained from A. Vidaver (22). PAT904(RP1), a strain of *P. aeruginosa* carrying plasmid RP1, was obtained from R. Olsen, who also supplied bacteriophages PRR1 and PRD1 (13, 14) that form plaques on many different species carrying RP1. *E. coli* CA274 carrying amber mutations in *lac* and *trp* was obtained from J. D. Smith (15). *P. aeruginosa* strain PAO1 was obtained from A. Emerick. *E. coli* strains K37 and K38 were obtained from N. Zinder. K37 contains a suppressor, *supD*, (3, 7, 24), whereas K38 has no suppressor.

Media. LC broth and agar was used for most experiments. This medium contains 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7.0. Bottom agar contained 1.5% agar and top agar was 2.5 ml of LC with 0.6% agar. Synthetic medium was M8 (17), with glucose or lactate as the carbon source

TABLE 1. *Strains used*

Designation	Relevant properties	Source/reference no.
P. aeruginosa		
PAT094 *rev-1* (RP1)	*str/amp tet kan*	R. Olsen (13)
PAO1	Prototroph	A. Emerick
PAO1(pLM2)	*kan amp(am) tet(am)*	This study
PAO1(pLM2)*supB*	*kan amp(am) tet(am) su*$^+$	This study
P. pseudoalcaligenes		
ERA	Sensitive to ϕ6h1	This study
ERA(pLM2)S4	*kan amp(am) tet(am) su*$^+$	This study
P. phaseolicola		
HB10Y	Sensitive to ϕ6	A. Vidaver (22)
E. coli		
CA274	HfrC *trp(am) lac(am)*	J. D. Smith (15)
CA274(RP1)	HfrC *trp(am) lac(am)/kan amp tet*	This study
CA274(pLM1)	HfrC *trp(am) lac(am)/kan amp(am) tet*	This study
CA274(pLM2)	HfrC *trp(am) lac(am)/kan amp(am) tet(am)*	This study
K37	Su-I *(supD)*	N. D. Zinder (3, 7, 24)
K38	Nonsuppressor	N. D. Zinder (3, 7, 24)

and amino acids as required. Ampicillin, kanamycin, and tetracycline resistances were assayed at 400, 50, and 25 μg/ml, respectively, for *E. coli, P. phaseolicola,* and *P. pseudoalcaligenes.* Concentrations of 2 mg/ml, 400 μg/ml, and 200 μg/ml, respectively, were used for *P. aeruginosa.* Ampicillin and kanamycin were generous gifts of Bristol Laboratories. Tetracycline was purchased from Sigma Chemical Co.

Mutagenesis of bacteria. Overnight cultures in LC broth were centrifuged and resuspended in 10 ml of M8 salts (17) and treated with 0.2 ml of ethyl methane sulfonate (EMS) for 40 min, centrifuged again, resuspended in LC broth, washed once more, and then incubated overnight in LC broth. Treatment was at 26 C for *P. phaseolicola* and at 37 C for all other strains. Mutagenesis with *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG) was as described by Adelberg et al. (1), using tris(hydroxymethyl)-aminomethane-maleate buffer, pH 6, and 200 μg of NTG per ml.

Mutagenesis of bacteriophages. PRR1 was treated with 0.05 M sodium nitrite in LC broth containing 0.25 M acetate, pH 4.4, for 1 h at 25 C (24). Survival was 2×10^{-5}. Mutagenized phage was plated on K37(RP1), and 1,000 plaques were picked with toothpicks and stabbed into lawns of K37(RP1) and K38(RP1). Two mutants were isolated that formed plaques on suppressor-containing strains but not on others. They were designated PRR1 *sus-1* and *sus-2*.

PRD1 was mutagenized with 25 μg of NTG per ml while infecting K37(RP1) by the procedure of Studier (19) for T7. The mutagenized phage was plated as described for PRR1, and mutants were designated PRD1 *sus-1* and *sus-2*. The reversion frequency of the amber mutants of PRR1 was approximately 10^{-3} and for those of PRD1, approximately 10^{-6}, consistent with the RNA and deoxyribonucleic acid respective compositions of the bacteriophages (13, 14). ϕ6hls was mutagenized by incubating virus for 3 days in tris(hydroxymethyl)aminomethane-maleate buffer, pH 6, (1) containing 1 mg of NTG per ml.

Transfer of RP1. Transfer of plasmids was usually as described by Olsen and Shipley (13). In many cases transfer was effected by simply cross-streaking overnight cultures of the donor and recipient strains on media containing kanamycin to select for plasmid-containing strains and the proper conditions to select against the donor strain. Transfer was at high frequency (greater than 0.01%), except in the case of transfers to *P. phaseolicola.* Transfers to this strain could be effected by plating approximately 10^9 donor and recipient cells per plate. Transfer frequency was about 1 in 10^9 cells. However, this frequency could be raised about 100-fold by mutagenizing the recipient cells with EMS.

Selection of amber mutations in drug resistance. RP1 was transferred from HB(RP1) to *E. coli* CA274 *su*$^-$ by selecting for kanamycin resistance at 37 C. CA274(RP1) was mutagenized with NTG, and 2×10^4 cells were plated on GL agar (11) containing kanamycin. These plates were replicated both with and without ampicillin (400 μg/ml). Forty ampicillin-sensitive colonies were isolated. Twenty-four were found to be penicillinase negative by a staining test (11). These were purified and cross-streaked against K37 and K38 on ampicillin-containing plates that lacked tryptophan. One of the isolated mutants transferred ampicillin resistance to K37 but not to K38 and was designated CA274 (pLM1). This strain was again mutagenized, grown overnight, reinoculated into fresh LC broth with 25 μg of tetracycline per ml, and grown for 30 min before adding 1,000 U of penicillin G per ml. After several hours, the culture lysed and was centrifuged and washed twice with LC broth and plated on LC agar plates. These plates were replica plated onto plates with and without tetracycline. Of 33 tetracycline-sensitive mutants, 2 transferred resistance to K37 but not to K38. One of these strains was designated CA274(pLM2).

The plasmid pLM2 is transferable and carries resistance to kanamycin and amber mutations in the genes for ampicillin and tetracycline resistance. The resistance to ampicillin in suppressor strains containing *supD* is temperature sensitive above 37 C.

Taxonomic classification of strain ERA. Standard techniques of identification (8, 23) were applied to strain ERA (Table 2).

RESULTS

General strategy for the preparation of a suppressor strain. The plasmid RP1 can transfer across generic lines in gram-negative bacteria (5, 13). In particular, it is easily transferred between pseudomonads and *E. coli* (5, 13). In addition, two bacteriophages, PRR1 and PRD1, that attack strains carrying RP1 have been isolated (13, 14). The original plan of this study was to transfer RP1 into *P. phaseolicola* HB1OY (called HB in this report), obtain many amino acid auxotrophic mutants, among which some would be due to nonsense mutations, isolate revertants of these mutants, and test their capacity as suppressors with amber mutants of phages PRR1 or PRD1.

TABLE 2. *Characteristics of strain ERA (8, 23)*

Test	Results and comments
Blood agar – 5% sheep defibrinated	Grayish opaque colonies, nonhemolytic
MacConkey agar	Lactose nonfermenter
Russell double sugar	Alkaline/no change
Fluorescence	Negative
Growth at 42 C	Positive
Indole broth	Negative
Cytochrome oxidase	Positive
Nitrate reduction	Positive
Nitrite reduction	Negative
Urease agar	Negative, pink, 72 h
Simmons citrate	Negative
OF glucose medium	
open tube	Alkaline
closed tube	No growth
OF maltose	Alkaline
OF fructose	Positive
OF sucrose	Alkaline
OF lactose	Alkaline
OF rhamnose	Alkaline
OF mannitol	Alkaline
OF xylose	Alkaline
OF 3% ethyl alcohol	Positive
o-nitrophenyl-β-D-galactopyranoside	Negative
Assimilation of D-glucose	Negative
Assimilation of D-fructose	Positive
Assimilation of L-glutamate	Positive
Assimilation of lactate	Positive
Assimilation of ethanol	Positive
Growth on cetrimide agar	Positive
Phenylalanine deaminase	Negative
Arginine dihydrolase	Positive
Gelatinase	Negative
Deoxyribonuclease	Negative
H_2S production	Negative
Tyrosine agar	No pigmentation
Flagella	1-2 polar

The transfer of RP1 to HB was accomplished. The frequency of transfer was found to be very low (about 10^{-8} to 10^{-9}) when *E. coli* CA274(RP1) was the donor and untreated HB was the recipient. However, the frequency of transfer was increased to about 10^{-6} after mutagenesis of HB with EMS. The effect of this mutagenesis persisted in that a strain of HB(RP1) could be cured of RP1 by selecting for phage PRD1-resistant mutants, and the cured strain showed a very high frequency of uptake of RP1 on subsequent tests ($>10^{-5}$).

Auxotrophic mutants of HB(RP1) were isolated as described above, and revertants were isolated and tested by cross-streaking for growth of the amber mutants of phage PRR1. None of the revertants grew the amber mutants of the phage. Ultimately, 180 amino acid auxotrophs of HB(RP1) were isolated, and approximately five revertants of each were isolated and tested with no success. Twenty double auxotrophic mutants were isolated from each of 15 single auxotrophs, and revertants were isolated from several of these. None was able to grow the amber mutants of PRR1.

The second plan for isolating suppressor mutants involved the preparation of double amber mutants for ampicillin and tetracycline resistance in RP1. Since the plasmid carries the genes for kanamycin, tetracycline, and ampicillin resistances, it should be possible to isolate a mutant plasmid that has amber mutations in the *tet* and *amp* genes and use the kanamycin resistance as a selection for transfer to HB. *E. coli* CA274(RP1) was mutagenized and tested as described above, and the resulting plasmid that conferred ampicillin and tetracycline resistances only on suppressor strains was designated pLM2.

Transfer of pLM2 to HB. The plasmid pLM2 could be transferred from CA274(pLM2) to HB by selecting for kanamycin resistance and tryptophan prototrophy, since the donor required tryptophan and HB did not. Transfer occurred at a frequency of 10^{-9}. The resulting strain HB(pLM2) was mutagenized with either EMS, NTG, or ultraviolet light, grown overnight in

broth, and then spread on plates containing 400 μg of ampicillin per ml and 25 μg of tetracycline per ml. No resistant colonies were found on plates containing both antibiotics, although approximately 10^{11} cells were plated in each case. When selection was applied for either ampicillin or tetracycline resistance, resistant colonies did appear, but these colonies did not support the growth of the PRR1 *sus* mutant. These experiments indicated that the formation or expression of suppressor mutations was either improbable or deleterious in HB. Strain CA274(pLM2) produced many mutants resistant to both ampicillin and tetracycline after EMS mutagenesis. Of 10 strains tested, all had become lac^+, indicating the presence of a suppressor, and all supported the growth of PRR1 *sus-1*.

Isolation of ERA. Since HB, the natural host for bacteriophage φ6, did not seem capable of producing suppressor mutations, we attempted to isolate a new host for the virus. Water from the East River was spread on agar plates, and approximately 300 colonies were picked, suspended in broth, and cross-streaked against φ6. Two strains were found to support the growth of the virus. One was designated strain ERA. It was found to be oxidase positive and motile, and it grew well at 42 C in complex media, and used lactate or amino acids as a source of carbon. The characteristics of ERA are identical to those of *P. pseudoalcaligenes* (Table 2) (8, 23).

Bacteriophage φ6 plated with an efficiency of plating (EOP) of 5×10^{-6} on ERA when compared with HB. However, the progeny of the successful plating on ERA subsequently had an EOP of 0.6 on ERA. The mutant virus was designated φ6h1. Growth of φ6h1 on HB did not change its EOP on ERA. Although ERA grows up to 42 C, the growth of φ6 derivatives does not occur above 27 C.

Transfer of pLM2 to ERA. The plasmid was easily transferred to ERA with a frequency of about 10^{-6}. ERA(pLM2) supported the growth of bacteriophage PRD1 but not that of PRR1. After mutagenesis with EMS and plating on ampicillin-tetracycline plates, mutants were obtained that were resistant to both antibiotics. These mutants were tested for their ability to support the growth of PRD1 *sus-1*. Of 100 mutants tested, only 10 supported the growth of the amber phage. Most of these mutants were extremely unstable. Plating out on nonselective media led to the appearance of a majority of nonresistant cells. One of the mutants designated ERA(pLM2)S4 was fairly stable and gave the highest EOP for PRD1 *sus-1* (Table 3). The EOP of φ6h1 was 100-fold lower on S4 than on ERA; therefore, an additional mutant of the virus was isolated that has an EOP of 0.6 on S4. This is called φ6h1s.

Nonsense mutants in φ6h1s. A preparation of φ6h1s was mutagenized by treatment with NTG at 1 mg/ml for 3 days at room temperature. The virus was then plated on S4, and plaques were picked with sterile toothpicks and stabbed into fresh plates of S4 and HB. Mutants were obtained with a frequency of 0.5% that grew on S4 and not on HB, HB(RP1), HB(pLM2), ERA, or ERA(pLM2). The analysis of these mutants is the subject of a report in preparation.

Transfer of pLM2 to *P. aeruginosa* strain PAO1. The transfer of the plasmid to strains of *P. aeruginosa* is made difficult by their natural high levels of resistance to ampicillin, tetracycline, and kanamycin. The concentration of kanamycin for transfer selection was 400 μg/ml. PAO1(pLM2) was mutagenized with EMS and spread on plates containing 2 mg of ampicillin and 200 μg of tetracycline per ml. Mutants resistant to both antibiotics were obtained and tested for the ability to support the growth of PRD1 *sus-1*. Of 20 mutants tested, 5 supported the amber mutant phage. Three of these mutants were designated PAO1(pLM2) *supA*, *supB*, and *supC*, respectively. The suppressor mutations in *P. aeruginosa* seem to be more stable than those in strain ERA. Table 3

TABLE 3. *Plating of amber mutants of PRD1 and PRR1 on various bacterial strains*[a]

Bacteria	PRD1 (PFU/ml)[b]	PRD1 *sus-1* (PFU/ml)	PRR1 (PFU/ml)	PRR1 *sus-1* (PFU/ml)
K38(RP1)	2.1×10^{10}	$<10^{3}$	1.9×10^{5}	6×10^{1}
K37(RP1)	2.0×10^{10}	2.5×10^{7}	9.3×10^{4}	1.1×10^{5}
PAO1(pLM2)	1.2×10^{11}	$<10^{3}$	1.2×10^{5}	1.2×10^{2}
PAO1(pLM2)*supB*	1.9×10^{11}	9.7×10^{7}	9×10^{4}	4×10^{1}
PAO1(pLM2)*supC*	1.4×10^{11}	6.2×10^{7}	6.5×10^{4}	2×10^{1}
ERA(pLM2)	8.3×10^{8}	$<10^{3}$	$<2 \times 10^{1}$	$<2 \times 10^{1}$
ERA(pLM2)S4	1.4×10^{9}	4.2×10^{5}	$<2 \times 10^{1}$	$<2 \times 10^{1}$

[a] Plates were prepared for LC agar with 2.5-ml overlay at 37 C for PRD1 and at 25 C for PRR1.
[b] PFU, Plaque-forming units.

shows the relative plating characteristics of the various amber phage mutants on the suppressor strains discussed in this report. It should be noted that the amber mutant of PRD1 was able to grow on suppressor strains of *E. coli*, *P. aeruginosa*, and *P. pseudoalcaligenes*. Although the results are not shown, the EOP of PRD1 on *E. coli* K37 and K38 and on *P. pseudoalcaligenes* was at least 1,000-fold lower at 25 C than at 37 C. This difference was not seen with *P. aeruginosa*. On the other hand, although PRR1 plated with a higher EOP at 37 C than at 25 C, the amber mutant PRR1 *sus-1* had a higher EOP at 25 C than at 37 C. This mutant did not seem to be suppressed at 25 C by the *P. aeruginosa* strains that supported the growth of PRD1 *sus-1*. *P. aeruginosa* PAO1-(pLM2) *susB* did seem to suppress PRR1 *sus-1* to a slight degree at 37 C.

DISCUSSION

The primary goal of this project, the isolation of a suppressor host for bacteriophage $\phi 6$, was accomplished. However, some of the secondary results will be of value in other systems. The plasmid bearing two amber mutants in drug resistance should be of use in the isolation of suppressor mutants in the various genera that can carry RP1. In this report we have demonstrated its utility for *E. coli*, *P. pseudoalcaligenes*, and *P. aeruginosa*. An amber mutation in ampicillin resistance of plasmid R1 has been used in isolating nonsense suppressors on the *Enterobacteriaceae* (9). Suppressor mutations have not been described previously in pseudomonads, and our system should be of special usefulness in the study of the physiology of the pseudomonads and their bacteriophages. In principle, it should be possible to isolate nonsense suppressor mutations in all species that can carry RP1. Similarly, the amber mutant of PRD1 should facilitate the testing of the suppressor activity in these strains.

The reason for our lack of success in isolating nonsense suppressors in HB is not known. The most likely possibilities are that either there is a paucity of dispensible transfer RNA genes that can form suppressors, with suppressor mutations then being lethal (18), or suppressors when formed do not express themselves. The latter would be the case if the peptide chain termination system worked so efficiently that the suppressor transfer RNA could not compete with the termination system. Mutants that are, perhaps, of this type have been described by Elseviers and Gorini (2). In these mutants, suppression is diminished by mutations mapping in the region of ribosomal protein structural genes. Another possibility would be that the amber codon UAG is used in normal termination, as has been found for some phages (21).

In the case of suppressor mutations in ERA and *P. aeruginosa*, the instability may be due to the same effects described above. The effect does not seem to be due to the plasmid since the suppressors isolated in *E. coli* using the same system are stable.

The isolation of ERA was fortuitous, since it appears that host strains for $\phi 6$ are not common. The host range mutants of $\phi 6$ that grow on ERA still give high yields on HB, and their plating efficiency on ERA remains high after passage on HB. This indicates that the host range limitation is not due to host modification and restriction. A suppressor strain isolated from ERA has been found suitable for the isolation of nonsense mutants of $\phi 6$. This is of special interest because this bacteriophage has a genome of three pieces of double-stranded RNA and an envelope that contains lipids. The study of the morphogenesis of this virus, which should be greatly facilitated by the isolation of amber mutants, should elucidate many elements in the biosynthesis of double-stranded RNA and in the formation of membrane-like structures.

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